DEVELOPING A MODEL SYSTEM FOR *Staphylococcus aureus* RESPIRATORY INFECTION IN CYSTIC FIBROSIS PATIENTS

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by

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DECLARATION

I declare that the work reported in this thesis is entirely my own and has been conducted at Kingston University and St. George`s Hospital, United Kingdom.

This thesis has not been submitted, in whole or in part, for any other degree at this or any other university.

Christianne Micallef

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'Like the waves make towards the pebbled shore,
So do our minutes hasten to their end;
Each changing place with that which goes before,
In sequent toil all forwards do contend.'

William Shakespeare, Sonnet 60.

Abstract

For the first time, an *in vitro* cystic fibrosis (CF) artificial sputum model (ASM) was found to support the growth and survival of a clinical epidemic strain of meticillin-resistant *Staphylococcus aureus* (EMRSA16-252). Specific components, which included mucin, DNA and others, were removed from ASM and the physiological impact of this was fully explored using viable counts and light microscopy. As CF patients are known to develop cystic fibrosis-related diabetes or CFRD, glucose was added to ASM (GASM), to explore the physiological impact of glucose on the growth and survival MRSA252. Total RNA was extracted from the corresponding log phases of MRSA252 grown in brain heart infusion (BHI) as a laboratory control, as well as ASM and GASM. RNA was extracted in order to conduct microarray analysis. MRSA252 DNA was used as a control. RNA (from the samples) was labelled with Cy5 and control DNA was labelled with Cy3. Once labelled and amplified, the Cy5/Cy3 mixture was then purified and hybridised onto an array containing seven sequenced *S. aureus* genomes (N315, Mu50, MW2, MRSA252, MSSA476, COL and NCTC8325). The array was then scanned and the raw results were analysed. Differentially expressed genes in ASM equalled 15% of the MRSA252 sequenced genome and those for GASM amounted to 21%. Normalised results showed that 14 capsular polysaccharide genes were up-regulated significantly in both ASM and GASM, as well as genes coding for enzymes involved in carbohydrate and vitamin metabolism. Genes coding for surface proteins such as *spa* and *lrgA* and *lrgB*, responsible for peptidoglycan metabolism, were significantly down-regulated. A similar gene expression pattern was observed with GASM, although the total number of genes which were differentially regulated in GASM was greater than for ASM. The microarray data was validated using RT-PCR. Samples of unmodified liquid human CF sputum were then used as a growth medium for MRSA252. These were found to support the growth and survival of this bacterium. RT-PCR was used to investigate gene expression in the liquid human samples and the results compared to those obtained with the *in vitro* models, ASM and GASM. Capsular genes were found to be up-regulated in both the human samples as well as the *in vitro* media. The work conducted in this thesis presents data which suggest that one mechanism involved in *Staphylococcus aureus*-CF respiratory infection may be mediated through genes encoding capsular polysaccharides and offers a possible target for future anti-staphylococcal therapy in cystic fibrosis.

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LIST OF ABBREVIATIONS

ABBREVIATION	DEFINITION
Agr	accessory gene regulator
AIP	auto-inducing peptide
Arl	autolysis-related locus
ASL	airway surface liquid
ASM	artificial sputum medium
BHI	brain heart infusion
BHIA	brain heart infusion agar
BHI+M	brain heart infusion + 1%w/v mucin
CA-MRSA	community-acquired meticillin-resistant *Staphylococcus aureus* (BAN Nomenclature)
Can	collagen
CAT	catalase
CC	clonal complex
cDNA	complementary DNA
CF	cystic fibrosis
CFRD	cystic fibrosis-related diabetes
CFTR	cystic fibrosis transmembrane conductance regulator
CHIP	chemotaxis inhibitory protein
Clf	clumping factor
Coa	coagulase
CoNS	coagulase-negative staphylococci
CP	capsular serotype
Ct	cycle threshold
dsDNA	double-stranded DNA
DTPA	diethylenetriaminepentacetic acid
FAME	fatty acid esterifying enzyme
Fnb	fibronectin-binding protein
GASM	ASM + 10mM glucose
GISA/VISA	glycopeptide-intermediately resistant *S. aureus*/vancomycin-intermediately resistant *S. aureus*
HAI	hospital-acquired infection
HAEC	human airway epithelial cells
Ica	intracellular adhesion locus
IgG	immunoglobulin G
IL-8	interleukin-8
MGEs	mobile genetic elements
MLST	multilocus sequence typing
MOPS	3-(N-morpholino)-propanesulfonic acid

MPO	myeloperoxidase
MRSA	meticillin-resistant *Staphylococcus aureus* (BAN Nomenclature)
MSCRAMMS	microbial surface components recognizing adhesive matrix molecules
NE complex	neutrophil elastase-α-1-protease inhibitor complex
Nuc	nuclease
ORCC	outward rectifying chloride channel
PCL	periciliary layer
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PVL	Panton-Valentin leukocidin
PMN	polymorphonuclear neutrophil
QRT-PCR	quantitative RT-PCR
Rot	repressor of toxins
RT-PCR	reverse transcription polymerase chain reaction
Sak	staphylokinase
Sar	staphylococcal accessory regulator
SCC	staphylococcal cassette chromosome
SCV	small colony variant
SNPs	single nucleotide polymorphisms
Spa	protein A
SSTIs	skin and soft tissue infections
TE	tris EDTA
Tm	melting temperature
TNFα	tumour necrosis factor α
TSB	tryptone soya broth

CHAPTER 1
GENERAL INTRODUCTION

SECTION 1.1: GENERAL OVERVIEW

Staphylococcus aureus is currently one of the world's most important human pathogens and has been defined as a 'super-bug' by worldwide media. Combating this pathogen is now a recognized priority in most healthcare budgets, including those of the United Kingdom. With the onset of meticillin-resistant *Staphylococcus aureus* (MRSA), the UK Government has determined a course of action to decrease the rates of infection and would like to reduce the number of MRSA cases by 50% over a three year period (Spiegelhalter, 2005).

This bacterium possesses an impressive arsenal of virulence factors which actively aid in the colonization of the human host. These include cell surface factors, which facilitate binding to cells, and exotoxins which collectively assist in destroying tissues, permitting effective invasion and penetration. Typical diseases caused by this bacterium include superficial infections and serious, deep-seated infections, commonly occurring in immunocompromised individuals often requiring hospitalization.

Penicillin-resistant strains of *S. aureus* date back to the 1950s (Grundmann *et al.,* 2006). The first MRSA actually emerged in 1960, soon after the marketing of meticillin (Lindsay and Holden, 2004; Grundmann *et al.,* 2006). However, MRSA strains did not spread quickly, not until the late 1980s to mid-1990s. These strains have now firmly established themselves in all hospitals and are one of the most feared cause of hospital-acquired infection (HAI) worldwide (Lindsay and Holden, 2004). An added concern is the recent emergence of Community-Acquired MRSA (CA-MRSA). This is found in otherwise healthy individuals in the community, without any evident predisposing factors and manifests predominantly in skin and soft-tissue infections, although potentially fatal, invasive disease in affected individuals has been recorded (Lindsay and Holden, 2004; Deresiński , 2005).

Thus, understanding the pathogenesis of *S. aureus* in association with typical infections is fundamental, in order to promote more targeted and innovative therapy. *Staphylococcus aureus* was the primary cause of pulmonary infections in CF patients, until the 1950s (Rao *et al.*, 1998).

With the introduction of anti-staphylococcal antibiotics, *P. aeruginosa* replaced *S. aureus* as the most predominant pathogen overall, accounting for more than 60% of pulmonary infections (Rao *et al.*, 1998).

This chapter aims to review the current available literature on *S. aureus* and cystic fibrosis, with special emphasis on the role of *S. aureus* in CF lung infections. Section 1.2, describes the general characteristics of *S. aureus* and presents a wide array of virulence factors possessed by this organism. *Staphylococcus aureus*, as a human pathogen, is fully discussed and the various phenotypic and genotypic characteristics of this bacterium are presented. Subsections on the regulation of specific genes by global regulators, such as *agr* and *sar* ensue, together with information on small colony variants, nasal carriage, *S. aureus* genome and typing systems, as well as the occurrence of different types of *S. aureus* antibiotic resistant strains. Section 1.3 discusses CF in detail. Information on the aetiology of the disease, the various hypotheses governing the observations seen in CF patients and the clinical manifestations of this disease are portrayed. *Staphylococcus aureus* respiratory infection in CF patients is then discussed. In addition, the occurrence of diabetes in these patients may have an impact on patient quality of life, as well as mortality and the link between glucose in respiratory secretions and infections is also presented.

SECTION 1.2: *Staphylococcus aureus*

Section 1.2.1: Characteristics of *Staphylococcus aureus*

In 1880, Sir Alexander Ogston, an eminent Scottish surgeon was studying the role of micrococci in acute inflammatory states and consequently described *S. aureus* in his work, "Über Abscesse" (Macdonald and Smith, 1981).

Staphylococcus aureus is a Gram-positive coccus, ~ 1μm in diameter and morphologically appears in groups, arranged in grape-like clusters, but may also occur as single cells or pairs, especially when observed in pathological specimens. Staphylococci are salt-tolerant and can be selectively isolated from materials such as faeces and food by use of media containing 7-10% sodium chloride (Greenwood *et al.*, 1994).

As with other staphylococci, *S. aureus* is catalase-positive, divides in more than one plane to form irregular grape-like clusters, capable of aerobic and anaerobic metabolism and occur widely on the surfaces of man and other vertebrate animals (Greenwood *et al.*, 1994).

Table 1.1: Distinctive Characteristics of *Staphylococcus aureus* (Adapted from Barrow and Feltham, 1999; Greenwood *et al.*,1994).

- Non-sporing, non-motile, oxidase-negative, generally non-capsulate
- Growth on blood media, nutrient agar or milk agar results in the appearance of colonies, with a diameter of 2-3mm
- Colonies may be pigmented, appearing golden-yellow, fawn or cream
- Production of coagulase (clearly diagnostic of *S. aureus* presence)
- Production of acetoin and acid from a number of sugars such as lactose, maltose, sucrose and trehalose
- Production of thermostable nucleases that break down DNA
- Production of a surface-associated protein (clumping factor or bound coagulase) that reacts with fibrinogen

Section 1.2.2: Virulence Factors of *S. aureus*

Many virulence factors which actively play a role in *S. aureus* pathogenesis have been studied and although many of the mechanisms are known, many more play an unknown role in the infectious process. Surface associated factors, extracellular enzymes and toxins are directly involved in attachment and adherence, tissue degradation and penetration and cytotoxicity, respectively (Wright III and Novick, 2003). Table 1.2 includes a summary of the main virulence factors which may be found in *S. aureus*.

Table 1.2: Virulence Factors of *Staphylococcus aureus*
(Adapted from Projan and Novick *et al.*, 1997, Greenwood *et al.*, 1994)

CLASS OF VIRULENCE FACTOR	TYPE OF VIRULENCE FACTOR	GENE
FACTORS INVOLVED IN ATTACHMENT	Clumping factor,	*clfA, B*
	Fibronectin-binding protein,	*fnbA, B*
	Fibrinogen-binding protein	*fbpA*
	Collagen-binding protein	*cna*
	Coagulase	*cga*
	Protein A	*spa*
FACTORS INVOLVED IN HOST DEFENCES EVASION	Enterotoxins (A, B, C1-3, D, E, G)	*sea-h*
	Staphylokinase	*sak*
	Epidermolytic toxins (A and B),	*eta, etb*
	Toxic Shock Syndrome Toxin 1 (TSST1)	*tst*
	Panton-Valentine Leukocidin	*lukF-PV, lukS-PV*
	V8 Protease	*sasP*
	Lipase	*geh*
	Fatty Acid modifying enzyme (FAME)	
	Capsular polysaccharides (1,5,8)	*cap1,cap5* and *cap8* loci
	Hyaluronidase	*hysA*
	α-toxin	*hla*
	β-haemolysin	*hlb*
	γ-haemolysin	*hlgA, hlgB, hlgC*
	δ-haemolysin	*hld*
FACTORS INVOLVED IN INVASION/TISSUE DESTRUCTION	Phospholipase C	*plc*
	Elastase	*sepA*
	Deoxyribonucleases	*DNase*

In essence, virulence factors can be subdivided into two major categories:

- Surface-associated factors: provide mechanisms for adherence, attachment and immune evasion,
- Secreted factors: cause tissue destruction and are used to counteract host cell responses.

Section 1.2.2.1 Surface Proteins: Adhesins and Immune Evasion Factors

Staphylococcal surface proteins facilitate the adherence to host and bacterial cells, extracellular matrix and inert surfaces. *Staphylococcus aureus* possesses a large number of such proteins, termed as MSCRAMMS or Microbial Surface Components Recognizing Adhesive Matrix Molecules; these share many structural and biological features and also, certain domains are common (Wright III and Novick, 2003; Hook and Foster, 2000). Staphylococcal surface proteins are covalently anchored to the cell wall by the action of sortase A (SrtA). This is a transpeptidase, which acts by catalyzing the exchange of the threonine, in the conserved Leu-Pro-X-Thr-Gly (LPXTG) motif, for a glycine residue present in the pentaglycine cross-bridge (Mazmanian *et al.*, 1999; Wright III and Novick, 2003).

Section 1.2.2.1.1 Protein A

Protein A (encoded by *spa*) is a staphylococcal surface protein, whose collective actions may serve to conceal the organism from the innate immune system at the critical early time in infection, when bacterial cell numbers are low. Protein A contributes to *S. aureus* virulence, as observed by Patel *et al.*, (1987). As a result of *spa* function, the bacterium is thus coated with IgG in the orientation opposite to that required for IgG function. Thus, there is a direct inhibition of phagocytosis, which may aid in disguising the organism against the innate immune system, thereby preventing opsonization-dependent activation of the complement cascade (Wright III and Novick, 2003).

Section 1.2.2.1.2 Fibrinogen Binding Proteins

The fibrinogen binding proteins are known as clumping factors and are encoded by *clfA* and *clfB* genes. These are responsible for the well-known characteristic of bacterial clumping in the presence if human plasma. Clumping factor A and clumping factor B are structurally related but bind to different sites on the fibrinogen molecule and also promote binding to fibrinogen coated biomaterials (Hook and Foster, 2000; Wright III and Novick, 2003). Binding of clumping factor A to fibrinogen is inhibited by Ca^{2+} and exhibits binding characteristics akin to the platelet $\alpha_{IIb}\beta_3$ integrin (O`Connell *et al.*, 1998). Clumping factor A also binds to platelets and is a potent competitor of platelet fibrinogen binding and aggregation (Siboo *et al.*, 2001). Platelet aggregation, mediated by fibrinogen, stimulates the release of antimicrobial peptides. Inhibition of platelet aggregation by clumping factor A, is a potential means of avoiding these peptides. Clumping factor B is expressed primarily during the post-exponential phase of growth, whilst clumping factor A appears to be expressed throughout growth *in vitro* (Foster and Hook, 1998; Wright III and Novick, 2003).

Section 1.2.2.1.3 Fibronectin Binding Proteins

Fibronectin binding proteins, encoded by *fnbA* and *fnbB* genes mediate the binding of *S. aureus* to fibronectin. Fibronectin binding protein A, also binds fibrinogen. Fibronectin and fibrinogen are found predominantly in the extracellular matrix and are required fundamentally during wound healing. Also, they are abundantly absorbed to foreign surfaces, such as catheters and prosthetic devices. This implies that the fibronectin binding proteins may have a major role in the establishment of *S. aureus* following entry into the subcutaneous tissues or encounter with implanted foreign bodies (Wright III and Novick, 2003). Mongodin *et al.* (2002) assessed whether fibronectin-binding protein (FnBP)-deficient strains of *S. aureus* compared to FnBP-possessing strains, demonstrated differences in binding capacities to human airway epithelial cells (HAEC). The researchers found that FnBP-deficient strains showed a five-fold lower decrease in adherence when compared to undifferentiated HAEC.

Section 1.2.2.1.4 Collagen-binding protein

A collagen-binding protein (encoded by *cna*) has been shown to contribute towards virulence in *S. aureus* deep tissue infections, typically, osteomyelitis, keratitis and septic arthritis (Wright III and Novick, 2003). The *cna* gene is encoded on by a 'pathogenicity islet' and highest transcription is obtained during exponential growth (Gillaspy *et al.*, 1997). Thus, the presence of this gene confers adherence to collagen. As fibronectin can also bind to collagen, this may indicate that strains encoding *cna* may be able to bind collagen indirectly via a fnb-fibronectin bridge (Wright III and Novick, 2003).

Section 1.2.2.1.5 Capsule

The polysaccharide capsule in *S. aureus* is usually very thin, (<0.05μm) and referred to as a microcapsule. More than 90% of all clinical isolates in fact, usually produce a capsule. Eleven serotypes have been identified and these are antigenic. Serotypes 5 and 8 are the most common and together account for 80% of clinical isolates (Wright III and Novick, 2003).

Section 1.2.2.2 Secreted Proteins: Enzymes & Toxins

Staphylococcus aureus strains produce a large array of extracellular enzymes and toxins, most of which play a role in pathogenesis. These include a number of enzymes, such as coagulase, staphylokinase, proteases and lipases, as well as toxins, such as, alpha, beta and gamma haemolysins (Greenwood *et al.*, 1994; Projan and Novick *et al.*, 1997, Wright III and Novick, 2003).

Section 1.2.2.2.1 Coagulase

As referred to earlier on in this chapter (Table 1.1), coagulase (encoded by *coa* gene) is an important distinguishing feature of *S. aureus* from other types of staphylococci, (referred to as coagulase-negative staphylococci or CoNS). This enzyme binds to prothrombin forming staphylothrombin, which acts on fibrinogen converting it to fibrin (Kawabata *et al.*, 1985) In addition, coagulase binds to fibrinogen (it should be specified that coagulase is distinctive from clumping factor or fibrinogen-binding protein) and eight serotypes of this enzyme has been described. Coagulase is negatively controlled by the accessory gene regulator (*agr*). In wild-type bacteria, coagulase is produced in early exponential growth

phase but experiments have shown that *agr* as well as the staphylococcal accessory regulator (*sae*) may have a positive feedback on *coa* (Arvidson, 2000).

Section 1.2.2.2.2 Staphylokinase

Encoded by the *sak* gene, staphylokinase activates plasminogen and is positively regulated by *agr*. This indicates that staphylokinase is not produced at the same time as coagulase. Staphylokinase forms a 1:1 stoichiometric complex with plasmin, having a high plasminogen-activating activity (Arvidson, 2000). This enzyme is a potent activator of plasminogen, as well as being a strong thrombolytic agent. Essentially, *Sak* is not an enzyme but forms a 1:1 complex with plasmin that has high plasminogen-activating activity. Staphylokinase also binds to plasminogen but this complex is apparently inactive. Both coagulase and staphylokinase production are reciprocally regulated *in vitro*, with coagulase being produced early in the growth phase and staphylokinase post-exponentionally (Wright III and Novick, 2003).

Section 1.2.2.2.3 Extracellular Evasion Factors

Chemotaxis Inhibitory Protein or CHIP, is a small extracellular protein which interferes with the mobilization of PMNs and blocks the early activation of the C5a complement cascade. This protein is encoded by a common temperate phage which also variably encodes enterotoxin A and staphylokinase and insertionally inactivates *hlb* (Wright III and Novick, 2003). *Eap*, another secreted protein, has also been found to prevent neutrophil recruitment (Wright III and Novick, 2003).

Section 1.2.2.2.4 Proteases

Extracellular enzymes with proteolytic activity produced by *S. aureus* include a number of enzymes from three different families: serine-, metallo- and thiol (cysteine) proteases.

Serine protease (also known as the V8 protease), encoded by *SspA*, is one of the most important proteases in staphylococci. This protease can cleave the fibrinogen binding proteins and other staphylococcal adhesions, thus enabling spreading of the organism, once released from attachment sites (McGavin *et al.*, 1997). Protease activity on extracellular matrix components may generate fragments that bind these adhesions and interfere with secondary adhesion to the same substrates. The V8 protease can also cleave the heavy

chains of all human immunoglobulin classes, enabling inactivation of antibodies. In addition, it also cleaves human α1-proteinase inhibitor, which is the major inhibitor of elastase, released from polymorphonuclear neutrophils (PMNs) upon phagocytosis of invading microorganisms. Serine protease is efficiently inhibited by α2-macroglobulin, which is a protease inhibitor found in plasma. The uncontrolled activity of host elastase may contribute to tissue damage and degradation of proteins involved in host defense. *Staphylococcus aureus* produces a variety of other proteases, whose potential roles in pathogenesis are not well determined, however at least one has strong activity on elastin (Wright III and Novick, 2003).

Section 1.2.2.2.5 Lipases, Esterases and FAME

Three lipid esterases with different substrate specificities and a fatty acid monoesterifying enzyme (FAME) are secreted by *S. aureus*. *Geh* lipase (most known), is a serine esterase which can hydrolyze long-chain triaglycerols, shorter water-soluble triaglcyerols and tweens. Products of lipase action are long chain free fatty acids which impair the host immune system and are also bactericidal for *S. aureus*. FAME is a fatty acid monoesterifying enzyme that inactivates the bactericidal fatty acids produced in infected tissues by bacterial lipase action and directly by host cells, esterifying them to cholesterol. It has been found that FAME-producing strains are more virulent in a murine model of infection (Mortensen *et al.*, 1992). Lipases may promote interstitial spreading of the organisms and may also be important for bacterial nutrition (Wright III and Novick, 2003).

Section 1.2.2.2.6 Toxins

Section 1.2.2.2.6.1 Alpha-haemolysin

Alpha-haemolysin (encoded by the *hla* gene) expressed by *S. aureus*, has been the most studied of all the cytotoxins. The *agr* system controls the synthesis of the alpha-haemolysin toxin and this is synthesized during the late exponential phase of growth in a batch culture. Environmental factors may also play a role in *hla* expression. Alpha-haemolysin monomers bind with the membrane of a target cell, forming cylindrical heptamers. Hence, the oligomeric form of the protein enables lysis of eukaryotic cells. The majority of *S. aureus* strains produce alpha-toxin and this is both neurotoxic as well as dermonecrotic and was found to induce apoptosis (Dinges *et al.*, 2000; Menzies and Kourteva, 2000; Wright III and Novick, 2003). This exotoxin can be lethal to several cell types. Typically, these

include erythrocytes, mononuclear immune cells, epithelial and endothelial cells and platelets. Also, prostaglandin (and/or leukotriene) release can be initiated by *hla* (Bonach and Foster, 2000; Wright III and Novick, 2003).

Section 1.2.2.2.6.2 Beta-Haemolysin

Beta-haemolysin (also referred to as sphingomyelinase C) was first identified in 1935 and is encoded by *hlb*. This exotoxin is produced in high amounts, predominantly with animal isolates. Beta-haemolysin was found to have phosphorylase c activity, requiring the presence of Mg^{2+} and is limited with the specificity for sphingomyelin and lyso-phosphatidylcholine (Bonach and Foster, 2000; Dinges *et al.*, 2000; Wright III and Novick, 2003).

Section 1.2.2.2.6.3 Delta Haemolysin

Delta haemolysin (encoded by the *hld* gene) is a 26-amino acid amphiphilic peptide, encoded within the *agr* locus and produced by most other staphylococci, as well as *S. aureus*. Being heat stable, delta-haemolysin also has surfactant properties and is lytic for many types of membranes, including those of erythrocytes, organelles and even bacterial protoplasts, probably disrupting membranes by its surfactant action (Bonach and Foster, 2000; Wright III and Novick, 2003). As with alpha-haemolysin, delta-haemolysin is also controlled by *agr* and the highest expression in broth culture is found in the late-exponential (post-exponential) phase (Dinges *et al.*, 2000).

Section 1.2.2.2.6.4 Gamma Haemolysin and Leukocidin

Gamma haemolysin and Panton-Valentin leukocidin (PVL) are bicomponent toxins, essentially products of three genes, namely *hlgA* (LukS-like), *hlgC* (LukS-like) and *hlgB* (LukF). Hence, these combine to form two distinct synergohymenotropic bicomponent toxins: each composed of one LukS-like and one LukF subunit (Cooney *et al.*, 1993; Gillet *et al.*, 2002). More than 99% of clinical *S. aureus* isolates carry the *hlg* locus, which encodes a pair of these subunits that form gamma haemolysin. Only 2-5% encode a second LukS-like subunit that combines with the common LukF or a second LukF to form PVL (Wright III and Novick, 2003). Gamma-haemolysin is not identifiable on blood agar plates, due to the inhibitory effect of agar on toxin activity (Dinges *et al.*, 2000). The toxins affect

neutrophils and macrophages and gamma-haemolysin is additionally able to lyse many varieties of mammalian erythrocytes (Dinges *et al.*, 2000). PVL can stimulate and lyse neutrophils and macrophages and this has been associated with cutaneous infections and most recently has been implicated in a fulminant necrotizing pneumonia: all of the 16 isolates, thus far implicated in this syndrome are community-acquired MRSA (CA-MRSA) and incidentally are all in the *agr* group III (Bonach and Foster, 2000; Wright III and Novick, 2003). Boubaker *et al.* (2004) described the incidence of PVL-mediated skin infections in a school outbreak in Switzerland. Children affected, as well as family contacts were found to be nasal carriers of the implicated PVL *S. aureus* clone. In a retrospective study, Robert *et al.* (2005), found 12 French laboratories that possessed PVL-MRSA cases were less than 1% during 2000-2003. However 3/12 hospitals had rates of more than 2%.

Holmes *et al.* (2005) investigated the incidence of PVL genes in 515 isolates obtained from around England and Wales. A total of 1.6% had the PVL locus. PVL genes were detected in strains from EMRSA-16 (ST36) and EMRSA-15 (ST22). Morgan (2005) has reported that in addition to skin and soft tissue infections, which are rarely life-threatening, PVL-related *S. aureus* infection (which may be MSSA or MRSA) may include more serious clinical manifestations, such skin sepsis, leukocidin-purpura fulminans (mortality rate of 60%) and necrotising pneumonia (mortality rate of *ca.*75%). Garnier *et al.* (2006) described the occurrence of a new, PVL-positive CA-MRSA clone having the staphylococcal cassette chromosome (SCC) *mec* type V cassette in France which caused fatal necrotising pneumonia in a 59-year old woman with no previous significant medical history. Welinder-Olsson *et al.* (2008) reported the occurrence of PVL-positive MRSA present in skin infections, found in two previously-healthy males living in Sweden. These patients were found to be infected with the *spa*-type strain, t034 , thought to be of animal origin, however no animal contacts were found in either of these index cases.

Section 1.2.2.2.6.5 Toxins with Superantigen effects

Staphylococcus aureus strains may produce one or several exoproteins, possessing superantigen activity and these include: toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH and SEI), the exfoliative toxins (ETA and ETB) and leukocidin. They act primarily by preventing host immune responses to *S. aureus* in the host. The pyrogenic toxin superantigens or PTSAgs, include the staphylococcal enterotoxins, as well as TSST-1. TSST-1 was known previously

as staphylococcal pyrogenic exotoxin C and/or staphylococcal enterotoxin F (Dinges *et al.*, 2000). These cause staphylococcal toxinoses (diseases wholly attributable to a single toxin), including staphylococcal scarlet fever, food poisoning, exfoliative dermatitis (scalded skin syndrome) and toxin shock syndrome. PTSAgs are potent T-cell mitogens and bind to major histocompatibility complex class II (MHC II) proteins, which stabilize the interaction with CD4 T-cell receptor β-chains resulting in Th1 type response stimulating the proliferation and the secretion of various interleukins and cytokines. (Wright III and Novick, 2003).

Section 1.2.3: Regulatory Pathways in *Staphylococcus aureus*

Section 1.2.3.1: The *Agr* System

Staphylococci possess an *agr* locus, or an accessory gene regulator, which is a complex, polycistronic regulatory locus (Fig 1.1) and is the principle regulator of virulence-factor staphylococcal gene expression (Novick *et al.*, 1993). A peptide pheromone which is produced by the *Staphylococcus* and is encoded by *agr* is also responsible for the further activation of *agr*, by means of a quorum sensing system, which, once a threshold is reached, proceeds to upregulate production of certain toxins and proteases (Lindsay, 2000). As extracellular levels reach a threshold level or quorum, they are detected by the *agr* signal transduction system, activating the gene regulation response. It is known that peptides synthesized by various staphylococcal species and subspecies actually inhibit the single transduction step. These inhibitory peptides may possibly contribute to counteracting *agr*-induced pathogenesis, supplementing current antibiotic therapy (Lindsay, 2000). The *agr* quorum-sensing system is known to decrease the expression of a number of cell surface proteins and increases the expression of many secreted virulence factors in the transition from late-exponential growth to stationary phase *in vitro* (Yarwood and Schlievert, 2003).

The *agr* locus incorporates the P2 and P3 promoters which give rise to RNA II and RNA III transcripts, respectively. The P2 operon essentially encodes four proteins which initiate the *agr*-sensing mechanism. These include: *agrA*, *agrC*, *agrD* and *agrB*. The *agrB* is a transmembrane protein with a number of functions. Essentially, *agrB* is involved in processing the *agrD* into an octapeptide as well as producing and modifying accordingly, an autoinducing peptide (AIP), which in turn binds to *agrC*. The *agrA* and *agrC* form a bi-component system in which *agrC* (which is a transmembrane component) binds to extracellular AIP. AIP modulates the activity of *agrA* (intracellular) which is termed, a response regulator. During late exponential phase, *agr* expression is at its highest and *agrA*-mediated activity increases P2 and P3 transcription. It has been documented that differences in sequences of *agrB*, *agrD*, and *agrC* has given rise to at least four separate agr specificity groups. Indeed, the AIP of one group inhibits the expression of *agr* of the other groups. Levels of RNAIII are increased when P3 transcription is up-regulated (Yarwood and Schlievert, 2003). RNAIII encodes the *hld* gene which leads to the formation of the toxin δ-haemolysin. In addition, RNAIII is known to be involved in

increasing transcription and also in some cases translation of other virulence factors, such as TSST-1 and other haemolysins (Novick *et al.*, 1993; Recsei *et al.*, 1986).

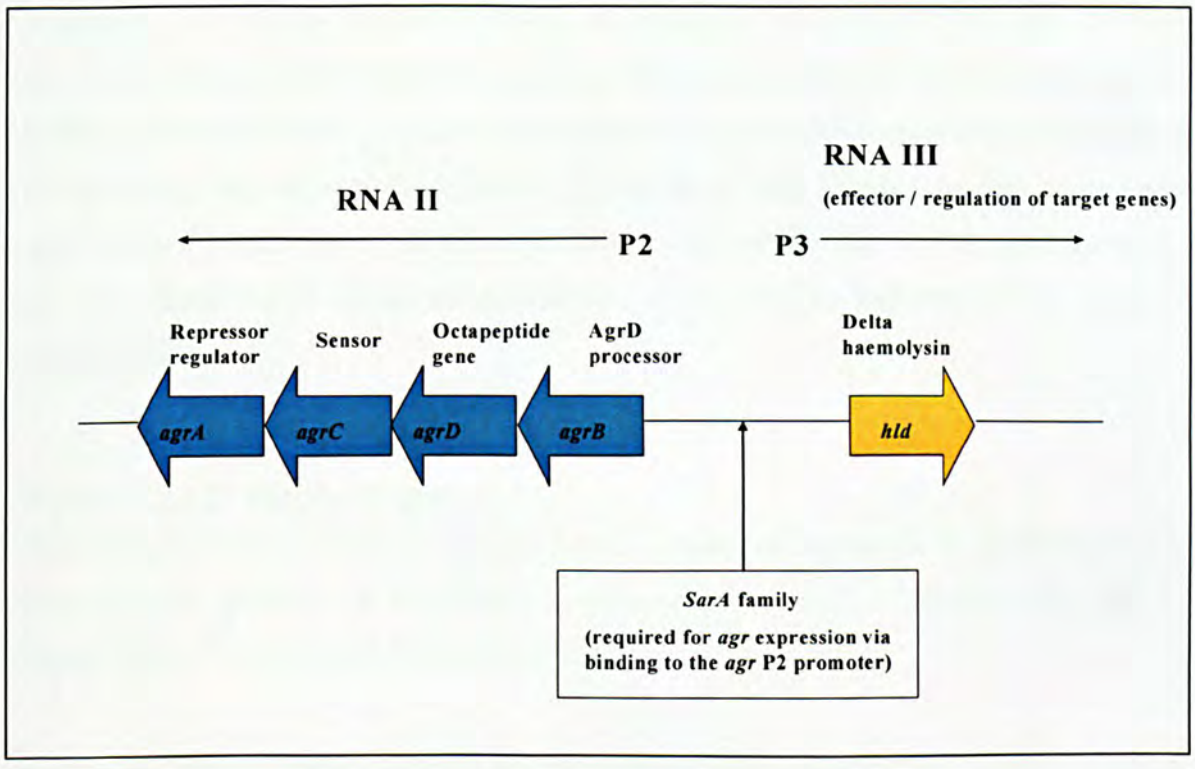

Fig 1.1: The *agr* divergent operon in *Staphylococcus aureus* (Adapted from Yarwood and Schlievert, 2003).

Elevated salt concentration is known to downregulate toxin production by a mechanism independent of *agr* (Chan and Foster, 1998a; Lindsay and Foster, 1999). Despite these laboratory studies, it is not known if these gene expression patterns also occur *in vivo* or whether they are laboratory artefact. It has also been suggested that the laboratory strains used for these experiments are not typical of those strains that cause disease. The *agr* or accessory gene regulator controls the synthesis of the alpha-haemolysin toxin and this is synthesized during the late exponential phase of growth in a batch culture. Environmental factors may also play a role in *agr* expression. Alpha-haemolysin monomers bind with the membrane of a target cell, forming cylindrical heptamers. Hence, the oligomeric form of the protein enables lysis of eukaryotic cells. The majority of *S. aureus* strains produce alpha-toxin and this is both neurotoxic as well as dermonecrotic (Dinges *et al.*, 2000; Wright III and Novick, 2003). *In vitro*, the expression of many of these factors is carefully regulated by external triggers such as quorum sensing and by salt concentration.

Kahl *et al.* (2003) found that the *agr* specificity group distribution of persistent *S. aureus* clones recovered from airways of CF patients were not any different from those of isolates recovered from various clinical infections (endocarditis, osteomyelitis, skin and soft tissue infections, device-related infections, chronic otitis media, pneumonia and central nervous system infection) as well as healthy nasal carriers. The *agr* group specificity was not found to be directly related with CF clones of *S. aureus* of both infected as well as colonised individuals. The success of CF clones in terms of co-colonization and/or infection with *S. aureus*, prevalence of clones, or persistence, appeared to be independent of *agr* group specificity.

Section 1.2.3.2: The *Sar* System

The staphylococcal accessory regulatory (*sar*) family of regulators in staphylococci are DNA-binding proteins. It has been documented that *sarA* is essential for full *agr* transcription (Yarwood and Schlievert, 2003).

Chan and Foster (1998b) created a *sarA* knockout strain and specifically investigated the impact of *sarA* on different virulence factors. The authors found that major proteases which are repressed by *sarA* include the V8 protease and a novel metalloprotease. In addition, *hla* is activated by *sarA*. Indeed, *sarA* was found to up-regulate both the *tst* gene as well as the staphylococcal enterotoxin B gene (*seb*) and this was confirmed by both transcriptional gene fusion and Western blotting.

Pratten *et al.* (2001) investigated the effect of *S. aureus agr* and *sar* mutants to surface binding. Results showed that the *sarA* mutant adhered better to glass than the wild type or *agrA* mutant. Increased adherence to fibronectin-coated glass was observed for all mutants and wild type strains.

Section 1.2.3.3: Additional regulatory *S. aureus* mechanisms

Isolates taken directly from CF patients suggest *S. aureus* can undergo a phenotypic switch to a more mucoid form. A genotypic switch to an SCV form induced by aminoglycosides has also been reported (Kahl *et al.*, 1998), and these mutants grow more slowly and secrete less alpha-haemolysin. SCVs appear as small colonies (or pin-point) when grown on solid media and normal *S. aureus* can also grow in the presence of SCVs (Seligman, 2006). They also exhibit a lack of pigmentation (Sadowska *et al.*, 2002).

Additional regulatory pathways in *S. aureus* include the repressor of toxins (*rot*) and alternative sigma factor B (Yarwood and Schlievert, 2003). Moisan *et al.* (2006) studied the transcriptional virulence factors of small colony variants (SCVs) from CF patients, as well as in a *hemB* mutant and a prototype *S. aureus* strain (Newbould). The researchers found that genes upregulated in patient SCVs, as well as the laboratory-derived *hemB* strain were involved in glycolysis and fermentation pathways. They all also found that *agr* was not activated in SCVs and in fact, there was decreased expression of *hla* (which is *agr*-dependent). In addition, *sigB* was activated in SCVs which upregulated genes such as capsular biosynthesis genes and surface-associated proteins. The authors postulated that there may be a possible role of intracellular persistence of SCVs in mammalian cells.

Other regulatory circuits include *sae*, *arl* and a series of proteins having homology to *sarA* (Said-Salim *et al.*, 2003). Said-Salim *et al.* (2003) investigated the transcriptional profile of a *rot agr* double mutant to that of its *agr* parental strain using microarrays. The authors found that *rot* has positive and negative effects on the expression of *S. aureus* genes. Also, *rot* and *agr* have opposite effects on specific genes. For instance, *hlb* (encodes β-haemolysin) and *geh* (encodes lipase) are repressed in the presence of *rot* whilst *agr* up-regulates expression of these genes. *Rot* up-regulates staphylococcal cell surface proteins such as *spa* (encodes Protein A). Hence, it was postulated that *rot* has a significant role in the initial infection process which involves the production of cell surface components.

Section 1.2.4: Small Colony Variants (SCVs)

One of the salient features of SCVs is the colony size, which appears as one-tenth of the original isogenic strain. SCVs occur in a wide range of bacterial species (not only with staphylococci) e.g. *Pseudomonas aeruginosa*, *Burkholderia cepacia* and coliforms (Proctor *et al.*, 2006). In general, SCVs isolated from clinical specimens include two main groups: SCVs exhibiting deficiencies in electron transport and SCVs that are deficient in thymidine synthesis. In addition to reduced colony size, general features observed in *S. aureus* SCVs include: decreased pigmentation and haemolytic activity and a reduction in coagulase production and respiration (Proctor *et al.*, 2006). On a microscopic level, *S. aureus* SCVs Gram-stains appear as pleomorphic cocci and electron microscopy revealed large cocci with incomplete or multiple cross-walls, indicating impaired cell-wall separation (Proctor *et al.*, 2006).

Sadowska *et al.* (2002) compared properties of *S. aureus* SCVs and their parent strains. The researchers suggested that the ability of granulocytes to uptake SCV *S. aureus* forms is decreased and the SCV susceptibility to the bactericidal action of cationic peptides is low. In addition to gentamicin, other positively charged bactericidal compounds, such as protamine, are able to select for SCVs. Increased salt concentration (similar to that present in CF patients) competitively inhibits cationic peptide activity and the inhibitory effect is dependent on the properties of the target microbe.

Samuelsen *et al.* (2005) investigated the effect that lactoferricin B (antimicrobial peptide) has on *S. aureus* SCVs. As indicated earlier, SCVs occur as different types which are divided into 2 major groups consisting of:

a) Electron-transport deficient SCVs which are haemin or menadione auxotrophs and
b) Thymidine-auxotrophs.

Bacterial metabolic processes rely on cytochromes and the electron transport chain for activities such as peptidoglycan synthesis, protein synthesis and amino acid transport. The absence of an electron transport chain also results in a reduction of the transmembrane potential which is needed for the action of antimicrobials such as aminoglycosides and this infers that such agents have limited effect on SCVs. Thymidine-auxotrophs result from long-term antibiotic therapy with trimethoprim/sulfamethoxazole. Sulphonamides and

trimethoprim ultimately inhibit thymidine and DNA synthesis by inhibiting specific reactions in the tetrahydrofolic acid pathway. These types of SCVs survive such antimicrobial therapy by obtaining thymidine directly from the environment. Samuelsen *et al.* (2005) concluded that all types of SCVs are resistant to lactoferricin B.

Section 1.2.5: Nasal Carriage

Staphylococcus aureus occurs asymptomatically in around 30% of people and is commonly sited in the anterior nares, but other carriage sites may be possible (Klutymans *et al.*, 1997). Nilsson and Ripa (2006) compared throat and anterior nares as *S. aureus* carriage sites and concluded that the throat proved to be a preferential carriage site to the anterior nares. Alternative colonisation sites may include mucous membranes e.g. pharynx, vagina and the perineum. Also, certain skin conditions such as eczema and psoriasis can contribute towards *S. aureus* colonisation (Polgreen and Herwaldt, 2004).

Section 1.2.6: Clinical Diseases caused by *S. aureus*

These can be divided primarily into two major classes which include superficial and deep-seated infections. Superficial infections include pustules, boils, carbuncles, impetigo, abscesses, styes, blepharitis, whitlow, sycosis barbae, conjunctivitis and wound infections. Deep-seated infections include septicaemia, endocarditis, pyaemia, pneumonia and osteomyelitis. *Staphylococcus aureus* infections may be pyogenic or toxin-mediated. Pyogenic infections may include both superficial as well as deep-seated infections. Typical pyogenic infections include boils, carbuncles, wound infections, abscesses, impetigo, mastitis, septicaemia, osteomyelitis and pneumonia. Toxin-mediated infections include scalded skin syndrome/Ritter's disease (exfoliatin-mediated infection), pemphigus neonatorum, toxic shock syndrome and food poisoning (Greenwood *et al.*, 1994).

Section 1.2.7: *Staphylococcus aureus* Genome Structure

The *S. aureus* genome essentially possesses a set of genes that form the backbone of all known *S. aureus* strains (Stephens *et al.*, 2006). Nine genome sequences have been produced for *S. aureus*. These include: N315 and Mu50 (VISA) and are hospital-acquired MRSA, MW2 is a community-acquired MRSA, MRSA252 is an epidemic hospital acquired strain (EMRSA-16), MSSA476 is a community-acquired meticillin-susceptible strain and COL is an early MRSA strain originally isolated in the 1960s and NCTC8325, a laboratory strain also has been elucidated. In addition, FPR3757, a representative from the USA300 strain, which is CA-MRSA, possessing PVL toxin and also RF122, a bovine *S. aureus* isolate, causing mastitis, have been sequenced (Lindsay and Holden, 2007). At least 75% of core genes are present in all the *S. aureus* strains. In addition to genes which are essential for growth and survival, there are virulence genes specific only to *S. aureus*. However, the occurrence of small-scale sequence genes may have a dramatic impact on gene expression and protein function (Lindsay and Holden, 2006).

Sequence variations in the core genomes are due to single nucleotide polymorphisms (SNPs). The effect of the SNP depends on its nature and position, and may only lead to a change in the amino acid encoded and giving rise to a mutant allele, if present as a non-synonymous substitution. Multilocus sequence typing (MLST) has been used to detect SNPs in core genomes (Lindsay and Holden, 2006).

The staphylococcal genome also incorporates an accessory genome which contains genes which code for functions such as virulence, drug and metal resistance to substrate utilisation. A large number of these regions present in the accessory genome are termed mobile genetic elements (MGEs). MGEs include bacteriophages, *S. aureus* Pathogenicity Islands (SaPIs), SCC, plasmids and transposons. These may be transferred horizontally between bacteria. Virulence genes have been observed to occur on phages and SaPI whilst resistance genes depend on SCC, plasmids and transposons for transfer. Bacterial horizontal gene transfer occurs via three mechanisms: conjugation, transduction and transformation. In *S. aureus* most horizontal gene transfer occurs via transduction with a bacteriophage (Lindsay and Holden, 2006).

Section 1.2.8: *Staphylococcus aureus* Typing Systems

A number of *S. aureus* typing systems are currently available for epidemiological purposes. Pulsed-field gel electrophoresis (PFGE), a whole genome typing method, is the most common technique used. MLST is based on specific gene sequences and though highly reproducible, requires highly skilled personnel to use properly and is still largely a research tool to date. Ribotyping, randomly amplified polymorphic DNA analysis (RAPD), as well as *spa* (Protein A) typing are other systems which may be utilised epidemiologically (Bannerman and Peacock, 2007).

MLST uses seven housekeeping genes, consisting of carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqil*). Forward and reverse primers designed from these housekeeping genes are used in a PCR, in order to generate amplified fragments. Sequences obtained are assigned allele numbers. For a single isolate, alleles at each of the seven loci define the allelic profile, corresponding to a sequence type (ST) and these in turn, form part of a clonal cluster, or CC (Enright *et al.*, 2000).

The European Union HARMONY project sought to establish a common European protocol for PFGE typing (Murchan *et al.*, 2003). As a means to investigate the best molecular techniques to monitor MRSA, Cookson *et al.* (2007) analysed representative MRSA isolates from 11 EU countries. The techniques used included: PFGE, MLST and

spa-typing, with PCR analysis of the staphylococcal chromosome cassette, containing the *mec* genes involved in meticillin resistance (SCC*mec*). Results showed that all the methods used provided a high level of discrimination but the need for a common strategic approach was evident. The authors concluded that ideally, strains should first be typed using PFGE and then isolates selected for clonal investigation by MLST and SCC*mec*. Alternatively, *spa* typing could be used instead of PFGE, however, *spa* investigates only a small region of the *S. aureus* chromosome and if recombination occurs between unrelated clones, this would not be detected with *spa* typing. PFGE and MLST target multiple sites on the genome. MLST and *spa* are portable systems and results can be compared to global databases.

Grundmann *et al.* (2002), used MLST, PFGE, RAPD and phage typing to investigate the discriminatory potential of these techniques when discerning different *S. aureus* strains obtained from human carriers. The authors found that PFGE showed the best ability to discriminate between strains, followed by MLST. RAPD, which is based on DNA fingerprints, generated from a single primer, also had a high discriminatory power, although less than PFGE and MLST. Phagetyping was found to be inferior to the DNA-based approaches. Melles *et al.* (2007) used MLST, PFGE and amplified fragment length polymorphism (AFLP) in order to type *S. aureus* isolates and investigate whether these typing methods yielded similar results. AFLP is essentially a whole genome typing technique but also takes into account, the 'accessory genetic elements' along with the genome-core polymorphisms. AFLP was found to distinguish all the strains and both AFLP and PFGE were found to have the highest discriminatory powers. The authors stated that MLST should not be advocated for the epidemiological spread of *S. aureus* strains within a limited time period.

Typing methods which detect MRSA using specific gene sequences, such as the RM test (Cockfield *et al.*, 2007) have been developed, in addition to commercially-available molecular-based MRSA identification techniques (Francois *et al.*, 2007).

Section 1.2.9: Emergence of MRSA/GISA

Between the 1980s and 1990s, the epidemic MRSA strains which appeared in England and Wales were EMRSA-1, EMRSA-15 and EMRSA-16. Blood culture MRSA positive isolates increased from 5% in 1991 to 42% in 2000, with higher proportions in infants (Khairulddin *et al.*, 2004). MRSA strains possess an extra Penicillin-Binding Protein (PBP 2`/PBP 2a) which is encoded by the *mecA* gene. The *mecA* gene is carried on a large section of DNA termed the staphylococcal cassette chromosome (SCC*mec*). This PBP 2` is a transpeptidase which functions by continuing to cross-link peptidoglycan, producing a strong cell wall, despite other PBPs (PBP1, PBP2, PBP3, PBP4) which become non-functionable due to inactivation by β-lactam antibiotics (Chambers, 1997; Livermore, 2000).

Wielders *et al.* (2002) compared MSSA and MRSA isolates originating from across the globe and results were in accordance with the idea of horizontal transfer as an essential mode of transfer of the *mecA* gene from MRSA to MSSA. β-lactam antibiotics target the transpeptidase domain (TP) of PBPs. These membrane-bound D,D-peptidases are related to serine proteases which are transpeptidases and transglycosylates, aiding in cross-linking the peptidoglycan cell wall and helping to preserve its integrity (Chambers, 1997; Mallorqui-Fernandez *et al.*, 2004). The *mecA* gene complex consists of *mecA* together with regulator genes, *mecI* and *mecR*, and is found in the genomic island, the staphylococcal cassette chromosome *mec* (SCC*mec*) that makes up 1-2% of the *S. aureus* genome (Deresinski, 2005).

EMRSA-16 is resistant to penicillin, erythromycin, kanamycin, neomycin and ciprofloxacin. It shows variable resistance to gentamicin and is still susceptible to tetracycline and streptomycin. Antibacterial agents which would still be effective against EMRSA-16 include rifampicin and fusidic acid, though combination therapy is cardinal to prevent emergence of resistance. EMRSA-15 strains are resistant to penicillin and ciprofloxacin. Some isolates may show erythromycin resistance. These two strains have been much more successful than earlier strains in worldwide dissemination but the exact reason has not been deciphered yet. The fact that EMRSA-15 and EMRSA-16 are both resistant to ciprofloxacin may be a contributing factor (Livermore, 2000).

Fluit *et al.* (2001) compared MSSA and MRSA isolates from the European SENTRY study and found that MRSA accounted for 25% of all the isolates and was observed to be more prevalent in southern Europe, primarily in intensive care departments. Countries with the highest MRSA levels included Portugal and Italy whilst the lowest were the Netherlands and Switzerland. In addition, only a small proportion of the MSSA isolates proved to be multi-drug resistant whilst the majority of MRSA isolates were indeed multi-drug resistant. The epidemiology of MRSA in Western Australia was explored by Dailey *et al.* (2005) from 1983 to 2002, after an initial MRSA outbreak in Perth in 1982. In the 1980s, the MRSA rate was 0.4%. MRSA rates in Western Australia rose greatly in 1989 from 14% to 94% in 1998. In 1998, a total of 6.4% MRSA notified cases were classified as EMRSA and this figure rose to 24.4% in 2002. Also, CA-MRSA in Western Australia overall, accounted for 94% of cases. A total of 97% of these isolates were SCC *mec* type IV positive whilst 3% were SCC*mec* type V. It was also observed that ciprofloxacin resistance increased during the study period, mainly due to increases in the cases of UK EMRSA-15 and EMRSA-16.

Pastila *et al.* (2004) described an MRSA epidemic due to E1 in the greater Helsinki region, Finland, which peaked from 1993 to 1995. Donnio *et al.* (2004) studied the possible differences between SCC types and antibiotic resistance for 14 MRSA strains, from a French hospital during the period, 1992-2002. The authors found that the resistance profile was associated with an SCC type. Essentially, 4 patterns corresponded to SCC type I or type I A and 9 to SCC type IV or IV A (one isolate was not typed). PFGE patterns showed that SCC type I or I A isolates belonged to a single lineage. This incorporated most epidemic clones which possess the SCC type IVA. However, isolates with SCC type IV or IVA were associated with different PFGE clusters. SCC type IV or IV A isolates were susceptible to more antibiotics when compared to type I or I A strains.

The occurrence of Glycopeptide-Intermediately Resistant *S. aureus* strains (GISA) has been documented in a number of countries, mainly Japan, USA and France and the mechanism of resistance resides in the thickened bacterial cell wall which prevents the glycopeptide molecules from finding their target site (Livermore, 2000). The mechanism of glycopeptide resistance involves the *vanA* operon (Walsh and Amyes, 2004). This leads to the formation of the precursor D-alanyl D-lactate, in contrast to the site of action for glycopeptides which is D-alanyl D-alanine. This modified precursor does not affect

peptidoglycan synthesis and its integrity, as the lactate moiety is lost during transpeptidation. A thick cell wall consistent with vancomycin intermediate susceptibility may imply that the bacteria are also resistant to disinfectants which include both chemical and physical methods (Ruef, 2004).

Song *et al.* (2004) screened 1,357 MRSA isolates from Asian countries and found that 4.3% were heterointermediately resistant to vancomycin (hVISA). Plipat *et al.* (2005) challenged 109 MRSA strains with increasing vancomycin concentrations and found that 74% of these strains developed unstable heteroresistance.

Section 1.2.10: Emergence of Community-acquired MRSA (CA-MRSA)

CA-MRSA has been documented to affect previously healthy adolescents and adults. CDC guidelines for diagnosis of a CA-MRSA positive patient state that only if the MRSA is isolated from an outpatient, or the MRSA positive culture results within 48 hours of hospital admission, can this be termed CA-MRSA. The patient who has CA-MRSA should not have been hospitalised (or had surgical procedures and/or dialysis) a year prior to infection and should not live in a nursing home, or a caring institution (Rybak and LaPlante, 2005). During the period 2000-2002, Zindermann *et al.* (2004) reported an outbreak of CA-MRSA in military recruits. CA-MRSA has also been found in soccer teams (Huijsdens *et al.*, 2006).

Vandenesch *et al.* (2003) investigated the occurrence of genetic markers in 117 worldwide CA-MRSA isolates. Results showed that a type IV SCC*mec* cassette featured in all the CA-MRSA strains, as well as the PVL locus. Other toxin genes varied according to the region of origin. PVL genes have been found in almost all CA-MRSA strains, causing worldwide epidemic furunculosis (Zetola *et al.*, 2005).

O'Donoghue and Boost (2004) investigated MRSA in the community in Hong Kong. Nasal MRSA carriage proved to be 1.4% and there was an association with healthcare environment. In addition, strains isolated from healthcare workers were multi-drug resistant whilst strains from non-hospital workers were only meticillin-resistant.

Buck *et al.* (2005) reported on the characteristics of CA-MRSA and skin and soft tissue infections (SSTIs) in Minnesota from 2000-2003. From a total of 730 isolates, 79% were due to SSTIs, whilst invasive disease amounted to 9%. Patient factors such as smoking, immunosuppressive therapy, emphysema or injected drug use contributed to invasive disease. These patients may have had additional healthcare exposures to MRSA which may have indeed placed them at risk of developing HA-MRSA. Invasive isolates also demonstrated less sensitivity to antimicrobial agents such as clindamycin and ciprofloxacin.

Chavez-Bueno *et al.* (2005) investigated the clindamycin resistance in CA-MRSA isolates of paediatric cases from 1999 to 2002 in Dallas, Texas. Isolates tested were erythromycin-resistant and clindamycin-susceptible. Results showed that inducible clindamycin resistance was observed in 93% of isolates tested in 1999, 64% of isolates in 2000, 23% of isolates in 2001 and only 7% in 2002. Non-inducible strains possessed the *msr* (A) gene. The authors concluded that a clonal shift was obtained in the CA-MRSA strains, phenotype was associated with their sequence type and inducible methylase resistance decreased during the study period.

Furuno *et al.* (2005) conducted a study in Baltimore, USA which examined the MRSA and vancomycin-resistant enterococci (VRE) co-colonisation rate. Cultures were obtained from patients during a 2-year interval, in medical and surgical care units of a tertiary-care hospital (with 29 beds in total). A total of 2.7% patients were found to be co-colonised. Factors contributing to co-colonisation included male sex, age, admission to medical intensive care unit and having antimicrobial therapy up to a year before, during a previous admission. This finding is very interesting as the only VRSA reported until now are known to possess the *van*A gene which is believed to have been acquired by conjugation from a VRE to an MRSA.

Van Griethuysen *et al.* (2003) reported 7.6% of MRSA isolates, originating from the Netherlands, to have decreased susceptibility to glycopeptides when tested with E Test technology. Howe *et al.* (2004) tested 101 MRSA worldwide isolates for vancomycin susceptibility. Results showed that vancomycin reduced susceptibility was found in many successful epidemic lineages (including UK EMRSA-3,15,16), however no clear clonal pattern emerged.

Section 1.2.11: MRSA in Animals

It has been documented recently that MRSA can occur in animals and transmission between animals and man is possible. Baptiste *et al.* (2005) found that dogs may act as reservoirs for MRSA. Indeed, this implies a possible risk for owners and veterinary staff who come into contact with infected animals, implying that MRSA can also be a zoonotic disease. Voss *et al.* (2005) investigated the incidence of MRSA in pig farmers in the Netherlands and found this to be >760 times the rate of patients admitted to Dutch hospitals. A study by van Leeuwen *et al.* (2005) showed that *S. aureus* invasive infections occurring in pet animals and humans usually have a common genetic background. In 2007, it was reported that an individual working closely with cows possessed the same strain of MRSA present in a number of these cows with subclinical mastitis (Juhász-Kaszanyitzky *et al.*, 2007). In addition, a non-typeable MRSA strain emerged in the Netherlands in 2003 and van Loo *et al.* (2007) confirmed that this strain originated from an animal population. This strain, ST 398 (according to MLST), was responsible for more than 20% of MRSA found in the Netherlands (van Loo *et al.*, 2007).

SECTION 1.3: CYSTIC FIBROSIS

Until the start of 20th century, cystic fibrosis (CF) was not recognized as a disease. The various symptoms exhibited by CF patients were merely seen as separate, unrelated incidents. In 1912, an English physician, Archibald Garrod, noted the occurrence of steatorrhoea in several individuals, belonging to the same family. This term refers to the occurrence of fatty stools and this feature was undoubtedly an effect of CF (Harris and Super, 1995).

Following this finding, Guido Fanconi, a Swiss paediatrician, described children with CF in 1928 and again in 1936. He also clearly distinguished CF from coeliac disease. In 1938, Dorothy Anderson in Baltimore published her observations, giving an almost complete account of CF, with an additional paper in 1946, describing the treatment of this disease with a high-calorie, high-protein, low-fat diet, with pancreatin supplementation. In 1948, Anderson and Paul Di Sant`Agnese also noted that CF patients were particularly susceptible to chest infections caused by staphylococci (Harris and Super, 1995).

Following the 1948 heatwave in New York, Di Sant`Agnese noted that the majority of children brought to casualty departments with heat prostration were in fact CF patients. Di Sant`Agnese observed that CF patients exhibited greatly elevated levels of sodium and chloride in their sweat which persisted, even after the heat wave was over (Davis, 2006). Elevated sodium and chloride levels have become the gold standard for diagnosing CF. Consequently, physiologists Gibson and Cooke, recognized the fact that a standard technique for this diagnosis was essential. They formulated a procedure for collecting sweat for CF testing and described a method of stimulating sweat production and collection (Harris and Super, 1995).

Cystic fibrosis is a pleiotropic, autosomal recessive disorder of the exocrine glands, affecting a number of organ systems which include the pancreas, intestine and the respiratory tract (Choi *et al.*, 2001). Clinical signs and symptoms are varied (Larson and Cohen, 2005) and complications such as chronic obstructive pulmonary disease (COPD) and exocrine pancreatic insufficiency result. This disease is typified by an abnormality in the sweat chloride concentration (Merck, 2005).

It is the most common genetic disease in Caucasians, with a frequency of 1 in 2500 live births. The carrier rate in this population would be 4% or 10 million people worldwide (Rajan and Saiman, 2002; Ratjen and Döring, 2003). Fig 1.2 illustrates CF genetic inheritance.

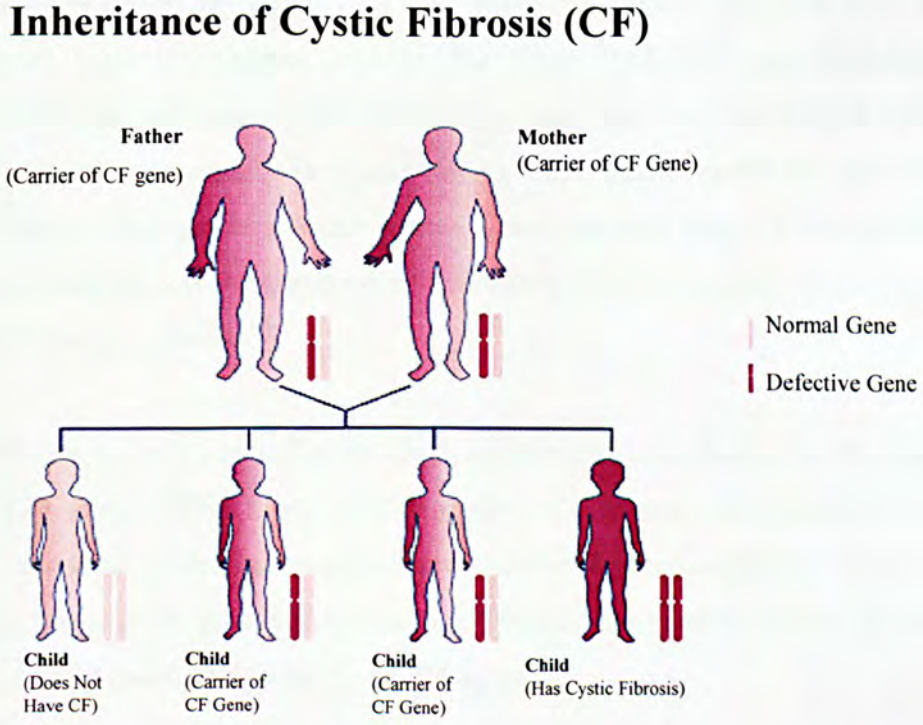

Fig 1.2: Model showing genetic inheritance of cystic fibrosis (Reproduced from http://www.nhlbi.nih.gov).

Due to CF, epithelial cell ion transport abnormalities result. These in turn, lead to pancreatic insufficiency and chronic, progressive, pulmonary disease, characterized by early onset of airway inflammation and infection. The median estimated age of affected

individuals has increased over the years. In the 1960s, a child with CF lived to *circa* 10 years. This has more than trebled and reached *ca* 32 years currently (McColley, 2004).

The increase in survival may stem from a series of factors which mainly include: (McColley, 2004)

- Treatment in specialist care centres,
- Changing food patterns to a widespread adoption of a liberal fat diet,
- Improved physiotherapy techniques for airway clearance,
- Targeted antibiotic therapy for chronic pulmonary infection and resultant exacerbations of infections.

Progressive pulmonary disease leading to respiratory failure remains the cause of death in the overwhelming majority of people with CF (McColley, 2004). Survival rates of CF patients, obtained from US sources showed that from 1985-1999, the mortality rate decreased to 61% for patients aged 2 to 5, 70% for patients aged 6-10 and 45% for patients aged 11 to 15. However, mortality rates had only a slight improvement for patients >15 years of age. While both genders demonstrated better survival rates, it was noted that female survival remained consistently poorer than male survival between 2 and 20 years of age (Goss and Rosenfeld, 2004).

Essentially, CF results from a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR is an ATP-dependent Cl^- channel that mediates cAMP-mediated Cl^- secretion in apical membranes of epithelia, predominantly those in the pancreas, lungs, testis, sweat glands and intestines (Hodson and Geddes, 2000; Galietta and Moran, 2004). Fig 1.3 overleaf depicts the CFTR channel.

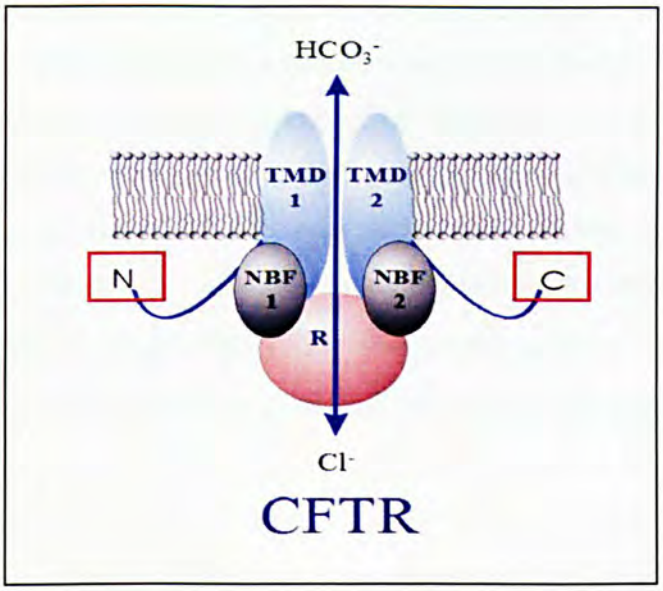

Fig 1.3: Schematic diagram of the CFTR channel. This figure is representative of the CFTR protein channel, having 2 transmembrane domains (TMD), 2 nucleotide-binding fractions (NBF) connected to nitrogen (N) and carbon (C) termini and a central regulatory centre (R) (Reproduced from http://www. stanford.edu).

The CFTR gene forms part of the ABC transporter supergene family. The ATP Binding Cassette (ABC) transporters perform essential functions such as nutrient uptake, toxin elimination and cell-to-cell communication in eukaryotes and bacteria. These transporters are responsible for the ATP-dependent transport of an exceptionally diverse set of solutes across biological membranes, such as sugars, amino acids, lipids, proteins, chromophores and drugs (Dorwart *et al.*, 2004).

The CFTR gene was discovered in 1989 and encodes a 1480 amino-acid protein. CFTR protein is encoded by a large *circa* 250 kB gene found on the long arm of chromosome 7 and more than 1,200 mutations of this protein have been described. The most common mutation is the ΔF508 (Class 2), caused by a deletion of phenylalanine at position 508 (Kumar and Clarke, 1994; Hodson and Geddes, 2000; McKone and Aiken, 2004). This mutation accounts for almost 70% of disease-causing alleles (McKone and Aiken, 2004). It is recorded in literature that 82% of CF patients have the F508del in Denmark and only 32% of the CF population in Turkey (Ratjen and Döring, 2003). The association between CFTR genotype and phenotype is not easily defined; only a minority of CFTR mutations

are related to a milder clinical phenotype, usually due to preservation of pancreatic function. These phenotypic variations might result from the mutation effect on the CFTR gene. Very low levels of CFTR protein would be associated with a severe phenotype whilst intermediate levels, would be have a milder effect (McKone and Aiken, 2004). Indeed, Smith and Kirk (2005) reported a case of a 39-year old woman who was diagnosed with CF at 5 years of age, developed a lower respiratory tract infection at 10 years but since then was never treated for her CF. This female patient desired to clarify her CF status and her genotype was found to be ΔF508(9T)/5T. This genotype is less severe than the overt ΔF508 and has been associated with a number of clinical phenotypes but no clinical features.

As a result of mutations in the CFTR gene, recurrent lung infections occur. In essence, these infections may be facilitated due to decreased killing and bacterial clearance as well as an intrinsic hyperinflammation (Starner and McCray, 2005). Table 1.3 lists different CFTR mutation types which may occur.

Table 1.3: Mutation Classes of CFTR protein (Adapted from: Ratjen and Döring, 2003; McKone and Aiken, 2004; Rowe *et al.*, 2005)

TYPE OF MUTATION	DESCRIPTION
Class 1	Mutations altering the *production* of the protein. These mutations result in the total or partial absence of the protein.
Class 2	Mutations altering cellular *maturation* of the protein. Most common due to ΔF508.
Class 3	Mutations disturbing the *regulation* of Cl^- channel. These mutations are frequently situated in the ATP-binding domain.
Class 4	Mutations altering *conduction* of Cl^- channel.
Class 5	Mutations altering *stability of mRNA*.
Class 6	Mutations altering the *stability of mature CFTR protein*.

Staphylococcus aureus is one of the first bacteria to infect CF patients, followed by others such as *Pseudomonas aeruginosa* and *Burkholderia* spp (Webb *et al.*, 1998; Ratjen, 2001; Heijerman, 2005; Davies, 2006). Emerging bacterial pathogens include *Stenotrophomonas maltophilia* and *Alcaligenes xylosoxidans* (Hutchinson and Govan, 1999; Hutchinson *et al.*, 2000; Chmiel and Davies, 2003).

Section 1.3.1: Alterations exhibited in airway structures due to CF disease

The respiratory tract mucosa is covered by a liquid layer referred to as the airway surface liquid (ASL). The ASL consists of the periciliary layer (PCL) and a mucus, gel-like layer. The PCL forms the liquid layer, surrounding the cilia between the cell surface and the mucus layer. The mucus is in turn positioned between the PCL and the airway lumen (Perez-Vilar and Boucher, 2004). Mucus acts as both a physical and chemical barrier, promoting attachment of particles and organisms. A fundamental function of the mucus layer is to capture inhaled particles during the clearance process from the airways. Since the mucus exhibits turbulent flow, particulate matter is incorporated into the mucus and hence trapped during clearance. In addition, mucin molecules possess carbohydrate epitopes which ensure low affinity binding to most particles. Also, mucus may act as a reservoir by storing and releasing liquid (Boucher, 2004).

Normal ASL is hypotonic and contains antimicrobial peptides and defensins. Defensins are synthesized by human neutrophils on skin as well as various mucosal epithelia (Peschel and Collins, 2001). These are cationic peptides which disrupt microbial membranes (Chilvers and O'Callaghan, 2000). It has been noted that epithelial cells produce β-defensins (types 1 and 2) (Rastogi *et al.*, 2001). However, these are inactive when salt concentration increases, as in CF. Dysfunction of CFTR results in hypertonicity of ASL This, in turn, may have an impact on mucociliary defenses, inactivating these antimicrobial peptides and facilitating microbial infections (Chilvers and O'Callaghan, 2000). The PCL is thought to lack mucin, whilst the mucus layer contains soluble compounds which are mixed with mucin polymers and aggregates of mucins and other high molecular-weight glycoproteins. Foreign bodies and particles become trapped in the ASL, where the action of cilia helps in removing them from the airway surfaces. The occurrence of mucus plugs, containing mucins, as well as bacteria and polymorphonuclear cells, blocking the lower

airways in the lung of CF patients may suggest that synthesis of 'abnormal' mucins is a critical characteristic of this disease (Perez-Vilar and Boucher, 2004).

Submucosal glands are present under the surface lung epithelium and consist of a large number of branching secretory tubules that terminate in a collecting duct, which leads into a ciliated duct that opens into the airway surface. These secretory tubules are composed of two main secretory cells which include mucous cells, containing mucin-filled granules as well as serous cells. The latter possess electron-dense secretory granules which may be implicated in glandular liquid and ion secretion (Inglis and Wilson, 2005). The submucosal glands produce other components, in addition to mucin. These include protease inhibitors, secretory IgA, lysozyme, lactoferrin, and peroxidase. The serous cells present in the submucosal glands are considered to be involved in primary defence mechanisms in the airway and exhibit a high degree of CFTR expression. The occurrence of CFTR in secretory granules of serous cells suggests that CFTR contributes to the secretion of glycoproteins. Absence of CFTR may result in changes to the macromolecular composition of the serous cell secretions, affecting mucus viscosity and possibly mucociliary clearance (Terheggen-Lagro *et al.*, 2005).

Blockage of the exocrine glands by copious amounts of mucus is the principle reason for morbidity and mortality in CF patients. Sticky mucus blocks the distal airways and submucosal glands, which express CFTR. Glandular ductular dilation occurs as a result and infiltration of neutrophils also contributes to the features seen in CF. In addition, glandular hyperplasia in submucusal regions is a characteristic feature together with peribronchiolar inflammation and scar tissue (Rowe *et al.*, 2005)

Airway cells may contain cilia which beat in an aqueous environment termed 'sol', originating from the lung. The alveolar volume is much larger than the airway surface volume and the airway epithelial cells have been postulated to absorb a large proportion of the fluid. Sodium is absorbed through the apical membrane and the Na-K ATPase co-transporter pumps out the sodium from the cell, water following by osmosis. A similar process is thought to occur in sweat glands (Greening, 2000).

The CFTR regulates both the epithelial sodium channel as well as the Outward Rectifying Chloride Channel (ORCC). A direct result of a malfunctioning or absent CFTR in the airway epithelium produces an increase in the sodium and water influx and a stunted outward flow of chloride and water. These effects facilitate adherence and hence colonisation of pathogens, as the ambient becomes dehydrated (Greening, 2000).

Studies have indicated that CF lung disease progression is characterized by an early and a later phase. In the early phase, there is increased dehydration in the ASL, accompanied by altered pH and levels of glutathione. The later phase is characterized by bacterial infection and neutrophil invasion, leading to increased ASL concentrations of myeloperoxidase and hypochlorous acid. Both *in vivo* data and studies with normal and CF bronchial epithelial cells differentiated *in vitro* suggest a reduction of pH between 0.3 and 0.7 units in CF mucus. This implies a change in pH from the *circa* pH of 7.2 in normal mucus (Perez-Vilar and Boucher, 2004).

A review by Chmiel and Davis (2003) described the environment present in the initial stages in CF lung infection. Levels of the antibacterial molecule nitric oxide (NO) are decreased in CF. In addition, there is increased asialo GM_1 molecules which act as binding sites for bacteria. Once bacteria invade, production of virulence factors coupled with the biofilm environment encourage proliferation. An exacerbated inflammatory response due to infection contributes towards the persistence of infection. Elevated levels of IL-8 and leukotriene B_4 trigger neutrophil infiltration which result in the secretion of mediators such as elastase. This inhibits host defences, prevents active opsonophagocytosis, promotes mucociliary stasis and facilitates increased damage to airway structures.

Diagnosis of CF can be supplemented by genotyping of the most common CFTR mutations. These vary with different geographical locations. CF sputum has been found to contain increased amounts of iron and ferritin, and iron-regulatory cytokines such as IL-1ß and TNF-α, as well as elevated microalbumin levels. Indeed, Reid *et al.* (2004) showed that iron availability in CF is essential to *P. aeruginosa* and established that iron, as well as iron-regulatory cytokines are important in CF lung disease. Adult CF patients were assessed during an acute exacerbation and analysed repeatedly after 12 days of antibiotic treatment. These were compared to stable CF and COPD patients, as well as normal individuals.

Dysfunction of CFTR in CF may affect neutrophils. Neutrophil functions such as cytokine production, migration, phagocytosis and apoptosis indeed seem to be altered in CF (Terheggen-Lagro *et al.*, 2005). Certain cytokines and inflammatory mediators are known to be involved in the pathogenesis of CF lung lesions. Wolter *et al.* (1999) selected Interleukin-8 (IL-8), tumour necrosis factor alpha (TNF-α), neutrophil elastase-α-1-protease inhibitor complex (NE complex), protein and α-1-protease inhibitor (α-1-PI) and measured the concentrations in CF serum and sputum. Samples were collected from patients during both a period of well-being as well as during respiratory exacerbations. Results showed that protein, NE complex and α-1-PI levels increased in sputum during respiratory exacerbations, but decreased when antibiotics were started. IL-8 and TNF-α levels were very high in sputum but were not affected by the CF clinical status. In serum, IL-8 and TNF-α levels were largely undetectable. The authors concluded that there is a high degree of immunological activity in adult CF lungs but measuring sputum levels of cytokines and inflammatory mediators does aid identifying acute respiratory phases (Wolter *et al.*, 1999). Simpson *et al.* (1999) investigated the effect of elafin on *P. aeruginosa* and *S. aureus*. Elafin is present in the lung and produced by neutrophils. It is an elastase-specific inhibitor with antimicrobial effects. The authors showed that 2.5μM of elafin was sufficient to kill 93% of *P. aeruginosa* whilst 25 μM was required to kill 48% of *S. aureus* organisms. McMichael *et al.* (2005) cultured mouse tracheal epithelial cells and when an adenovirus-mediated gene transfer system was used with increased expression of elafin, this reduced *S. aureus* load. In addition, the authors found that the elafin decreased neutrophils and bronchoalveolar lavage levels of myeloperoxidase (MPO) and tumour necrosis factor.

It has been hypothesised that polymorphonuclear cells (PMN) which enter the lumen of the infected airways undergo activation and release toxic oxygen metabolites. Myeloperoxidase (MPO), an enzyme which transforms H_2O_2 into highly toxic oxygen metabolites, has been detected in CF patients. Worlitzsch *et al.* (1998) compared H_2O_2 levels in breath condensates of 63 CF patients from six German CF clinics and 51 normal individuals. They demonstrated that H_2O_2 levels were similar in CF patients and normal subjects. They also detected concentrations and activities of catalase (CAT) and MPO in 38 CF sputa. The addition of hydrogen peroxide to *in vitro* cells pre-incubated with CF

sputum did not induce cytotoxicity even when CAT was removed from sputum. Also, sputum MPO, in the presence of H_2O_2 failed to inactivate α1-proteinase inhibitor. However, pre-incubation of MPO with sulphated glycoconjugates or DNA totally inhibited its cytotoxic effect. Finally, the authors concluded catalase, sulphated glycoconjugates and DNA may prevent MPO-mediated oxygen radical generation in CF sputum.

Fig 1.4 below represents the key differences between a healthy lung epithelial cell cell and a CF-affected lung epithelial cell.

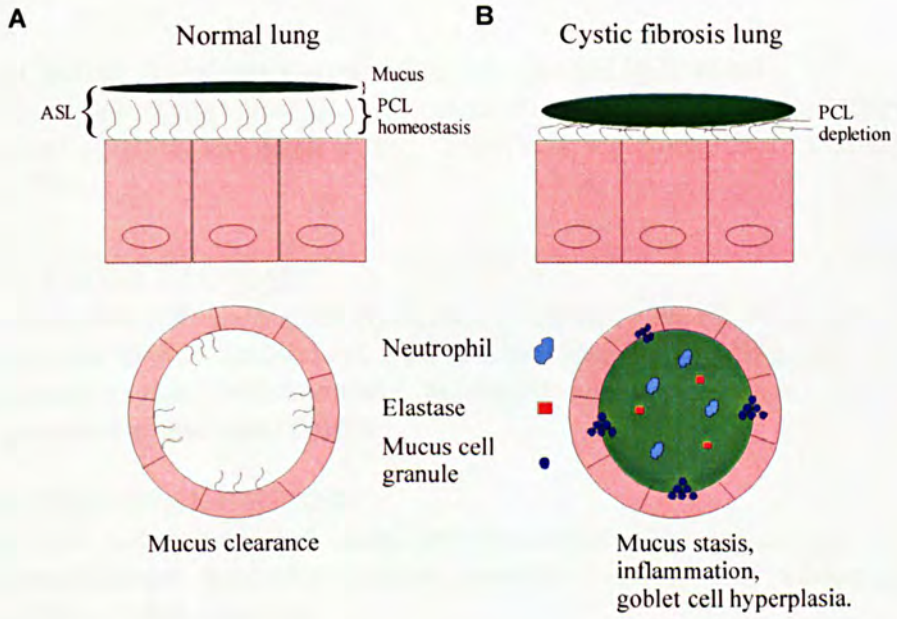

Fig 1.4: Diagrammatic representation of a normal, healthy epithelial lung cell (A) vs a cystic fibrosis lung epithelial cell (B). In (A) PCL homeostasis is apparent with clear airway and without mucus accumulation. In (B), there is PCL depletion and inflammatory mediators are present, together with increased neutrophils and copious amounts of mucus, resulting in airway blockade. ASL: airway surface epithelium; PCL: periciliary layer (Adapted from http://www. nature.com).

Section 1.3.2 Clinical Manifestations of CF

A clinical diagnosis of CF is still based on the observations of Di Sant`Agnese *et al.* (1953). A sweat chloride concentration of <40mmol/L is considered normal and further investigations are initiated only in the presence of CF symptoms. Typically, a sweat chloride concentration of >60mmol/L on repeated analysis, is clearly diagnostic of CF. However, up to 5% of cases can result as false negatives. A diagnosis of CF can be supplemented by genotyping of the most common CFTR mutations. These vary with geographical locations. When the range falls within 40-60mmol/L, genotyping is the best method to assess whether the patient has CF (Ratjen and Döring, 2003). Table 1.4 describes the clinical manifestations of CF disease.

Table 1.4: Clinical Manifestations of CF in an affected individual

Clinical signs and symptoms of CF, predominantly affecting the respiratory and gastrointestinal systems, are listed in this table (Adapted from Ratjen and Döring, 2003; Solomon, 2006).

CHRONIC AIRWAY DISEASE Airway colonisation with pathogens (typically, *S. aureus*, mucoid *P. aeruginosa*), chronic productive cough, airway obstruction, recurrent/persistent pneumonia, recurrent/persistent wheezing, haemoptysis, bronchiectasis, persistent abnormalities on chest radiograph, clubbing, pansinusitis and nasal polyps.
GASTROINTESTINAL DISEASE Meconium ileus, failure to thrive, distal intestinal obstruction syndrome, rectal prolapse, pancreatic insufficiency, pancreatitis, biliary cirrhosis, oedema with hypoproteinaemia and deficiency of fat-soluble vitamins.

SECTION 1.4: RESPIRATORY INFECTIONS IN CF PATIENTS

Pulmonary disease is a widely recognised cardinal complication in CF. At birth, the lungs appear normal but with the acquisition of bacterial invaders and subsequent infection, eradication becomes very difficult. It has been recorded that during the first year of life different bacteria, even enteric pathogens, may be present but as the child develops, two main organisms prevail i.e. *S. aureus* and *P. aeruginosa*. *Staphylococcus aureus* seems to be present and then is eliminated but at times may re-appear. At present, MRSA is being commonly isolated in CF centres. *Pseudomonas aeruginosa* however becomes the key player, as it is the one which persists until adulthood and may be present in CF lungs up to 80% of patients (Nelson, 2005).

Moore *et al.* (2005) investigated the association between numbers of culturable microbes from the lungs of CF patients and microbial loading. The authors also examined qualitative combinations of the microflora present in an Irish CF adult centre, in order to explore possible ecological interactions between the different microbial flora. From a cohort of 34 patients, examination of sputum samples revealed that these patients possessed between 1 to 3 taxa, 53% of patients were colonised by one organism, 38% by two and 4% by three organisms in their sputum. On comparing microbial cell density to number of taxa present, the authors failed to find the data statistically significant. Nonetheless, patients with a single Gram-negative infection exhibited significantly higher cell log counts when compared to those with mixed Gram-negative and Gram-positive infections. A number of hypotheses have been put forward, relating CF and progressive lung disease and these are included in Table 1.5 overleaf.

Table 1.5: Hypotheses linking mutations in CFTR to respiratory infections

HYPOTHESIS	DETAILS
Inflammation-First Hypothesis	Inflammation is present in airways of a CF patient in the first months of life before infection (Ratjen and Döring, 2003). Donaldson and Boucher (2003) however suggest that CF lung is sterile and uninflamed at birth and remains so until acquisition of infection. Once infection starts the CF inflammatory response is very intense. Despite this, infection persists. In addition, serial infections may give rise to cell biologic adaptations, such as expansion of endoplasmic reticulum which promotes a more heightened inflammatory response.
Cell-Receptor Hypothesis	CF organelles have a different pH to normal cells, resulting in decreased sialysation of glycoconjugates on CF epithelial cell membranes. Normal CFTR binds *P. aeruginosa* and destroys it. However mutated CFTR is unable to perform this function and the pathogen is left free to multiply (Ratjen and Döring, 2003). A tetrasaccharide present on asialoGM_1 molecules (a receptor for many bacterial respiratory pathogens) has been reported on CF apical epithelial cells- this is responsible for increased binding of bacteria such as *P. aeruginosa* and *S. aureus* on these cells (Imundo *et al.*, 1995; Ratjen and Döring, 2003).
Salt-Defensin Hypothesis	Hypothesis based on assumption that CF airways epithelial cells have very similar properties to sweat glands: i.e. increased luminal salt concentration. Defensins are antimicrobial host defence proteins which are inactivated by salt concentrations >50mmol/L. Thus, bacteria can freely multiply on respiratory epithelial cell surfaces of CF patients, leading to infections (Ratjen and Döring, 2003).
Isotonic Fluid Depletion/Anoxic Mucus Hypothesis	Abnormal absorption of sodium from airway lumen together with malfunction of CFTR which results in failure to secrete Cl^-, leads to water/volume depleted periciliary liquid. Mucus viscosity is increased as water loss increases, preventing mucociliary clearance. The resulting environment, with microaerophilic conditions and a thick mucus plug, enhances bacterial growth and favours development of mucoidal cell-types (Ratjen and Döring, 2003; Heijerman, 2005).

Micro-organisms which contribute largely to secondary infection in CF lung disease include respiratory viruses, typically respiratory syncytial virus and influenza virus, as well as *Haemophilus influenzae* and *Aspergillus fumigatus*, *Mycobacterium* spp., (not *Mycobacterium tuberculosis*), *Stenotrophomonas maltophilia* and *Alcaligenes*

xylosoxidans. In essence, this plethora of invading pathogens may occur as a direct result of the increasing life span of CF patients as well as the excessive use of antibiotics (Miller and Gilligan, 2003).

Moore *et al.* (2004) analysed sputum from 138 adult CF patients. The authors found that CF patients are able to produce vast numbers of bacteria in sputum, reaching levels of 10^9 organisms daily for a patient. Bacteria isolated included also *S. aureus* at a rate of 18.1%. This indicated that indeed CF patients may actually act as a risk for other patients and the importance of personal hygiene measures should be ensured.

Section 1.4.1: *Staphylococcus aureus* respiratory infections in CF patients

A review by Rajan and Saiman (2002) stated that though *S. aureus* and *Haemophilus influenzae* are the most common bacteria to be isolated during the first decade of life in a CF patient, *P. aeruginosa* may indeed be the first pathogen to be isolated from infants.

Data from the US CF National Patient Registry showed that in 2000, among 1000 infants with <2 years of age, bacterial pathogens isolated included *H. influenzae* (19%), *S. aureus* and *P. aeruginosa* (29%), with *S. aureus* having the highest infection rate of 42% (Saiman, 2004). Other important bacterial pathogens included *S. maltophilia* (7%) and *B. cepacia* (<1%) (Saiman, 2004). A study reported in a review by Ratjen (2001) found that when CF patients taken from a German database were analysed, it emerged that continuous anti-staphylococcal therapy was associated with an increased rate of *P. aeruginosa* acquisition, especially during the initial 6 years of life. Lyczak *et al.* (2003) discussed whether the early presence of *S. aureus* in the airways could predispose to infections with other pathogenic agents. Prior to the adoption and administration of anti-staphylococcal antibiotics, *S. aureus* was indeed the most important pathogen in CF, however no recent studies have addressed this question properly.

Thomas *et al.* (1998) reported that between 1965-1997, adult CF patients who became colonized with MRSA amounted to 26. The researchers, working at the Royal Brompton Hospital (RBH), London concluded that MRSA colonization or infection during this period was relatively uncommon with a rate of 2.7%. Indeed, the first case of MRSA was reported in 1982 and the highest incidence rate was between January and July 1997 with nine cases.

The authors concluded that there is a lack of important clinical consequences from MRSA colonization in CF patients and the risk of MRSA infection is low.

Miall *et al.* (2001) focused on MRSA in CF children. MRSA did not aggravate respiratory function but there seemed to be a negative effect on growth. The authors found a prevalence MRSA rate of 3% in the paediatric population studied. These children needed more intravenous antibiotic treatment and were found to have a worse chest X-ray appearance, than control children. A twelve-year retrospective study by Solis *et al.* (2003) in Liverpool, UK, also investigated the prevalence of MRSA in the CF paediatric population. The authors determined whether MRSA had a clinical impact on these children and evaluated an MRSA eradication protocol for these patients. Cultures were taken from nose, rectum, oral cavity and indwelling devices. Patients who were found to be positive were treated with 5 days of oral/nebulised vancomycin. Also, as part of the anti-staphylococcal policy advocated, flucloxacillin which is known to affect the patient's flora was substituted with cephradine.
In addition, a stringent anti-staphylococcal hygiene policy was ensured in MRSA carriers. This did not solely concern handwashing but was extended to indwelling devices. From a total of 108 children, 6.5% were found to be colonised with MRSA, with a median age of MRSA acquisition being 73 months. All the MRSA strains were found to be sensitive to vancomycin, teicoplanin and gentamicin. The authors concluded that when MRSA is found in a CF patient, efforts must be made to eradicate it. MRSA might result in spread amongst other patients (CF and non-CF) and also may limit the chances of a CF patient having lung transplantation, as colonisation or infection with MRSA is normally a contra-indication to the procedure in a number of transplant units (Solis *et al.*, 2003).

Steinkamp *et al.* (2005) found that MRSA prevalence was lower in Germany than in the UK. This study incorporated 12 German CF centres (1419 patients). From 213 specimens with emerging bacteria isolated from 145 different patients, only 5.2% of specimens were found to contain MRSA.

The risk factors for MRSA acquisition in CF patients was investigated by Nadesalingam *et al.* (2005). This retrospective analysis involved 15 CF patients who were positive for MRSA. Thirty age-sex-matched controls were used for comparison with MRSA subjects. In essence, four main factors were associated with MRSA, the most significant being the

number of in-patient days. In addition, the number of days of therapy with ciprofloxacin as well as cephalosporins and also chronic colonisation with *Aspergillus* spp., were, also found to have a significant effect or MRSA acquisition.

Renders *et al.* (1997) investigated the prevalence of *S. aureus* genotypes from six unrelated CF patients and 6 pairs of CF siblings, using arbitrary primed polymerase chain reaction (AP-PCR) assays. In 5 of 6 cases, identical isolates were shared by CF siblings at a specific time. This finding may imply intra-family transmission or the presence of a common environmental source. The fact that in most of the CF sibling pairs different genotypes of *S. aureus* caused the ultimate long-term colonization, indicates that despite regular cross-colonization, patient characteristics play an important role in selecting the *S. aureus* strain best adapted to the affected lung.

Denis *et al.* (2002) reported the occurrence of vancomycin-intermediately resistant *S. aureus* strains from three patients in a Belgian hospital. One case was a female 18-year old cystic-fibrosis patient who had been colonised with MRSA since 1993. She experienced three separate pulmonary exacerbations due to MRSA during winter 1998-1999 and was also co-infected with *P. aeruginosa*. Eventually the patient was cleared of MRSA infection with the use of continuous vancomycin infusion and fusidic acid, followed by an oral combination of fusidic acid and minocycline. During the subsequent 2 years, only vancomycin-susceptible MRSA was isolated from her sputum.

Serisier *et al.* (2004) reported the first eradication of an MRSA infection from an adult male CF patient, using oral linezolid. The patient initially presented with an exacerbation of lung disease and was co-colonised with *P. aeruginosa*. Subsequent analysis showed that the MRSA isolated was MRSA-16, exhibiting high mupirocin resistance and positive for *mec*A and *mup*A genes. In addition, chlorhexidine washes and cream were also applied to the nares. The patient was monitored until 18 months later and routine sputum cultures did not reveal any MRSA.

Table 1.6 includes studies discussing respiratory bacterial pathogens other than *S. aureus*, which may occur in CF respiratory infections.

Table 1.6: Respiratory bacterial pathogens in addition to *Staphylococcus aureus* known to affect CF patients

SOURCE	FINDINGS
Campana *et al.* 2004	Bacterial isolates were obtained from 163 CF patients in Florence, Italy and hospital environment. Results showed that 43% of patients were colonised with *Pseudomonas aeruginosa*, 3.6% by *Burkholderia cepacia* complex and 7% by MRSA. (*B. cepacia* spp includes at least 9 genomovars, with *Burkholderia cenocepacia* (genomovar III) and *Burkholderia multivorans* (genomovar II) increasingly associated with CF lung disease).
*Campana *et al.* 2005	*In a national Italian study of *B. cepacia* complex in CF patients, 61.1% belonged to the *B. cenocepacia*.
Dobbin *et al.* 2004	A retrospective study in Sydney, Australia, during the period 1989-2002 analysed survival rates post-lung transplantation. From 65 patients who were listed for lung transplantation with 11 of these died before transplant and 6 had pan-resistant *P. aeruginosa*. From 54 transplanted patients, 30 had at least 1 pan-resistant microbe before transplant and in 28 cases this included *P. aeruginosa*. One year survival rate was 92% with a median survival of *ca* 5 years. Patients continued to acquire multiresistant bacteria, even after transplantation.
#Hutchinson and Govan, 1999	#In this review, *Stenotrophomonas maltophilia* is mentioned as an emerging pathogen in CF.
Hutchinson *et al.* 2000	The authors investigated the inflammatory potential of a number of Gram-negative species (*Alcaligenes xylosoxidans*, *Ralstonia eutropha*, *Bordetella hinzii*, *Ralstonia pickettii*, *B. cepacia*, *Ralstonia solanacearum*, *Ralstonia paucula*) which have been found increasingly in CF patients. Except for *S. maltophilia*, the lipopolysaccharide (LPS) obtained from all other bacteria up-regulated the expression of the pro-inflammatory cytokines studied.
Lambiase *et al.* 2006	This study sought to establish the incidence and prevalence of Gram-negative bacteria and assess the antibiotic profiles. Sputum samples were obtained from a Regional CF Reference Centre in Naples, Italy. Results showed that 40% of patients had *P. aeruginosa*, 7% *Burkholderia cepacia* complex, 11% *Stenotrophomonas maltophilia* and 7% *Alcaligenes xylosoxidans*. Bacteria which were multi-drug resistant included *P. aeruginosa* and *Burkholderia cepacia* complex. Ceftazidime was the only efficacious β-lactam whilst aminoglycosides and quinolones were ineffective.
Rogers *et al.* 2005	Sputum samples from 17 CF patients attending Southampton University Hospital, UK were used to obtain PCR products and then amplified, using terminal restriction fragment length polymorphism (T-RFLP) and reverse transcription terminal restriction fragment length polymorphism (RT-T-RFLP). *P. aeruginosa* was present and also seen to be metabolically active. The authors also found other organisms present in CF sputum which had not previously been linked to CF. *B. cepacia* complex was detected with RT-RFLP but was absent from the T-RFLP profile and this implies that the bacteria were probably present in low numbers however they were highly active metabolically.

Section 1.4.2: Biofilm environment in the CF Lung

Conditions in a CF lung offer an ideal environment for possible biofilm formation with bacteria. A biofilm may be defined as a group of microbial cells that is irreversibly associated (hence implying that it is not removed by gentle rinsing) with a particular surface (which can be animate or inanimate). This aggregate of cells is enclosed in a matrix of extracellular polysaccharide material (EPS), allowing growth and survival in a sessile environment (Prakash *et al.*, 2003).

In such a situation, the microbial cells which are irreversibly attached to a substratum/interface or to each other are embedded in a matrix of extracellular polymeric substances which they have synthesized and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002). Biofilms may occur on a large number of inanimate as well as living surfaces. These include living tissues, and indwelling medical devices, such as catheters. Typically, *S. aureus* may form biofilms on prosthetic heart valves, artificial hip prosthesis, central venous catheters and intrauterine devices (Donlan, 2002).

The biofilm enables survival of the bacterial community in adverse conditions. Indeed, a number of factors differ between biofilm-forming microbes and their free-living counterparts: namely growth rate, composition and increased resistance to biocides, antibiotics and antibodies. In addition, there is up regulation and/or down regulation of approximately 40% of genes (Prakash *et al.*, 2003).

An ideal environment for the initiation of a biofilm can be provided by the solid-liquid interface between a surface and an aqueous medium. However, a definite illustration of the attachment cannot be obtained without considering the effects of the substratum, conditioning films forming on the substratum, hydrodynamics and characteristics of the aqueous medium, as well as properties of the cell surface (Donlan, 2002). A number of parameters play a fundamental role for the proliferation and maintenance of a biofilm. For instance, the physicochemical characteristics of the surface, together with the surface area, have great implications on the rate and extent of microbial cell attachment. Others may include the hydrodynamics of the substratum/liquid interface, the conditioning film which

coats the medium, pH, nutrient levels, iron, oxygen, ionic strength, temperature and properties of the cell (Donlan, 2002).

Effectively, in CF, there is a deficiency of water, which hinders the upward flow of the mucus layer, hence preventing the normal expulsion of particles from the airways. Also, decreased secretion and increased absorption of electrolytes lead to dehydration and thickening of secretions covering the respiratory epithelium (Donlan and Costerton, 2002). By exhibiting intercellular signalling or quorum-sensing systems, staphylococci are able to produce cell-to-cell communication, as well as regulate numerous colonization and virulence factors in the transition from late-exponential growth to stationary phase in *vitro*. In addition to *agr*, the repressor of toxins (*rot*) and the alternative sigma factor B also play a role in *S.aureus* quorum-sensing. In fact, these have been shown to affect the expression of a large number of virulence-associated genes. Both *rot* and sigma factorB counter *agr* activity. In essence, the quorum response in staphylococci during infection occurs within the context of a complex regulatory network that continually modifies either *agr* activity itself or its downstream effects (Yarwood and Schlievert, 2003; Yarwood *et al.*, 2004). Biofilm-associated infections have fundamental clinical importance as they are generally resistant to antibiotic therapy and clearance by host defences. Biofilm-induced staphylococcal infections include endocarditis, osteomyelitis, implanted device-related infections and even some skin infections.

Effectively, two stages of staphylococcal biofilm formation have been described:

a) Attachment of cells to a surface: This stage of biofilm formation is likely to be mediated in part by cell wall-associated adhesions, including the MSCRAMMs.
b) Cell multiplication and formation of a mature, multi-layered, structured community: Associated with the production of extracellular factors, including the polysaccharide intercellular adhesion (PIA) component of the extracellular matrix. Detachment of cells from the established biofilm may permit spreading of staphylococci to colonize new sites (Yarwood and Schlievert, 2003; Yarwood *et al.*, 2004).

SECTION 1.5: DIABETES AND CYSTIC FIBROSIS

Diabetes may occur in CF patients due to damage to exocrine tissue, which leads to glucose intolerance (Finkelstein *et al.,* 1988). Cystic Fibrosis-Related Diabetes (CFRD) has a negative impact on CF patients by increasing morbidity and mortality (Jefferies *et al.,* 2005). Finkelstein *et al.* (1988) retrospectively studied the prevalence of diabetes mellitus in 448 CF patients. Insulin-dependent diabetes mellitus (IDDM) developed in 7.6% of the population and these were found to have decreased survival rate when compared to their CF non-diabetic counterparts. In fact, fewer than 25% of patients reached 30 years of age, compared to 60% of non-diabetics. Clinical deterioration of the CF diabetic patients was seen 2 years prior to the onset of overt diabetes. The average age of IDDM-CF diagnosis was 19.8 years. Also, the total glycosylated haemoglobin (HbA_1) was found to be higher in the CF population when compared to normal individuals. This was even higher in the IDDM-CF patients cohort when compared to the non-diabetic CF patients. Female CF patients also had higher HbA_1 when compared to the male CF children. For CF patients exhibiting impaired HbA_1 metabolism, this indicated that it would lead to progressive clinical deterioration.

Milla *et al.* (2000) investigated a cohort of 152 CF patients, without diabetes, over a period of 4 years. They assessed the patients for glucose tolerance and classified them as having normal glucose tolerance, impaired glucose tolerance and CFRD without fasting hyperglycaemia. Parameters such as lung function and body mass index (BMI) for each patient were monitored and recorded. Results showed that all groups experienced a decrease in lung function during the study period. However, there was a direct correlation between patients who were glucose intolerant and a decrease in pulmonary function, which was independent of any other additional patient factors. The highest rate of decline in lung function parameters was seen in the CFRD patient group without fasting hyperglycaemia.

CFRD survival was also explored in a US CF population numbering 1,081 patients. This study found that being a female CFRD patient implied decreased survival, when compared to male counterparts or female CF patients, without CFRD (Milla *et al.*, 2005). Possible reasons may include the joint effects of female hormones and diabetes together with an increased inflammatory environment. Alternatively, a lack of androgens might negatively

affect the catabolic effects of insulin deficiency. Milla *et al.* (2005) also suggest that the presence of diabetes may indicate additional (unidentified) factors which play a part.
Sims *et al.* (2005) conducted a separate study which compared CFRD patients with normoglycaemic CF patients. Female CFRD patients proved to have significant decreased lung function when compared to male normoglycaemic patients. Interestingly, the effects due to diabetes were not apparent until a year after the diagnosis was made.

Philips *et al.* (2003) conducted a study which sought to establish whether glucose was present in normal respiratory secretions. The study included healthy volunteers, non-diabetic individuals with acute viral rhinitis and diabetics. Results showed that glucose was absent from healthy persons but was found in 50% of healthy individuals with those with viral rhinitis, as well as in 90% of diabetics. Philips *et al.* (2005) working at St George's Hospital, United Kingdom, discovered that the presence of glucose in bronchial aspirates of intubated patients, predisposed to the development of MRSA respiratory colonisation and/or infection. Baker *et al.* (2006) found that hyperglycaemia, in the context of acute exacerbations of chronic obstructive pulmonary disease, was indeed associated with increased isolation of pathogens, such as *S. aureus* from sputum.

In summary, *S. aureus* with its large array of virulence factors and complex regulatory networks is typically the first bacterium to colonise and infect CF patients paving the way for other pathogens, such as *P. aeruginosa*. The biochemical and cellular abnormalities present in CF airways, which may include: copious anoxic mucus, increased inflammatory mediators, low defensins, high salt concentrations, accumulation of DNA and proteins from neutrophil breakdown, as well as presence of glucose in CF secretions (particularly evident in diabetic CF patients), might facilitate the proliferation of this bacterium.

SECTION 1.6: AIMS OF PHD THESIS

This project aims to investigate the pathogenic effects of *S. aureus* growth and survival in cystic fibrosis sputum. The development of a model system, closely mimicking the typical environment found in an *in vivo* CF patient, forms the cornerstone of this work.

Section 1.6.1: Overall Hypothesis

The fundamental question to be addressed is, whether a model for *Staphylococcus aureus*-CF lung infection can be developed in a microbiology laboratory setting. The synthetic model should be easily reproducible and resemble, both in consistency, as well as in components, human cystic fibrosis sputum. This model, will then be used to monitor the growth and survival of *S. aureus* and to analyse the likely impact of specific model components on *S. aureus*. The strain of *S. aureus* to be used will be MRSA 252, a clinical epidemic MRSA-16 strain. The model will be challenged by altering environmental parameters, including glucose in the medium (simulating diabetes in CF patients) and this should presumably have a direct impact on the growth, survival and gene expression of MRSA 252.

Section 1.6.2: Main Targets

The main targets of this study include:

- Establishing a reproducible, *S. aureus*-CF model system by investigating the growth and survival of MRSA252 in this medium
- Altering specific components of the medium and monitoring the physiological effects on *S. aureus* growth and survival, using viable colony counts and light microscopy
- Using microarray technology to investigate differences in gene expression induced in the MRSA252 genome when this bacterial strain is grown in laboratory media and also in the *in vitro* sputum medium
- Attempting growth and survival techniques for MRSA252 in human CF sputum; if adequate growth and survival patterns are obtained, gene expression of target genes, using reverse-transcription polymerase chain reaction (RT-PCR).

CHAPTER 2
MATERIALS AND METHODS

SECTION 2.1: GROWTH AND SURVIVAL EXPERIMENTS

Section 2.1.1: Source of Bacteria and Confirmatory Tests

The MRSA-16 strain 252 (ST36 and CC30) was obtained from the Department of Cellular and Molecular Medicine, St. George's, University of London and was stored in brain heart infusion broth (BHI) with added 20% glycerol and kept at -80°C in 1.5ml Eppendorf® tubes.

Preparation of Brain Heart Infusion Agar CM0375 (Oxoid, Basingstoke, UK)

This agar was used for overnight growth of MRSA252 and was the medium selected for viable counts. Quality control on each batch was done by incubating ten sterile Brain Heart Infusion Agar (BHIA) plates in a static incubator (37°C) prior to using the media for any experiment. This was done for all solid media used throughout thesis.

Formula:	g/l
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Sodium chloride	5.0
Glucose	2.0
Disodium phosphate	2.5
Agar	10.0

pH: 7.4 ± 0.2

Five hundred millitres of distilled water were transferred from a measuring cylinder to a beaker and 23.5g of agar powder was measured using an electronic balance. The agar was suspended in the water and the mixture placed in 500ml flasks. The flasks were autoclaved at 121°C at 15psi for 15minutes. After autoclaving, at about 50°C, the liquid agar was poured directly, ensuring aseptic technique, into Petri dishes. These were left to set overnight and then stored in a fridge at 2-8°C.

Preparation of Brain Heart Infusion Broth CM0225 (Oxoid, Basingstoke, UK)

This liquid medium was used with added 20% glycerol in order to store MRSA at -80°C. Brain Heart Infusion (BHI) was one of the media used in the Survival and Growth Assays and a control for ASM and CFS experiments.

Formula:	g/l
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5

pH: 7.4 ± 0.2

A litre of distilled water was transferred from a measuring cylinder to a beaker and 37g of BHI powder was measured using an electronic balance. The powder was dissolved in the water and adequate mixing ensured. Aliquots of the BHI were placed either in 20ml containers or else in 50ml flasks as controls, or with added mucin as test stock solutions. These were autoclaved at 121°C at 15psi for 15 minutes. Once cooled, all containers were kept in a fridge at 2-8°C.

Once the MRSA was revived, confirmatory tests were performed to prove that the strain was indeed MRSA. These included: coagulase test (Fluka; Buchs, Switzerland) and meticillin-resistance (Mast Diagnostics; Merseyside, United Kingdom), testing. For the coagulase test, a drop of distilled water was placed on a glass microscope slide. A heavy suspension of the bacterium was then prepared, using, a fresh, overnight pure culture. A disc (Fluka®) was taken aseptically and placed on the slide. The disc was rubbed into the suspension, using the tip of a sterile loop. A second drop of water was then added and the entire suspension mixed once more. A positive test for *S. aureus* was obtained, when macroscopic clumping occurred within 3 seconds.

In order to confirm meticillin resistance, a Mueller-Hinton (Oxoid, Basingstoke, UK) agar plate, supplemented with 2% NaCl was swabbed with MRSA252 (obtained from a purity plate on BHIA), at a 1:10 dilution of 0.5McFarland. A 5μg meticillin disc was obtained aseptically and transferred onto the centre of the plate (BSAC Guidelines, 2008). This was kept in an incubator at 30°C for 24hrs. A zone diameter ≤ 14mm was considered meticillin-resistant. *Staphylococcus aureus* Oxford 6571 MSSA strain was used as a control for meticillin-susceptibility.

Preparation of Mueller Hinton Agar CM0337 (Oxoid, Basingstoke, UK)

Formula:	g/l
Beef, dehydrated infusion form	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

pH: 7.3 ± 0.1

This medium was only used to confirm meticillin resistance. A litre of distilled water was placed in a abeaker and 38 g of Mueller Hinton agar were dissolved. In addition, 20g of NaCl were also added. The mixture was sterilised by autoclaving at 121°C at 15psi for 15minutes and poured in Petri dishes with a depth of 4mm.

Section 2.1.2: Absorbance of MRSA252 in BHI with added 1% mucin

A pre-culture was prepared from an overnight pure culture on a BHIA plate; three colonies of MRSA252 were transferred directly into 25mls of BHI and were grown for three hours.

Since the mucin concentration in the normal human lung ranges from 0.5 to 5.0%, (Bolister *et al.*, 1991), a 1% mucin (Sigma, Poole, UK) solution was utilised for these experiments. A stock solution of autoclaved 100mls BHI with added 1% mucin w/v (BHI+M) was prepared and an aliquot of 20mls transferred to a separate sterile container. This was left to acclimatise to 37°C, in a static incubator. The inoculated solution containing BHI+M and MRSA252 was placed in a shaking incubator at 37°C (80rpm).

Absorbance readings were then obtained as follows:

a) Using Sterile BHI as blank: a reading of sterile BHI+ M was taken and another reading for the test sample.
b) Using sterile BHI as blank: a 1:10 dilution reading was taken, for both sterile BHI+ M and the test sample (diluent used was sterile BHI).
c) Using sterile BHI+M as blank: a reading for the test sample was taken. A colony count (counts were done using BHIA and in duplicate) was taken at each time interval (0, 3, 6, 24hrs), with different dilutions. Mean bacterial counts were calculated and these were taken from duplicate counts on BHIA and from the lowest dilution producing the closest counts. The test sample was incubated in a rotating incubator at 80rpm at 37°C, throughout the entire experiment.

Section 2.1.3: Survival studies of MRSA252 in artificial mucin-containing media and controls

Preparation of Nutrient Broth CM0001 (Oxoid, Basingstoke, UK)

Nutrient broth was mixed with 1% w/v mucin as a test sample and was also used, without the mucin, as a control.

Formula	g/l
'Lab-Lemco' Powder	1.0
Yeast Extract	2.0
Peptone	5.0
Sodium Chloride	5.0

pH: 7.4 ± 0.2

A litre of distilled water was transferred from a measuring cylinder to a beaker and 13g of NB powder measured using an electronic balance. Adequate mixing was ensured and this was then dispensed into a 50ml flask with the mucin and without (in a separate 50ml flask) as a control. These were then autoclaved at 121°C at 15psi for 15 minutes. Once having cooled, these were stored at 2-8°C.

Preparation of Ringer Solution BR0052 (Oxoid, Basingstoke, UK)

Ringer solution was used as a diluent (for viable counts) in the Survival and Growth Experiments. It was also one of the media tested with mucin and as a control.

Formula	g/l
Sodium chloride	2.25
Potassium chloride	0.105
Calcium chloride $6H_2O$	0.12
Sodium bicarbonate	0.05
pH: 7.0	

In order to prepare a quarter-strength Ringer Solution, 1 tablet was dissolved in 500ml of distilled water. This was then dispensed into 1ml sterile centrifuge tubes for diluting purposes or in 50ml flasks, with mucin as test sample or without mucin as a control. These were autoclaved at 121°C at 15psi for 15 minutes and once cooled, stored at 2-8°C.

Initially, stock solutions for water + 1% mucin, BHI + 1% mucin and Ringers + 1% mucin, were prepared. pH readings for the stock solutions, were taken using a pH meter (Infolab WTW Series), before autoclaving (Table 3.1). Prior to utilising the mucin-containing media from stock solutions, these were stirred to avoid particles of mucin settling at the bottom of the container and ensuring that all the mucin was in solution. From an overnight, pure MRSA252 culture on BHIA (incubated aerobically at 37°C), three colonies were selected and transferred directly to 25ml BHI, in a pre-sterilised 250ml conical flask.
The inoculated BHI was agitated lightly and placed in a rotating incubator at 37°C. The culture was incubated for 5 hours to achieve late exponential phase. At late exponential phase, an absorbance reading for the pre-culture, at a wavelength of 600nm was taken.

Aliquots of 5mls each were taken from stock solutions (kept at 2-8°C) of controls and mucin-containing media (BHI, Water and Ringers). These were warmed to 37°C, prior to use. An aliquot of 1ml was taken from the pre-culture and transferred directly into each of the control and mucin-containing media (each experiment included the test sample and respective control), and immediately vortexed and 1ml of each transferred to 9mls of Ringers diluent as a 1:10 dilution. A series of such dilutions (up to 10^{-6}) were prepared. This was done for both test and control.

The time when pre-culture was added to the test media was taken as being 0hrs. Mean bacterial viable counts were estimated for both tests and controls (viable counts were done in duplicate). The tests and controls were incubated for 1 hour in a standing incubator at 37°C. After 1 hr, 1ml aliquots were taken as described above and counts taken. This was repeated at 3 and 24 hours. Results were read, using a colony counter (Stuart® Colony Counter). Each experiment with test and control was conducted in triplicate.

Section 2.1.4: Growth Studies of MRSA252 in BHI, BHI and 1% mucin, Ringers and Ringers with 1% mucin

An overnight culture (incubated aerobically at 37°C on BHIA) of MRSA252 was obtained and three colonies were transferred directly in pre-autoclaved BHI (volume of 25mls) in a 250ml conical flask. This was left for 2-3 hrs in a shaking incubator and the OD_{600nm} was then obtained. An absorbance reading was obtained for the pre-culture.

An aliquot (10mls) of pre-autoclaved BHI + 1% mucin was placed in a universal container, with a BHI control (left in a static incubator to acclimatise at 37°C, as all stock solutions were kept at 2-8°C). This was repeated for the Ringers and Ringers + mucin. On placing the pre-culture into the test and control solutions, these samples were vortexed to ensure adequate mixing. Dilutions were then prepared, using Ringers as diluent. The OD_{600nm} at Time 0hrs was then obtained, 100μL aliquots were plated in duplicate onto BHIA and left overnight for viable counts. Tests and controls were then re-incubated for 3hrs, aerobically at 37°C in a shaking incubator (80rpm). As for 0hrs, absorbance readings and viable counts were obtained. The steps were repeated for the 6hr and 24hr time intervals.

One experiment using the same pre-culture was done with BHI + mucin and Ringers + mucin, together with controls, whilst a second experiment testing growth in BHI + mucin was performed using another pre-culture to give two separate BHI + mucin and controls. This was done to investigate whether using the same pre-culture would produce more consistent results.

Section 2.1.5: Cystic Fibrosis Artificial Sputum Medium (ASM) Experiments

Section 2.1.5.1: Preparation of ASM

Formula (Ghani and Soothill, 1997; Sriramulu *et al.*, 2005):

Gastric hog mucin (Mucin Type II Sigma®, Poole, UK)	5g
DNA (herring sperm; Sigma®)	4g
DTPA (Fluka®, UK)	0.0059g
NaCl (Oxoid, Basingstoke, UK)	5g
KCl (Fluka®)	2.2g
Alanine (Sigma®)	0.250g
Arginine (Sigma®)	0.250g
Asparagine (Sigma®)	0.250g
Aspartic acid (Sigma®)	0.250g
Cysteine (Sigma®)	0.250g
Glutamine (Sigma®)	0.250g
Glutamic acid (Sigma®)	0.250g
Glycine (Sigma®)	0.250g
Histidine (Sigma®)	0.250g
Isoleucine (Sigma®)	0.250g
Leucine (Sigma®)	0.250g
Lysine (ICN®)	0.250g
Methionine (Sigma®)	0.250g
Phenylalanine (Sigma®)	0.250g
Proline (Sigma®)	0.250g
Serine (Sigma®)	0.250g
Threonine (Sigma®)	0.250g
Tryptophan (Sigma®)	0.250g
Tyrosine (Sigma®)	0.250g
Valine (Sigma®)	0.250g
Distilled Water	1000ml
Egg Yolk Emulsion (Oxoid)	5ml

The amino acids were individually weighed and dissolved in 1L of distilled water. All other components were also weighed and added, ensuring adequate mixing. The pH was adjusted to 6.9 by adding crystalline TRIS/L [tris (hydroxymethyl) aminomethane] (BDH, UK) and using a pH meter (Infolab WTW Series®). The liquid mixture was then autoclaved at 121°C at 15psi for 15mins and once this had cooled to room temperature, the egg yolk emulsion was added aseptically and stirred to ensure adequate mixing. This stock solution was then kept at 2-8°C.

Section 2.1.5.2: MRSA 252 Growth Studies in ASM

An overnight culture of MRSA252 was obtained (at 37°C). Three colonies were taken and placed in a pre-autoclaved conical (250ml) flask containing 25ml ASM (control); another three colonies were placed in a separate conical flasks with 25ml of ASM +/- components. Prior to inoculation, both flasks containing the broths were left to equilibrate to 37°C. On transferring the colonies into the media in the flasks, these were swirled gently. Aliquots (100µl) were taken from each flask and serial dilutions set up for viable counts at time 0hrs. This was done for the BHI control and all the ASM tests and controls. The diluent used was Ringers solution. The flasks were kept in a rotating incubator at 80rpm (37°C) and sampling subsequently occurred at times 0, 2, 4, 6, 8, 24 and 48hrs. Viable counts were read with a Stuart® colony counter.

Section 2.1.5.3: Statistical Analyses

Statistical analyses such as mean, standard deviation and standard error of the mean were calculated using Microsoft Excel. One-way ANOVA and post hoc Tukey`s multiple comparison test were done using the SPSS statistical package Version 14.

Section 2.1.5.4: MRSA 252 Light Microscopy Studies in ASM

TheGram-stain was prepared as follows (modified from Collins and Lyne, 1984): a direct sample containing MRSA252 was obtained by using a sterile 10µl loop to transfer an equal amount of inoculated, diluted ASM (10^{-1} dilution) onto a clean glass slide. Throughout all experiments and repeats, for each time interval, the same procedure was followed. The inoculated slide was then allowed to dry in air and then passed through a Bunsen flame for fixation. The slide was then flooded with methyl violet stain (Pro-Lab® Diagnostics, Cheshire, UK) and washed with distilled water. It was then flooded with Lugol's iodine (Pro-Lab® Diagnostics) and distilled water applied, as previously. Ethanol was diluted to 70% (BDH, VWR International Ltd., Leicester, UK) and used as a decolourisation agent and distilled water was used again to remove the stain. Dilute carbol fuschin (Pro-Lab® Diagnostics) was then applied as a counterstain, followed by distilled water, which was used in order to wash the slide. The slide was then gently blotted dry and examined using an oil immersion 100X objective lens on a Carl-Zeiss® Axioskop light microscope. Images were captured with a Nikon DN100 digital camera and EclipseNet® software. Scale bars were imported from the software which had been previously calibrated to a stage micrometer.

Section 2.1.5.5: MRSA 252 Protease Experiments in ASM

For the protease experiments, 12% zymogram (casein) gels were chosen (Chan and Foster, 1998; Lindsay and Foster, 1999) and pre-cast gels were purchased (Bio-Rad, Hemel Hempstead, UK), in order to ensure reproducibility throughout the experiments. To investigate protease activity in MRSA 252, samples were grown to 8, 24 and 48hrs (as described previously in this chapter) in ASM. An aliquot of 10mls was taken from each and these were centrifuged at 3000g. The resulting supernatant was carefully removed and stored in 1ml aliquots at -80°C. Sterile ASM was chosen as a negative control (this should not show any protease activity) and this was centrifuged at 3000g, and the supernatant retained as for the ASM samples. The positive control used in these experiments was a *sarA::kan* strain/PC1839 (kindly donated by Dr Jodi Lindsay); this is known to be a protease (V8 serine protease) producer. This strain was grown in BHI (see Lindsay and Foster, 1999) and harvested at 24hrs. All samples and controls were stored at -80°C.

During these experiments, samples were used 'neat', meaning directly from the supernatants and also at increasing concentrations. Concentration was achieved by using Vivapore® (VivaScience, Stonehouse, United Kingdom) which was used in order to concentrate the samples to achieve 5X, 10X, 50X and 100X concentrated samples. All these processes were performed at 4°C.

Sample Preparation:

Supernatant: 12µl

Tris-Glycine SDS Sample Buffer (Novex®, Invitrogen, Paisely, UK): 15µl (2X)

Deionized water: 3µl

The samples and controls were then loaded onto the pre-cast gel, once this had been carefully mounted and secured in the Bio-Rad® (Hemel Hempstead, UK) Miniprotean II cell. Also, 10µl of Colourburst® Electrophoresis Wide Range 6.5-205 kDa Markers (Sigma) were loaded at one end of the gel. The molecular weights of these markers ranged from 8-220kDa and the different colours appearing on gel indicated these molecular weights. Tris-Glycine SDS Running Buffer (X1) was prepared (prior to initiating the experiment) by adding 100ml of 10X Novex® Tris-Glycine SDS Running Buffer to 900ml of deionized water. The upper and lower buffer chambers of Bio-Rad® Miniprotean II cell were loaded with the running buffer (1X). The lid was securely fastened onto the chamber, connected to a Bio-Rad® power-pack and left to run for approximately 90 minutes at 145V. The set-up was inspected (during the 90 minutes run time) to ensure that the current was active and to assess the dye-front distance. Meanwhile, Novex® Zymogram Renaturing Buffer (10X) and Novex® Zymogram Developing Buffer (10X) were diluted at 1:9 with deionized water (100ml of each buffer required per one or 2 gels).

Once electrophoresis was complete and the dye front reached the end of the gel, the gel was then developed as follows:

1. The gel was removed and incubated in Novex® Zymogram Renaturing Buffer (1X) for 30 minutes at room temperature with gentle agitation.
2. The Novex® Zymogram Renaturing Buffer was then decanted and Novex® Zymogram Developing Buffer (1X) added to the gel.

3. The gel was left to equilibrate for 30 minutes at room temperature with gentle agitation.
4. The buffer was then decanted and fresh Novex® Zymogram Developing Buffer (1X) was added.
5. The gel was then incubated at 37°C, overnight, for maximum sensitivity.
6. After incubation, the gel was rinsed in 100ml of deionised water, three times for 5 minutes each and the last rinse was discarded.
7. Staining was done using Novex® SimplyBlue Safestain (a branded Coomassie stain). Protease activity appeared as clear bands against a dark background. Approximately, 25ml of SimplyBlue Stain® was added (enough to cover the gel) and this was left for 1hr at room temperature (22°C) with gentle agitation.
8. The stain was then discarded and the gel was washed with 100ml of deionised water for a minimum of 1-2hrs. Longer wash times enhanced the band intensity and reduced the background. Prior to analysis, a second water wash (100ml), using deionised water, was applied.
9. Finally, the gels were placed in a transparent plastic folder and analysed using an hp® scanner.

SECTION 2.2: MICROARRAY EXPERIMENTS

Section 2.2.1: Preparation of samples for Microarray Analysis

Section 2.2.1.1: DNA Extraction

Initially, an overnight culture of MRSA 252 was prepared on BHIA. Then, 1 colony was taken and placed in a sterile microcentrifuge (Eppendorf®) tube containing 1ml sterilised Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK). The TSB recipe is provided below.

Preparation of Tryptone-Soya Broth CM0129 (Oxoid, Basingstoke, UK)

Tryptone-Soya Broth (TSB) was needed for the preparation of MRSA252 stock DNA for microarray analysis. Hence, 30g of TSB powder was initially added to 1L distilled water and mixed well. Then 50ml aliquots were measured and sterilised by autoclaving at 121°C for 15 minutes.

Formula	g/l
Pancreatic Digest of casein	17.0
Papaic digest of soyabean meal	3.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.5
Glucose	2.5

pH: 7.3 ± 0.2

A minimum of 10 Eppendorf® tubes were prepared simultaneously in order to provide enough DNA stock for all the microarrays. These were left overnight in a shaking incubator at 150 revs/min at 37°C. The Eppendorf® tubes were then centrifuged at highest speed for three minutes to obtain a pellet (approx 50mg) and the supernatant was then discarded. Four hundred microlitres of Spheroblast® buffer (Edge Biosystems, Gaithersburg, MD, USA) was then added to the pellet and vortexed at highest speed to re-suspend the pellet. Six microlitres of lysostaphin (Sigma, Dorset, UK) were added to the mixture. This was then incubated for 20 minutes at 37°C in a water-bath. One hundred microlitres of Lysis 1® (Edge Biosystems) was added, followed by 100µl of Lysis 2® (Edge Biosystems). Using a pipette, the contents were mixed well and incubated for 5

minutes at 65°C. One hundred microlitres of Advamax Beads® (Edge Biosystems) and 100µl of Extraction Buffer® (Edge Biosystems) were then added and vortexed for 30 seconds. The mixture was centrifuged in a microcentrifuge (Eppendorf®) at the highest speed for 3 minutes and the supernatant was then transferred to a clean 2ml tube. An equal volume (*ca* 700µl) of isopropanol (BDH) was added and the tube was inverted several times, in order to ensure adequate mixing. The DNA was pelleted by centrifugation for 10 seconds. It was then washed with 70% ethanol (BDH) and left to dry in air (*ca* 1hr). DNA was then re-suspended in 50µl TE buffer (Sigma) and left in a water-bath at 50°C for 1hr (or even longer). The DNA concentrations were then measured using a NanoDrop® ND-1000 UV-Visible Spectrophotometer; 1.5µl of filtered water was used initially as a blank and then, 1.5µl of sample fluid was measured. Every sample was blanked prior to measuring DNA concentration.

Section 2.2.1.2: RNA Extractions

Initially, a 50ml Falcon® tube was prepared for each sample and this contained 20ml of RNAProtect Bacteria Reagent® (Qiagen, West Sussex, UK). A 10ml aliquot of bacterial culture was transferred directly from the culture flask into the RNAProtect®. The time intervals when RNA preparations were required, included: 3hr for BHI + MRSA 252, 8hrs for ASM + MRSA 252 and 6hrs for MRSA 252. This data was obtained from the Results section in Chapter 3.

The samples were mixed immediately by vortexing for 5 seconds. The tubes were then incubated at room temperature (15-25°C) for 5 minutes and centrifuged for 25 minutes at 3260g. The supernatant was then discarded. Residual supernatant was removed by gently dabbing the inverted tube once onto a paper towel. The pellets were then stored for a maximum of -70°C for up to 4 weeks before further processing.

RNA from the thawed pellets was extracted using an RNAEasy-Mini® kit (Qiagen). Prior to actual processing of samples, 10µl of β-mercaptoethanol (Fluka Biochemika, Buchs, Switzerland) for every ml of RLT® buffer (Qiagen) was added. This was mixed well and stored at room temperature for up to 1 month. Next, a mixture of X1 TE® buffer (Promega, Southampton, UK), 200µg/ml of lysostaphin (Sigma) and 40µg/ml of lysozyme (Sigma) was prepared. The DNase reagents were then prepared (for the elimination of DNA

contaminants): DNase I® powder was mixed with water by adding 550µl of RNase-free® water (Qiagen), using a syringe to the powder, and the solution was mixed delicately (without vortexing). Aliquots were then made up and stored in the -20°C freezer. The RPE® buffer (Qiagen) was prepared by adding 1 volume of RPE® (Qiagen) to 4 volumes of ethanol (BDH).

Sample Processing:

Once having thawed the frozen tube of each sample at room temperature (15-25°C), the pellet was washed with 1ml of 1X TE® buffer (Promega) which amounted to *ca* 10X the volume of the pellet. The tube was then centrifuged at 5000g for 10 minutes. The supernatant was totally discarded. The pellet was re-suspended in 200µl of TE containing 200µg/ml of lysostaphin RNAase-free and 40µg/ml of Proteinase K® (i.e 20µl of lysostaphin 2mg/ml (in TE 1X) + 1µl PK of 10mg/ml + 179µl TE). The mixture was vortexed for 10 seconds (until all aggregates disappeared) and incubated for 1hr 30minutes at 37°C with vortexing every 15 minutes.

Safe-Lock® tubes (Hybaid, Basingstoke, UK) were placed in ice and the Safe-lock® particles were removed from the tubes to make a total weight of 1.7-1.8mg per tube. An aliquot equal to 700µl of RLT® (Qiagen) buffer was added to the sample. The lid was screwed on strongly to avoid leaking during vortexing with the Hybaid® Ribolyser and samples were vortexed for 10 seconds. The contents were transferred into the Safe-Lock® tubes and placed in ice. After 2 minutes they were placed in the Tissue-Lyser® (speed: 6.5 and for 15 seconds). This was done for 4 times in total and the tube was placed in ice for 2 minutes between each time interval and centrifuged at maximum speed for 10 seconds. An aliquot equal to 760µl of the supernatant was then transferred into a new tube. Next, 590µl of 80% ethanol (BDH) were mixed using a pipette and 700µl of lysate was transferred onto an RNeasy Mini® (Qiagen) pink column and into a centrifuge tube. This was centrifuged for 15 seconds at ≥8000g and the elutant was eliminated and 250µl of buffer RW1 (Qiagen) was added and centrifuged at 15 seconds at 8000g. The elutant was then eliminated. In a separate tube, 10µl of DNase I® (Qiagen) + 70 µl RDD® (Qiagen) buffer were mixed without vortexing and the mixture transferred directly to the centre of the column and incubated for 15 minutes at 20-30°C. An amount equal to 350µl of RW1® (Qiagen) buffer was placed on the column and after 5 minutes at room temperature, this

was then centrifuged for 15 seconds at 8000g. The resulting elutant and the collecting tube were eliminated.

A new (labelled) tube was placed under the column and 500µl of RPE® (Qiagen) buffer were added. The tube was centrifuged for 15 seconds at 8000g and the elutant eliminated and 500µl of RPE® (Qiagen) buffer was placed onto the column and centrifuged for 2 minutes at 8000g. The elutant and the collecting tube were discarded. A new tube (with a number and reference for each tube) was placed under the column and centrifuged for 1 minute at maximum speed. A new tube was placed under the column and 30µl of RNase® free water (Qiagen) was added directly at the centre of the column. After 1 minute the tube was centrifuged for 15 seconds at maximum speed (≥8000g). The tube was removed and placed in ice, the column was extracted and previous tube was conserved in ice. This was done for three consecutive times.

Finally, the concentration of RNA was measured using a Nanodrop ND1000® Spectrophotometer; 1.5µl of filtered water (Sigma) was placed initially as a blank and then, 1.5µl of sample fluid was measured. Each sample was blanked prior to measuring RNA concentration. The purity of the RNA measured was assessed according to the A_{260} /A_{280} ratio, where A= Absorbance, which should be ≥2. RNA solutions with a very low ratio were taken to be consistent with significant contamination and were immediately discarded (Roche Applied Science Manual, 2002).

Section 2.2.1.3: Bioanalyzer Experiments

The Agilent 2100 Bioanalyzer® was used to determine the purity of the RNA. Also, concentrations measured by both the Nanodrop® and Bioanalyzer® should be identical or very similar. Any discrepancies could indicate potential contamination of the RNA and analysing RNA by both methods prior to hybridisation ensures a high quality RNA (Reagent Kit Guide RNA 6000 Nano® Assay Agilent Technologies, Edition 2003).

Initially, the Bioanalyzer® was decontaminated as follows:

- First, 350µl RNaseZap® (Ambion, Applied Biosystems, Warrington, UK) was placed into the wells of the electrode cleaner. This was then placed in the Agilent

2100 Bioanalyzer®. The lid was closed and the electrode cleaner was left for 1minute to act. The lid was then opened and the electrode cleaner was removed.

- Another electrode cleaner was selected and the wells were filled with 350µl RNase-free water (Sigma). This was then placed in the Agilent 2100 Bioanalyzer® and left for 10 seconds with the lid closed. The lid was then opened for 10 seconds prior to closing the lid, ensuring that the water present on the electrodes evaporated. Both the RNaseZap® and the RNase-free water were removed from the electrodes after use.

Determining the RNA purity:

1. All reagents were allowed to equilibrate to room temperature for 30 minutes prior to use. Then 550µl of RNA 6000 Nano® gel matrix was placed into the top receptable of a spin filter. The spin filter was placed in a microcentrifuge (Eppendorf®) and spun for 10 minutes at 1500g. The filter was then discarded and 65µL of filtered gel was placed in 0.5ml RNase-free microcentrifuge (Eppendorf®) tubes. The aliquots were stored at 4°C and used within a month of preparation.
2. The Gel-Dye Mix was prepared next: the dye concentrate is light sensitive and so this was protected from light by applying foil around the tube. The RNA 6000 Nano® dye concentrate was vortexed for 10 seconds and spinned down. Then 1µl of dye was added to a 65µl aliquot of filtered gel. The tube was vortexed thoroughly and adequate mixing of gel and dye was ensured. This was kept in the dark at 4°C. Prior to use, the tube was spun for 10 minutes at room temperature at 13000g. The gel-dye mix was used within 24 hours.
3. A new RNA Nano® chip was obtained from a sealed bag and placed on the Chip Priming Station. An aliquot of the gel-dye mix equal to 9µl was placed at the bottom of the 1st well marked "G". The Priming Station was closed and left for 30 seconds and then the plunger was released. After 5 seconds, the plunger was pulled back to the 1ml position. The Chip Priming Station was opened and 9µl aliquots placed in 2 other wells marked "G". Next, 5µl of the RNA 6000 Nano® Marker was placed into the ladder well as well as into all sample wells.

4. Samples as well as the RNA 6000® ladder were heated in a heat block (Perkin-Elmer®) at 70°C for 2 minutes. An aliquot equal to 1µl of the ladder was placed in the ladder well and 1µl of each sample placed in the sample wells. Once this was done, the chip was transferred into the IKA® vortexer at 2400rpm and vortexed for 1 minute. The chip was then transferred directly into the Agilent 2100 Bioanalyzer®. Using the software the option for total RNA prokaryotic NANO Series II® was chosen and the run initiated. Once the run was completed, the Bioanalyzer® was decontaminated as described initially.

Section 2.2.2: RNA vs DNA Microarray Protocol

In order to conduct gene expression studies, it was deemed essential to produce an RNA vs DNA microarray protocol. This enables future comparison with similar data obtained from other studies and so RNA vs DNA is indeed more effective for comparative genomics.

Before starting the microarray experiments, both DNA and RNA concentrations were re-measured using the NanoDrop® ND-1000 UV-Visible Spectrophotometer and aliquots equal to 2µg for DNA and 4µg for RNA were prepared for each microarray. These amounts were kept constant for all the microarrays done (BµG@S Bacterial Microarray Group at St. George's: Microarray Protocols for RNA vs DNA Labelling and Hybridisation).

Preparing Cy3 labelled DNA:

Components	Amount
DNA	2µg
Random primers at 3µg/µl (Invitrogen)	1µl
Filtered distilled water (Sigma)	30.5µl

The DNA sample was then heated for 5 minutes at 95°C in a heat block (Perkin-Elmer®) and snapped cool on ice. Then it was briefly centrifuged. In addition, the following were added:

10X React2 Buffer® (Invitrogen)	5µl
dNTP at 5mM dA/G/TTP, 2mM dCTP (Invitrogen)	1µl

Cy3 dCTP (Amersham Biosciences, Buckinghasmshire, UK)	1.5µl
Klenow® at 3-9U/µl (Invitrogen)	1µl

The mixture was left in a heat block at 37°C in the dark for 90 minutes.

Preparing Cy5 labelled RNA:

Components	Amount
RNA	4µg
Random Primers 3µg/µl (Invitrogen)	1µl
Filtered Distilled Water (Sigma)	up to 11µl

The sample was heated at 95°C for 5 minutes, snapped cool on ice and centrifuged briefly after.

The following components were added immediately afterwards:

5X First Strand Buffer® (Invitrogen)	5µl
Dithiothreitol (DTT) at 100mM (Invitrogen)	2.5µl
dNTPs at 5mM dA/G/TTP, 2mM dCTP (Invitrogen)	2.3µl
Cy5 dCTP Fluorolink® (Amersham Biosciences)	1.7µl
Superscript II® at 200U/µl (Invitrogen)	2.5µl

The sample was then incubated in the dark at 25°C for 10 minutes, followed by another 90 minutes at 42°C. The microarray slide to be used was then hybridised. The pre-hybridisation solution was first prepared and left to equilibrate in an incubator to 65°C during the labelling reaction.

The contents of the pre-hybridisation solution included:

20X SSC (Sigma)	8.75ml
20% SDS (National Diagnostics, East Riding, Yorkshire, UK)	250µl
Bovine Serum Albumin at 100mg/ml (Sigma Aldrich)	5ml
Double-distilled water	up to 50ml

This solution was placed in a Coplin jar and the microarray slide was left inside at 65°C for 20 minutes. The slide was then rinsed in 400ml of distilled water for 1 minute, followed by another rinse in 400ml of propan-2-ol for 1 minute and the slide was then centrifuged in a 50ml centrifuge tube at 1500rpm for 5 minutes (label down). This was then stored in the dark in the dust-free box until hybridisation (<1hour).

A Qiagen MinElute® Purification kit was obtained. The Cy3 and Cy5 labelled samples were combined in one single tube and 375µl Buffer PB® (Qiagen) was added. The mixture was added to the MinElute® column in a collection tube and centrifuged at 13,000rpm for 1 minute. The flow-through was then discarded and the column was placed back into the same collection tube, 500µl of buffer PE® (Qiagen) was added to the column and it was centrifuged at 13,000rpm for 1 minute. The flow-through was discarded and the column replaced into the same collection tube. Then, 250µl of buffer PE was added to the MinElute® column and centrifuged at 13000rpm for 1 minute. The flow-through was discarded and placed in the MinElute® column back into the same collection tube. The tube and column were centrifuged at 13,000rpm for an additional 1 min to remove residual ethanol. The column was then transferred into a fresh 1.5ml tube and 33µl of filtered water was added to the centre of the membrane and allowed to stand for 1 minute. This was then centrifuged for 1minute.

Wash A was then prepared and left in a sealed bottle to incubate at 65°C together with an empty staining trough.

Wash A

20X SSC (Sigma)	20ml
20% SDS (National Diagnostics)	1ml
Distilled Water (Sigma)	to 400ml

The Hybridisation step:

The freshly hybridised microarray slide was then placed to a hybridisation cassette and 2 15µl aliquots of filtered distilled water were placed in the wells inside the cassette.

In a new 0.5ml tube, the purified Cy3/Cy5 labelled cDNA sample was mixed with the hybridisation solution in the following manner:

For Lifterslip® (VWR International, Leicester, UK) 22x50mm:

Cy3/Cy5 labelled cDNA sample	29.2µl
Filtered 20X SSC (Sigma)	9.0µl
Filtered 2% SDS (National Diagnostics)	6.8µl

The Lifterslip® (VWR International) was placed carefully over the arrayed area of the slide, ensuring that the LifterSlip® (VWR International) bars were face down and that the array surface was intact, with no scratches.

Hybridisation solution was heated at 95°C for 2 minutes. The tube contents were allowed to cool slightly and this was then centrifuged. Whilst still warm, the hybridisation solution was carefully pipetted under one corner of the LifterSlip® (VWR International), allowing the solution to be drawn completely across the array by capillary action. Any excess hybridisation solution was pipetted under the opposite corner of the LifterSlip® (VWR International). The hybridisation cassette (Corning®) was sealed and submerged in a water bath at 65°C in the dark for 16-20 hours.

Preparation of Wash B:

20X SSC (Sigma)	1.2ml
Distilled water (Sigma)	upto 400ml

Wash A was placed in the staining trough and Wash B (left at room temperature) was placed in 2 other troughs. The slide was then removed from the hybridisation cassette and washed carefully in Wash A until the LifterSlips® (VWR International) were displaced. Once this was done, the slide was then placed in a slide rack and agitated for a further 2 minutes. This was then followed by another wash inside Wash B Trough 1 for 2 minutes. The slide was then transferred to Wash B Trough 2 and agitated for another 2 minutes.

It was then placed in a 50ml centrifuge tube and centrifuged at 1,500rpm for 5 minutes to dry the slide. Once this was done, the slide was scanned using an Affymerix® Scanner (Genetic Microsystems GMS 418).

Section 2.2.3 Microarray Software Analysis

The scanned arrays were analysed first using Imagene® and then BlueFuse® (BlueGnome, Cambridge, UK) software. All the 'raw' data was placed into GeneSpring® GX 7.3.1 Expression Analysis (Agilent® Technologies, Silicon Genetics, USA) and resulting data was then normalised against the laboratory control, i.e. BHI. Normalised data was subsequently analysed statistically using one-way ANOVA and the multiple testing correction, Benjamini and Hochberg False Discovery Rate test. A list of different classes of genes which were differentially regulated were then transferred from GeneSpring® into Microsoft Excel®. Functional classes were assigned to each gene, primarily using the http:// www.genedb.org website.

Fig 2.1 below is a representation of the various experimental procedures followed, in order to produce the microarray results presented in Chapter 4.

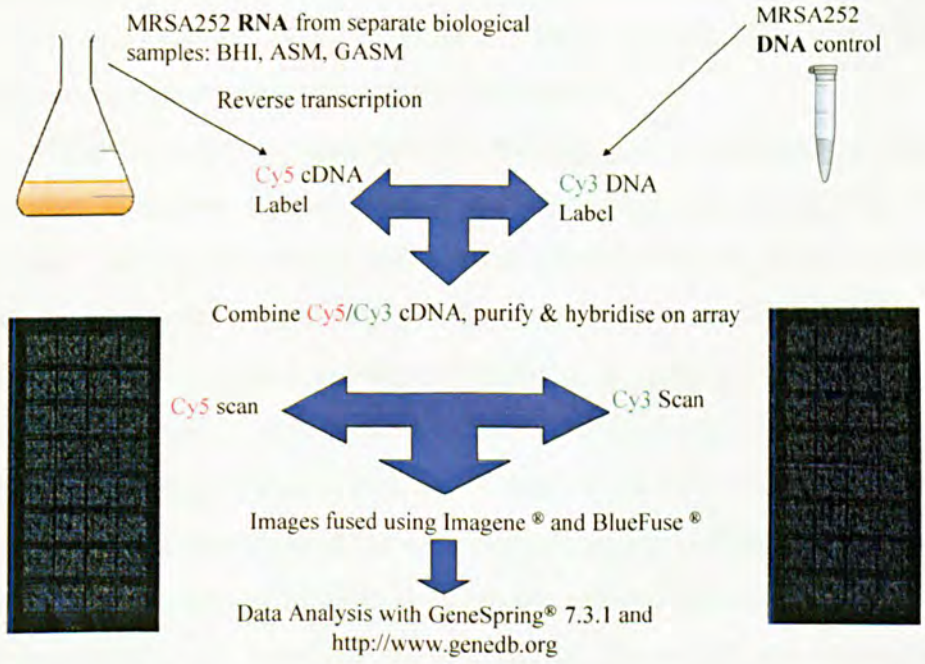

Fig 2.1: A schematic representation of the steps involved in these microarray experiments. This figure illustrates the steps from the initial RNA and DNA isolation from biological samples, labelling, purification, hybridisation to data analysis of the scanned Cy5 and Cy3 images.

SECTION 2.3: RT-PCR EXPERIMENTS

Section 2.3.1: Primer Design

Twelve genes were selected after analysing the normalised microarray data and these were divided into 3 types: up and down-regulated, as well as reference genes. Each gene required a forward and reverse primer and these were designed as follows:

1) The open reading frame of interest (ORF) was obtained from the sequenced MRSA 252 genome in http:// www.genedb.org using the ARTEMIS option

2) The gene option was then chosen

3) FASTA format was advocated and this result was then copied to MS Word and labelled with the respective gene

4) Since sequences were not immediately evident in the http://www.genedb.org website for the reference genes, these were found using the BLAST option from NCBI in PubMed. Once the sequence was blasted, this confirmed the gene of interest was the one selected.

5) The sequence was then placed in the http://ecom.mwgdna.com website and a number of options for the forward and reverse primers were given. Additional data also included the length, GC content (increase in GC yields an increase in melting point) and Tm (melting point). Once the primers of interest are selected, these were placed through the Sigma® website, in order to check whether primer-dimers formed and once these are confirmed not to occur, respective primers were selected and ordered directly. A primer-dimer can be defined essentially as an extra double-peak which appears in the dissociation curve (in addition to the normal peak of the primer melting point) and is due to the forward and reverse primers actually forming an amplicon in the absence of template. This occurrence would give a false cycle threshold (Ct) and so a No Primer Control (NPC) is normally included with a sample to be tested. Sequences for Forward (F) and Reverse (R) primers for each gene are provided in Table 2.1.

Table 2.1: List of genes and related primer sequences

PRIMER NAME	SEQUENCE (5`-3`)
16Sr RNA (F)	GAAAGCCACGGCTAACTAC
16Sr RNA (R)	TCAGACTTAAAAAACCGCCTAC
gyrB (F)	AAAAGGTATTATGGCGGCAC
gyrB (R)	CGGCTAATTTACCTGGAAGAC
lrgB (F)	CACCAGCCAAAGAATTAGGAC
lrgB (R)	GCTACAAAGACAGGCACAAC
spa (F)	AGAAGCAACCAGCAAACC
spa (R)	AATAACGCTGCACCTAACG
hld (F)	AAGGAAGGAGTGATTTCAATGG
hld (R)	GTGAATTTGTTCACTGTGTCG
icaB (F)	CGCAGAAAACTATAGCCTATCC
icaB (R)	CATTGGAGTTCGGAGTGAC
icaA (F)	TTAAGTATGAACCGCTTGCC
icaA (R)	AGTAATACTTCGTGTCCCCC
clfA (F)	CTGAACAACCTGATGAGCC
clfA (R)	ATCTGAATTAGAATCGCTGCC
capE (F)	TGATGTGCGTGATAGTCAAAG
capE (R)	CACTGCCTCAACTGGAAAG
capD (F)	TGACACATCCTGAAATGACAC
capD (R)	ACAAATACTTCGCCACCTTC
capC (F)	ACGGTCCGACAAATGAAAC
capC (R)	CGAGGATGTAAGTGATGTGATG
lrgA (F)	CCTATGCCTGCATCAGTAATC
lrgA (R)	GCTGGTACGAAGAGTAAGCC

Table 2.2: Primer Master Stock Dilution Chart (where TE= Tris-EDTA Buffer, Promega®)

PRIMER NAME	1X TE DILUENT (µL) FOR A 50µM STOCK CONCENTRATION
16Sr RNA (F)	1140
16Sr RNA (R)	920
gyrB (F)	1020
gyrB (R)	840
lrgB (F)	860
lrgB (R)	900
spa (F)	1000
spa (R)	980
hld (F)	1040
hld (R)	840
icaB (F)	880
icaB (R)	980
icaA (F)	1100
icaA (R)	580
clfA (F)	1040
clfA (R)	1000
capE (F)	940
capE (R)	1260
capD (F)	1140
capD (R)	1220
capC (F)	1280
capC (R)	960
lrgA (F)	1160
lrgA (R)	940

Section 2.3.2: Primer Optimization

Prior to starting the QRT-PCR, it was necessary to perform primer optimizations. For each primer, Forward (F) and Reverse (R), a number of different concentration ratios were set up. This was all done in a clean room and Class 2 microbiological safety cabinet.

Procedure:

From an original primer stock, 200µl of Forward (F) and Reverse (R) primers were prepared, i.e. concentration of 50µM (Table 2.2).

For each particular gene, the following final concentrations were used: 50nM/300nM, 50nM/100nM, 150nM/150nM, 100nM/50nM and 300nM/50nM having these corresponding ratios: 1F:6R, 1F:2R, 1F:1R, 2F:1R and 6F:1R were set up. Fig 2.2 depicts a typical PCR plate (AB gene®, Epsom, UK) set-up.

A Master Mix of the following components was then prepared as follows:

2x SYBR Mix:	12.5 µl
Primers (F+R):	2.5 µl
ROX (reference dye) 1:200 dilution	0.5 µl
Template:	1µl equivalent to 50ng for 1 reaction
RNase-free, DNAse-free dH_2O:	8.5 µl
Total Reaction Volume:	25µl

ROX dilution was prepared from an initial concentration of 1mM. A 1:200 working stock dilution was prepared and 0.5µl taken for each reaction. Care was ensured to keep the solution away from light, as it is light-sensitive, so the ROX dilution tube was wrapped in foil.

In each well, 21.5 µl of master mix was placed (SYBR® Green + water + ROX). Then a primer mix for each gene was added, except for Row 11. Instead 2.5 µl of double distilled water was added. The plate was covered with stick-on caps (AB gene®) and the DNA template added to rows 1-11 and 1 µl row 12 add water. Also the Primer Mix was added to Row 12, equal to 1:1 ratio. Finally, the plate was mixed in a centrifuge for 2 minutes and placed in a Stratagene® MX3005P, using the SYBR Green option, with ROX as reference dye. Then using the Setup option, all wells were labelled accordingly and the thermal profile was then adjusted to include a 15 minute enzyme activation at 95°C (Fig 2.3). Each run was set at 2.5hrs. Results were analysed using MXPro® Software.

WELL	1	2	3	4	5	6	7	8	9	10	11	12
A-*16SrRNA*	1F:6R	1F:6R	1F:2R	1F:2R	1F:1R	1F:1R	2F:1R	2F:1R	6F:1R	6F:1R	NPC	NTC
B-*gyrB*	1F:6R	1F:6R	1F:2R	1F:2R	1F:1R	1F:1R	2F:1R	2F:1R	6F:1R	6F:1R	NPC	NTC
C-*lrgA*	1F:6R	1F:6R	1F:2R	1F:2R	1F:1R	1F:1R	2F:1R	2F:1R	6F:1R	6F:1R	NPC	NTC
D-*lrgB*	1F:6R	1F:6R	1F:2R	1F:2R	1F:1R	1F:1R	2F:1R	2F:1R	6F:1R	6F:1R	NPC	NTC
E-*spa*	1F:6R	1F:6R	1F:2R	1F:2R	1F:1R	1F:1R	2F:1R	2F:1R	6F:1R	6F:1R	NPC	NTC
F-*hld*	1F:6R	1F:6R	1F:2R	1F:2R	1F:1R	1F:1R	2F:1R	2F:1R	6F:1R	6F:1R	NPC	NTC
G-*icaA*	1F:6R	1F:6R	1F:2R	1F:2R	1F:1R	1F:1R	2F:1R	2F:1R	6F:1R	6F:1R	NPC	NTC
H-*icaB*	1F:6R	1F:6R	1F:2R	1F:2R	1F:1R	1F:1R	2F:1R	2F:1R	6F:1R	6F:1R	NPC	NTC

Fig 2.2: Typical QPCR plate set up (F: Forward Primer; R: Reverse Primer; NPC: No primer control; NTC: no template control).
This figure illustrates a typical 96-well plate used in the RT-PCR experiments. Different forward and reverse primer concentrations, with respective controls, for each gene to be investigated are portrayed.

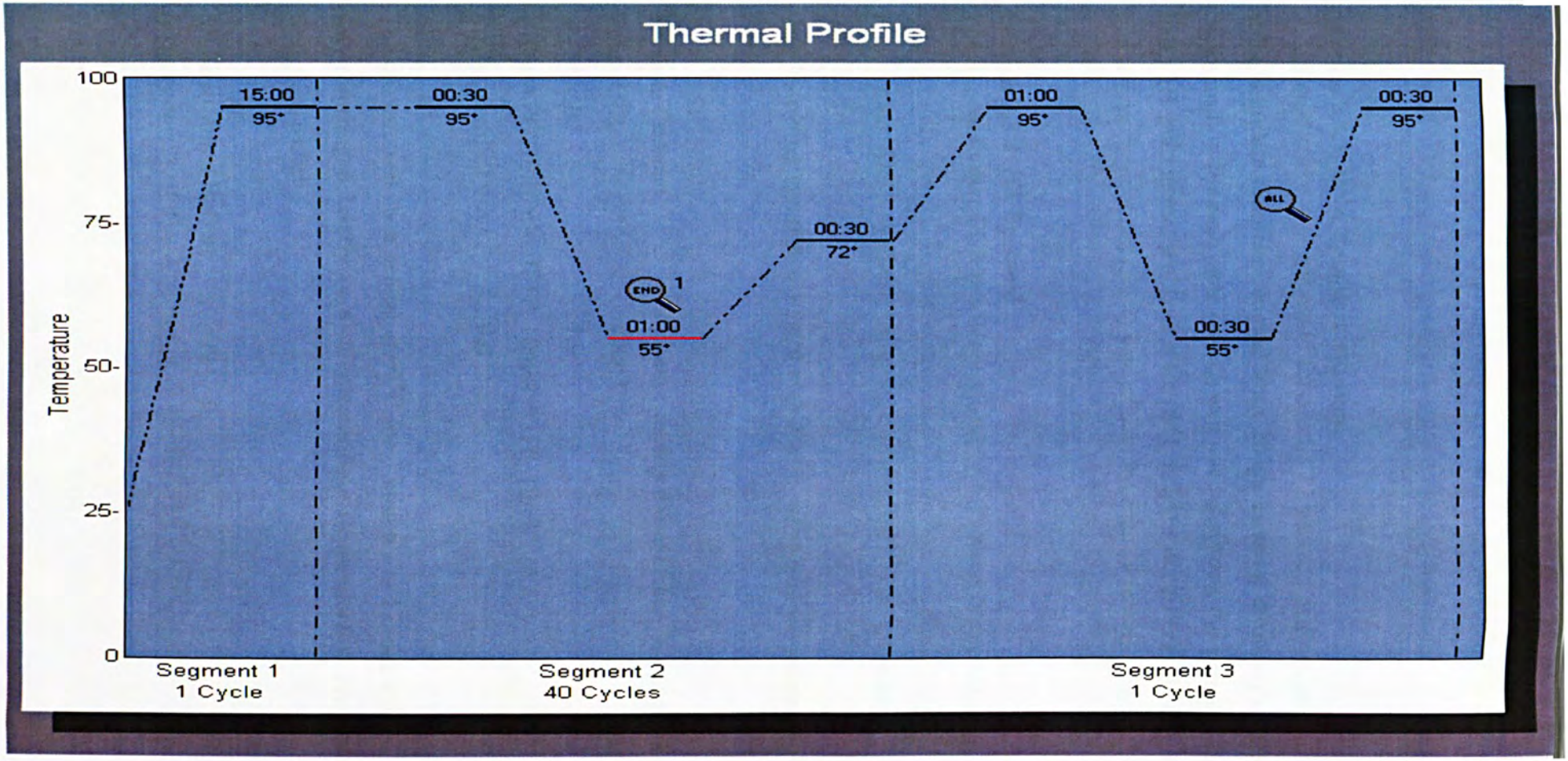

Fig 2.3: Thermal Profile Set-up for SYBR Green with ROX as the reference dye.
Segment 1 represents the enzyme activation phase; Segment 2 typical PCR reactions and Segment 3 represents the melting point or Tm.

Section 2.3.3: DNA Template dilutions

A standard curve was generated for all the 12 genes. Each sample was done in duplicate, using template dilutions ranging from 200ng in serial dilutions of 1:10. The primer-mix concentration for each gene was chosen after analyzing the previous results and all genes had a 1:1 ratio primer pair except for *hld* (1F:2R) and *clfA* (1F: 6R). A non-template (NTC) and non-primer control (NPC) was present for each gene with every plate run.

Section 2.3.4: RT Protocol

For all quantitative PCR reactions, a 2-step RT Protocol was used.

First-strand cDNA synthesis using Superscript II RT® (Invitrogen):

The following components were added to a nuclease-free microcentrifuge (Eppendorf®) tube:

Random Primers:	250ng (initially diluted from 3ug/μl stock concentration)
Total RNA:	400ng
dNTP Mix:	1μl
(10mM dCTP, 10mM dTTP, 10mM dGTP, 10mM dATP)	
Sterile, distilled water:	9μl

The mixture was then heated to 65°C for 5 minutes and quick chilled on and the mixture was centrifuged briefly in a microcentrifuge (Eppendorf®). The following components were then added:

5X First-Strand Buffer:	4μl
0.1M DTT:	2μl

The contents were then carefully mixed and incubated at 25°C for 2 minutes. Finally, 1µl of SuperScript II RT® (Invitrogen) was added and mixed by pipetting upwards and downwards. The mixture was then placed in a ThermoHybaid® thermocyler and tube incubated at the following temperatures:

- 25°C for 10 minutes,
- 42°C for 50 minutes and
- Heated at 70°C for 15 minutes.

The resultant cDNA was kept at 2-8°C and used as stock for all the experiments. One RT was sufficient for all subsequent experiments for this chapter. In addition, cDNA was diluted 1:10 because *16SrRNA* Ct values initially were <1 and did not produce the characteristic sigmoid curve (as in Chapter 5: Fig 5.4a). By diluting cDNA, Ct values were >1 and constant for all the samples and in all the experiments. No RT controls were required on each plate run, due to possible variation in fluorescence. Hence for no RT controls, RNA dilutions were set up for each particular sample (equivalent to the theoretical amount which would be present if no RT occurred i.e. 2ng) and also no template controls for each gene combination were included with each plate run. For each assay, 3 biological replicates and 3 technical replicates of each condition were included for all the samples, i.e. BHI, ASM and GASM. These samples were the same ones used for the microarray experiments (Chapter 4) and this was considered essential in order to validate the microarray results.

Section 2.3.5: Method of Analysis

Relative fold differences were calculated by importing individual Ct values from the Stratagene software, MXPro®, directly into MS Excel®. The 2 reference genes chosen i.e. *16SrRNA* and *gyrB* were used to normalize sample Ct values. In addition, all normalised Ct values were also normalised to the BHI Ct values, as this was the condition to which ASM and GASM were also normalised in the microarray analysis in Chapter 4. This was done according to the mathematical derivation: $2^{-\Delta\Delta Ct}$ (Stratagene® Manual, 2005).

Using SPSS Version 14, the 2-tailed, paired, Student t test was then utilised to investigate differences between BHI and ASM and BHI and GASM Ct values. Statistical significance was achieved when $p \leq 0.05$.

SECTION 2.4: EXPERIMENTS USING HUMAN CYSTIC FIBROSIS SPUTA

Section 2.4.1: γ-radiation sterilization of ASM

Three colonies of MRSA252 were taken from an overnight BHIA culture and transferred to a plastic sterile MSU-container, containing 25ml of pre-autoclaved ASM. This was repeated with a second container having 50ml of ASM. A control for each 25ml and 50ml aliquots was prepared, consisting of 25ml or 50ml sterile ASM without any inoculum. Viable counts were estimated for each aliquot at time 0hrs (25ml container: 4.33×10^6 cfu/ml whilst 50ml container: 1.58×10^6 cfu/ml). All containers (with and without MRSA252) were subjected to γ-radiation from the Cs 137 source at St. George's Hospital, London, at 50Gy for 357 seconds. Viable counts were obtained after radiation exposure.

Section 2.4.2: Ethics committee approval

Ethics Committee approval was granted by Kingston University and also separately by the Royal Brompton Hospital (RBH), London, for the acquisition of human sputum CF samples. These samples were received after routinely processing by the microbiology laboratory for pathogen detection and had they not been used for the present study would have been discarded.

Section 2.4.3: Human CFS Selection

Initially, 41 coded patient samples were obtained from the RBH, once ethics approval was obtained. Each of the samples contained glass beads and was diluted with Ringer's solution. All samples were stored at -20°C prior to usage. However, most of these contained tightly compacted sputum and these were deemed unworkable (as, even after vigorous vortexing, the sputum was still very compact and it was not feasible for viable count estimations) and were not used for the study. Eighteen samples were observed to contain relatively liquid sputum and experiments proceeded on these. Age and sex of patients was provided with each sample.

Section 2.4.4: Screening of CFS for micro-organisms

The first step was to establish whether the CFS samples contained micro-organisms. Therefore, 20µl aliquots of CFS were transferred to a BHI agar plate and growth of bacteria was observed. In addition, 20µl of CFS placed on the selective medium, mannitol salt agar (MSA), to check for *S. aureus* growth. All plates were left for 48 hrs and observed every 24 hours and observations noted.

Preparation of Mannitol Salt Agar CM0085 (Oxoid,Basingstoke, UK)

Mannitol Salt Agar (MSA) was used as a selective medium for the isolation of *Staphylococcus aureus* when human cystic fibrosis samples were being screened. In addition, MSA was used for colony counts when MRSA 252 was grown in patient sputa.

Formula	g/l
'Lab-Lemco' Powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

A proportion of agar powder equal to 55.5g was suspended in distilled water and boiled to dissolve completely. Sterilization by autoclaving was achieved at 121°C for 15 minutes. Adequate mixing was ensured before pouring into sterile plastic Petri dishes.

Section 2.4.5: Monitoring for residual antimicrobial activity in CFS

To check for any residual antibiotic activity in the CFS, a grid plate was designed and placed on the surface of a square Petri-dish (measuring 12cm by 12cm) containing Mueller-Hinton agar (MHA). From overnight cultures of the following bacteria: *Staphylococcus aureus* Oxford 6571 strain, MRSA252, *Escherichia coli* ATCC 11775 and *Pseudomonas aeruginosa* ATCC 10145, grown on BHI agar, bacteria were diluted in Ringer's solution and made up to 10^5 cfu/ml (x 1000 dilution of 0.5 McFarland). An aliquot equal to 100µl of the diluted 0.5Mc Farland was transferred onto the MHA plate and this was swabbed with the respective equivalent Mc Farland bacterial dilution. Then,

10µl (in duplicate) from each patient sample was transferred onto the square plate from each sample and incubated aerobically at 37°C.

Section 2.4.6: Growth Experiments of MRSA252 in CFS

Once the initial screening experiments and antibiotic activity assays were completed and results analysed, two human CFS were selected for further investigation (i.e. Pt 21 and Pt 14). Thus, 1ml from each sample was obtained and transferred to a sterile Falcon tube (12ml) and inoculated with MRSA252 from an overnight culture on BHI agar. The samples were then incubated aerobically at 37°C in a shaker, at 80rpm. Prior to initiating the experiments, the pH of each sample was measured using pre-autoclaved pH paper (Johnson test papers, London, UK). This was performed prior to inoculating with MRSA252.

Growth of MRSA252 in the respective human samples was monitored as mentioned previously (Chapter 3). However, MSA was used instead of BHI for colony count estimations and at each time interval, 10µl aliquots of CFS culture were used and these were serially diluted using Ringers solution for the time intervals: 0,2,4,6,8,24 and 48 hrs.

Section 2.4.7: Light Microscopy Experiments of MRSA252 in CFS

A loopful (equal to 10µl) from the direct CFS culture was taken at the individual time points and placed on a clean glass slide. Each slide contained sample from each test condition, i.e. ASM, Pt21 and Pt14 and this was done with each growth experiment. Each slide was left in air to dry and a Gram-stain was then performed (as described earlier in this chapter in Section 2.1.5.4) and subsequently analysed using Carl-Zeiss® Axioskop light microscope. Digital images were captured with a Nikon DN100 digital camera and an EclipseNet® software package.

Section 2.4.8: RNA Extraction

MRSA252 was grown to exponential phase in the 2 respective samples. Exponential phase was achieved at 3hrs for Pt21 and 4hrs for Pt14. A 50ml Falcon tube was prepared containing 2ml of RNAProtect® Bacteria Reagent (Qiagen®). An aliquot of bacterial culture equal to 1ml was then transferred directly from the culture into the RNAProtect®

and samples treated as described previously. RNA was extracted from the patient samples, as detailed earlier in (Section 2.2.1.2).

Section 2.4.9: RT-PCR

The same primer sequences (and respective concentrations) used in the RT-PCR experiments (described in Section 2.3) for BHI, ASM and GASM were utilised in this chapter. RT-PCR was also set up in the same manner as previously described and 1:10 diluted cDNA aliquots used for the ensuing PCR (for BHI and Pt21). For Pt14, cDNA was diluted 1:5. BHI RNA from Chapter 5 was used for these experiments as a control. No RT controls were also set up to reflect the theoretical amount of RNA which would be present if an RT did not occur (0.25ng for BHI and Pt21 and 0.50ng for Pt14). NTC controls were included for each gene, with each assay.

Section 2.4.10: On-Column DNase Digestion using the RNase-Free DNase® Set

Owing to the low yield of RNA in the human samples, a second-DNAse treatment was performed on the eluted RNA. This was done for Pt14 RNA samples as Pt21 RNA yielded higher concentrations. The low-yield RNA sample was placed onto an RNeasy® Min spin column (Qiagen®) which had been previously placed in a 2ml collection tube. The lid was closed gently and centrifuged at 14secs at 8000g. The flow-through was discarded. An aliquot equal to 350µl RW1 Buffer was added to the RNeasy® spin column and this was centrifuged at 8000g for 15secs to wash the spin column membrane. The flow-through was then discarded. After, 10µl of DNase I stock solution was added to 70µl RDD Buffer and mixed by gently inverting the tube. It was then centrifuged briefly to mobilise residual liquid from the sides of the tube. The DNase I incubation mix (80µl) was added directly to the RNeasy® spin column membrane and incubated at room temperature (20-30°C) for 10 min. Then, 350µl of Buffer RW1 was added to the RNeasy® spin column and left to stand for 5 min. This was then centrifuged for 15secs at 8000g and both flow-through and tube were discarded. The RNeasy Mini® spin column was placed in a new 2ml collection tube and 500µl Buffer RPE was added to the RNeasy Mini® spin column. The lid was then closed gently and tube centrifuged for 15 seconds at 8000g, to wash the spin column membrane. The flow-through was discarded and 500µl Buffer RPE was added to the RNeasy Mini® column. The lid was closed gently and tube centrifuged for 2 min at 8000g to wash the spin column membrane. After centrifugation, the RNeasy Mini® spin column

was carefully removed from the collection tube so that the spin column did not touch the flow-through, otherwise carryover of ethanol would have occurred. The spin column was then placed in a new 2ml collection tube and the old collection tube containing the flow-through, discarded. The tube was then centrifuged at full speed for 1 min. The RNeasy® spin column was placed in a new 1.5ml tube and 30µl of RNase-free® water added directly to the spin column membrane. The lid was closed gently and centrifuged for 1 min at 8000g to elute RNA.

Section 2.4.11: Methods of Analysis

Statistical analyses for the mean bacterial counts were calculated using MS Excel statistics tools and SPSS Version 14.

Statistical analyses for RT-PCR results were estimated using SPSS Version 14.

CHAPTER 3
GROWTH AND SURVIVAL STUDIES OF MRSA252 IN MUCIN MEDIA

SECTION 3.1: INTRODUCTION

Section 3.1.1: Composition of Cystic Fibrosis sputum

Mucus covers mucosal surfaces and acts as a protective barrier, with its most important function being that of shielding epithelial cells. Due to the biochemical abnormalities exhibited in cystic fibrosis (CF) sufferers, these patients exhibit very viscous and sticky mucus (Hansson *et al.*, 2005). The resulting CF secretions offer an ideal environment for bacterial proliferation. In addition, there is increased sulphation of the glycoconjugates which may have an impact on the viscoelastic properties of the mucus (Quraishi *et al.*, 1998). Mucus is also referred to as airway surface liquid (ASL) and consists of water, salts and a number of macromolecules including mucins, proteoglycans, lipids, as well as other proteins (Lillehoj and Chul Kim, 2002).

Section 3.1.2: Mucins in CF sputum

As noted above, mucus is composed in part of mucins, as well as other components. Mucins are essentially high molecular weight glycoproteins, containing one or more multiple protein domains that are sites of extensive O-glycan attachment. Effectively, the composition of these glycoproteins is dominated by carbohydrate which can amount, in some cases, to 80% of the weight of the molecule (Thornton and Sheehan, 2004). Mucins exist in two forms, which include transmembrane and polymeric mucins, as well as a secreted monomeric mucin: MUC7 (Hansson *et al.*, 2005). In humans, transmembrane mucins include MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC16 and MUC17. The polymeric gel-forming mucins include five known mucins present in the human genome: MUC2, MUC5AC, MUC5B, MUC6 and MUC19, all having large sequence similarities in their N- and C-temini (Hansson *et al.*, 2005).

The environment in CF ASL has a pH which is around 6. This acidic environment is adequate for cleaving MUC2. MUC5AC has the same cleavable sequence and so it is possible that this normal respiratory mucin can be cleaved in such an environment (Hansson *et al.*, 2005). CF ASL appears much thinner than normal ASL, thus preventing the mucus gel from floating on the cilia (Hansson *et al.*, 2005).

Gupta and Jentoft (1992) purified tracheobronchial mucin samples from both control individuals and CF patients, using gel filtration chromatography and density gradient centrifugation. Results showed that normal secretions contained high molecular weight mucins whilst CF secretions contained relatively small amounts of high molecular weight mucins, as well as higher amounts of lower molecular weight mucin fragments. The latter could be due to products of protease digestion. Experimental reduction of the disulfide bonds present in the mucins (from both control and CF) yielded in both cases, 2000 kDa fragments. On treating these fragments with trypsin, 300 kDa fragments were obtained from both normal and disease conditions. Also, when unreduced mucin was treated with trypsin, 2000 kDa fragments which could be converted to 300 kDa subunits were obtained. Gupta and Jentoft (1992) also noted that the composition of high molecular weight fractions in CF mucins do not seem to differ from those of normal individuals.

Trivier *et al.* (1997) studied the binding capacity of a mucoid and non-mucoid strain of *S. aureus* with human bronchial mucins, using a radiolabelled based assay. They showed that the non-mucoid strain exhibited a threefold binding capacity to the bronchial mucins when compared to the other, mucoid strain. In addition, when the bacterial cells were treated with lysostaphin and the surface components extracted and challenged with the human mucins, it was revealed that with the non-mucoid strain, three mucin-bonding proteins were obtained but with the mucoid strain only one mucin binding protein was found. The mucoid isolate was a known capsule producer and the authors postulated that the capsule might have limited the binding capacity of the mucoid *S. aureus* strain. This study also showed that the binding capabilities of *S. aureus* to human mucins varied with different strains.

Holmen *et al.* (2004) investigated the mucins produced by second-passage primary human bronchial epithelial cell cultures derived from non-CF and CF patients. Both CF and non-CF cultures produced MUC5B, predominantly. In disease states such as CF, MUC2, which is an intestinal mucin, is produced in limited amounts in the airways (Holmen *et al.*, 2004). Using quantitative Western blotting, Thornton and Sheenan (2004) found that MUC5AC and MUC5B were the predominant gel-forming mucins in sputum and MUC2 was also present but in limited quantities, compared to the other 2 mucins. Higher proportions of MUC5B were detected in CF sputa with a low charge form. The researchers hypothesised that MUC5AC, a product of goblet cells, may have the principal mechanical function of

facilitating ciliary clearance of mucus, whilst MUC5B produced from the glands, may form a gel basis, its main function involving clearance of specific pathogens or other irritants.

Schulz *et al.* (2005) investigated human bronchial explants (after lung transplantation) and used cadavers (without lung disease) as controls. The researchers studied the components of human bronchial tissue from patients with severe lung disease, as well as those with non-CF lung disease and controls without lung disease. Results revealed that MUC5B was the principle protein component of the submucosal secretions and there was no difference between the different conditions. MUC5AC and MUC5B were both detected in the secretions, MUC5B being the predominant mucin present. A recent review by Rubin (2007) stated that CF sputum contains fewer mucins (both MUC5B and MUC5AC) than sputum from normal controls and this could be due to mucins being depolymerised by proteases in CF secretions.

Section 3.1.3: Additional components of CF sputum

CF sputum also contains water, ions, immunological proteins/peptides, proteoglycans, glycoproteins, enzymes and lipids. In addition, glycosylated domains of membrane/tethered mucins shed from the cell surface are present and these, together with proteoglycans, probably contribute to the viscoelastic properties of the airway mucus (Perez-Vilar and Boucher, 2004). Different lipid profiles are present in normal individuals and others with mucus hypersecretory conditions. Healthy individuals tend to have cholesterol and some phospholipids but no glycolipids. Individuals exhibiting hypersecretion of mucus have glycolipids as the major lipid (Lillehoj and Chul Kim, 2002). CF sputum also contains polymorphonuclear neutrophils (PMNs) (Donlan and Costerton, 2002) and nuclear DNA from destroyed leucocytes is also present and accounts for 4% of sputum CF solids (Dwyer, 2005).

Sahu and Lynn (1978) showed that lipids amount to *ca.* 30% of CF sputum, with phosphatidylcholine, as the major lipid component present. Additional lipid components included phosphatidylethanolamine and phosphatidylglycerol, in relatively large quantities. In lesser quantities, hexosyl ceramides, sphingomyelin and phosphatidylinositol were

present. Also, other lipids found included lysophosphatidylcholine and lysophosphatidylethanolamine.

Section 3.1.4: Main Aims

The main focus of this chapter was to establish a reproducible, artificial sputum medium (ASM) mimicking human CF sputum and adequately supporting the growth and survival of MRSA252. Once this is fully established, the effect of eliminating or adding specific components in ASM will be investigated. Physiological characteristics of MRSA252 in ASM will be investigated and elucidated, using light microscopy and protease activities, using zymography.

SECTION 3.2: MATERIALS AND METHODS

Section 3.2.1: Source of Bacteria and Confirmatory Tests

The strain MRSA252 which was used in all the experiments throughout this thesis was obtained from a library of clinical *S. aureus* strains retained at St George's Hospital and originating from the Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford. Further details are provided in Chapter 2: Materials and Methods: Section 2.1.1.

Section 3.2.2: Investigating absorbance of MRSA252 in BHI with added 1% mucin

A detailed account of the materials and methods used in this initial pilot study are provided in Chapter 2: Materials and Methods: Section 2.1.2.

Section 3.2.3: Survival studies of MRSA252 in artificial mucin-containing media and controls

A protocol for these experiments is provided in Chapter 2: Materials and Methods: Section 2.1.3.

Section 3.2.4: Growth Studies of MRSA252 in BHI, BHI and 1% mucin, Ringers and Ringers with 1% mucin

Details for the materials and methods used in these growth studies can be found in Chapter 2: Materials and Methods: Section 2.1.4.

Section 3.2.5: Cystic Fibrosis Artificial Sputum Medium (ASM) Experiments

Chapter 2: Section 2.1.5 and sub sections 2.1.5.1 and 2.1.5.2 provide a full account on the preparation of ASM and the ensuing growth experiments.

Section 3.2.5.1: Statistical Analyses

Statistical analysis was performed as detailed in Chapter 2: Section 2.1.5.3.

Section 3.2.5.2: MRSA 252 Microscopic and Protease Studies in ASM

Chapter 2: Section 2.1.5.4 and Section 2.1.5.5 provide a detailed account of the methodology used for these experiments.

SECTION 3.3: RESULTS

Section 3.3.1: Results for Survival and Growth of MRSA 252 in mucin- supplemented media

It was observed that the presence of mucin in the BHI produced a murky, opaque solution (even when this was diluted) and particles tended to sediment out. This immediately indicated that aborbance was not a reliable indicator of bacterial growth. Also, at 0hr, the absorbance of MRSA252 in the BHI + mucin was noted to be much higher than for BHI alone, for a similar number of bacteria (Figures 3.1-3.2).

Figures 3.1 and 3.2 illustrate the association between absorbance and mean bacterial counts when MRSA252 was grown in BHI and BHI supplemented with 1% mucin (BHI+M) for a 24-hour period. These results were taken from two independent experiments (in duplicate) and the means calculated. It can be observed for Fig 3.1 that absorbance decreases after reaching a maximum and this would be consistent with what typically happens in the stationary phase and phase of decline, where mean bacterial counts decrease steadily. In Fig 3.2, absorbance increases steadily throughout the experiments and does not decrease. This strongly suggested that absorbance is not a good measure of bacterial counts in the BHI + M. Since viable counts are a true measure of bacterial growth whilst absorbance measures both viable and non-viable bacteria, it was decided that viable mean bacterial counts would be used for future experiments.

Figures 3.3-3.5 represent the survival of MRSA252 in BHI, Ringers and Water with added 1% mucin and these were compared to the respective controls. As can be seen in these histograms, in all the different media conditions with added mucin, MRSA252 survived comparatively well and showed growth, when compared to the controls.

Thus, it can be deduced that mucin is not inhibitory to MRSA252, although higher bacterial numbers were obtained in the controls. Mean bacterial viable counts were estimated from duplicate count readings of at least three independent experiments.

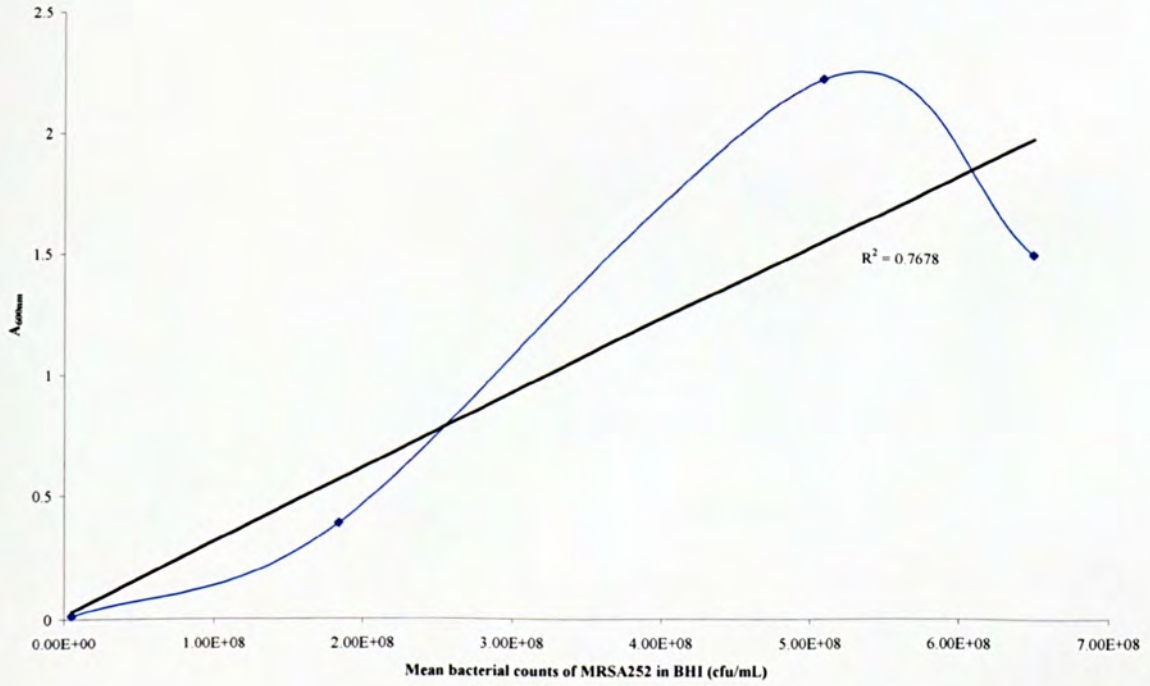

Fig 3.1: Absorbance vs Mean bacterial counts (cfu/ml) for MRSA252 in BHI (Blue line indicates the actual observations whilst the black line is the line of best fit)

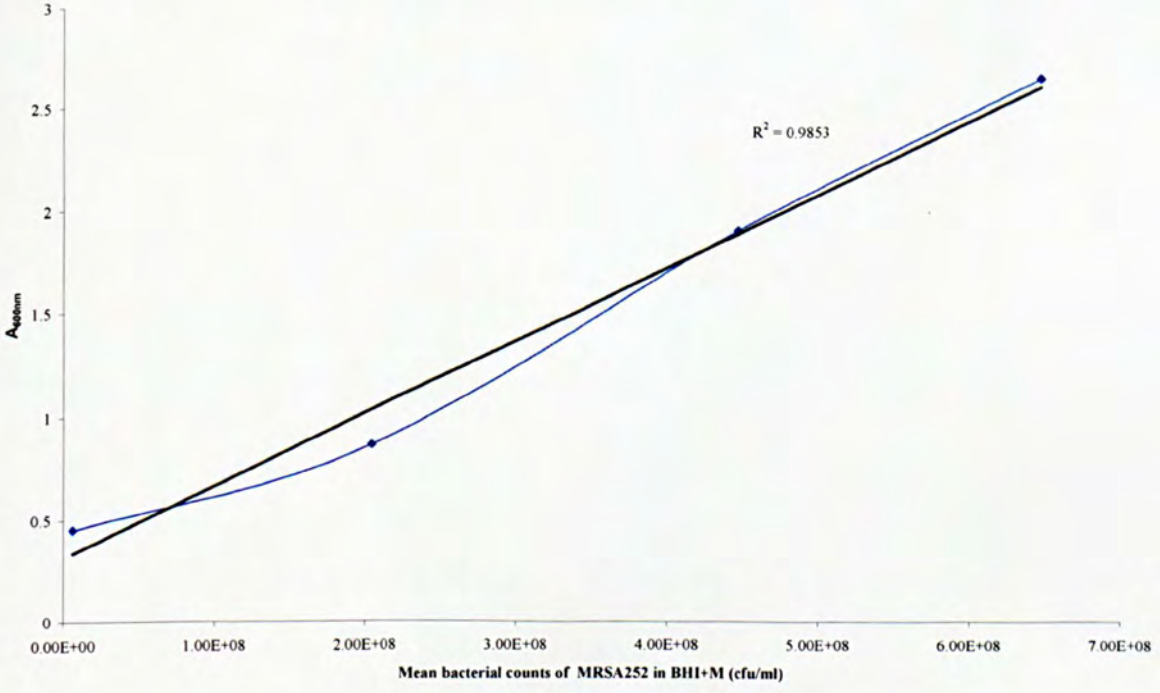

Fig 3.2: Absorbance vs Mean bacterial counts (cfu/ml) for MRSA252 in BHI+M (Blue line indicates the actual observations whilst the black line is the line of best fit).

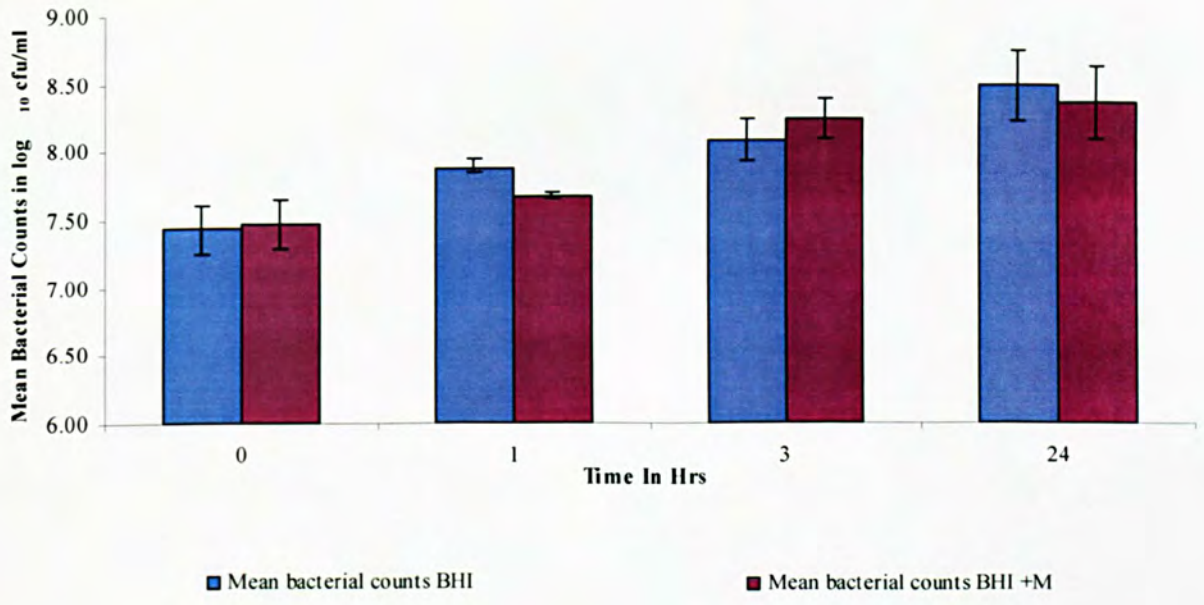

Fig 3.3: Survival of MRSA252 in BHI and BHI + M (Bar: Standard Error of Mean).

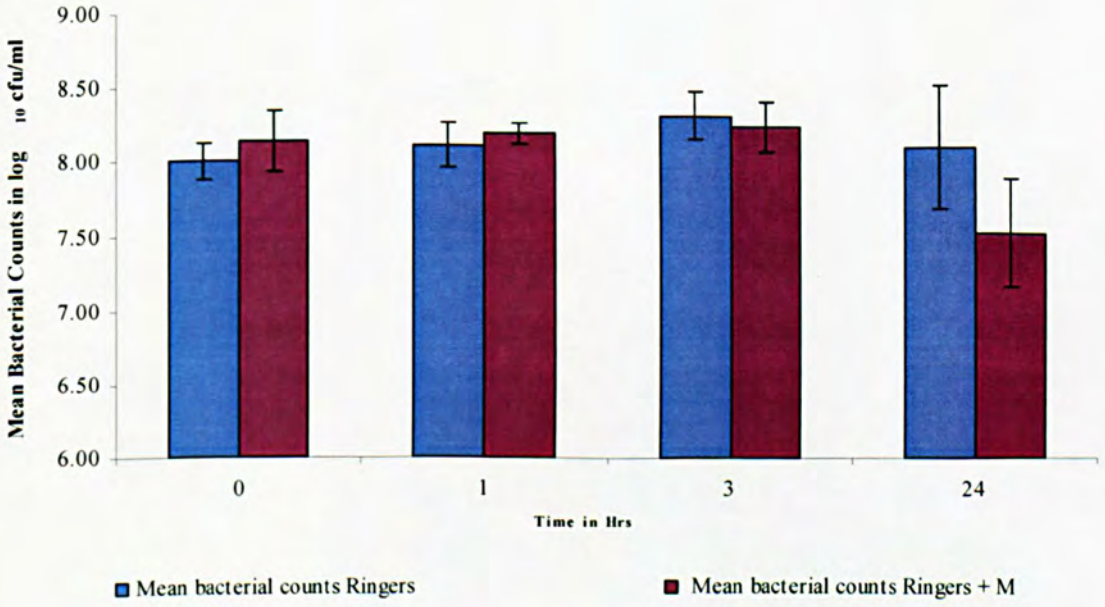

Fig 3.4: Survival of MRSA252 in Ringers and Ringers + M (Bar: Standard Error of Mean)

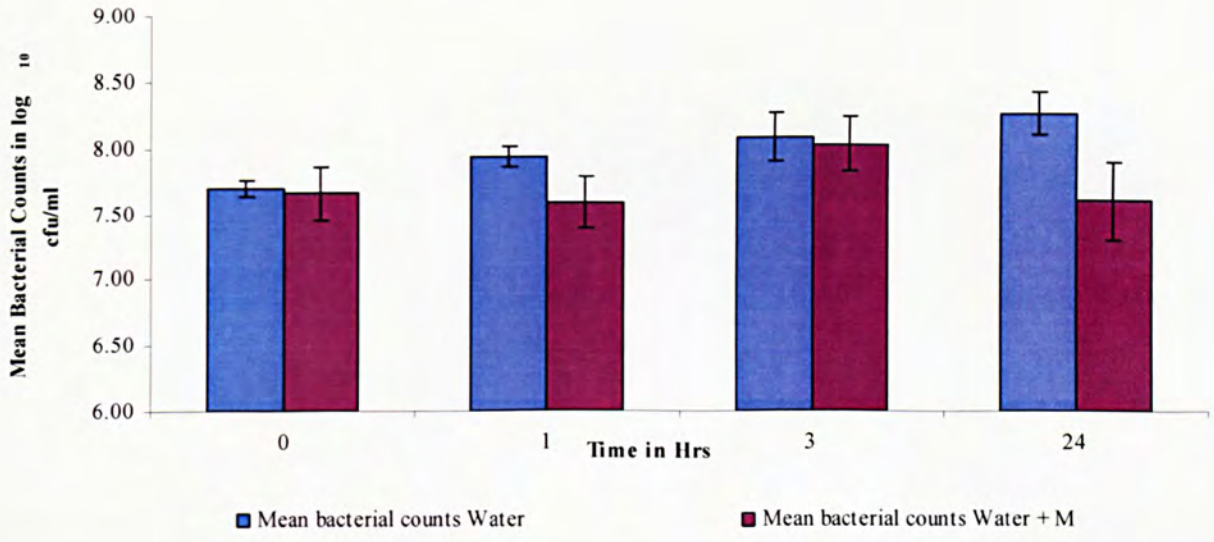

Fig 3.5: Survival of MRSA252 in Water and Water + M (Bar: Standard Error of Mean)

Fig 3.6 overleaf shows growth of MRSA 252 in the presence of mucin, as well as in BHI, the control medium. The presence of mucin is not inhibitory to *S. aureus* growth and survival. Indeed, the mean bacterial levels at all the time intervals, in both mucin-supplemented BHI and BHI control, are quite similar.

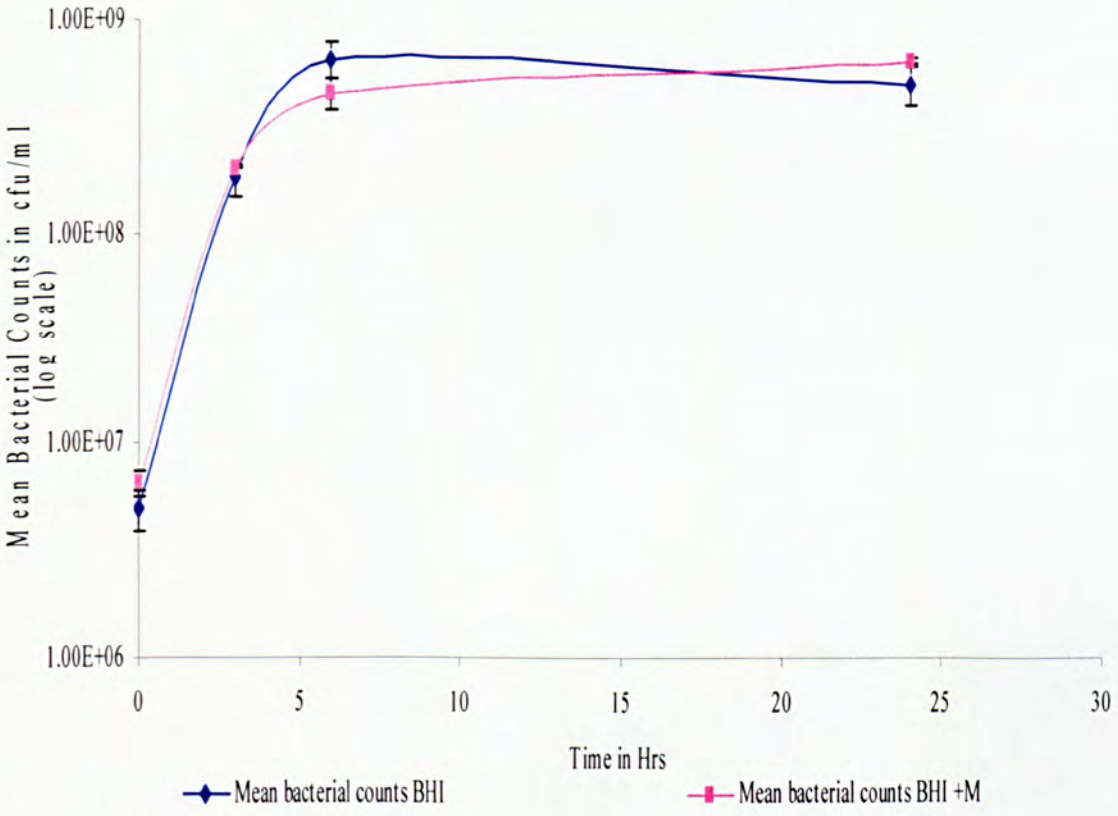

Fig 3.6: Growth of MRSA252 in BHI + M with BHI as control. Averages calculated from two independent experiments (Bar: Standard Error of Mean).

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The table below (Table 3.1) presents pH values for all the mucin media and respective controls.

Table 3.1: pH Readings for sterile mucin-containing media and controls

Media	pH
Ringers	4.76
Ringers + Mucin	3.80
Water	7.05
Water + Mucin	3.65
BHI	7.07
BHI + Mucin	6.70

Section 3.3.2: Results for Growth of MRSA 252 in ASM

Mean bacterial counts were calculated from three independent experiments in all ASM studies. The figure below (Fig 3.7) compares the growth of MRSA252 in BHI and ASM and it can be clearly observed that MRSA252 grows and survives well in ASM, when compared to an enriched laboratory medium, such as BHI. The bacterial growth rate in ASM was observed to be much slower than in BHI.

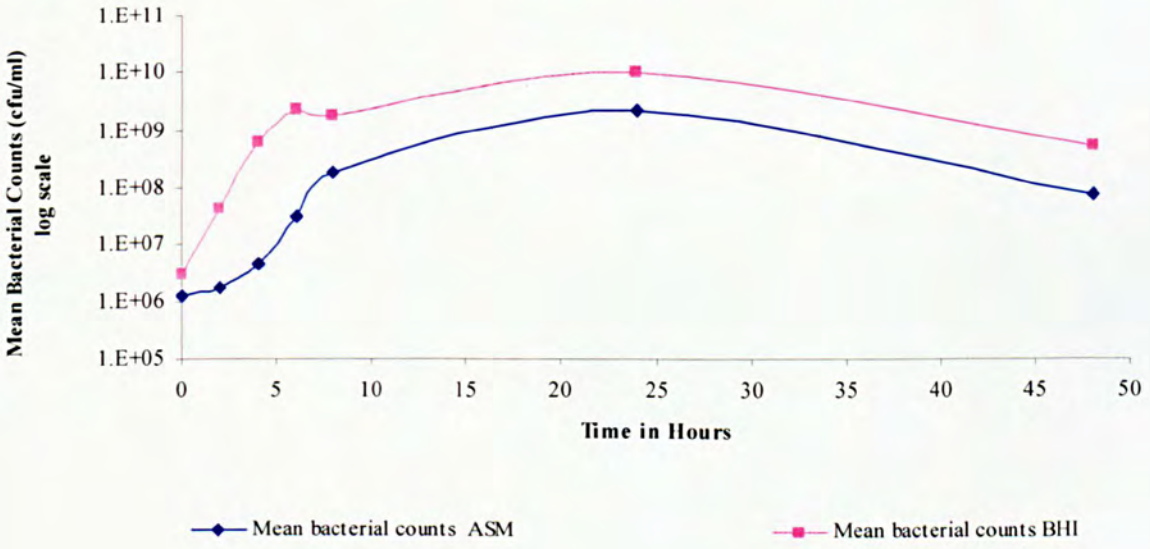


Fig 3.7: Growth of MRSA252 in ASM vs BHI (Bar: Standard Error of Mean)

Fig 3.8 demonstrates that the removal of mucin from ASM was consistent with dramatically reduced survival and no growth of MRSA252 occurred. Fig 3.9 demonstrates that the absence of the amino acids in ASM impacted negatively on the survival of MRSA252.

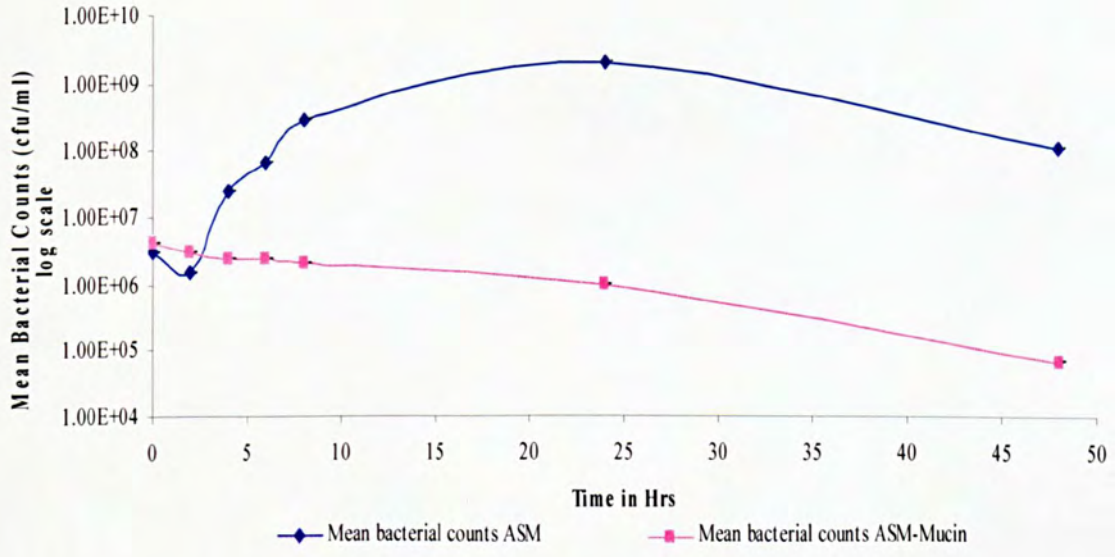


Fig 3.8: Growth of MRSA252 in ASM vs ASM-Mucin (Bar: Standard Error of Mean).

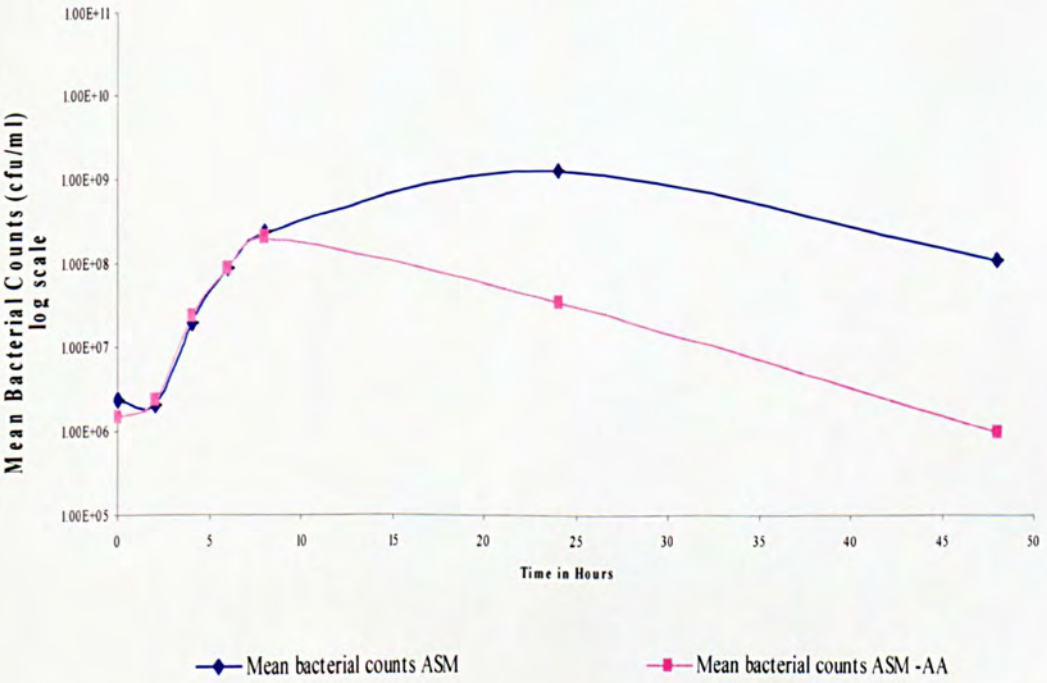


Fig 3.9: Growth of MRSA252 in ASM vs ASM-AA (Bar: Standard Error of Mean)

Cystic fibrosis-related diabetes mellitus (CFRD) may be an added problem to CF patient management (Jefferies *et al.*, 2005; Finkelstein *et al.*, 1988) and so glucose was added aseptically to ASM (at 10mmol/L), in order to simulate a diabetic state. The addition of glucose to ASM (GASM) resulted in an initial rapid growth spurt and stationary phase was attained faster than for the ASM control (Fig 3.10).

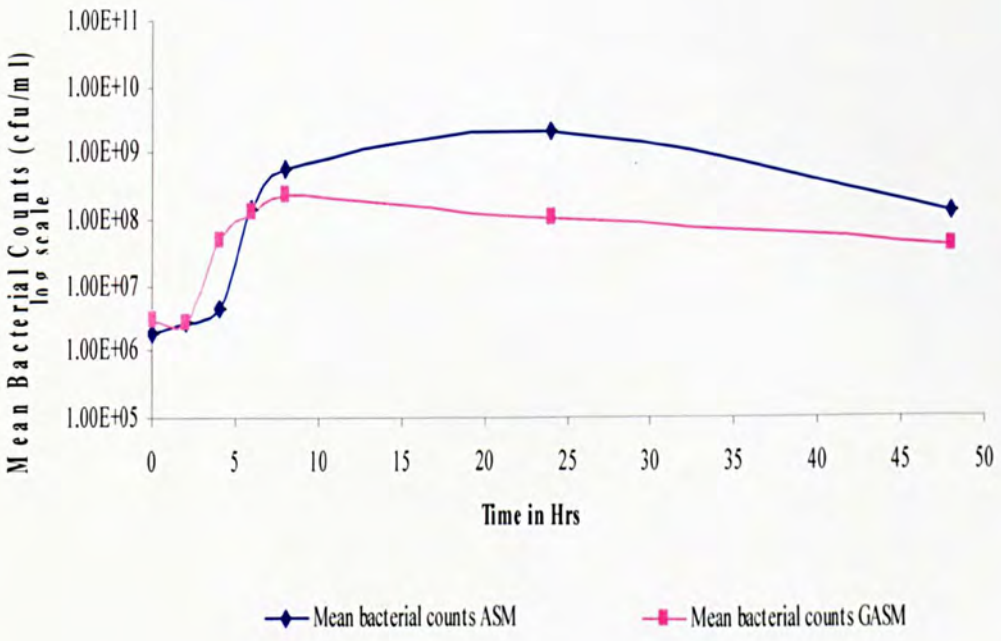


Fig 3.10: Growth of MRSA252 in ASM vs GASM (Bar: Standard Error of Mean)

Removal of sodium chloride from ASM did not seem to have an effect on the immediate growth of MRSA252 (Fig 3.11).

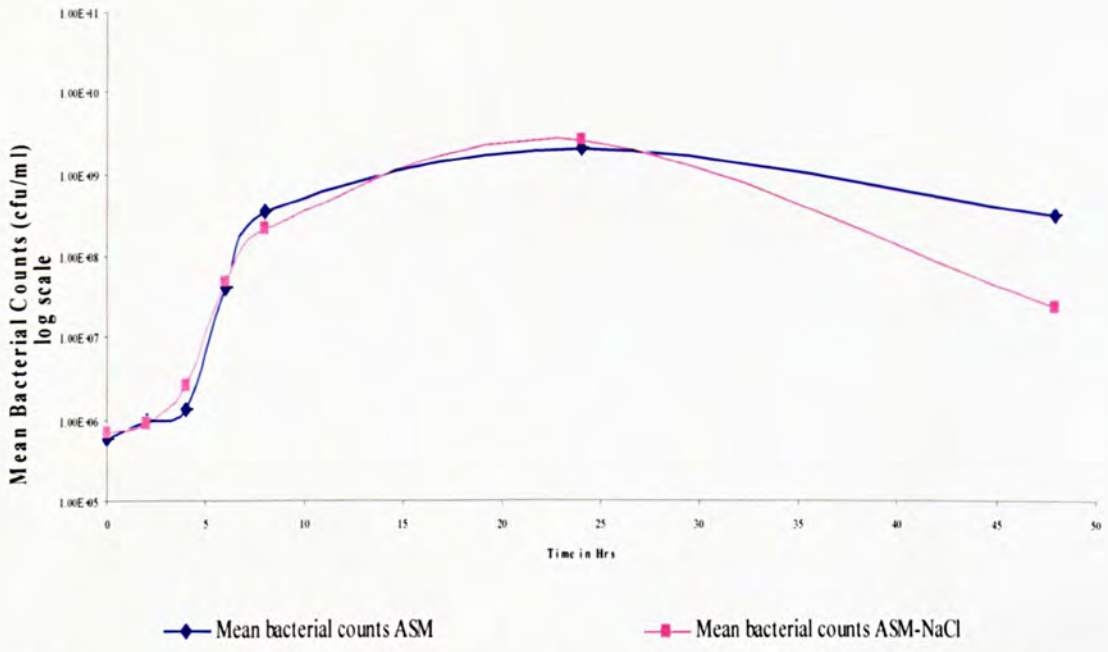


Fig 3.11: Growth of MRSA252 in ASM vs ASM-NaCl (Bar: Standard Error of Mean)

Figures 3.12-3.13 shows the effect of removing DNA and DTPA components respectively from the medium; without DNA, MRSA 252 grew slightly better than in ASM control but with the removal of DTPA, a slight decrease in the growth was apparent. Overall, neither DTPA nor DNA appeared essential for the growth and survival of MRSA252.

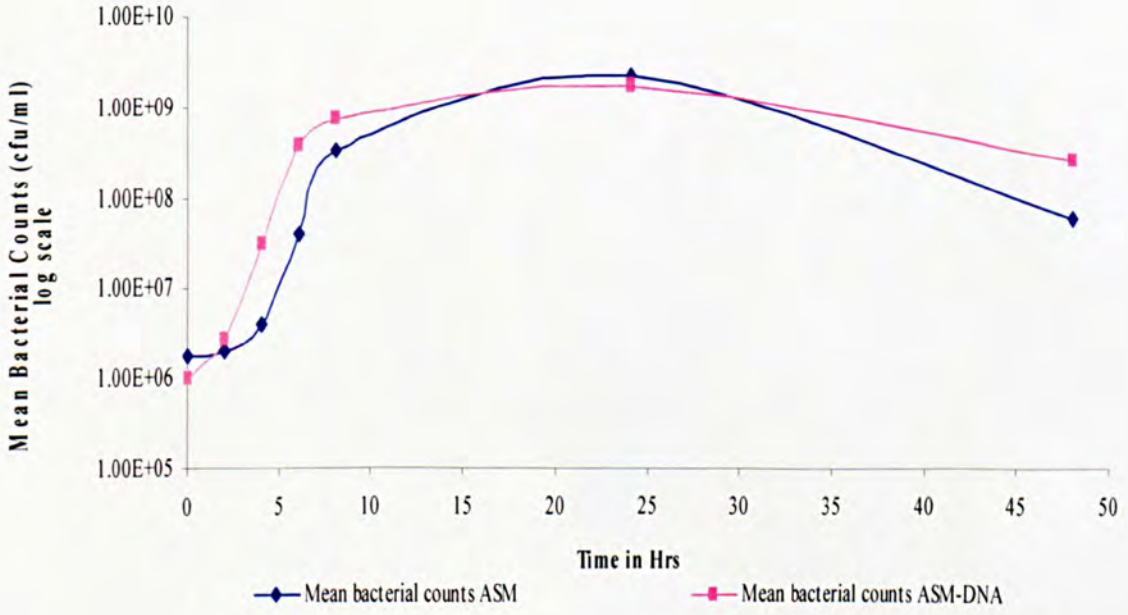


Fig 3.12: Growth of MRSA252 in ASM vs ASM-DNA (Bar: Standard Error of Mean)

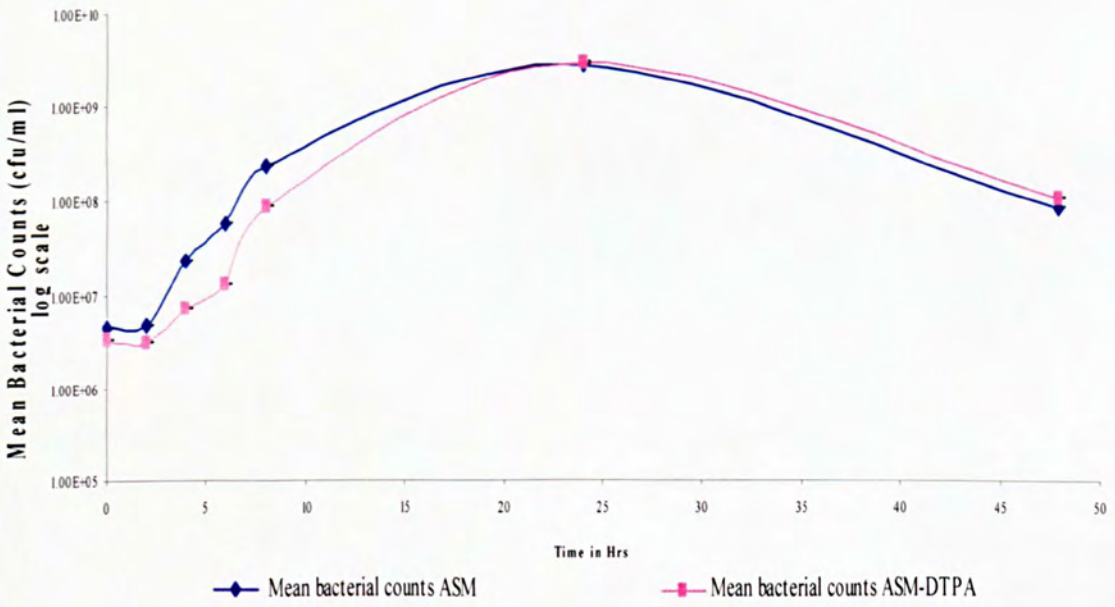


Fig 3.13: Growth of MRSA252 in ASM vs ASM-DTPA (Bar: Standard Error of Mean).

Section 3.3.3: Results for microscopic analysis

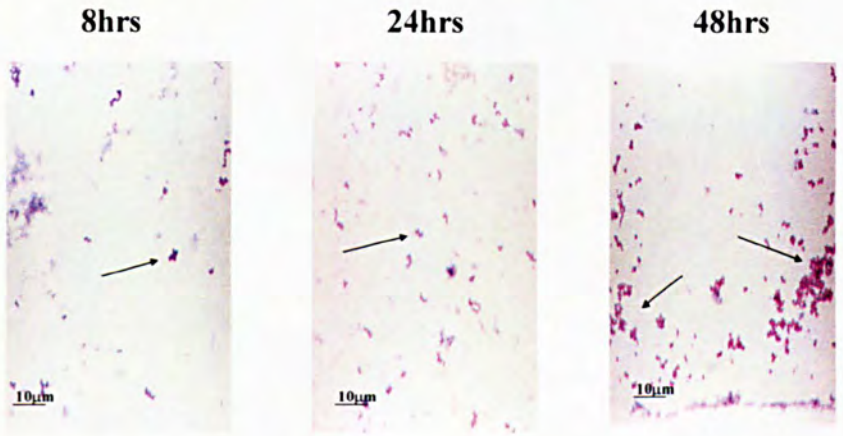


Fig 3.14: Images of Gram-stain preparations of MRSA252 growing in ASM at 8, 24 and 48 hr time intervals (Bar indicates 10µm).
Arrows indicate prescence of cocci, which were noted to be increasing in number with increasing time-intervals. Cocci appeared pink/red rather than dark-purple, as in a typical Gram-positive stain. The latter was a common occurrence when MRSA252 was grown in ASM media. In addition, clearing of the peripheral material (proteinaceous) present was observed with increasing time-intervals, when MRSA252 was grown in ASM.

Additional images of Gram-stain preparations for MRSA252 growing in BHI and all the modified ASM media can be found in Appendix I, Disc One.

Section 3.3.4: Results for V8 serine protease activity with zymogram gels

It was observed that a decrease in proteinaceous material occurred when MRSA252 was grown in the ASM media, especially evident from 8 to 48hrs, suggesting possible production of proteases. Thus, in order to explore this further, casein zymogram gels were used for protease activity detection. The negative control consisted of sterile ASM (in order to confirm sterility an aliquot equal to 100µl was placed onto BHIA plates in duplicate and this was incubated aerobically at 37°C: absence of colonies was consistent with sterility). The positive control (i.e. a V8 serine protease-producing mutant) was also obtained as described in the Materials and Methods section. Initially, gels were loaded with samples retrieved from MRSA252 growing in ASM at 8, 24 and 48hrs. However, as can be visualized in Fig 3.15, no protease activity was observed in the tests. No activity was seen in the negative control but a prominent band was obtained with the positive control.

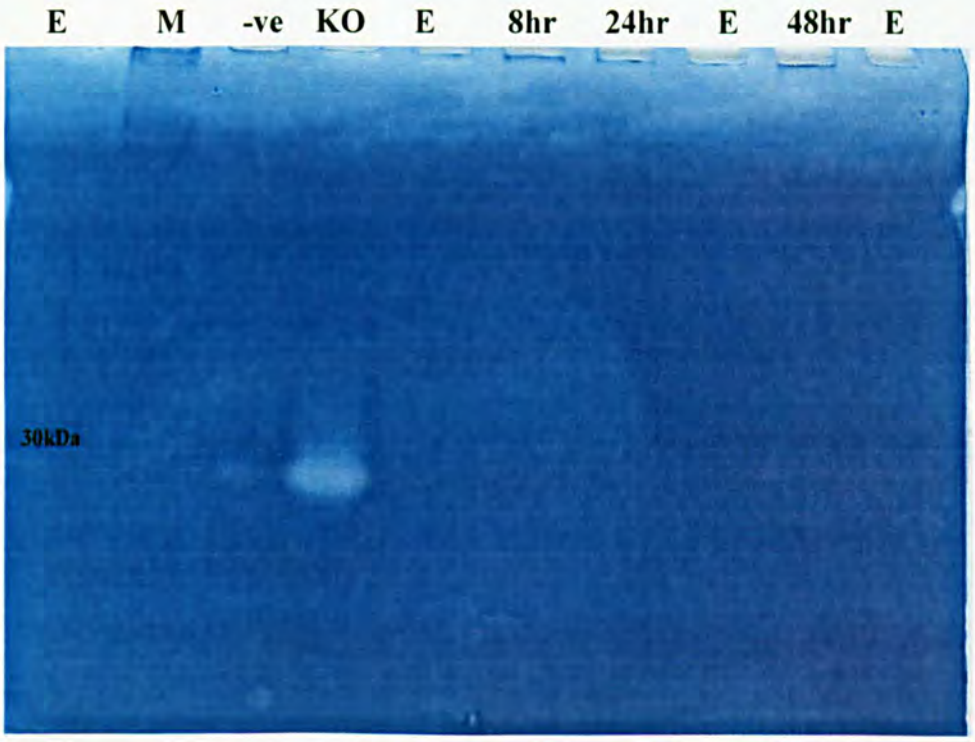


Fig 3.15: Absence of V8 serine protease activity in MRSA252 grown in ASM at 8, 24 and 48hrs (E: denotes empty well; M: Colourburst® Electrophoresis Markers; -ve: sterile ASM and negative control; KO: *sarA* knock-out mutant, which is a V8 serine protease producer; 8, 24 and 48hrs: indicate time intervals when MRSA252 growing aerobically in ASM was harvested. This experiment was repeated at least 5 times and this figure is a representation of these experiments.)

Since no protease activity in the test samples was evident, it was decided to concentrate the samples. However the markers, negative and positive controls were not concentrated. Indeed, concentrating the positive control would probably have led to an increase the band size and this could have given rise to diffuse protease activity, which would potentially distort any ensuing results.

Since microscopic analysis yielded abundant clearance with the 24hr and 48hr, but not so evident in the 8hr time interval, it was decided to concentrate initially on the 24hr time interval. Using a concentrator (as described in Chapter 3 Materials and Methods section), 24hr samples were concentrated to 5X and 10X initially and also to 50X and 100X. No visible protease activity was observed when MRSA252 was grown in ASM at 8, 24 and 48hrs, even when samples were concentrated to 100X.

Section 3.3.5: Statistical Analyses

One-way ANOVA and Tukey`s, post-hoc, multiple comparative analysis tests were used to analyse data obtained when MRSA252 was grown in BHI, ASM, GASM and modified ASM media at different time-intervals.

In BHI vs ASM, no significant statistical difference in mean bacterial counts was obtained when MRSA252 was grown in both media from 0 to 48 hours, indicating that the bacterial numbers in each medium was indeed comparable between the two. This was similar for ASM vs ASM-NaCL, ASM vs ASM-DTPA and ASM vs ASM-DNA.

In ASM vs GASM, a significant difference in mean bacterial numbers was obtained at the 24hour time-interval ($p = >0.001$). For ASM vs ASM-Mucin, at 24 hours, mean bacterial counts in the 2 media were highly significant different ($p = >0.001$). Also, in ASM vs ASM-AA, the mean bacterial counts in ASM at 24hours where significantly different to those in ASM-AA at 24 hours and this was similar for ASM at 48hours vs ASM-AA at 48hours ($p = >0.001$).

SECTION 3.4: DISCUSSION

The principle aim of this chapter was to attempt to create a reproducible medium comprising typical components found in human cystic fibrosis sputum, supporting the growth and survival of *S. aureus*. Artificial Sputum Medium (ASM) was found to be a reliable medium for the growth and survival of the epidemic clinical strain used for these experiments, that is, MRSA252.

The preliminary experiments revealed that recording viable counts was a much more reliable and accurate method of assessing MRSA252 growth and survival, rather than absorbance. In addition, when the experiments with BHI, Ringers and Water and added 1% mucin were conducted (Section 3.4.1), to assess the effect of the mucin component on *S. aureus*, it was revealed that MRSA252 actually did survive and grow, with bacterial numbers being similar to the controls, without mucin. As detailed, in the introduction to this chapter, mucins are found in CF lung secretions and researchers have investigated the effect of these on CF pathogens. CF sputum also is known to contain other components, such as DNA. Thus, these preliminary experiments ascertained that MRSA252 survives and also grows in the presence of one of the known components of human CF sputum, i.e. mucin.

The pH in the CF airway is known to be slightly acidic, typically approximately pH 6 (Hansson *et al.*, 2005, Sriramulu *et al.*, 2005). The mucin used for the experiments in this chapter, was porcine gastric mucin (this was chosen as it is a commercially available mucin and has been used previously in other studies) which may have contributed to the low pH found on addition of mucin to the media (Table 3.1). However, the low pH did not negatively affect the survival of MRSA252 and this is indeed interesting, since the ideal pH range for growth of staphylococci is usually within 7.0-7.7 pH range (Bayani and Azanza, 2005). From the results obtained in Fig 3.3 -3.6, it can be hypothesised that the mucin component might have a nutritious role for the growth and survival of MRSA252.

Ghani and Soothill (1997) initially devised a basic medium similar to CF bronchial secretions. Constituents of this medium included mucin, herring sperm DNA, diethylenetriaminepentaacetic acid (DTPA), hydroxymethylaminomethane (Tris) used to adjust pH of the mixture at *ca.* 6.9, sodium chloride and potassium chloride in water. After autoclaving, egg yolk (representing lecithin) was added aseptically. This biofilm medium was used with additional antibiotics, such as gentamicin, ceftazidime and rifampicin in order to study the survival of *P. aeruginosa* with and without antibiotics. Sriramulu *et al.* (2005) used a modified version of this medium to investigate microcolony formation of *P. aeruginosa* (without antibiotics). They modified the Ghani and Soothill (1997) medium to include 20 amino acids and investigated the effect of removing specific components of the medium on microcolony formation. Sriramulu *et al.* (2005) concluded that *P. aeruginosa* growing in this modified ASM represented a chronic state of infection in the CF lung.

Infections such as those evident in CF patients are often polymicrobial. Mashburn *et al.* (2005) used a rat dialysis membrane model to investigate the effect of growing *P. aeruginosa* alone and in co-culture with *S. aureus*. Gene expression of *P. aeruginosa* grown with *S. aureus*, revealed that genes responsible for iron regulation were suppressed. The authors hypothesised that *P. aeruginosa* actually utilises *S. aureus* as a source of iron for growth. Palmer *et al.* (2005) subsequently utilised human CF sputum, as well as non-CF controls, as a growth medium for *P. aeruginosa*. The sputum chosen for the experiments was positive for *P. aeruginosa* and did not contain antibiotics. The sputum samples were lyophilized overnight and sterilized. Lyophilised powdered sputum was then suspsended in MOPS minimal medium, sonicated and used for subsequent experiments. Although the results of this study overall were interesting, processing the sputum may have altered its components, implying that the growth patterns of *P. aeruginosa* investigated in this study were not truly representative of those occurring in human CF sputum.

From the results obtained with the ASM medium (Section 3.3.2) it was shown that ASM (containing 0.5% mucin w/v) adequately supported the growth and survival of MRSA252. As evidenced by the preliminary experiments conducted prior to using ASM, MRSA252 could tolerate, grow and survive higher mucin concentrations (1% w/v). The removal of mucin from ASM (Fig 3.8) however, had a negative effect on the bacteria, as instead of following the characteristic lag, exponential, stationary and phase of decline, a survival slope was obtained. One-way ANOVA and Tukey`s multiple comparative tests indicated

that when mean bacterial counts in ASM-Mucin and ASM were compared, counts for ASM 24hr were significantly different from counts of ASM-Mucin at 24hr and the rest of the ASM-Mucin mean counts for each time interval of 0, 2, 4, 6, 8, 24 and 48hr ($p = <0.0001$). This strongly suggests that mucin plays a fundamental role in the growth and survival of *S. aureus* when growing in ASM. It might also indicate that mucins are a source of nutrients for the bacteria present and thus their presence during a CF lung infection may actually enhance *S. aureus* growth and survival.

The antimicrobial effects of Lavasept® (containing polihexanide) against MRSA and MSSA strains were investigated by Ansorg *et al.* (2003), who demonstrated that mucin prevents antimicrobial activity. Furthermore, the addition of 0.5% and 1% mucin showed reduced the anti-staphylococcal efficacy of the polihexanide by 90%. Bolister *et al.* (1991) examined the diffusion rates of beta-lactam antibiotics through purified extracellular alginate, (derived from *P. aeruginosa*) and also in mucins, obtained from CF patients. Diffusion of ticarcillin through 1% w/v mixtures of alginate and purified mucus glycoprotein derived from CF sputa, demonstrated that at equal concentrations, alginate was the principle barrier to antibiotic penetration. However, on increasing the mucin concentration to 4% w/v (which is physiologically nearer to the concentration found in CF sputa), the diffusion of ticarcillin was decreased to a much greater extent than with the 1% w/v alginate. This barrier effect on antibiotic penetration was noted to increase when additional components, such as protein and DNA (typically present in CF sputa), were also present.

Previous studies have shown that *P. aeruginosa*, another important bacterial pathogen found in CF lung infections, grows and survives in ASM, an *in vitro* medium closely resembling CF sputum (Ghani and Soothill, 1997; Sriramulu *et al.*, 2005). Fig 3.7 illustrates the growth and survival of MRSA252 in ASM and BHI is used as a control. MRSA252 is observed to grow and survive comparatively well when compared to BHI. Tukey's multiple comparison tests yielded no significant differences between mean counts in ASM and BHI ($p = >0.05$). Conversely, when all 20 amino acids were removed from ASM, mean bacterial counts at ASM 24hrs and ASM 48hrs were significantly different to ASM-AA values ($p = <0.05$; Tukey's multiple comparative tests). Indeed, the initial increase in bacterial numbers observed in the ASM-AA could have been due to 'carry-over' of amino acids from the stationary phase, BHI inoculum. This finding emphasizes

the importance of amino acids for the survival of MRSA252. Indeed, amino acids are known to be present at higher concentrations in exacerbations of CF and with decreased lung function, when compared than in normal sputum (Thomas *et al.*, 2000).
Furthermore, Watson *et al.* (1998), conducted starvation-survival studies with *S. aureus* strain 8325-4 and found that the removal of amino acids from the chemically defined medium (CDM) had a negative impact on the development of a starvation-survival state of the bacterium. In fact, bacterial cultures lost their viability and were non-culturable after a week, whereas bacteria growing in the medium with added amino acids survived for more than 20 days.

The removal of sodium chloride, DNA and DTPA from ASM in this study did not significantly effect the growth and survival of MRSA252. This is surprising since CF secretions contain elevated NaCl levels when compared with healthy individuals (*ca* ≥60mmol/L denotes CF) and *S. aureus* tolerates high salt concentrations ranging from 7-10% NaCl (Greenwood *et al.*, 1994). Lindsay and Foster (1999) showed that high NaCl levels influence gene expression, the *hla* transcript is greatly reduced, whilst serine protease transcript levels are increased.

Ghani and Soothill (1997) utilised DTPA instead of lactoferrin as an iron binder, due to its low cost. However, the results presented in this chapter did not show any significant difference between growth of MRSA252 in ASM and ASM-DTPA ($p = >0.05$).

Sadeghi *et al.* (1994) investigated Gram-stain usage for identifying bacterial pathogens in CF sputa. From 287 respiratory samples, *S. aureus* could be correctly identified in 86.3% of cases although Gram-negative bacteria, such as *P. aeruginosa* had higher detection rates (98%). Overall, the authors concluded that theGram-stain was a reliable test for detecting the presence of pathogens in CF sputum.

Reed *et al.* (1996) conducted a meta-analysis to evaluate the sensitivity and specificity of the sputum Gram-stain in community-acquired pneumococcal pneumonia. The authors reviewed MEDLINE published articles from 1966-1993 and concluded that theGram-stain may produce misleading results in community-acquired pneumonia, in a clinical setting, thereby leading to inaccurate treatment. Problems identified included failure of patients to produce adequate sputum, different thresholds for test positivity and interpreter variability.

In this chapter, Gram-stain microscopy studies revealed that with increasing bacterial numbers, clearing of the extracellular and organic materials present in ASM increased. Although aliquots were taken at each time interval from 0, 2, 4, 6, 8, 24 and 48hrs, it was generally at the 8 to 48hr time intervals the Gram-positive cocci could be observed clearly. Also, in some instances, the characteristic blue/purple colour of the Gram-positive cocci appeared red/pinkish. This could have been due to smaller numbers of bacteria being present in the 10µl aliquot, excessive alcohol decolourisation or else, possible changes induced in the bacterial peptidoglycan cell wall. Also, the prescence of capsular material around the bacterial peptidoglycan cell wall could have prevented penetration of the crystal violet stain, yielding the pink/red colour observed.
Ideally, transmission electron microscopy should have been used, along with the Gram-stain in order to fully investigate the appearance of *S. aureus* in ASM. Previous studies have utilised electron microscopy to elucidate structural abnormalities in small colony variants (Bulger and Bulger, 1967) and others (Giesbrecht *et al.*, 1998) for penicillin-induced structural aberrations in the staphylococcal cell wall. Gram-stain pleomorphims have been described in staphylocci SCVs (Proctor *et al.*, 2006).

Results for the protease experiments showed that V8 serine protease activity was not evident in MRSA252 growing in ASM at 8, 24 and 48hrs. A hypothesis for this occurrence could be this particular protease was actually down-regulated and so V8 serine protease activity was not apparent. The clear band obtained with the positive control was around 33kDa in size and denoted V8 serine protease, as in the study which previously used this same strain of the bacterium (Lindsay and Foster, 1999).

A marked difference between the *S. aureus sarA* mutant and MRSA252 was in the growth medium. Hastie *et al.* (1983) obtained *P. aeruginosa* strains from CF patients and on growing these in TSB, found protease activity. An interesting observation was made by Saravia-Otten *et al.* (1997) who found that when stationary phase *S. aureus* cells were transferred to fresh tryptone-soya broth (TSB) medium, they were still able to produce fibronectin-binding (*fnb*) transcripts. However, when stationary phase cells were placed in BHI for 1hr (i.e growth to early exponential phase), then placed in fresh TSB, this effect was abolished. The authors postulated that when stationary phase bacteria are transferred to fresh medium, they retain the ability to produce *fnb* but when cells are in early exponential phase, this is not the case.

The *sarA* mutant, used in the protease experiments in this chapter, was grown in BHI, whilst the MRSA252 was grown in ASM. The growth rate patterns between MRSA252 in BHI and ASM are different, MRSA252 growing more slowly in ASM than in BHI. This might indicate specific gene expression changes in the MRSA252 genome, as a response to growth in ASM, which is nutritionally less favourable than BHI. Further experiments using microarray and also RT-PCR technologies (Chapter 4 and 5) aim to investigate precisely whether there are differences in gene expression patterns between the two media and also other ASM media (ASM + 10mM glucose: GASM).

SECTION 3.5: CONCLUSIONS

The main target of this chapter, was to explore the physiological and microscopic behaviour of *S. aureus* in an *in vitro* medium which closely resembles human CF sputum. This aim was fully achieved, as *S. aureus*, typified by the clinical epidemic strain MRSA252, was observed to grow and survive well in ASM, when compared to an enriched laboratory medium such as BHI.

Preliminary studies initially that mucin was not inhibitory to MRSA252 growth and survival, despite the lower pH detected, whenever mucin was added to the medium.
In this chapter, specific components were removed from or added to ASM and MRSA252 growth and survival was monitored for each condition.

For the first time, it was revealed that:

- Mucin is fundamental for growth and survival of MRSA252 in ASM
- Amino acids are needed for survival of MRSA252 in ASM
- Glucose may enhance the initial growth of MRSA252 in ASM
- DNA and DTPA do not appear essential and are not inhibitory for the growth and survival of MRSA252 in ASM
- Sodium chloride may have an effect on the survival of this organism in ASM.

Gram-stain preparations showed Gram-positive cocci proliferating with increasing time intervals in ASM, representing the first observations of *S. aureus* growing in ASM on a microscopical level. In addition, this chapter raised questions regarding possible differences in gene expression of MRSA252 growing in BHI, ASM and GASM. This will be fully investigated in the following chapters.

CHAPTER 4

TRANSCRIPTOME PROFILING OF MRSA252 GROWING IN BHI, ASM AND GASM

SECTION 4.1: INTRODUCTION

Gene expression may be defined as, "the overall process by which a gene produces its product and the product carries out its function" (Russell, 2006). This chapter investigates the gene expression of MRSA252 in three different media: brain heart infusion (BHI), an *in vitro* artificial sputum medium (ASM) and GASM, which is ASM supplemented with glucose, using microarray technology.

DNA microarrays are tools which detect pre-labelled (usually with fluorescent dyes) nucleic acids present in typical biological samples. Such tools are highly specific and detect not only the presence but also signal the abundance of the nucleic acids present, which in turn hybridise specifically to a DNA template. A microarray is physically a solid surface, which may consist of glass, silicon, nitrocellulose, or less frequently, a nylon membrane. It contains specific DNA molecules which have been chemically bonded at specific sites. An array location, or probe, may be found at different sites on the array, as replicates. Each probe is highly specific and binds only to the corresponding mRNA molecules, which is turn correspond to a specific gene. Probes consist of PCR products or oligonucleotides (Baldwin *et al.*, 1999; Richmond *et al.*, 1999; Winzeler *et al.*, 1999; Cummings and Relman, 2000; Conway and Schoolnik, 2003; Russell, 2006).

Fig 4.1 describes the basic steps involved from the production of a microarray, from using for instance, glass or silicone, down to computer-based data analysis. Fig 4.2 further illustrates these steps pictorially.

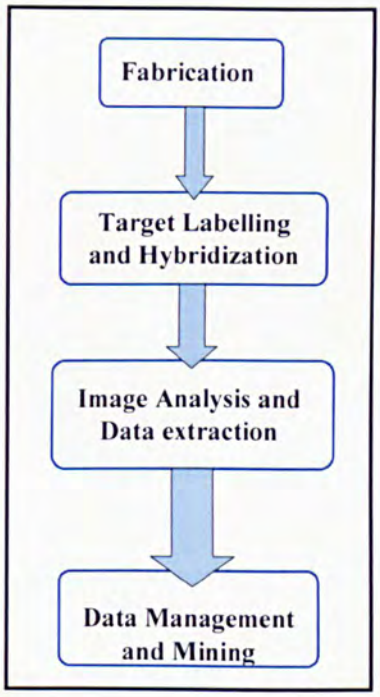


Fig 4.1: Flowchart showing the steps involved from the initial production of a specific microarray, to the final step of data analysis (Adapted from Duggan *et al.*, 1999).

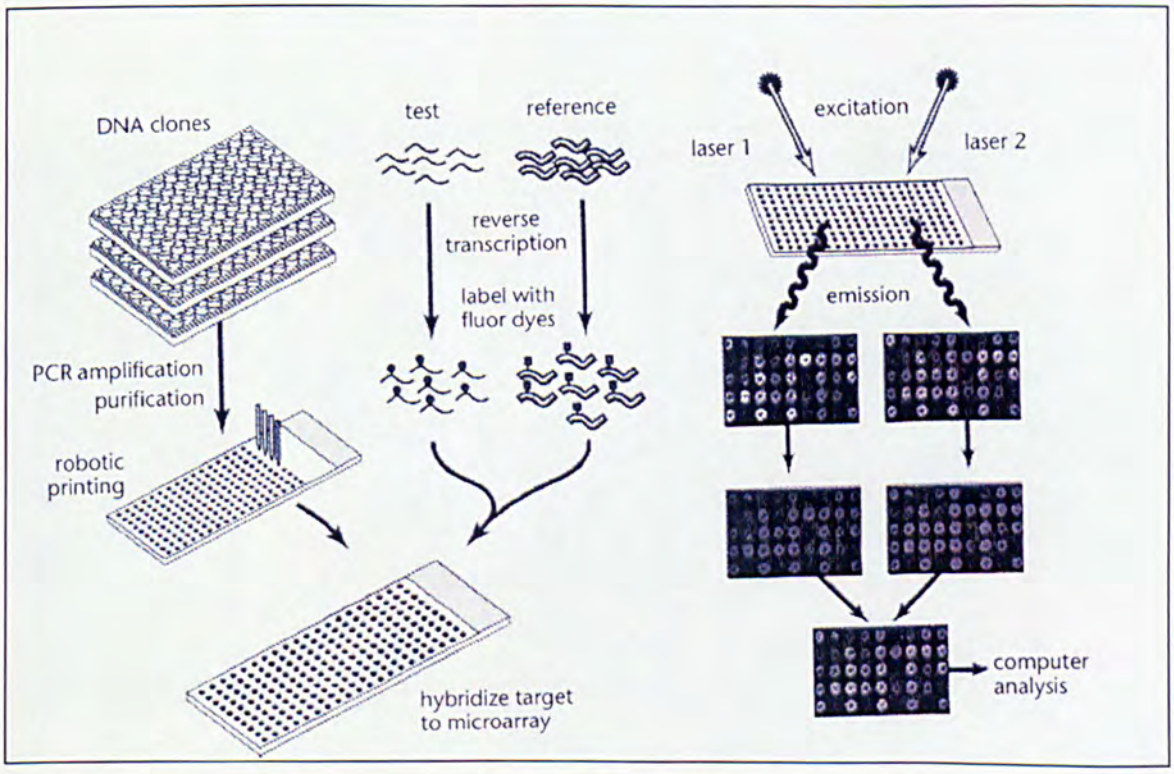


Fig 4.2: A schematic representation of a typical cDNA microarray experiment (Reproduced from Duggan *et al.*, 1999).

Microarray analysis can provide the suitable answer, when exploring transcriptomic bacterial gene expression during a particular event, especially when the bacterium is exposed to a specific environment (Rhodius *et al.*, 2002; Conway and Schoolnik, 2003). This approach enables analysis of the entire genome, rather than just focussing on limited gene activities, which could lead to a disjointed image being created.

Additional points to be considered when RNA is used in microarray experiments, concern the quality of RNA used. Since mRNA has a very short half-life, the extraction of RNA should be done swiftly and RNA concentrations ascertained prior to the microarray experiment. Also, in order to enable effective comparison between different RNA samples from different experiments, it is advisable that genomic DNA be used as the reference channel (Hinton *et al.*, 2004). This also offers quality control for the spots on the array, as genomic DNA also hybridises (Hinton *et al.*, 2004).

Section 4.1.1: Main Aims

The findings in Chapter 3 led to the production of a reproducible, *in vitro* artificial sputum system (ASM), which actively supports the growth and survival of MRSA252.

The aims of this chapter were to conduct microarray experiments on MRSA252 in exponential phase, growing in BHI, ASM and glucose supplemented ASM, (GASM). By obtaining RNA from MRSA252, transcriptomic gene expression would be conducted, using microarrays. In order to confirm the transcriptomic profiles in the different media, gene expression using ten specific genes (found to be differentially regulated in ASM and GASM, when compared to BHI) would then be investigated using RT-PCR.

SECTION 4.2: MATERIALS AND METHODS

Section 4.2.1: Preparation of samples for Microarray Analysis

Section 4.2.1.1: DNA Extraction

A detailed account of the DNA extraction method adopted for these experiments is provided in Chapter 2: Materials and Methods, Section 2.2.1.1.

Section 4.2.1.2: RNA Extractions

A complete protocol of the RNA extractions conduced in this chapter is provided in Chapter 2: Materials and Methods, Section 2.2.1.2.

Section 4.2.1.3: Bioanalyzer Experiments

The Agilent 2100 Bioanalyzer® was used in order to check the RNA purity, prior to performing the microarray experiments. The entire procedure can be viewed in Chapter 2: Materials and Methods, Section 2.2.1.3.

Section 4.2.2: RNA vs DNA Microarray Protocol

The RNA vs DNA microarray protocol used for these microarray experiments is fully described in Chapter 2: Materials and Methods, Section 2.2.2.

Section 4.2.3 Microarray Software Analysis

The arrays were scanned using an Affymerix® Scanner (Genetic Microsystems GMS 418). The scanned arrays were analysed first using Imagene® and then BlueFuse® (BlueGnome, Cambridge, UK) software. Further details are provided in Chapter 2: Materials and Methods, Section 2.2.3.

SECTION 4.3: RESULTS

The results demonstrated up-regulated MRSA252 genes in ASM, with a fold difference ≥ 1.3 when normalised to BHI (Appendix II, Table 1, CD One) and down-regulated MRSA252 genes in ASM with a fold ≥1.3 (Appendix II, Table 2, CD One). Up-regulated MRSA252 genes in ASM amounted to 11% of the total genome and down-regulated MRSA252 genes in ASM amounted to 9% of the total genome. Taking MRSA252 genes in ASM which were differentially regulated by ≥2 fold (p= 0.05), up-regulated genes were equal to 9% of the total MRSA252 genome, whilst down-regulated genes were equal to 6% of the genome, with this same fold (Table 4.1).

MRSA252 genes in GASM which were differentially regulated in a similar manner, when normalised to BHI, follow suit (Appendix II, Table 3, CD One, for up-regulated genes and Appendix II, Table 4, CD One for down-regulated genes). Up-regulated MRSA252 genes in GASM amounted to 15% of the entire MRSA252 genome with a fold difference of ≥1.3 (p= 0.05) and down-regulated genes 14% of the genome, with this same fold. Taking a cut-off fold of ≥ 2.0 (p= 0.05), 12% of MRSA252 genes in GASM were observed to be up-regulated, whilst 9% were down-regulated with this stringent cut-off value. Analysing the global MRSA252 gene expression profile of ASM vs BHI, it was apparent that the capsular genes (amounting to 14 in all) were highly up-regulated with *capC* having the highest fold of 64. Genes encoding for the tricarboxyclic acid (TCA) cycle enzymes were seen to be highly up-regulated as well (Appendix II). These included *citC*, *citZ*, *citB* and *citK* with 11.6, 9.9, 8.1 and 6.8 fold respectively. Highly down-regulated MRSA252 genes in ASM, normalised to BHI, included *lrgA* and *lrgB* encoding holin-like proteins (with 10.8 and 4.9 fold respectively). Also, *spa*, encoding Protein A, was down-regulated by 9.2 and *sbi* encoding an IgG-binding protein with 3.6.

Functional classes of each gene are provided alongside the gene description in Appendix II and differentially-regulated genes, divided into respective functional classes may be viewed in Fig 4.3 for normalised MRSA252 genes in ASM and GASM.

Putative, conserved hypothetical and hypothetical proteins accounted for the highest proportions of genes expressed and a similar pattern was observed for GASM genes normalised to BHI (Fig 4.3).

Table 4.1: Differentially regulated MRSA252 genes in ASM and GASM vs BHI

A 100% was taken to be equivalent to 2,671 genes, as stated in Holden *et al.*, (2004); Genes with a cut-off fold value of ≥1.3 and ≥2.0 (p= 0.05) were compared in ASM and GASM, when these were normalised to BHI.

DIFFERENTIAL REGULATION OF MRSA252 GENES	GENES (%)	FOLD VALUE
MRSA252 genes up-regulated in ASM	11	≥1.3
	9	≥2.0
MRSA252 genes down-regulated in ASM	9	≥1.3
	6	≥2.0
MRSA252 genes up-regulated in GASM	15	≥1.3
	12	≥2.0
MRSA252 genes down-regulated in GASM	14	≥1.3
	9	≥2.0

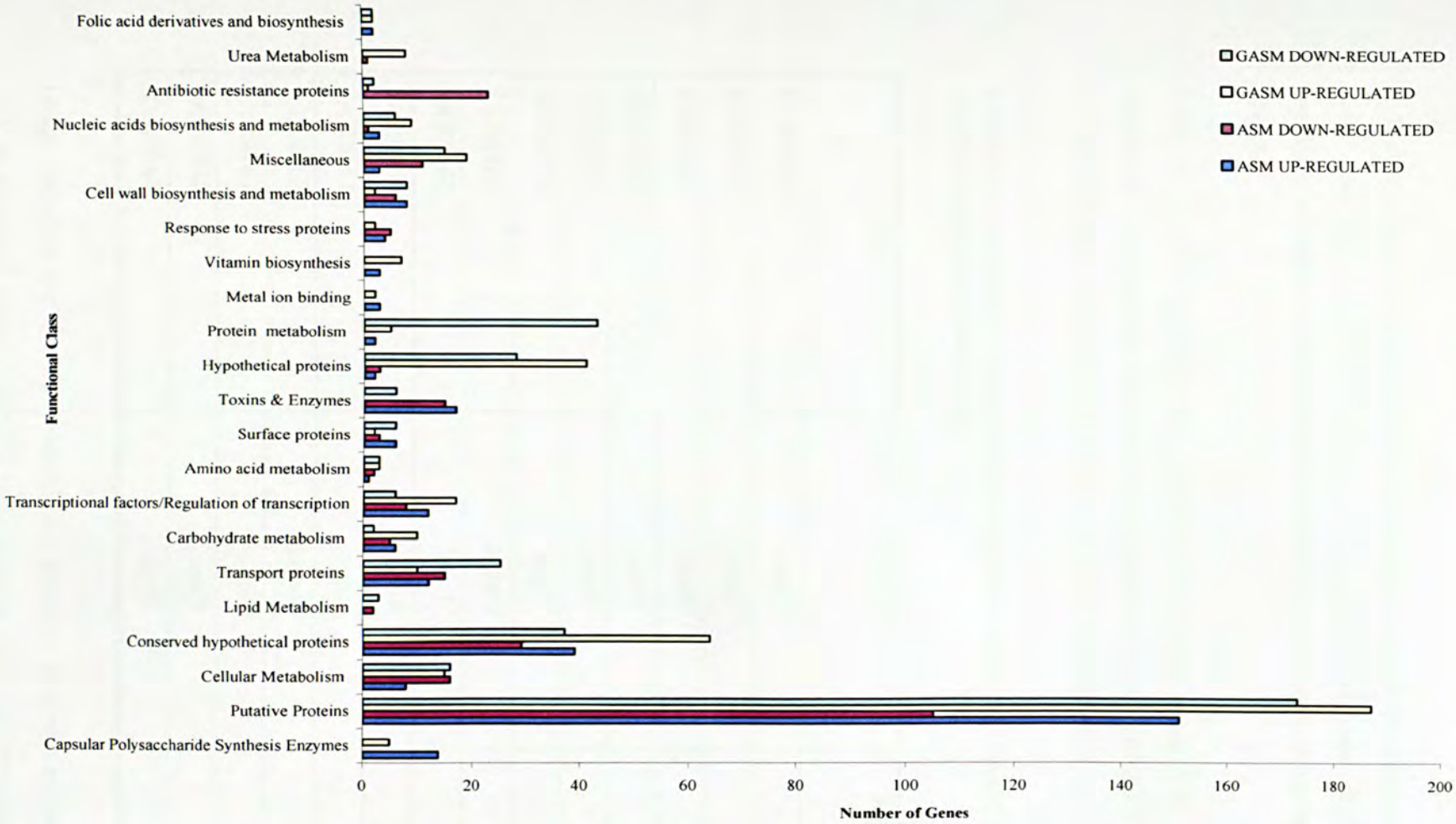


Fig 4.3: Functional classes of up-regulated and down-regulated genes differentially expressed in ASM and GASM, normalised to BHI. All normalised MRSA252 genes in the media (≥1.3 fold with p=0.05) were manually entered in http://www.genedb.org, e (Wellcome Trust, Sanger Institute, Cambridge, UK) and functional classes were assigned accordingly to each gene.

A higher proportion of capsular polysaccharide synthesis enzymes were up-regulated in ASM, compared to GASM (Table 4.1). Folic acid derivatives and its' synthesis were up-regulated in ASM but an equal proportion of genes up and down-regulated were seen with GASM.

Table 4.2 below includes the individual capsular genes with the respective folds. Additional information on MRSA252 genes in GASM (which includes some capsular synthesis genes) normalised to BHI is provided in Appendix II.

Table 4.2: List of the 14 capsular synthesis enzyme MRSA252 genes up-regulated in ASM, when normalised to BHI.

ORF	GENE	FOLD EXPRESSION INCREASE
SAR0153	*capC*	64
SAR0154	*capD*	33.3
SAR0155	*capE*	24.5
SAR0157	*capG*	16.7
SAR0152	*capB*	15.3
SAR0156	*capF*	14.2
SAR0158	*cap8H*	9.8
SAR0162	*capL*	8.6
SAR0161	*cap8K*	8.5
SAR0151	*capA*	8.4
SAR0163	*capM*	6.9
SAR0164	*capN*	6.2
SAR0165	*capO*	4.6
SAR0166	*capP*	3.8

Genes encoding for vitamin biosynthesis, response to stress and metal ion binding were only up-regulated when MRSA252 was grown in ASM. All genes in these categories had a level of increased expression which was >2 fold. *RocD*, (coding for ornithine transferase with transaminase activity and pyridoxal phosphate binding) had a fold increase of 11.6.

Response to stress proteins included catalase (4.1 fold), *asp23* (2.4 fold) and *spoVG* (3.3 fold). A metal ion binding gene, SAR0721 was noted to be up-regulated five fold.

When considering MRSA252 genes in GASM vs BHI, genes required for urea metabolism were up-regulated in GASM, but not in ASM (Table 4.6). In fact, the entire urease operon was up-regulated with >3 fold expression. As for ASM, genes belonging to metal ion binding, response to stress and vitamin biosynthesis were only up-regulated in GASM. In total, 7 genes coding for vitamin biosynthesis were up-regulated at >2 fold. These included: *ribE* (21.7 fold), *ribA* (19.2 fold), *rocD* (17.2 fold), *ribH* (10.9 fold), *ribD* (10.5 fold), SAR2619 (5.5 fold) and SAR0523 (2.9 fold). Genes encoding for metal ion binding proteins, included SAR0135 or *sodM*, encoding superoxide dismutase with a 6.2 fold expression (this was absent from ASM list of normalised genes). Genes encoding for response to stress proteins, included catalase at 6.8 fold as well as *spoVG* (2.6 fold), similar to ASM.

Down-regulated MRSA252 in GASM, normalised to BHI included *lrgA*, which was the most down-regulated gene for GASM, at 9.7 fold. Other down-regulated genes, similarly to ASM, included *lrgB*, *spa* and *sbi* at 8.0, 6.2 and 4.8 fold.

Table 4.3: List of genes postulated to be affected by the *arlRS* operon and present/absent in ASM and GASM normalised gene lists. NDE denotes no differential expression.

ORF	GENE	FOLD EXPRESSION IN ASM	FOLD EXPRESSION IN GASM
SAR1427	***arlR***	2.9	NDE
SAR1426	***arlS***	2.4	NDE
SAR1136	***Hla***	-2.0	-2.0
	Hlb	NDE	NDE
	Lipase	1.8 (*geh;* SAR0317)	-2.1 (SAR 2106; phospholipase C precursor)
	Coagulase	2.3(SAR0222v); 2.5 (SAR0222)	2.9 (*coaE;* SAR1767)
SAR1905	**serine protease**	NDE	-2.6
SAR0114	*Spa*	-9.2	-6.2

Table 4.4: Selected MRSA252 genes differentially expressed in ASM and GASM, normalised to BHI which have a direct or indirect effect on the *lrgAB* operon. NDE indicates that no differential expression observed.

ORF	GENE	FOLD EXPRESSION IN ASM	FOLD EXPRESSION IN GASM
SAR0259	*lrgA*	-10.8	-9.7
SAR0260	*lrgB*	-4.9	-8.0
SAR0257	*lytS*	-2.5	-2.7
SAR0258	*lytR*	-2.0	-3.2
SAR1427	*arlR*	2.9	NDE
SAR1426	*arlS*	2.4	NDE
SAR0625	*sarA*	2.3	NDE
SAR2152	*sigB*	2.0	NDE

Table 4.5: Up-regulated genes from the *icaADBC- icaR* operon in ASM and GASM. NDE indicates no differential gene expression observed.

ORF	GENE	FOLD EXPRESSION IN ASM	FOLD EXPRESSION IN GASM
SAR2747	*icaA*	4.3	1.6
SAR2748	*icaD*	3.0	NDE
SAR2749	*icaB*	3.1	1.8
SAR2746	*icaR*	NDE	2.7

MRSA252 differentially regulated genes in ASM vs GASM were also explored (Appendix II, Table 4.5). In all, 387 genes were differentially regulated. However the fold differences were not dramatically different as for ASM vs BHI and GASM vs BHI. Of the 387 genes, only 28 were down-regulated ≥2 fold (p= 0.05) in ASM and 90 genes were up-regulated ≥2 fold (p= 0.05) in ASM. The most down-regulated ASM gene was SAR2437 with a 4.6 fold. Capsular genes were highly up-regulated in ASM, when compared to GASM, which were also up-regulated but to a much lesser degree than ASM, with *capC* being up-regulated by a factor of 64 (similar to the situation observed with BHI). This was also up-regulated in GASM by a factor of 8.3.

When MRSA252 genes in GASM, were compared to those in ASM, 61 genes were found to be down-regulated by ≥2 fold whilst with this same stringent cut-off value, 116 genes were up-regulated. The gene most up-regulated in GASM was SAR2297 by a factor of 22.8. Conversely this was down-regulated by 2.2 fold in ASM.

SAR1049, SAR1050 and SAR1051 were up-regulated in GASM by 14.6, 19.3 and 19.1 fold and in ASM by 4.6, 5.8 and 4.6 fold. Fig 4.4 below depicts this gene locus. Also, *cysJ* (encoding a putative sulfite reductase [NADPH] flavoprotein alpha-component) was up-regulated by 8.2 fold in GASM and only 1.04 fold by ASM. The urease operon, encoded by *ure* was observed to up-regulated in GASM when compared to ASM and this may be viewed in Fig 4.5. Fig 4.6 depicts the urea cycle and metabolism of amino groups and denotes the position of specific MRSA252 enzymes used in this biochemical pathway.

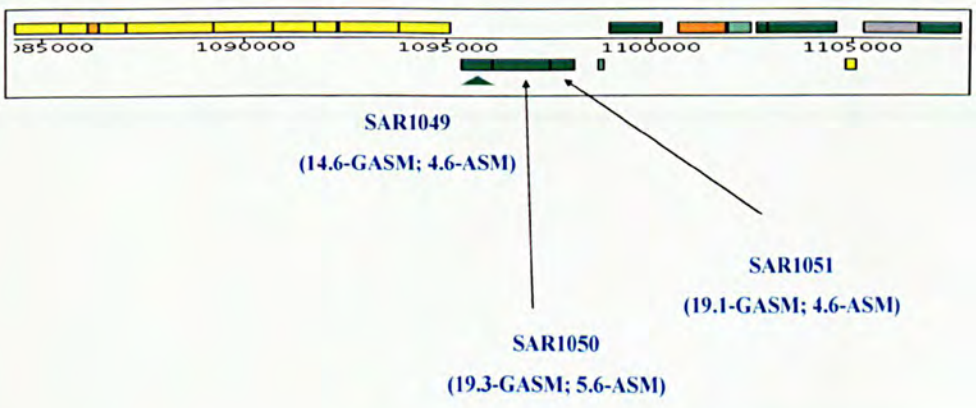


Fig 4.4: Schematic represention of a gene locus in the MRSA252 genome showing ORFs SAR1049, SAR1050 and SAR1051. These genes were found to be amongst the most highly up-regulated in GASM vs BHI and GASM vs ASM. SAR1049 codes for a putative cobalt transport protein, SAR1050 encodes an ABC transporter ATP-binding protein and SAR1051 encodes a putative membrane protein. Fold expression is provided in brackets for both GASM and ASM. The size of the genes (in nucleotides) are indicated by the numbers provided (Adapted from http://www.genedb.org).

Table 4.6: Up-regulation of urease enzyme genes in GASM vs ASM.

ORF	GENE	FOLD EXPRESSION IN ASM	FOLD EXPRESSION IN GASM
SAR2372	*ureA*	1.5	5.1
SAR2373	*ureB*	1.2	3.7
SAR2374	*ureC*	1.3	3.6
SAR2375	*ureE*	1.3	3.2
SAR2376	*ureF*	1.2	3.6
SAR2378	*ureD*	1.7	3.9

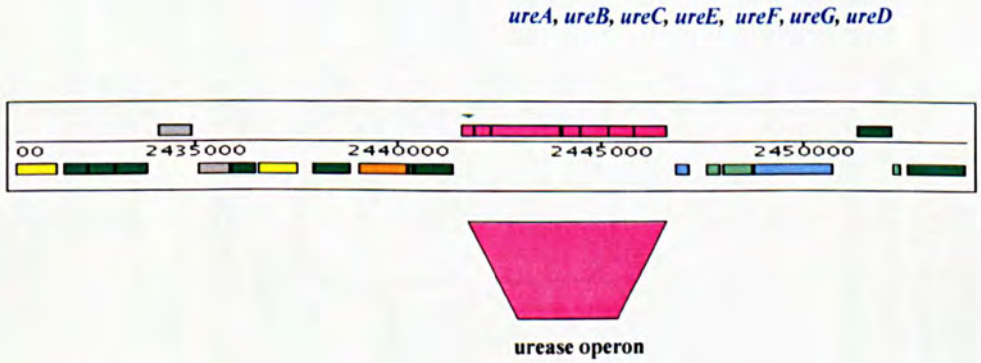


Fig 4.5: The urease operon (encoded by 7 genes: *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, *ureG* and *ureD*) in MRSA252. The small green arrow denotes the start of the operon with *ureA* and the entire urease operon can be seen to have been up-regulated in GASM vs BHI with a fold ≥3 (p= 0.05). The size of the gene (in nucleotides) is indicated by the numbers provided (Adapted from http://www.genedb.org).

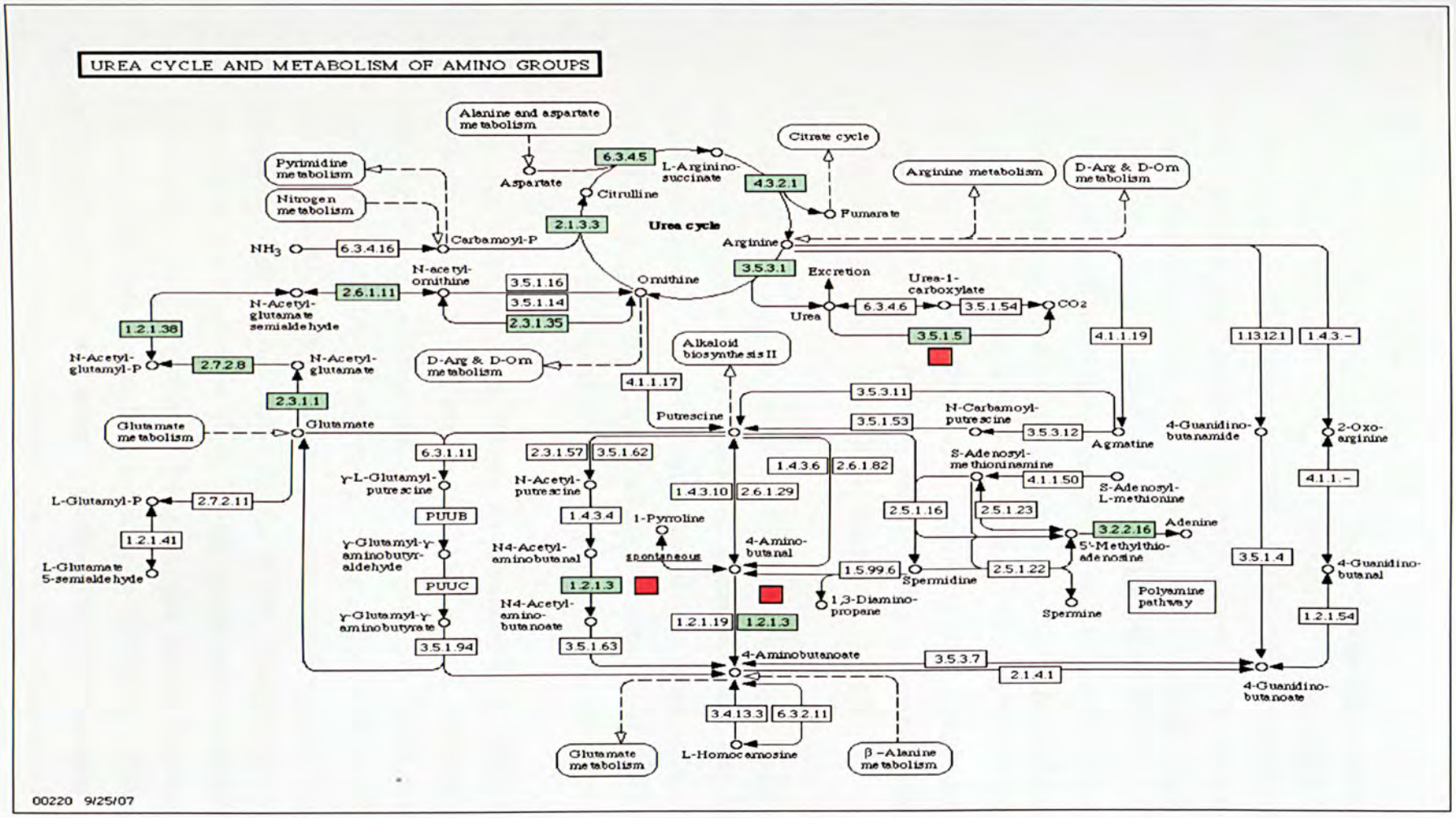


Fig 4.6: The urea cycle and metabolism of amino groups. Green boxes indicate enzymes that are present in MRSA252 and the red boxes show up-regulation of genes coding for these enzymes in GASM vs BHI. EC: 3.5.1.5 represents *ureA* (SAR2372), up-regulated by 5.1 fold and EC: 1.2.1.3 represents SAR0169, encoding a putative aldehyde dehydrogenase with an 11.5 fold increase in expression (Adapted from KEGG or Kyoto Encyclopedia of Genes and Genomes obtained from http://www.genome.jp/kegg/).

SECTION 4.4: DISCUSSION

The advent of microarrays has greatly facilitated bacterial comparative genomics however, this is highly dependent on the design and quality of the microarray as well as the number of genes present (Witney *et al.*, 2005). The microarray used for these experiments was a whole genome gene specific PCR-product based DNA microarray (Dr Adam Witney, personal communication). It was based on seven *S. aureus* whole-genome sequences and contains up to 3,623 probes. These included MRSA252 (an epidemic hospital-acquired MRSA16 clinical strain, originating from a fatal bacteraemia from a UK hospital), N315 (a hospital MRSA strain from Japan), Mu50 (a Japanese hospital vancomycin-intermediately resistant MRSA), COL (a 1961 MRSA strain), 8325 (an MSSA laboratory strain), MW2 (a community-acquired MRSA strain, originating from a fatal paediatric bacteraemia from the US and PVL toxin positive) and MSSA476 (community-acquired MSSA strain from the UK) (Lindsay and Holden, 2004; Witney *et al.*, 2005).

MRSA252 belongs to the epidemic EMRSA-16 clone (proven by PFGE and MLST ST36) and it is resistant to a number of antibiotics, including penicillin, ciprofloxacin, erythromycin and meticillin but sensitive to fusidic acid, rifampicin, tetracycline, trimethoprim, gentamicin and amikacin. It consists of 2,902,619 bp in size which corresponds to 2,671 genes or predicted protein-coding sequences (Holden *et al.*, 2004).

This discussion mainly addresses the significance of differentially expressed genes, primarily in ASM vs BHI and also in GASM vs BHI. ASM and GASM differ from each other, only by the addition of a single component to GASM, which is glucose. Capsular genes were seen to have been highly up-regulated in ASM vs BHI and the significance of this observation is fully tackled. Also, the *lrgAB* operon, found to be highly down-regulated in ASM vs BHI and in GASM vs BHI, is adequately discussed. In addition, this discussion also focuses on exploring the significance of specific transcriptional factors, such as *agr*, *sar* and *sigB* known to affect and control virulence factor production in *S. aureus*. Other focal points in this discussion include *clp* proteins, *spa*, *clfA* and the *ica* operon. Finally, the last section links the main findings in this chapter, with microarray studies in the literature, conducted in a cystic fibrosis setting with *S. aureus* primarily but also with *P. aeruginosa*, another key bacterium afflicting CF patients.

Capsular Genes

A review by O'Riordan and Lee (2004) stated that 11 *S. aureus* capsular serotypes have been reported. Capsular serotypes 5 (CP5) and 8 (CP8) are mostly seen clinically. The authors referred to studies which showed that in iron-deplete media and with NaCl concentrations up to 5% (as in ASM), this promotes capsule formation. Capsule formation starts in the exponential growth phase and maximises in the post-exponential phase. The global regulator *agr* positively regulates capsule formation. In this chapter, approximately 4% of all up-regulated MRSA252 genes in ASM, encoded capsular synthesis enzymes.

The *cap* gene clusters of both *cap5* (capsular Type 5) and cap8 (capsular Type 8) are composed of 16 ORFs. Most of these genes are thought to be involved in amino sugar synthesis, whilst the rest may include functions such as capsule-chain length regulation, polymerization, transport and sugar transfer (Sau *et al.*, 1997).

Pöhlmann-Dietze *et al.* (2000) investigated the influence of *S. aureus* strain Newman CP5, *agr* and bacterial growth phase on bacterial adherence to human endothelial cells. The authors found that capsulated strains produce less adherence then unencapsulated ones. They proposed that CP may inhibit bacterial adherence by masking the presence of an adhesin which is produced postexponentially and is *agr*-independent. A separate study by Luong *et al.* (2002) explored CP8 expression and *agr* and *sarA* activities in the *S. aureus* strain Becker. The results showed that maximal CP8 production occurred during early stationary phase and did not increase after late stationary phase. The same effect on capsule production was observed with different *agr* groups of *S. aureus* strains. In *agr* and *agr sarA* mutants, CP8 levels were minimal, inferring that *agr* activates CP8 production. In contrast, with the *sarA* mutant, CP8 production increased in early exponential phase, decreased to minimal levels in mid-exponential phase and increased from the exponential-stationary transition phase, to maximal levels in late stationary phase.

Table 4.2 in the Results section describes the individual capsular genes with the respective folds and the capsule type 8 is observed, as reported previously by other researchers (Lindsay *et al.*, 2006) for MRSA252 (ST36 and CC30). Fig 4.7 below provides a schematic diagram depicting the position of the capsular gene locus in MRSA252.

Albus *et al.* (1988) obtained 170 *S. aureus* isolates from CF patients as well as healthy individuals and studied the production of CF5 and CP8 using monoclonal antibodies. From all isolates, 85% of staphylococcal isolates produced CPs with 77% of these being CP8. Examination of a *S. aureus* CF isolate in CF sputum revealed that CPs were not only produced in cultures *in vitro* but also *in vivo*.

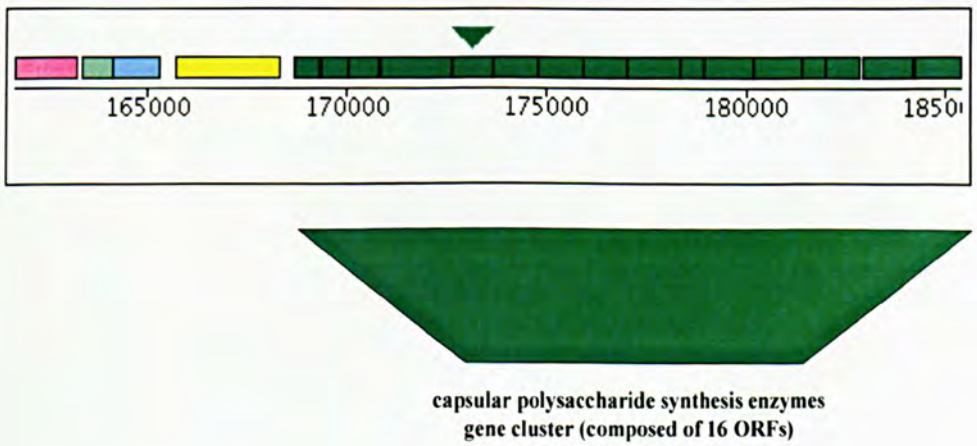


Fig 4.7: Schematic diagram of the capsular polysaccharide synthesis enzyme gene cluster in MRSA252. The small green arrow indicates *capE*. The 16ORFs can be seen in green and start with *capA*, *capB*, *capC*, *capD (*upstream from *capE).* Downstream from *capE*: *capF*, *capG*, *capH*, *capI*, *capJ*, *capK*, *capL*, *capM*, *capN*, *capO* and *capP*. The size of the genes (in nucleotides) are indicated by the numbers provided (Adapted from http://www.genedb.org).

Stringfellow *et al.* (1991) studied whether the capsular serotype 5, *S. aureus* Reynolds strain was affected by typical components in synthetic media. The authors found that CP5 production was linked to energy availability and energy source. Carbohydrate concentration and carbon/nitrogen ratio did not seem to have any effect on CP5 production. Dassy and Fournier (1996) investigated the influence of respiratory activity on *S. aureus* Reynolds strain CP5 production in the presence of different concentrations of dissolved oxygen and nitrite, as well as the effect of a number of metabolic inhibitors. The authors found that CP5 was always produced during the exponential growth phase with all the culture conditions (including when the metabolic inhibitors were added) but this was not the same with the post-exponential phase. The researchers also referred to previous studies which stated that CP production is pH-dependent and ideally for CP production, pH should range from 6-9. At a pH 8, CP production is inhibited. Watts *et al.* (2005) found that *S. aureus* CP5 and CP8 produced different virulence profiles in a mouse model, with CP5 exhibiting higher virulence.

Microarrary gene expression was conducted on a number of vancomycin-intermediate and resistant *S. aureus* strains originating from the blood and a heart valve of a patient who was administered long-term vancomycin therapy (JH1-parent strain: vancomycin-intermediately resistant). One particular strain recovered from the patient`s blood (JH9) was vancomycin-resistant (MIC: 8.0 μg/ml) and the researchers revealed that capsular genes were up-regulated when this strain was compared to JH1, when each was grown to exponential phase (McAleese *et al.*, 2006).

Kampen *et al.* (2005) found that expression of capsular types 5 or 8 in *S. aureus* prevented opsonophagocytic killing of the bacteria, as well as respiratory burst induction of neutrophils, in a bovine model. However, this effect was reversed, when serotype-specific antisera were added to the model. This finding is very interesting, especially in the light of the current results, observed in this chapter, as this might imply that vaccination of CF patients against capsular types 5 and 8 may confer resistance to *S. aureus* infection. Herbert *et al.* (2001) revealed that CF *S. aureus* strains expressed CP8 both *in vitro* and *in vivo*, however air supplemented with 5% CO_2 could depress capsule production.

Iron

During an infection, bacteria need to obtain iron from the host, in order to survive. This may prove to be a difficult task, especially during an acute phase of infection when hypoferremia may occur (low iron). Many pathogenic bacteria have developed siderophores to counteract this and low iron levels stimulate bacterial virulence factor production (Litwin and Calderwood, 1993).

Lindsay and Riley (1994) investigated the effect of growth of *S. aureus* and coagulase-negative staphylococci (CoNS) isolates in iron-restricted environments. *Staphylococcus aureus* was found to survive very well in iron-deplete environment whilst CoNS exhibited very poor growth. The authors found that *S. aureus* showed altered patterns on SDS-PAGE, unlike CoNS which did not exhibit such patterns. Also when tested with chrome azurol S universal method for detection of siderophores, *S. aureus* produced iron-regulated siderophores when grown in Fe-restricted conditions. The ASM used in this current research contains diethylenetriaminepentacetic acid (DTPA), which acts as an iron chelator.

The current results show that the low iron in the medium did not inhibit *S. aureus* growth and survival. Genes involved in iron-binding activities (as detailed in http://www.genedb.org) included SAR2393 (putative bifunctional protein) and SAR0218 (putative pyruvate formate-lyase activating enzyme) and these were up-regulated by 3.7 and 2.9 fold respectively. However 4 genes encoding iron transport proteins were down-regulated. These included SAR0790 (*sstD*; lipoprotein), SAR1065 (putative polypeptide deformylase 2), SAR1268 (conserved hypothetical protein), SAR0618 (putative transport system lipoprotein) and SAR2368 (putative ferrichrome-binding lipoprotein precursor) and were down-regulated by -3.0, -1.9, -1.8, -1.8 and -1.8 fold respectively.

These findings may suggest that as the bacteria are in exponential phase, the mechanisms eliciting iron transport or siderophore production have not yet been fully activated. Also, it may be that certain hypothetical, conserved or putative proteins which were up-regulated may have an iron-uptake function. Alternatively, MRSA 252 may have another optimised mechanism by which it captivates iron which is as yet unknown.

Interestingly, in the GASM vs BHI list of normalised genes, *fur*, encoding an iron uptake regulatory protein was observed to be up-regulated by 3.7 fold. This gene is a known iron regulator of iron homeostasis in a variety of bacteria (Horsburgh *et al.*, 2001). *Fur* was

absent from the normalised ASM gene lists, indicating that it was not differentially regulated, when compared to BHI. Horsburgh *et al.* (2001) showed that *fur* was the main regulator controlling iron supply. With the aid of *perR* (peroxide stress regulator), this gene was able to control catalase-dependent oxidative stress resistance. In this chapter, *perR* was not differentially regulated in both ASM and GASM normalised gene lists, however catalase was up-regulated in both ASM and GASM by 4.1 and 6.8 fold respectively. In GASM, *sodM* (encoding superoxide dismutase, which is known to counteract superoxide radicals) was up-regulated 6.2 fold. This gene was not differentially regulated in the normalised ASM gene lists. Dubrac and Touati (2000) found that *fur* actually induced *sodB* (encoding superoxide dismutase) expression in *E. coli* and this process was iron-dependent. Li *et al.* (2009) investigated the effect of iron-limiting conditions on *Streptococcus suis*, using a selective capture of transcribed sequences (SCOTS) technique and qRT-PCR. Results showed that in addition to a number of virulence factors e.g. *cpdB* (a cell surface protein, substrate of sortase A), *fur* was also up-regulated.

Horsburgh *et al.* (2001) specifically tested *S. aureus* mutants for *fur* and *fur perR* in a murine skin abscess infection model and revealed that these showed a significant reduction in pathogenicity. This finding clearly indicates that increased expression of *fur* may produce increased virulence in *S. aureus*.

From the results shown in this Chapter, it can be speculated that the presence of glucose in GASM may contribute to the up-regulation of *fur* and as a result, may promote iron-regulated activities. Therefore, glucose (in GASM) may initiate *fur*-dependent oxidative stress mechanisms, as well virulence mechanisms in MRSA252. Since GASM is simulating glucose in the CF sputum, the above observation is indeed quite important; the prescence of glucose in CF airways, may up-regulate *fur* and increase virulence in *S. aureus*. This could help explain, in part, the increased morbidity and mortality observed in diabetic CF patients, as discussed in Chapter 1.

Transcriptional regulators

A review by Bonner *et al.* (2004) described the regulation of virulence gene expression in *S. aureus*. This is modulated primarily by regulatory transcription factors. These regulatory components form part of complex networks which in turn, interact specifically with target gene promoters. In essence, most of these factors occur as two-component systems, which consist of a sensor protein which is a histidine kinase and a response regulator protein. Examples of such systems include the well-characterised accessory gene regulator or *agr*, autolysis-related locus or *arlRS*, *lytSR* and others such as *S. aureus* exprotein expression or *saeRS* and staphylococcal respiratory response or *srrAB*.

Fig 4.8 describes the interactions between different transcriptional regulators and virulence factor production in MRSA252.

Chan and Foster (1998a) investigated environmental effects on *agr* and *sar*, *hla*, *spa* and *tst* genes. The authors found that *agr* and *sar* were not affected by the presence of 1M NaCl or 20mM of sucrose, however *spa*, *tst* and *hla* were highly down-regulated. This observation is similar to the microarray results obtained in this chapter. Down-regulation of *spa* and *hla* was observed in ASM and GASM. In fact, *spa* was greatly repressed in both ASM and GASM, with 9.2 and 6.2 fold increased expression, respectively. Interestingly, *hla* was repressed two fold in both ASM and GASM, possibly indicating that the effect of glucose does not have an impact on *hla* gene expression in the exponential phase. Maximal *hla* expression is known to concur in the late-exponential to stationary transition phase of growth (cited in Chan and Foster, 1998a). However, ASM, BHI and GASM MRSA252 cultures in this current study were all taken from exponential phase.

Chan and Foster (1998b) examined the role of *sarA* in virulence factor production and revealed that proteases such as V8 serine protease and a novel metalloprotease were the major extracellular proteins repressed by *sarA*. Also, *hla* is up-regulated by *sarA*, as well as toxic shock syndrome toxin I (*tst*) and staphylococcal enterotoxin B (*seb*).

In this chapter, *sarA* was up-regulated in ASM by 2 fold and the SAR1905 serine protease was found to be down-regulated in GASM by 2.6 fold but no differential regulation was obtained for ASM for this protease.

In a review by Kong *et al.* (2006), it was reported that the autoinducing peptide (AIP), is encoded inside the *agrD* gene and thought to be produced as a larger protein, later modified to a smaller version by *agrB*. Wright *et al.* (2005) evaluated AIP in the *agr* system. AIPs from different staphylococci act as inhibitors of the *agr* systems. The authors revealed that during the evolution of staphylococci, *agr* divergence occurred at the start of evolutionary development and likely came before the present nucleotide polymorphisms which are currently used in genotyping.

The *sarA* locus is an important global regulator of virulence genes in *S. aureus*. It is known to down-regulate *spa* and protease genes. The *sarA* locus consists of 3 transcripts: *sarA* P1, *sarA* P2 and *sarA* P3 and these lead to the synthesis of *sarA*. Liu *et al.* (2006) deduced the crystal structure of *sarA* and found it to possess a DNA-binding region and a putative divalent cation binding pocket for gene function.

Heyer *et al.* (2002) investigated the potential of *agr* and *sarA* mutants to induce invasive lung infection in a mouse model. Virulence activity was assessed on the ability of the mutants to cause pneumonia and to increase interleukin-8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF) expression in the mouse respiratory epithelial cells. Results showed that in a neonatal mouse, *agr* and *sarA* were associated with invasive, fatal, pulmonary infection and *sarA* was specifically required to cause bacteraemia. The *agr* and *sarA* mutants were still able to cause pneumonia and to stimulate IL-8 expression. However *agr* and *sarA* mutants induced less epithelial GM-CSF expression. In addition, MSCRAMM mutants, (lacking fibronectin-binding proteins or clumping factor A, which binds fibrinogen) could not stimulate GM-CSF. This infers that conserved staphylococcal components, such as peptidoglycan are indeed sufficient to stimulate inflammation and cause pneumonia, but *agr* and *sarA* regulators are fundamental for the synchronization of invasive staphylococcal lung infection.

Xiong *et al.* (2004) investigated *agr*, *sarA* and *fnbA* activities in *in vitro* and *in vivo* rabbit endocarditis models using a Newman *S. aureus* parent strain. *SarA* was found to repress whilst *agr* up-regulated *fnbA* expression in both models. In addition, *fnbA* expression was still evident in the absence of both regulators.

In this chapter, it was noted in the GASM vs BHI normalised gene lists, the gene *rot*, (repressor of toxins) was positively expressed by 2.1 fold. However, *rot* was not differentially expressed in ASM. It might be postulated that the addition of glucose to ASM, i.e. GASM, might have triggered positive expression of *rot*, in comparison to BHI. Saïd-Salim *et al.* (2003) investigated the role of the gene *rot*, repressor of toxins in *S. aureus* virulence. This gene is known to show homology to *sar*. The authors found, using microarrays, that on comparing a *rot agr* double mutant to its *agr* parental strain, *rot* demonstrated global regulatory actively and opposed the action of *agr* on specific genes, such as *hla* and *hlb*, encoding haemolysin A and B respectively. However it was also found to up-regulate virulence genes such as *spa*. Interestingly, urease genes and also capsular genes were found to be down-regulated by *rot*, whereas, in the GASM vs BHI normalised gene lists, capsular genes were up-regulated, as well as genes encoding the entire urease operon.

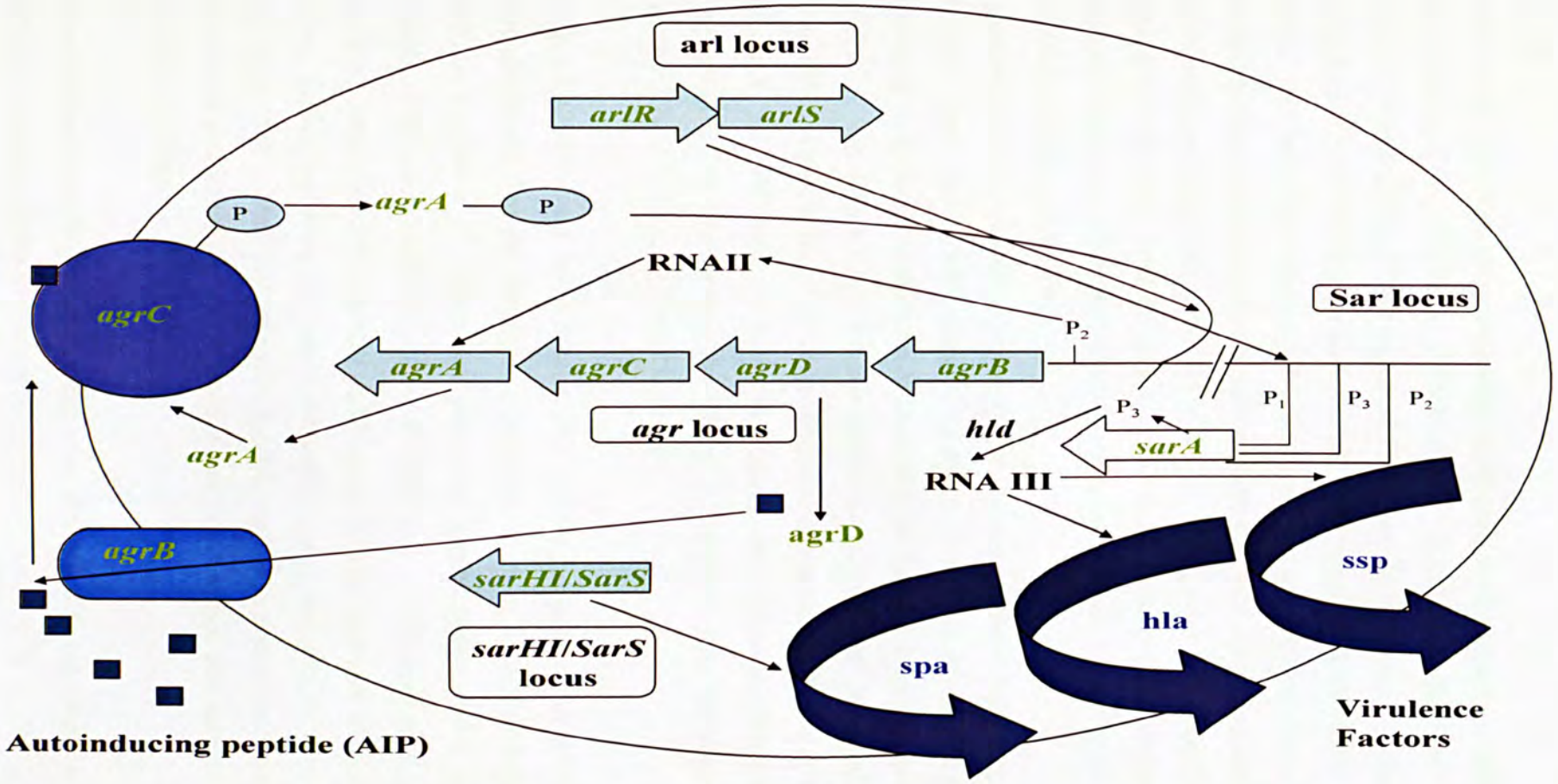


Fig 4.8: Schematic representing the positive interactions between different transcriptional regulators in *Staphylococcus aureus* and the production of virulence factors: *arlS-arlR*, a two-component regulator and the transcriptional global regulators *agr* and *sar* (Adapted from Fournier *et al.*, 2001 and Bronner *et al.*, 2004).

Sigma B

Bischoff *et al.*, (2004) conducted a microarray analysis of the transcriptional profiles of *S. aureus* strains COL (MRSA), GP268 (*rsbU*$^{+}$ strain) and Newman (MSSA). These researchers revealed that σ^B up-regulated 198 genes by a factor $\geq$ 2.0 and 53 were down-regulated in at least 2 of the strains studied. Gene products found to be under σ^B control included cell envelope biosynthesis, intermediary metabolism (such as carbohydrate metabolism which includes glycolysis, tricarboxyclic acid cycle and amino acid and lipid metabolism) and signalling pathways (such as DNA synthesis, modification and repair mechanisms and RNA production and regulation). Other researchers reported that *sigB* upregulates clumping factor and coagulase and is required for meticillin-resistance (cited in Karlsson-Kanth *et al.*, 2006).

Up-regulated genes included capsular polysaccharide synthesis enzymes, *clfA*, *clpL*, *sarA* , *sigB*, *rsbW* and *spoVG*. Interestingly, all these genes were up-regulated also in ASM in this chapter and increased expression indicated in Table 4.2 for capsular genes and was 8.7, 3.1, 2.3, 2.0, 1.8, 3.3 fold for the others, respectively. In addition, the 2-component system, *arlRS* was also found to be up-regulated in the Bischoff *et al.* (2004) study, as for ASM (*arlR*: 2.9 fold; *arlS*: 2.4 fold). In essence, the authors proposed that σ^B controls a large regulon and is an important modulator of virulence gene expression and is likely to act against RNAIII, the *agr* effector molecule.

Senn *et al.* (2005) went on to explore the various components of the σ^B operon. These researchers found that the operon is transcribed from at least 2 differentially controlled promoters, a putative σ^A –dependent promoter, named $sigB_{P1}$ giving rise to a transcript covering sa2059-sa2058-*rsbU-rsbV-rsbW-sigB* and a σ^B–dependent promoter, $sigB_{P3}$, producing a transcript covering *rsbV-rsbW-sigB*. A third promoter was also proposed to be involved, $sigB_{P2}$, including the *rsbU-rsbV-rsbW-sigB*. Interestingly, the authors also found that in an *in vivo* guinea pig infection model, *sigB* was transcribed at similar rates to *in vitro* cultures at 2 and 8 days post-inoculation. This implies that *sigB* activity plays a role in *in vivo* infectivity.

The *sar* operon contains 3 promoter elements (P1, P2, P3) and Deora *et al.* (1997) found that P3 is dependent on the alternative transcription factor, σ^{SB}.

Chan *et al.* (1998) showed that *sigB* was involved in acid-adaptive responses, resistance to hydrogen peroxide and heat shock recovery (54°C). Reference is made in their study, to the fact that a low pH environment is one of the major killing methods adopted by human neutrophils.

A recent study by Meier *et al.* (2007) investigated the association of capsular gene expression, σ^B, *arlRS* and the *yabJ-spoVG* locus in a Newman *S. aureus* strain (MSSA). They concluded that removal of σ^B was consistent with down-regulation of *capA* and this effect was also seen when *arlR* was inactivated. This is strikingly similar to the findings in this chapter, as genes encoding capsular synthesis enzymes were found to have elevated expression, and *arlR* had a 2.9 fold increased expression whilst σ^B was increased by 2.0 fold in ASM. Fig 4.9 below represents the *yabJ-spoVG* gene locus in MRSA252.

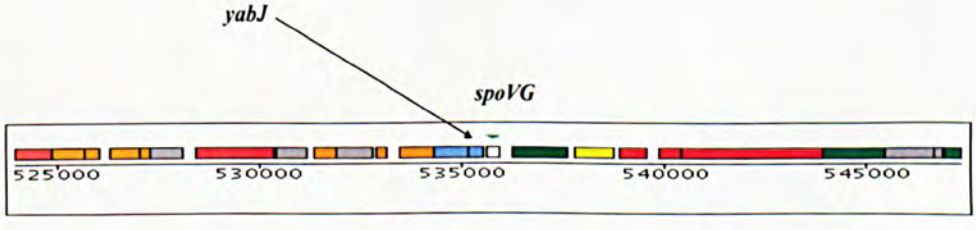


Fig 4.9: Position of *yabJ-spoVG* locus in MRSA252. *SpoVG* is indicated by a green arrow and *yabJ* is found adjacent and upstream to *spoVG*. The size of the gene (in nucleotides) is indicated by the numbers provided (Adapted from http://www.genedb.org).

SpoVG was originally located in *Bacillus subtilis* and found to assist sporulation but in certain non-sporulating bacteria, this gene can still found and it is thought to be involved in more general regulatory cell functions. The *yabJ* gene belongs to the YigF protein family and this is thought to be involved in many biological processes but its exact function is unknown (cited in Meier *et al.*, 2007)

Meier *et al.* (2007) showed that *spoVG-yabJ* locus enhances capsular production, although to a lesser degree than other regulators, such as *arlRS* and σ^B. In this work, *spoVG* was up-regulated by 3.3 and 2.6 in ASM and GASM respectively, whilst *yabJ* increased by 2.9 and 2.6 fold, respectively, in ASM and GASM. Table 4.3 in the Results section includes a list of normalised genes in ASM and GASM and thought to be affected by the *arlR-arlS* operon.

Fournier *et al.* (2001) investigated the effect of *arlR and arlS* mutations on *S. aureus* virulence factors. They discovered that the *arl* operon plays a role in virulence, by suppressing the production of *hla* (coding for α-haemolysin), *hlb* (coding for β-haemolysin), lipase, coagulase and serine protease. The authors emphasized the down-regulation of *spa* (coding for protein A) mostly by the *arlRS* bi-component regulator. Also, it was apparent that the *arl* locus was actually dependent on *sarA* and *agr* activity.
In Table 4.3, the differential regulation folds for each of these genes obtained in this chapter can be viewed. *Spa* was one of the most down-regulated genes in both ASM and GASM normalised values. However, no evidence of differential regulation of the *arlRS* operon was observed in GASM, whereas in ASM, this was found to be up-regulated.

Clp proteins

It has been reported that the *clp* proteolytic complexes are essential for virulence and for survival under stress conditions, in several pathogenic bacteria. These proteins adapt to multiple stresses by degrading accumulated misfolded proteins (Frees *et al.*, 2003; Michel *et al.*, 2006). Frees *et al.* (2003) conducted studies with *clpP* and *clpX* mutants (derived from parent *S. aureus* 8325-4) to investigate the effect of stress factors on virulence. They found that in a mouse skin abscess model both *clpP* and *clpX* were needed for virulence.
Oxidative stress conditions, provided by H_2O_2, were tested against *clpP* and *clpX* mutants. Results showed that both mutants were increasingly sensitive when compared to the parent strain. When exposed to varying temperature conditions, inactivation of *clpX* improved

survival under heat shock conditions and reduced growth rate at lower temperatures, whereas inactivation of *clpP* reduced survival at high temperatures and decreased the growth rate at low temperatures.

Frees *et al.* (2003) also investigated the effects of *clpX* and *clpP* on *hla* gene transcription. *Hla* expression in the *clpP* and *clpX* mutants was greatly reduced in the post exponential phase. Finally, the *agr* effector molecule RNAIII and also AIP (autoinducing peptide), were reduced in *clpP* and *clpX* mutants. In this chapter, *clpB* (SAR0938) was up-regulated 5.1 fold and *clpL* (SAR2628) 3.1X in ASM whilst in GASM, *clpB* (SAR0938) and *clpC* (SAR0528) were up-regulated 3.8 and 3.4 respectively and *clpL* (SAR2628) down-regulated by 2.6.

Michel *et al.* (2006) constructed a Δ*clpP* mutant from NCTC 8325 (a laboratory-derived MSSA strain) and studied the differences between the two strains using microarrays. This study showed that *clpP* had a powerful regulatory effect on *S. aureus* genes involved in virulence and response to stress. In addition, the Δ*clpP* mutant decreased all capsular synthesis enzymes, as well as *lrgAB* genes but *clfA* expression was increased. This is interesting and shows that *clpP* may have a direct role in controlling these gene activities, however in the current chapter *clpP* was not differentially regulated when MRSA252 was grown to exponential phase in both ASM and GASM and normalised to BHI.

Frees *et al.* (2005) showed that *clpP* and *clpX* are required for *spa* transcription. Recently they also found that *clpX* mediates *spa* expression through *sarS* independent and dependent mechanisms. In addition, *clfA* levels were found to be unaffected by *agr*, *clpX*, *clpP* and *SarA* as others had proved before but *clfB* levels were reduced by *agr*, *sarA* and *clpX* and *clpP*.

LrgAB operon

The position of the *lrgAB* operon in MRSA252 is depicted in Fig 4.10 and differentially regulated normalised genes in ASM and GASM which have a direct or indirect effect on the *lrgAB* operon, are included in Table 4.4, in the Results section.

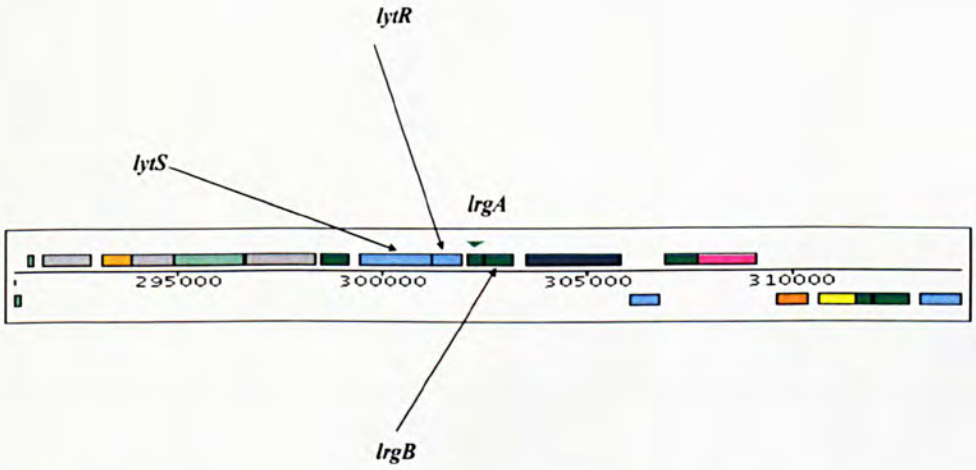


Fig 4.10: The *lrgAB* and *lytSR* loci in MRSA252. The size of the gene (in nucleotides) is indicated by the numbers provided (Adapted from http://www.genedb.org).

Brunskill and Bayles (1996a) revealed the existence of 2 genes, *lytS* and *lytR* which form a two–component regulatory sequence. Transmission electron microscopy was used to examine, a *lytS* mutant, KB300, which was found to exhibit increased lysis in liquid culture (15 to 20%), whilst parent strains appeared normal. Mutant cells had a rough and diffuse appearance, when compared to the smooth surface of the parental strain (NCTC 8325-4). The rough appearance was associated with defects in the expression of murein hydrolases. Also, KB300 demonstrated a higher rate of autolysis, compared with the parent strain. Brunskill and Bayles (1996b) investigated the DNA sequence immediately downstream from the *lytR* and found the ORFs: *lrgA* and *lrgB*. They proposed that these genes may be involved in cell wall metabolism.

In a review by Bayles (2000), it was reported that an *lrgAB* mutant strain (KB345) caused increased murein hydrolase activity in the extracellular fraction. Also, when KB345 and the parental strain were examined for penicillin sensitivity, it was observed that KB345 actually decreased tolerance of late-exponential phase cells to the killing of penicillin. Bayles (2000) suggests that during the early exponential phase, there is low *lrgAB* expression and so cells are penicillin-susceptible, whereas during late-exponential phase, *lrgAB* expression is increased and this gives rise to penicillin tolerance. The author stated that the *lrgAB* operon is clearly involved in *S. aureus* cell wall metabolism. It seems apparent that there is a subtle balance between the PBPs, responsible for peptidoglycan polymerization and the murein hydrolases, which are important for cell wall expansion, septum formation and daughter cell separation. Overall, it is highly indicative that there is a delicate balance between the two groups of enzymes, which must be controlled adequately in order to prevent cell autolysis.

Groicher *et al.* (2000) proposed that *lrgA* and *lrgB* function in a similar way to an antiholin, which inhibits murein hydrolase export and not as a protease or a transcription factor due to their high hydrophobicity. The authors revealed that the expression of the *lrgAB* operon was consistent with inhibition of penicillin-induced *S. aureus* killing, independent of cell-lysis, following exponential phase which also concurred with maximal expression. In addition, others found that *lrgAB* was down-regulated by σ^B when in the stationary phase (Rice *et al.*, 2004).

In this chapter, it was observed that in ASM *sigB* (the gene encoding σ^B as cited in Bischoff *et al.*, 2004) was up-regulated 2 fold whilst *lrgA* and *lrgB* were down-regulated by 10.8 and 4.9 fold, respectively but no differential *sigB* regulation was evident in GASM.

Fujimoto *et al.* (2000) showed that *lytSR* as well as *agr* and *sar* had a positive regulatory impact on *lrgAB*. Also, they stated that the effects of *agr* and *sar* on *lrgAB* were mediated through an a *lytSR*-independent pathway. The *lrgAB* operon is situated downstream from the *lytSR* operon and *lytSR* gene products have been found to activate *lrgAB* transcription.

Renzoni *et al.* (2006) studied an isogenic pair of strains: strain 14-4 (teicoplanin-resistant MRSA) and MRGR3 (teicoplanin-susceptible MRSA). They revealed that 14-4 exhibited decreased expression of the autolysis negative regulators, *lytSR* and *lrgAB*. Also when compared to MRGR3, 14-4 demonstrated increased expression of negative autolysis regulators, such as *arlRS*, *mgrA* and *sarA* and reduced expression of positive regulators, *agr* RNAII and *agr* RNAIII. These authors also stated that capsular polysaccharide genes were up-regulated with 14-4 and this is akin to what was observed in this chapter for both ASM and GASM.

In this chapter, with ASM, *lrgA* and *lrgB* were down-regulated 10.8 and 4.9 fold respectively and also with GASM a similar situation was observed, with 9.7 and 8.0 fold for *lrgA* and *lrgB*. Also, *lytS* and *lytR* were down-regulated by 2.5 and 2.0 fold in ASM and 2.7 and 3.2 in GASM. In addition, *arlR* and *arlS* were up-regulated by 2.9 and 2.4 fold respectively in ASM and *sarA* by 2.3 fold. No differential regulation for these last three genes was observed in GASM. Thus, it can hypothesized that MRSA252 growing in ASM may utilise similar mechanisms to the teicoplanin-resistant *S. aureus* strain 14-4. Also the presence of glucose (as evidenced in GASM) does not alter *lrgAB* down-regulation but could perhaps suppress *sarA* and *arlRS*.

Protein A (*spa*) and clumping factor (*clfA*)

In this chapter, *spa* which encodes Protein A, was found to be down-regulated in both ASM and GASM by 9.2 and 6.2 fold respectively. Gomez *et al.* (2004) showed that tumour-necrosis factor α (TNF) acts as a receptor for Protein A and the interaction between protein A and TNF receptor 1 (TNFR1-expressed in epithelial cells) is pivotal for staphylococcal pneumonia.

A study by Karlsson *et al.* (2001) investigated the interaction between Protein A (*spa*), fibronectin-binding (*fnbA, B*) proteins and extracellular proteases in a *sarA* mutants. It has been recorded in literature that *S. aureus* possesses a number of proteases: serine protease or V8 protease (*sspA*), cysteine protease (*sspB*) and *sspC* , encoding a cytoplasmic protein of unknown function. These form part of the same operon. In addition, a metalloprotease, named aureolysin (*aur*) and another cysteine protease, termed staphopain (*scp*) are also

formed. Hence, *sarA aur* and *sarA ssp* knockout mutants were produced. Mutants were shown to produce 10-20 fold more *spa* than the parent *S. aureus* strain.
The authors showed that inactivation of *ssp* operon resulted in approximately the same increase in cell-wall associated Protein A as inactivation of *aur* did. They also deduced that *sspA* was the most important enzyme in the degradation of Protein A (Karlsson *et al.*, 2001).

Higgins *et al.* (2006) investigated the anti-phagocytic effects of protein A (*spa*) and clumping factor A (*clfA*) using human PMNLs, in the presence of human serum opsonins. Mutant strains Newman *spa*, Newman *clfA* and Newman *spa clfA* were obtained from the parent Newman (MSSA) strain. Increased phagocytic uptake of all mutant and parent Newman strains was observed with increasing concentrations of serum. This showed that the process was dependent on serum opsonins. The parent Newman strain was phagocytosed by approximately 46% of PMNLs when challenged with 1% normal human serum. The *spa* and *clfA* mutants were phagocytosed by approximately 45% of PMNLs at this serum concentration, whereas the *spa clfA* double mutant was taken up by 72% of PMNLs. All this demonstrated that both protein A and *clfA* can contribute to the inhibition of phagocytosis of *S. aureus* Newman and that the absence of one factor was compensated for by the presence of the other. Absence of both *spa* and *clfA* contributed to a significant increase in phagocytic uptake. The authors further explored the anti-phagocytic properties of *clfA* and *spa*, using *Lactococcus lactis* as a Gram-positive surrogate host. They revealed that *clfA* inhibited phagocytosis, even in the absence of fibrinogen but demonstrated increased anti-phagocytic effect in the presence of fibrinogen. Protein A had strong anti-phagocytic effects which were fibrinogen-independent (Higgins *et al.*, 2006).

Vaudaux *et al.* (2002) observed *clfA* and *fnb A, B* levels in *S. aureus hemB* mutants (indicating auxotrophy for haemin) exhibiting SCV phenotypes. Flowcytometry studies were used to assess adhesion potential to fibrinogen (for *clfA*) and fibronectin (for *fnb*). The authors found that levels were much higher than that of the isogenic parent strain and correlated with the increased surface display of these adhesions. Real-time qRT-PCR was used to analyse the transcript levels of *clfA* and *fnb* genes, of the *hemB* mutants vs parents 8325-4 and 8325-4G (defective in *agr* RNAIII production). Results showed that *clfA* and *fnb* expression was increased in the *hemB* mutants when compared to the parent isogenic

strains. Restoration of the gene levels to those akin to the parent strains was observed once the *hemB* mutation was reversed, or the growth medium was supplemented with haemin. Also, the authors noted that the increased expression levels of *clfA* and *fnb* genes occurred independently from *agr*.

In this chapter, *clfA* was one of the most up-regulated genes in both ASM and GASM (Appendix II), with 8.7 and 5.4-fold increased expression in ASM and GASM respectively. Also, the microarray results did not produce any significant evidence of *agr* or *fnb* activity in ASM. However, in GASM, which differs from ASM (solely by the inclusion of 10mM glucose) *agr* activity is apparent, since *agrC*, *agrIII*, *RNAIII* and *agrA* are up-regulated by 3.5, 2.6, 2.5 and 1.8-fold respectively. Also, *fnb* was down-regulated by 1.9-fold.

Wolz *et al.* (2002) studied *clfA* levels in sessile and planktonic bacteria grown *in vitro* and in an *in vivo* device-associated guinea-pig model of infection of *S. aureus* strains, Newman, Reynolds and RN6390. Total RNA for the Light Cycler reverse transcription polymerase chain reaction (RT-PCR) experiments were obtained from different growth phases and *gyrB* was selected as a normalizer gene. Results showed that *clfA* levels were higher in the planktonic state for strains Newman and Reynolds but *clfA* levels in RN6390 were much less than for the other 2 strains and also no difference was seen between planktonic and sessile states. In addition, *clfA* levels were higher in the post-exponential phase and stationary phase when compared to early exponential phase. In the *in vivo* model, *clfA* levels in the devices were lower than those *in vitro* grown to stationary phase in the Newman and Reynolds strains. In addition, during the initial phase of infection, *clfA* mRNA levels increased and remained constant after 96hr post-inoculation. However, *clfA* transcript levels in the unattached bacteria of the exudates never exceeded the level of *clfA* transcripts in the sessile bacteria attached to glass beads, in contrast to the *in vitro* results. An obvious increase in *clfA* quantities in the sessile bacteria was noted after 144 hours post-inoculation. In both models, it was observed that maximal *clfA* transcript levels were achieved late during growth *in vitro* and *in vivo*.

The σ^B–dependent ability of *S. aureus clfA* and *fnbA* to bind to fibrinogen and fibronectin was assessed by Enteza *et al.* (2005) in a rat model of endocarditis. A Fluorescent-Activated Cell Sorter (FACS) was used *in vitro* in order to monitor *clfA* expression in *S.*

aureus mutants possessing differing abilities to produce σ^B levels. FACS detected higher *clfA* levels with mutants having higher σ^B activities but limited activities for mutants with low σ^B activities. A *clfA*-negative strain did not produce any signal on FACS, implying that changes observed were due to *clfA* activity solely. Adherence to platelet-fibrin clots was also monitored in the *in vivo* endocarditis model and *S. aureus* mutants exhibiting high level σ^B activities had the highest binding affinities. Northern blotting analyses showed that *clfA* transcripts, as well as *fnb* were positively influenced by σ^B. In the rat model, higher σ^B production at 16hr post-inoculation yielded increased bacterial densities in both aortic vegetations and spleens. Spleens were affected at a much higher frequency than the aortic vegetations and spleen positive cultures were correlated with increased σ^B activities. This effect was reversed at 72hr, indicating that although *in vitro*, σ^B appears to increase the adhesion of *S. aureus* to various host cell-matrix proteins *in vitro*, it has limited effect on the pathogenesis in this endocarditis model. It also seems that σ^B has an initial effect on bacterial density in the early stages of infection but does not continue with increased disease progression.

Ica operon

Fig 4.11 overleaf represents a schematic diagram of the *ica* operon in MRSA252 and Table 4.5 in the Results section includes actual folds for differentially expressed normalised genes in ASM and GASM.

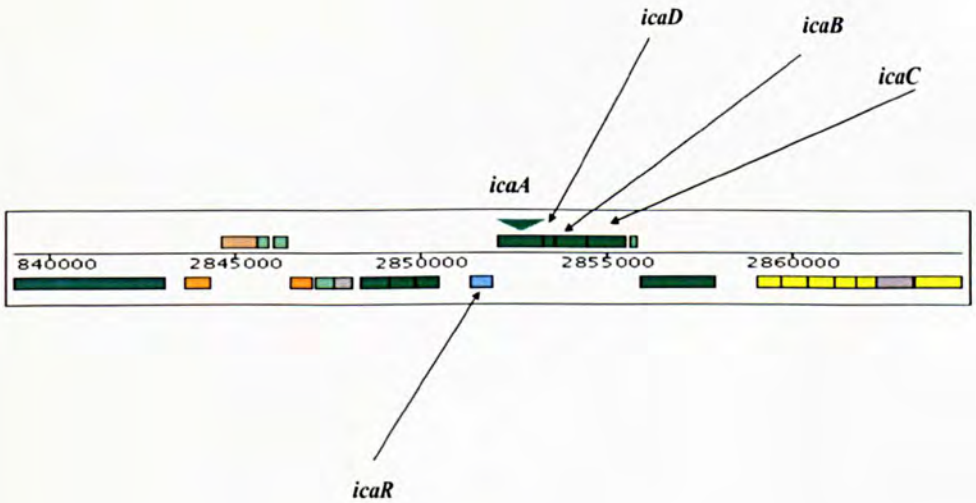


Fig 4.11: The *ica* operon in MRSA252. *icaR* is transcribed in the opposite direction to *icaADBC*, as shown above. The size of the genes is indicated by the numbers present (in nucleotides) (Adapted from http://www.genedb.org).

Cramton *et al.* (1999) investigated the role of the *ica* locus in biofilm formation. They analysed several *S. aureus* strains and found that all possessed the *ica* locus and this was associated with the ability to form biofilms *in vitro*. A deletion of *ica* produced an inability for biofilm formation, polysaccharide interceullar adhesion (PIA) production (required for biofilm formation) and N-acetylglucosaminyltransferase activity (which is used as a substrate during biofilm formation). A recent review by O` Gara (2007) stated that a change in the bacterial environment such as NaCl or ethanol, added to a growth medium was sufficient to activate the *ica* operon. Also, *icaA* is a transmembrane protein, requiring *icaD* gene for optimal activity and it is homologous to N-acetyl-glucosaminyltransferases. Once *icaAD* is co-expressed with *icaC*, longer oligomer chains are produced. *IcaC* is probably also involved in translocation of the growing polysaccharide to the cell surface. *icaB*, which is a surface-attached protein, is then responsible for de-acetylation of the poly-N-acetylglucosamine molecule. *IcaR* is divergently transcribed and located upstream of the *icaADBC* operon and has been reported to repress the *ica* operon in *S. epidermidis.*

In this chapter, *icaA*, *icaD* and *icaB* were up-regulated in ASM, suggesting that there may be biofilm formation in the ASM. However *icaB* was not differentially regulated. In addition, in GASM, *icaA* and *icaB* were only slightly up-regulated (1.6 and 1.8 respectively). In GASM, *icaR* was up-regulated by 2.7 fold and this may indicate that in fact, the *ica* operon was being repressed by *icaR* in this medium.

Ulrich *et al.* (2007) investigated the association between the *ica* operon, PIA production and the *SrrAB* (staphyloccoccal respiratory response) regulator. The authors found that when *S. aureus* were challenged with human neutrophils using a phagocytosis assay, 68% of PIA-producing wild-type cells survived but only 19% of mutant *srrAB* cells did, under anaerobic conditions. No protection was observed, with both *S. aureus* and *S. epidermidis* producing PIA in aerobic conditions. Mandell (1974) had assessed the bactericidal activity of human polymorphonuclear neutrophils (PMNs) on a variety of bacteria. Anaerobic PMNs were unable to kill 2 *S. aureus* strains (Wood 46 and 502a) within 1 hour. Specifically, >90% of *S. aureus* strain 502a were killed by aerobic PMNs, whereas only 50% were killed by anaerobic PMNs.

Possible role of specific genes in CF

Goerke *et al.* (2000) analysed gene regulation of clinical *S. aureus* isolates from CF patients in CF sputum and *in vitro* and also analysed *S. aureus* strains which were *agr*+ and *hla*, *spa* deficient (*in vitro*). The authors investigated the transcription of RNAIII, *agr*, *spa* and *hla*. They found that RNA III, the effector molecule of *agr* was poorly expressed in CF sputum. Also there was no correlation between cell density and RNAIII transcript. In all samples the bacterial numbers were lower than the threshold for *agr* activation *in vitro* (10^9 cfu/ml). Protein A (encoded by *spa*) was down-regulated by *agr in vitro*. Only minimal amounts of *spa* were detected both in the exponential phase as well as post-exponentially. Alpha-haemolysin (encoded by *hla*) expression, known to be activated by *agr in vitro* was also investigated and found to yield erratic expression. The authors concluded that *agr* was not activated in chronic *S. aureus* CF lung infections. They postulated that *agr* may be activated only in certain stages of infection. In CF chronic lung infections, *S. aureus* is located in the viscous mucus present in the airways and infection does not have systemic consequences. It is also worth mentioning that since bacterial numbers in the specimens were lower, this might have elicited other regulatory pathways and also this might indicate that *agr* was still inactive.

The results obtained for ASM in the current study suggest that *agr* was not positively regulated compared with BHI and *spa* and *hla* levels were down-regulated. However other transcriptional facts such as *sarA*, *sigB*, *arlS* and *arlR* and *mscL* were up-regulated. In all, 16 transcriptional factors were activated, in total whilst 12 were repressed (Fig 4.3).

Moisan *et al.* (2006) performed DNA microarray transcriptional analysis of clinical small colony variants (SCVs) isolated from CF patients. These were compared to a laboratory SCV (*hemB* mutant) and 2 prototype *S. aureus* strains (Newbould and ATCC 29740). The genes found to be most commonly upregulated in both CF SCVs and *hemB* were involved in glycolysis and fermentation pathways. Also these strains were all found to have low levels of *hla*. Many genes under the influence of *sigB* were seen to be positively regulated in clinical SCVs and the pattern was different to that observed with the *hemB* and prototype strains. Moisan *et al.* (2006) also observed that there was an up-regulation of capsular genes, *SarA* and *clfA* with the clinical SCVs. This occurrence is strikingly similar to the findings in this chapter, where capsular biosynthesis genes were the most up-

regulated and also both *sarA* and *clfA* were up-regulated. In addition, *sigB* was one of the transcriptional regulators found to be positively regulated (2-fold) and *hla* and *nucl* were down-regulated. Interestingly, (in Moisan *et al.*, 2006) clinical SCVs which were obtained from patient sputum and throat and subsequently cultured in BHI had very similar growth patterns to the MRSA252 grown in ASM, as both exhibited much slower growth rates when compared to MRSA 252 in BHI as well as to the ATCC 29740 and Newbould strains in the authors' study. Also *nucl* (which is down-regulated by *sigB*) was also down-regulated in Moisan's study in both *hemB* and the CF strains. In addition, the authors found that stress-associated genes such as *asp23*, *spoVG* were up-regulated with the clinical SCVs.

This finding is also similar to the results obtained in this chapter, where both these genes were also observed to be up-regulated in MRSA252, grown in ASM. This effect is known to be positively regulated by *sigB*. Also, the holin-like proteins, *lrgA* and *lrgB* which are murein hydrolases, were down-regulated in the *hemB* mutant but up-regulated in the clinical SCVs. In this chapter, both *lrgA* and *lrgB* were down-regulated. The *hemB* mutant also exhibited repression of *lytM* and *atl* which were in fact up-regulated in the clinical SCVs. *SigB* is known to up-regulate these genes and in this chapter, both genes were seen to have been positively regulated. The authors concluded that *agr* does not play a significant role in the regulation of SCV virulence, contrary to *sigB* which seems to have a definite impact on SCV physiology in a CF *in vivo* setting.

Chatterjee *et al.* (2007) explored the metabolic gene expression profile of clinical *S. aureus* SCVs derived from CF patients, which were auxotrophic for thymidine. Their results revealed alterations in the tricarboxylic acid cycle (TCA), as well as acetic acid metabolism. During the post-stationary growth phase, thymidine-dependent SCVs exhibited a reduction in TCA function, thereby preventing entry into the death phase. In addition, the TCA cycle is required for amino acid metabolism and thus, the absence of a fully functioning TCA, inhibits amino acid catabolism. These observations might indicate how SCVs continue to persist and survive in the CF lung.

A recent study by Voggu *et al.* (2006) presented data which showed that non-pathogenic staphylococcal species, such as *S. gallinarum*, *S. carnosus* and *S. piscifermentans* were

resistant to the action of respiratory inhibitors, typically hydrogen cyanide, pyocyanin and quinoline N-oxides, produced by *P. aeruginosa*. These products are indeed lethal to pathogenic staphylococcal species such as *S. aureus* and *S. epidermidis*.

Voggu *et al.* (2006) revealed that the resistant phenotype was due to the expression of *cydAB* genes which code for a cytochrome *bd* quinol oxidase, which is insensitive to cyanide and pyocyanin. In fact, implementing a *S. carnosus*-specific *cydB* in *S. aureus*, whilst knocking out the *S. aureus cydB*, provided resistance to the *P. aeruginosa* toxins. The researchers proposed that the non-pathogenic staphylococcal species are co-inhabitants of environments also occupied by *Pseudomonas* spp. As a result, a resistant phenotype evolved in the non-pathogenic staphylococci, permitting the co-habitation of *Pseudomonas* spp and non-pathogenic staphylococcal species.

McNamara and Proctor (2006) discussed the significance of Voggu *et al.*'s study (2006), referring to the fact that the CF milieu is known to house both *P. aeruginosa* and *S. aureus*. The entry of *P .aeruginosa* into the lung does not eradicate *S. aureus* but on the contrary, it seems to encourage the isolation of *S. aureus* SCVs. SCVs are cytochrome-deficient, implying that they are insensitive to the respiratory toxins produced by *P. aeruginosa*.

Chang *et al.* (2005) conducted microarray analysis of *P. aeruginosa* challenged with H_2O_2. Results showed that DNA repair proteins and catalases were produced in response to the oxidative stress. Also, iron regulation genes in the bacterium were down-regulated. In this chapter, response to stress proteins-encoding genes including catalase, were up- regulated. Also, iron-regulating proteins were down-regulated for ASM.

Anderson *et al.* (2006) investigated the stress responses of *S. aureus* UAMS-1 (clinical osteomyelitis strain) by using Affymetrix® Genechips. The *S. aureus* strain was grown in BHI and cells in mid-exponential phase were used throughout experiments. The authors observed transcriptomic changes to cold and heat shock, stringent (due to mupirocin) and SOS (induced by mitomycin C) response-inducing conditions. For every stress response, a distinctive biological process, antibiotic characteristics and specific virulence factors were observed. Interestingly, with heat shock an LPXTG motif-containing gene and members of the urease system were up-regulated. In stringent conditions *alt* (autolysin), *geh* (lipase), *icaA* and *icaB* (intracellular adhesion locus) and *sspA-sspC* (extracellular proteases) were up-regulated. In this chapter, it was observed that *atl*, *icaA* and *icaB*, *geh* and *sspB* genes

were induced. Also when MRSA252 genes in GASM vs ASM were compared, genes encoding for urease enzymes were found to be up-regulated in GASM and also ASM (Results section).

The up-regulation of the urease operon in MRSA252 (Table 4.6) was noted when glucose was added to ASM (GASM). *Helicobacter pylori,* a Gram-negative pathogenic bacterium, which inhabits the human stomach, (a highly acidic environment), is a known urease producer. Urease production is known to aid the survival of the bacterium in this inhospitable environment (Kusters *et al.*, 2006). Since it grows in oxidative stress conditions, catalase, *sodB* and *fur* gene products are produced in this organism. It can also catabolize glucose. The ability of *H. pylori* to survive in the gastric environment is highly dependent on the production of urease enzymes which not only help reduce acidity and counteract acid stress but possesses a role in virulence (Kusters *et al.*, 2006). A review by Scott Algood and Cover (2006) stated that *H. pylori* mutants for urease production and flagella (the latter help bacteria penetrate the mucus layer) were unable to colonize animal models.

It can be therefore hypothesized that the up-reguluation of the urease operon in GASM may prompt *S. aureus* to produce specific factors which could help not only counteract acid and oxidative stresses but also play a role in enhancing virulence.

Mashburn *et al.* (2005) studied the transcriptome of *P. aeruginosa* in a rat dialysis membrane peritoneal model, alone as well as with the presence of *S. aureus*. *In vitro* cultures were also set up for comparison. Results showed that 5% of all *P. aeruginosa* genes were differentially expressed by at least 5-fold when grown in monoculture *in vivo*, with genes involved in iron acquisition and growth in low oxygen environments activated. In co-culture, the iron regulatory genes in *P. aeruginosa* were repressed. This indicated that *S. aureus* acts as an iron source for *P. aeruginosa*. Classes of genes induced *in vivo* with *P. aeruginosa* monoculture included amino acids biosynthesis and metabolism, biosynthesis of cofactors, energy metabolism, transport of small molecules as well as transcriptional factors. In this current study, all genes belonging to these classes were also seem to be induced when compared to the BHI control although amino acid biosynthesis and metabolism were also repressed in our study. This could potentially be explained by the abundance of all 20 amino acids in ASM. Also, the authors observed that genes

encoding proteins involved in amino acid metabolism and transport of amino acids, typically aromatic and branched chain amino acids were largely increased in the *in vivo* model. This finding indicated that *P. aeruginosa* is using amino acids as a source of carbon.

Similar data was obtained in this chapter, although amino acid metabolism proteins were also down-regulated, possibly due to an abundance of amino acids or protein biosynthesis activities.

Using Affymetrix® GeneChip microarrays, Palmer *et al.* (2005) examined the *P. aeruginosa* transcriptome when this bacterium was grown in CF sputum and compared to growth in a glucose-medium. In CF sputum, genes involved in branched chain and aromatic amino acid catabolism were greatly induced whilst genes involved in biosynthesis of these amino acids were down-regulated. Genes involved in glucose transport and metabolism were also repressed.

Salunkhe *et al.* (2005) compared transcriptome profiles of Liverpool epidemic strains of *P. aeruginosa* (LES), LES431, LES400 with each other and with the laboratory strain PAO1. *P. aeruginosa* was grown in Luria broth and late-exponential phase bacteria were used. Cultures were also challenged with H_2O_2 in order to induce a stress response. LES400 exhibited down-regulation of a large cluster of ribosomal proteins. A similar effect was seen with the results presented in this chapter as 14 proteins involved in ribosomal synthesis or modification were repressed.

SECTION 4.5: CONCLUSIONS

This chapter has presented a detailed analysis of differentially regulated genes, when exponential phase MRSA252 was grown in ASM and GASM and normalised to BHI, as a laboratory control.

Genes belonging to a number of functional classes, were seen to be differentially regulated. Functional classes included: capsular polysaccharide synthesis enzymes, putative proteins, cellular metabolism, conserved hypothetical proteins, lipid metabolism, transport proteins, carbohydrate metabolism, transcriptional factors and their regulation, amino acid metabolism, surface proteins, toxins and enzymes, hypothetical proteins, protein metabolism, metal ion binding, vitamin biosynthesis, response to stress proteins, nucleic acids biosynthesis and metabolism, antibiotic resistance proteins, urea metabolism, cell wall biosynthesis and metabolism and folic acd derivatives and biosynthesis.

For both ASM and GASM (which differed from ASM, only by the addition of one component added to ASM, i.e. 10mM sterile glucose), the most interesting finding was that capsular genes were seen to have been highly up-regulated. Also, clumping factor (*clfA*) was observed to be highly expressed. In ASM, *sigB* was observed to be up-regulated and this agreed with previous studies in the literature (as discussed previously in this chapter). These proposed that during *S. aureus* lung infection in CF, *sigB* was the transcriptional regulator thought to be operative, rather than the more well known *agr* system. Also, in ASM and GASM, highly down-regulated genes included the *lrgAB* operon and *spa*, encoding Protein A. This finding also supported the observations of other researchers, as reported earlier in this chapter.

It was also revealed that the addition of glucose, represented by GASM, resulted in an overall increase of differentially regulated normalised genes, when compared with ASM. There was an overall increase of 3% in GASM up-regulated genes, over ASM, with a cut-off value of ≥ 2 fold. The same percentage of 3%, was obtained with down-regulated genes at this same cut-off value. In addition, genes encoding *rot*, *sodM* and urease enzymes and vitamin and co-factor synthesis were observed to have been positively regulated in GASM, but little or no differential regulation was evident with ASM.

CHAPTER 5

RT-PCR OF SIGNIFICANT GENES OF MRSA252 IN BHI, ASM AND GASM

SECTION 5.1: INTRODUCTION

Reverse transcription polymerase chain reaction (RT-PCR) is a quantitative technique which is an excellent tool for gene expression studies (Freeman *et al.*, 1999). In general, studies in the literature which use DNA microarrays to study gene expression, utilise real-time PCR of significant gene sequences in order to confirm whether the pattern observed with the microarrays were similar (Palmer *et al.*, 2005; Anderson *et al.*, 2006, Moisan *et al.*, 2006, Roberts *et al.*, 2006). Thus, after having analysed the microarray data (Chapter 4), a number of significant genes which were differentially regulated were chosen and quantitative RT-PCR (QRT-PCR) was performed.

Prior to commencing the PCR, a 2-step QRT-PCR protocol was selected. The main advantage of a 2-step protocol over a 1-step protocol lies in the fact that the RT step is done separately and the same stock of cDNA is used for all the subsequent reactions. Additional advantages and disadvantages of each technique are listed in Table 5.1 below.

Table 5.1: Advantages and Disadvantages of a 1-step and 2-step QRT-PCR protocol (Baker *et al.*, ABgene®)

ONE-STEP QRT-PCR PROTOCOL	TWO-STEP QRT-PCR PROTOCOL
ADVANTAGES	**ADVANTAGES**
1) Easy to perform and fast	1) Very sensitive
2) Reduction in pipetting errors	2) Many primer options for RT
3) PCR primers and conditions are easily optimised	3) Master cDNA stock can be prepared for subsequent PCR experiments
	4) Provides accurate quantitation
	5) Quality control of RT and PCR can be conducted
	6) RT and PCR processes can be individually optimised
	7) Increased applications
DISADVANTAGES	**DISADVANTAGES**
1) Errors may build up systematically	1) Time consuming
2) RT step cannot be truly validated	2) Higher cost in consumables and plastics
3) No master cDNA stock	3) More pipetting steps and hence increased potential for pipetting errors and contamination
4) Less sensitive	
5) Primer choice is limited	

A number of real time RT-PCR techniques can be used. SYBR® Green was chosen for these experiments, due to its relatively low cost compared to others such as TaqMan® and Molecular Beacons.

SYBR® Green is a fluorescent dye which binds specifically to the minor groove of dsDNA (Pfaffl, 2001). Once DNA is denatured, the SYBR® Green molecules are present in the solution but do not fluoresce and so do not emit any signal. However, once primers start annealing and the extension phase, followed by polymerisation proceeds, the SYBR® Green molecules bind non-selectively to all dsDNA products and emit fluorescence (Fig 5.1 below illustrates this concept). The amount of fluorescence which is significantly above background is then converted to the cycle threshold (Ct) and this relates directly to the fact that the SYBR® Green binds directly to the double-stranded DNA products of amplicons which are formed. The Ct is inversely proportional to the DNA concentration, implying that a higher Ct means a lower concentration. NTC or No Template Controls are normally included as controls with each run and these provide useful checks for any contaminating DNA, which would give a false Ct reading.

Section 5.1.1: Main Aims

The microarray results reported in Chapter 4 were validated in this chapter, using QRT-PCR. The reason behind this was to reinforce the previous findings using a selection of MRSA252 genes which were differentially regulated in ASM and GASM, when normalised to BHI. Comparative results between the two methods were sought and obtained.

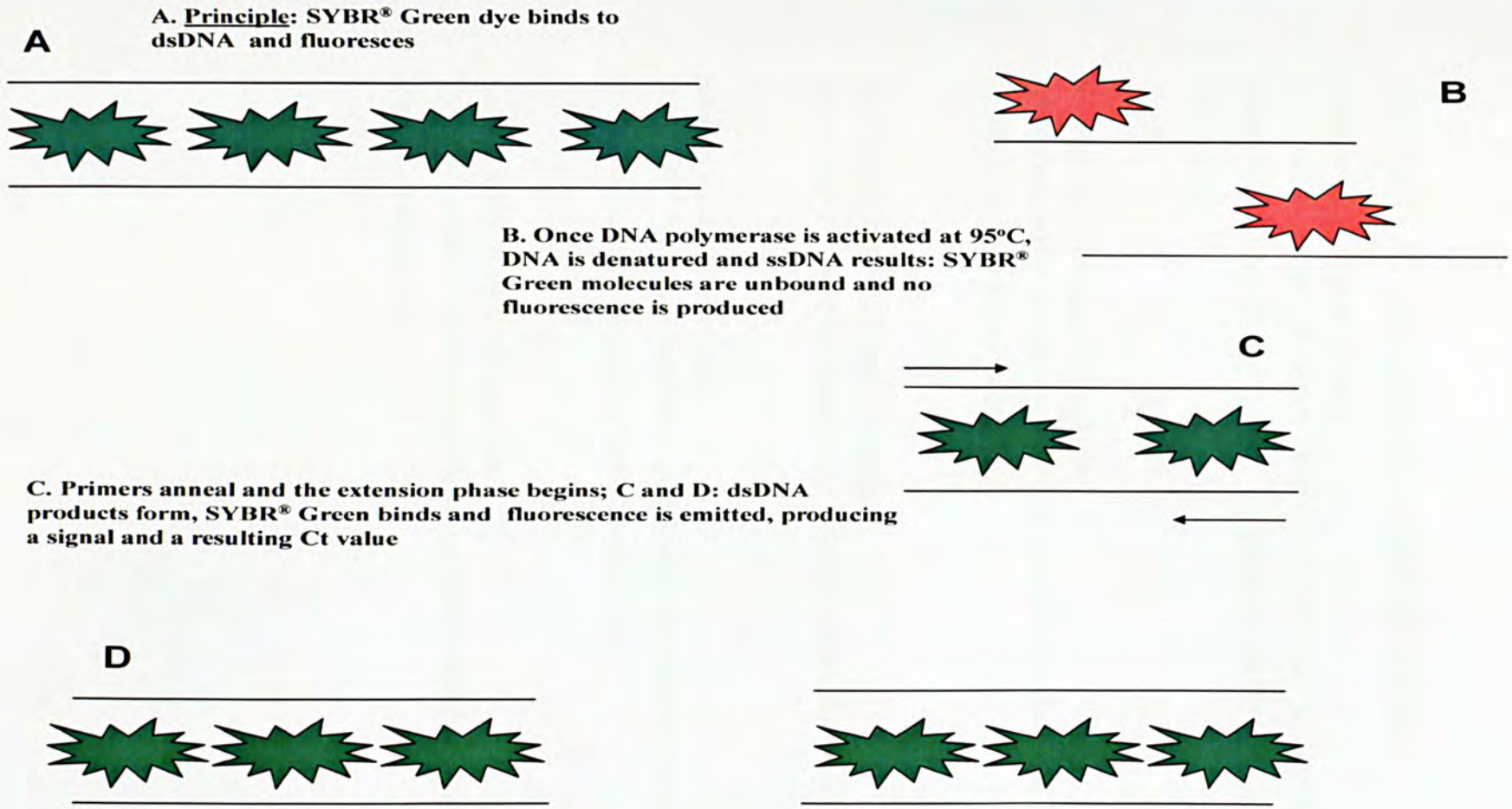


Fig 5.1: A schematic representation of the mode of action of SYBR® Green. SYBR® Green is a non-specific dye which binds only to dsDNA (A). During a PCR reaction, once DNA is denatured (B), DNA separates into ssDNA strands. SYBR® Green is present in solution but does produce any fluorescence. Once primers anneal and extension begins (C), the SYBR® Green binds to dsDNA and a signal is emitted (D).

SECTION 5.2: MATERIALS AND METHODS

Section 5.2.1: Primer Design

A detailed account of primer design and selection, as well as a list of all the primer sequences utilised in this chapter are provided in Chapter 2: Materials and Methods, Section 2.2.1.

Section 5.2.2: Primer Optimization

Primer optimizations were done according to the procedure described earlier in Chapter 2: Materials and Methods, Section 2.2.2.

Section 5.2.3: DNA Template dilutions

These were prepared as described in Chapter 2: Materials and Methods, Section 2.2.3.

Section 5.2.4: RT Protocol

For the RT reaction, a two step protocol was used and the method adopted, fully described in Chapter 2: Materials and Methods, Section 2.2.4.

Section 5.2.5: Method of Analysis

This was conducted according to the procedure described in Chapter 2: Materials and Methods, Section 2.2.5.

SECTION 5.3: RESULTS

As stated in Section 5.2 Material and Methods, prior to working with total RNA samples, it was essential to optimise the primers first. Fig 5.2a shows a typical amplification plot with different primer ratios for *capE*. Such plots were done with all other 11 genes, including the reference genes, *16SrRNA* and *gyrB*. To eliminate the possibility of primer-dimer formation, the dissociation curve for each amplification was viewed simultaneously (Fig 5.2b) and thus, the Ct (cycle threshold) for all the primer ratios was taken and graphs plotted using MS Excel (Fig 5.3 a-c). The Ct is defined as that cycle where the fluorescence signal is significantly above background. The primer ratio having the least Ct was consequently chosen. For most genes, the 1F:1R ratio was chosen. However, with some cases such as *hld* and *clfA* it was necessary to choose other ratios such as 1(F):2(R) and 1(F):6(R). Also, Fig 5.4 represents an example of a standard curve, using DNA as template for optimisation.

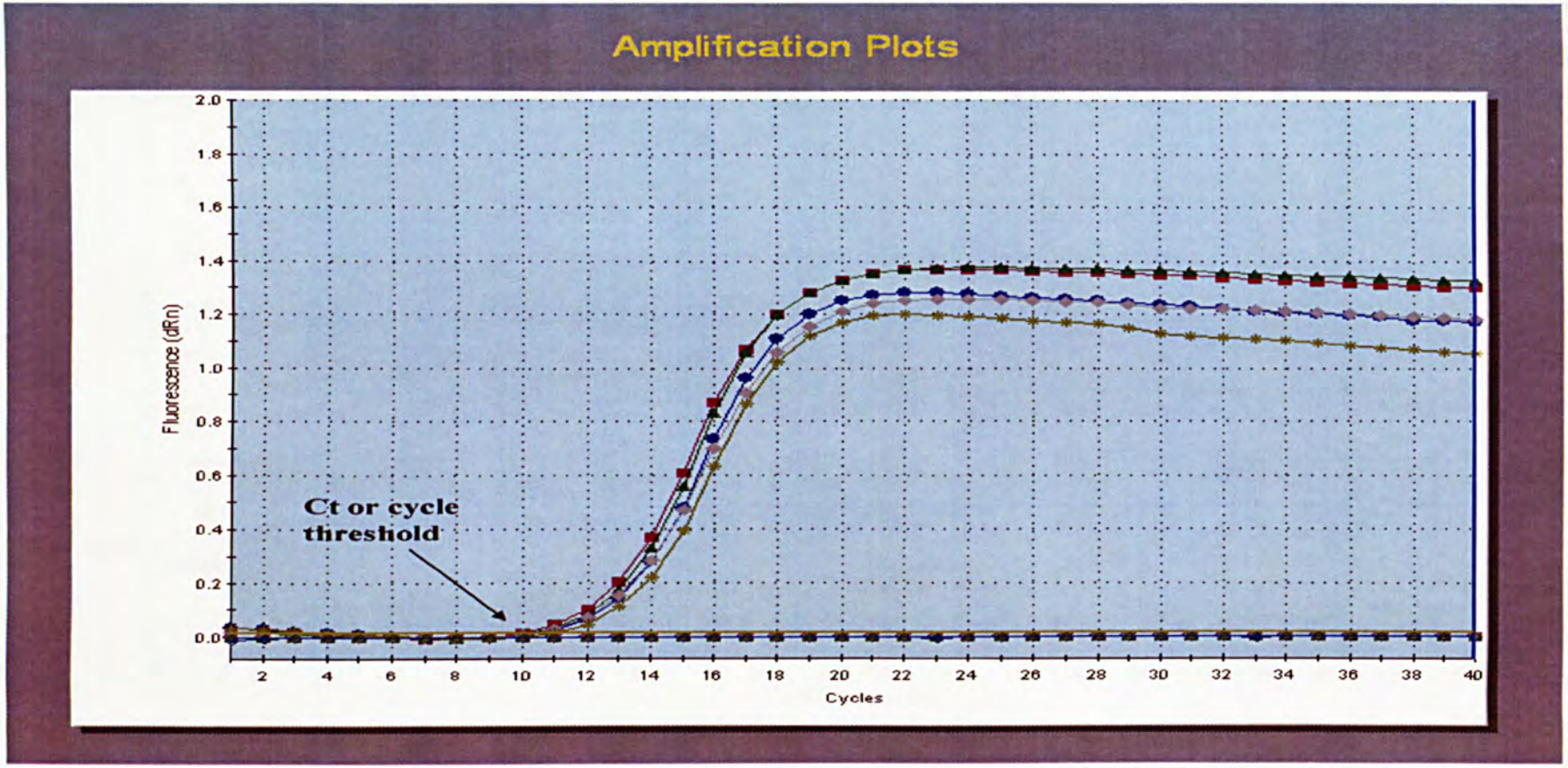


Fig 5.2a: CapE primer ratios Amplification Plot : yellow line- cap E 6 (F): 1(R); grey line- 2(F):1(R); blue line- 1(F):6(R), red- 1(F):2(R) and green: 1(F):1(R). Arrow indicates the Cycle threshold (Ct) and Ct for the 1(F):1(R) concentration produces the best Ct.

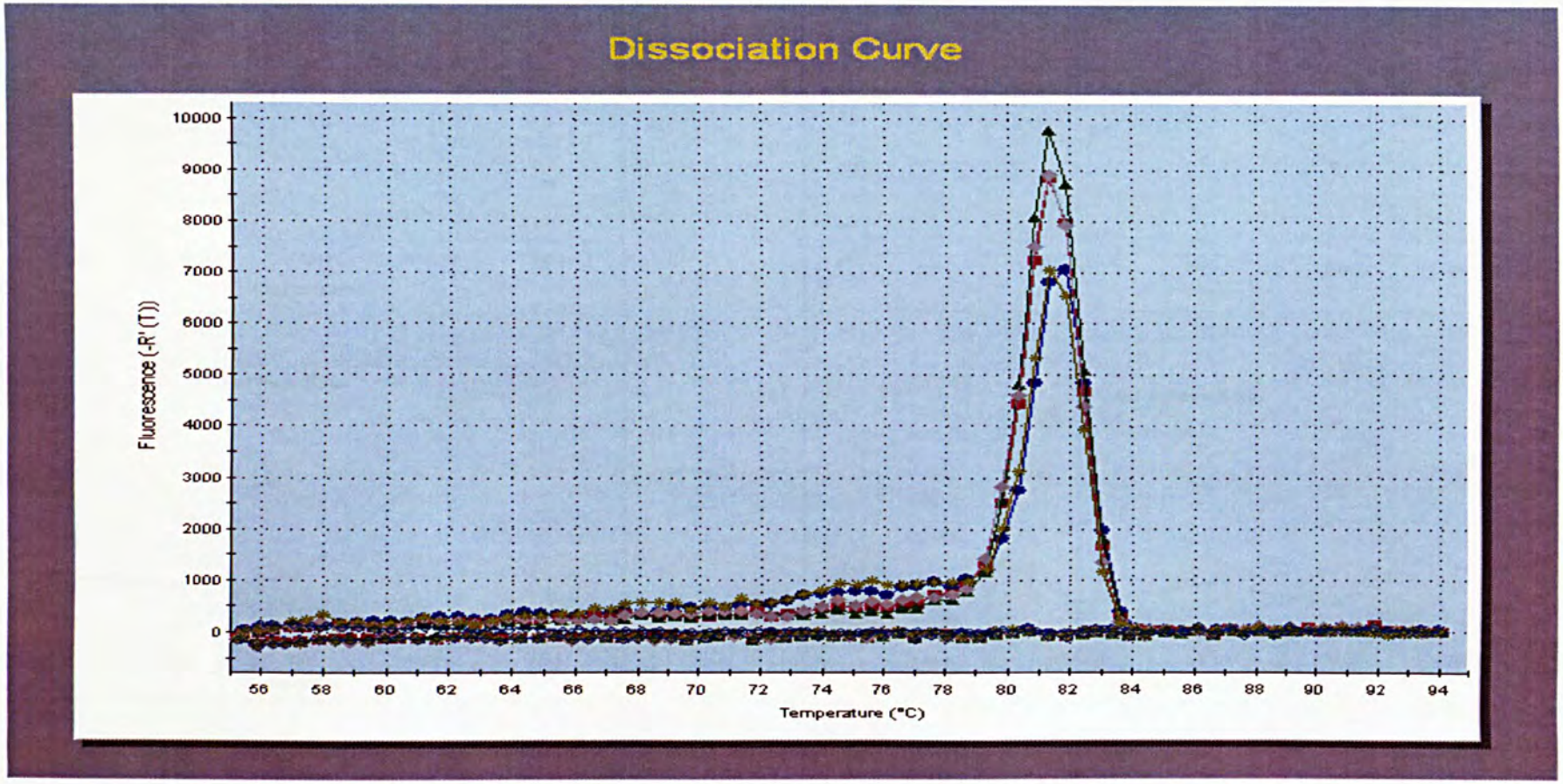
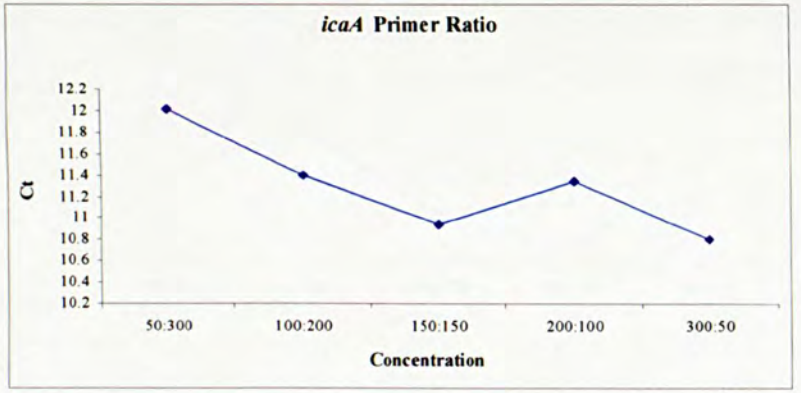
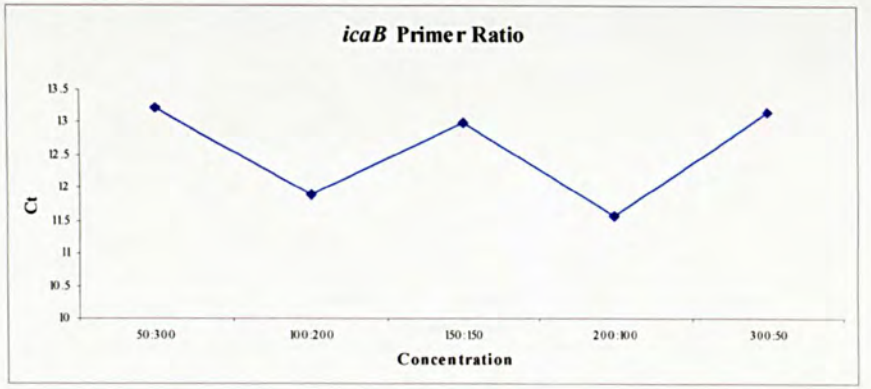
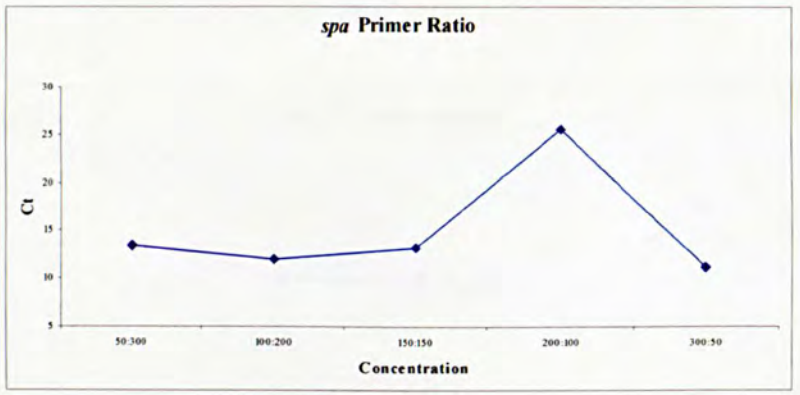


Fig 5.2b: CapE primer ratios Dissociation Curve: Melting point is 81°C: yellow line- cap E 6 (F): 1(R); grey line- 2(F):1(R); blue line- 1(F):6(R), red- 1(F):2(R) and green: 1(F):1(R). All primer concentrations yield one melting point and no primer-dimers are observed.







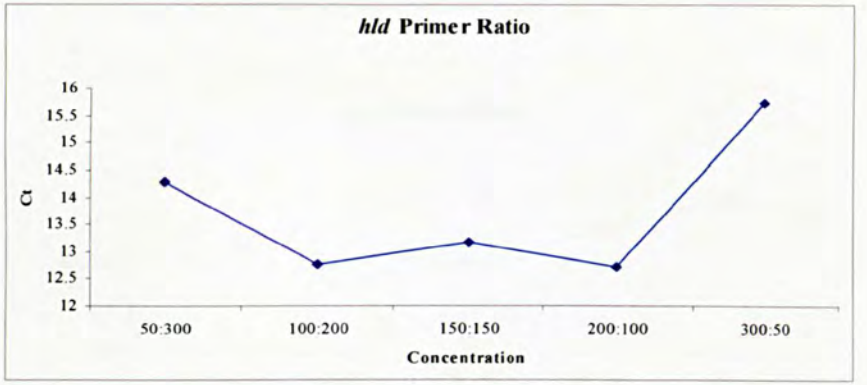
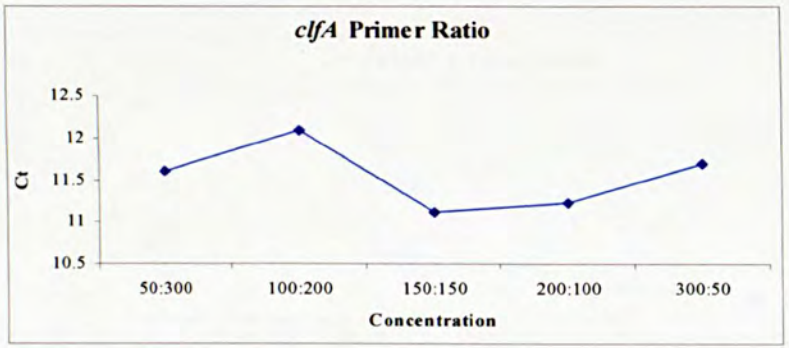
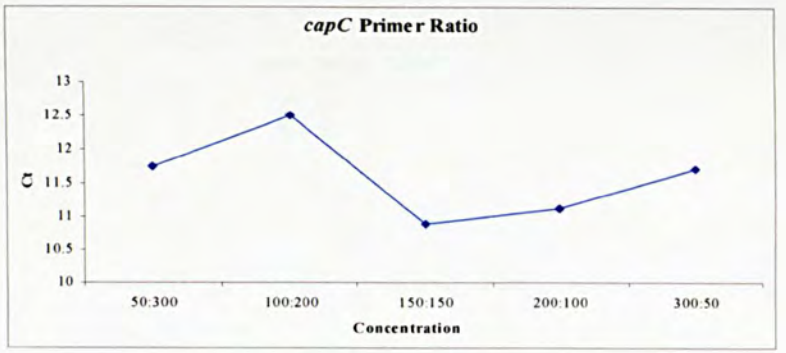
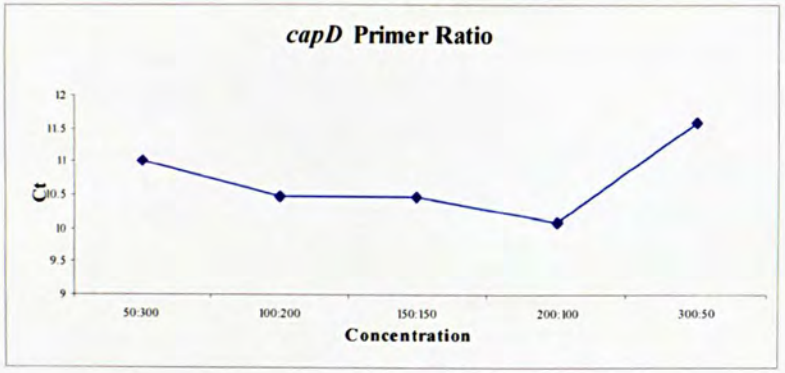


Fig 5.3a: Ct values vs forward and reverse primer concentrations (Forward: Reverse primer ratio in nM) of *icaA*, *icaB*, *spa* and *hld* genes This figure presents Ct vs Primer (forward and reverse) concentration values of individual genes. Primer ratios with the lowest Ct (or the 150:150 concentration if Ct values were very similar e.g *spa*) were chosen for ensuing experiments.







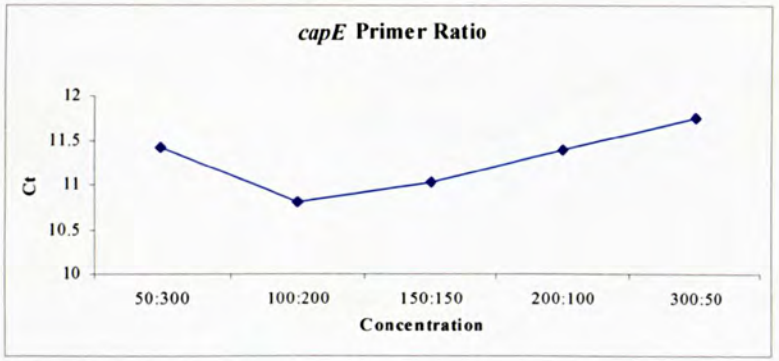


Fig 5.3b: Ct values vs forward and reverse primer concentrations (Forward: Reverse primer ratio in nM) of *clfA*, *capC*, *capD* and *capE* genes. This figure presents Ct vs Primer (forward and reverse) concentration values of individual genes. Primer ratios with the lowest Ct (or the 150:150 concentration if Ct values were very similar e.g *capD*) were chosen for ensuing experiments.

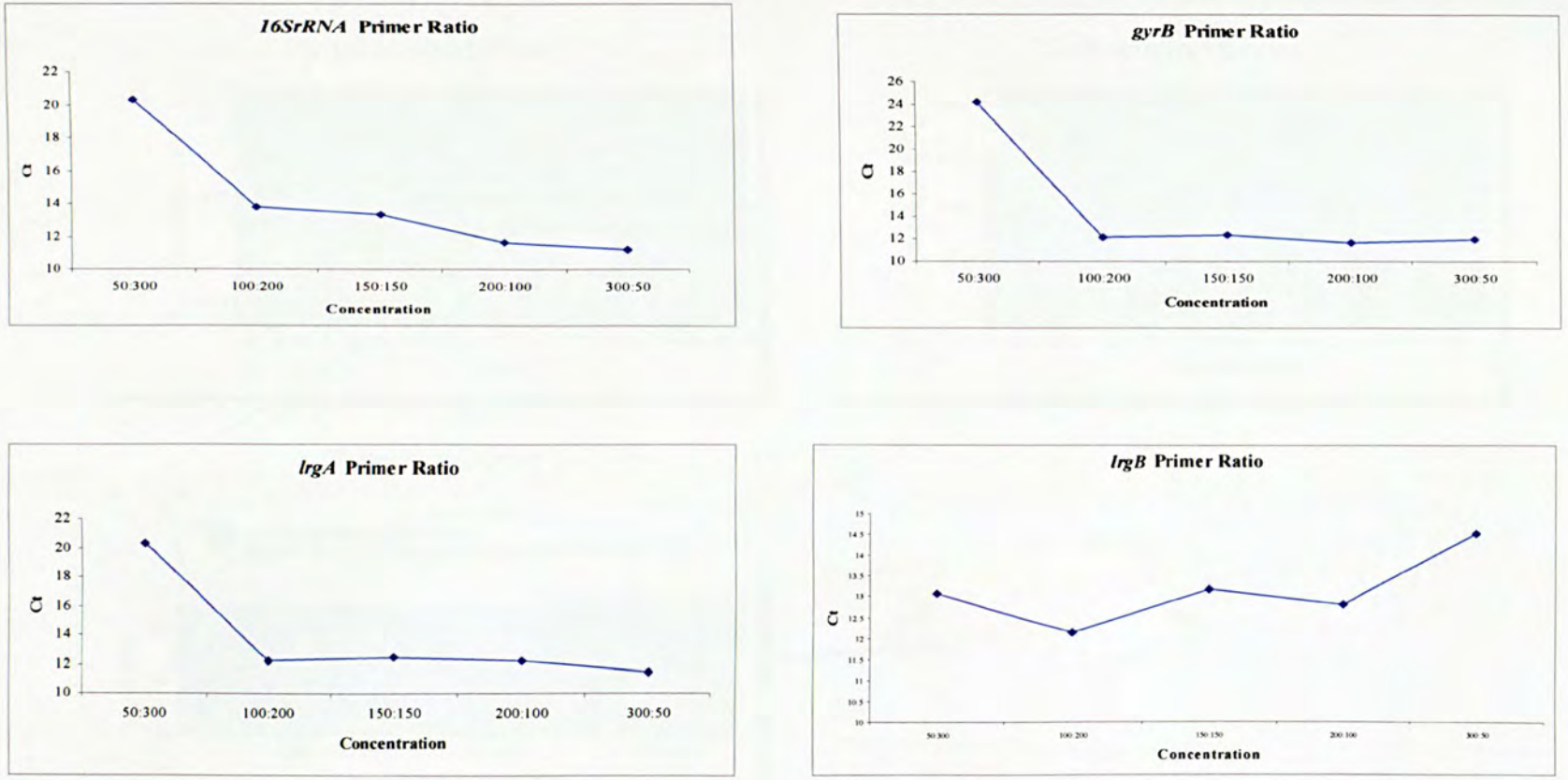


Fig 5.3c: Ct values vs forward and reverse primer concentrations (Forward: Reverse primer ratio in nM) of *16SrRNA, gyrB*, *lrgA* and *lrgB* genes. This figure presents Ct vs Primer (forward and reverse) concentration values of individual genes. Primer ratios with the lowest Ct (or the 150:150 concentration if Ct values were very similar e.g *lrgA*) were chosen for ensuing experiments.

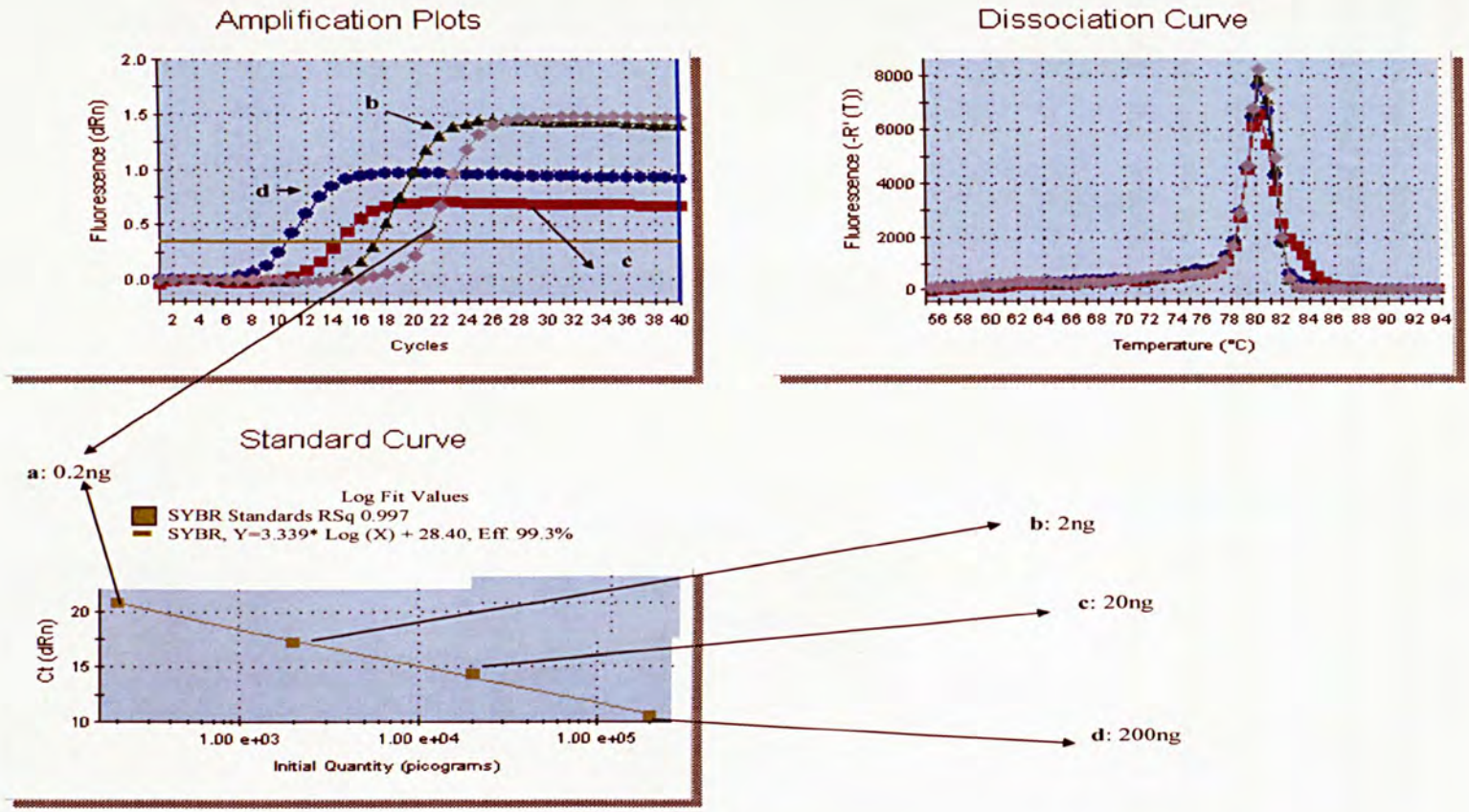


Fig 5.4: Example of a standard curve for *capC*, using DNA as the sample template for optimisation (r^2 =0.997 and efficiency=99.3%)

Prior to initializing experiments using RNA samples, an assay was set up which sought to prove that the levels of the normaliser genes, *gyrB* and *16SrRNA* were indeed constant across all the samples. This was deemed an essential step before commencing the assays with the genes of interest. Once Ct values were confirmed as constant for all BHI, ASM and GASM samples, experiments were allowed to proceed further.

In Fig 5.5 the relative fold differences of the genes of interest investigated in the RT-PCR experiments are presented. Table 5.2 and Fig 5.6 compare fold differences between the microarray and RT-PCR experiments in the same genes.

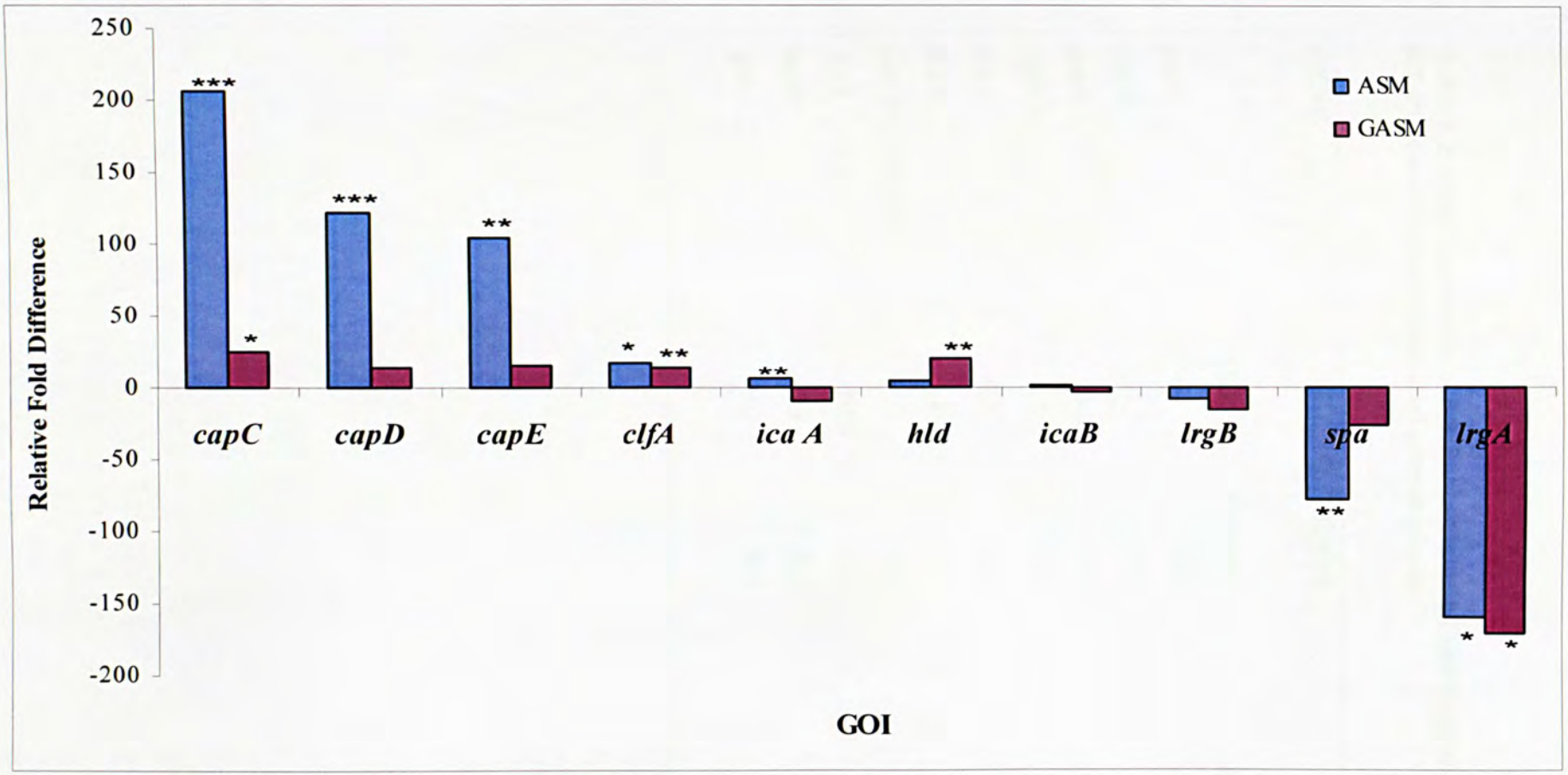


Fig 5.5: Relative fold differences of the Gene of Interest (GOI) (*** Represents statistical significance using the 2-tailed, paired student t test and p= ≤0.001; ** when p= ≤0.01 and * when p=≤0.05)

Table 5.2: Fold Expression of MRSA 252 in ASM and GASM in microarrays vs RT-PCR during the exponential growth phase.

GENE	MICROARRAYS		RT-PCR	
	ASM	GASM	ASM	GASM
capC	64	8.3	206.5	24.9
capD	33.3	4.4	121.9	14.7
capE	24.5	3.6	105.2	15.0
clfA	8.7	5.4	16.9	14.0
icaA	4.3	1.6	6.8	-9.4
icaB	3.1	1.8	1.8	-3.0
hld(RNAIII/*agr*)		2.5	5.0	20.2
lrgA	-10.8	-9.7	-160.2	-171.1
lrgB	-4.9	-8.0	-7.6	-15.5
spa	-9.2	-6.2	-78.6	-26.0

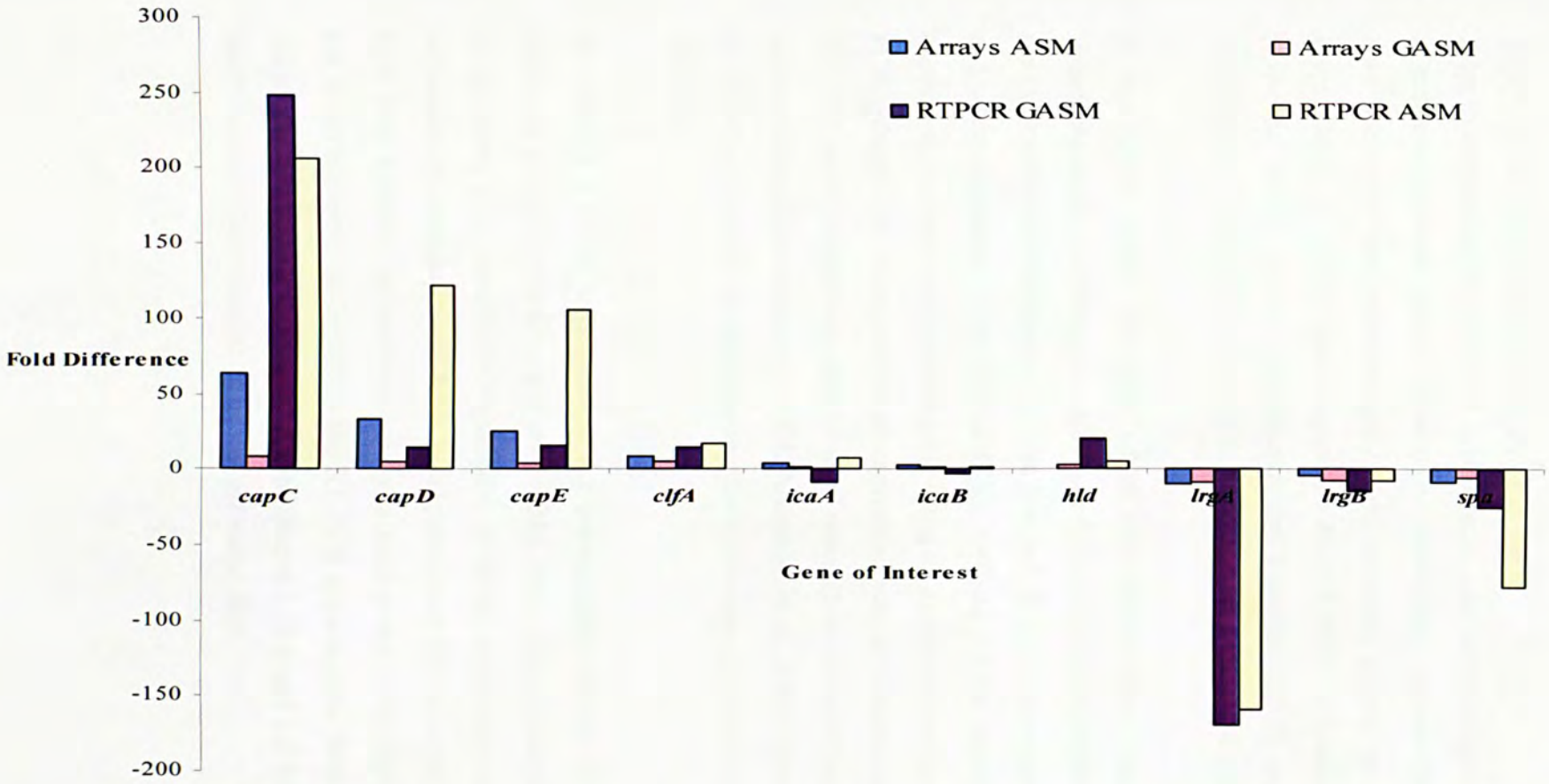


Fig 5.6: Graph comparing the fold differences obtained with microarrays and RT-PCR for MRSA252 grown in BHI, ASM and GASM during the exponential growth phase. Fold difference for ASM and GASM are normalised against BHI control. Specific values for each gene can be seen in Table 5.4.

SECTION 5.4: DISCUSSION

Prior to conducting the RT-PCR experiments, two reference genes were selected, in order to produce validated results. Most other researchers, such as Goerke *et al.* (2000, 2001) have used only one reference gene, *gyr*, encoding gyrase, as a normaliser gene when investigating *S. aureus* gene expression by RT-PCR in human cystic fibrosis sputum. However, others, such as Sabersheikh and Saunders (2004) and Eleaume and Jabbouri (2004) used *16SrRNA* as a normaliser gene in *S. aureus* gene expression studies.

In the current study, ten genes which were differentially expressed when MRSA252 (exponential growth phase) was grown in ASM and GASM (when normalised to BHI from the microarray results-Chapter 4) were selected. A selection of genes which were highly up and down-regulated were included, as well as others which were not very highly expressed. The aim was to investigate, using an alternative method to microarrays, i.e. RT-PCR, whether the same pattern of expression was achieved as in the microarray results. RT-PCR is the method adopted by most researchers for confirming gene expression in *S. aureus* studies (Beenken *et al.*, 2004, Anderson *et al.*, 2006, Luong *et al.*, 2006, Roberts *et al.*, 2006) and also for *P. aeruginosa* gene expression (Chang *et al.*, 2005 and Palmer *et al.*, 2005).

In both Fig 5.5 and 5.6 and Table 5.2 above, genes coding for capsular polysaccharide synthesis enzymes (*capC*, *capD* and *capE*) were up-regulated (normalised to BHI) and most values were statistically significant. Both the microarrays and RT-PCR showed up-regulation of capsular genes. Also, up-regulation of *clfA* in ASM and GASM was detected with both methods. In addition, *icaA* and *icaB* genes were slightly up-regulated in ASM but down-regulated in GASM in the RT-PCR experiments. With the microarrays, GASM values for these genes were only slightly above 1 (1.6 and 1.8 fold increases respectively) and these were below the stringent cut-off value of 2.

The *lrgA* gene was down-regulated in both ASM and GASM with both methods and this finding was highly significant. *LrgB* and *spa* were also down-regulated in both media and both methods.

Although individual values for relative fold difference when compared to BHI were different between the 2 techniques, this is may have resulted from RT-PCR being very sensitive and requiring minimal cDNA amounts. However, overall, the pattern of expression was similar with both methods and highly up and down-regulated genes were observed to be statistically significant.

Goerke *et al.* (2000) studied gene expression of *agr*, *spa* and *hla* of *S. aureus* isolates present in human CF sputum, using RT-PCR. Low expression levels of *spa* and *hla* were observed. RNAIII levels which was used as a marker for *agr* activity were found to be minimal, indicating that *agr* may not play such a pivotal role in controlling virulence mechanisms in *S. aureus* CF lung infections. However, the authors also stated that the results may have been influenced by the bacterial density which was lower than considered necessary for *agr* activation *in vitro*, i.e. 10^9cfu/ml.

A similar pattern was observed in the present study, where MRSA252 grown to exponential phase in ASM. Here, both *spa* (validated with RT-PCR) and *hla* levels were decreased, whilst no RNAIII or any *agr* seen in the microarray results (Chapter 4).

SECTION 5.5: CONCLUSIONS

As indicated above, RT-PCR is a very sensitive tool for analysing RNA transcripts and is ideal in conditions where RNA levels are restricted. However, the number of genes studied are much fewer than with microarrays. Nonetheless, the results in Chapter 5 indicate that the data obtained using 2 different techniques, i.e. microarrays (Chapter 4) and RT-PCR (Chapter 5), yielded similar patterns. Also, these results helped to validate the previous results obtained with the microarrays (Chapter 4).

From the results obtained from both Chapter 4 and Chapter 5, it may be postulated that since genes coding for capsular proteins are up-regulated, MRSA252 produces a capsule to enhance proliferation during the initial stages of infection in a CF lung infection. The presence of glucose in CF secretions (as represented by GASM) does not deter the production of capsule, since capsule coding genes are also up-regulated in this medium, although not to the same extent as for ASM. Also, when grown in GASM, or without glucose, ASM, MRSA 252 may initiate gene mechanisms in order to prevent murein hydrolases from destroying the peptidoglycan cell wall which could have a negative impact on bacterial proliferation (as evidenced by the down-regulation of the *lrgAB* operon in both ASM and GASM).

Finally, the results obtained in this chapter, suggest that RT-PCR is the ideal method for adoption when RNA concentrations are limited. It was also the technique chosen to investigate whether RNA transcript levels are similar to those obtained with ASM and GASM when MRSA252 was grown directly in human CF sputum samples (Chapter 6).

CHAPTER 6

MRSA252 Growth Physiology and Gene Expression in CFS

SECTION 6.1: INTRODUCTION

A recent review by Stone and Saiman (2007) reported that in the US in 2005, respiratory tract infections in cystic fibrosis patients due to *S. aureus* amounted to 51.8%, with the highest prevalence observed in children and individuals ≤ 17 years of age. Souza *et al.* (2007) obtained oropharyngeal swabs from neonates in a CF out-patient hospital clinic in Brazil and *S. aureus* was the most frequently isolated bacterium (76% of patients). Kidd *et al.* (2006) investigated MRSA colonization of CF patients from an adult Australian CF clinic using PFGE. MRSA isolates collected from 21 patients during a two-year period, MRSA isolates from non-CF patients (these patients were recruited from health institutions in Southeast Queensland) and isolates from CF patients who were chronic carriers of MRSA, from the same hospital were studied. The authors revealed that 11 from the 21 patients possessed 1 of the 2 MRSA strains which were known to be endemic at the hospital but >47% of the MRSA isolates were different to the endemic strains. Analysis of the MRSA from chronic carriers indicated that patients retained the same strain and this indicated clonal stability over time.

Ren *et al.* (2007) investigated the differences in disease severity between CF patients having MRSA and those with MSSA. The researchers retrieved data related to patient respiratory tract cultures from the Epidemiological Study of CF (a large North American CF study). Over a 1 year period, data from 20,451 patients was reviewed and 7.5% of CF patients had *S. aureus* only. FEV1 in MRSA patients was significantly lower than in MSSA patients, implying increased airflow obstruction. In addition, MRSA positive patients had increased chance of hospitalization and treatment with oral, inhaled and intravenous antibiotics. This effect was mirrored in people ≥18 years of age. Similar observations have been reported by other investigators (Miall *et al.*, 2001; Nadesalingam *et al.*, 2005).

Observations in the above studies indicate a fascinating relationship between the respiratory environment of the CF patient and the ability of *S. aureus* to exploit it. This calls for further exploration of the human CF sputum ambient in relation to *S. aureus* and this is the aim of Chapter 6. Indeed, the previous chapters in this thesis have focussed on the production of a model system for *S. aureus* infection in a cystic fibrosis lung using ASM, an artificial medium which has been shown to closely resemble human cystic fibrosis sputum (Ghan and Soothill, 1997; Sriramulu *et al.*, 2005). However, the need for

comparison of the activity exhibited by MRSA252 when grown in ASM and GASM (which simulates a diabetic state) with the human CFS samples, was the main focus of this chapter.

Section 6.1.1: Main Aims

The initial goal was to compare growth physiology of MRSA252 in CFS with ASM and microscopic studies were also performed (similarly to Chapter 3). Once the appropriate growth phases were identified, gene expression studies ensued. The technique employed in this chapter for the analysis of gene expression, based on previous observations (Chapter 5), is real-time PCR. RT-PCR is an important diagnostic tool in the bacteriology laboratory (Pitt and Saunders, 2000) and offers an ideal opportunity for the investigation of specific gene sequences. This technique was used for the investigation of 10 MRSA252 genes, when MRSA252 was grown to exponential phase in cystic fibrosis patient sputum. The results for the patient samples were compared to the ASM and GASM results obtained in the previous chapters.

SECTION 6.2: MATERIALS AND METHODS

Section 6.2.1: γ-radiation sterilization of ASM

The procedure for sterilisation of pre-inoculated ASM with MRSA252 and pre-autoclaved (control) ASM can be found in Chapter 2 Materials and Methods, Section 2.4.1.

Section 6.2.2: Ethics Committee

The ethics committee approval required was obtained as discussed in Chapter 2 Materials and Methods, Section 2.4.2.

Section 6.2.3: Human cystic fibrosis sputum experiments

A detailed account of all the materials and methods pertaining to these experiments can be found in Chapter 2 Materials and Methods, Section 2.4.3-2.4.11.

SECTION 6.3: RESULTS

Table 6.1 below indicates how the human samples were treated. In all, 41 samples were obtained from RBH. However, 23 of these proved to be too compact to enable viable count estimations. Such compact sputum (which remained so even after the action of vigorous vortexing), necessitated enzymatic action. This was beyond the scope and aims of this chapter, as utilising such components would have dramatically altered the CFS, as resulting proteolytic activity would have undoubtedly altered the CFS, presenting a major bias and leading to false results. More than half of the CFS samples (56%) would have required enzymatic digestion. These problematic samples were excluded from this study.

From the 18 samples screened for growth on BHIA and MSA, 28% yielded *S. aureus*. This indicated that these samples could not be used for growth of MRSA252 and subsequent gene testing, as viable count estimations were determined using MSA and differentiation between MRSA252 and colonies arising from the *S. aureus* present in the sample would not have been possible. Other CFS samples contained moulds and were immediately discarded if mould was apparent in the liquid sample or later, when grown on BHIA. Some CFS samples showed profuse growth on BHIA and it could not be assessed whether these were external contaminants or derived from patient lung. Some CFS samples contained antibiotics, as when challenged with a panel of Gram-positive and Gram-negative bacteria, some showed zones of inhibition. Thus, the presence of antibiotics might have interfered with MRSA252 and specifically affected gene expression, so these samples were not used either. Most of the CFS liquid samples (33%) were heavily contaminated with micro-organisms and these samples were also eliminated from the study.
Finally this screening process, presented 2 samples which could be used for growth of MRSA252. One sample (Pt14) yielded no growth for both BHIA and MSA and also when challenged with the different bacteria, did not produce any zones of inhibition. Another sample (Pt21) yielded growth of a pure culture on BHIA. This was confirmed twice. Gram-staining and light microscopy revealed a yeast (Fig 6.4).

The following tables and figures demonstrate the results obtained in this chapter. Each table and figure has a full legend giving details of the results pertaining to the initial screening process and the patient samples investigated in this chapter.

Table 6.1: Results for Patient Sample Screening

CF samples Received from RBH	41
Containing tightly compacted sputum which would need protease digestion in addition to glass beads and would be a potential bias	23
Containing liquid human CF sputum	18
*Containing *S. aureus*, hence exhibiting confluent growth on MSA	5[¶] (28%)
Containing moulds	4
Exhibiting a zone of inhibition for 1 or >1 of the bacteria tested (i.e. *E. coli*, MSSA (*S. aureus* Oxford 6571 strain), MRSA252 and *P. aeruginosa*)	3[§]
Containing >3 colony types on BHIA and/or possible contaminants	6
Exhibiting no growth on BHIA or MSA	1
Having <2 colony types on BHIA	1

* Indicates % CFS samples yielding *S. aureus*, from the 18 samples which were screened; [¶] 1 CFS sample yielded moulds and had *S. aureus*; [§] 1 CFS had a zone of inhibition for MRSA252 and had >3 colony types on BHIA.

Table 6.1 above presents the results obtained in the initial patient sample screening experiments. Table 6.2 below illustrates patient details retrieved from CFS samples which yielded *S. aureus*. It can be observed that 60% of these patients were males and the overall (both sexes) average age range was 20.2 years (Fig 6.1).

Table 6.2: Analysis of *S. aureus* positive CFS

CFS APPEARANCE	AGE (years)	GENDER
Clear-White	20	M
Opaque-White	19	M
Clear-White	20	M
Clear-White	29	F
Clear-White	13	F

Table 6.3, shows pH values of the human samples selected for gene expression studies, compared to ASM.

Table 6.3: pH values for ASM, Pt 14 and Pt 21 and cultures prior to inoculating with MRSA252

Medium	pH
ASM	**7.0**
Pt 14 CFS	**8.0**
Pt 21 CFS	**7.4**

Table 6.4 below contains patient data relating to CFS sample consistency, age and gender of Pt14 and Pt21.

Table 6.4: CFS Details of Pt 14 and Pt 21

SAMPLE	CFS APPEARANCE	AGE (years)	GENDER
Pt 14	Clear-White	19	F
Pt 21	Opaque-White	43	F
ASM (control and pre-autoclaved)	Light Yellow	-	-

Fig 6.1 overleaf shows images taken from BHIA and MSA plates containing microbial growth resulting from direct CFS sampling. A and C show confluent growth on BHIA whilst B shows no growth on MSA and D shows *S. aureus* growth on MSA. MSA exhibits a colour change when *S. aureus* grows, from red to yellow, due to *S. aureus* fermenting mannitol and thus, leading to a decrease in pH which causes the phenol red indicator present in the medium to change colour to yellow.

Fig 6.2 illustrates MHA plates of *Pseudomonas aeruginosa* ATCC 10145 (E), *Escherichia coli* ATCC 11775 (F), MRSA252 (G) and *Staphylococcus aureus* Oxford 6571 (H), containing the grid set-up used for antibiotic screening.

Fig 6.3 shows yeasts (budding can be visualized) present in Pt21 CFS sample, prior to MRSA252 inoculation. This image is the result of a Gram-stained preparation taken from a pure culture of the yeasts growing on BHIA. This stain was repeated twice on separate slides and each time a pure culture of yeasts, sometimes with budding, was observed.

Fig 6.4 illustrates typical growth curve obtained when MRSA252 was grown in ASM, Pt14 and Pt21 sputa. These are results of the average of 2 independent experiments.

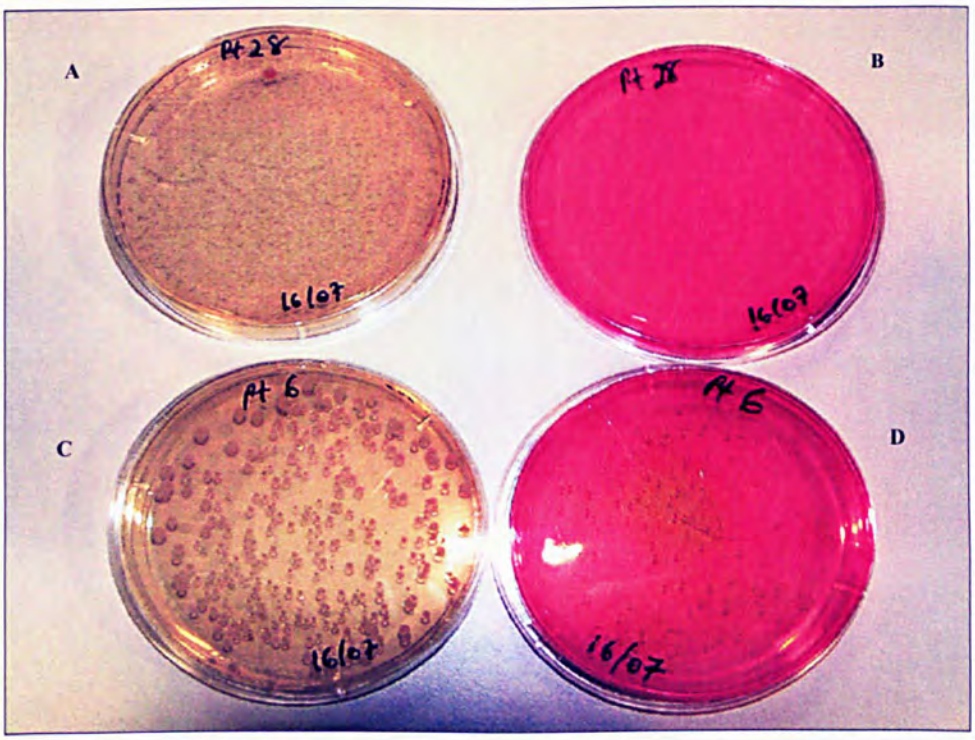


Fig 6.1: Direct growth resulting from CFS on BHIA (A, C) and isolation of *S. aureus* on MSA (D) and B shows no growth on MSA.

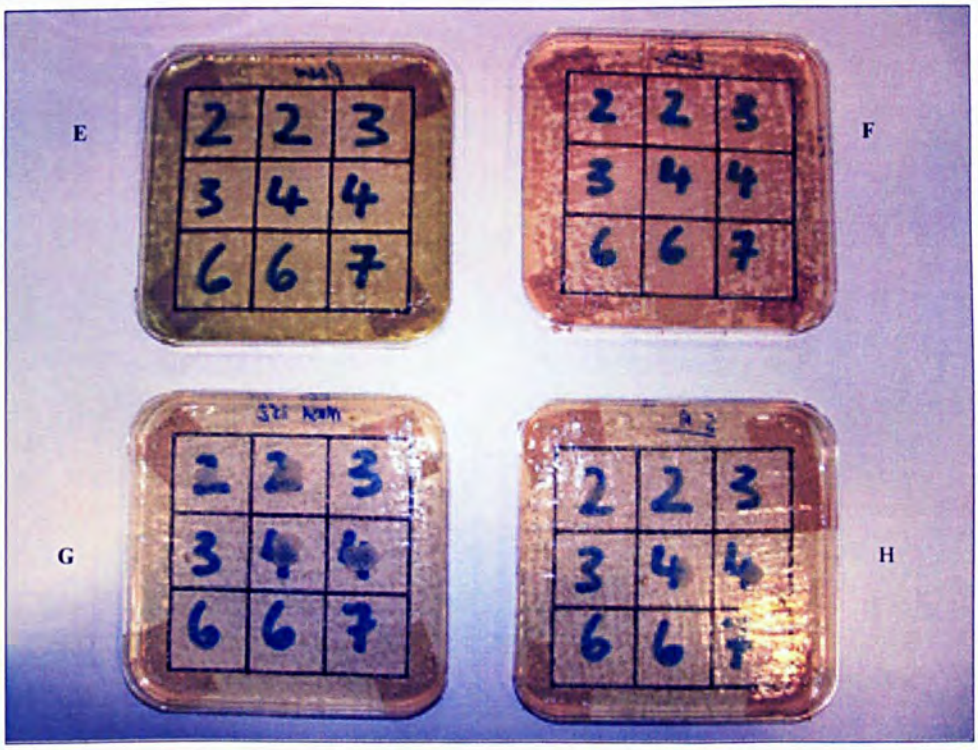


Fig 6.2: Bacterial growth on MHA, in the presence of CFS. Each square inside the main grid represents different patient samples and 2 (10µl) aliquots were taken from each patient sputum sample. The numbers on the grid indicate coded patient samples. (E: *Pseudomonas aeruginosa* ATCC 10145; F: *Escherichia coli* ATCC 11775; G: MRSA252 and H *Staphylococcus aureus* Oxford 6571).

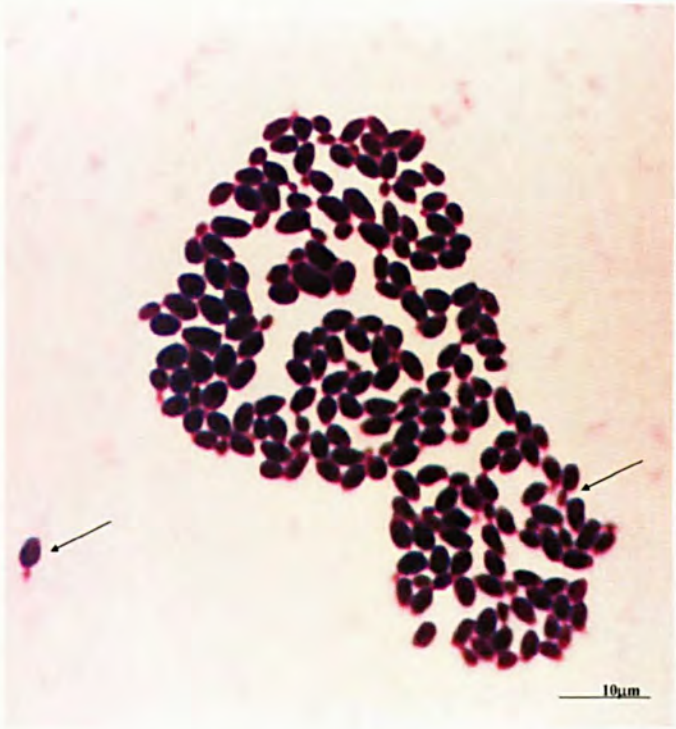


Fig 6.3: Isolation of yeasts from Pt 21 CFS prior to the inoculation with MRSA252 These large colonies appeared on BHIA, when Gram-stained and budding can be observed, as indicated by arrows (Bar: 10µm).

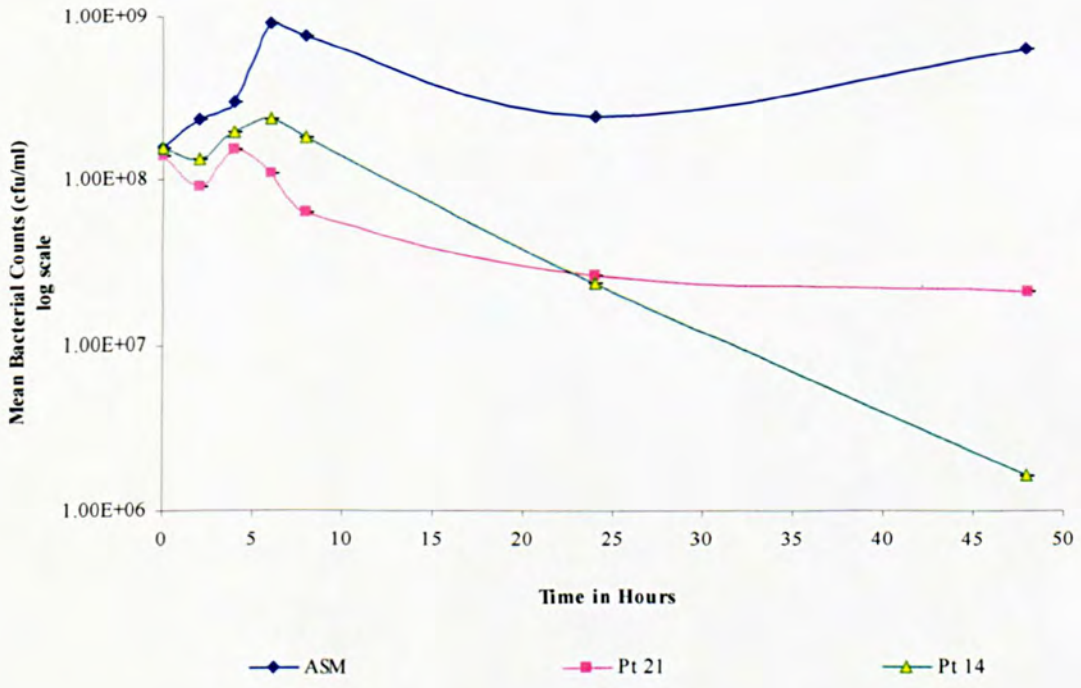


Fig 6.4: Growth of MRSA 252 in ASM, Pt21 and Pt14 CFS (Bar indicates standard error of mean)

Fig 6.5 (A-C) shows Gram-stain preparations taken from MRSA252 cultures in Pt21 sputum. As with the ASM, initial time intervals produced pinkish cocci but with increasing time intervals, the colour of the cocci appeared to be much darker and purple/blue colour.

Fig 6.6 (A-C) demonstrates Gram-stain preparations obtained from MRSA252 growing in Pt14 sputum. The images show that this sputum, as with Pt21 was indeed heterogeneous and indicated that when comparing one CF sputum sample belonging to one patient with another this fact must be taken into account. Also, cocci appear mostly pink.

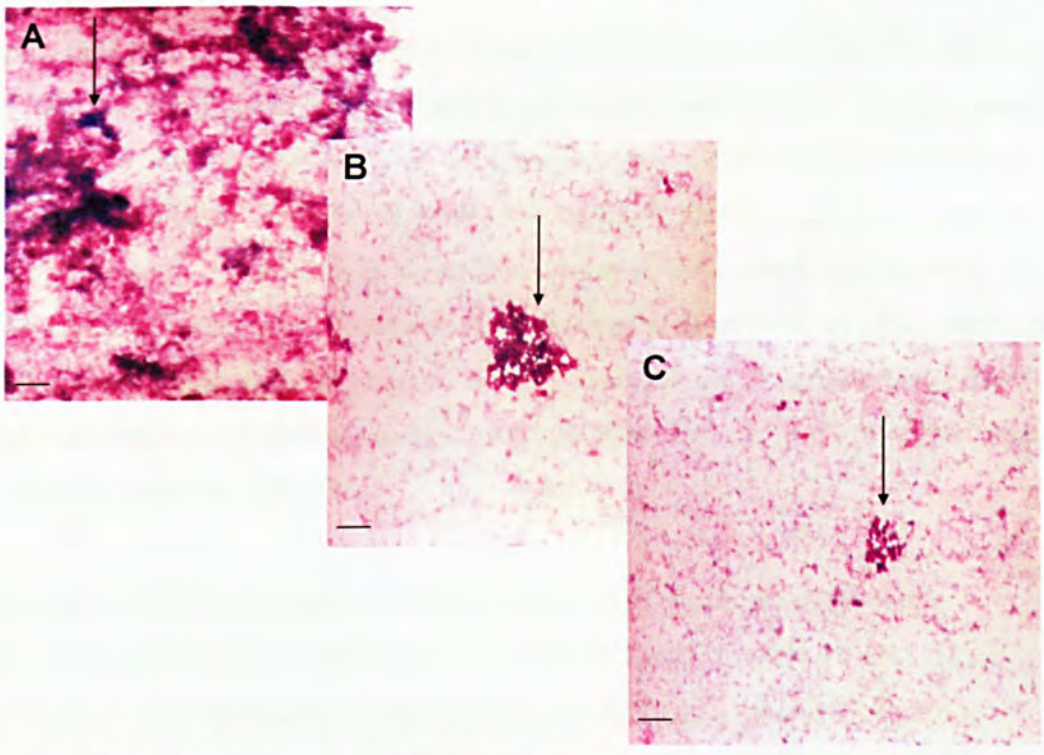


Fig 6.5 A-C: Gram-stain images of MRSA252 growing in Pt21 sputum at 8hrs(A), 24hrs(B) and 48hrs(C) (Bar indicates 10μm). This figure shows Gram-positive cocci (indicated by the black arrows), appearing in clusters, at increasing time-intervals.

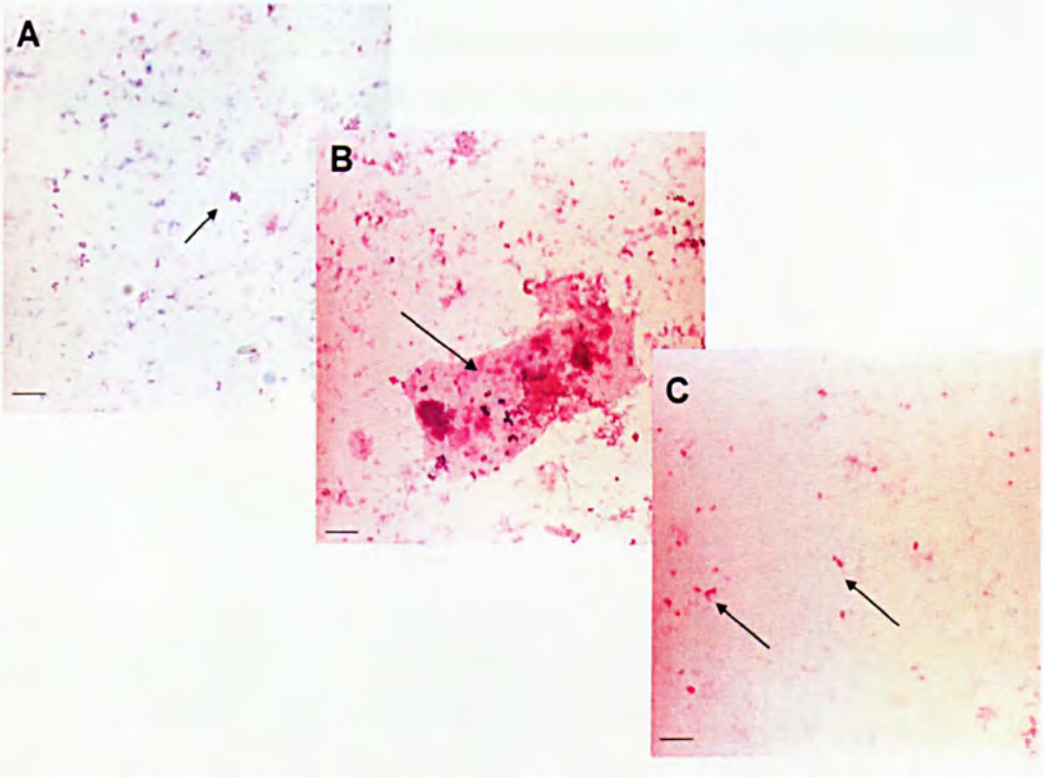


Fig 6.6 A-C: Gram-stain images of MRSA252 growing in Pt14 sputum at 8hrs(A), 24hrs(B) and 48hrs(C) (Bar indicates 10μm). Gram-positive cocci, (appearing pink/red in A and C and indicated by the black arrows), at increasing time-intervals. In B, the cocci can be seen in cellular debris, indicating the heterogenous composition of the sputum.

Fig 6.7 shows the results of gene expression when MRSA252 was grown in Pt21 sputum, using RT-PCR. Ten GOIs were analysed and it can clearly seen that the capsular genes, (*capC*, *capD capE*) were up-regulated, in addition to *clfA*. *LrgAB* operon, *hld* and *spa* genes were down-regulated. Fig 6.8 illustrates the results obtained when this strain was grown in Pt14 sputum. Relative fold differences were overall much higher with this sample. The capsular genes were observed to be up-regulated as well as *clfA*, although there are differences between the 2 samples, notably with the *icaAB* operon being highly up-regulated and the *LrgAB* operon, being downregulated. The *hld* and *spa* genes were down-regulated in both Pt21 and Pt14.

Fig 6.9 compares the results obtained in Chapter 5 when MRSA252 was grown to exponential phase in ASM and GASM to Pt21 and RT-PCR for the same 10 genes was performed. Fig 6.10 gives a schematic representation of the gene expression of the ten MRSA252 genes investigated in the ASM, GASM, Pt21 and Pt14 (and normalised to BHI). The aim of this diagram is to portray which genes were up and down-regulated, without taking note of the fold expression. Indeed, Table 6.5 includes all the fold expression differences of these 10 genes, when gene expression of MRSA252 in each medium, i.e Pt21, Pt14, ASM and GASM was normalised to BHI.

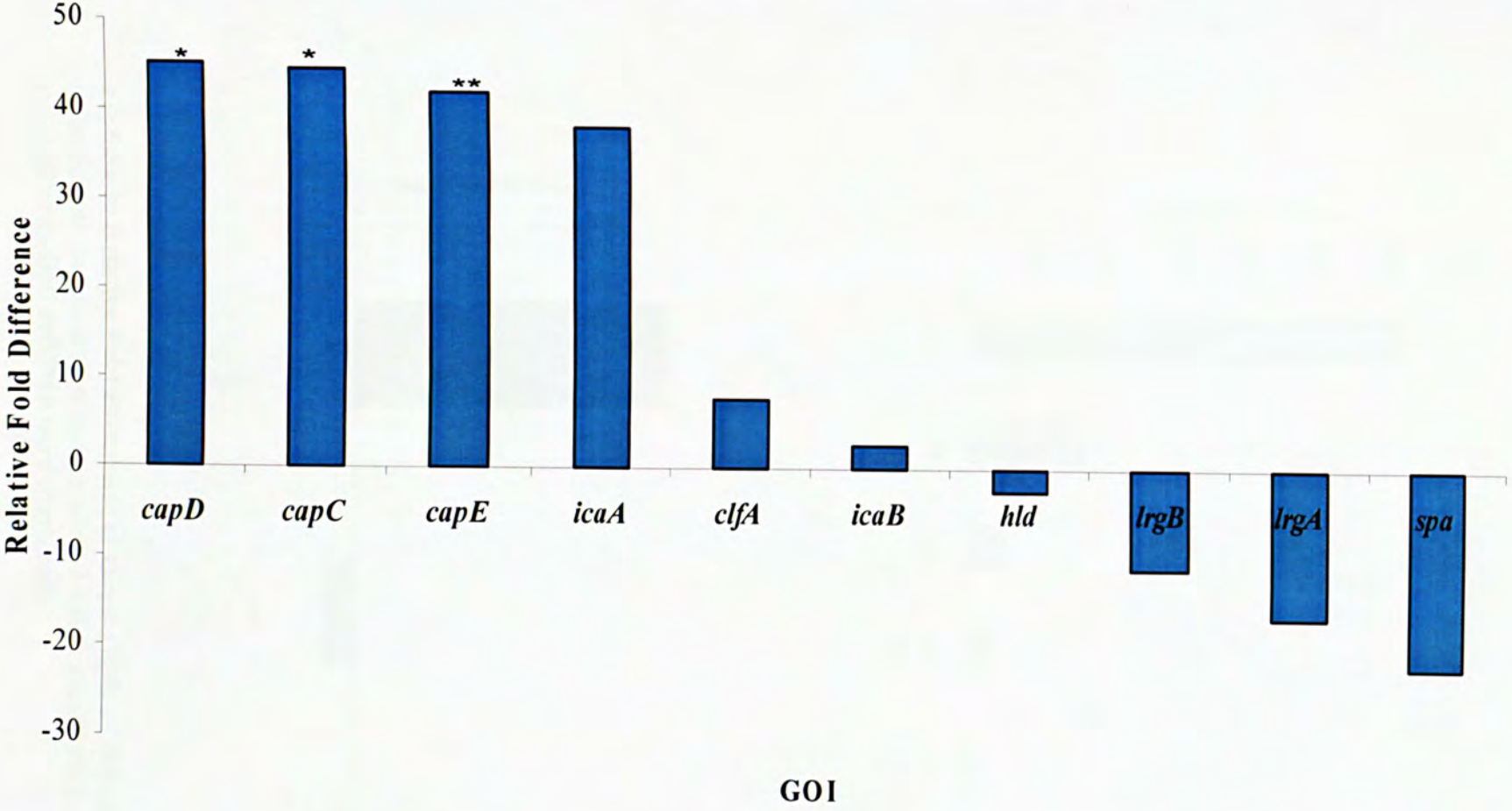
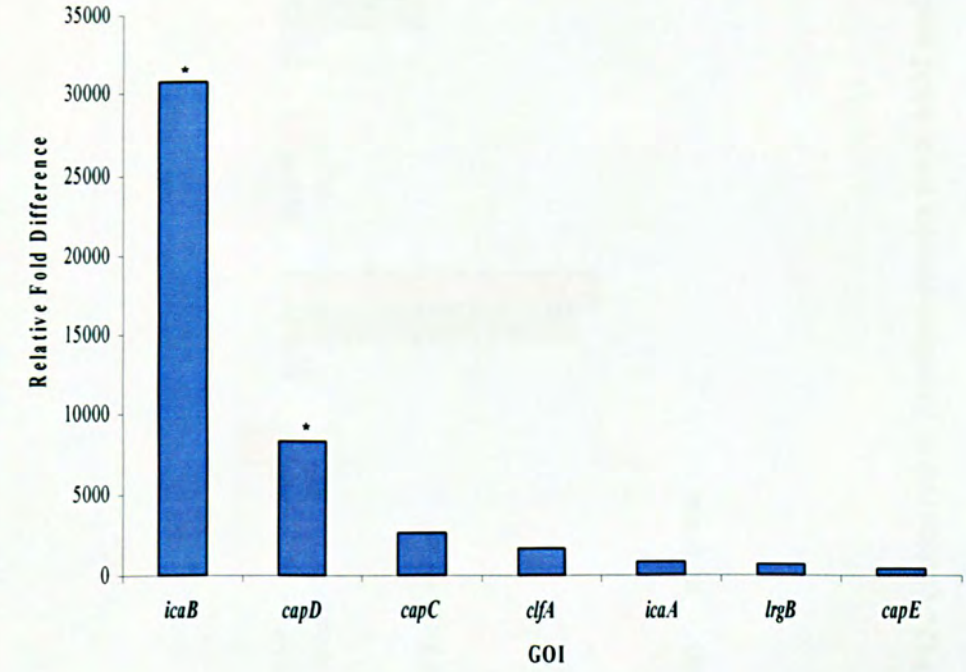


Fig 6.7: Relative Fold Difference of target Genes of Interest (GOIs) of MRSA252 in Pt21 CFS (**Represents statistical significance when 2-tailed, paired student t test was performed and $p \leq 0.01$, and * when $p \leq 0.05$, when BHI and Pt21 were compared).



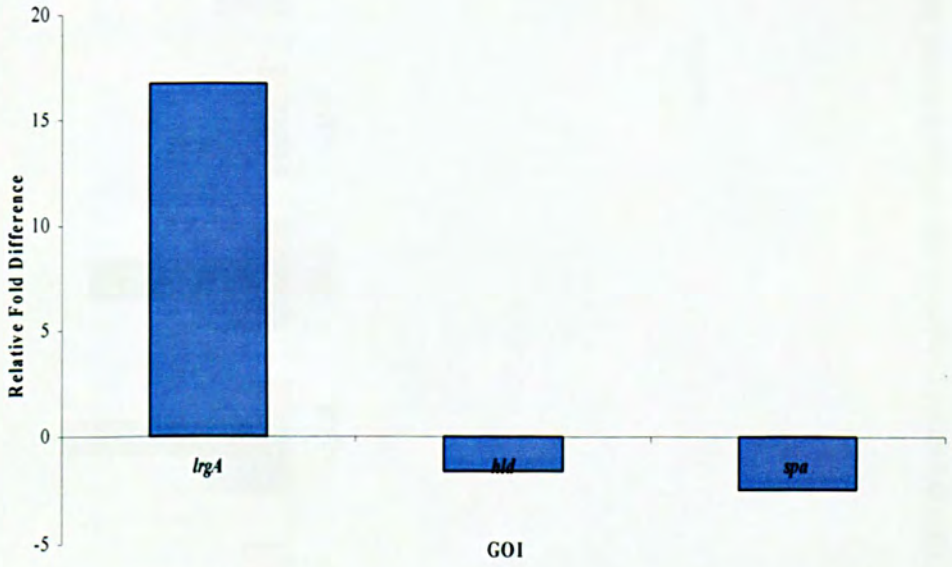


Fig 6.8a-b: Relative Fold Difference of target GOIs of MRSA252 in Pt14 CFS
(*Represents statistical significance when 2-tailed, paired student t test was performed and $p \leq 0.05$ when BHI and Pt14 were compared).

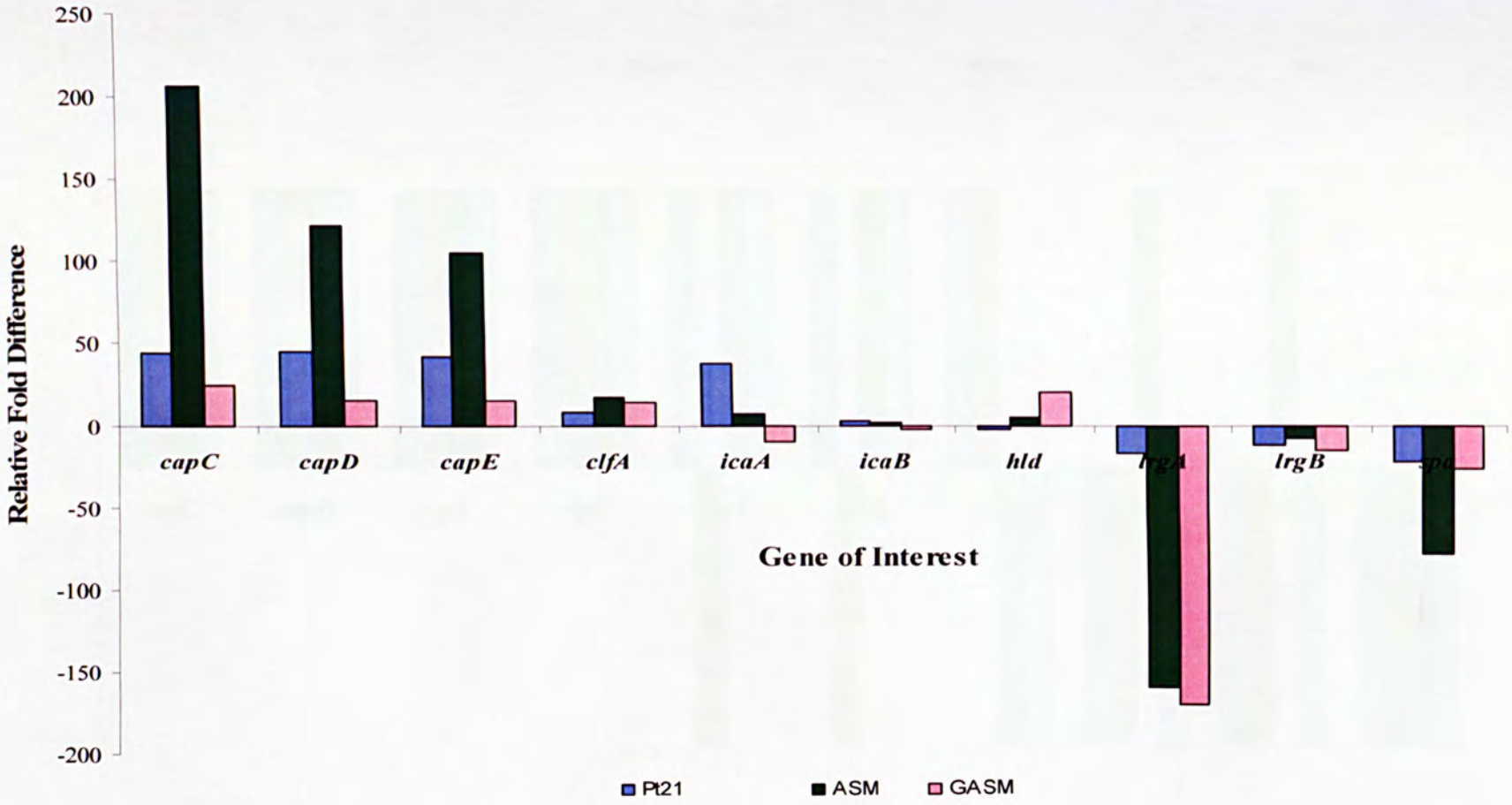


Fig 6.9: Relative Fold Difference of target Gene of Interest of MRSA252 in Pt21 compared to previous results with ASM and GASM.

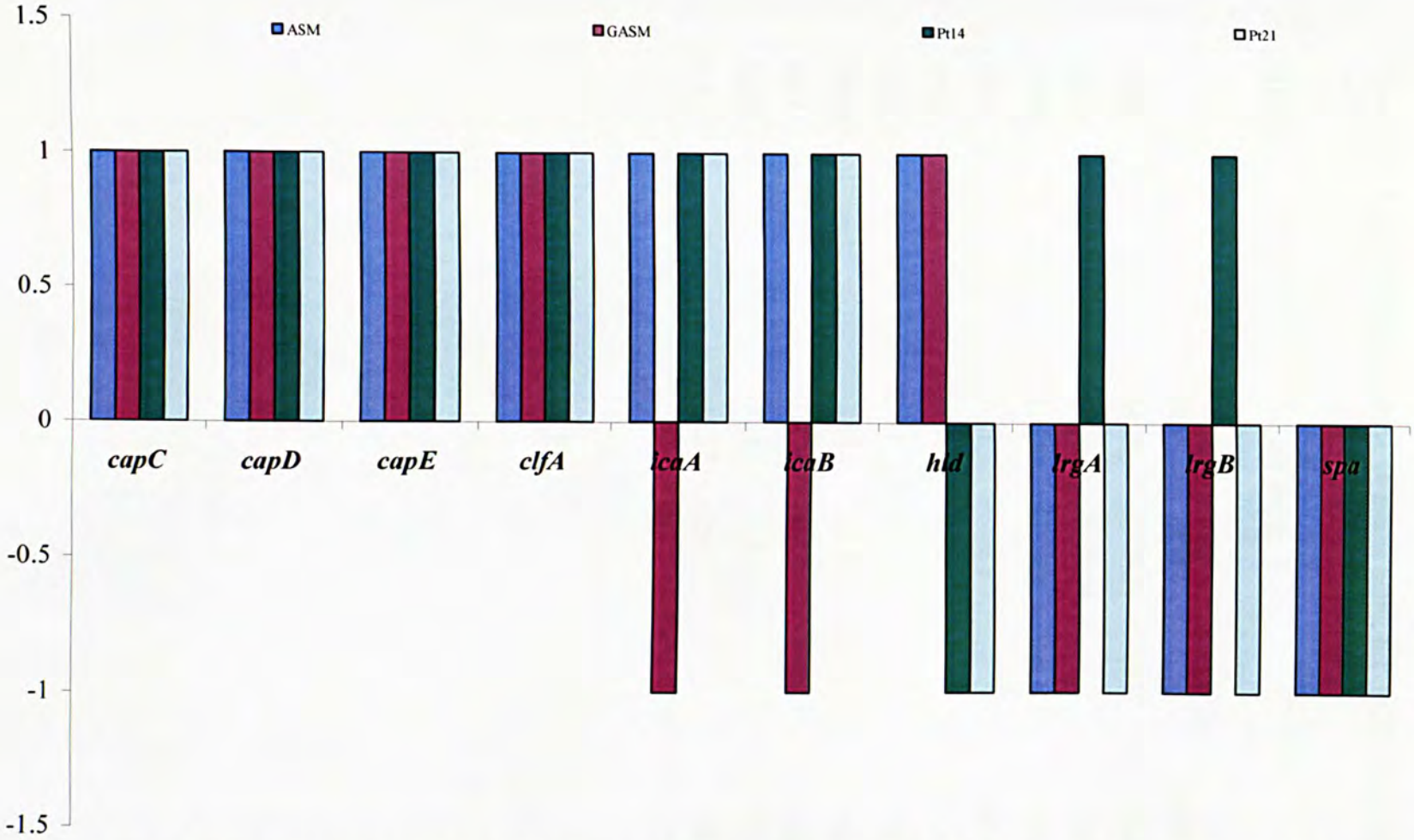


Fig 6.10: Schematic graph showing the 10 genes investigated in Chapter 5 and 6 using RT-PCR and differential MRSA252 gene expression in ASM, GASM, Pt14 and Pt21. Up-regulated genes were assigned an arbitrary +1 whilst down-regulated genes -1, in order to enable direct comparison between different media but not taking into consideration the actual relative fold difference. (Actual fold difference can be viewed in the previous figures earlier, in this chapter. Legend: ASM: blue, GASM: burgundy, Pt14: green and Pt21: light green).

Table 6.5: Fold differences of the 10 genes using RT-PCR Pt21, Pt14, ASM and GASM.

GENE	RT-PCR			
	Pt21	**Pt14**	**ASM**	**GASM**
capC	44.20	2599.77	206.5	24.9
capD	45.20	8378.64	121.9	14.7
capE	41.88	412.29	105.2	15.0
clfA	7.75	1605.9	16.9	14.0
icaA	38.05	764.04	6.8	-9.4
icaB	2.52	30679.77	1.8	-3.0
hld(RNAIII/*agr*)	-2.44	-1.56	5.0	20.2
lrgA	-16.75	16.71	-160.2	-171.1
lrgB	-11.42	717.0	-7.6	-15.5
spa	-22.37	-2.50	-78.6	-26.0

SECTION 6.4: DISCUSSION

In the Introduction to this chapter, it was reported that the occurrence of MRSA in CF respiratory cultures is consistent with increased patient morbidity, leading to decreased airflow obstruction, hospitalization and antibiotic treatment. Therefore, exploring the possible genetic effects of MRSA in such an environment was considered the focal point of this chapter.

The main aims of this chapter were to obtain human cystic fibrosis sputum from CF patients and attempt to grow MRSA252 in this sputum. The rationale was based on the premise that if MRSA252 showed characteristic growth physiology in the sample, gene expression analysis using RT-PCR would then be performed.

Prior to actually utilizing human CFS, a pilot experiment using δ–radiation to sterilise inoculated ASM with MRSA252 was conducted. The fundamental reason for this pilot experiment was that the components of the CFS remained as much as possible unaltered and akin to the *in vivo* situation, that is, human CF sputum. Sleigh and Timbury (1997) mentioned cobalt-60 as a suitable δ–radiation source. However, at St George's Hospital, Caesium-137 was the δ–radiation source of choice, as cobalt-60 is not used any longer here (Dr Vincent Ang, personal communication). Autoclaving, which would have been straightforward to perform and much less hazardous than δ–radiation, was not selected as this would have induced protein coagulation, denaturation (Alcamo, 2001; Widmer and Frei, 2007) and therefore destruction of protein structure. Consequently, this was not considered as a method of sterilisation, as this would not truly represent the CFS environment.

In the radiation experiment, it was revealed post-radiation, that the pre-inoculated ASM yielded viable bacteria at 2.12×10^8 cfu/ml in the 25ml container and 3.0×10^7 cfu/ml in the 50ml container, clearly indicating that the radiation dose was not sufficient to kill the bacteria and indeed, bacterial growth was noted (from $x10^6$ cfu/ml for both 25ml and 50ml containers at time 0hr to $x10^8$ cfu/ml for 25ml and $x10^7$ cfu/ml with the 50ml container, after radiation exposure).

This experiment clearly showed that sterilisation by δ–radiation should not be advocated to pre-sterilize human CFS. Literature searches revealed that an adequate δ–radiation for bacterial sterilisation was in the order of kGy, rather than Gy, as was used for this experiment (Roberts, 1998; FDA, 1998). At St George`s Hospital, the source available could only be used at the fixed radiation dose, i.e. 50Gy and for the fixed times, due to inherent security measures (Mr Eamon Lane, Onyvax® representative. Personal communication). Indeed, if suitable facilities had been available, such a procedure would have enabled testing of most of the CFS samples obtained from the RBH, but this was not the case. Thus, from, the 41 CFS samples which were obtained from the RBH and were screened, it was found that more than half (59%) could not be used for the purpose of the gene expression experiments as proteolytic enzymes would have had to be employed in order to break up the compacted sputum. From the remaining liquid samples, 28% yielded *S. aureus*.

In this chapter, mannitol salt agar (MSA) was chosen as the selective medium to select for *S. aureus* by producing a colour change. Sharp and Searcy (2006) evaluated MSA as a selective medium for direct detection of *S. aureus* and found that > 98% of *S. aureus* were correctly isolated and identified. MSA was also used by Garske *et al.* (2004) to isolate *S. aureus* directly from adult CF patients.

In the current study results showed that 5 out of 18 CFS liquid samples yielded *S. aureus* (28%). Three were males, aged 20 years, 19 years and 20 years (average age of 19.7 years) and 2 were females (one aged 29 years and one 13 years) with an average age of 21 years.

The capsular synthesis genes *capC*, *capD* and *capE* were up-regulated in all the samples tested, which were, ASM, GASM, Pt14 and Pt21. This may indicate that when MRSA252 is infecting a CF patient`s lungs, the initial mechanisms involved includes the capsule. The *clfA* gene was up-regulated in ASM, GASM, Pt21 and Pt14 whilst *spa* was down-regulated for all 4 culture conditions.

These findings are strikingly similar to a study conducted with strains isolated from CF patients which found that capsular genes as well as *clfA*, were up-regulated in SCVs isolated from CF patients (Moisan *et al.*, 2006). When considering the different patient samples, i.e. Pt14, Pt21 as well as the *in vitro* models, i.e. ASM and GASM, some variations occurred in the gene expression profile with *hld*, *lrgAB* and *icaAB*. The *hld* gene

(associated with *agr* activity) was down-regulated in Pt14, Pt21 and GASM but not in ASM. This was similar to the findings of Goerke *et al.* (2000), who detected levels of *agr* (through RNAIII, the effector molecule) from *S. aureus* CF-infected sputum and concluded that in CF, *agr* is not an important regulatory system involved in virulence factor production.

Other variations included the *lrgAB* operon, which was down-regulated in ASM, Pt21 and GASM but up-regulated in Pt14. When extracting RNA from human CFS samples it was noted that the RNA yield was much lower than for ASM and GASM. In fact, levels for Pt14 were lower than for Pt21. In order to maximise the RNA yield, an on-column DNase digestion was performed for Pt14 samples. This lead to larger Ct values being obtained in the RT-PCR (Dr Ken Laing. Personal communication) and hence, possible discrepancies. The RNA yield obtained with Pt14 may also have occurred due to the high pH observed with pre-MRSA252 inoculation of the sputum (pH 8). Pt21 had a pH of 7.4, pre-MRSA252 inoculation. The pH in CF is ca 6.9 (Perez-Vilar and Boucher, 2004).

With the *icaAB* operon, up-regulation was seen with Pt14, Pt21 and ASM but with GASM this was down-regulated. This might indicate that glucose does affect specific gene expression and is an interesting observation as CF patients are known to be pre-disposed to developing diabetes. Diabetes could possibly lead to accumulation of glucose in body fluids, possibly producing a direct effect on bacterial growth and gene expression.

SECTION 6.5: CONCLUSIONS

In this chapter, several important observations were made:

Initially it was observed that *Staphylococcus aureus*, specifically MRSA16-252, can grow and survive adequately in human cystic fibrosis sputum, even in the presence of other organisms, such as yeasts (Pt21). This consolidates the general view in literature that this organism is highly versatile and thus, an extremely successful human pathogen.

Patients undoubtedly possess different components in their sputum which in turn has a direct impact on *S. aureus* growth, survival and gene expression profile (as seen with Pt14 and Pt21 and also with ASM and GASM, the *in vitro* models).

Using RT-PCR, capsular genes and clumping factor A (*clfA*) were found to be up-regulated when MRSA252 was grown in Pt14 and Pt21 sputa as well as ASM and GASM. This implies that when MRSA252 infects or colonizes human CF sputum (as seen with both Pt14 and Pt21), these genes are positively regulated. Such observations may elicit further research, which if the above is confirmed, may promote development of future therapies, targeted for CF patients. These would be targeted to specifically counteract the de-activation of the capsular and *clfA* genes, thereby attempting to arrest *S. aureus* and MRSA proliferation and persistence in these patients.

CHAPTER 7

GENERAL DISCUSSION

SECTION 7.1: INTRODUCTION

Initially this thesis examined the physiological behaviour of a clinical epidemic strain, MRSA16-252 in an *in vitro* medium, artificial sputum medium, ASM, closely resembling human cystic fibrosis sputum. The impact of various components present in, or added to the ASM, on the bacterium was assessed. Transcriptomic gene expression was then investigated, using the clinical MRSA strain grown in ASM, GASM (ASM with added glucose) and a laboratory control, brain heart infusion (BHI). Variations in gene expression patterns when MRSA252 was grown in different media, were then studied. Finally, human cystic fibrosis (CF) sputum was used as a growth medium for MRSA252 and gene expression patterns subsequently analysed. This final chapter of the thesis will analyse each of the major 4 aims on which this thesis has been progressively based and assess whether the findings presented in this thesis support or refute these aims.

SECTION 7.2: AIMS OF THE PHD THESIS RE-VISITED

Section 7.2.1: Can *Staphylococcus aureus* grow and survive in an *in vitro* cystic fibrosis sputum medium?

In Chapter 3, preliminary studies explored whether mucin, which is known to be a component of the thick and viscous mucus in CF patients, contributing to clogging of the airways in CF airways (Lillehoj and Chul Kim, 2002), allowed adequate growth and survival of MRSA252. The results obtained in this chapter, showed that MRSA252 could grow and survive adequately in the presence of mucin, although media containing mucin had a lower pH than controls.

An artificial cystic fibrosis sputum medium, proposed and investigated by Ghani and Soothill, (1997) and later modified by Sriramulu *et al.* (2005) to ASM, had been used to study *Pseudomonas aeruginosa* growth and survival and gene expression. This bacterium, was found to grow and survive adequately in this medium. For the first time, MRSA252 was grown in the ASM and the growth pattern analysed and compared with that obtained for a laboratory control, i.e. brain heart infusion (BHI). The results demonstrated that ASM supported the growth and survival of MRSA252 and a characteristic bacterial growth curve was also obtained. Growth of MRSA252 in ASM was observed to occur at a slower rate than in the enriched BHI laboratory medium.

Section 7.2.2: Do individual components present in the *in vitro* medium (ASM), contribute to growth and survival of MRSA252?

In order to investigate this, specific components constituting ASM, such as mucin, DNA, amino acids, the iron binder DTPA and sodium chloride, were removed from ASM and others added, such as glucose (GASM). These experiments were conducted and the results fully discussed in Chapter 3. MRSA252 was grown in these modified media and the growth patterns were assessed using viable counts and light microscopy. The results demonstrated that on removal of mucin, MRSA252 did not exhibit the characteristic growth curve and instead a survival curve was obtained. This demonstrated that mucin was probably contributing as a nutrient source when in ASM and the removal of mucin significantly impacted on the growth of this bacterium. When all the 20 amino acids were removed from the ASM, MRSA252 showed initial growth, however survival was severely reduced as the growth curve lacked the characteristic stationary phase and phase of decline. The addition of glucose was consistent with an initial increase in growth, which then tapered down to levels similar to those seen in the control. The removal of DNA, DTPA and NaCl did not significantly affect the growth and survival of this organism. The mere removal or addition of some but not all specific components in the ASM, may thus drastically alter the growth and survival of MRSA252.

Section 7.2.3: Does *Staphylococcus aureus* show an altered whole genomic expression profile when grown in ASM, GASM and BHI?

In Chapter 4, transcriptomic gene expression profiling of MRSA252 in exponential phase, growing in BHI, ASM and GASM, was performed using microarray technology. Gene expression profiles were normalised to BHI, enabling direct comparison of genes which were differentially expressed. Capsular genes in ASM and also GASM were found to be highly up-regulated, when compared to BHI. The results obtained in Chapter 4 demonstrated that the genes of MRSA252 in ASM were up-regulated by 9% of the total MRSA252 genome, whilst down-regulated genes formed a proportion equal to 6% of the genome (fold difference ≥ 2; $p = 0.05$). Genes differentially expressed in GASM were regulated similarly to those in ASM. Using a stringent cut-off value of ≥ 2.0 fold ($p = 0.05$), 12% of GASM genes were seen to be up-regulated and 9% of MRSA252 genes in GASM were down-regulated. All the differentially expressed genes were individually assessed and assigned specific classes, using http://www.genedb.org and these included: capsular polysaccharide synthesis enzymes, putative proteins, general metabolic enzymes (includes

cellular, lipid, carbohydrate, protein, nucleic acids), conserved hypothetical and hypothetical proteins, transcriptional factors, transport proteins, surface proteins, vitamin biosynthesis, metal ion binding, toxin and enzymes, response to stress proteins, cell wall and folic acid metabolism. Differentially regulated, normalised genes of MRSA252 in ASM and GASM were also compared and the effect of glucose fully assessed. The results obtained in Chapter 4 are similar to the observations of Moisan *et al.* (2006), who dealt with the transcriptomic analysis of clinical small colony variants (SCVs) as well as laboratory SCVs. The clinical strains were obtained from patient throat and sputum and cultured in BHI. Both the strains in the study by Moisan *et al.* (2006) and the MRSA252 growing in ASM and GASM showed slower growth rates, when compared with MRSA252 grown in BHI in this thesis and with ATCC29740 and Newbould, investigated by Moisan *et al.* (2006). These researchers proposed that *sigB* is the main regulator in *S. aureus*-CF respiratory infection. The results obtained in Chapter 4, which were further validated in Chapter 5, also suggest that this is the case in this thesis. Of particular interest, was the observation that Moisan *et al.* (2006) revealed that capsular genes as well as stress-associated genes such as *asp23* and *spoVG* were up-regulated with the clinical SCVs. This finding is also similar to the results obtained in this chapter, where these genes were also positively regulated in MRSA252 grown in ASM. A sample of the differentially regulated genes observed in Chapter 4 were later analysed by another technique, i.e. RT-PCR and the results confirmed the previous observations (Chapter 5).

Section 7.2.4: Can *Staphylococcus aureus* grow and survive in unmodified human cystic fibrosis sputum?

Finally, in Chapter 6, it was revealed that growing *S. aureus* in unmodified human CF sputum could be achieved and also *S. aureus* grew and survived adequately in the presence of yeasts (which are isolated in CF patients). pH differences in human CF sputum may impair growth and survival of MRSA252 and also alter gene expression and Chapter 6 showed that capsular genes were also up-regulated when exponential phase MRSA252 grew in different human CF samples, matching the *in vitro* observations seen earlier.

SECTION 7.3: SUMMARY OF MAIN FINDINGS

This work has intricately investigated the physiological effect of growing a clinical MRSA strain (MRSA252) in *in vitro* cystic fibrosis sputum media (ASM and modified ASM, GASM), comparing growth and survival patterns to an enriched laboratory medium (BHI). Growth of MRSA252 was observed to occur at a slower rate in ASM and GASM, when compared to BHI. The effect of removing specific components from ASM was also explored. Removal of mucin produced a survival curve and this indicated that mucin was probably an important nutritional resource for the bacterium in ASM. Also, removal of the amino acids did show initial growth, however survival was drastically reduced. Removal of sodium chloride, DNA and DTPA did not have any significant adverse effect on MRSA252 growth and survival. Once the growth and survival patterns of MRSA252 in ASM and GASM were found to be reproducible and possessed the growth phases exhibited during a typical bacterial growth cycle, gene expression studies, using microarray technology ensued. This high throughput technique revealed an interesting differential gene expression pattern, when MRSA252 grown to exponential phase in BHI, ASM and GASM were analysed. MRSA252 genes expressed when growing in ASM and GASM were normalised to BHI. Capsular genes and clumping factorA (*clfA*) were observed to be highly up-regulated in ASM and GASM, whilst *lrgAB* genes were down-regulated in both media.The increased expression of the transcriptional factor, *sigB* in ASM may imply that when *S.aureus* infects CF airways, virulence production is mediated by *sigB*, rather than *agr*. Growth of MRSA252 was also successfully conducted in unmodified human CF sputum. Gene expression studies were also performed using RT-PCR on exponential-phase MRSA252 growing in two CF sputa (originating from 2 different CF human patients) and compared to ASM and GASM. Capsular genes, as well as clumping factor A were up-regulated in both CF sputum samples as well as the *in vitro* media.

SECTION 7.4: FUTURE WORK

To supplement the current findings, transcriptomic analysis of stationary phase MRSA252 growing in ASM, GASM and BHI should be explored and the results compared with those observed in Chapter 4 (where MRSA252 was grown to exponential phase in ASM, GASM and BHI). In addition, since this thesis focuses only on one particular strain, i.e, MRSA252, it would be interesting to investigate whether variations exist between different *S. aureus* strains. This would involve repeating the experiments conducted in this thesis, using ideally fully characterised clinical *S. aureus* strains (both meticillin-resistant and meticillin-sensitive). In addition, a large number of human CF samples could be screened for antibiotics and those unmodified liquid sputa deemed to be antibiotic-free (ideally 100 samples), used as growth media for MRSA252 as well as other clinical *S. aureus* strains, which might also include SCVs. Gene expression of the ten genes studied in Chapter 6 (especially capsular synthesis and *lrgAB* genes) could be performed and the gene expression profile compared with the results obtained in this thesis.

Since *P. aeruginosa* is often found in sputum samples from CF sufferers and known to increase morbidity in these patients, a study exploring the growth and survival of both *S. aureus* and *P. aeruginosa* in ASM, GASM and unmodified human sputum is also advocated and transcriptomic gene expression can be attempted, if adequate growth and survival is observed.

These prospective experiments would provide further insight into the basic biological effects induced, when different strains are grown in ASM and GASM and compared to BHI. In addition to increasing scientific knowledge, this would also enable the study of the complex genetic pathways evoked when *S. aureus* is grown in ASM and GASM. Such studies might contribute to the development of future antimicrobial therapies. In contrast to conventional antimicrobials, these therapies could target specific genes, such as the capsular genes (*cap*) and clumping factor A (*clfA*) and might help control and possibly eradicate, *S. aureus* respiratory infection in CF patients.

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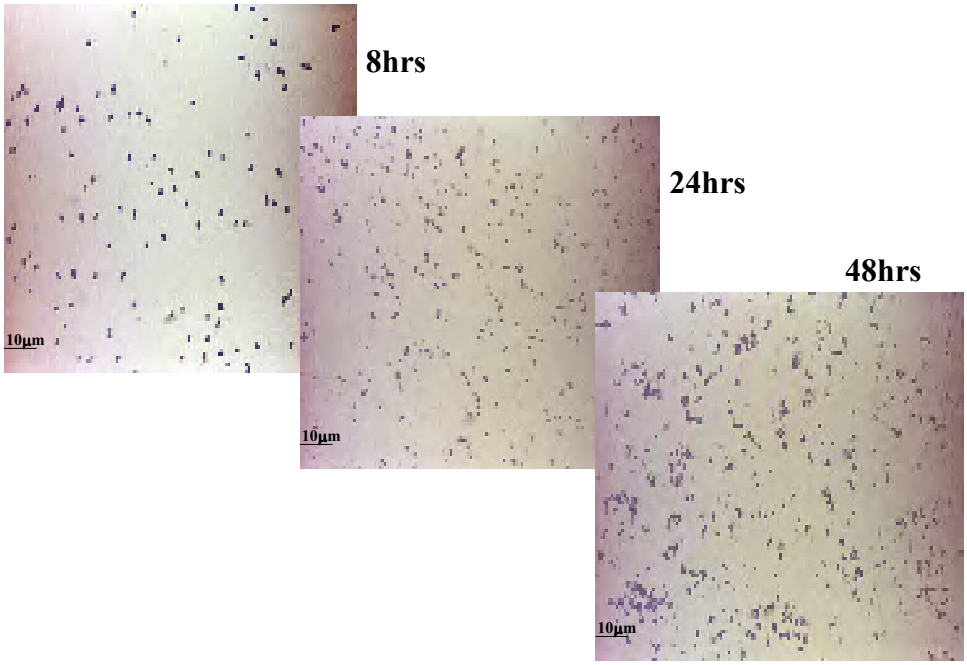
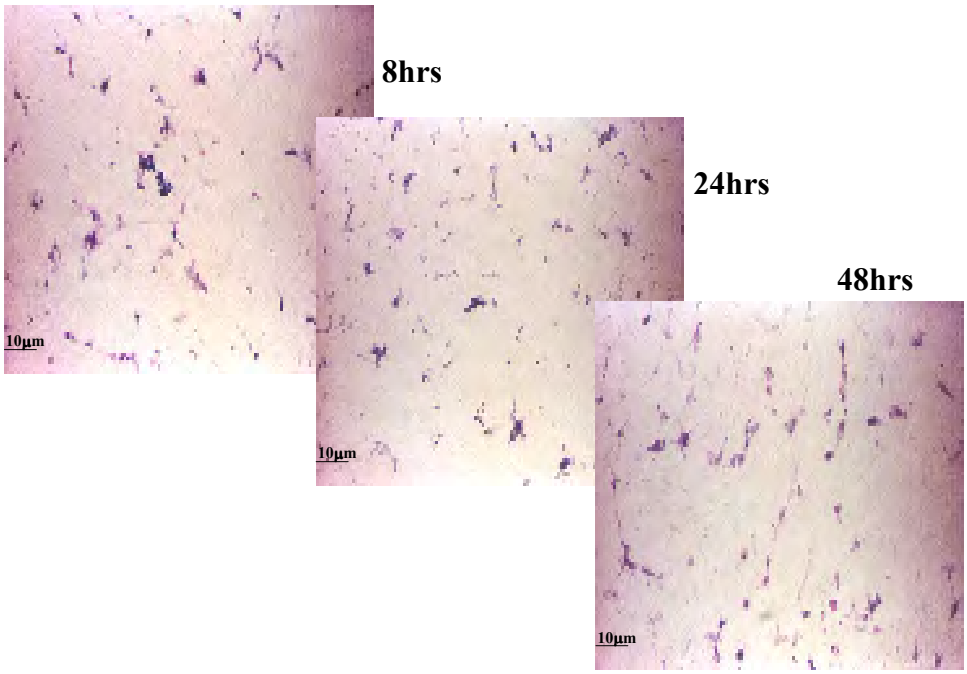
APPENDIX I: LIGHT MICROSCOPY IMAGES

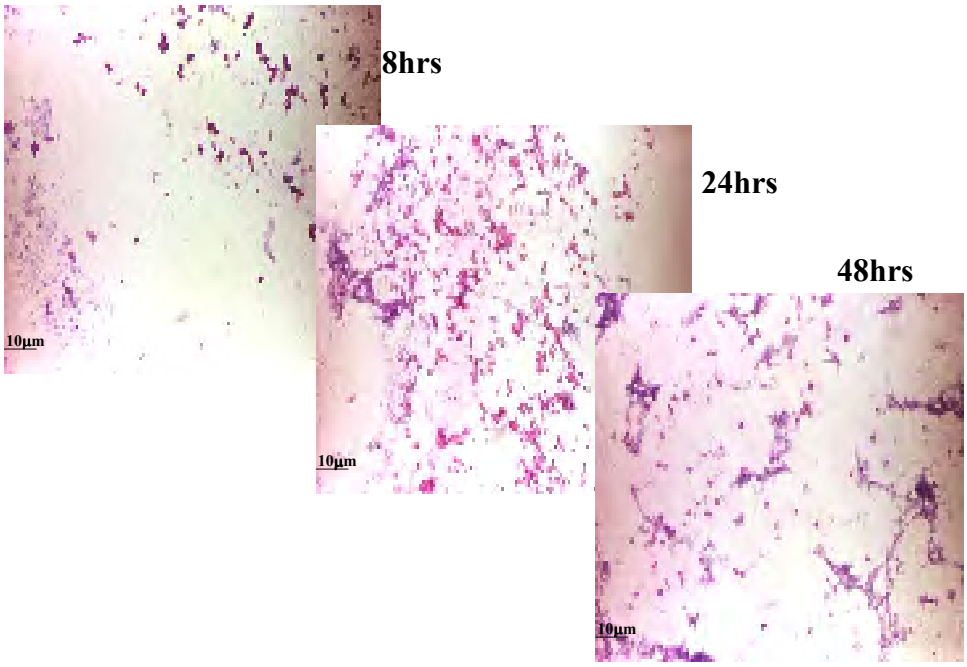
BHI



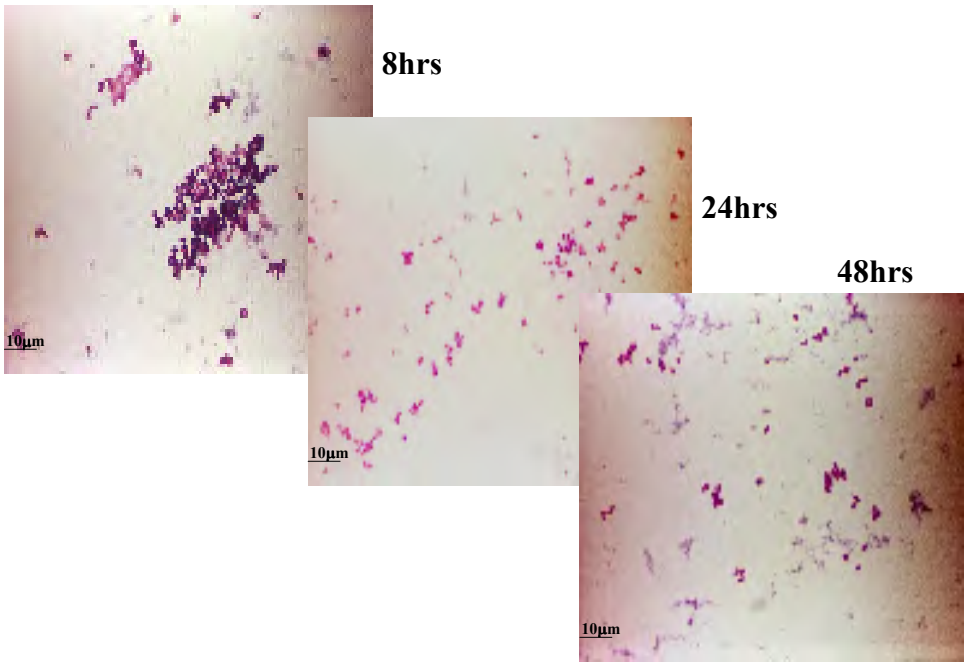
ASM-Mucin



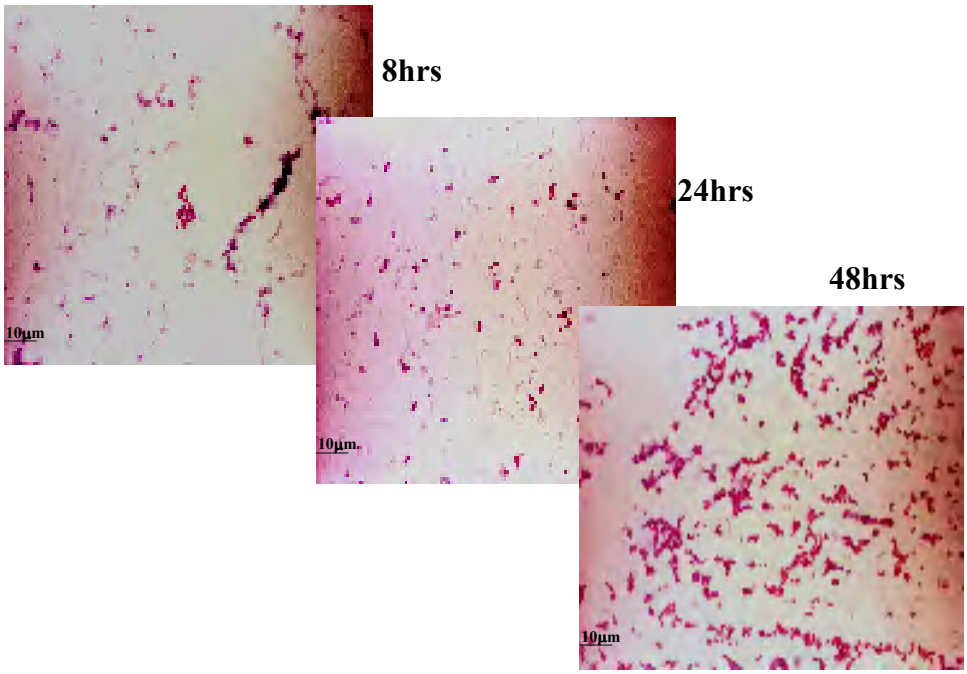
ASM-AA


8hrs
24hrs
48hrs
10µm
10µm
10µm

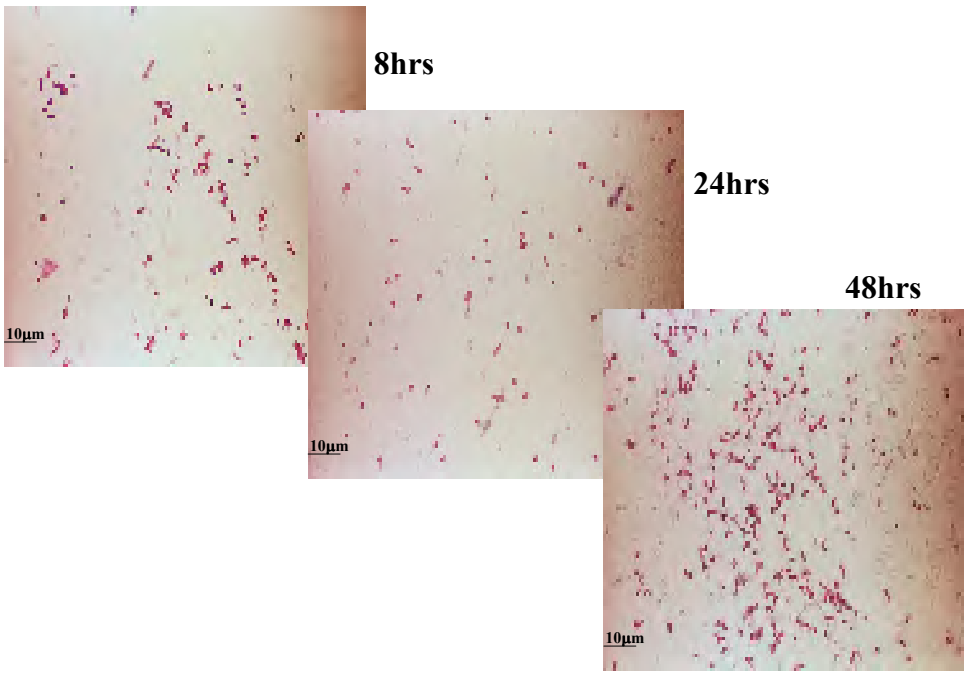
GASM


8hrs
24hrs
48hrs
10µm
10µm
10µm

ASM -NaCl



ASM-DNA



ASM-DTPA

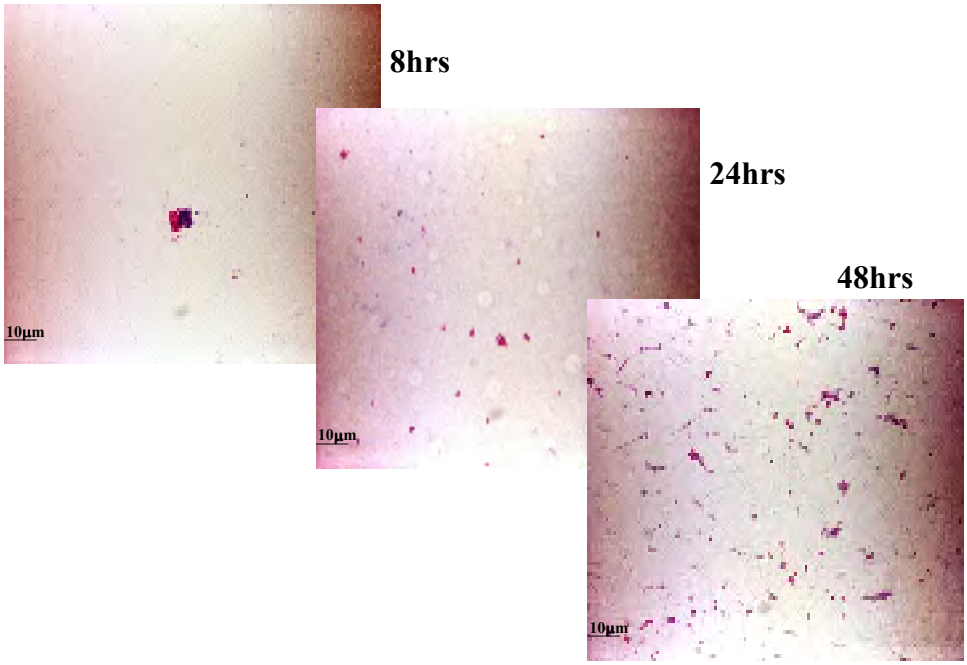


Table 1: Up-regulated ASM genes, normalised to BHI, using one-way ANOVA and posthoc Benjamini and Hochberg tests ($p \leq 0.05$). The corresponding ORF (Open Reading Frame), gene name and protein function are listed. The table is arranged according to the most up-regulated gene with the highest fold difference.

ORF	GENE	PROTEIN (FUNCTION)	FOLD
SaMRSA252-0153 (2L7)	**capC**	capsular polysaccharide synthesis enzyme	**64.0**
SaMRSA252-2295 (6N17)		putative exported protein	**36.6**
SaMRSA252-0154 (2M7)	**capD**	capsular polysaccharide synthesis enzyme	**33.3**
SaMRSA252-0155 (2N7)	**capE**	capsular polysaccharide synthesis enzyme	**24.5**
SaMRSA252-0225 (2E16)	**fadD**	putative acyl-CoA dehydrogenase (involved in electron transport)	**23.0**
SaMRSA252-0169 (2L9)		putative aldehyde dehydrogenase (oxidoreductase activity; metabolism)	**17.9**
SaMRSA252-0157 (2P7)	**capG**	capsular polysaccharide synthesis enzyme	**16.7**
SaMRSA252-0152 (2K7)	**capB**	capsular polysaccharide synthesis enzyme	**15.3**
SaMRSA252-0156 (2O7)	**capF**	capsular polysaccharide synthesis enzyme	**14.2**
SaMRSA252-0191 (2J12)		conserved hypothetical protein	**14.2**
SaMRSA252-0223 (2C16)	**fadA**	putative thiolase (involved in degradative pathways e.g. fatty acid beta-oxidation)	**12.8**
SaMRSA252-0193 (2L12)		sucrose-specific PTS tranporter protein (membrane component-functions as sugar transport system)	**12.7**
SaMRSA252-0227 (2G16)	**fadX**	putative acetyl-CoA transferase (fatty acid metabolism; CoA-transferase activity)	**12.4**
SaMRSA252-1773 (5B14)	**citC**	isocitrate dehydrogenase (carbohydrate metabolism; TCA cycle)	**11.6**
SaMRSA252-0919	**rocD**	ornithine aminotransferase (transaminase activity and pyridoxal phosphate binding)	**11.6**

(3A17)			
SaMRSA252-0226 (2F16)	**fadE**	putative acyl-CoA synthetase (metabolism and catalytic activity)	**11.4**
SaMRSA252-0192 (2K12)		conserved hypothetical protein	**11.4**
SaMRSA252-0194 (2M12)		RpiR family transcriptional regulator (metabolism and regulation of transcription)	**10.8**
SaMRSA252-1787 (5H15)	**ald1**	alanine dehydrogenase 1(oxidoreductase activity and electron transport)	**10.5**
SaMRSA252-0920 (3B17)		putative NAD-specific glutamate dehydrogenase (oxidoreductase activity and amino acid metabolism)	**10.4**
SaMRSA252-1774 (5C14)	**citZ**	citrate synthase II (carbohydrate metabolism: TCA)	**9.9**
SaMRSA252-0158 (2I8)	**cap8H**	capsular polysaccharide synthesis enzyme	**9.8**
SaMRSA252-0224 (2D16)	**fadB**	putative fatty oxidation complex protein (oxidoreductase activity; fatty acid metabolism)	**9.1**
SaMRSA252-0405 (2L14)		hypothetical protein	**8.8**
SaMRSA252-1614 (5G6)	**gcvT**	putative aminomethyltransferase (glycine metabolism)	**8.7**
SaMRSA252-0842 (2F7)	**clfA**	clumping factor	**8.7**
SaMRSA252-0162 (2M8)	**capL**	capsular polysaccharide synthesis enzyme	**8.6**
SaMRSA252-0161 (2L8)	**cap8K**	capsular polysaccharide synthesis enzyme	**8.5**
SaMRSA252-0151 (2J7)	**capA**	capsular polysaccharide synthesis enzyme	**8.4**
SaMRSA252-0577 (3G11)	**proP**	putative proline/betaine transporter (integral to membrane; transporter activity)	**8.3**
SaMRSA252-1362 (4M11)	**citB**	aconitate hydratase (carbohydate metabolism; TCA cycle)	**8.1**
SaMRSA252-1849 (5E23)		proline dehydrogenase (proline catabolism; glutamate biosynthesis)	**7.9**
SaMRSA252-1871 (5K14)	**pckA**	phosphoenolpyruvate carboxykinase (ATP binding; gluconeogenesis)	**7.8**
SaMRSA252-1222 (1K6)		putative succinyl-CoA ligase (catalytic activity; metabolism)	**7.4**
SaMRSA252-2688		hypothetical protein	**7.3**

(7I18)			
SaMRSA252-2109 (6J7)		putative peptidase (hydrolase; proteolysis)	**7.3**
SaMRSA252-0921 (3C17)	**glpQ**	putative glycerophosphoryl diester phosphodiesterase (glycerol metabolism)	**7.2**
SaMRSA252-1612 (5E6)		putative glycine cleavage system P-protein (lyase activity; amino acid metabolism)	**7.2**
SaMRSA252-2420 (7B9)		arginase family protein (metal ion binding)	**7.1**
SaMRSA252-1121 (4H5)	**sdhA**	putative succinate dehydrogenase flavoprotein subunit (oxidoreductase activity; electron transport)	**7.1**
SaMRSA252-2634 (7E23)		aldehyde dehydrogenase family protein (oxidoreductase activity; metabolism)	**7.0**
SaMRSA252-0163 (2N8)	**capM**	capsular polysaccharide synthesis enzyme	**6.9**
SaMRSA252-1811 (5G18)	**acsA**	acetyl-coenzyme A synthetase (catalytic activity; metabolism)	**6.8**
SaMRSA252-2559 (7C14)		putative short chain dehydrogenase (oxidoreductase activity; metabolism)	**6.8**
SaMRSA252-1425 (4C19)	**odhA, citK**	2-oxoglutarate dehydrogenase E1 component (oxidoreductase activity; metabolism)	**6.8**
SaMRSA252-2469 (7K3)		conserved hypothetical protein (Flavin mononucleotide binding))	**6.7**
SaMRSA252-0624 (3L5)		putative esterase	**6.7**
SaMRSA252-1221 (1J6)		putative CoA synthetase protein (catalytic activity; metabolism)	**6.6**
SaMRSA252-1684 (5J3)		conserved hypothetical protein	**6.6**
SaMRSA252-2275 (6J15)		putative membrane protein	**6.4**
SaMRSA252-0211 (2F14)		conserved hypothetical protein (myo-inositol catabolism)	**6.4**
SaMRSA252-0048 (1F6)		putative membrane protein -partial (integral to membrane)	**6.3**
SaMRSA252-0164 (2O8)	**capN**	capsular polysaccharide synthesis enzyme	**6.2**
SaMRSA252-0855 (2B9)		hypothetical protein	**6.0**
SaMRSA252-0208 (2C14)		putative sugar transport system permease	**5.9**

SaMRSA252-1050 (3J21)		ABC transporter ATP-binding protein (ATP binding/ATPase activity)	**5.7**
SaMRSA252-2647 (7H24)		putative membrane protein	**5.6**
SaMRSA252-1686 (5L3)		putative biotin carboxyl carrier protein of acetyl-CoA carboxylase	**5.4**
SaMRSA252-1810 (5F18)	**fhs**	formate--tetrahydrofolate ligase (folic acid and derivative biosynthesis)	**5.4**
SaMRSA252-1424 (4B19)	**odhB**	dihydrolipoamide succinyltransferase E2 component of 2-oxoglutarate dehydrogenase complex (acyltransferase activity; metabolism)	**5.3**
SaMRSA252-0996 (3M14)		conserved hypothetical protein	**5.2**
SaMRSA252-2388 (7D5)		putative exported protein	**5.2**
SaMRSA252-0938 (3D19)	**clpB**	putative ATPase subunit of an ATP-dependent protease (ATP binding; protein metabolism)	**5.1**
SaMRSA252-2558 (7B14)		conserved hypothetical protein	**5.1**
SaMRSA252-2274 (6I15)		putative membrane protein	**5.1**
SaMRSA252-2646 (7G24)		putative phytoene dehydrogenase related protein (oxidoreductase activity; electron transport)	**5.1**
SaMRSA252-0721 (1N16)		multicopper oxidase protein (oxidoreductase activity; copper ion binding)	**5.0**
SaMRSA252-2668 (7M15)		conserved hypothetical protein	**4.9**
SaMRSA252-0171 (2N9)		hypothetical protein	**4.8**
SaMRSA252-0312 (1A15)		putative N-acetylneuraminate lyase (metabolism)	**4.8**
SaMRSA252-1683 (5I3)		putative membrane protein	**4.8**
SaMRSA252-1685 (5K3)		putative biotin carboxylase subunit of acetyl-CoA carboxylase (urea cycle and biosynthesis of arginine and pyrimidines)	**4.7**
SaMRSA252-1879		putative lipoprotein	**4.7**

(5J15)			
SaMRSA252-2544 (7L12)		ABC transporter ATP-binding protein (ATP-binding/ATPase activity)	**4.7**
SaMRSA252-2661 (7N14)		putative hydrolase	**4.7**
SaMRSA252-1120 (4G5)	**sdhC**	putative succinate dehydrogenase cytochrome b558	**4.6**
SaMRSA252-0305 (1B14)		putative membrane protein	**4.6**
SaMRSA252-2645 (7F24)		putative glycosyl transferase	**4.6**
SaMRSA252-1049 (3I21)		putative cobalt transport protein (cobalamin biosynthesis)	**4.6**
SaMRSA252-0165 (2P8)	**capO**	capsular polysaccharide synthesis enzyme	**4.6**
SaMRSA252-2561 (7E14)		conserved hypothetical protein (aromatic compound metabolism)	**4.6**
SaMRSA252-1051 (3K21)		putative membrane protein	**4.6**
SaMRSA252-2543 (7K12)		putative membrane protein	**4.5**
SaMRSA252-1880 (5K15)		putative membrane protein	**4.4**
SaMRSA252-2780 (8C5)		putative membrane protein	**4.4**
SaMRSA252-2747 (8C1)	**icaA**	glucosaminyltransferase	**4.3**
SaMRSA252-2695 (7P18)	**nrdD**	anaerobic ribonucleoside-triphosphate reductase (catalytic activity; metabolism)	**4.3**
SaMRSA252-2395 (7C6)		inositol monophosphatase family protein	**4.3**
SaMRSA252-2728 (7I23)		preprotein translocase SecA subunit-like protein (membrane component)	**4.2**
SaMRSA252-1152 (4E9)		acetyltransferase (GNAT) family protein (transcription and DNA repair)	**4.2**
SaMRSA252-0985 (3J13)		conserved hypothetical protein (RNA metabolism)	**4.2**
SaMRSA252-0306 (1C14)		ABC transporter ATP-binding protein (ATPbinding/ATPase activity)	**4.2**
SaMRSA252-0210		putative oxidoreductase (oxidoreductase activity; electron transport)	**4.2**

(2E14)			
SaMRSA252-2596 (7H18)		conserved hypothetical protein	**4.1**
SaMRSA252-1344 (4K9)		catalase (response to oxidative stress)	**4.1**
SaMRSA252-1037 (3M19)	**folD**	FolD bifunctional protein (catalytic activity; folic acid and derivative biosynthesis)	**4.1**
SaMRSA252-0170 (2M9)		putative cation efflux system protein (membrane component involved in removal of divalent metal ions from cells)	**4.0**
SaMRSA252-1766 (5C13)	**gap2**	glyceraldehyde 3-phosphate dehydrogenase 2 (glycolysis)	**3.8**
SaMRSA252-1785 (5F15)		metallo-beta-lactamase superfamily protein	**3.8**
SaMRSA252-0166 (2I9)	**capP**	capsular polysaccharide synthesis enzyme	**3.8**
SaMRSA252-2182 (6B16)	**thiD**	putative phosphomethylpyrimidine kinase (thiamine pyrophosphate synthesis pathway)	**3.8**
SaMRSA252-2393 (7A6)		putative bifunctional protein (iron and molybdenum ion binding; electron transport)	**3.7**
SaMRSA252-0189 (2P11)		putative thiamine pyrophosphate enzyme (magnesium ion binding; thiamine pyrophosphate binding)	**3.7**
SaMRSA252-0311 (1H14)		sodium:solute symporter family protein (membrane transporter activity)	**3.7**
SaMRSA252-0806 (2C3)		putative S30EA family ribosomal protein	**3.7**
SaMRSA252-0279 (2B23)		conserved hypothetical protein	**3.7**
SaMRSA252-0952 (3B21)	**oppF**	putative oligopeptide transport ATP-binding protein	**3.7**
SaMRSA252-2181 (6A16)	**thiM**	putative hydroxyethylthiazole kinase (thiamine biosynthesis)	**3.7**
SaMRSA252-0675 (3N11)		putative exported protein (cell wall catabolism)	**3.6**
SaMRSA252-2568 (7D15)		hypothetical protein	**3.6**
SaMRSA252-2210 (6F19)		aldehyde dehydrogenase family protein (oxidoreductase activity; metabolism)	**3.6**

SaMRSA252-1190 (1K2)		putative membrane protein	**3.6**
SaMRSA252-0207 (2B14)		putative sugar transport system permease (membrane; transporter function)	**3.6**
SaMRSA252-1688 (5N3)		conserved hypothetical protein	**3.6**
SaMRSA252-0824 (2D5)		putative malolactic enzyme (oxidoreductase activity)	**3.5**
SaMRSA252-0867 (2F10)		hypothetical protein (nucleic acid binding; DNA modifcation)	**3.5**
SaMRSA252-0825 (2E5)		conserved hypothetical protein (nucleotide-sugar metabolism)	**3.5**
SaMRSA252-0188 (2O11)		putative isochorismatase (catalytic activity; metabolism)	**3.5**
SaMRSA252-0333 (1F17)	**glpT**	putative glycerol-3-phosphate transporter (major facilitator superfamily)	**3.5**
SaMRSA252-1788 (5A16)		putative universal stress protein (response to stress)	**3.5**
SaMRSA252-2180 (6H15)	**thiE**	putative thiamine-phosphate pyrophosphorylase (thiamine biosynthesis)	**3.4**
SaMRSA252-2589 (7A18)		putative transporter protein (major facilitator superfamily)	**3.4**
SaMRSA252-0629 (3I6)		phage integrase family protein (DNA binding)	**3.4**
SaMRSA252-2514 (7N8)		putative 8-amino-7-oxononanoate synthase (transaminase activity; biosynthesis/metabolism)	**3.4**
SaMRSA252-0951 (3A21)	**oppD**	putative oligopeptide transport ATP-binding protein (nucleotide-triphosphate activity; nucleotide binding)	**3.4**
SaMRSA252-0821 (2A5)		conserved hypothetical protein	**3.4**
SaMRSA252-1837 (5A22)		putative exported protein	**3.3**
SaMRSA252-0850 (2E8)		hypothetical protein	**3.3**
SaMRSA252-0609 (3M3)		conserved hypothetical protein (NADPH-dependent FMN reductase)	**3.3**
SaMRSA252-0953 (3C21)		transport system extracellular binding lipoprotein (transporter activity)	**3.3**

SaMRSA252-0499 (3A2)	**spoVG**	stage V sporulation protein G	**3.3**
SaMRSA252-0209 (2D14)		putative oxidoreductase (oxidoreductase activity; electron transport)	**3.3**
SaMRSA252-0661 (3P9)		putative dihydroxyacetone kinase (glycerol metabolism)	**3.2**
SaMRSA252-1965 (6F1)		ThiJ/PfpI family protein (transcriptional regulation)	**3.2**
SaMRSA252-2413 (7D8)		putative short chain dehydrogenase (oxidoreductase activity; metabolism)	**3.2**
SaMRSA252-2120 (6M8)		conserved hypothetical protein (oxidoreductase activity; electron transport)	**3.1**
SaMRSA252-0882 (2D12)		putative membrane protein	**3.1**
SaMRSA252-0841 (2E7)		putative acetyltransferase	**3.1**
SaMRSA252-2628 (7G22)	**clpL**	putative ATP-dependent protease ATP-binding subunit ClpL (ATP binding; nucleotide-excision repair)	**3.1**
SaMRSA252-2749 (8E1)	**icaB**	intercellular adhesion protein B (carbohydrate metabolism)	**3.1**
SaMRSA252-2739 (7K24)		conserved hypothetical protein	**3.0**
SaMRSA252-0558 (3C9)		conserved hypothetical protein (catalytic activity; coenzyme binding; cellular metabolism)	**3.0**
SaMRSA252-0840 (2D7)		putative membrane protein	**3.0**
SaMRSA252-2392 (7H5)		conserved hypothetical protein	**3.0**
SaMRSA252-1772 (5A14)	**phoP**	alkaline phosphatase synthesis transcriptional regulatory protein - (regulation of transcription: DNA-dependent)	**3.0**
SaMRSA252-2748 (8D1)	**icaD**	intercellular adhesion protein D	**3.0**
SaMRSA252-0498 (3H1)	**yabJ**	putative regulatory protein	**2.9**
SaMRSA252-0757 (1J21)		putative glucosyl transferase (biosynthesis of disaccharides, oligosaccharides and polysaccharides)	**2.9**

SaMRSA252-0218 (2E15)		putative pyruvate formate-lyase activating enzyme (iron ion binding)	**2.9**
SaMRSA252-1026 (3J18)	**atl**	bifunctional autolysin precursor (peptidoglycan metabolism)	**2.9**
SaMRSA252-2224 (6D21)	**pyn; pdp**	putative pyrimidine-nucleoside phosphorylase (transferase activity; metabolism)	**2.9**
SaMRSA252-0299 (1E13)		hypothetical protein	**2.9**
SaMRSA252-2655 (7P13)		putative glyoxalase	**2.9**
SaMRSA252-1427 (4E19)	**arlR**	response regulator protein (regulation of transcription)	**2.9**
SaMRSA252-2727 (7P22)		hypothetical protein (biosynthesis of disaccharides, oligosaccharides, polysaccharides)	**2.9**
SaMRSA252-0883 (2E12)		putative dioxygenase (oxidoreductase; electron transport)	**2.8**
SaMRSA252-0111 (2J2)		putative myosin-crossreactive antigen	**2.8**
SaMRSA252-0744 (1M19)		putative DNA photolyase	**2.8**
SaMRSA252-2541 (7I12)		putative carboxylesterase	**2.8**
SaMRSA252-0334 (1G17)		putative dioxygenase	**2.8**
SaMRSA252-0944 (3B20)		putative exported protein	**2.8**
SaMRSA252-2276 (6K15)	**opuD2**	glycine betaine transporter 2 (membrane; transporter activity)	**2.8**
SaMRSA252-2184 (6D16)		putative exported protein (probable transglycosylase enzyme)	**2.8**
SaMRSA252-0735 (1L18)		putative exported protein	**2.8**
SaMRSA252-2119 (6L8)		membrane anchored protein	**2.8**
SaMRSA252-2610 (7E20)		putative L-serine dehydratase, alpha chain (gluconeogenesis)	**2.8**
SaMRSA252-0464 (2N21)		putative exported protein (cell wall catabolism)	**2.7**
SaMRSA252-0390 (1E24)		putative lipoprotein (metalloprotease activity; Zn ion binding)	**2.7**

SaMRSA252-0575 (3E11)		putative 6-phospho-3-hexuloisomerase (sugar binding; carbohydrate metabolism)	**2.7**
SaMRSA252-2454 (7L1)	**mqo1**	putative malate:quinone oxidoreductase 1 (citric acid cycle)	**2.7**
SaMRSA252-2648 (7I13)	**ssaA**	secretory antigen precursor (cell wall metabolism)	**2.7**
SaMRSA252-1564 (4N24)		hypothetical protein (partial)	**2.7**
SaMRSA252-0849 (2D8)		hypothetical protein	**2.7**
SaMRSA252-0309 (1F14)		putative membrane protein	**2.7**
SaMRSA252-2694 (7O18)	**nrdG**	putative anaerobic ribonucleotide reductase activating protein	**2.6**
SaMRSA252-2611 (7F20)		putative L-serine dehydratase, beta chain (gluconeogenesis)	**2.6**
SaMRSA252-0280 (2C23)		putative membrane protein	**2.6**
SaMRSA252-0660 (3O9)		putative dihydroxyacetone kinase (glycerol metabolism)	**2.6**
SaMRSA252-2010 (6B7)		hypothetical protein	**2.6**
SaMRSA252-0557 (3B9)		putative L-ribulokinase (carbohydrate metabolism)	**2.6**
SaMRSA252-1687 (5M3)		conserved hypothetical protein	**2.5**
SaMRSA252-0222 (2A16)	**coa**	staphylocoagulase precursor [conserved region]	**2.5**
SaMRSA252-1814 (5B19)	**ccpA**	catabolite control protein A (regulation of transcription-DNA-dependent)	**2.5**
SaMRSA252-0273 (2D22)	**lytM**	peptidoglycan hydrolase (metalloendopeptidase activity;proteolysis)	**2.5**
SaMRSA252-2660 (7M14)		conserved hypothetical protein (metabolism)	**2.5**
SaMRSA252-1274 (1O12)	**glpF**	putative glycerol uptake facilitator protein (transport)	**2.5**
SaMRSA252-0574 (3D11)		putative hexulose-6-phosphate synthase ('de novo' pyrimidine base biosynthesis)	**2.5**
SaMRSA252-2444		putative membrane protein	**2.5**

(7B12)			
SaMRSA252-2642 (7D24)	**crtN**	squalene synthase (oxidoreductase activity; electron transport)	**2.5**
SaMRSA252-0630 (3J6)		putative NADH-Ubiquinone/plastoquinone (complex I) oxidoreductase protein (ATP synthesis coupled electron transport)	**2.5**
SaMRSA252-2233 (6E22)	**czrA; rzcA**	zinc and cobalt transport repressor protein (regulation of transcription-DNA-dependent)	**2.5**
SaMRSA252-0138 (2M5)	**deoD1**	putative purine nucleoside phosphorylase (nucleoside metabolism)	**2.5**
SaMRSA252-1857 (5E24)		putative exported protein (peptidoglycan metabolism)	**2.5**
SaMRSA252-2560 (7D14)		putative transport protein	**2.4**
SaMRSA252-2517 (7I9)		putative dethiobiotin synthetase	**2.4**
SaMRSA252-1426 (4D19)	**arlS**	sensor kinase protein (signal transduction)	**2.4**
SaMRSA252-1289 (4M2)		putative exported protein	**2.4**
SaMRSA252-2740 (7L24)		conserved hypothetical protein	**2.4**
SaMRSA252-2273 (6P14)	**asp23**	alkaline shock protein 23 (possible role in stress response)	**2.4**
SaMRSA252-2781 (8D5)	**vraD**	ABC transporter ATP-binding protein (ATP-binding/ATPase activity)	**2.4**
SaMRSA252-0813 (2B4)	**uvrA**	excinuclease ABC subunit A (ATP-binding/ATPase activity)	**2.4**
SaMRSA252-2612 (7G20)		putative membrane protein	**2.4**
SaMRSA252-0206 (2A14)		putative extracellular sugar-binding lipoprotein (transporter activity)	**2.4**
SaMRSA252-1290 (4N2)		putative exported protein (possible role in peptidoglycan hydrolysis)	**2.4**
SaMRSA252-0277 (2H22)		putative exported protein	**2.4**
SaMRSA252-0950 (3H20)	**oppC**	putative oligopeptide transport system permease protein (transporter activity)	**2.3**
SaMRSA252-0625 (3M5)	**sarA**	staphylococcal accessory regulator A	**2.3**

SaMRSA252-1440 (4B21)	**thyA;thyE**	thymidylate synthase	**2.3**
SaMRSA252-2752 (8G1)		conserved hypothetical protein	**2.3**
SaMRSA252-0222v (2B16)	**coa**	staphylocoagulase precursor [variable region]	**2.3**
SaMRSA252-2667 (7L15)		hypothetical protein	**2.3**
SaMRSA252-2602 (7F19)		glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein	**2.2**
SaMRSA252-2223 (6C21)		putative membrane protein	**2.2**
SaMRSA252-2656 (7I14)		conserved hypothetical protein (regulation of nitrogen utilization)	**2.2**
SaMRSA252-0839 (2C7)		putative lipoprotein	**2.2**
SaMRSA252-2245 (6G23)		putative transcriptional antiterminator (regulation of transcription, DNA-dependent)	**2.2**
SaMRSA252-0128 (2K4)		putative membrane protein	**2.2**
SaMRSA252-2665 (7J15)		conserved hypothetical protein	**2.2**
SaMRSA252-1360 (4K11)	**mscL**	large-conductance mechanosensitive channel (ion channel activity; possible role in regulation of osmotic pressure changes within the cell)	**2.2**
SaMRSA252-0147 (2N6)		putative nucleotidase (nucleotide catabolism)	**2.2**
SaMRSA252-0734 (1K18)		conserved hypothetical protein	**2.2**
SaMRSA252-2228 (6H21)		conserved hypothetical protein	**2.2**
SaMRSA252-0308 (1E14)		PfkB family carbohydrate kinase (thiamine pyrophsophate synthesis)	**2.2**
SaMRSA252-2696 (7I19)		putative transporter protein (citrate transporter)	**2.2**
SaMRSA252-1813 (5A19)		histone deacetylase family protein (regulation of transcription)	**2.2**
SaMRSA252-1703 (5L5)		putative oxygenase	**2.2**

SaMRSA252-1616 (5A7)		putative membrane protein	**2.2**
SaMRSA252-0868 (2G10)		putative thioredoxin	**2.2**
SaMRSA252-0205 (2H13)		putative ABC transporter, ATP-binding protein	**2.1**
SaMRSA252-2726 (7O22)		conserved hypothetical protein	**2.1**
SaMRSA252-0581 (3B12)		putative ketoacyl-CoA thiolase	**2.1**
SaMRSA252-1615 (5H6)		putative shikimate kinase (amino acid biosynthesis)	**2.1**
SaMRSA252-2189 (6A17)		putative membrane protein	**2.1**
SaMRSA252-0555 (3H8)	**kbl**	putative 2-amino-3-ketobutyrate coenzyme A ligase (pyridoxal phosphate binding; transaminase activity)	**2.1**
SaMRSA252-1387 (4F14)	**femA**	factor essential for expression of methicillin resistance (antibiotic resistance)	**2.1**
SaMRSA252-0313 (1B15)		putative ROK family protein (transcriptional repressors)	**2.1**
SaMRSA252-2624 (7C22)		putative exported protein (cell wall metabolism)	**2.1**
SaMRSA252-2619 (7F21)		thiamine pyrophosphate enzyme (Mg ion binding; thiamin pyrophosphate binding)	**2.1**
SaMRSA252-0092 (1H11)		putative hydratase (metabolism)	**2.1**
SaMRSA252-2567 (7C15)		putative short chain dehydrogenase (oxidoreductase activity; metabolism)	**2.0**
SaMRSA252-2152 (6M12)	**sigB**	RNA polymerase sigma-B factor	**2.0**
SaMRSA252-2443 (7A12)	**tcaR**	MarR family regulatory protein (regulation of transcription)	**2.0**
SaMRSA252-0776 (1M23)		ABC transporter permease protein (transport)	**2.0**
SaMRSA252-2467 (7I3)		conserved hypothetical protein	**2.0**
SaMRSA252-2734 (7N23)	**sasA**	putative serine rich repeat containing protein (LPXTG surface protein; cell surface component)	**2.0**

SaMRSA252-1458 (4D23)		conserved hypothetical protein	**2.0**
SaMRSA252-2394 (7B6)		putative exported protein (cell envelope-related transcriptional attenuator domain)	**2.0**
SaMRSA252-1478 (4P13)	**ndk**	putative nucleoside diphosphate kinase (ATP binding)	**2.0**
SaMRSA252-1439 (4A21)	**dfrB**	dihydrofolate reductase type I (nucleotide biosynthesis)	**2.0**
SaMRSA252-0877 (2G11)		conserved hypothetical protein	**2.0**
SaMRSA252-2782 (8E5)	**vraE**	ABC transporter permease protein	**2.0**
SaMRSA252-0749 (1J20)		putative exported protein	**1.9**
SaMRSA252-0631 (3K6)		putative membrane protein	**1.9**
SaMRSA252-0576 (3F11)		putative haloacid dehalogenase-like hydrolase (catalytic activity; metabolism)	**1.9**
SaMRSA252-0965 (3F22)		conserved hypothetical protein (cell redox homeostasis)	**1.9**
SaMRSA252-1048 (3P20)	**purD**	putative phosphoribosylamine--glycine ligase	**1.9**
SaMRSA252-0580 (3A12)		putative AMP-binding enzyme	**1.9**
SaMRSA252-0837 (2A7)	**smpB**	putative tmRNA-binding protein (protein biosynthesis)	**1.9**
SaMRSA252-2689 (7J18)		hypothetical protein	**1.9**
SaMRSA252-2546 (7N12)		putative lipoprotein	**1.9**
SaMRSA252-0602 (3O2)		putative membrane protein	**1.9**
SaMRSA252-0876 (2F11)		ABC transporter ATP-binding protein	**1.8**
SaMRSA252-2256 (6H24)		conserved hypothetical protein	**1.8**
SaMRSA252-0638 (3J7)		putative membrane protein (major facilitator superfamily)	**1.8**
SaMRSA252-0317 (1F15)	**geh**	lipase precursor	**1.8**

SaMRSA252-2386 (7B5)		putative dehydrogenase (glycerol-3-phosphate catabolism)	**1.8**
SaMRSA252-2153 (6N12)	**rsbW**	anti-sigma B factor (ATP binding)	**1.8**
SaMRSA252-2666 (7K15)		hypothetical protein	**1.8**
SaMRSA252-2528 (7L10)		putative amino acid permease (transport)	**1.8**
SaMRSA252-2635 (7F23)		putative acetyltransferase	**1.8**
SaMRSA252-2641 (7C24)		putative aminotransferase (biosynthesis)	**1.8**
SaMRSA252-0598 (3K2)	**mvaK2**	phosphomevalonate kinase (ATP binding;phosphorylation)	**1.8**
SaMRSA252-2737 (7I24)		conserved hypothetical protein	**1.8**
SaMRSA252-2778 (8A5)		putative nickel transport protein (metal ion binding/transport)	**1.7**
SaMRSA252-1017 (3I17)		putative menaquinone biosynthesis bifunctional protein (thiamin pyrophosphate binding)	**1.7**
SaMRSA252-0931 (3E18)		putative membrane protein	**1.7**
SaMRSA252-2729 (7J23)		hypothetical protein	**1.7**
SaMRSA252-0140 (2O5)	**deoC1**	deoxyribose-phosphate aldolase	**1.7**
SaMRSA252-0113 (2L2)	**lldP1**	L-lactate permease 1(lactate transport)	**1.7**
SaMRSA252-0949 (3G20)	**oppB**	putative oligopeptide transport system permease protein (transporter activity)	**1.7**
SaMRSA252-2277 (6L15)		putative zinc-binding dehydrogenase	**1.7**
SaMRSA252-0314 (1C15)		putative transcription regulator (regulation of transcription, DNA-dependent)	**1.7**
SaMRSA252-0939 (3E19)		LysR family regulatory protein (regulation of transcription, DNA-dependent)	**1.7**
SaMRSA252-1220 (1I6)	**rnhB**	putative ribonuclease HII (RNA binding)	**1.6**
SaMRSA252-1021 (3M17)	**sspB**	cysteine protease precursor (proteolysis)	**1.6**

SaMRSA252-0733 (1J18)		conserved hypothetical protein	**1.6**
SaMRSA252-2730 (7K23)		conserved hypothetical protein	**1.6**
SaMRSA252-2396 (7D6)		DeoR family regulatory protein	**1.6**
SaMRSA252-0836 (2H6)	**rnr**	putative ribonuclease R (RNA binding)	**1.6**
SaMRSA252-0838 (2B7)		putative membrane protein	**1.5**
SaMRSA252-1577 (5B2)		hypothetical protein	**1.5**
SaMRSA252-0862 (2A10)		putative thioredoxin	**1.5**
SaMRSA252-2366 (7F2)		BioY family protein	**1.5**
SaMRSA252-2731 (7L23)		conserved hypothetical protein (pseudogene)	**1.4**
SaMRSA252-2735 (7O23)		conserved hypothetical protein	**1.4**
SaMRSA252-0740 (1I19)		putative cobalamin synthesis protein	**1.3**
SaMRSA252-0860 (2G9)		putative type I 3-dehydroquinase (aromatic amino acid family biosynthesis)	**1.3**

Table 4.2: Down-regulated ASM genes, normalised to BHI, using one-way ANOVA and posthoc Benjamini and Hochberg tests ($p \leq 0.05$). The corresponding ORF (Open reading frame), gene name and protein function are listed. The table is arranged according to the most down-regulated gene with the highest fold difference.

ORF	GENE	PROTEIN (FUNCTION)	FOLD
SaMW2-0754 (10A9)		hypothetical protein	**-22.2**
SaMRSA252-0259 (2F20)	**lrgA**	holin-like protein (possible role in murein hydrolysis and penicillin tolerance)	**-10.8**
SaMRSA252-0114 (2M2)	**spa**	immunoglobulin G binding protein A precursor (surface protein)	**-9.2**
SaMRSA252-2131 (6P9)		conserved hypothetical protein	**-7.3**
SaMRSA252-2504 (7L7)		extracellular solute-binding lipoprotein (transport)	**-6.4**
SaMRSA252-2438 (7D11)		putative exported protein	**-5.8**
SaMRSA252-0260 (2G20)	**lrgB**	holin-like protein (possible role in murein hydrolysis and penicillin tolerance)	**-4.9**
SaMRSA252-1060 (3L22)		putative membrane protein	**-4.9**
SaMRSA252-2437 (7C11)		putative transport protein	**-4.6**
SaMRSA252-2503 (7K7)		transport system membrane protein (major facilitator superfamily)	**-4.6**
SaMRSA252-1454 (4H22)		putative membrane protein-pseudogene (metallopeptidase activity/Zn ion binding/proteolysis)	**-4.6**
SaMRSA252-2502 (7J7)		ABC transporter ATP-binding protein (ATP binding/ATPase activity)	**-4.1**
SaMRSA252-0172 (2O9)		conserved hypothetical protein	**-4.0**
SaMRSA252-2488 (7M5)	**nasE**	assimilatory nitrite reductase small subunit (oxidoreductase ativity; electron transport)	**-3.9**
SaMRSA252-0752		putative phosphofructokinase (thiamine pyrophosphate synthesis)	**-3.9**

(1M20)			
SaMRSA252-1133 (4D7)		putative membrane protein	**-3.8**
SaMRSA252-2489 (7N5)	**nasD**	nitrite reductase large subunit (disulfide oxidoreductase activity; electron transport)	**-3.7**
SaMRSA252-0906 (3D15)		conserved hypothetical protein (catalytic activity)	**-3.7**
SaMRSA252-2508 (7P7)	**sbi**	IgG-binding protein	**-3.6**
SaMRSA252-2132 (6I10)		putative membrane protein	**-3.6**
SaMRSA252-0905 (3C15)		putative transporter protein (Sodium ion transport/regulation of pH)	**-3.6**
SaMRSA252-1237 (1J8)		putative phosphatidate cytidylyltransferase (phospholipid biosynthesis)	**-3.6**
SaMRSA252-2470 (7L3)		putative exported protein	**-3.6**
SaMRSA252-1010 (3J16)		putative membrane protein	**-3.5**
SaMRSA252-2799 (8C7)	**rnpA**	ribonuclease P protein component (ribonuclease activity/tRNA processing)	**-3.5**
SaMRSA252-2320 (6P20)	**rplF**	50S ribosomal protein L6 (structural constituent of ribosome)	**-3.5**
SaMRSA252-2476 (7J4)	**narT**	nitrite transport protein (major facilitaor superfamily)	**-3.3**
SaMRSA252-0401 (2P13)		putative sodium:dicarboxylate symporter protein	**-3.3**
SaMRSA252-2709 (7N20)	**clfB**	fibrinogen and keratin-10 binding surface anchored protein (cell surface protein)	**-3.3**
SaMRSA252-0926 (3H17)	**spsA**	putative signal peptidase Ia (proteolysis)	**-3.1**
SaMRSA252-0694 (1P13)		putative exported protein	**-3.1**
SaMRSA252-0753 (1N20)	**fruA**	PTS transport system, fructose-specific IIABC component (sugar porter activity)	**-3.1**
SaMRSA252-0212 (2G14)		putative membrane protein (C-terminal protein amino acid methylation)	**-3.1**
SaMRSA252-2168 (6E14)		putative helicase (ATP binding; helicase activity; nucleic acid binding)	**-3.1**

SaMRSA252-1235 (1P7)	**frr**	ribosome recycling factor (protein biosynthesis)	**-3.0**
SaMRSA252-0790 (2C1)	**sstD**	lipoprotein (iron ion transport activity)	**-3.0**
SaMRSA252-1052 (3L21)		hypothetical protein	**-3.0**
SaMRSA252-2316 (6L20)	**rplO**	50S ribosomal protein L15 (protein biosynthesis)	**-3.0**
SaMRSA252-1059 (3K22)		putative cytochrome ubiquinol oxidase (membrane component; oxidoreductase activity; electron transport)	**-3.0**
SaMRSA252-1777 (5F14)	**pfkA**	6-phosphofructokinase (glycolysis)	**-2.9**
SaMRSA252-1127 (4F6)		putative exported protein	**-2.9**
SaMRSA252-2328 (6P21)	**rplP**	50S ribosomal protein L16 (protein biosynthesis)	**-2.9**
SaMRSA252-0234 (2F17)	**ldh1**	L-lactate dehydrogenase 1(oxidoreductase activity; TCA intermediate metabolism)	**-2.9**
SaMRSA252-1450 (4D22)	**tdcB**	putative threonine dehydratase (pyridoxal phosphate dependent enzyme; metabolism)	**-2.9**
SaMRSA252-0827 (2G5)	**gapR**	glycolytic operon regulator (carbohydrate binding; transcription regulator activity)	**-2.8**
SaMRSA252-2020 (6D8)		putative membrane protein	**-2.8**
SaMRSA252-1186 (1O1)		conserved hypothetical protein (transcription, DNA-dependent)	**-2.8**
SaMRSA252-0543 (3D7)	**rplA**	50S ribosomal protein L1(protein biosynthesis)	**-2.8**
SaMRSA252-2321 (6I21)	**rpsH**	30S ribosomal protein S8 (protein biosynthesis)	**-2.8**
SaMRSA252-0363 (1C21)	**ssb**	putative single-strand DNA-binding protein (single-stranded DNA binding)	**-2.7**
SaMRSA252-0787 (1P24)	**sstA**	FecCD transport family protein (membrane; transporter activity)	**-2.7**
SaMRSA252-1759 (5L12)	**rpmI**	50S ribosomal protein L35 (protein biosynthesis)	**-2.7**
SaMRSA252-2219 (6G20)		hypothetical protein	**-2.7**

SaMRSA252-1204 (1I4)		conserved hypothetical protein	**-2.7**
SaMRSA252-0910 (3H15)	**mnhE**	Na+/H+ antiporter subunit (integral to membrane; cation transport)	**-2.6**
SaMRSA252-0173 (2P9)		putative ABC transporter ATP-binding protein (ATP binding; ATPase activity)	**-2.6**
SaMRSA252-1715 (5P6)	**apt**	adenine phosphoribosyltransferase (nucleoside metabolism)	**-2.6**
SaMRSA252-0408 (2O14)	**guaB**	putative inosine-5'-monophosphate dehydrogenase (catalytic activity)	**-2.6**
SaMRSA252-0946 (3D20)	**fabH**	putative 3-oxoacyl-[acyl-carrier-protein] synthase III (fatty acid synthesis)	**-2.6**
SaMRSA252-2324 (6L21)	**rplX**	50S ribosomal protein L24	**-2.6**
SaMRSA252-0789 (2B1)	**sstC**	ABC transporter ATP-binding protein (ATP binding/ATPase activity)	**-2.6**
SaMRSA252-2315 (6K20)	**secY**	preprotein translocase SecY subunit (protein secretion)	**-2.5**
SaMRSA252-0257 (2D20)	**lytS**	autolysin sensor kinase protein (2component signal transduction (photorelay); cell wall organization and biogenesis	**-2.5**
SaMRSA252-0452 (2J20)		putative NADH-Ubiquinone/plastoquinone (complex I) protein (ATP synthesis coupled electron transport)	**-2.5**
SaMRSA252-0897 (3C14)	**dltD**	putative lipoteichoic acid biosynthesis protein (biosynthesis of D-alanyl-lipoteichoic acid)	**-2.5**
SaMRSA252-1236 (1I8)	**uppS**	undecaprenyl pyrophosphate synthetase (transferase activity; metabolism)	**-2.5**
SaMRSA252-0344 (1H18)		putative Sec-independent protein translocase protein (protein transporter activity)	**-2.5**
SaMRSA252-0454 (2L20)		conserved hypothetical protein	**-2.5**
SaMRSA252-2004 (6D6)		putative membrane protein	**-2.5**
SaMRSA252-2678 (7O16)		putative ketopantoate reductase (glycerol-3-phosphate catabolism)	**-2.5**
SaMRSA252-2344 (6P23)		putative membrane protein	**-2.5**
SaMRSA252-1836		putative peptidase (metallopeptidase activity; proteolysis)	**-2.4**

(5H21)			
SaMRSA252-1939 (5L22)		putative response regulator (regulation of transcription, DNA-dependent)	**-2.4**
SaMRSA252-0174 (2I10)		putative lipoprotein	**-2.4**
SaMRSA252-1174 (4C12)	**pyrR**	putative pyrimidine operon regulatory protein (nucleoside metabolism)	**-2.4**
SaMRSA252-0912 (3B16)	**mnhC**	Na+/H+ antiporter subunit	**-2.4**
SaMRSA252-0542 (3C7)	**rplK**	50S ribosomal protein L11(protein biosynthesis)	**-2.4**
SaMRSA252-2308 (6L19)	**rplQ**	50S ribosomal protein L17 (protein biosynthesis)	**-2.4**
SaMRSA252-0455 (2M20)		putative membrane protein	**-2.4**
SaMRSA252-1776 (5E14)	**pyk**	pyruvate kinase (phosphorylation)	**-2.4**
SaMRSA252-2675 (7L16)	**panD**	putative aspartate 1-decarboxylase precursor (alanine biosynthesis)	**-2.4**
SaMRSA252-0894 (3H13)	**dltA**	D-alanine-D-alanyl carrier protein ligase (catalytic activity; metabolism)	**-2.4**
SaMRSA252-2003 (6C6)		conserved hypothetical protein	**-2.4**
SaMRSA252-1134 (4E7)		hypothetical protein	**-2.4**
SaMRSA252-0914 (3D16)	**mnhA**	Na+/H+ antiporter subunit (electron transport)	**-2.4**
SaMRSA252-1419 (4E18)		putative branched-chain amino acid transporter protein (branched chain aliphatic amino acid transport)	**-2.4**
SaMRSA252-2669 (7N15)		putative dihydroorotate dehydrogenase ('de novo' pyrimidine base biosynthesis)	**-2.4**
SaMRSA252-2369 (7A3)		conserved hypothetical protein (electron transport)	**-2.3**
SaMRSA252-2618 (7E21)	**glcB**	PTS system, glucose-specific IIABC component (sugar porter activity)	**-2.3**
SaMRSA252-2500 (7P6)		putative lipoprotein	**-2.3**
SaMRSA252-1762	**thrS**	threonyl-tRNA synthetase (protein biosynthesis)	**-2.3**

(5O12)			
SaMRSA252-0791 (2D1)		hypothetical protein (Zn ion binding)	**-2.3**
SaMRSA252-1599 (5H4)		putative geranyltranstransferase (isoprenoid biosynthesis)	**-2.3**
SaMRSA252-2351 (6O24)		hypothetical protein (regulation of transcription)	**-2.3**
SaMRSA252-0788 (2A1)	**sstB**	FecCD transport family protein (membrane; transporter activity)	**-2.3**
SaMRSA252-2455 (7M1)	**lldP2**	putative L-lactate permease 2 (lactate transport)	**-2.3**
SaMRSA252-0546 (3G7)		conserved hypothetical protein (methyltransferase activity)	**-2.3**
SaMRSA252-1083 (4B1)		BipA family GTPase (GTP binding)	**-2.3**
SaMRSA252-2216 (6D20)	**rpoE**	DNA-directed RNA polymerase delta subunit (DNA binding;transcription)	**-2.3**
SaMRSA252-2484 (7I5)	**narJ**	respiratory nitrate reductase delta chain (nitrate reductase activity; electron transport)	**-2.3**
SaMRSA252-1206 (1K4)	**fabD**	putative malonyl CoA-acyl carrier protein transacylase (transferase activity; metabolism)	**-2.3**
SaMRSA252-1239 (1L8)	**proS**	prolyl-tRNA synthetase (protein biosynthesis)	**-2.2**
SaMRSA252-1601 (5B5)		putative exodeoxyribonuclease VII large subunit (DNA catabolism)	**-2.2**
SaMRSA252-1602 (5C5)		putative N utilization substance protein B (regulation of transcription; DNA-dependent)	**-2.2**
SaMRSA252-0063 (1E8)		hypothetical protein	**-2.2**
SaMRSA252-2341 (6M23)	**glcU**	glucose uptake protein (carbohydrate transport)	**-2.2**
SaMRSA252-2314 (6J20)	**adk**	adenylate kinase	**-2.2**
SaMRSA252-2215 (6C20)	**pyrG**	putative CTP synthase (pyrimidine nucleotide biosynthesis)	**-2.2**
SaMRSA252-0407 (2N14)	**pbuX**	putative xanthine permease (transporter activity)	**-2.2**
SaMRSA252-0256 (2C20)	**scdA**	cell wall metabolism protein	**-2.2**

SaMRSA252-1743 (5L10)	**valS**	valyl-tRNA synthetase (tRNA aminoacylation for protein translation)	**-2.2**
SaMRSA252-0799 (2D2)		putative membrane protein	**-2.2**
SaMRSA252-0909 (3G15)	**mnhF**	Na+/H+ antiporter subunit (membrane;ion transport)	**-2.2**
SaMRSA252-1711 (5L6)	**hisS**	histidyl-tRNA synthetase (tRNA aminoacylation for protein translation)	**-2.2**
SaMRSA252-1805 (5B18)		putative protease (proteolysis)	**-2.2**
SaMRSA252-0013 (1E2)		putative membrane protein	**-2.2**
SaMRSA252-1758 (5K12)	**rplT**	50S ribosomal protein L20 (protein biosynthesis)	**-2.1**
SaMRSA252-0890 (3D13)		conserved hypothetical protein	**-2.1**
SaMRSA252-2439 (7E11)		TetR family regulatory protein (regulation of transcription, DNA dependent)	**-2.1**
SaMRSA252-0681 (3K12)		conserved hypothetical protein	**-2.1**
SaMRSA252-2499 (7O6)		putative lipoprotein	**-2.1**
SaMRSA252-1778 (5G14)	**accA**	acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha (fatty acid biosynthesis)	**-2.1**
SaMRSA252-1605 (5F5)	**accB; fabE**	biotin carboxyl carrier protein of acetyl-CoA carboxylase	**-2.1**
SaMRSA252-1111 (4F4)	**pheS**	putative phenylalanyl-tRNA synthetase alpha chain (phenylalanyl-tRNA aminoacylation)	**-2.1**
SaMRSA252-2621 (7H21)		putative membrane protein	**-2.1**
SaMRSA252-1404 (4F16)		ABC transporter ATP-binding protein (ATP binding/ATPase activity)	**-2.0**
SaMRSA252-0360 (1H20)		putative GTP-binding protein	**-2.0**
SaMRSA252-1551 (4I23)		hypothetical phage protein	**-2.0**
SaMRSA252-1136 (4F7)	**hla**	alpha-hemolysin precursor-pseudogene (haemolysis of red blood cells)	**-2.0**
SaMRSA252-2335	**rplC**	50S ribosomal protein L3 (protein biosynthesis)	**-2.0**

(6O22)			
SaMRSA252-1486 (4P14)	**cmk**	cytidylate kinase (nucleic acid metabolism)	**-2.0**
SaMRSA252-1207 (1L4)	**fabG**	3-oxoacyl-[acyl-carrier protein] reductase (oxidoreductase activity; metabolism)	**-2.0**
SaMRSA252-0366 (1F21)		putative integrase (family of phage integrase-DNA integration/DNA recombination)	**-2.0**
SaMRSA252-1243 (1P8)		conserved hypothetical protein	**-2.0**
SaMRSA252-2361 (7A2)	**modC**	putative molybdenum transport ATP-binding protein	**-2.0**
SaMRSA252-1780 (5A15)		NAD-dependent malic enzyme (oxidoreductase activity acting on the CH-OH group of donors NAD or NADP as acceptor)	**-2.0**
SaMRSA252-0652 (3O8)	**pbp4**	penicillin-binding protein 4 (proteolysis)	**-2.0**
SaMRSA252-0490 (2P24)		tetrapyrrole (corrin/porphyrin) methylase family protein (methyltransferase activity; metabolism)	**-2.0**
SaMRSA252-0563 (3H9)		putative deaminase (hydrolase activity; Zn ion binding)	**-2.0**
SaMRSA252-2424 (7F9)		putative aldose 1-epimerase (galactose metabolism)	**-2.0**
SaMRSA252-0891 (3E13)		haloacid dehalogenase-like hydrolase (catalytic activity; metabolism)	**-2.0**
SaMRSA252-1396 (4F15)		putative membrane protein	**-2.0**
SaMRSA252-1173 (4B12)		putative RNA pseudouridylate synthase (RNA binding)	**-2.0**
SaMRSA252-2796 (8H6)	**gidB**	putative glucose inhibited division protein B (cell cycle)	**-2.0**
SaMRSA252-0258 (2E20)	**lytR**	autolysin response regulator protein (2-component signal transduction system; regulation of transcription, DNA-dependent)	**-2.0**
SaMRSA252-1789 (5B16)	**ackA**	acetate kinase (phosphorylation; metabolism)	**-2.0**
SaMRSA252-1843 (5G22)	**leuS**	leucyl-tRNA synthetase (tRNA aminoacylation for protein translation)	**-2.0**

SaMRSA252-2207 (6C19)		putative thymidine kinase (ATP binding)	**-2.0**
SaMRSA252-0351 (1G19)	**thl;thlA**	acetyl-CoA acetyltransferase (lipid metabolism or synthesis)	**-2.0**
SaMRSA252-1719 (5L7)	**tgt**	queuine tRNA-ribosyltransferase (queuosine biosynthesis)	**-1.9**
SaMRSA252-2117 (6J8)	**groES**	hsp10-a 10 kDa chaperonin (protein folding)	**-1.9**
SaMRSA252-2338 (6J23)		xanthine/uracil permeases family protein (transporter activity)	**-1.9**
SaMRSA252-0456 (2N20)		putative membrane protein	**-1.9**
SaMRSA252-0803 (2H2)		conserved hypothetical protein (possible role in fatty acid transport or metabolism)	**-1.9**
SaMRSA252-0911 (3A16)	**mnhD**	Na+/H+ antiporter subunit (ATP synthesis coupled with electron transport)	**-1.9**
SaMRSA252-1215 (1L5)	**rimM**	16S rRNA processing protein (ribosome biogenesis)	**-1.9**
SaMRSA252-2001 (6A6)		staphopain protease (proteolysis)	**-1.9**
SaMRSA252-0979 (3D24)		putative membrane protein	**-1.9**
SaMRSA252-1923 (5L20)	**hemY**	putative protoporphyrinogen oxidase (oxidoreductase activity; electron transport)	**-1.9**
SaMRSA252-1604 (5E5)	**accC**	biotin carboxylase subunit of acetyl-CoA carboxylase (urea cycle and biosynthesis or arginine and/or pyrimidines)	**-1.9**
SaMRSA252-1803 (5H17)		PTS system IIBC component (sugar transport)	**-1.9**
SaMRSA252-0983 (3H24)		hypothetical protein	**-1.9**
SaMRSA252-1065 (3P22)		putative polypeptide deformylase 2 (peptide deformylase activity; iron and ion binding; protein biosynthesis)	**-1.9**
SaMRSA252-0646 (3I8)	**tagA**	teichoic acid biosynthesis protein (biosynthesis of teichoic acid)	**-1.9**
SaMRSA252-1804 (5A18)		putative acyltransferase (acyltransferase activity; metabolism)	**-1.9**
SaMRSA252-2170		D-alanine--D-alanine ligase (peptidoglycan biosynthesis)	**-1.9**

(6G14)			
SaMRSA252-0084 (1H10)		hypothetical protein	**-1.9**
SaMRSA252-0016 (1H2)	**dnaC**	DnaB-like helicase (DNA replication)	**-1.9**
SaMRSA252-0908 (3F15)	**mnhG**	Na+/H+ antiporter subunit	**-1.9**
SaMRSA252-0934 (3H18)		putative haloacid dehalogenase-like hydrolase (catalytic activity; metabolism)	**-1.9**
SaMRSA252-0357 (1E20)		putative DNA-binding protein (DNA binding)	**-1.8**
SaMRSA252-1720 (5M7)	**queA**	S-adenosylmethionine: tRNA ribosyltransferase-isomerase (quenosine biosynthesis)	**-1.8**
SaMRSA252-2411 (7B8)		putative transport protein (Na+: H antiporter; sodium ion transport; regulation of pH	**-1.8**
SaMRSA252-1677 (5M2)		putative membrane protein	**-1.8**
SaMRSA252-1268 (1I12)		conserved hypothetical protein (iron ion binding; catalytic activity)	**-1.8**
SaMRSA252-0664 (3K10)		putative membrane protein (branched chain aliphatic amino acid transport)	**-1.8**
SaMRSA252-2340 (6L23)		acetyltransferase (GNAT) family protein	**-1.8**
SaMRSA252-1793 (5F16)	**thiI**	putative thiamine biosynthesis protein (thiamin biosynthesis)	**-1.8**
SaMRSA252-0618 (3N4)		putative transport system lipoprotein (iron ion transporter)	**-1.8**
SaMRSA252-1634 (5C9)		putative endonuclease (DNA repair)	**-1.8**
SaMRSA252-0599 (3L2)		hypothetical protein	**-1.8**
SaMRSA252-1598 (5G4)	**argR**	arginine repressor (regulation of transcription, DNA-dependent)	**-1.8**
SaMRSA252-1635 (5D9)		putative helicase (nucleic acid binding)	**-1.8**
SaMRSA252-1674 (5J2)		putative GTPase (GTP binding)	**-1.8**
SaMRSA252-0343 (1G18)		putative Sec-independent protein translocase protein	**-1.8**

SaMRSA252-0494 (3D1)		ribosomal RNA adenine dimethylase (rRNA modification)	**-1.8**
SaMRSA252-2670 (7O15)		putative membrane protein	**-1.8**
SaMRSA252-0782 (1K24)		conserved hypothetical protein (aromatic compound biosynthesis)	**-1.8**
SaMRSA252-1838 (5B22)		RNA pseudouridine synthase (RNA binding)	**-1.8**
SaMRSA252-1091 (4B2)		putative membrane protein	**-1.8**
SaMRSA252-2368 (7H2)		putative ferrichrome-binding lipoprotein precursor (iron ion transporter activity)	**-1.8**
SaMRSA252-0506 (3H2)		putative tetrapyrrole (corrin/porphyrin) methylases (methyltransferase activity; metabolism)	**-1.8**
SaMRSA252-0892 (3F13)		D-isomer specific 2-hydroxyacid dehydrogenase (L-serine biosynthesis)	**-1.7**
SaMRSA252-1794 (5G16)		aminotransferase class-V protein (transaminase activity)	**-1.7**
SaMRSA252-2397 (7E6)		putative membrane protein	**-1.7**
SaMRSA252-0916 (3F16)		putative cyclophilin type peptidyl-prolyl cis-trans isomerase (protein folding)	**-1.7**
SaMRSA252-1757 (5J12)		conserved hypothetical protein	**-1.7**
SaMRSA252-0982 (3G24)		hypothetical protein	**-1.7**
SaMRSA252-2391 (7G5)		putative N-acetylmuramoyl-L-alanine amidase (peptidoglycan catabolism)	**-1.7**
SaMRSA252-1596 (5E4)	**bfmBC**	putative dihydrolipoamide dehydrogenase (disulfide oxidoreductase activity; electron transport)	**-1.7**
SaMRSA252-1241 (1N8)		conserved hypothetical protein	**-1.7**
SaMRSA252-1272 (1M12)	**mutL**	DNA mismatch repair protein MutL (ATP binding)	**-1.7**
SaMRSA252-0657 (3L9)	**fhuA**	ferrichrome transport ATP-binding protein (ATP binding/ATPase activity)	**-1.7**
SaMRSA252-1366 (4I12)		topoisomerase IV subunit B (involved in DNA modification; DNA metabolism)	**-1.7**

SaMRSA252-0367 (1G21)		DNA-binding protein (DNA binding)	**-1.7**
SaMRSA252-0686 (3P12)		putative transposase -pseudogene (transposition, DNA-mediated)	**-1.7**
SaMRSA252-0518 (3D4)	**lysS**	lysyl-tRNA synthetase (ATPbinding; aminoacyl-tRNA ligase activity; nucleic acid binding)	**-1.7**
SaMRSA252-1389 (4H14)		conserved hypothetical protein (pseudogene)	**-1.7**
SaMRSA252-1846 (5B23)		conserved hypothetical protein	**-1.7**
SaMRSA252-2795 (8G6)		putative DNA-binding protein (DNA binding)	**-1.7**
SaMRSA252-1844 (5H22)		putative membrane protein	**-1.7**
SaMRSA252-2477 (7K4)		conserved hypothetical protein	**-1.7**
SaMRSA252-0384 (1H23)		hypothetical protein	**-1.7**
SaMRSA252-0450 (2P19)		putative cobalamin synthesis protein	**-1.7**
SaMRSA252-0648 (3K8)	**tagG**	teichoic acid ABC transporter permease protein	**-1.7**
SaMRSA252-2707 (7L20)		putative regulatory protein (transcription factor activity)	**-1.7**
SaMRSA252-0925 (3G17)		putative membrane protein	**-1.7**
SaMRSA252-1334 (4I8)	**nucI**	thermonuclease (nucleic acid binding)	**-1.6**
SaMRSA252-1466 (4D24)		conserved hypothetical protein (nucleobase, nucleoside, nucleotide and nucleic acid metabolism)	**-1.6**
SaMRSA252-1195 (1P2)		putative protein phosphatase (catalytic activity)	**-1.6**
SaMRSA252-1716 (5I7)		putative single-stranded-DNA-specific exonuclease (nucleic acid binding)	**-1.6**
SaMRSA252-1094 (4E2)		putative exported protein (glycerol metabolism)	**-1.6**
SaMRSA252-0586 (3G12)	**ung**	putative uracil-DNA glycosylase	**-1.6**

SaMRSA252-1096 (4G2)		conserved hypothetical protein	**-1.6**
SaMRSA252-2363 (7C2)	**modA**	putative molybdate-binding lipoprotein precursor (transporter activity)	**-1.6**
SaMRSA252-1833 (5E21)		putative methyltransferase	**-1.6**
SaMRSA252-1267 (1P11)		conserved hypothetical protein	**-1.6**
SaMRSA252-1097 (4H2)		putative methylase	**-1.6**
SaMRSA252-1699 (5I5)		conserved hypothetical protein	**-1.6**
SaMRSA252-1167 (4D11)		conserved hypothetical protein (RNA binding)	**-1.6**
SaMRSA252-2261 (6L13)		putative membrane protein	**-1.6**
SaMRSA252-1723 (5P7)		putative DNA-binding protein (amino acid metabolism)	**-1.6**
SaMRSA252-2204 (6H18)		conserved hypothetical protein	**-1.6**
SaMRSA252-1341 (4P8)		haloacid dehalogenase-like hydrolase (catalytic activity; metabolism)	**-1.6**
SaMRSA252-1110 (4E4)		SpoU rRNA Methylase family protein (RNA processing)	**-1.5**
SaMRSA252-2671 (7P15)		conserved hypothetical protein	**-1.5**
SaMRSA252-2711 (7P20)	**arcC**	carbamate kinase (amino acid biosynthesis)	**-1.5**
SaMRSA252-0562 (3G9)		putative deoxyadenosine kinase protein (nucleobase, nucleoside, nucleotide and nucleic acid metabolism)	**-1.5**
SaMRSA252-2139 (6P10)		conserved hypothetical protein	**-1.5**
SaMRSA252-1607 (5H5)		putative peptidase (metallopeptidase activity; proteolysis)	**-1.5**
SaMRSA252-2495 (7K6)		hypothetical protein	**-1.5**
SaMRSA252-0677 (3P11)		AraC family regulatory protein -pseudogene (regulation of transcription, DNA-dependent)	**-1.5**

SaMRSA252-1756 (5I12)		hypothetical protein	**-1.5**
SaMRSA252-2703 (7P19)		response regulator protein (2-component signal transduction system; regulation of transcription, DNA-dependent)	**-1.5**
SaMRSA252-0485 (2K24)	**holB**	putative DNA polymerase III, delta' subunit	**-1.5**
SaMRSA252-1369 (4L12)		transcription antiterminator (regulation of transcription, DNA-dependent)	**-1.5**
SaMRSA252-1783 (5D15)		putative DNA-binding protein	**-1.4**
SaMRSA252-2435 (7A11)		hypothetical protein	**-1.4**
SaMRSA252-0758 (1K21)	**saeS**	histidine kinase protein (signal transduction)	**-1.4**
SaMRSA252-1581 (5F2)		conserved hypothetical protein	**-1.4**
SaMRSA252-2118 (6K8)		putative membrane protein	**-1.3**

Table 4.3: Up-regulated GASM genes, normalised to BHI, using one-way ANOVA and posthoc Benjamini and Hochberg tests ($p \leq 0.05$). The corresponding ORF (Open reading frame), gene name and protein function are listed. The table is arranged according to the most up-regulated gene with the highest fold difference.

ORF	GENE	PROTEIN (FUNCTION)	FOLD
SaMRSA252-2297 (6P17)	**SAR2297**	putative acetolactate synthase (thiamin pyrophosphate binding)	**22.8**
SaMRSA252-0129 (2L4)	**SAR0129**	putative short chain dehydrogenase (metabolism; oxidoreductase activity)	**21.9**
SaMRSA252-1852 (5H23)	**ribE**	riboflavin synthase alpha chain (riboflavin synthesis)	**21.7**
SaMRSA252-1050 (3J21)	**SAR1050**	ABC transporter ATP-binding protein (transport)	**19.3**
SaMRSA252-1851 (5G23)	**ribA**	riboflavin biosynthesis protein (riboflavin biosynthesis)	**19.2**
SaMRSA252-1051 (3K21)	**SAR1051**	putative membrane protein	**19.1**
SaMRSA252-0919 (3A17)	**rocD**	ornithine aminotransferase (pyridoxal phosphate binding)	**17.2**
SaMRSA252-1773 (5B14)	**citC**	isocitrate dehydrogenase (carbohydrate metabolism; oxidoreductase activity)	**15.5**
SaMRSA252-2296 (6O17)	**SAR2296**	conserved hypothetical protein	**15.5**
SaMRSA252-1774 (5C14)	**citZ**	citrate synthase II (carbohydrate metabolism)	**15.3**
SaMRSA252-1049 (3I21)	**SAR1049**	putative cobalt transport protein (cobalamin biosynthetic process)	**14.6**
SaMRSA252-0920 (3B17)	**SAR0920**	putative NAD-specific glutamate dehydrogenase (amino acid metabolism; oxidoreductase activity)	**14.5**
SaMRSA252-0169 (2L9)	**SAR0169**	putative aldehyde dehydrogenase (metabolism; oxidoreductase activity)	**11.5**
SaMRSA252-1425 (4C19)	**odhA**	2-oxoglutarate dehydrogenase E1 component (metabolism; oxidoreductase activity)	**11.0**
SaMRSA252-1850 (5F23)	**ribH**	6,7-dimethyl-8-ribityllumazine synthase (riboflavin biosynthetic process)	**10.9**

SaMRSA252-1853 (5A24)	**ribD**	bifunctional riboflavin biosynthesis protein (riboflavin biosynthetic process)	**10.5**
SaMRSA252-1120 (4G5)	**sdhC**	putative succinate dehydrogenase cytochrome b558	**10.2**
SaMRSA252-2697 (7J19)	**SAR2697**	conserved hypothetical protein	**10.2**
SaMRSA252-2295 (6N17)	**SAR2295**	putative exported protein	**10.1**
SaMRSA252-1614 (5G6)	**gcvT**	putative aminomethyltransferase (glycine catabolic process)	**10.0**
SaMRSA252-1121 (4H5)	**sdhA/citF**	putative succinate dehydrogenase flavoprotein subunit (electron transport; oxidoreductase activity)	**9.6**
SaMRSA252-0378 (1B23)	**SAR0378**	hypothetical protein	**9.0**
SaMRSA252-1424 (4B19)	**odhB/citM**	dihydrolipoamide succinyltransferase E2 component of 2-oxoglutarate dehydrogenase complex (metabolism; acyltransferase activity/protein binding)	**9.0**
SaMRSA252-0996 (3M14)	**SAR0996**	conserved hypothetical protein	**8.7**
SaMRSA252-0806 (2C3)	**SAR0806**	putative S30EA family ribosomal protein	**8.6**
SaMRSA252-0577 (3G11)	**proP**	putative proline/betaine transporter	**8.6**
SaMRSA252-0405 (2L14)	**SAR0405**	hypothetical protein	**8.4**
SaMRSA252-2596 (7H18)	**SAR2596**	conserved hypothetical protein	**8.3**
SaMRSA252-0153 (2L7)	**capC**	capsular polysaccharide synthesis enzyme	**8.3**
SaMRSA252-2698 (7K19)	**cysJ**	putative sulfite reductase [NADPH] flavoprotein alpha-component - (electron transport; FMN binding; oxidoreductase activity)	**8.2**
SaMRSA252-1810 (5F18)	**fhs**	formate--tetrahydrofolate ligase (folic acid & derivative biosynthetic process)	**8.1**
SaMRSA252-0661 (3P9)	**SAR0661**	putative dihydroxyacetone kinase (glycerol metabolism)	**8.1**
			7.7
SaMRSA252-1787 (5H15)	**ald1**	alanine dehydrogenase 1 (aromatic amino acid family biosythesis; shikimate pathway)	
SaMRSA252-0334 (1G17)	**SAR0334**	putative dioxygenase	**7.5**

SaMRSA252-1612 (5E6)	**SAR1612**	putative glycine cleavage system P-protein (amino acid metabolism)	**7.5**
SaMRSA252-2120 (6M8)	**SAR2120**	conserved hypothetical protein (electron transport; oxidoreductase activity)	**7.4**
SaMRSA252-2278 (6M15)	**SAR2278**	putative zinc-binding dehydrogenase	**7.1**
SaMRSA252-1222 (1K6)	**SAR1222**	putative succinyl-CoA ligase (metabolism; catalytic activity)	**7.1**
SaMRSA252-1492 (4N15)	**SAR1492**	ferredoxin	**6.9**
SaMRSA252-1344 (4K9)	**SAR1344**	catalase (response to oxidative stress)	**6.8**
SaMRSA252-0660 (3O9)	**SAR0660**	putative dihydroxyacetone kinase (glycerol metabolism)	**6.6**
SaMRSA252-0400 (2O13)	**SAR0400**	nitroreductase family protein (electron transport; oxidoreductase activity)	**6.5**
SaMRSA252-2602 (7F19)	**SAR2602**	glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein	**6.4**
SaMRSA252-0227 (2G16)	**fadX**	putative acetyl-CoA transferase	**6.2**
SaMRSA252-0558 (3C9)	**SAR0558**	conserved hypothetical protein (biosynthetic process; nucleotide-sugar metabolic process)	**6.2**
SaMRSA252-0135 (2J5)	**sodM**	superoxide dismutase (superoxide metabolic process; metal ion binding)	**6.2**
SaMRSA252-0420 (2J16)	**SAR0420**	putative membrane protein	**5.9**
SaMRSA252-1265 (1N11)	**SAR1265**	putative pyruvate flavodoxin/ferredoxin oxidoreductase (electron transport; oxidoreductase activity)	**5.9**
SaMRSA252-1037 (3M19)	**folD**	FolD bifunctional protein [includes: methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase] (folic acid & derivative biosynthetic process)	**5.9**
SaMRSA252-2717 (7N21)	**isaB**	immunodominant antigen B	**5.9**
SaMRSA252-0225 (2E16)	**fadD**	putative acyl-CoA dehydrogenase (electron transport; oxidoreductase activity)	**5.7**
SaMRSA252-1152 (4E9)	**SAR1152**	acetyltransferase (GNAT) family protein (N-acetyltransferase activity)	**5.6**
SaMRSA252-0526 (3D5)	**SAR0526**	conserved hypothetical protein (nucleotide-excision repair; DNA binding; nuclease activity)	**5.5**
SaMRSA252-2180	**thiE**	putative thiamine-phosphate pyrophosphorylase (thiamin biosynthetic process)	**5.5**

(6H15)			
SaMRSA252-2619 (7F21)	**SAR2619**	thiamine pyrophosphate enzyme (thiamin pyrophosphate binding)	**5.5**
SaMRSA252-1849 (5E23)	**SAR1849**	proline dehydrogenase (amino acid metabolism)	**5.4**
SaMRSA252-0842 (2F7)	**clfA**	clumping factor (cell surface-virulence)	**5.4**
SaMRSA252-2621 (7H21)	**SAR2621**	putative membrane protein (lrgA family)	**5.3**
SaMRSA252-1221 (1J6)	**SAR1221**	putative CoA synthetase protein (metabolism; catalytic activity)	**5.3**
SaMRSA252-1266 (1O11)	**SAR1266**	conserved hypothetical protein (thiamin pyrophosphate)	**5.3**
SaMRSA252-0525 (3C5)	**SAR0525**	putative DNA-binding protein (family of firmicute transcriptional repressor of classIII stress genes; CtsR)	**5.2**
SaMRSA252-2372 (7D3)	**ureA**	urease gamma subunit (nitrogen compound metabolic process)	**5.1**
SaMRSA252-2181 (6A16)	**thiM**	putative hydroxyethylthiazole kinase (thiamin biosynthetic process)	**4.9**
SaMRSA252-1811 (5G18)	**acsA**	acetyl-coenzyme A synthetase (metabolism; catalytic activity)	**4.7**
SaMRSA252-1153 (4F9)	**SAR1153**	conserved hypothetical protein	**4.7**
SaMRSA252-2182 (6B16)	**thiD**	putative phosphomethylpyrimidine kinase (role in thiamin pyrophosphate phosphate pathway)	**4.7**
SaMRSA252-2290 (6I17)	**SAR2290**	aldo/keto reductase family protein (oxidoreductase activity)	**4.6**
SaMRSA252-2408 (7G7)	**SAR2408**	PTS system, arbutin-like IIBC component (transport)	**4.6**
SaMRSA252-1415 (4A18)	**SAR1415**	hypothetical protein	**4.6**
SaMRSA252-0653 (3P8)	**SAR0653**	ABC transporter ATP-binding protein (transport)	**4.5**
SaMRSA252-1312 (4I5)	**SAR1312**	hypothetical protein	**4.5**
SaMRSA252-2224 (6D21)	**pyn**	putative pyrimidine-nucleoside phosphorylase (metabolism; transferase activity)	**4.4**
SaMRSA252-1879 (5J15)	**SAR1879**	putative lipoprotein	**4.4**
SaMRSA252-0154 (2M7)	**capD**	capsular polysaccharide synthesis enzyme	**4.4**
SaMRSA252-1880 (5K15)	**SAR1880**	putative membrane protein	**4.3**

SaMRSA252-1871 (5K14)	**pckA**	phosphoenolpyruvate carboxykinase (gluconeogenesis)	**4.2**
SaMRSA252-0662 (3I10)	**SAR0662**	conserved hypothetical protein (carbohydrate transport)	**4.2**
SaMRSA252-2771 (8B4)	**SAR2771**	conserved hypothetical protein	**4.1**
SaMRSA252-0985 (3J13)	**SAR0985**	conserved hypothetical protein (RNA metabolic process)	**4.1**
SaMRSA252-2279 (6N15)	**SAR2279**	putative exported protein	**4.0**
SaMRSA252-2256 (6H24)	**SAR2256**	conserved hypothetical protein	**4.0**
SaMRSA252-2454 (7L1)	**mqo1**	putative malate:quinone oxidoreductase 1 (carbohydrate metabolism)	**4.0**
SaMRSA252-2734 (7N23)	**sasA**	putative serine rich repeat containing protein (cell surface protein)	**4.0**
SaMRSA252-1288 (4L2)	**SAR1288**	putative lipoprotein	**4.0**
SaMRSA252-1190 (1K2)	**SAR1190**	putative membrane protein	**3.9**
SaMRSA252-2378 (7B4)	**ureD**	urease accessory protein UreD (nitrogen compound metabolic process)	**3.9**
SaMRSA252-0223 (2C16)	**fadA**	putative thiolase (fatty acid metabolism)	**3.9**
SaMRSA252-0399 (2N13)	**ahpC**	alkyl hydroperoxide reductase subunit C (possible role in antioxidant activity)	**3.9**
SaMRSA252-1610 (5C6)	**SAR1610**	lipoate-protein ligase A protein (protein modification; catalytic activity)	**3.9**
SaMRSA252-1573 (5F1)	**xerD**	integrase/recombinase (DNA integration/DNA recombination)	**3.8**
SaMRSA252-1585 (5B3)	**malR**	maltose operon transcriptional repressor (regulation of transcription)	**3.8**
SaMRSA252-2637 (7H23)	**copA**	putative copper importing ATPase A (metabolism; metal ion transport)	**3.8**
SaMRSA252-0938 (3D19)	**clpB**	putative ATPase subunit of an ATP-dependent protease (protein metabolic process)	**3.8**
SaMRSA252-1574 (5G1)	**fur**	iron uptake regulatory protein (regulation of transcription)	**3.7**
SaMRSA252-2583 (7C17)	**gntK**	putative gluconokinase (carbohydrate metabolism)	**3.7**
SaMRSA252-0379 (1C23)	**SAR0379**	hypothetical protein	**3.7**

SaMRSA252-1684 (5J3)	**SAR1684**	conserved hypothetical protein	**3.7**
SaMRSA252-1362 (4M11)	**citB**	aconitate hydratase (carbohydrate metabolism)	**3.7**
SaMRSA252-0226 (2F16)	**fadE**	putative acyl-CoA synthetase (possible role in fatty acid metabolism)	**3.7**
SaMRSA252-0707 (1K15)	**SAR0707**	putative replication-associated protein (pseudogene)	**3.7**
SaMRSA252-2373 (7E3)	**ureB**	urease beta subunit (nitrogen compound metabolic process)	**3.7**
SaMRSA252-1493 (4O15)	**SAR1493**	putative membrane protein	**3.6**
SaMRSA252-1423 (4A19)	**SAR1423**	hypothetical protein	**3.6**
SaMRSA252-0969 (3B23)	**SAR0969**	conserved hypothetical protein	**3.6**
SaMRSA252-2568 (7D15)	**SAR2568**	hypothetical protein	**3.6**
SaMRSA252-0335 (1H17)	**SAR0335**	putative luciferase-like monooxygenase	**3.6**
SaMRSA252-0855 (2B9)	**SAR0855**	hypothetical protein	**3.6**
SaMRSA252-2374 (7F3)	**ureC**	urease alpha subunit (urea metabolism)	**3.6**
SaMRSA252-2359 (7G1)	**moaB**	putative molybdenum cofactor biosynthesis protein B (cofactor biosynthesis)	**3.6**
SaMRSA252-2376 (7H3)	**ureF**	urease accessory protein UreF (nitrogen compound metabolism)	**3.6**
SaMRSA252-0155 (2N7)	**capE**	capsular polysaccharide synthesis enzyme	**3.6**
SaMRSA252-1326 (4I7)	**SAR1326**	conserved hypothetical protein (amino acid metabolism)	**3.6**
SaMRSA252-1686 (5L3)	**SAR1686**	putative biotin carboxyl carrier protein of acetyl-CoA carboxylase (biotin-requiring enzyme)	**3.5**
SaMRSA252-1645 (5F10)	**cdd**	cytidine deaminase (hydrolase activity; zinc ion binding)	**3.5**
SaMRSA252-2125 (6J9)	**agrC**	autoinducer sensor protein	**3.5**
SaMRSA252-0279 (2B23)	**esxA**	conserved hypothetical protein (possible role in virulence)	**3.5**
SaMRSA252-2291 (6J17)	**SAR2291**	MerR family regulatory protein (regulation of transcription)	**3.5**
SaMRSA252-2469 (7K3)	**SAR2469**	conserved hypothetical protein (flavin mononucleotide binding)	**3.5**

SaMRSA252-2545 (7M12)	**SAR2545**	conserved hypothetical protein (enzymatic function)	**3.5**
SaMRSA252-0152 (2K7)	**capB**	capsular polysaccharide synthesis enzyme	**3.5**
SaMRSA252-1683 (5I3)	**SAR1683**	putative membrane protein	**3.4**
SaMRSA252-0168 (2K9)	**SAR0168**	putative membrane protein	**3.4**
SaMRSA252-2380 (7D4)	**SAR2380**	hypothetical protein	**3.4**
SaMRSA252-2582 (7B17)	**gntP**	putative gluconate permease (transport)	**3.4**
SaMRSA252-0528 (3F5)	**clpC**	putative stress response-related Clp ATPase (nucleotide-excision repair; protein metabolic process)	**3.4**
SaMRSA252-1864 (5L13)	**SAR1864**	putative transaldolase (carbohydrate metabolism)	**3.3**
SaMRSA252-2517 (7I9)	**SAR2517**	putative dethiobiotin synthetase	**3.3**
SaMRSA252-2389 (7E5)	**SAR2389**	putative D-isomer specific 2-hydroxyacid dehydrogenase (L-serine biosynthetic process)	**3.3**
SaMRSA252-0667 (3N10)	**SAR0667**	putative acetyltransferase	**3.3**
SaMRSA252-0622 (3J5)	**SAR0622**	putative exported protein	**3.3**
SaMRSA252-0584 (3E12)	**SAR0584**	hypothetical protein	**3.3**
SaMRSA252-1417 (4C18)	**SAR1417**	putative membrane protein	**3.3**
SaMRSA252-0031 (1F4)	**pre/mob**	plasmid recombination enzyme	**3.3**
SaMRSA252-2688 (7I18)	**SAR2688**	hypothetical protein (antibiotic biosynthesis process)	**3.3**
SaMRSA252-2396 (7D6)	**SAR2396**	DeoR family regulatory protein (regulation of transcription, DNA-dependent)	**3.2**
SaMRSA252-0824 (2D5)	**SAR0824**	putative malolactic enzyme (NAD binding; oxidoreductase activity)	**3.2**
SaMRSA252-0917 (3G16)	**SAR0917**	putative S1 RNA binding domain (RNA binding)	**3.2**
SaMRSA252-1788 (5A16)	**SAR1788**	putative universal stress protein (response to stress)	**3.2**
SaMRSA252-2223 (6C21)	**SAR2223**	putative membrane protein	**3.2**
SaMRSA252-2375	**ureE**	urease accessory protein UreE (protein complex assembly; urea metabolism)	**3.2**

(7G3)			
SaMRSA252-2364 (7D2)	**SAR2364**	FdhD/NarQ family protein (electron transport)	**3.1**
SaMRSA252-0565 (3B10)	**SAR0565**	conserved hypothetical protein (electron transport)	**3.1**
SaMRSA252-0970 (3C23)	**SAR0970**	protozoan/cyanobacterial globin family protein (oxygen transport)	**3.1**
SaMRSA252-1964 (6E1)	**SAR1964**	putative transglycosylase (peptidoglycan biosynthetic process)	**3.1**
SaMRSA252-0527 (3E5)	**SAR0527**	putative phosphotransferase (enzymatic)	**3.1**
SaMRSA252-0944 (3B20)	**SAR0944**	putative exported protein	**3.0**
SaMRSA252-2360 (7H1)	**moeB**	putative molybdopterin synthase sulfurylase (catalytic activity)	**3.0**
SaMRSA252-2371 (7C3)	**SAR2371**	putative membrane protein (urea transport)	**3.0**
SaMRSA252-2377 (7A4)	**ureG**	urease accessory protein UreG	**3.0**
SaMRSA252-0299 (1E13)	**SAR0299**	hypothetical protein	**3.0**
SaMRSA252-0965 (3F22)	**SAR0965**	conserved hypothetical protein (electron transport; oxidoreductase activity)	**2.9**
SaMRSA252-1739 (5P9)	**tnpA2**	transposase A 2 (DNA integration; DNA recombination; DNA binding)	**2.9**
SaMRSA252-1632 (5A9)	**mreB**	ABC transporter permease protein (transport)	**2.9**
SaMRSA252-2356 (7D1)	**mobB**	putative molybdopterin-guanine dinucleotide biosynthesis protein B (Mo-molybdopterin cofactor biosynthesis)	**2.9**
SaMRSA252-0839 (2C7)	**SAR0839**	putative lipoprotein	**2.9**
SaMRSA252-1685 (5K3)	**SAR1685**	putative biotin carboxylase subunit of acetyl-CoA carboxylase (metabolism; purine base biosynthetic process)	**2.9**
SaMRSA252-1582 (5G2)	**zwf**	putative glucose-6-phosphate 1-dehydrogenase (glucose metabolism)	**2.9**
SaMRSA252-2615 (7B21)	**SAR2615**	putative exported protein	**2.9**
SaMRSA252-0523 (3A5)	**SAR0523**	SNO glutamine amidotransferase family protein (possible role in pyridoxine biosynthesis)	**2.9**
SaMRSA252-0301 (1F13)	**SAR0301**	putative membrane protein	**2.9**

SaMRSA252-2547 (7O12)	**SAR2547**	putative membrane protein (glutamate biosynthesis)	**2.9**
SaMRSA252-1814 (5B19)	**ccpA**	catabolite control protein A (regulation of transcription, DNA-dependent)	**2.9**
SaMRSA252-2684 (7M17)	**fda**	fructose-bisphosphate aldolase class I (glycolysis)	**2.9**
SaMRSA252-1703 (5L5)	**SAR1703**	putative oxygenase	**2.9**
SaMRSA252-2263 (6N13)	**SAR2263**	putative membrane protein	**2.9**
SaMRSA252-1767 (5D13)	**coaE**	putative dephospho-CoA kinase (ATP binding)	**2.9**
SaMRSA252-1418 (4D18)	**SAR1418**	conserved hypothetical protein	**2.9**
SaMRSA252-2293 (6L17)	**SAR2293**	peptidase family M23/M37 protein (proteolysis)	**2.8**
SaMRSA252-2520 (7L9)	**SAR2520**	hypothetical protein	**2.8**
SaMRSA252-1643 (5D10)	**SAR1643**	putative recombination protein O (DNA recombination; DNA repair)	**2.8**
SaMRSA252-2653 (7N13)	**SAR2653**	hypothetical protein	**2.8**
SaMRSA252-0336 (1A18)	**SAR0336**	NADH-dependent FMN reductase (pseudogene)	**2.8**
SaMRSA252-1150 (4C9)	**SAR1150**	antibacterial protein (pathogenesis)	**2.8**
SaMRSA252-1079 (3N24)	**SAR1079**	putative manganese transport protein (transport)	**2.8**
SaMRSA252-0419 (2I16)	**SAR0419**	hypothetical protein	**2.8**
SaMRSA252-0818 (2G4)	**trxB**	putative thioredoxin reductase (electron transport)	**2.8**
SaMRSA252-2393 (7A6)	**SAR2393**	putative bifunctional protein (electron transport)	**2.8**
SaMRSA252-0385 (1A24)	**SAR0385**	putative membrane protein	**2.8**
SaMRSA252-2365 (7E2)	**SAR2365**	acetyltransferase (GNAT) family protein	**2.8**
SaMRSA252-2210 (6F19)	**SAR2210**	aldehyde dehydrogenase family protein (metabolism; oxidoreductase activity)	**2.8**
SaMRSA252-1858	**SAR1858**	hypothetical protein	**2.8**

(5F24)			
SaMRSA252-2460 (7J2)	**SAR2460**	putative acetyltransferase (GNAT) family protein	**2.8**
SaMRSA252-2611 (7F20)	**SAR2611**	putative L-serine dehydratase, beta chain (gluconeogenesis)	**2.8**
SaMRSA252-2746 (8B1)	**icaR**	ica operon transcriptional regulator (regulation of transcription, DNA-dependent)	**2.7**
SaMRSA252-1055 (3O21)	**SAR1055**	hypothetical protein	**2.7**
SaMRSA252-2467 (7I3)	**SAR2467**	conserved hypothetical protein	**2.7**
SaMRSA252-0624 (3L5)	**SAR0624**	putative esterase	**2.7**
SaMRSA252-2121 (6N8)	**SAR2121**	putative carbon-nitrogen hydrolase (nitrogen compound metabolism)	**2.7**
SaMRSA252-2357 (7E1)	**moeA**	putative molybdenum cofactor biosynthesis protein (cofactor biosynthesis)	**2.7**
SaMRSA252-1953 (5J24)	**SAR1953**	AhpC/TSA family protein	**2.7**
SaMRSA252-1151 (4D9)	**SAR1151**	putative haloacid dehalogenase-like hydrolase (metabolism; catalytic activity)	**2.7**
SaMRSA252-1333 (4P7)	**SAR1333**	putative membrane protein	**2.7**
SaMRSA252-1772 (5A14)	**phoP**	alkaline phosphatase synthesis transcriptional regulatory protein (regulation of transcription, DNA-dependent)	**2.7**
SaMRSA252-0495 (3E1)	**veg**	conserved hypothetical protein	**2.7**
SaMRSA252-0555 (3H8)	**kbl**	putative 2-amino-3-ketobutyrate coenzyme A ligase (biosynthesis)	**2.7**
SaMRSA252-0040 (1G5)	**mecR1**	methicillin resistance protein MecR1(cell wall biosynthesis)	**2.7**
SaMRSA252-1279 (4L1)	**SAR1279**	conserved hypothetical protein (mRNA processing)	**2.6**
SaMRSA252-2731 (7L23)	**SAR2731**	conserved hypothetical protein (pseudogene)	**2.6**
SaMRSA252-0663 (3J10)	**SAR0663**	hypothetical protein	**2.6**
SaMRSA252-0721 (1N16)	**SAR0721**	multicopper oxidase protein (copper ion binding; oxidoreductase activity)	**2.6**
SaMRSA252-2610 (7E20)	**SAR2610**	putative L-serine dehydratase, alpha chain (gluconeogenesis)	**2.6**
SaMRSA252-1081 (3P24)	**SAR1081**	inositol monophosphatase family protein (inositol or phosphatidylinositol phosphte activity)	**2.6**
SaMRSA252-agrIII	**agrIII**	Class III accessory gene regulator (agr) locus [3'agrB-agrD-5'agrC]	**2.6**

(8E7)			
SaMRSA252-1251 (1P9)	**SAR1251**	conserved hypothetical protein	**2.6**
SaMRSA252-1914 (5L19)	**SAR1914**	hypothetical protein (pseudogene)	**2.6**
SaMRSA252-0499 (3A2)	**spoVG**	stage V sporulation protein G (response to stress)	**2.6**
SaMRSA252-0711 (1M15)	**SAR0711**	putative replication initiation protein (pseudogene)	**2.6**
SaMRSA252-0498 (3H1)	**yabJ**	putative regulatory protein (possible role in purine biosynthesis)	**2.6**
SaMRSA252-0637 (3I7)	**SAR0637**	sodium/hydrogen exchanger family protein (regulation of pH)	**2.5**
SaMRSA252-0966 (3G22)	**SAR0966**	regulatory protein	**2.5**
SaMRSA252-rnaIII (8F7)	**rnaIII**	rnaIII accessory gene regulator (agr) locus	**2.5**
SaMRSA252-0054 (1D7)	**tnpA1**	transposase A 1 (DNA integration; DNA recombination; DNA binding)	**2.5**
SaMRSA252-2420 (7B9)	**SAR2420**	arginase family protein (urea cycle)	**2.5**
SaMRSA252-2366 (7F2)	**SAR2366**	BioY family protein	**2.5**
SaMRSA252-1197 (1J3)	**SAR1197**	conserved hypothetical protein	**2.5**
SaMRSA252-1791 (5D16)	**SAR1791**	putative thiol peroxidase	**2.5**
SaMRSA252-1633 (5B9)	**mreA**	ABC transporter ATP-binding protein (transport)	**2.5**
SaMRSA252-2715 (7L21)	**SAR2715**	arginine repressor family protein (regulation of transcription, DNA-dependent)	**2.5**
SaMRSA252-1954 (5K24)	**SAR1954**	putative glutamate-1-semialdehyde 2,1-aminomutase (pyridoxal phosphate binding)	**2.5**
SaMRSA252-0796 (2A2)	**SAR0796**	putative glycerate kinase (organic acid phosphorylation)	**2.4**
SaMRSA252-1435 (4E20)	**SAR1435**	PTS system, glucose-specific IIA component (transport)	**2.4**
SaMRSA252-0901 (3G14)	**SAR0901**	conserved hypothetical protein	**2.4**
SaMRSA252-1688 (5N3)	**SAR1688**	conserved hypothetical protein	**2.4**
SaMRSA252-2354	**moaD**	putative molybdopterin-synthase small subunit (sulfur metabolism)	**2.4**

(7B1)			
SaMRSA252-1416 (4B18)	**SAR1416**	putative acylphosphatase (acylphosphatase activity)	**2.4**
SaMRSA252-1784 (5E15)	**SAR1784**	putative universal stress protein (response to stress)	**2.4**
SaMRSA252-1769 (5F13)	**polA**	DNA polymerase I (DNA replication)	**2.4**
SaMRSA252-1054 (3N21)	**SAR1054**	conserved hypothetical protein	**2.4**
SaMRSA252-1891 (5M16)	**SAR1891**	putative exported protein (pseudogene)	**2.4**
SaMRSA252-2514 (7N8)	**SAR2514**	putative 8-amino-7-oxononanoate synthase (biosynthesis; metabolism)	**2.4**
SaMRSA252-1336 (4K8)	**SAR1336**	hypothetical protein	**2.4**
SaMRSA252-2612 (7G20)	**SAR2612**	putative membrane protein	**2.4**
SaMRSA252-2277 (6L15)	**SAR2277**	putative zinc-binding dehydrogenase	**2.4**
SaMRSA252-1085 (4D1)	**SAR1085**	conserved hypothetical protein	**2.4**
SaMRSA252-2522 (7N9)	**SAR2522**	putative glycerate kinase (organic acid phosphorylation)	**2.4**
SaMRSA252-2337 (6I23)	**SAR2337**	putative membrane protein	**2.4**
SaMRSA252-1615 (5H6)	**SAR1615**	putative shikimate kinase (amino acid biosynthesis)	**2.4**
SaMRSA252-2353 (7A1)	**mobA**	putative molybdopterin-guanine dinucleotide biosynthesis protein A	**2.4**
SaMRSA252-1459 (4E23)	**SAR1459**	conserved hypothetical protein	**2.3**
SaMRSA252-0918 (3H16)	**SAR0918**	NADH:flavin oxidoreductase / NADH oxidase family protein (electron transport; oxidoreductase activity)	**2.3**
SaMRSA252-1644 (5E10)	**era**	putative GTP-binding protein (nucleic acid binding)	**2.3**
SaMRSA252-0813 (2B4)	**uvrA**	excinuclease ABC subunit A (ATP binding/ATPase activity)	**2.3**
SaMRSA252-0862	**SAR0862**	putative thioredoxin (electron transport)	**2.3**

(2A10)			
SaMRSA252-0280 (2C23)	**SAR0280**	putative membrane protein	**2.3**
SaMRSA252-2127 (6L9)	**SAR2127**	putative fructokinase (carbohydrate metabolism)	**2.3**
SaMRSA252-1436 (4F20)	**SAR1436**	conserved hypothetical protein	**2.3**
SaMRSA252-2730 (7K23)	**SAR2730**	conserved hypothetical protein	**2.3**
SaMRSA252-1360 (4K11)	**mscL**	large-conductance mechanosensitive channel (transport)	**2.3**
SaMRSA252-1611 (5D6)	**SAR1611**	putative membrane protein	**2.3**
SaMRSA252-2392 (7H5)	**SAR2392**	conserved hypothetical protein	**2.3**
SaMRSA252-0807 (2D3)	**secA**	preprotein translocase SecA subunit (protein import)	**2.3**
SaMRSA252-0921 (3C17)	**glpQ**	putative glycerophosphoryl diester phosphodiesterase (glycerol metabolism)	**2.3**
SaMRSA252-1706 (5O5)	**SAR1706**	conserved hypothetical protein (transcriptional regulator)	**2.3**
SaMRSA252-0706 (1J15)	**SAR0706**	putative membrane protein	**2.3**
SaMRSA252-0682 (3L12)	**SAR0682**	hypothetical protein	**2.3**
SaMRSA252-1987 (6C4)	**SAR1987**	putative membrane protein	**2.3**
SaMRSA252-1391 (4A15)	**SAR1391**	hypothetical protein	**2.3**
SaMRSA252-2729 (7J23)	**SAR2729**	hypothetical protein	**2.3**
SaMRSA252-0167 (2J9)	**SAR0167**	conserved hypothetical protein (antibiotic biosynthetic process)	**2.3**
SaMRSA252-1589 (5F3)	**gnd**	6-phosphogluconate dehydrogenase, decarboxylating (pentose phosphate shunt)	**2.2**
SaMRSA252-0569 (3G10)	**SAR0569**	putative glycosyl transferase (biosynthetic process)	**2.2**
SaMRSA252-1363 (4N11)	**SAR1363**	conserved hypothetical protein (catalytic activity)	**2.2**
SaMRSA252-2428 (7B10)	**SAR2428**	putative membrane protein	**2.2**

SaMRSA252-0724 (1I17)	**cadC**	putative cadmium efflux system accessory protein (regulation of transcription, DNA-dependent)	**2.2**
SaMRSA252-1018 (3J17)	**SAR1018**	putative hydrolase	**2.2**
SaMRSA252-0600 (3M2)	**SAR0600**	pyridine nucleotide-disulphide oxidoreductase protein (electron transport)	**2.2**
SaMRSA252-1647 (5H10)	**SAR1647**	conserved hypothetical protein	**2.2**
SaMRSA252-2395 (7C6)	**SAR2395**	inositol monophosphatase family protein (inositol or phosphatidylinositol phosphte activity)	**2.2**
SaMRSA252-0854 (2A9)	**SAR0854**	hypothetical protein	**2.2**
SaMRSA252-1437 (4G20)	**msrA2**	peptide methionine sulfoxide reductase II (protein metabolic process)	**2.2**
SaMRSA252-0581 (3B12)	**SAR0581**	putative ketoacyl-CoA thiolase	**2.2**
SaMRSA252-2641 (7C24)	**SAR2641**	putative aminotransferase (biosynthesis)	**2.2**
SaMRSA252-2687 (7P17)	**SAR2687**	putative AMP-binding enzyme (metabolism)	**2.2**
SaMRSA252-2399 (7G6)	**SAR2399**	putative transcription regulator (carbohydrate metabolism; regulation of transcription, DNA-dependent)	**2.2**
SaMRSA252-1198 (1K3)	**SAR1198**	putative ribulose-phosphate 3-epimerase (carbohydrate metabolism)	**2.2**
SaMRSA252-1641 (5B10)	**SAR1641**	putative DNA-binding protein [pseudogene]; (regulation of transcription, DNA-dependent)	**2.2**
SaMRSA252-2358 (7F1)	**moaC**	putative molybdenum cofactor biosynthesis protein C	**2.1**
SaMRSA252-0445 (2K19)	**SAR0445**	putative lipoprotein	**2.1**
SaMRSA252-0812 (2A4)	**uvrB**	excinuclease ABC subunit B (nucleotide-excision repair)	**2.1**
SaMRSA252-1057 (3I22)	**ptsI**	phosphoenolpyruvate-protein phosphotransferase (carbohydrate metabolism)	**2.1**
SaMRSA252-0151 (2J7)	**capA**	capsular polysaccharide synthesis enzyme	**2.1**
SaMRSA252-2225 (6E21)	**deoC2**	putative deoxyribose-phosphate aldolase (nucleoside metabolism)	**2.1**
SaMRSA252-0147 (2N6)	**SAR0147**	putative nucleotidase (nucleotide catabolism)	**2.1**

SaMRSA252-2515 (7O8)	**bioB**	putative biotin synthase (biotin biosynthesis)	**2.1**
SaMRSA252-1086 (4E1)	**SAR1086**	conserved hypothetical protein	**2.1**
SaMRSA252-1203 (1P3)	**recG**	ATP-dependent DNA helicase (ATP binding/helicase activity/nucleic acid binding)	**2.1**
SaMRSA252-2274 (6I15)	**SAR2274**	putative membrane protein	**2.1**
SaMRSA252-2780 (8C5)	**SAR2780**	putative membrane protein	**2.1**
SaMRSA252-2728 (7I23)	**SAR2728**	preprotein translocase SecA subunit-like protein (protein binding)	**2.1**
SaMRSA252-1877 (5P14)	**SAR1877**	AMP-binding enzyme (metabolism)	**2.1**
SaMRSA252-1884 (5O15)	**SAR1884**	hypothetical protein	**2.1**
SaMRSA252-1847 (5C23)	**rot**	repressor of toxins	**2.1**
SaMRSA252-1591 (5H3)	**SAR1591**	putative membrane protein	**2.1**
SaMRSA252-0802 (2G2)	**SAR0802**	putative membrane protein	**2.1**
SaMRSA252-1379 (4F13)	**SAR1379**	putative peptidase	**2.1**
SaMRSA252-2451 (7I1)	**SAR2451**	putative membrane protein (regulation of transcription-DNA-dependent)	**2.1**
SaMRSA252-2516 (7P8)	**bioA**	putative adenosylmethionine-8-amino-7-oxononanoate aminotransferase (pyridoxal phosphate binding)	**2.1**
SaMRSA252-0381 (1E23)	**SAR0381**	hypothetical protein	**2.1**
SaMRSA252-1053 (3M21)	**SAR1053**	putative membrane protein	**2.1**
SaMRSA252-1260 (1I11)	**SAR1260**	conserved hypothetical protein (Mo-molybdopterin cofactor biosynthesis)	**2.1**
SaMRSA252-1350 (4I10)	**SAR1350**	putative membrane protein	**2.1**
SaMRSA252-0305 (1B14)	**SAR0305**	putative membrane protein	**2.1**
SaMRSA252-2546 (7N12)	**SAR2546**	putative lipoprotein	**2.1**
SaMRSA252-0559 (3D9)	**SAR0559**	putative aminotransferase (metabolism)	**2.1**

SaMRSA252-1785 (5F15)	**SAR1785**	metallo-beta-lactamase superfamily protein	**2.1**
SaMRSA252-0380 (1D23)	**SAR0380**	hypothetical protein	**2.0**
SaMRSA252-2413 (7D8)	**SAR2413**	putative short chain dehydrogenase	**2.0**
SaMRSA252-2655 (7P13)	**SAR2655**	putative glyoxalase	**2.0**
SaMRSA252-1183 (1L1)	**SAR1183**	conserved hypothetical protein	**2.0**
SaMRSA252-2379 (7C4)	**sarR**	staphylococcal accessory regulator A homologue	**2.0**
SaMRSA252-0580 (3A12)	**SAR0580**	putative AMP-binding enzyme (metabolism)	**2.0**
SaMRSA252-0020 (1D3)	**yycH**	putative exported protein	**2.0**
SaMRSA252-1985 (6A4)	**SAR1985**	putative exonuclease	**2.0**
SaMRSA252-2532 (7P10)	**SAR2532**	hypothetical protein	**2.0**
SaMRSA252-0448 (2N19)	**SAR0448**	hypothetical protein	**2.0**
SaMRSA252-2369 (7A3)	**SAR2369**	conserved hypothetical protein (electron transport)	**2.0**
SaMRSA252-0735 (1L18)	**SAR0735**	putative exported protein	**2.0**
SaMRSA252-1782 (5C15)	**SAR1782**	conserved hypothetical protein	**2.0**
SaMRSA252-1813 (5A19)	**SAR1813**	histone deacetylase family protein	**2.0**
SaMRSA252-2640 (7B24)	**SAR2640**	D-isomer specific 2-hydroxyacid dehydrogenase (L-serine biosynthesis; metabolism)	**2.0**
SaMRSA252-2658 (7K14)	**SAR2658**	TetR family regulatory protein (regulation of transcription, DNA-dependent)	**2.0**
SaMRSA252-0869 (2H10)	**SAR0869**	hypothetical protein	**2.0**
SaMRSA252-0876 (2F11)	**SAR0876**	ABC transporter ATP-binding protein- (ATP binding/ATPase activity/nucleoside-triphosphatase activity/ nucleotide binding)	**2.0**
SaMRSA252-1766	**gap2**	glyceraldehyde 3-phosphate dehydrogenase 2 (glycolysis)	**2.0**

(5C13)			
SaMRSA252-1816 (5D19)	**SAR1816**	putative membrane protein	**2.0**
SaMRSA252-0986 (3K13)	**SAR0986**	putative membrane protein	**2.0**
SaMRSA252-1707 (5P5)	**SAR1707**	putative ATPase (ATP binding; nucleoside-triphosphatase activity; nucleotide binding)	**1.9**
SaMRSA252-0987 (3L13)	**SAR0987**	conserved hypothetical protein (biosynthetic process)	**1.9**
SaMRSA252-1261 (1J11)	**recA**	recombinase A (DNA metabolic process)	**1.9**
SaMRSA252-1422 (4H18)	**SAR1422**	conserved hypothetical protein	**1.9**
SaMRSA252-0128 (2K4)	**SAR0128**	putative membrane protein	**1.9**
SaMRSA252-0821 (2A5)	**SAR0821**	conserved hypothetical protein	**1.9**
SaMRSA252-0757 (1J21)	**SAR0757**	putative glucosyl transferase	**1.9**
SaMRSA252-1965 (6F1)	**SAR1965**	ThiJ/PfpI family protein	**1.9**
SaMRSA252-2294 (6M17)	**SAR2294**	hypothetical protein	**1.9**
SaMRSA252-2275 (6J15)	**SAR2275**	putative membrane protein	**1.9**
SaMRSA252-2394 (7B6)	**SAR2394**	putative exported protein	**1.9**
SaMRSA252-1458 (4D23)	**SAR1458**	conserved hypothetical protein	**1.9**
SaMRSA252-2617 (7D21)	**SAR2617**	hypothetical protein	**1.9**
SaMRSA252-0021 (1E3)	**yycI**	putative exported protein	**1.9**
SaMRSA252-1837 (5A22)	**SAR1837**	putative exported protein	**1.9**
SaMRSA252-0867 (2F10)	**SAR0867**	hypothetical protein (DNA modification)	**1.9**
SaMRSA252-0744 (1M19)	**SAR0744**	putative DNA photolyase	**1.9**
SaMRSA252-0683 (3M12)	**SAR0683**	LysR family regulatory protein (regulation of transcription, DNA-dependent)	**1.9**

SaMRSA252-2352 (6P24)	**moaA**	putative molybdenum cofactor biosynthesis protein A	**1.9**
SaMRSA252-2128 (6M9)	**scrB**	sucrose-6-phosphate hydrolase (carbohydrate metabolism)	**1.9**
SaMRSA252-2627 (7F22)	**SAR2627**	putative 6-O-methylguanine DNA methyltransferase (DNA repair)	**1.9**
SaMRSA252-1048 (3P20)	**purD**	putative phosphoribosylamine--glycine ligase (purine base biosynthesis)	**1.9**
SaMRSA252-0743 (1L19)	**SAR0743**	putative sodium:sulfate symporter protein (transport)	**1.8**
SaMRSA252-2262 (6M13)	**SAR2262**	putative UTP--glucose-1-phosphate uridylyltransferase (metabolism)	**1.8**
SaMRSA252-2618 (7E21)	**glcB**	PTS system, glucose-specific IIABC component (sugar transport)	**1.8**
SaMRSA252-1876 (5O14)	**SAR1876**	hypothetical protein	**1.8**
SaMRSA252-1696 (5N4)	**SAR1696**	conserved hypothetical protein	**1.8**
SaMRSA252-0048 (1F6)	**SAR0048**	putative membrane protein (partial)	**1.8**
SaMRSA252-1017 (3I17)	**SAR1017**	putative menaquinone biosynthesis bifunctional protein	**1.8**
SaMRSA252-2749 (8E1)	**icaB**	intercellular adhesion protein B (carbohydrate metabolism)	**1.8**
SaMRSA252-0583 (3D12)	**SAR0583**	hypothetical protein	**1.8**
SaMRSA252-1651 (5D11)	**SAR1651**	putative membrane protein	**1.8**
SaMRSA252-0669 (3P10)	**SAR0669**	putative response regulator protein (regulation of transcription, DNA-dependent)	**1.8**
SaMRSA252-0024 (1H3)	**SAR0024**	conserved hypothetical protein	**1.8**
SaMRSA252-2126 (6K9)	**agrA**	autoinducer sensor protein response regulator protein	**1.8**
SaMRSA252-0668 (3O10)	**SAR0668**	hypothetical protein	**1.8**
SaMRSA252-1640 (5A10)	**SAR1640**	conserved hypothetical protein	**1.8**
SaMRSA252-1593 (5B4)	**bfmB**	lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex (metabolism)	**1.8**

SaMRSA252-2724 (7M22)	**SAR2724**	isochorismatase family protein (metabolism)	**1.7**
SaMRSA252-2656 (7I14)	**SAR2656**	conserved hypothetical protein (regulation of nitrogen utilisation)	**1.7**
SaMRSA252-2589 (7A18)	**SAR2589**	putative transporter protein	**1.7**
SaMRSA252-1783 (5D15)	**SAR1783**	putative DNA-binding protein	**1.7**
SaMRSA252-1890 (5L16)	**SAR1890**	putative exported protein	**1.7**
SaMRSA252-1488 (4J15)	**SAR1488**	putative pyridine nucleotide-disulphide oxidoreductase (electron transport)	**1.7**
SaMRSA252-1084 (4C1)	**SAR1084**	conserved hypothetical protein	**1.7**
SaMRSA252-0820 (2H4)	**SAR0820**	conserved hypothetical protein	**1.7**
SaMRSA252-1571 (5D1)	**SAR1571**	conserved hypothetical protein	**1.7**
SaMRSA252-0560 (3E9)	**SAR0560**	haloacid dehalogenase-like hydrolase (metabolism)	**1.7**
SaMRSA252-1289 (4M2)	**SAR1289**	putative exported protein	**1.7**
SaMRSA252-1171 (4H11)	**SAR1171**	glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein	**1.7**
SaMRSA252-0929 (3C18)	**SAR0929**	conserved hypothetical protein (DNA repair)	**1.7**
SaMRSA252-2727 (7P22)	**SAR2727**	hypothetical protein (biosynthesis)	**1.7**
SaMRSA252-1583 (5H2)	**SAR1583**	AraC family regulatory protein (regulation of transcription, DNA-dependent)	**1.7**
SaMRSA252-0756 (1I21)	**SAR0756**	aldo/keto reductase family protein (oxidoreductase activity)	**1.7**
SaMRSA252-1371 (4N12)	**SAR1371**	putative membrane protein	**1.7**
SaMRSA252-2657 (7J14)	**SAR2657**	hypothetical protein	**1.6**
SaMRSA252-0928 (3B18)	**SAR0928**	conserved hypothetical protein	**1.6**
SaMRSA252-0930	**SAR0930**	fumarylacetoacetate (FAA) hydrolase family protein (metabolism)	**1.6**

(3D18)			
SaMRSA252-2661 (7N14)	**SAR2661**	putative hydrolase	**1.6**
SaMRSA252-0994 (3K14)	**SAR0994**	putative 5'-nucleotidase (nucleotide catabolism)	**1.6**
SaMRSA252-2747 (8C1)	**icaA**	glucosaminyltransferase (transferase activity)	**1.6**
SaMRSA252-1569 (5B1)	**rluB**	ribosomal large subunit pseudouridine synthase B (RNA processing)	**1.6**
SaMRSA252-0092 (1H11)	**SAR0092**	putative hydratase (metabolism)	**1.6**
SaMRSA252-2735 (7O23)	**SAR2735**	conserved hypothetical protein	**1.6**
SaMRSA252-2558 (7B14)	**SAR2558**	conserved hypothetical protein	**1.6**
SaMRSA252-0638 (3J7)	**SAR0638**	putative membrane protein	**1.6**
SaMRSA252-2778 (8A5)	**SAR2778**	putative nickel transport protein (metal ion transport)	**1.6**
SaMRSA252-1441 (4C21)	**SAR1441**	conserved hypothetical protein	**1.6**
SaMRSA252-0582 (3C12)	**SAR0582**	hypothetical protein	**1.6**
SaMRSA252-2299 (6K18)	**SAR2299**	hypothetical protein	**1.6**
SaMRSA252-0191 (2J12)	**SAR0191**	conserved hypothetical protein	**1.5**
SaMRSA252-2434 (7H10)	**SAR2434**	hypothetical protein	**1.5**
SaMRSA252-2660 (7M14)	**SAR2660**	conserved hypothetical protein (metabolism)	**1.5**
SaMRSA252-2659 (7L14)	**SAR2659**	putative short chain dehydrogenase (metabolism; oxidoreductase activity)	**1.5**
SaMRSA252-0508 (3B3)	**SAR0508**	putative cell division protein (cell cycle)	**1.5**
SaMRSA252-0934 (3H18)	**SAR0934**	putative haloacid dehalogenase-like hydrolase (metabolism; catalytic activity)	**1.5**
SaMRSA252-0570 (3H10)	**SAR0570**	conserved hypothetical protein	**1.5**
SaMRSA252-0852	**SAR0852**	putative membrane protein	**1.4**

(2G8)			
SaMRSA252-1392 (4B15)	**SAR1392**	putative oligopeptide transporter ATPase (ATP binding; ATPase activity; nucleoside-triphosphatase activity; nucleotide binding)	**1.4**
SaMRSA252-0382 (1F23)	**SAR0382**	putative terminase small subunit (DNA packaging)	**1.4**
SaMRSA252-1388 (4G14)	**femB**	putative methicillin resistance expression factor (peptidoglycan biosynthetic process)	**1.4**
SaMRSA252-0621 (3I5)	**SAR0621**	putative hydrolase	**1.4**
SaMRSA252-2259 (6J13)	**SAR2259**	putative membrane protein	**1.4**
SaMRSA252-2512 (7L8)	**SAR2512**	putative membrane protein	**1.4**
SaMRSA252-2702 (7O19)	**SAR2702**	sensor kinase protein (ATP binding)	**1.4**
SaMRSA252-2544 (7L12)	**SAR2544**	ABC transporter ATP-binding protein (ATP binding/ATPase activity/nucleoside-triphosphatase activity/ nucleotide binding)	**1.3**

Table 4.4: Down-regulated GASM genes, normalised to BHI, using one-way ANOVA and posthoc Benjamini and Hochberg tests ($p \leq 0.05$). The corresponding ORF (Open reading frame), gene name and protein function are listed. The table is arranged according to the most down-regulated gene with the highest fold difference.

ORF	GENE	PROTEIN (FUNCTION)	FOLD
SaMRSA252-0259 (2F20)	**lrgA**	holin-like protein (export murein hydrolases)	**-9.7**
SaMRSA252-1010 (3J16)	**SAR1010**	putative membrane protein	**-9.2**
SaMRSA252-2383 (7G4)	**SAR2383**	putative exported protein	**-8.4**
SaMRSA252-0212 (2G14)	**SAR0212**	putative membrane protein (amino acid metabolism)	**-8.3**
SaMRSA252-0260 (2G20)	**lrgB**	holin-like protein (export murein hydrolases)	**-8.0**
SaMRSA252-2650 (7K13)	**isaA**	immunodominant antigen A (cellular component; extracellular region)	**-7.5**
SaMRSA252-1454 (4H22)	**SAR1454**	putative membrane protein [pseudogene] (proteolysis)	**-6.7**
SaMRSA252-2488 (7M5)	**nasE**	assimilatory nitrite reductase small subunit (electron transport; oxidoreductase activity)	**-6.3**
SaMRSA252-0114 (2M2)	**spa**	immunoglobulin G binding protein A precursor (cell wall catabolism; immunoglobulin binding)	**-6.2**
SaMRSA252-0172 (2O9)	**SAR0172**	conserved hypothetical protein	**-6.0**
SaMRSA252-2669 (7N15)	**SAR2669**	putative dihydroorotate dehydrogenase (pyrimidine base biosynthesis)	**-6.0**
SaMRSA252-2131 (6P9)	**SAR2131**	conserved hypothetical protein	**-5.9**
SaMRSA252-1060 (3L22)	**SAR1060**	putative membrane protein	**-5.9**
SaMRSA252-2504 (7L7)	**SAR2504**	extracellular solute-binding lipoprotein (transport)	**-5.9**
SaMRSA252-2329 (6I22)	**rpsC**	30S ribosomal protein S3 (translation)	**-5.5**
SaMRSA252-2487 (7L5)	**SAR2487**	tetrapyrrole (corrin/porphyrin) methylase family protein (metabolism)	**-5.5**
SaMRSA252-2331	**rpsS**	30S ribosomal protein S19 (translation)	**-5.4**

(6K22)			
SaMRSA252-2322 (6J21)	**rpsN**	30S ribosomal protein S14 (translation)	**-5.3**
SaMRSA252-2486 (7K5)	**narG**	nitrate reductase alpha chain (molybdenum ion binding; oxidoreductase activity)	**-5.3**
SaMRSA252-2328 (6P21)	**rplP**	50S ribosomal protein L16 (translation)	**-5.1**
SaMRSA252-1938 (5K22)	**SAR1938**	putative DNA-binding protein (DNA binding)	**-4.9**
SaMRSA252-2319 (6O20)	**rplR**	50S ribosomal protein L18 (translation)	**-4.9**
SaMRSA252-2324 (6L21)	**rplX**	50S ribosomal protein L24 (protein biosynthesis)	**-4.9**
SaMRSA252-1993 (6A5)	**gatC**	glutamyl-tRNA amidotransferase subunit C (regulation of translational fidelity)	**-4.8**
SaMRSA252-2508 (7P7)	**sbi**	IgG-binding protein	**-4.8**
SaMRSA252-2321 (6I21)	**rpsH**	30S ribosomal protein S8 (translation)	**-4.8**
SaMRSA252-2327 (6O21)	**rpmC**	50S ribosomal protein L29 (translation)	**-4.8**
SaMRSA252-2473 (7O3)	**SAR2473**	putative exported protein	**-4.7**
SaMRSA252-1178 (4G12)	**pyrAA**	putative carbamoyl-phosphate synthase, pyrimidine-specific, small chain- (nitrogen compound metabolic process)	**-4.5**
SaMRSA252-2438 (7D11)	**SAR2438**	putative exported protein	**-4.5**
SaMRSA252-2485 (7J5)	**narH**	nitrate reductase beta chain	**-4.4**
SaMRSA252-2489 (7N5)	**nasD; nirB; nasBC**	nitrite reductase large subunit (electron transport; oxidoreductase activity)	**-4.4**
SaMRSA252-2325 (6M21)	**rplN**	50S ribosomal protein L14 (translation)	**-4.3**
SaMRSA252-1179 (4H12)	**pyrAB**	putative carbamoyl-phosphate synthase, pyrimidine-specific, large chain- (metabolism; biosynthesis of arginine, purine base and pyrimidine base)	**-4.2**
SaMRSA252-2334 (6N22)	**rplD**	50S ribosomal protein L4 (translation)	**-4.2**

SaMRSA252-1361 (4L11)	**opuD1**	glycine betaine transporter 1 (transport)	**-4.2**
SaMRSA252-1132 (4C7)	**SAR1132**	hypothetical protein	**-4.1**
SaMRSA252-1450 (4D22)	**tdcB**	putative threonine dehydratase (metabolism; possible role in pyridoxine synthesis)	**-4.0**
SaMRSA252-1759 (5L12)	**rpmI**	50S ribosomal protein L35 (translation)	**-4.0**
SaMRSA252-1174 (4C12)	**pyrR**	putative pyrimidine operon regulatory protein (nucleoside metabolism)	**-4.0**
SaMRSA252-1052 (3L21)	**SAR1052**	hypothetical protein	**-4.0**
SaMRSA252-2472 (7N3)	**gltT**	putative proton/sodium-glutamate symport protein (transport)	**-3.9**
SaMRSA252-2320 (6P20)	**rplF**	50S ribosomal protein L6 (translation)	**-3.9**
SaMRSA252-1237 (1J8)	**SAR1237**	putative phosphatidate cytidylyltransferase (phospholipid biosynthesis)	**-3.9**
SaMRSA252-2333 (6M22)	**rplW**	50S ribosomal protein L23 (protein biosynthesis)	**-3.8**
SaMRSA252-2677 (7N16)	**panB**	putative 3-methyl-2-oxobutanoate hydroxymethyltransferase (pantothenate biosynthesis)	**-3.8**
SaMRSA252-0542 (3C7)	**rplK**	50S ribosomal protein L11(translation)	**-3.8**
SaMRSA252-2335 (6O22)	**rplC**	50S ribosomal protein L3 (translation)	**-3.7**
SaMRSA252-2799 (8C7)	**rnpA**	ribonuclease P protein component (tRNA processing)	**-3.7**
SaMRSA252-1404 (4F16)	**SAR1404**	ABC transporter ATP-binding protein (ATP-binding; ATPase activity; nucleoside triphosphate; nucleotide binding)	**-3.7**
SaMRSA252-1760 (5M12)	**infC**	translation initiation factor IF-3 (translational initiation)	**-3.6**
SaMRSA252-2317 (6M20)	**rpmD**	50S ribosomal protein L30 (translation)	**-3.6**
SaMRSA252-0946 (3D20)	**fabH**	putative 3-oxoacyl-[acyl-carrier-protein] synthase III (fatty acid biosynthesis)	**-3.6**
SaMRSA252-1091 (4B2)	**SAR1091**	putative membrane protein	**-3.6**
SaMRSA252-2709	**clfB**	fibrinogen and keratin-10 binding surface anchored protein (cell surface component)	**-3.6**

(7N20)			
SaMRSA252-0118 (2I3)	**sirA**	lipoprotein (iron ion transport)	**-3.5**
SaMRSA252-2132 (6I10)	**SAR2132**	putative membrane protein	**-3.5**
SaMRSA252-2676 (7M16)	**panC**	putative pantoate--beta-alanine ligase (pantothenate biosynthesis)	**-3.5**
SaMRSA252-2315 (6K20)	**secY**	preprotein translocase SecY subunit (protein secretion)	**-3.4**
SaMRSA252-2207 (6C19)	**SAR2207**	putative thymidine kinase (ATP binding; thymidine kinase activity)	**-3.4**
SaMRSA252-0545 (3F7)	**rplL**	50S ribosomal protein L7/L12 (translation)	**-3.4**
SaMRSA252-0544 (3E7)	**rplJ**	50S ribosomal protein L10 (ribosome biogenesis & assembly)	**-3.4**
SaMRSA252-2476 (7J4)	**narT**	nitrite transport protein	**-3.4**
SaMRSA252-2470 (7L3)	**SAR2470**	putative exported protein	**-3.4**
SaMRSA252-2675 (7L16)	**panD**	putative aspartate 1-decarboxylase precursor (alanine biosynthesis)	**-3.3**
SaMRSA252-0790 (2C1)	**sstD**	lipoprotein (iron ion transport)	**-3.3**
SaMRSA252-1716 (5I7)	**SAR1716**	putative single-stranded-DNA-specific exonuclease (nucleic acid binding)	**-3.3**
SaMRSA252-0406 (2M14)	**xpt**	putative xanthine phosphoribosyltransferase (nucleoside metabolism)	**-3.3**
SaMRSA252-2316 (6L20)	**rplO**	50S ribosomal protein L15 (translation)	**-3.3**
SaMRSA252-0407 (2N14)	**pbuX**	putative xanthine permease (transport)	**-3.3**
SaMRSA252-0110 (2I2)	**SAR0110**	putative Na+/Pi-cotransporter protein (transport)	**-3.2**
SaMRSA252-2484 (7I5)	**narJ**	respiratory nitrate reductase delta chain (electron transport)	**-3.2**
SaMRSA252-2215 (6C20)	**pyrG**	putative CTP synthase (pyrimidine nucleotide biosynthesis)	**-3.2**
SaMRSA252-0258 (2E20)	**lytR**	autolysin response regulator protein (regulation of transcription, DNA-dependent)	**-3.2**
SaMRSA252-1717 (5J7)	**secF**	putative protein-export membrane protein (protein transport)	**-3.2**
SaMRSA252-0173 (2P9)	**SAR0173**	putative ABC transporter ATP-binding protein (ATP binding; ATPase activity; nucleosidetriphosphate activity; nucleotide binding)	**-3.2**
SaMRSA252-2308 (6L19)	**rplQ**	50S ribosomal protein L17 (translation)	**-3.1**
SaMRSA252-1451 (4E22)	**ald2**	alanine dehydrogenase 2 (electron transport)	**-3.1**

SaMRSA252-2323 (6K21)	**rplE**	50S ribosomal protein L5 (translation)	**-3.1**
SaMRSA252-0787 (1P24)	**sstA**	FecCD transport family protein (transport)	**-3.1**
SaMRSA252-2437 (7C11)	**SAR2437**	putative transport protein	**-3.1**
SaMRSA252-0543 (3D7)	**rplA**	50S ribosomal protein L1 (translation)	**-3.1**
SaMRSA252-1715 (5P6)	**apt**	adenine phosphoribosyltransferase (nucleoside metabolism)	**-3.1**
SaMRSA252-1605 (5F5)	**accB; fabE**	biotin carboxyl carrier protein of acetyl-CoA carboxylase	**-3.0**
SaMRSA252-2386 (7B5)	**SAR2386**	putative dehydrogenase (glycerol-3-phosphate catabolism)	**-3.0**
SaMRSA252-1719 (5L7)	**tgt**	queuine tRNA-ribosyltransferase (queuosine biosynthesis; tRNA modification)	**-3.0**
SaMRSA252-1059 (3K22)	**SAR1059**	putative cytochrome ubiquinol oxidase (electron transport; oxidoreductase activity)	**-3.0**
SaMRSA252-1762 (5O12)	**thrS**	threonyl-tRNA synthetase (translation)	**-3.0**
SaMRSA252-2314 (6J20)	**adk**	adenylate kinase (nucleobase, nucleoside, nucleotide and nucleic acid metabolism)	**-3.0**
SaMRSA252-2195 (6G17)	**atpF**	putative ATP synthase subunit b (ATP synthesis coupled proton transport)	**-3.0**
SaMRSA252-1449 (4C22)	**SAR1449**	amino acid permease (amino acid transport)	**-2.9**
SaMRSA252-0781 (1J24)	**SAR0781**	putative proton-dependent oligopeptide transport protein (oligopeptide transport)	**-2.9**
SaMRSA252-2490 (7O5)	**SAR2490**	conserved hypothetical protein (possible role in cobalamin biosynthesis)	**-2.9**
SaMRSA252-0905 (3C15)	**SAR0905**	putative transporter protein	**-2.9**
SaMRSA252-1758 (5K12)	**rplT**	50S ribosomal protein L20 (protein biosynthesis)	**-2.9**
SaMRSA252-2341 (6M23)	**glcU**	glucose uptake protein (carbohydrate transport)	**-2.9**
SaMRSA252-0546 (3G7)	**SAR0546**	conserved hypothetical protein	**-2.9**
SaMRSA252-0632 (3L6)	**SAR0632**	putative membrane protein	**-2.8**
SaMRSA252-1074 (3I24)	**SAR1074**	putative ABC transport system permease protein (transport)	**-2.8**

SaMRSA252-0789 (2B1)	**sstC**	ABC transporter ATP-binding protein (ATP-binding; ATPase activity; nucleoside triphosphatase; nucleotide binding)	**-2.8**
SaMRSA252-1227 (1P6)	**SAR1227**	conserved hypothetical protein	**-2.8**
SaMRSA252-2330 (6J22)	**rplV**	50S ribosomal protein L22 (translation)	**-2.8**
SaMRSA252-0363 (1C21)	**ssb**	putative single-strand DNA-binding protein (DNA binding)	**-2.8**
SaMRSA252-1235 (1P7)	**frr**	ribosome recycling factor (translation)	**-2.8**
SaMRSA252-2194 (6F17)	**atpH**	putative ATP synthase delta chain (ATP synthesis coupled proton transport)	**-2.8**
SaMRSA252-1419 (4E18)	**SAR1419**	putative branched-chain amino acid transporter protein (aliphatic amino acid transport)	**-2.8**
SaMRSA252-1366 (4I12)	**grlB; parE**	topoisomerase IV subunit B (DNA metabolism)	**-2.8**
SaMRSA252-1836 (5H21)	**SAR1836**	putative peptidase (proteolysis)	**-2.8**
SaMRSA252-0359 (1G20)	**SAR0359**	conserved hypothetical protein	**-2.8**
SaMRSA252-0318 (1G15)	**SAR0318**	hypothetical protein	**-2.8**
SaMRSA252-0362 (1B21)	**rpsF**	30S ribosomal protein S6 (translation)	**-2.7**
SaMRSA252-0618 (3N4)	**SAR0618**	putative transport system lipoprotein (iron ion transport)	**-2.7**
SaMRSA252-0257 (2D20)	**lytS**	autolysin sensor kinase protein (cell wall organisation and biogenesis)	**-2.7**
SaMRSA252-1431 (4A20)	**SAR1431**	putative acetyltransferase (N-acetyltransferase activity)	**-2.7**
SaMRSA252-0344 (1H18)	**SAR0344**	putative Sec-independent protein translocase protein (protein transport)	**-2.7**
SaMRSA252-2318 (6N20)	**rpsE**	30S ribosomal protein S5 (translation)	**-2.7**
SaMRSA252-1604 (5E5)	**accC**	biotin carboxylase subunit of acetyl-CoA carboxylase (metabolism)	**-2.7**
SaMRSA252-0383 (1G23)	**SAR0383**	hypothetical protein	**-2.7**
SaMRSA252-2439 (7E11)	**SAR2439**	TetR family regulatory protein (regulation of transcription, DNA-dependent)	**-2.7**

SaMRSA252-0982 (3G24)	**SAR0982**	hypothetical protein	**-2.7**
SaMRSA252-2107 (6P6)	**SAR2107**	putative leukocidin F subunit (haemolysis of red blood cells)	**-2.6**
SaMRSA252-2628 (7G22)	**clpL**	putative ATP-dependent protease ATP-binding subunit ClpL (nucleotide-excision repair)	**-2.6**
SaMRSA252-1720 (5M7)	**queA**	S-adenosylmethionine:tRNA ribosyltransferase-isomerase (queuosine biosynthesis)	**-2.6**
SaMRSA252-2680 (7I17)	**ldh2**	L-lactate dehydrogenase 2 (TCA cycle intermediate metabolism)	**-2.6**
SaMRSA252-1465 (4C24)	**asnS**	putative asparaginyl-tRNA synthetase (tRNA aminoacylation for protein translation)	**-2.6**
SaMRSA252-0216 (2C15)	**SAR0216**	putative lipoprotein	**-2.6**
SaMRSA252-2033 (6A10)	**SAR2033**	putative membrane protein	**-2.6**
SaMRSA252-2332 (6L22)	**rplB**	50S ribosomal protein L2 (translation)	**-2.6**
SaMRSA252-0174 (2I10)	**SAR0174**	putative lipoprotein	**-2.6**
SaMRSA252-2505 (7M7)	**SAR2505**	putative transport system protein	**-2.6**
SaMRSA252-1147 (4H8)	**SAR1147**	putative membrane protein	**-2.6**
SaMRSA252-2411 (7B8)	**SAR2411**	putative transport protein	**-2.6**
SaMRSA252-1905 (5K18)	**SAR1905**	serine protease (proteolysis)	**-2.6**
SaMRSA252-2344 (6P23)	**SAR2344**	putative membrane protein	**-2.6**
SaMRSA252-2430 (7D10)	**SAR2430**	putative permease (glutamate transport)	**-2.6**
SaMRSA252-1675 (5K2)	**SAR1675**	conserved hypothetical protein	**-2.5**
SaMRSA252-2231 (6C22)	**SAR2231**	putative mannose-6-phosphate isomerase (carbohydrate metabolism)	**-2.5**
SaMRSA252-2219 (6G20)	**SAR2219**	hypothetical protein	**-2.5**
SaMRSA252-0176	**SAR0176**	conserved hypothetical protein	**-2.5**

(2K10)			
SaMRSA252-0614 (3J4)	**SAR0614**	conserved hypothetical protein	**-2.5**
SaMRSA252-0220 (2G15)	**SAR0220**	putative membrane protein (glycerol metabolism)	**-2.5**
SaMRSA252-1127 (4F6)	**SAR1127**	putative exported protein	**-2.5**
SaMRSA252-0256 (2C20)	**scdA**	cell wall metabolism protein	**-2.5**
SaMRSA252-1239 (1L8)	**proS; drpA**	prolyl-tRNA synthetase (protein translation)	**-2.5**
SaMRSA252-0912 (3B16)	**mnhC**	Na+/H+ antiporter subunit	**-2.5**
SaMRSA252-1912 (5J19)	**SAR1912**	transposase (partial)	**-2.5**
SaMRSA252-2310 (6N19)	**rpsK**	30S ribosomal protein S11 (translation)	**-2.5**
SaMRSA252-2503 (7K7)	**SAR2503**	transport system membrane protein (transport)	**-2.5**
SaMRSA252-2494 (7J6)	**SAR2494**	putative membrane protein	**-2.5**
SaMRSA252-2349 (6M24)	**SAR2349**	MarR family regulatory protein (regulation of transcription, DNA-dependent)	**-2.4**
SaMRSA252-1456 (4B23)	**SAR1456**	conserved hypothetical protein	**-2.4**
SaMRSA252-0079 (1C10)	**SAR0079**	putative protein kinase (protein amino acid phosphorylation)	**-2.4**
SaMRSA252-2198 (6B18)	**atpI**	putative ATP synthase protein I	**-2.4**
SaMRSA252-1793 (5F16)	**thiI**	putative thiamine biosynthesis protein (thiamin biosynthesis)	**-2.4**
SaMRSA252-0586 (3G12)	**ung**	putative uracil-DNA glycosylase	**-2.4**
SaMRSA252-0319 (1H15)	**SAR0319**	NADH:flavin oxidoreductase / NADH oxidase family protein (electron transport; oxidoreductase activity)	**-2.4**
SaMRSA252-2192 (6D17)	**atpG**	ATP synthase gamma chain (ATP synthesis coupled proton transport)	**-2.4**
SaMRSA252-2168 (6E14)	**SAR2168**	putative helicase (ATP binding; helicase activity; nucleic acid binding)	**-2.4**
SaMRSA252-1448 (4B22)	**SAR1448**	transporter protein	**-2.4**

SaMRSA252-2209 (6E19)	**rho**	transcription termination factor (ATP synthesis coupled proton transport; transcription termination)	**-2.4**
SaMRSA252-0287 (2C24)	**SAR0287**	hypothetical protein	**-2.4**
SaMRSA252-1711 (5L6)	**hisS**	histidyl-tRNA synthetase (protein translation)	**-2.3**
SaMRSA252-1556 (4N23)	**SAR1556**	putative regulatory protein (DNA binding)	**-2.3**
SaMRSA252-0059 (1A8)	**ccrB**	site-specific recombinase (DNA recombination; regulation of transcription)	**-2.3**
SaMRSA252-2493 (7I6)	**SAR2493**	putative nitrite transporter (transport)	**-2.3**
SaMRSA252-1429 (4G19)	**SAR1429**	putative membrane protein	**-2.3**
SaMRSA252-0783 (1L24)	**SAR0783**	putative membrane protein	**-2.3**
SaMRSA252-1551 (4I23)	**SAR1551**	hypothetical phage protein	**-2.3**
SaMRSA252-1075 (3J24)	**SAR1075**	putative ABC transport system permease protein (transport)	**-2.3**
SaMRSA252-1134 (4E7)	**SAR1134**	hypothetical protein	**-2.3**
SaMRSA252-1898 (5L17)	**hsdS**	putative type I restriction modification DNA specificity protein [conserved region] (DNA modification)	**-2.3**
SaMRSA252-1600 (5A5)	**SAR1600**	putative exodeoxyribonuclease VII small subunit (DNA catabolism)	**-2.3**
SaMRSA252-0686 (3P12)	**SAR0686**	putative transposase [pseudogene] (DNA-mediated transposition)	**-2.3**
SaMRSA252-2626 (7E22)	**mvaS**	3-hydroxy-3-methylglutaryl coenzyme A synthase (acetyl CoA metabolism)	**-2.3**
SaMRSA252-0914 (3D16)	**mnhA**	Na+/H+ antiporter subunit (ATP synthesis coupled electron transport)	**-2.3**
SaMRSA252-2787 (8H5)	**SAR2787**	hypothetical protein	**-2.3**
SaMRSA252-0357 (1E20)	**SAR0357**	putative DNA-binding protein (DNA binding)	**-2.3**
SaMRSA252-0408 (2O14)	**guaB**	putative inosine-5'-monophosphate dehydrogenase (catalytic activity)	**-2.3**
SaMRSA252-0983 (3H24)	**SAR0983**	hypothetical protein	**-2.2**
SaMRSA252-0630 (3J6)	**SAR0630**	putative NADH-Ubiquinone/plastoquinone (complex I) oxidoreductase protein -	**-2.2**

		(ATP synthesis coupled transport)	
SaMRSA252-1368 (4K12)	**SAR1368**	sodium:alanine symporter family protein (Sodium ion transport)	**-2.2**
SaMRSA252-1430 (4H19)	**murG**	putative UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide)pyrophosphoryl-undecaprenol N-acetylglucosamine transferase (carbohydrate metabolism; lipid glycosylation)	**-2.2**
SaMRSA252-1693 (5K4)	**SAR1693**	putative O-methyltransferase	**-2.2**
SaMRSA252-2313 (6I20)	**infA**	translation initiation factor IF-1 (translational initiation)	**-2.2**
SaMRSA252-0386 (1B24)	**SAR0386**	hypothetical protein	**-2.2**
SaMRSA252-0511 (3E3)	**hpt**	putative hypoxanthine phosphoribosyltransferase (nucleoside metabolism)	**-2.2**
SaMRSA252-0631 (3K6)	**SAR0631**	putative membrane protein	**-2.2**
SaMRSA252-0150 (2I7)	**adhE**	putative aldehyde-alcohol dehydrogenase (metabolism)	**-2.2**
SaMRSA252-1401 (4C16)	**SAR1401**	ABC transporter permease protein (transport)	**-2.2**
SaMRSA252-0615 (3K4)	**argS**	putative arginyl-tRNA synthetase (arginyl-tRNA aminoacylation; cysteinyl-tRNA aminoacylation)	**-2.2**
SaMRSA252-0099 (1F12)	**SAR0099**	conserved hypothetical protein (tRNA processing)	**-2.2**
SaMRSA252-0384 (1H23)	**SAR0384**	hypothetical protein	**-2.2**
SaMRSA252-1863 (5K13)	**SAR1863**	putative membrane protein	**-2.2**
SaMRSA252-1757 (5J12)	**SAR1757**	conserved hypothetical protein	**-2.2**
SaMRSA252-1111 (4F4)	**pheS**	putative phenylalanyl-tRNA synthetase alpha chain (phenylalanyl-tRNA aminoacylation)	**-2.2**
SaMRSA252-2338 (6J23)	**SAR2338**	xanthine/uracil permeases family protein (transport)	**-2.2**
SaMRSA252-1236 (1I8)	**uppS**	undecaprenyl pyrophosphate synthetase (metabolism)	**-2.2**
SaMRSA252-0393 (1H24)	**SAR0393**	hypothetical protein	**-2.2**
SaMRSA252-0652 (3O8)	**pbp4**	penicillin-binding protein 4 (proteolysis)	**-2.2**
SaMRSA252-0017 (1A3)	**purA**	putative adenylosuccinate synthetase (purine nucleotide biosynthetic process)	**-2.2**

SaMRSA252-2397 (7E6)	**SAR2397**	putative membrane protein	**-2.2**
SaMRSA252-1234 (1O7)	**pyrH**	putative uridylate kinase (amino acid biosynthesis)	**-2.2**
SaMRSA252-2651 (7L13)	**SAR2651**	putative membrane protein	**-2.2**
SaMRSA252-2197 (6A18)	**atpB**	ATP synthase subunit a (proton transport)	**-2.2**
SaMRSA252-1148 (4A9)	**SAR1148**	putative DNA-binding protein	**-2.2**
SaMRSA252-0851 (2F8)	**SAR0851**	putative exported protein	**-2.2**
SaMRSA252-0235 (2G17)	**SAR0235**	putative PTS transport system, IIBC component (phosphoenolpyruvate-dependent sugar phosphotransferase system transport)	**-2.1**
SaMRSA252-0343 (1G18)	**SAR0343**	putative Sec-independent protein translocase protein	**-2.1**
SaMRSA252-1991 (6G4)	**gatB**	glutamyl-tRNA amidotransferase subunit B (translation)	**-2.1**
SaMRSA252-0913 (3C16)	**mnhB**	Na+/H+ antiporter subunit	**-2.1**
SaMRSA252-0633 (3M6)	**SAR0633**	putative NADH-Ubiquinone/plastoquinone (complex I) oxidoreductase protein (ATP synthesis coupled electron transport)	**-2.1**
SaMRSA252-2382 (7F4)	**SAR2382**	putative transcriptional regulator (regulation of transcription, DNA-dependent)	**-2.1**
SaMRSA252-0562 (3G9)	**SAR0562**	putative deoxyadenosine kinase protein (nucleobase, nucleoside, nucleotide and nucleic acid metabolism)	**-2.1**
SaMRSA252-1992 (6H4)	**gatA**	glutamyl-tRNA amidotransferase subunit A	**-2.1**
SaMRSA252-0847 (2B8)	**nuc**	thermonuclease precursor (nucleic acid binding)	**-2.1**
SaMRSA252-2134 (6K10)	**SAR2134**	ABC transporter ATP-binding protein (ATP binding; ATPase activity; nucleoside-triphosphatase activity; nucleotide binding)	**-2.1**
SaMRSA252-1794 (5G16)	**SAR1794**	aminotransferase class-V protein (metabolism)	**-2.1**
SaMRSA252-0968 (3A23)	**pepB**	putative oligopeptidase (proteolysis)	**-2.1**
SaMRSA252-2388 (7D5)	**SAR2388**	putative exported protein	**-2.1**

SaMRSA252-0517 (3C4)	**folK**	putative 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase- (folic acid and derivative biosynthesis)	**-2.1**
SaMRSA252-0890 (3D13)	**SAR0890**	conserved hypothetical protein	**-2.1**
SaMRSA252-0891 (3E13)	**SAR0891**	haloacid dehalogenase-like hydrolase (metabolism)	**-2.1**
SaMRSA252-1718 (5K7)	**SAR1718**	putative exported protein	**-2.1**
SaMRSA252-2106 (6O6)	**SAR2106**	phospholipase C precursor [partial]	**-2.1**
SaMRSA252-0695 (1I14)	**SAR0695**	putative membrane protein	**-2.1**
SaMRSA252-1445 (4G21)	**SAR1445**	putative membrane protein	**-2.1**
SaMRSA252-1272 (1M12)	**mutL**	DNA mismatch repair protein MutL (DNA mismatch repair)	**-2.1**
SaMRSA252-1642 (5C10)	**SAR1642**	putative glycyl-tRNA synthetase (tRNA aminoacylation for protein translation)	**-2.1**
SaMRSA252-1674 (5J2)	**SAR1674**	putative GTPase	**-2.0**
SaMRSA252-0215 (2B15)	**SAR0215**	putative sensor kinase protein (2-component signal transduction system)	**-2.0**
SaMRSA252-0490 (2P24)	**SAR0490**	tetrapyrrole (corrin/porphyrin) methylase family protein (metabolism)	**-2.0**
SaMRSA252-1374 (4A13)	**SAR1374**	putative membrane protein	**-2.0**
SaMRSA252-2529 (7M10)	**SAR2529**	sodium/hydrogen exchanger family protein (regulation of pH)	**-2.0**
SaMRSA252-2678 (7O16)	**SAR2678**	putative ketopantoate reductase (glycerol-3-phosphate catabolism)	**-2.0**
SaMRSA252-2788 (8A6)	**SAR2788**	putative exported protein	**-2.0**
SaMRSA252-0634 (3N6)	**SAR0634**	putative membrane protein (cation transport)	**-2.0**
SaMRSA252-1819 (5G19)	**SAR1819**	FtsK/SpoIIIE family protein	**-2.0**
SaMRSA252-1723 (5P7)	**SAR1723**	putative DNA-binding protein (metabolism)	**-2.0**
SaMRSA252-0761 (1N21)	**SAR0761**	putative lipoprotein	**-2.0**

SaMRSA252-2502 (7J7)	**SAR2502**	ABC transporter ATP-binding protein (ATP binding; ATPase activity; nucleoside-triphosphatase activity; nucleotide binding)	**-2.0**
SaMRSA252-0360 (1H20)	**SAR0360**	putative GTP-binding protein	**-2.0**
SaMRSA252-0801 (2F2)	**SAR0801**	putative glycosyl transferase (lipid metabolism)	**-2.0**
SaMRSA252-0469 (2K22)	**SAR0469**	putative membrane protein	**-2.0**
SaMRSA252-0760 (1M21)	**SAR0760**	putative membrane protein	**-2.0**
SaMRSA252-0947 (3E20)	**fabF**	3-oxoacyl-[acyl-carrier-protein] synthase II (fatty acid biosynthesis)	**-2.0**
SaMRSA252-1073 (3P23)	**SAR1073**	putative ABC transport ATP-binding protein (ATP binding; ATPase activity; nucleoside-triphosphtase activity; nucleotide binding)	**-2.0**
SaMRSA252-0355 (1C20)	**SAR0355**	Cys/Met metabolism PLP-dependent enzyme (amino acid metabolism)	**-2.0**
SaMRSA252-1136 (4F7)	**hla**	alpha-hemolysin precursor [pseudogene] (haemolysis of host red blood cells)	**-2.0**
SaMRSA252-0981 (3F24)	**SAR0981**	conserved hypothetical protein	**-2.0**
SaMRSA252-2193 (6E17)	**atpA**	ATP synthase alpha chain (ATP biosynthesis; ATP synthesis coupled proton transport)	**-2.0**
SaMRSA252-0307 (1D14)	**SAR0307**	putative membrane protein	**-2.0**
SaMRSA252-2385 (7A5)	**SAR2385**	putative Na+/H+ antiporter (regulation of pH; Na ion transport)	**-1.9**
SaMRSA252-2139 (6P10)	**SAR2139**	conserved hypothetical protein	**-1.9**
SaMRSA252-2580 (7G16)	**fnb**	fibronectin-binding protein precursor [conserved region] (cell adhesion)	**-1.9**
SaMRSA252-1939 (5L22)	**SAR1939**	putative response regulator (regulation of transcription, DNA-dependent)	**-1.9**
SaMRSA252-2265 (6P13)	**SAR2265**	putative membrane protein (nucleoside binding)	**-1.9**
SaMRSA252-0590 (3K1)	**SAR0590**	putative membrane protein	**-1.9**
SaMRSA252-1334 (4I8)	**nucI; nucH**	thermonuclease (nucleic acid binding)	**-1.9**
SaMRSA252-2345 (6I24)	**SAR2345**	AcrB/AcrD/AcrF family protein	**-1.9**

SaMRSA252-1820 (5H19)	**SAR1820**	conserved hypothetical protein (tRNA binding)	**-1.9**
SaMRSA252-0474 (2P22)	**SAR0474**	putative glycosyl hydrolase (carbohydrate metabolism)	**-1.9**
SaMRSA252-1282 (4O1)	**SAR1282**	conserved hypothetical protein (amino acid metabolism)	**-1.9**
SaMRSA252-1922 (5K20)	**SAR1922**	hypothetical protein	**-1.9**
SaMRSA252-2391 (7G5)	**SAR2391**	putative N-acetylmuramoyl-L-alanine amidase (peptidoglycan catabolism)	**-1.9**
SaMRSA252-1195 (1P2)	**SAR1195**	putative protein phosphatase (catalytic activity)	**-1.9**
SaMRSA252-0782 (1K24)	**SAR0782**	conserved hypothetical protein (biosynthetic process)	**-1.9**
SaMRSA252-1671 (5O1)	**SAR1671**	conserved hypothetical protein (biosynthetic process)	**-1.9**
SaMRSA252-0696 (1J14)	**SAR0696**	putative exported protein	**-1.9**
SaMRSA252-0117 (2P2)	**sirB**	putative siderophore transport system permease (transport)	**-1.9**
SaMRSA252-0485 (2K24)	**holB**	putative DNA polymerase III, delta' subunit	**-1.9**
SaMRSA252-0098 (1E12)	**SAR0098**	acetyltransferase (GNAT) family protein (n-acetyltransferase activity)	**-1.9**
SaMRSA252-0452 (2J20)	**SAR0452**	putative NADH-Ubiquinone/plastoquinone (complex I) protein (ATP synthesis coupled electron transport)	**-1.9**
SaMRSA252-2096 (6M5)	**SAR2096**	putative anti repressor (DNA binding)	**-1.9**
SaMRSA252-2495 (7K6)	**SAR2495**	hypothetical protein	**-1.9**
SaMRSA252-1253 (1J10)	**SAR1253**	conserved hypothetical protein (regulation of transcription, DNA-dependent)	**-1.9**
SaMRSA252-0617 (3M4)	**SAR0617**	putative DNA repair protein (base-excision repair)	**-1.9**
SaMRSA252-0610 (3N3)	**SAR0610**	putative acetyltransferase (N-acetyltransferase activity)	**-1.9**
SaMRSA252-1186 (1O1)	**SAR1186**	conserved hypothetical protein (transcription, DNA-dependent)	**-1.9**
SaMRSA252-2449 (7G12)	**SAR2449**	hypothetical protein	**-1.9**

SaMRSA252-2342 (6N23)	**SAR2342**	putative membrane protein	**-1.9**
SaMRSA252-0697 (1K14)	**SAR0697**	ABC transporter ATP-binding protein (ATP binding; ATPase activity; nucleoside-triphosphatase activity; nucleotide binding)	**-1.9**
SaMRSA252-0911 (3A16)	**mnhD**	Na+/H+ antiporter subunit (ATP synthesis coupled electron transport)	**-1.9**
SaMRSA252-1002 (3K15)	**SAR1002**	putative membrane protein	**-1.8**
SaMRSA252-0455 (2M20)	**SAR0455**	putative membrane protein	**-1.8**
SaMRSA252-1138 (4H7)	**SAR1138**	putative transposase (transposition, DNA-mediated)	**-1.8**
SaMRSA252-2142 (6K11)	**ilvH**	acetolactate synthase isozyme III small subunit [partial] (metabolism;amino acid binding)	**-1.8**
SaMRSA252-1217 (1N5)	**rplS**	50S ribosomal protein L19 (protein biosynthesis)	**-1.8**
SaMRSA252-2722 (7K22)	**SAR2722**	putative membrane protein (transport)	**-1.8**
SaMRSA252-2791 (8D6)	**SAR2791**	putative membrane protein (DNA binding)	**-1.8**
SaMRSA252-2674 (7K16)	**SAR2674**	conserved hypothetical protein (proteolysis)	**-1.8**
SaMRSA252-0187 (2N11)	**SAR0187**	putative branched-chain amino acid transport system carrier protein (branched-chain aliphatic amino acid transport)	**-1.8**
SaMRSA252-2187 (6G16)	**fabZ**	putative hydroxymyristoyl-(acyl carrier protein) dehydratase	**-1.8**
SaMRSA252-0765 (1J22)	**SAR0765**	conserved hypothetical protein	**-1.8**
SaMRSA252-0412 (2K15)	**SAR0412**	conserved hypothetical protein (pseudogene)	**-1.8**
SaMRSA252-2477 (7K4)	**SAR2477**	conserved hypothetical protein	**-1.8**
SaMRSA252-1634 (5C9)	**SAR1634**	putative endonuclease (DNA repair)	**-1.8**
SaMRSA252-1860 (5H24)	**SAR1860**	hypothetical protein	**-1.8**
SaMRSA252-1255	**SAR1255**	putative protease	**-1.8**

(1L10)			
SaMRSA252-2542 (7J12)	**SAR2542**	putative transport protein	**-1.8**
SaMRSA252-2346 (6J24)	**fmhB**	putative peptidoglycan pentaglycine interpeptide biosynthesis protein (peptidoglycan biosynthesis)	**-1.8**
SaMRSA252-0353 (1A20)	**metE**	5-methyltetrahydropteroyltriglutamate--homocyst eine methyltransferase (methionine biosynthesis)	**-1.8**
SaMRSA252-0894 (3H13)	**dltA**	D-alanine-D-alanyl carrier protein ligase (metabolism)	**-1.8**
SaMRSA252-2789 (8B6)	**SAR2789**	putative subtilase family protease (proteolysis)	**-1.8**
SaMRSA252-0356 (1D20)	**SAR0356**	Cys/Met metabolism PLP-dependent enzyme (amino acid metabolism)	**-1.8**
SaMRSA252-1846 (5B23)	**SAR1846**	conserved hypothetical protein	**-1.8**
SaMRSA252-0846 (2A8)	**SAR0846**	putative exported protein	**-1.8**
SaMRSA252-2442 (7H11)	**tcaA**	teicoplanin resistance associated membrane protein	**-1.8**
SaMRSA252-2311 (6O19)	**rpsM**	30S ribosomal protein S13 (translation)	**-1.7**
SaMRSA252-1264 (1M11)	**SAR1264**	conserved hypothetical protein	**-1.7**
SaMRSA252-0906 (3D15)	**SAR0906**	conserved hypothetical protein	**-1.7**
SaMRSA252-0925 (3G17)	**SAR0925**	putative membrane protein	**-1.7**
SaMRSA252-1692 (5J4)	**SAR1692**	putative peptidase (proteolysis)	**-1.7**
SaMRSA252-2003 (6C6)	**SAR2003**	conserved hypothetical protein	**-1.7**
SaMRSA252-0221 (2H15)	**SAR0221**	hypothetical protein	**-1.7**
SaMRSA252-0483 (2I24)	**tmk**	putative thymidylate kinase (tTDP biosynthesis)	**-1.7**
SaMRSA252-2458 (7P1)	**SAR2458**	acetyltransferase (GNAT) family protein (N-acetyltransferase activity)	**-1.7**
SaMRSA252-1475 (4M13)	**aroA**	3-phosphoshikimate 1-carboxyvinyltransferase	**-1.7**
SaMRSA252-1599	**SAR1599**	putative geranyltranstransferase (isoprenoid biosynthesis)	**-1.7**

(5H4)			
SaMRSA252-2251 (6C24)	**SAR2251**	putative transposase (transposition, DNA-mediated)	**-1.7**
SaMRSA252-0524 (3B5)	**nupC**	nucleoside permease (transport)	**-1.7**
SaMRSA252-1455 (4A23)	**SAR1455**	putative exported protein	**-1.7**
SaMRSA252-0979 (3D24)	**SAR0979**	putative membrane protein	**-1.7**
SaMRSA252-1844 (5H22)	**SAR1844**	putative membrane protein	**-1.7**
SaMRSA252-1158 (4C10)	**mraY**	phospho-N-acetylmuramoyl-pentapeptide-transferase (lipid metabolism)	**-1.7**
SaMRSA252-2004 (6D6)	**SAR2004**	putative membrane protein	**-1.7**
SaMRSA252-1756 (5I12)	**SAR1756**	hypothetical protein	**-1.7**
SaMRSA252-2221 (6A21)	**SAR2221**	putative peptidase (proteolysis)	**-1.7**
SaMRSA252-0515 (3A4)	**folP**	dihydropteroate synthase (folic acid and derivative biosynthesis)	**-1.7**
SaMRSA252-1697 (5O4)	**alaS**	putative alanyl-tRNA synthetase (alanyl-t-RNA aminoacylation)	**-1.7**
SaMRSA252-0086 (1B11)	**SAR0086**	hypothetical protein	**-1.7**
SaMRSA252-2233 (6E22)	**czrA**	zinc and cobalt transport repressor protein (regulation of transcription, DNA-dependent)	**-1.7**
SaMRSA252-2774 (8E4)	**cna**	collagen adhesin precursor (collagen binding)	**-1.7**
SaMRSA252-1635 (5D9)	**SAR1635**	putative helicase (ATP binding; helicase activity; nucleic acid binding)	**-1.6**
SaMRSA252-1750 (5K11)	**hemC**	porphobilinogen deaminase (porphyrin biosynthesis)	**-1.6**
SaMRSA252-2001 (6A6)	**SAR2001**	staphopain protease (proteolysis)	**-1.6**
SaMRSA252-0943 (3A20)	**SAR0943**	conserved hypothetical protein	**-1.6**
SaMRSA252-1095 (4F2)	**SAR1095**	conserved hypothetical protein	**-1.6**
SaMRSA252-0771	**SAR0771**	conserved hypothetical protein	**-1.6**

(1P22)			
SaMRSA252-1913 (5K19)	**SAR1913**	hypothetical protein	**-1.6**
SaMRSA252-1110 (4E4)	**SAR1110**	SpoU rRNA Methylase family protein (RNA processing)	**-1.6**
SaMRSA252-0619 (3O4)	**SAR0619**	FecCD transport family protein (transport)	**-1.6**
SaMRSA252-2204 (6H18)	**SAR2204**	conserved hypothetical protein	**-1.6**
SaMRSA252-0988 (3M13)	**murE**	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-dia minopimelate ligase (biosynthesis)	**-1.6**
SaMRSA252-1653 (5F11)	**SAR1653**	conserved hypothetical protein (catalytic activity; Fe ion binding)	**-1.6**
SaMRSA252-0516 (3B4)	**folB**	dihydroneopterin aldolase (folic acid and derivative metabolism)	**-1.6**
SaMRSA252-1602 (5C5)	**SAR1602**	putative N utilization substance protein B (regulation of transcription, DNA-dependent)	**-1.6**
SaMRSA252-2340 (6L23)	**SAR2340**	acetyltransferase (GNAT) family protein (N-acetyltransferase activity)	**-1.6**
SaMRSA252-2441 (7G11)	**tcaB**	teicoplanin resistance associated membrane protein	**-1.6**
SaMRSA252-0766 (1K22)	**SAR0766**	glutamine amidotransferase class-I protein (catalytic activity)	**-1.6**
SaMRSA252-0456 (2N20)	**SAR0456**	putative membrane protein	**-1.6**
SaMRSA252-0636 (3P6)	**SAR0636**	putative membrane protein (pseudogene)	**-1.6**
SaMRSA252-0616 (3L4)	**SAR0616**	hypothetical protein	**-1.5**
SaMRSA252-0563 (3H9)	**SAR0563**	putative deaminase (hydrolase activity; Zn ion binding)	**-1.5**
SaMRSA252-1838 (5B22)	**SAR1838**	RNA pseudouridine synthase (RNA processing)	**-1.5**
SaMRSA252-1743 (5L10)	**valS**	valyl-tRNA synthetase (tRNA aminoacylation for protein translation)	**-1.5**
SaMRSA252-1778 (5G14)	**accA**	acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha (fatty acid biosynthesis)	**-1.5**
SaMRSA252-0764 (1I22)	**SAR0764**	putative 6-pyruvoyl tetrahydropterin synthase	**-1.5**
SaMRSA252-0770 (1O22)	**SAR0770**	conserved hypothetical protein	**-1.5**

SaMRSA252-2772 (8C4)	**pcp**	pyrrolidone-carboxylate peptidase (proteolysis)	**-1.5**
SaMRSA252-1749 (5J11)	**hemD**	uroporphyrinogen III synthase (heme and porphyrin biosynthesis)	**-1.5**
SaMRSA252-0365 (1E21)	**SAR0365**	hypothetical protein	**-1.5**
SaMRSA252-0097 (1D12)	**SAR0097**	putative DNA-binding protein (regulation of transcription, DNA-dependent)	**-1.5**
SaMRSA252-2792 (8E6)	**SAR2792**	putative membrane protein	**-1.5**
SaMRSA252-1672 (5P1)	**SAR1672**	conserved hypothetical protein	**-1.5**
SaMRSA252-2170 (6G14)	**SAR2170**	D-alanine--D-alanine ligase (peptidoglycan biosynthesis)	**-1.5**
SaMRSA252-0291 (2G24)	**SAR0291**	putative membrane protein	**-1.5**
SaMRSA252-2387 (7C5)	**SAR2387**	putative membrane protein	**-1.5**
SaMRSA252-0611 (3O3)	**SAR0611**	putative phosphohydrolase (catalytic activity)	**-1.5**
SaMRSA252-0963 (3D22)	**SAR0963**	putative transposase (transposition, DNA-mediated)	**-1.5**
SaMRSA252-0529 (3G5)	**radA**	putative DNA repair protein (nucleoside-triphosphatase activity; nucleotide binding)	**-1.4**
SaMRSA252-0198 (2A13)	**SAR0198**	ABC transporter ATP-binding protein (ATP binding; ATPase activity; nucleoside-triphosphatase activity; nucleotide binding)	**-1.4**
SaMRSA252-1621 (5F7)	**SAR1621**	hypothetical protein (transport)	**-1.4**
SaMRSA252-0881 (2C12)	**SAR0881**	putative membrane protein	**-1.4**
SaMRSA252-0394 (2I13)	**SAR0394**	phosphoglycerate mutase family protein	**-1.4**
SaMRSA252-2671 (7P15)	**SAR2671**	conserved hypothetical protein	**-1.4**
SaMRSA252-1680 (5O2)	**SAR1680**	hypothetical protein	**-1.4**
SaMRSA252-0374 (1F22)	**SAR0374**	hypothetical protein	**-1.4**
SaMRSA252-0845 (2H7)	**SAR0845**	putative exported protein	**-1.3**

SaMRSA252-0967 (3H22)	**SAR0967**	hypothetical protein	**-1.3**
SaMRSA252-0959 (3H21)	**SAR0959**	putative oligopeptide transport system permease protein (pseudogene)	**-1.3**
SaMRSA252-2548 (7P12)	**SAR2548**	putative membrane protein	**-1.3**
SaMRSA252-0665 (3L10)	**SAR0665**	putative esterase	**-1.3**

APPENDIX II: MICROARRAY NORMALIZED DATA

Table 4.5: Differentially regulated ASM vs GASM genes normalised to BHI, using one-way ANOVA and posthoc Benjamini and Hochberg tests ($p \leq 0.05$).

ORF	GENE	FOLD ASM	FOLD GASM
SaMRSA252-0013 (1E2)	**SAR0013**	0.46468106	0.8918642
SaMRSA252-0016 (1H2)	**dnaC**	0.53992015	0.8762845
SaMRSA252-0022 (1F3)	**yycJ**	0.729247	1.4204
SaMRSA252-0024 (1H3)	**SAR0024**	0.874725	1.7696714
SaMRSA252-0048 (1F6)	**SAR0048**	6.287764	1.814429
SaMRSA252-0101 (1H12)	**SAR0101**	1.1625729	0.6440747
SaMRSA252-0109 (2P1)	**SAR0109**	1.2635933	0.7634881
SaMRSA252-0110 (2I2)	**SAR0110**	1.5920168	0.3095714
SaMRSA252-0111 (2J2)	**SAR0111**	2.8357062	0.3729886
SaMRSA252-0112 (2K2)	**SAR0112**	0.61468023	0.13412818
SaMRSA252-0113 (2L2)	**lldP1**	1.7183038	0.8133743
SaMRSA252-0150 (2I7)	**adhE**	1.3430483	0.45129952
SaMRSA252-0151 (2J7)	**capA**	8.371157	2.1304312
SaMRSA252-0152 (2K7)	**capB**	15.33862	3.465002
SaMRSA252-0153 (2L7)	**capC**	64.001526	8.281368
SaMRSA252-0154 (2M7)	**capD**	33.27507	4.371519
SaMRSA252-0155 (2N7)	**capE**	24.455772	3.5634563
SaMRSA252-0156 (2O7)	**capF**	14.19307	2.9339175
SaMRSA252-0158 (2I8)	**cap8H**	9.755827	2.175777
SaMRSA252-0163 (2N8)	**capM**	6.9461827	1.7309104
SaMRSA252-0164 (2O8)	**capN**	6.1501455	1.7297816
SaMRSA252-0187 (2N11)	**SAR0187**	1.1260557	0.54936343
SaMRSA252-0191 (2J12)	**SAR0191**	14.18649	1.5319127
SaMRSA252-0192 (2K12)	**SAR0192**	11.392867	1.1757923
SaMRSA252-0193 (2L12)	**SAR0193**	12.702764	1.4399105
SaMRSA252-0194 (2M12)	**SAR0194**	10.818244	1.2772143
SaMRSA252-0204 (2G13)	**SAR0204**	2.7809114	0.9627453
SaMRSA252-0206 (2A14)	**SAR0206**	2.3606691	1.1021621
SaMRSA252-0207 (2B14)	**SAR0207**	3.5640159	1.3266948
SaMRSA252-0208 (2C14)	**SAR0208**	5.8903646	1.3699492
SaMRSA252-0209 (2D14)	**SAR0209**	3.2597508	1.1438533
SaMRSA252-0210 (2E14)	**SAR0210**	4.1923976	1.0522432
SaMRSA252-0211 (2F14)	**SAR0211**	6.3751216	1.1716555
SaMRSA252-0212 (2G14)	**SAR0212**	0.32571846	0.12021539
SaMRSA252-0213 (2H14)	**uhpT**	2.4304507	0.9366067
SaMRSA252-0215 (2B15)	**SAR0215**	1.1194017	0.48859078
SaMRSA252-0216	**SAR0216**	1.3707523	0.3855865

(2C15)			
SaMRSA252-0218 (2E15)	**SAR0218**	2.9170387	0.25630042
SaMRSA252-0219 (2F15)	**SAR0219**	1.8668467	0.1562219
SaMRSA252-0220 (2G15)	**SAR0220**	1.3579767	0.3952986
SaMRSA252-0221 (2H15)	**SAR0221**	0.95297754	0.5812597
SaMRSA252-0222 (2A16)	**coa**	2.5444415	0.87432736
SaMRSA252-0222v (2B16)	**coa**	2.300444	0.7413258
SaMRSA252-0235 (2G17)	**SAR0235**	1.3265718	0.4652363
SaMRSA252-0251 (2F19)	**SAR0251**	0.44368398	0.5944115
SaMRSA252-0276 (2G22)	**SAR0276**	0.96288073	0.5861894
SaMRSA252-0277 (2H22)	**SAR0277**	2.3515658	1.0785847
SaMRSA252-0287 (2C24)	**SAR0287**	1.0098027	0.42515013
SaMRSA252-0291 (2G24)	**SAR0291**	1.2181065	0.67379504
SaMRSA252-0293 (1A13)	**SAR0293**	1.4962282	0.86213666
SaMRSA252-0294 (1B13)	**SAR0294**	1.6146489	0.856953
SaMRSA252-0295 (1C13)	**SAR0295**	1.4573473	0.99920726
SaMRSA252-0305 (1B14)	**SAR0305**	4.6145506	2.0549307
SaMRSA252-0306 (1C14)	**SAR0306**	4.2110868	1.6459296
SaMRSA252-0307 (1D14)	**SAR0307**	0.86055624	0.5120401
SaMRSA252-0308 (1E14)	**SAR0308**	2.1710634	0.8401185
SaMRSA252-0309 (1F14)	**SAR0309**	2.6541984	0.8357773
SaMRSA252-0310 (1G14)	**SAR0310**	1.6844846	0.73628896
SaMRSA252-0312 (1A15)	**SAR0312**	4.833047	1.3346497
SaMRSA252-0313 (1B15)	**SAR0313**	2.104855	0.9634332
SaMRSA252-0314 (1C15)	**SAR0314**	1.6627566	0.8750828
SaMRSA252-0317 (1F15)	**geh**	1.8109337	0.9805433
SaMRSA252-0318 (1G15)	**SAR0318**	0.7792553	0.36207578
SaMRSA252-0319 (1H15)	**SAR0319**	0.8426718	0.41777495
SaMRSA252-0325 (1F16)	**SAR0325**	1.5491469	0.7274927
SaMRSA252-0330 (1C17)	**SAR0330**	0.7637386	1.1472974
SaMRSA252-0333	**glpT**	3.478413	1.1045313

(1F17)			
SaMRSA252-0334 (1G17)	**SAR0334**	2.7958033	7.5052075
SaMRSA252-0335 (1H17)	**SAR0335**	0.977215	3.6095557
SaMRSA252-0336 (1A18)	**SAR0336**	1.0571787	2.821556
SaMRSA252-0378 (1B23)	**SAR0378**	1.86775	8.969302
SaMRSA252-0379 (1C23)	**SAR0379**	0.860358	3.7094965
SaMRSA252-0380 (1D23)	**SAR0380**	0.94656414	2.0422332
SaMRSA252-0381 (1E23)	**SAR0381**	0.93379253	2.073526
SaMRSA252-0382 (1F23)	**SAR0382**	0.9475221	1.3964286
SaMRSA252-0390 (1E24)	**SAR0390**	2.7000778	0.6112165
SaMRSA252-0392 (1G24)	**SAR0392**	2.3658993	0.69770944
SaMRSA252-0400 (2O13)	**SAR0400**	2.0147393	6.542723
SaMRSA252-0419 (2I16)	**SAR0419**	1.1223867	2.7930872
SaMRSA252-0426 (2P16)	**SAR0426**	1.0509486	0.31987083
SaMRSA252-0450 (2P19)	**SAR0450**	0.6034885	0.98016745
SaMRSA252-0454 (2L20)	**SAR0454**	0.4030579	1.0825
SaMRSA252-0468 (2J22)	**SAR0468**	1.0756818	0.7147368
SaMRSA252-0469 (2K22)	**SAR0469**	1.0397065	0.5024951
SaMRSA252-0506 (3H2)	**SAR0506**	0.56996506	1.1402757
SaMRSA252-0507 (3A3)	**SAR0507**	0.7301069	1.1886168
SaMRSA252-0508 (3B3)	**SAR0508**	0.74043894	1.5028244
SaMRSA252-0517 (3C4)	**folK**	0.73597705	0.47621188
SaMRSA252-0559 (3D9)	**SAR0559**	1.0344523	2.0505857
SaMRSA252-0565 (3B10)	**SAR0565**	0.87295514	3.1392572
SaMRSA252-0569 (3G10)	**SAR0569**	1.1602532	2.2392614
SaMRSA252-0572 (3B11)	**SAR0572**	0.8244016	1.4366922
SaMRSA252-0574 (3D11)	**SAR0574**	2.5007913	1.2052732
SaMRSA252-0583 (3D12)	**SAR0583**	0.95329225	1.7891588
SaMRSA252-0584 (3E12)	**SAR0584**	1.0583587	3.3078778
SaMRSA252-0590 (3K1)	**SAR0590**	0.95498496	0.5169464
SaMRSA252-0598 (3K2)	**mvaK2**	1.7587502	0.9208038
SaMRSA252-0609 (3M3)	**SAR0609**	3.304547	1.3392518
SaMRSA252-0616 (3L4)	**SAR0616**	1.1040673	0.6487962
SaMRSA252-0618 (3N4)	**SAR0618**	0.5560844	0.36583415
SaMRSA252-0626 (3N5)	**SAR0626**	1.4602909	0.653484
SaMRSA252-0629 (3I6)	**SAR0629**	3.417953	1.0389584
SaMRSA252-0630 (3J6)	**SAR0630**	2.4843543	0.44590223
SaMRSA252-0631 (3K6)	**SAR0631**	1.8996552	0.45092213

SaMRSA252-0632 (3L6)	**SAR0632**	1.5209657	0.35138708
SaMRSA252-0633 (3M6)	**SAR0633**	1.4893664	0.46969733
SaMRSA252-0634 (3N6)	**SAR0634**	1.5399393	0.49455386
SaMRSA252-0635 (3O6)	**SAR0635**	1.2378716	0.663227
SaMRSA252-0636 (3P6)	**SAR0636**	1.2435576	0.6400808
SaMRSA252-0663 (3J10)	**SAR0663**	0.89870083	2.6295123
SaMRSA252-0667 (3N10)	**SAR0667**	0.9990628	3.3208873
SaMRSA252-0675 (3N11)	**SAR0675**	3.621745	0.8221818
SaMRSA252-0680 (3J12)	**SAR0680**	0.6512578	1.2809916
SaMRSA252-0681 (3K12)	**SAR0681**	0.47230715	1.1786345
SaMRSA252-0682 (3L12)	**SAR0682**	1.0270505	2.265806
SaMRSA252-0699 (1M14)	**SAR0699**	0.9939254	1.4632269
SaMRSA252-0704 (1P14)	**SAR0704**	0.72204727	1.2806602
SaMRSA252-0707 (1K15)	**SAR0707**	1.2780637	3.666898
SaMRSA252-0715 (1P15)	**SAR0715**	0.70893186	1.4935954
SaMRSA252-0734 (1K18)	**SAR0734**	2.1742618	1.2069931
SaMRSA252-0752 (1M20)	**SAR0752**	0.25659445	0.59146357
SaMRSA252-0763 (1P21)	**SAR0763**	1.3698791	0.69622207
SaMRSA252-0764 (1I22)	**SAR0764**	1.6964288	0.65571976
SaMRSA252-0765 (1J22)	**SAR0765**	1.2290485	0.5534975
SaMRSA252-0772 (1I23)	**SAR0772**	0.73885334	1.4307061
SaMRSA252-0776 (1M23)	**SAR0776**	2.0287447	0.98375595
SaMRSA252-0799 (2D2)	**SAR0799**	0.4613194	0.69853044
SaMRSA252-0802 (2G2)	**SAR0802**	0.9659037	2.0883722
SaMRSA252-0803 (2H2)	**SAR0803**	0.51797	0.8881307
SaMRSA252-0807 (2D3)	**secA**	0.849651	2.288079
SaMRSA252-0811 (2H3)	**SAR0811**	0.72314346	1.0061675
SaMRSA252-0815 (2D4)	**lgt**	0.7778712	1.1221565
SaMRSA252-0821 (2A5)	**SAR0821**	3.3691092	1.9238251
SaMRSA252-0825 (2E5)	**SAR0825**	3.502403	1.4250748
SaMRSA252-0837 (2A7)	**smpB**	1.8648777	1.0639266
SaMRSA252-0840 (2D7)	**SAR0840**	3.0290346	1.0678478
SaMRSA252-0841 (2E7)	**SAR0841**	3.0662901	0.9958238
SaMRSA252-0849 (2D8)	**SAR0849**	2.6591408	0.8699937
SaMRSA252-0850 (2E8)	**SAR0850**	3.3150826	0.9235972
SaMRSA252-0882 (2D12)	**SAR0882**	3.0982707	0.9202519
SaMRSA252-0898 (3D14)	**SAR0898**	0.5002504	1.5868772
SaMRSA252-0901 (3G14)	**SAR0901**	0.8342097	2.4178452
SaMRSA252-0906 (3D15)	**SAR0906**	0.27218154	0.57793075
SaMRSA252-0921 (3C17)	**glpQ**	7.206343	2.2777202
SaMRSA252-0930	**SAR0930**	0.6580122	1.624189

(3D18)			
SaMRSA252-0931 (3E18)	**SAR0931**	1.7321172	0.6826681
SaMRSA252-0933 (3G18)	**SAR0933**	0.65305185	1.2815228
SaMRSA252-0934 (3H18)	**SAR0934**	0.54043883	1.5027121
SaMRSA252-0970 (3C23)	**SAR0970**	1.3282714	3.1005714
SaMRSA252-0975 (3H23)	**SAR0975**	0.77873516	1.4699986
SaMRSA252-0986 (3K13)	**SAR0986**	0.8763961	1.9587433
SaMRSA252-0992 (3I14)	**SAR0992**	0.7407757	0.4742173
SaMRSA252-1049 (3I21)	**SAR1049**	4.5883822	14.640269
SaMRSA252-1050 (3J21)	**SAR1050**	5.746336	19.28062
SaMRSA252-1051 (3K21)	**SAR1051**	4.555158	19.083761
SaMRSA252-1054 (3N21)	**SAR1054**	1.3044372	2.387946
SaMRSA252-1066 (3I23)	**SAR1066**	0.54897743	1.9485464
SaMRSA252-1079 (3N24)	**SAR1079**	0.35845894	2.7937887
SaMRSA252-1081 (3P24)	**SAR1081**	0.8043508	2.5910513
SaMRSA252-1084 (4C1)	**SAR1084**	1.1008649	1.6977886
SaMRSA252-1085 (4D1)	**SAR1085**	1.019481	2.363324
SaMRSA252-1086 (4E1)	**SAR1086**	0.83376646	2.1141768
SaMRSA252-1117 (4D5)	**SAR1117**	0.7898956	1.3995013
SaMRSA252-1138 (4H7)	**SAR1138**	0.79489857	0.544595
SaMRSA252-1144 (4F8)	**SAR1144**	1.4886609	0.73445994
SaMRSA252-1146 (4G8)	**SAR1146**	0.5942711	2.1793044
SaMRSA252-1151 (4D9)	**SAR1151**	1.2556902	2.7030556
SaMRSA252-1153 (4F9)	**SAR1153**	1.443294	4.6624227
SaMRSA252-1158 (4C10)	**mraY**	1.1389228	0.5946273
SaMRSA252-1168 (4E11)	**SAR1168**	0.7266735	1.5569783
SaMRSA252-1171 (4H11)	**SAR1171**	0.79100555	1.6801589
SaMRSA252-1174 (4C12)	**pyrR**	0.41208595	0.25032
SaMRSA252-1188 (1I2)	**priA**	0.88479745	1.620783
SaMRSA252-1197 (1J3)	**SAR1197**	0.8874229	2.4730105
SaMRSA252-1198 (1K3)	**SAR1198**	0.7461038	2.1706061
SaMRSA252-1203 (1P3)	**recG**	0.9336269	2.1120212
SaMRSA252-1225 (1N6)	**SAR1225**	0.71119165	0.2748725
SaMRSA252-1226 (1O6)	**topA**	1.0556458	0.6988616
SaMRSA252-1245 (1J9)	**infB**	0.5765104	1.0528471
SaMRSA252-1254 (1K10)	**SAR1254**	1.1771258	0.61312836
SaMRSA252-1261 (1J11)	**recA**	1.0044913	1.9427842
SaMRSA252-1265 (1N11)	**SAR1265**	1.0115081	5.920014
SaMRSA252-1266 (1O11)	**SAR1266**	0.48103064	5.258159
SaMRSA252-1267 (1P11)	**SAR1267**	0.6225463	1.3832681

SaMRSA252-1279 (4L1)	**SAR1279**	0.71481746	2.6435697
SaMRSA252-1284 (4I2)	**glnA**	0.9863276	0.51340914
SaMRSA252-1288 (4L2)	**SAR1288**	1.3689644	3.9565003
SaMRSA252-1312 (4I5)	**SAR1312**	1.4834946	4.456924
SaMRSA252-1317 (4N5)	**SAR1317**	1.4164617	4.440857
SaMRSA252-1326 (4I7)	**SAR1326**	0.92047817	3.5565271
SaMRSA252-1328 (4K7)	**SAR1328**	0.717452	1.3543725
SaMRSA252-1333 (4P7)	**SAR1333**	0.92974025	2.6899016
SaMRSA252-1338 (4M8)	**SAR1338**	1.6314249	0.5167573
SaMRSA252-1339 (4N8)	**thrC**	1.4996514	0.53818935
SaMRSA252-1341 (4P8)	**SAR1341**	0.64291674	1.1728145
SaMRSA252-1350 (4I10)	**SAR1350**	0.65999645	2.0569696
SaMRSA252-1361 (4L11)	**opuD1**	0.54965353	0.2408207
SaMRSA252-1363 (4N11)	**SAR1363**	1.3653331	2.2387235
SaMRSA252-1366 (4I12)	**grlB**	0.5918387	0.36008093
SaMRSA252-1368 (4K12)	**SAR1368**	0.7084515	0.44590306
SaMRSA252-1369 (4L12)	**SAR1369**	0.68764013	1.1949857
SaMRSA252-1371 (4N12)	**SAR1371**	0.87031615	1.6584486
SaMRSA252-1378 (4E13)	**SAR1378**	1.1249583	0.7057574
SaMRSA252-1380 (4G13)	**trpE**	1.215179	0.65280706
SaMRSA252-1396 (4F15)	**SAR1396**	0.50359505	1.134806
SaMRSA252-1399 (4A16)	**SAR1399**	1.753862	0.7480826
SaMRSA252-1400 (4B16)	**SAR1400**	1.4686277	0.6577418
SaMRSA252-1404 (4F16)	**SAR1404**	0.48823446	0.27051452
SaMRSA252-1415 (4A18)	**SAR1415**	1.0856936	4.550116
SaMRSA252-1416 (4B18)	**SAR1416**	0.8790099	2.4055398
SaMRSA252-1417 (4C18)	**SAR1417**	1.0661075	3.2639074
SaMRSA252-1418 (4D18)	**SAR1418**	0.9088879	2.8502138
SaMRSA252-1422 (4H18)	**SAR1422**	0.80868304	1.9379743
SaMRSA252-1423 (4A19)	**SAR1423**	1.3804976	3.6474955
SaMRSA252-1429 (4G19)	**SAR1429**	0.7309806	0.4303688
SaMRSA252-1431 (4A20)	**SAR1431**	0.70230114	0.3671887
SaMRSA252-1439 (4A21)	**dfrB**	1.963855	1.0251838
SaMRSA252-1440 (4B21)	**thyA**	2.3273325	0.8517861
SaMRSA252-1455 (4A23)	**SAR1455**	0.9027445	0.5917137
SaMRSA252-1456 (4B23)	**SAR1456**	0.67489475	0.41342312

SaMRSA252-1459 (4E23)	**SAR1459**	0.84245485	2.3415577
SaMRSA252-1466 (4D24)	**SAR1466**	0.61238414	1.3489516
SaMRSA252-1492 (4N15)	**SAR1492**	0.86510223	6.9110475
SaMRSA252-1543 (4I22)	**SAR1543**	0.63325864	0.2946172
SaMRSA252-1569 (5B1)	**rluB**	0.86726534	1.6020694
SaMRSA252-1571 (5D1)	**SAR1571**	0.9645274	1.6899518
SaMRSA252-1572 (5E1)	**SAR1572**	0.7804171	1.4855555
SaMRSA252-1573 (5F1)	**xerD**	1.2613442	3.8113089
SaMRSA252-1574 (5G1)	**fur**	1.2087466	3.735957
SaMRSA252-1589 (5F3)	**gnd**	0.6342126	2.2477493
SaMRSA252-1593 (5B4)	**bfmB**	0.7846024	1.7506498
SaMRSA252-1602 (5C5)	**SAR1602**	0.44708532	0.63315284
SaMRSA252-1610 (5C6)	**SAR1610**	1.4491228	3.864697
SaMRSA252-1611 (5D6)	**SAR1611**	0.7194571	2.2899566
SaMRSA252-1631 (5H8)	**zur**	0.74645513	1.3812275
SaMRSA252-1633 (5B9)	**mreA**	0.8142331	2.4687715
SaMRSA252-1640 (5A10)	**SAR1640**	0.84926766	1.7522871
SaMRSA252-1641 (5B10)	**SAR1641**	1.1069362	2.159852
SaMRSA252-1643 (5D10)	**SAR1643**	1.3235724	2.8229802
SaMRSA252-1644 (5E10)	**era**	1.1566461	2.312268
SaMRSA252-1645 (5F10)	**cdd**	1.8053241	3.516316
SaMRSA252-1693 (5K4)	**SAR1693**	0.76160365	0.44848996
SaMRSA252-1706 (5O5)	**SAR1706**	0.64109766	2.2770884
SaMRSA252-1707 (5P5)	**SAR1707**	1.00899	1.9465007
SaMRSA252-1712 (5M6)	**SAR1712**	0.73226786	0.44855282
SaMRSA252-1716 (5I7)	**SAR1716**	0.61630803	0.3027052
SaMRSA252-1717 (5J7)	**secF**	0.90324104	0.31227684
SaMRSA252-1732 (5I9)	**SAR1732**	0.67024153	1.086254
SaMRSA252-1769 (5F13)	**polA**	1.4180975	2.3993483
SaMRSA252-1776 (5E14)	**pyk**	0.41749287	0.70586896
SaMRSA252-1777 (5F14)	**pfkA**	0.33913073	0.63316107
SaMRSA252-1782 (5C15)	**SAR1782**	1.049404	2.0009587
SaMRSA252-1783 (5D15)	**SAR1783**	0.69296366	1.7152853
SaMRSA252-1833 (5E21)	**SAR1833**	0.6206138	1.096078
SaMRSA252-1837 (5A22)	**SAR1837**	3.324786	1.8899668
SaMRSA252-1858 (5F24)	**SAR1858**	0.80688614	2.758749
SaMRSA252-1863 (5K13)	**SAR1863**	0.7190963	0.45633465
SaMRSA252-1890 (5L16)	**SAR1890**	0.8495076	1.7078474
SaMRSA252-1891 (5M16)	**SAR1891**	1.1671354	2.3818016

SaMRSA252-1914 (5L19)	**SAR1914**	0.7735187	2.578354
SaMRSA252-1954 (5K24)	**SAR1954**	1.0411499	2.4540284
SaMRSA252-1963 (6D1)	**SAR1963**	0.743763	1.8899945
SaMRSA252-1964 (6E1)	**SAR1964**	0.8790183	3.0797398
SaMRSA252-1995 (6C5)	**SAR1995**	0.6552535	0.9611286
SaMRSA252-2010 (6B7)	**SAR2010**	2.5700839	0.86652184
SaMRSA252-2105 (6N6)	**int**	1.0930096	0.5389688
SaMRSA252-2106 (6O6)	**SAR2106**	0.8057229	0.48091838
SaMRSA252-2107 (6P6)	**SAR2107**	0.6458813	0.37886417
SaMRSA252-2108 (6I7)	**SAR2108**	1.1123862	0.44509915
SaMRSA252-2109 (6J7)	**SAR2109**	7.311793	1.160173
SaMRSA252-2119 (6L8)	**SAR2119**	2.775482	1.1478336
SaMRSA252-2121 (6N8)	**SAR2121**	1.1763545	2.724482
SaMRSA252-2128 (6M9)	**scrB**	1.0422435	1.8565555
SaMRSA252-2134 (6K10)	**SAR2134**	0.9495356	0.47409657
SaMRSA252-2153 (6N12)	**rsbW**	1.7983415	0.9171606
SaMRSA252-2184 (6D16)	**SAR2184**	2.7816274	0.7249816
SaMRSA252-2186 (6F16)	**SAR2186**	1.0739685	0.67882186
SaMRSA252-2188 (6H16)	**murA1**	1.2030783	0.5489191
SaMRSA252-2189 (6A17)	**SAR2189**	2.112052	1.0028553
SaMRSA252-2193 (6E17)	**atpA**	1.0871086	0.5119499
SaMRSA252-2194 (6F17)	**atpH**	0.7847439	0.35843208
SaMRSA252-2195 (6G17)	**atpF**	0.8050963	0.33882532
SaMRSA252-2198 (6B18)	**atpI**	0.9674913	0.41433123
SaMRSA252-2207 (6C19)	**SAR2207**	0.5110102	0.29248273
SaMRSA252-2213 (6A20)	**SAR2213**	0.4585006	0.76013404
SaMRSA252-2231 (6C22)	**SAR2231**	0.83040357	0.39282966
SaMRSA252-2233 (6E22)	**czrA**	2.4657042	0.60327
SaMRSA252-2245 (6G23)	**SAR2245**	2.1910203	0.6484807
SaMRSA252-2261 (6L13)	**SAR2261**	0.63114166	1.1607786
SaMRSA252-2263 (6N13)	**SAR2263**	1.1017318	2.8574197
SaMRSA252-2274 (6I15)	**SAR2274**	5.088405	2.1119266
SaMRSA252-2276 (6K15)	**opuD2**	2.788676	0.7115176
SaMRSA252-2279 (6N15)	**SAR2279**	1.8664368	4.0450864
SaMRSA252-2290 (6I17)	**SAR2290**	1.1485374	4.646416
SaMRSA252-2291 (6J17)	**SAR2291**	1.1662822	3.490584
SaMRSA252-2294 (6M17)	**SAR2294**	0.97685623	1.9172401

SaMRSA252-2296 (6O17)	**SAR2296**	1.1458637	15.48309
SaMRSA252-2297 (6P17)	**SAR2297**	0.46112514	22.774578
SaMRSA252-2299 (6K18)	**SAR2299**	0.8810989	1.5501517
SaMRSA252-2302 (6N18)	**SAR2302**	0.67431206	1.1052182
SaMRSA252-2330 (6J22)	**rplV**	0.82074946	0.35631707
SaMRSA252-2331 (6K22)	**rpsS**	0.49862272	0.18463457
SaMRSA252-2334 (6N22)	**rplD**	0.52386343	0.23684348
SaMRSA252-2337 (6I23)	**SAR2337**	1.0551952	2.3573105
SaMRSA252-2342 (6N23)	**SAR2342**	0.807362	0.5379894
SaMRSA252-2345 (6I24)	**SAR2345**	0.78560483	0.518235
SaMRSA252-2348 (6L24)	**SAR2348**	1.0615394	0.66864866
SaMRSA252-2356 (7D1)	**mobB**	0.9715027	2.928149
SaMRSA252-2359 (7G1)	**moaB**	1.1857909	3.578227
SaMRSA252-2361 (7A2)	**modC**	0.4991859	1.015381
SaMRSA252-2368 (7H2)	**SAR2368**	0.5682429	1.4254922
SaMRSA252-2369 (7A3)	**SAR2369**	0.42601746	2.006894
SaMRSA252-2371 (7C3)	**SAR2371**	1.2349043	3.0050142
SaMRSA252-2372 (7D3)	**ureA**	1.4874339	5.084831
SaMRSA252-2373 (7E3)	**ureB**	1.1750546	3.6501412
SaMRSA252-2374 (7F3)	**ureC**	1.3346074	3.5829763
SaMRSA252-2375 (7G3)	**ureE**	1.248785	3.1727018
SaMRSA252-2376 (7H3)	**ureF**	1.1946504	3.565164
SaMRSA252-2378 (7B4)	**ureD**	1.6539929	3.9222908
SaMRSA252-2379 (7C4)	**sarR**	0.6328243	2.0333102
SaMRSA252-2383 (7G4)	**SAR2383**	1.1446447	0.11971289
SaMRSA252-2385 (7A5)	**SAR2385**	1.6605369	0.51399887
SaMRSA252-2386 (7B5)	**SAR2386**	1.8012245	0.33154294
SaMRSA252-2387 (7C5)	**SAR2387**	1.4735298	0.6789819
SaMRSA252-2388 (7D5)	**SAR2388**	5.1713166	0.4759401
SaMRSA252-2396 (7D6)	**SAR2396**	1.6043496	3.2498379
SaMRSA252-2408 (7G7)	**SAR2408**	1.3095633	4.6447163
SaMRSA252-2420 (7B9)	**SAR2420**	7.1340647	2.501297
SaMRSA252-2434 (7H10)	**SAR2434**	0.82898635	1.5258524
SaMRSA252-2437 (7C11)	**SAR2437**	0.21577725	0.32191244
SaMRSA252-2444 (7B12)	**SAR2444**	2.491324	0.75838596
SaMRSA252-2451 (7I1)	**SAR2451**	0.82690036	2.081786
SaMRSA252-2472 (7N3)	**gltT**	0.6426751	0.25345513
SaMRSA252-2499 (7O6)	**SAR2499**	0.4724116	0.9743131
SaMRSA252-2500 (7P6)	**SAR2500**	0.42976326	0.94612026
SaMRSA252-2502 (7J7)	**SAR2502**	0.24529848	0.49952772
SaMRSA252-2503 (7K7)	**SAR2503**	0.21657951	0.40434593
SaMRSA252-2505 (7M7)	**SAR2505**	0.79907435	0.38875312
SaMRSA252-2522 (7N9)	**SAR2522**	1.0092856	2.3575854
SaMRSA252-2525 (7I10)	**SAR2525**	1.3237295	0.5373617
SaMRSA252-2528 (7L10)	**SAR2528**	1.7695054	1.02556

SaMRSA252-2539 (7O11)	**SAR2539**	0.7304307	2.3149438
SaMRSA252-2541 (7I12)	**SAR2541**	2.7962556	0.90368533
SaMRSA252-2542 (7J12)	**SAR2542**	0.9553454	0.5648206
SaMRSA252-2543 (7K12)	**SAR2543**	4.524537	0.98055327
SaMRSA252-2544 (7L12)	**SAR2544**	4.664336	1.3443356
SaMRSA252-2545 (7M12)	**SAR2545**	1.2099801	3.473688
SaMRSA252-2558 (7B14)	**SAR2558**	5.0922637	1.5767926
SaMRSA252-2559 (7C14)	**SAR2559**	6.7962766	1.6534109
SaMRSA252-2560 (7D14)	**SAR2560**	2.449029	0.9524388
SaMRSA252-2581 (7A17)	**SAR2581**	0.443871	1.2403115
SaMRSA252-2582 (7B17)	**gntP**	1.0354517	3.4062111
SaMRSA252-2583 (7C17)	**gntK**	1.1503041	3.7177267
SaMRSA252-2602 (7F19)	**SAR2602**	2.2497084	6.4499145
SaMRSA252-2618 (7E21)	**glcB**	0.42885956	1.8258067
SaMRSA252-2619 (7F21)	**SAR2619**	2.0832233	5.474948
SaMRSA252-2621 (7H21)	**SAR2621**	0.48751	5.2753344
SaMRSA252-2628 (7G22)	**clpL**	3.051173	0.37976193
SaMRSA252-2642 (7D24)	**crtN**	2.4902616	0.9802656
SaMRSA252-2645 (7F24)	**SAR2645**	4.6075287	0.8679969
SaMRSA252-2646 (7G24)	**SAR2646**	5.0779963	0.90271217
SaMRSA252-2647 (7H24)	**SAR2647**	5.569378	1.1717324
SaMRSA252-2648 (7I13)	**ssaA**	2.6725557	0.67489415
SaMRSA252-2650 (7K13)	**isaA**	1.6765643	0.13408762
SaMRSA252-2652 (7M13)	**SAR2652**	1.2931871	0.8769187
SaMRSA252-2668 (7M15)	**SAR2668**	4.876903	1.2021116
SaMRSA252-2669 (7N15)	**SAR2669**	0.4247543	0.1672614
SaMRSA252-2677 (7N16)	**panB**	0.60450274	0.2645785
SaMRSA252-2697 (7J19)	**SAR2697**	1.3697807	10.199416
SaMRSA252-2698 (7K19)	**cysJ**	1.0429815	8.20725
SaMRSA252-2699 (7L19)	**SAR2699**	0.7421925	1.6867834
SaMRSA252-2702 (7O19)	**SAR2702**	0.80438316	1.3696302
SaMRSA252-2703 (7P19)	**SAR2703**	0.6852052	1.2815194

SaMRSA252-2715 (7L21)	**SAR2715**	1.0546552	2.456144
SaMRSA252-2717 (7N21)	**isaB**	1.0285083	5.8660045
SaMRSA252-2726 (7O22)	**SAR2726**	2.1442962	1.2162427
SaMRSA252-2738 (7J24)	**SAR2738**	1.3490214	0.7138139
SaMRSA252-2739 (7K24)	**SAR2739**	3.0483608	1.119441
SaMRSA252-2740 (7L24)	**SAR2740**	2.3899858	0.6242509
SaMRSA252-2746 (8B1)	**icaR**	0.6319445	2.7494178
SaMRSA252-2747 (8C1)	**icaA**	4.3389544	1.60808
SaMRSA252-2748 (8D1)	**icaD**	3.0044913	1.3838906
SaMRSA252-2771 (8B4)	**SAR2771**	0.786412	4.1485305
SaMRSA252-2781 (8D5)	**vraD**	2.384115	0.7347819
SaMRSA252-2782 (8E5)	**vraE**	1.9555877	0.7751254
SaMRSA252-2784 (8G5)	**SAR2784**	1.2459375	0.600313
SaMRSA252-2789 (8B6)	**SAR2789**	0.880191	0.5669668
SaMRSA252-2791 (8D6)	**SAR2791**	0.83504206	0.54594046