THE MUTABILITY OF STAPHYLOCOCCAL BIOFILMS

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Intellectual Property and Publication Statements

The candidate confirms that the work submitted is her own and that the appropriate credit has been given where reference has been made to the work of others.

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Abstract

In their natural environment, bacteria are often found as sessile populations, known as biofilms, typified by surface adherence and extracellular matrix production. This growth phase confers broad spectrum antibiotic recalcitrance, through undefined mechanisms. This work sought to investigate a role for increased mutability ofstaphylococci in biofilms, as a contributing mechanism in biofilm antibiotic recalcitrance.

Initially, a novel biofilm model for use with staphylococci was established that utilised cellulose disks and incorporated human plasma to promote bacterial surface adherence. This system was validated by demonstrating antibiotic recalcitrance, dissemination by D-amino acids, and similarities with transcriptional profiles of other biofilm models.

Using this novel biofilm model, and the Sorbarod flow system, mutation frequencies (MFs) were determined for *Staphylococcus aureus* and *Staphylococcus epidermidis* in biofilms and compared with those in planktonic cultures. This revealed increases in MF of up to 68-fold compared with planktonic cultures. The role of oxidative stress in biofilm mutability was investigated by addition of antioxidants to biofilms. Here,MFs were reduced up to 5-fold, suggesting a role for oxidative damage. Transcriptional profiling of biofilms revealed upregulation of several genes involved in DNA repair, compared with planktonic cultures. Genes encoding antioxidant activity were also investigated; of these only *sodA* was upregulated. Staphyloxanthin biosysthetic genes, however, were downregulated.

During these studies, *S. aureus* biofilms were found to generate phenotypic variants. White variants (WVs) and large pale variants (LPVs) were mostly found amongst cells shed from biofilms. WVs had lost biofilm forming capacity and had mutations in the

alternative sigma factor, SigB. LPVs retained biofilm forming capacity, but the genetic basis of their variation remains undefined.

In summary, I have shown that staphylococci exhibit enhanced mutability in biofilms, accelerating the emergence of antibiotic resistance and morphological variants. This may result from oxidative stress, causing the enhanced accumulation of mutations within the genome.

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Abbrevations

AFM – Atomic force microscopy
Asc – Ascorbic acid
BHA – Brain heart infusion agar
BHB – Brain heart infusion broth
C – Celcius
CA-MRSA – Community acquired meticillin resistant *Staphylococcus aureus*
CBD – Calgary biofilm device
CDC – Centers for disease control and prevention
CDFF – Constant depth film fermenter
cDNA – complementary deoxyribonucleic acid
CF – Cystic fibrosis
cfu – Colony forming units
CM – Confocal microscopy
CNS – Coagulase negative staphylococci
CSP – Competence-stimulation peptide
DEPC - Diethylpyrocarbonate
DNA – Deoxyribonucleic acid
eDNA – extracellular DNA
EDTA – Ethylenediaminetetraacetic acid
EPS – Exopolysaccharide
F – Fertility
g – Standard gravity
hr(s) – Hour(s)
IS – Insertion sequence
l – litre
LBF – Line of best fit
LPV – Large pale variant
Mb - Megabases
mM - Millimolar
M - Molar
MF – Mutation frequency
mg - Milligram
MHA – Mueller Hinton agar
MHB – Mueller Hinton broth
MIC – Minimum inhibitory concentration
min(s) – Minute(s)
ml – Millilitre
mm – Millimetre
MMR – Methyl-directed mismatch repair
mRNA – Messenger ribonucleic acid
MRSA – Meticillin resistant *Staphylococcus aureus*

MSCRAMM – Microbial surface component recognising adhesive matrix molecules
NADH – Reduced nicotinamide adenine dinucleotide
NADPH – Reduced nicotinamide adenine dinucleotide phosphate
nm - Nanometre
NVE – Native valve endocarditis
OD – Optical density
ORF – Open reading frame
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
pH – Potential hydrogen
PIA – Polysaccharide intercellular adhesin
PNAG – Poly-*N*-acetyl glucosamine
RLU –Relative luminescence units
RNA – Ribonucleic acid
ROS – Reactive oxygen species
rpm – Revolutions per minute
RT-PCR – Real time polymerase chain reaction
SCV – Small colony variant
SOD – Superoxide dismutase
SSSS – Staphylococcal scalded skin syndrome
TA – Toxin-antitoxin
TAE – Tris-acetate-EDTA
Tau – Taurine
TCS – Two component system
TE – Tris-EDTA
tRNA – transfer ribonucleic acid
U – Enzymatic units
UDP – Uridine diphosphate
µg - Microgram
µl – Microlitre
µM – Micromolar
v/v – Volume/volume
Van – Vanillin
WV – White variant
w/v –Weight/volume

CHAPTER 1

1. Introduction

1.1. Bacterial biofilms

This thesis deals with mutability and oxidative stress within staphylococcal biofilms. In addition, the emergence of morphological variants from *S. aureus* biofilms and their role in biofilm dissemination have been investigated. Therefore, it is relevant to consider the literature on microbial biofilms.

In the mid-1800s, Robert Koch and colleagues helped generate the familiar microbiological techniques that allowed the isolation of pure bacterial cultures from complex mixed species environments. These cultures of free floating, or planktonic, cells have since proven invaluable for investigations concerning bacterial genetics, structure and metabolism, and elucidating the connection between pathogens and infectious disease. However, it is becoming increasingly apparent that the behaviour of planktonic cultures is not necessarily representative of that demonstrated by bacteria in their natural environments. Indeed, in their ecosystems, the vast majority of bacteria are found associated with surfaces as multicellular communities, or biofilms (Zobell, 1943; Zong *et al.*, 2005). These surface-attached communities are phenotypically differentiated from planktonic cultures by the formation of cellular aggregates, known as microcolonies, extracellular matrix production, which is often an exopolysaccharide (EPS) matrix, and a markedly slower growth rate (Lindsay & von Holy, 2006). Biofilms are found ubiquitously throughout the environment, from those lining the human gastrointestinal tract (Macfarlane, 2008) to those found in extreme climates such as deep-sea vents and hydrothermal springs (Taylor *et al.*, 1999; Yim *et al.*, 2006). Furthermore, evidence for their

existence has been identified in early fossil records, exemplified by the 3.2 billion year old hydrothermal rocks found to harbour fossilised filamentous biofilms (Rasmussen, 2000).

Biofilm structures were first observed on tooth surfaces by Van Leeuwenhoek, by utilising simple microscopes (Donlan, 2002). Zobell (1943) observed that the number of free floating microorganisms in seawater was significantly less than in the adherent population found on submerged surfaces . The advent of electron microscopy then allowed the visualisation of biofilms, from wastewater treatment plants, at high resolution (Jones *et al.*, 1969). Subsequently in 1978, following investigations into dental plaque and adherent populations in mountain streams, Costerton put forward the idea of "biofilms" as a mechanism to allow microorganisms to adhere to biotic and abiotic surfaces (Costerton *et al.*, 1978). The advent of biofilm studies then ensued, and as a result there has been increasing evidence for the presence of biofilms in the human host. Recent years have seen a profound increase in biofilm investigations, reflecting increasing reports of their involvement in infectious disease, with the National Institutes of Health now estimating that >80% of all infections in the developed world involve a biofilm component (NIH, 2002). It is important to state, however, that biofilms also form a protective part of human natural defences. In many cases biofilms formed by the resident microbiota provide colonisation resistance, to prevent colonisation by exogenous pathogens. For example, the polymicrobial biofilms attached to the intestinal epithelial lining in the human digestive tract, provide protection against food and waterborne pathogen infection (Lee *et al.*, 2000).

1.1.1. Biofilm development

1.1.1.1. Attachment

Bacteria undergo complex changes during the transition from a free-living lifestyle into a biofilm. This process occurs by several sequential steps (Figure 1.1). Prior to initial attachment of the cells, 'surface conditioning' is often required, whereby surfaces become coated with adsorbed bridging molecules (Lindsay & von Holy, 2006). For example, large cellular aggregates of *Pseudomonas aeruginosa* are formed on surfaces coated with mucin, a phenomenon not observed on surfaces coated with DNA or actin (Landry *et al.*, 2006). Similarly, the adherence of *S. epidermidis* to surfaces may be increased by the presence of surface-activated platelets (Wang *et al.*, 1993).

To bring about initial attachment, the bacterial cells must be in close proximity with the conditioned surface. This can be achieved by active motility via the use of flagella, a process demonstrated in *P. aeruginosa.* Indeed, mutants lacking a flagellum are unable to initiate surface colonisation, suggesting that these structures may be necessary for adherence (O'Toole & Kolter, 1998). Alternatively, the cells may be transported to the surface by processes such as liquid flow or Brownian motion (van Loosdrecht *et al.*, 1990). The initial attachment of bacteria is reversible, such that the adhering cells are in continuous exchange with the surrounding planktonic cells and continue to exhibit Brownian motion or self-propulsion. This is followed by irreversible binding due to the production of EPS and cell-to-cell bridges (Lindsay & von Holy, 2006). EPS production is necessary for the irreversible attachment of organisms such as *P. aeruginosa* and *S. epidermidis* (Davies & Geesey, 1995). Other organisms, however, may use specific receptors to anchor themselves to the surface. *Vibrio cholerae*, for example, utilises a mannose-sensitive haemagglutinin to achieve attachment on abiotic surfaces (Watnick *et al.*, 1999).

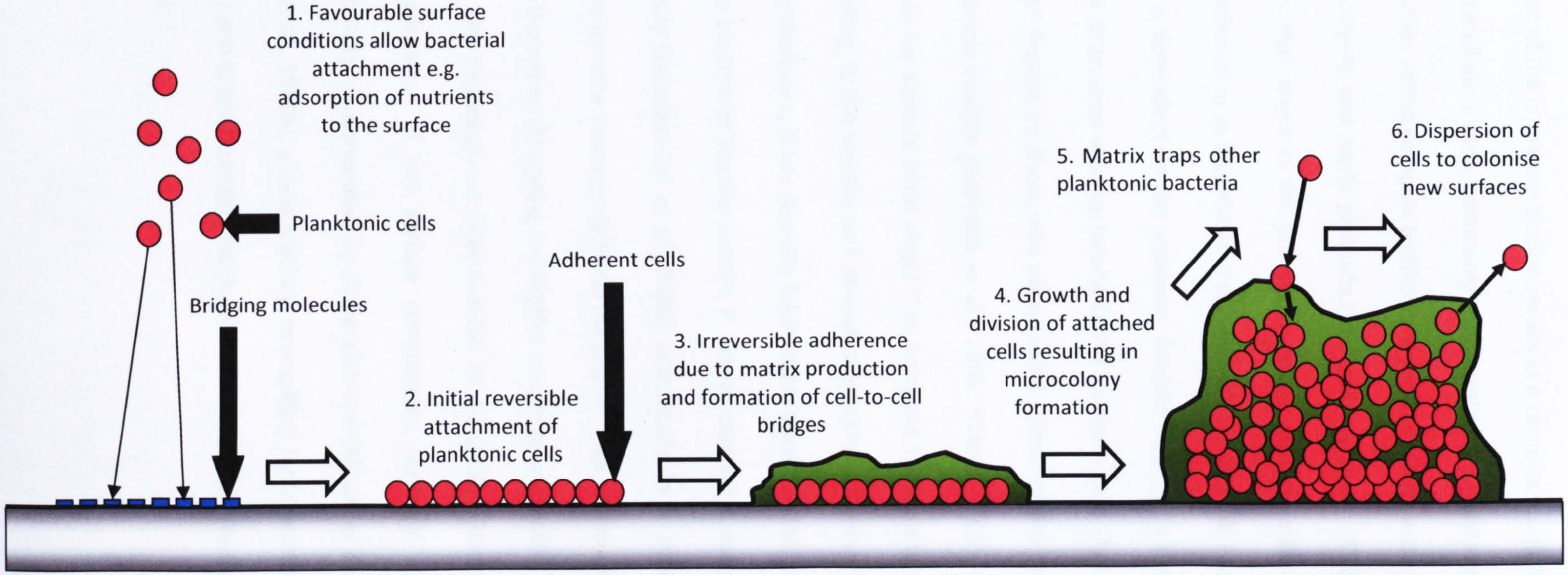

Figure 1.1 Bacterial differentiation and development from the planktonic phase into a biofilm community. The general steps of biofilm formation are shown.

1.1.1.2. Maturation and Dispersion

Maturation of the immature biofilm results in a complex multicellular structure surrounded by an extracellular matrix, commonly composed of EPS (Lawrence *et al.*, 1991). Indeed, the microcolonies within complex biofilms are surrounded by water channels, which transport water, nutrients and waste products (Massol-Deya *et al.*, 1995). Mature biofilm can also shed cells that revert to the planktonic phase, allowing colonisation of new surfaces, a process referred to as dispersion or dissemination (Parsek & Fuqua, 2004). The mechanism of which is dependent on the organism. *Proteus mirabilis*, a causative organism of urinary infections associated with catheters, forms swarmer cells. The significant increase in the number of flagella on these cells allows rapid spread of the organism over catheters and other medical devices (Sabbuba *et al.*, 2002; Rather, 2005). By contrast, *P. aeruginosa* synthesises an alginate lyase (AlgL) that functions to degrade alginate, the *P. aeruginosa* EPS, resulting in the release and spread of peripheral biofilm cells (Boyd & Chakrabarty, 1994). Furthermore, it has recently been established that the production of D-amino acids in mature biofilms of *Bacillus subtilis*, *P. aeruginosa* and *S. aureus*, may contribute to their disassembly (Kolodkin-Gal *et al.*, 2010). Specifically, in *B. subtilis* D-amino acids such as D-tyrosine become incorporated into the peptide side chains of the cell wall instead of the terminal D-alanine, disrupting the biofilm matrix by preventing anchoring of TasA amyloid fibres to the peptidoglycan (Kolodkin-Gal *et al.*, 2010). Other mechanisms of dispersion include alteration of cell surface components, such as an increase in cell surface hydrophobicity, demonstrated by *Escherichia coli* (Allison *et al.*, 1990b) and *P. aeruginosa* (Allison *et al.*, 1990a), gliding motility, exemplified by *Myxococcus xanthus* (Wolgemuth *et al.*, 2002) and quorum sensing mechanisms employed by *Xanthomonas campestris* (Dow *et al.*, 2003).

1.2. Staphylococci

Some of the most extensively studied organisms, in terms of biofilm formation, are the staphylococci. The staphylococci are Gram positive cocci, many of which form part of the natural flora of mammals. Coagulase negative staphylococci (CNS) such as *S. epidermidis*, for example, colonise the skin of the majority of the population, whilst the primary ecological niche for *S. aureus* is the anterior nares, where it permanently colonises approximately 20% of the population (Kluytmans *et al.*, 1997; O'Gara & Humphreys, 2001). In addition, *S. aureus* is a major cause of human infection in both the clinical and the community setting (Lowy, 1998). The severity of these infections may be partly attributed to the production of a variety of virulence factors in *S. aureus,* including coagulase, catalase, adhesins, toxins and capsule (Tenover & Gorwitz, 2006). Furthermore, the impact of this organism in the hospital setting is compounded by its propensity to develop antibiotic resistance. Meticillin-resistant *S. aureus* (MRSA), for example, gained notoriety in the hospital and other healthcare settings, where it has proven difficult to treat due to its resistance to β-lactams (David & Daum, 2010). Furthermore, the incidence of CA-MRSA (community acquired meticillin-resistant *S. aureus*) infections is on the increase, with these strains often affecting otherwise healthy individuals with no prior exposure to the healthcare setting (David & Daum, 2010). CA-MRSA infections are mostly of the skin and soft tissue, but necrotizing pneumonias and abscesses have also been reported (Fridkin *et al.*, 2005; Kaplan *et al.*, 2005; Purcell & Fergie, 2005; Thomas *et al.*, 2011). Efforts to elucidate the pathogenesis of CA-MRSA found that the majority of isolates harbour Panton-Valentine leukocidin and a variety of enterotoxins on several mobile genetic elements (Baba *et al.*, 2002; Vandenesch *et al.*, 2003). Furthermore, epidemic strains of CA-MRSA also secrete high levels of cytolytic peptides, known as phenol-soluble modulins, capable of lysing human neutrophils (Wang *et al.*, 2007).

S. aureus causes a broad range of infections, many of which are pyogenic and toxigenic in nature, including abscesses, food poisoning, impetigo, infective endocarditis and septicaemia (Tenover & Gorwitz, 2006). By contrast, CNS, such as *S. epidermidis*, are much less aggressive pathogens which rarely cause pyogenic infections in the healthy host, and the infections they do cause are not mediated by toxins (Heilmann & Peters, 2006). However, in the immunocompromised host, or those with indwelling medical devices (heart valves, shunts, plastic lines, *etc.*), *S. epidermidis* can become a frequent cause of infection (Raimundo *et al.*, 2002).

The majority of infections caused by staphylococci include a biofilm component. Staphylococcal biofilm formation is a multifactorial process that may differ between species and strains. For the purposes of this work, only biofilm formation by *S. epidermidis* and *S. aureus* will be discussed here.

1.2.1. Staphylococcal biofilm formation

1.2.1.1. Primary attachment

Primary attachment of cells to surfaces is the first step in biofilm formation, and in *S. epidermidis* and *S. aureus* this process is often achieved through interaction with host components via adhesins (Figure 1.2) (Arrecubieta *et al.*, 2007; Corrigan *et al.*, 2007; O'Neill *et al.*, 2008; Corrigan *et al.*, 2009). Furthermore, initial adhesion may also be influenced by surface chemistry, For example, hydrophobic surfaces promote biofilm formation in *S. epidermidis* (MacKintosh *et al.*, 2006; Patel *et al.*, 2007)

EPS of *S. epidermidis* biofilms, previously termed PS/A, polysaccharide intercellular adhesin (PIA) or Poly-β-(1,6)-*N*-acetylglucosamine, first isolated from rich cultures of *S. epidermidis*, is important for adherence, as antibodies raised against it prevent biofilm formation on silastic catheters by biofilm-positive strains (Tojo *et al.*, 1988).

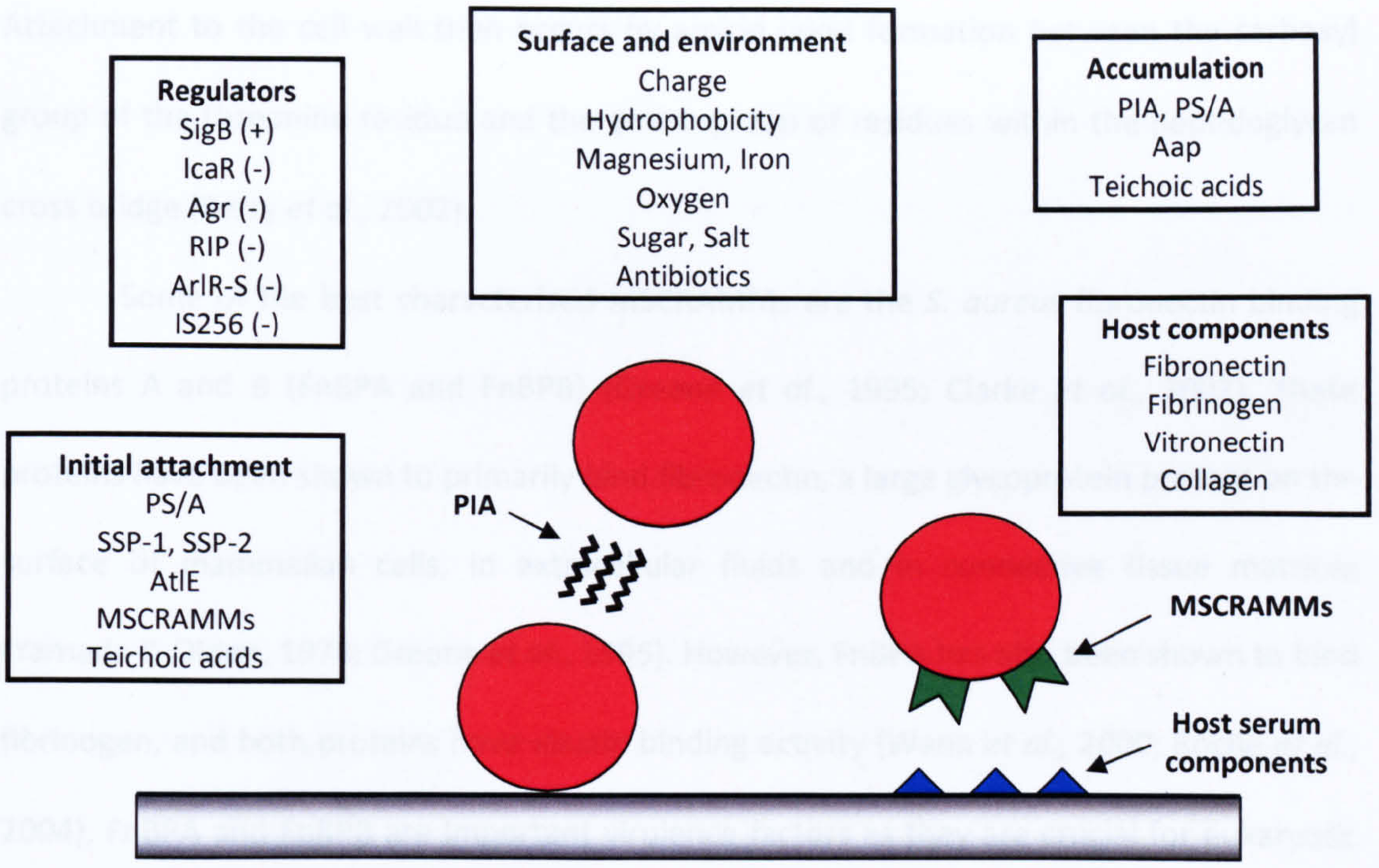

Figure 1.2 A summary of the bacterial, host and environmental components that influence biofilm formation in staphylococci. Adapted from Cramton & Götz (2004).

Staphylococci utilise a broad range of surface proteins that interact with host extracellular matrix molecules and serum proteins. These proteins, termed MSCRAMMs (microbial surface components recognising adhesive matrix molecules), interact with host components to bring about surface adhesion (Patti *et al.*, 1994). The most common host cellular components bound by MSCRAMMS are fibronectin, fibrinogen, collagen and elastin (Patti *et al.*, 1992; Greene *et al.*, 1995; McDevitt *et al.*, 1995; Keane *et al.*, 2007). However, binding of vitronectin, haemoglobin, antibodies and cellular lipids by some MSCRAMMs has also been demonstrated (Uhlen & Abrahmsen, 1989; Hussain *et al.*, 2001a; Huesca *et al.*, 2002; Mazmanian *et al.*, 2003). *S. aureus*, in particular, expresses many different MSCRAMMs that are usually covalently attached to the cell wall via a LPXTG motif (Navarre & Schneewind, 1994). Specifically, a membrane-anchored transpeptidase, known as sortase, cleaves the LPXTG sorting signal between the threonine and glycine residues.

Attachment to the cell wall then occurs by amide bond formation between the carboxyl group of the threonine residue and the amino group of residues within the peptidoglycan cross bridge (Perry *et al.*, 2002).

Some of the best characterised MSCRAMMs are the *S. aureus* fibronectin binding proteins A and B (FnBPA and FnBPB) (Greene *et al.*, 1995; Clarke *et al.*, 2002). These proteins have been shown to primarily bind fibronectin, a large glycoprotein present on the surface of mammalian cells, in extracellular fluids and in connective tissue matrices (Yamada & Olden, 1978; Greene *et al.*, 1995). However, FnBPA has also been shown to bind fibrinogen, and both proteins have elastin binding activity (Wann *et al.*, 2000; Roche *et al.*, 2004). FnBPA and FnBPB are important virulence factors as they are crucial for eukaryotic cell invasion (Sinha *et al.*, 1999). In terms of biofilm formation, these proteins have been implicated in the adherence of *S. aureus* to surfaces in EPS deficient mutants (O'Neill *et al.*, 2008).

The *S. aureus* clumping factor proteins ClfA and ClfB belong to the serine-aspartate repeat (Sdr) family of proteins and have both been implicated in *S. aureus* heart valve colonisation during bacterial endocarditis (Josefsson *et al.*, 1998; Rindi *et al.*, 2006). In *S. epidermidis*, fibrinogen binding proteins have also been identified, such as SdrG, (also known as Fbe) (Hall *et al.*, 2007). This protein also induces platelet activation and aggregation, and so may contribute to the virulence of *S. epidermidis* in infections such as endocarditis (Brennan *et al.*, 2009).

Staphylococci are also able to adhere to collagen via proteins such as SdrF in *S. epidermidis* and Cna in *S. aureus* (Zong *et al.*, 2005; Arrecubieta *et al.*, 2007). Such proteins contribute to the virulence of these organisms during infections such as osteomyelitis (Elasri *et al.*, 2002).

In *S. aureus*, the major autolysins of *S. aureus* (AtlA) and *S. epidermidis* (AtlE) are bifunctional proteins with amidase and glucosaminidase activity and are found associated with the septum during cell division (Yamada *et al.*, 1996). In addition, both of these enzymes have been implicated in biofilm formation. Indeed, a *S. epidermidis* AtlE mutant generated by transposon insertion was unable to form biofilms on polystyrene surfaces, but retained biofilm forming- capacity on glass surfaces (Heilmann *et al.*, 1996a). The *atlE* locus was subsequently found to encode a 148 kDa protein that is proteolytically modified to yield active 60 kDa amidase and 52 kDa glucosaminidase domains (Heilmann *et al.*, 1997) Furthermore, AtlE possesses polystyrene and vitronectin binding capacity and. upon deletion of *atlE*, *S. epidermidis* displays reduced virulence in a rat central venous catheter model, indicating the importance of this protein in the virulence of *S. epidermidis* (Rupp *et al.*, 2001). Similarly, an *atlA* deletion in *S. aureus* resulted in the formation of large cell clusters and loss of biofilm formation (Biswas *et al.*, 2006).

Teichoic acids are cell wall polymers that often contain alternating glycerol phosphate and ribitol phosphate groups (Baddiley *et al.*, 1961; Baddiley *et al.*, 1962) (Figure 1.3). These polymers have a significant role in staphylococcal initial adherence during biofilm formation. Specifically, a *S. aureus* mutant, defective in D-alanine incorporation into teichoic acids, was unable to adhere to polystyrene or glass surfaces due to a stronger negative surface charge causing repulsive electrostatic forces. Biofilm-forming capacity was restored with the addition of divalent cations to counteract the negative charge (Gross *et al.*, 2001). In *S. epidermidis* these polymers bind fibronectin, in combination with an unknown surface protein, suggesting a potential bridging function between the cells and fibronectin coated surfaces (Hussain *et al.*, 2001b).

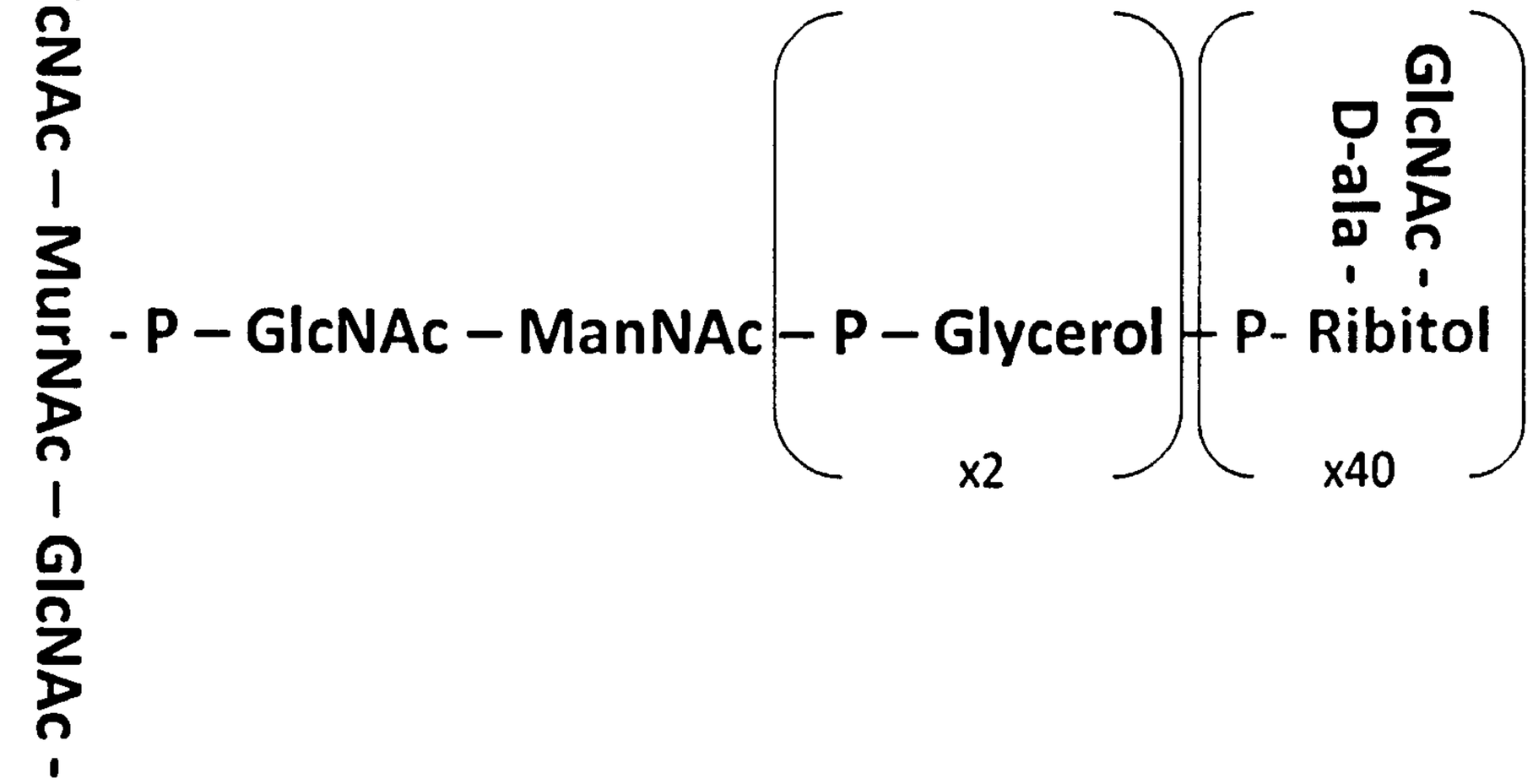

Figure 1.3 Structure of *S. aureus* cell wall teichoic acid. GlcNAc – N-acetylglucosamine, MurNAc – N-acetylmuramic acid, P – phosphate, ManNAc – N-acetylmannosamine, D-ala – D-alanine. Adapted from Xia *et al* (2010).

1.2.1.2. Maturation

Once attachment has occurred, the attached cells begin to divide and form microcolonies (Donlan, 2001a). Cell to cell adhesion then occurs, allowing maturation of the biofilm. One of the most typical features of a biofilm is the EPS component of the matrix, secreted by the adherent cells. In the case of staphylococci, this has gone by many names including slime-associated antigen, PS/A and PIA. For the purposes of this work, it will be referred to as PIA. This polymer, initially identified in *S. epidermidis*, is composed of poly-*N*-acetyl glucosamine (PNAG) and is also present in *S. aureus* (Mack *et al.*, 1994; Mack *et al.*, 1996; Cramton *et al.*, 1999). Synthesis of PIA has been attributed to the intercellular adhesion (*ica*) operon, which contains both a regulatory gene (*icaR)* and the biosynthetic genes *icaADBC* (Heilmann *et al.*, 1996b) (Figure 1.4). IcaA, C and D are membrane-associated proteins, whilst IcaB is mainly found in the extracellular fraction. IcaA possesses *N*-acetyl glucosaminetransferase activity,

utilising UDP-*N*-acetylglucosamine as a substrate, and only reaches optimal activity in the presence of IcaD (Gerke *et al.*, 1998). The role of IcaC remains largely unknown; however it has been hypothesised that it may serve to translocate PIA to the cell surface. Furthermore, the presence of IcaAD alone can only yield oligomers of up to a maximum of 20 residues, and IcaC is required for the synthesis of longer chains (Gerke *et al.*, 1998). IcaB deacetylates PIA, a process necessary for attachment of PIA to the cell surface and for biofilm formation (Vuong *et al.*, 2004).

In addition to PIA, proteinaceous structures and teichoic acids, biofilms formed by staphylococci are also dependent on the production of extracellular DNA (eDNA) which forms part of the biofilm matrix. In S. aureus, the production of eDNA via cell lysis is controlled on the Cid/Lrg system. Specifically, the *cidA* gene encodes a murein hydrolase and functions to lyse a proportion of the population to release genomic DNA (Rice *et al.*, 2007). Conversely, the *lrgAB* genes encode a proposed antiholin and function to inhibit cell lysis (Mann *et al.*, 2009). Furthermore, transcription from the *cidABC* and *lrgAB* operons is activated by CidR and the LytSR TCS, respectively (Yang *et al.*, 2005; Sharma-Kuinkel *et al.*, 2009). Subsequent investigation has also demonstrated that *S. aureus* beta toxin binds to the eDNA and other proteins to form a nucleoprotein matrix (Huseby *et al.*, 2010). Furthermore, although the production of PIA, by the expression of the *ica* operon, undoubtedly has an important role in biofilm formation in many staphylococcal strains, other studies have identified staphylococcal isolates lacking this operon that can still form biofilms (Ziebuhr *et al.*, 1997; Fitzpatrick *et al.*, 2002; Fitzpatrick *et al.*, 2005). Indeed, deletion of the *ica* operon in the human clinical isolate UAMS-1 had no effect on biofilm formation (Beenken *et al.*, 2004).

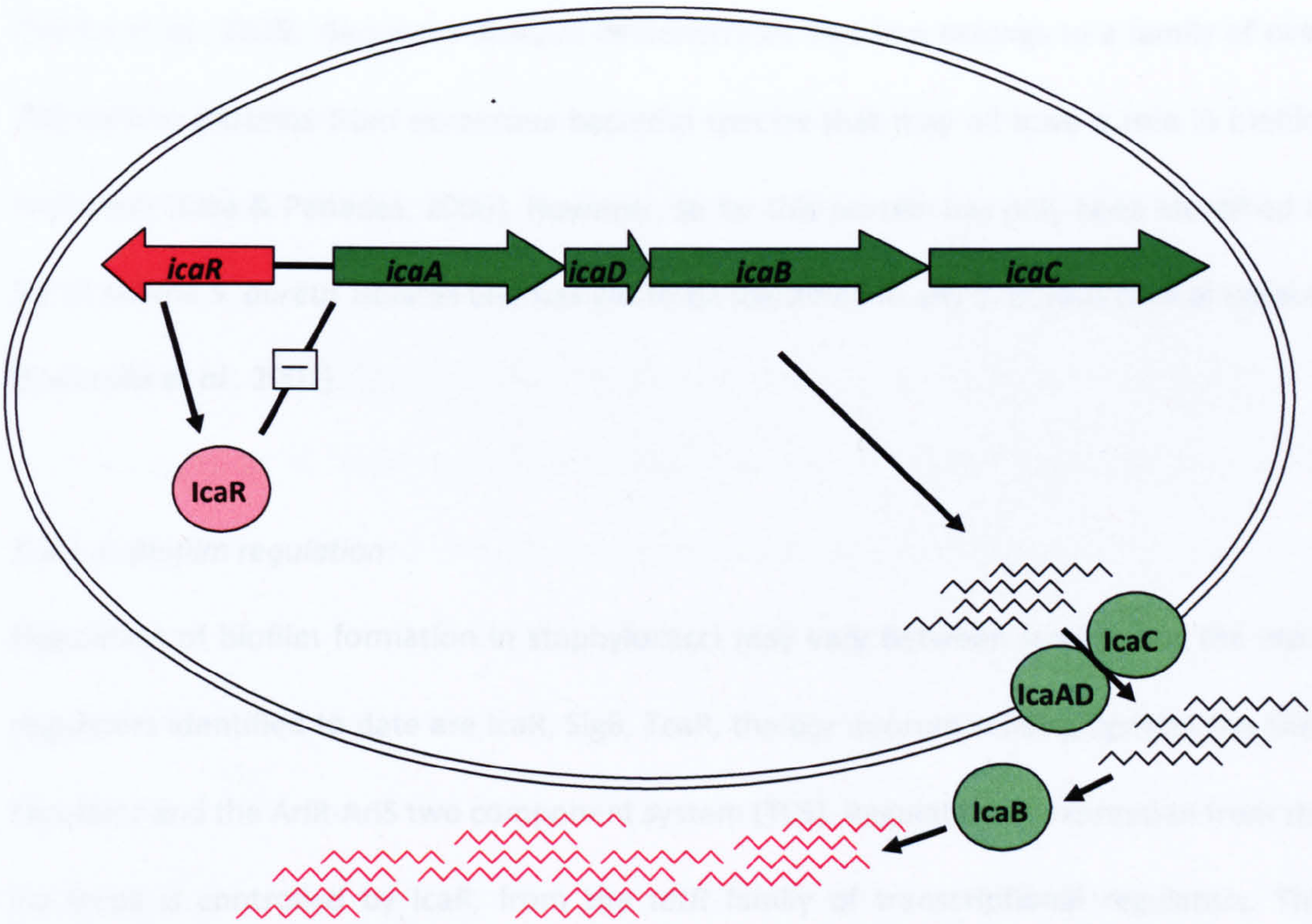

Figure 1.4 Production of PIA from the *ica* operon. IcaAD and IcaC are membrane-associated proteins that function to yield PNAG oligomers and translocate them across the membrane. IcaB then deacetylates the oligomers to associate them with the cell surface. Expression of IcaR results in down regulation of the *icaADBC* genes. Adapted from O'Gara (2007).

Several other genes have also been implicated in biofilm maturation. The accumulation associated protein (Aap), for example, was identified in a mutant strain of *S. epidermidis* derived from strain RP62A. This mutant, M7, lacks Aap and although capable of surface adhesion, is deficient in accumulation (Schumacher-Perdreau *et al.*, 1994; Hussain *et al.*, 1997). Interestingly, this strain was still able to produce PIA (Baldassarri *et al.*, 1996). The biofilm-associated protein (Bap), which was first identified in a bovine mastitis isolate of *S. aureus*, also confers biofilm forming capacity (Cucarella *et al.*, 2001). Mutations in the *ica* operon do not affect biofilm formation in this isolate (Cucarella *et al.*, 2004). Furthermore, a homologue of Bap has been identified in *S. epidermidis* and other CNS

(Tormo *et al.*, 2005). Sequence analysis demonstrates that Bap belongs to a family of over 100 surface proteins from numerous bacterial species that may all have a role in biofilm formation (Lasa & Penades, 2006). However, so far this protein has only been identified in 5% of bovine *S. aureus* isolates and has yet to be identified in any *S. aureus* clinical isolates (Cucarella *et al.*, 2001).

1.2.1.3. Biofilm regulation

Regulation of biofilm formation in staphylococci may vary between strains, but the main regulators identified to date are IcaR, SigB, TcaR, the *agr* quorum sensing system, the SarA regulator and the ArlR-ArlS two component system (TCS). Regulation of expression from the *ica* locus is controlled by IcaR, from the *tetR* family of transcriptional regulators. This transcriptional repressor, located upstream of *icaADBC,* binds to the promoter region near the *icaA* start codon (Conlon *et al.*, 2002a; Jefferson *et al.*, 2004). Activation of *icaADBC* can occur through environmental stimuli in an IcaR-dependent manner. For example, addition of sodium chloride or ethanol can repress IcaR, therefore enhancing expression from *icaADBC* (Conlon *et al.*, 2002b).

The teicoplanin-associated locus regulator (TcaR), of the MarR family of transcriptional regulators, has also been shown to regulate expression from the *ica* operon negatively, but deletion of this gene alone is not sufficient to prevent PIA production (Jefferson *et al.*, 2004). Further investigation revealed this regulation was due to the ability of TcaR to bind three 33 bp regions close to the IcaR binding region of the *ica* operon (Chang *et al.*, 2010). Furthermore, the alternative sigma factor, SigB, and its positive regulator RsbU, have been shown to regulate biofilm formation in *S. epidermidis* and *S. aureus* (Rachid *et al.*, 2000a; Knobloch *et al.*, 2001). In *S. epidermidis*, SigB controls expression from the *ica* operon by repressing transcription of *icaR* (Knobloch *et al.*, 2004).

In *S. aureus*, SigB induces biofilm formation under stress conditions, and SigB negative mutants are deficient in biofilm formation (Rachid *et al.*, 2000a). It has also been shown to control expression from the *agr* system in *S. aureus,* for maintaining *ica*-independent biofilms which lack PIA (Lauderdale *et al.*, 2009).

The accessory gene regulator (*agr*) system is a staphylococcal quorum sensing system (Recsei *et al.*, 1986; Otto *et al.*, 1998). The TCS conducting signal transduction for the *agr* system is composed of AgrA, the response regulator, and AgrC, the histidine kinase sensor (Lina *et al.*, 1998). Activation of the system occurs via an AgrD derivative, a thiolactone peptide pheromone, export of which is dependent on AgrB activation (Ji *et al.*, 1995). Many *S. aureus* clinical isolates carry mutations in the *agr* system, which confers enhanced ability to form biofilms on polystyrene *in vitro*, suggesting that such strains may be more able to colonise implanted devices *in vivo* (Vuong *et al.*, 2000). Furthmore, *agr* mutants have been shown to display reduced exotoxin production and increased surface protein production. Specifically, during colonisation expression of the *agr* system is minimal to allow surface adherence and biofilm formation. Expression is then increased, and via RNAIII, the main intracellular effector of the *agr* system, surface protein expression is reduced and exotoxin production is increased, potentially to aid nutrient acquisition *in vivo* (Recsei *et al.*, 1986). In contrast with *agr*, mutation of *sarA* in *S. aureus* results in loss of biofilm forming capacity due to an increase in the production of extracellular proteases and nucleases (Beenken *et al.*, 2010). Indeed, the addition of protease inhibitors and inactivation of nuclease, via a *nuc* mutation, in sarA mutants can restore biofilm formation (Tsang *et al.*, 2008). The autolysis-related (*arl)* locus in *S. aureus* is another TCS which influences biofilm formation. This locus encodes the predicted ArlR-ArlS sensor kinase-response regulator system. An *arlS* transposon insertional mutant of *S. aureus* displayed

enhanced polystyrene adherence compared with the wild-type parent (Fournier & Hooper, 2000).

In addition to IcaR and TcaR, insertion of IS*256*, an insertion sequence element, into the *ica* genes, *rsbU* of the *sigB* operon and *sarA* locus has been shown to regulate PIA production negatively, resulting in a biofilm-positive to biofilm-negative phenotypic switch in *S. epidermidis* (Conlon *et al.*, 2004). Similarly, insertion of IS*256* into genes involved in biofilm formation in *S. aureus* is also associated with a switch to a biofilm-negative phenotype. In this case this process is negatively regulated by SigB, as the number of copies of this insertion sequence on the chromosome, and therefore the rate of phenotypic switching, was significantly increased in mutants lacking this transcription factor (Valle *et al.*, 2007).

1.2.1.4. Environmental factors

In addition to the cellular factors discussed so far, many environmental stimuli also modulate biofilm formation. Indeed, enhanced expression from the *ica* operon occurs during anaerobic conditions, compared with aerobic incubation, in both *S. aureus* and *S. epidermidis* (Cramton *et al.*, 2001).

The availability of sugars also influences biofilm formation. The addition of D-glucose, saccharose, lactose, D-galactose, maltose and fructose to growth media, induce expression of PIA in *S. epidermidis* (Mack *et al.*, 1992). Similarly, the addition of ≥0.25% glucose (w/v) to brain-heart infusion broth enhances PIA production in *S. aureus* (McKenney *et al.*, 2000).

Metals such as iron, magnesium and calcium also affect biofilm production (Dunne & Burd, 1992; Deighton & Borland, 1993). *S. epidermidis* biofilm formation is increased in iron-limiting conditions (Deighton & Borland, 1993). In contrast, increasing concentrations

of magnesium or calcium increase biofilm formation in *S. epidermidis*, most likely through alteration of cell surface charge (Dunne & Burd, 1992). In a similar manner, the addition of alcohol results in increased PIA synthesis and biofilm formation. In particular, ethanol, isopropanol and n-propanol from skin disinfectants increase biofilm formation in *S. epidermidis* (Knobloch *et al.*, 2002).

Perhaps most worrying are reports implicating some antibiotics as biofilm inducers. For example, increased expression from the *ica* operon was observed in *S. epidermidis* in the presence of subinhibitory concentrations of tetracycline, quinupristin-dalfopristin and erythromycin (Rachid *et al.*, 2000b).

1.2.2. Staphylococcal biofilm infections

1.2.2.1. Medical devices

Modern medicine increasingly requires the use of indwelling medical devices to prolong and improve the quality of life for many individuals. These devices however, as with most medical interventions, are associated with side effects. One of the most common and serious of these is the potential for infection as these devices provide a platform for microbial colonisation and biofilm formation (Figure 1.5). Specifically, medical devices can become contaminated with bacteria which adhere and develop into biofilms thereby acting as a source of infection. Staphylococci, in particular, are able to adhere to numerous surface types and consequently a variety of indwelling devices. These include, but are not limited to, central nervous system shunts (Conen *et al.*, 2008), contact lenses and other ocular implants (Green *et al.*, 2008), endotracheal tubing (Estes & Meduri, 1995), intravascular devices (Sitges-Serra & Girvent, 1999), mechanical heart valves and cardiac pacemakers (Heimberger & Duma, 1989), urinary catheters (Weigel *et al.*, 2007) and replacement joints (Khardori & Yassien, 1995). The severity of the resulting infections

following medical device colonisation varies, depending on both the nature and location of the device and the immunological status of the individual.

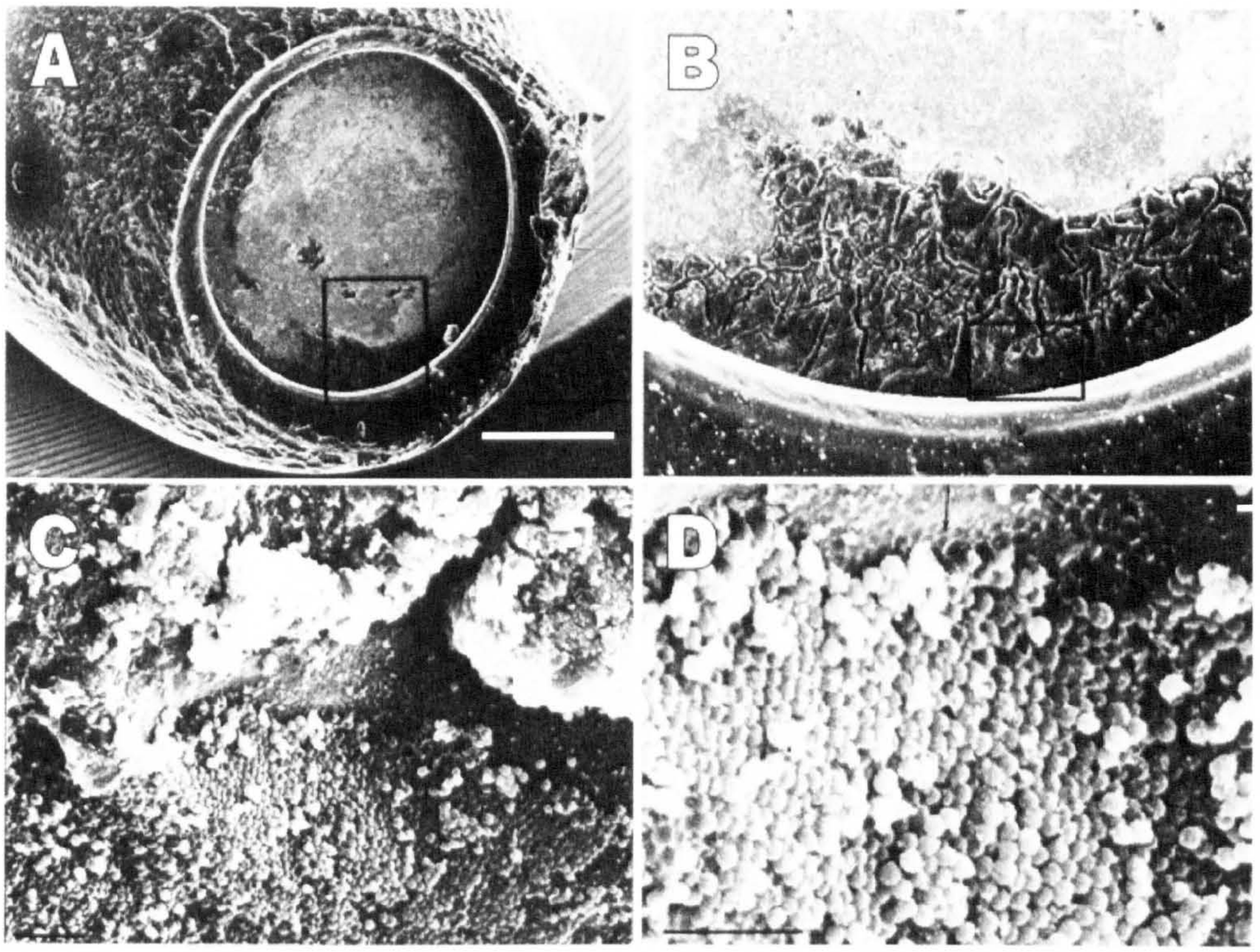

Figure 1.5 Scanning electron micrographs of a cardiac pacemaker lead colonised with *S. aureus* isolated from a patient who had suffered three episodes of *S. aureus* bacteraemia. **A** The colonised pacemaker tip (bar = 500 μm). **B** A magnified view of the quadrangle indicated in A (Bar = 50 μm). **C** A magnified view of the quadrangle indicated in B (Bar = 5 μm). **D** A magnified view of the same area as in C (Bar = 5 μm) (Marrie *et al.*, 1982).

Furthermore, following colonisation by a single species, the resulting mature biofilms found on indwelling medical devices are often polymicrobial in nature. Urinary catheters, for instance, are primarily colonised by *S. epidermidis, S. aureus, E. coli, P. mirabilis* or *Enterococcus faecalis*. During colonisation and development of the biofilm it remains a monoculture, but as the biofilm matures it can incorporate other species

including *Klebsiella pneumoniae*, *P. aeruginosa*, *Providencia stuartii* and *P. mirabilis* (Stickler, 1996).

1.2.2.2. Cutaneous infections

S. aureus is the causative organism of an array of cutaneous infections including pimples, impetigo, furuncles, carbuncles, cellulitis, abscesses and staphylococcal scalded skin syndrome (SSSS) (Tenover & Gorwitz, 2006). The role of biofilms in such infections remains poorly understood, although it has been established that *S. aureus* produces biofilms on damaged skin tissue and that this may be significant during the skin infection process (Akiyama *et al.*, 2002; Akiyama *et al.*, 2003). Furthermore, it has also been demonstrated in a mouse model, that biofilms of both *S. aureus* and *S. epidermidis* significantly inhibit re-epithelialisation, i.e. wound healing (Schierle *et al.*, 2009).

1.2.2.3. Native valve endocarditis (NVE)

NVE is a condition that results from bacteria or fungi entering the bloodstream and subsequent colonisation of the vascular endothelium of the heart valves (Donlan, 2001b). In the absence of any indwelling medical device or prior cardiac surgery, NVE is frequently associated with intravenous drug abuse, where right-sided valvular endocarditis, in particular, often occurs due to the introduction of organisms into the bloodstream (Robbins *et al.*, 1986). NVE typically develops on healthy heart valves, however damaged heart valves are also colonised as these abnormalities promote valvular thrombus formation (Weinstein & Schlesinger, 1974). A valvular thrombus is a mass of fibrin, platelets and erythrocytes that forms on the heart valve tissue (Riddle *et al.*, 1989 672). This acts as a point of attachment for bacterial cells resulting in the formation of a vegetation (Scheld *et al.*, 1978). Causative organisms of NVE form a diverse group ranging from viridans streptococci, enterococci,

pneumococci, staphylococci, including *S. aureus,* to Gram negative bacteria and fungi (Tunkel & Mandell, 1992). In the case of *S. aureus,* fibrinogen and fibronectin binding result in adherence to the thrombus and the subsequent vegetation formation (Que *et al.*, 2005). It has been suggested that cell wall teichoic acids of *S. aureus* may also have a role in the adherence to endothelial cells (Weidenmaier *et al.*, 2005).

1.2.2.4. Osteomyelitis

Osteomyelitis is the infection of bone or bone marrow as a result of the spread of microorganisms either haemotogenously or contiguously (Lew & Waldvogel, 1997). Haemotogenous osteomyelitis results in the seeding of the bone material with microorganisms from the bloodstream. Contiguous osteomyelitis, results from the introduction of microorganisms into the bone from surrounding tissues, such as penetrating tissue trauma, localised tissue infection or from a contaminated prosthetic joint (Lew & Waldvogel, 2004). Various species are capable of causing osteomyelitis in different age groups, but *S. aureus* remains the leading cause of osteomyelitis for all ages (Brady *et al.*, 2008).

1.2.2.5. Cystic fibrosis (CF)

CF is one of the most commonly inherited diseases in humans (O'Sullivan & Freedman, 2009). It is characterised by a decrease in electrolyte secretion plus an increase in the absorption of airway surface liquid by the lung epithelium, resulting in the formation of thick, dehydrated and sticky mucus (Reisin *et al.*, 1994; Matsui *et al.*, 1998). Increased mucus viscosity inhibits the normal mucociliary clearance mechanism by which cilia sweep mucus upwards to remove inhaled material from the lungs, thereby keeping them relatively sterile (Koch & Høiby, 1993). The deficiencies associated with CF allow the persistence of

pathogens in the lungs permitting colonisation and infection. *S. aureus* is among the first organisms to colonise the CF lung but can be controlled by antibiotic therapy. *Haemophilus influenzae* is also capable of colonisation and can coexist with *P. aeruginosa.* However as antibiotic therapy increases *P. aeruginosa* tends to predominate due to innate and acquired resistance to many antibiotic classes (May *et al.*, 1991). Other organisms reported as causing infection in the cystic fibrosis lung, particularly during the later stages of the disease, include non-tuberculous mycobacteria, members of the *Burkholderia cepacia* complex and *Stenotrophomonas maltophilia* (Isles *et al.*, 1984; Karpati *et al.*, 1994; Esther *et al.*, 2010)

1.3. Antibiotic recalcitrance in biofilms

Bacterial biofilms are highly refractory to inhibition and killing by most classes of antimicrobials including disinfectants and antibiotics (Figure 1.6). This phenomenon has been widely reported upon in the literature, and occurs in a broad spectrum of organisms. Currently, a general mechanism for this broad spectrum recalcitrance remains elusive. Several studies have demonstrated inhibition of antibiotic penetration by the biofilm matrix (Shigeta *et al.*, 1997), whilst others have attributed resistance to slow growth for reasons such as oxygen limitation (Debeer *et al.*, 1994). In addition, there are also reports of increased gene transfer and genetic mutation within biofilms that may promote the emergence of antibiotic resistant subpopulations (Cvitkovitch, 2004; Allegrucci & Sauer, 2008).

1.3.1. Limitation of antibiotic penetration

Inhibition of antibiotic penetration into the core of biofilms has been demonstrated for several organisms. The aminoglycosides amikacin and gentamicin, for example, cannot penetrate *P. aeruginosa* biofilms (Shigeta *et al.*, 1997). This may be due to the positively charged aminoglycosides binding to the negatively charged alginate (Gordon *et al.*, 1988). Furthermore, the penetration of oxacillin, cefotaxime and vancomycin through *S. aureus* and *S. epidermidis* biofilms is limited (Singh *et al.*, 2010b). However, the growth of *S. aureus* and *S. epidermidis* as a biofilm causes no inhibition of amikacin or ciprofloxacin penetration (Singh *et al.*, 2010b). These findings suggest that although antibiotic penetration limitation may contribute to the refractory nature of biofilm cultures, it does not account for all antibiotic recalcitrance observed in biofilms.

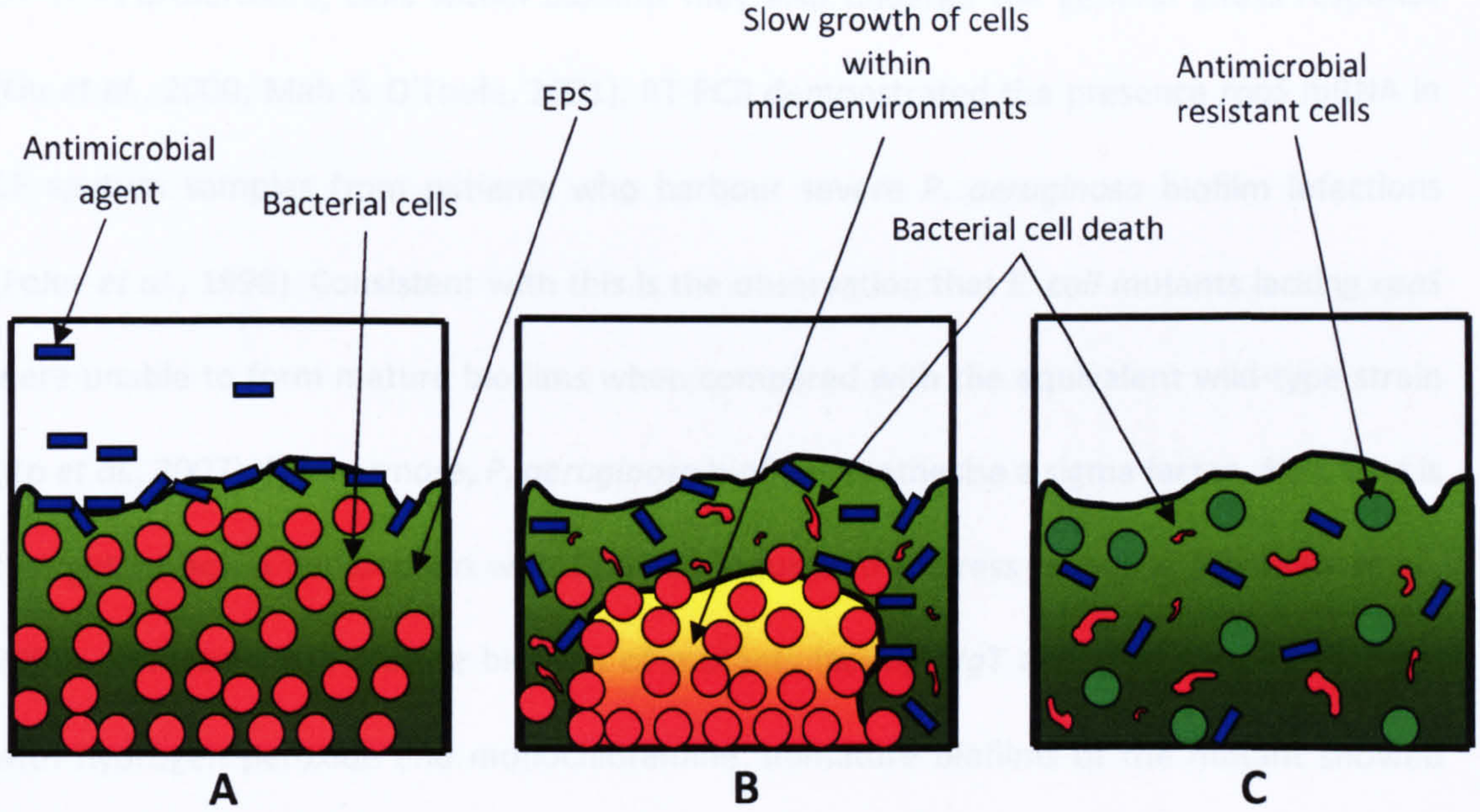

Figure 1.6 The proposed mechanisms by which biofilms resist killing by antibiotics and other antimicrobial agents. **A** Penetration limitation of the antibiotic by the matrix. **B** Microenvironments reduce bacterial growth resulting in recalcitrance. **C** Survival of phenotypically or genetically resistant organisms due to persistence, genetic transfer or mutation.

1.3.2. Microenvironments and slow growth

Mature biofilms harbour populations of cells with slower growth rates than planktonic cells (Wentland *et al.*, 1996). Cells with diminished growth rates are thought to exacerbate the recalcitrance of biofilms to antibiotics. *P. aeruginosa* biofilms, for example, show decreasing susceptibility to ciprofloxacin with decreasing growth rate (Evans *et al.*, 1991). One factor responsible for decreasing the growth rate of bacteria in biofilms may be oxygen depletion (Debeer *et al.*, 1994). This is demonstrated by *P. aeruginosa* biofilms where ciprofloxacin, carbenicillin, tobramycin, ceftazidime, chloramphenicol and tetracycline recalcitrance was observed as a result of the reduced growth rate of the bacterial cells following oxygen limitation (Borriello *et al.*, 2004).

Furthermore, cells within biofilms may also undergo the general stress response (Liu *et al.*, 2000; Mah & O'Toole, 2001). RT-PCR demonstrated the presence *rpoS* mRNA in CF sputum samples from patients who harbour severe *P. aeruginosa* biofilm infections (Foley *et al.*, 1999). Consistent with this is the observation that *E. coli* mutants lacking *rpoS* were unable to form mature biofilms when compared with the equivalent wild-type strain (Ito *et al.*, 2007). Furthermore, *P. aeruginosa* biofilms synthesise a sigma factor, AlgT, that is thought to act in conjunction with RpoS to regulate the stress response (Cochran *et al.*, 2000). Immature and mature biofilms of mutants lacking *algT* and *rpoS* were challenged with hydrogen peroxide and monochloramine. Immature biofilms of the mutant showed increased susceptibility to hydrogen peroxide, but retained recalcitrance to monochloramine when compared with the wild-type strain. In contrast, mature biofilms of both the mutant and wild-type were recalcitrant to both antimicrobial agents (Cochran *et al.*, 2000). This demonstrates that the stress response only provides partial protection against antimicrobial agents, consequently implying the existence of alternative recalcitrance mechanisms within bacterial biofilms.

1.3.3. Persistence

Bacterial persistence, originally identified in *S. aureus*, is a phenomenon whereby a small proportion (~10^{-6}) of a bacterial population is phenotypically tolerant to antibiotics (Bigger, 1944). These persister cells differ from genetically antibiotic resistant mutants as their tolerance to antibiotics is non-inheritable (Jayaraman, 2008). The mechanisms surrounding bacterial persistence remain unclear. However, the isolation of a high persister (*hip*) mutant of *E. coli* identified the *hip* set of genes as significant in this phenomenon (Moyed & Bertrand, 1983). Subsequent isolation and transcriptional analysis of other *hip* mutant persisters, such as the *hip A7* mutant of *E. coli,* identified increased expression of many genes, notably those encoding toxin-antitoxin (TA) modules (Keren *et al.*, 2004). TA

modules generally consist of two genes, one encoding a stable protein, or 'toxin', that functions to inhibit a specific cellular process, and an unstable protein or antisense RNA, otherwise known as an 'antitoxin', functioning to inhibit the action of the 'toxin' and autoregulate the expression of the TA module (Gerdes *et al.*, 2005). It has already been established that TA modules have an important role in plasmid maintenance (Faridani *et al.*, 2006), but their other roles in the bacterial cell are not completely understood. Nevertheless, there are reports of their involvement in programmed cell death (Aizenman *et al.*, 1996) and, perhaps most intriguingly, there is evidence for their role as inhibitors of macromolecular synthesis, leading to bacteriostasis (Christensen *et al.*, 2001). In this case, it has been postulated that inhibition of the synthesis of cellular macromolecules, such as DNA, RNA and protein, may lead to the "shielding" of antibiotic targets, resulting in insensitivity to said antibiotics.

The presence of persister cells within biofilms has been suggested to significantly contribute to their antibiotic recalcitrance. The *E. coli yafQ* mutant (part of the *dinJ-yafQ* TA module), for example, can form biofilms comparable to the parental stain. However, exposure to cefazolin or tobramycin results in up to 2400-fold reduction in cell survival compared to wild-type biofilms (Harrison *et al.*, 2009).

1.3.4. Genetic transfer and mutation

In addition to investigating phenotypic antibiotic recalcitrance, some studies have identified increased rates of genetic mutation and gene transfer, conferring antibiotic resistance (Cvitkovitch, 2004; Allegrucci & Sauer, 2008). In fact, it is thought that biofilms may represent an optimal environment for cell-to-cell processes to occur due to the close proximity of the cells to one another, resulting in increased diversity within the population.

1.3.4.1. Genetic transfer within biofilms

Bacteria can acquire foreign DNA by transformation, conjugation and transduction. Transduction is the process of DNA transfer between bacteria via bacteriophages. This is regarded as the most prevalent form of horizontal gene transfer in *S. aureus*, where phages such as ϕ11 and 80α transfer genes between strains (Cohen *et al.*, 1977; Christie *et al.*, 2010; Lindsay, 2010). Tranformation is the process whereby bacteria bind, absorb and propagate foreign DNA from the surrounding environment. This occurs at increased rates amongst biofilm cultures compared with equivalent planktonic cultures (Maeda *et al.*, 2006; Blokesch & Schoolnik, 2008). For example, biofilms of *V. cholerae* grown on chitin absorb extracellular DNA at a higher rate than planktonic cultures. This was shown, in part, to result from repression of the extracellular nuclease, Dns, upon adherence to chitin surfaces (Blokesch & Schoolnik, 2008). Similarly, nonconjugative transfer of plasmid DNA occurs at high frequencies in biofilms of *E. coli*, whereas equivalent planktonic cultures showed little or no transformation (Maeda *et al.*, 2006). Furthermore, DNA is an important structural component of the biofilm matrix (Tetz *et al.*, 2009). In streptococci the competence-stimulation peptide (CSP) cell-to-cell signal is involved in competence for transformation, biofilm formation and autolysis. CSP controls genes involved in extracellular DNA binding and uptake. Mutations within one such gene, *comGB*, not only reduce DNA binding and uptake but also reduce biofilm formation. To complement these findings the presence of DNase during growth of the wild-type strain reduced biofilm formation to that of the *comGB* mutant, suggesting that DNA forms a necessary structural component of the biofilm matrix (Petersen *et al.*, 2005).

Conjugation in Gram negative bacteria has been the most extensively characterised, where active transfer of genetic material occurs through direct contact via a fertility (F) factor. In its simplest form, this fertility factor takes the form of the F pilus,

which is found only in F+ strains. The majority of genes necessary for the assembly of the pilus and the transfer of genetic material are found on the *tra* operon on the F factor. The F pilus initiates contact between the cells and then draws them closer via contraction. Transfer of the plasmid, via *tra* encoded type IV secretory system, from donor to recipient, renders the recipient F+. The transfer of chromosomal DNA may also occur as a result of chromosomal integration and excision by the plasmid (Cvitkovitch, 2004). In Gram positive bacteria, the process of conjugation is not as well characterised, but based on sequence analysis of conjugative plasmids, significant similarities have been found in the transfer (*tra*) region, but with an absence of pilus-encoding genes (Berg *et al.*, 1998; Dougherty *et al.*, 1998; Grohmann *et al.*, 2003).

It is well established that the growth of bacteria on filters can significantly increase rates of conjugative transfer (Netherwood *et al.*, 1999). Not only is the chance of cell-to-cell contact increased, but it is also thought that some genes involved in conjugation may be upregulated upon surface adherence. To assess these processes, numerous studies have been conducted to identify increased conjugative gene transfer in biofilms. *E. faecalis*, for example, was shown to transfer a conjugative plasmid containing a tetracycline resistance marker approximately 100 times greater than the rate observed in planktonic cultures (Cvitkovitch, 2004).

Not only is it likely that conjugation occurs at higher frequencies in biofilm cultures, but some plasmids transferred via this mechanism actually enhance biofilm formation and allow the expansion of the population. For example, the conjugative plasmid pOLA52 encodes the *mrk* operon which is responsible for type 3 fimbriae expression. Mutations within this operon on the plasmid reduce biofilm formation in *E. coli* up to 100-fold and significantly reduce transfer frequencies (Burmolle *et al.*, 2008). Similarly, synergistic biofilm stimulation and formation was observed in a heterogeneous population of

uncharacterised *E. coli* strains in combination with the characterised *E.coli* K-12 strain. Although numerous mechanisms were likely to contribute to biofilm induction, the transfer of natural conjugative plasmids between strains occurred in the strains exhibiting the strongest biofilm stimulating effects (Reisner *et al.*, 2006).

1.3.4.2. Genetic mutation within biofilms

In addition to the transfer of genetic material between organisms in biofilms, it has also been established that bacteria growing within a biofilm can exhibit increased rates of mutation. *Streptococcus pneumoniae* biofilms, for instance, were shown to have up to 55-fold higher mutation frequencies than planktonic cultures, which led to the emergence of variants that differed in capsule production, cell attachment and biofilm formation (Allegrucci & Sauer, 2008). Similarly, increased MFs and the occurrence of double stranded DNA breaks have also been observed in *P. aeruginosa* biofilms, as a consequence of endogenous oxidative stress within the biofilm culture (Boles & Singh, 2008; Driffield *et al.*, 2008).

1.4. Primary objectives of this research programme

Currently, there is little standardisation of model systems for biofilm development, and many of the biofilm models available are either high-throughput but yield immature biofilms, or low-throughput yielding mature biofilms (Ceri *et al.*, 1999; Gander *et al.*, 2005). Most of these systems place limitations on experimental design, such as limitations on incubation time and the flexibility to incorporate additional conditions into the system. Therefore, a novel high-throughput method for generating mature staphylococcal biofilms will be developed. Specifically, cellulose membrane disks, incubated with human plasma, will be used as a substratum for biofilm formation (Foster & Hook, 1998). Following development, the model will be validated using a number of methods, namely antibiotic susceptibility testing, D-amino acid induced biofilm degradation, transcriptional profiling and microscopy. Specifically, these techniques will be employed to assess the characteristics of cultures grown using this system, and compare these data to those of previously published staphylococcal biofilms. This system will then be employed for investigations of staphylococcal biofilm mutability.

As previously described, biofilms of several bacterial genera have been shown to exhibit elevated mutability when compared with planktonic cultures (Allegrucci & Sauer, 2008; Driffield *et al.*, 2008). To assess whether this phenomenon is observed in staphylococcal biofilms, MFs of cultures grown using both static and flow biofilm models will be compared with planktonic cultures.

The mechanisms driving any observed increases in MF will be elucidated. Previous studies have identified a reduction in the expression of antioxidant enzymes in biofilm culture. This may result in an increase in intracellular reactive oxygen species, and subsequently an increase in MF. With this in mind, antioxidants will be added to biofilm cultures and the resulting MFs determined. In addition, DNA microarray analysis will also be

performed to reveal any changes in gene expression that may account for increases in MF in biofilm culture.

CHAPTER 2

2. Materials and Methods

2.1. Bacterial strains

Bacterial strains used throughout this study are described in Table 2.1.

Table 2.1 Bacterial strains used in this study.

Strain name	Organism	Comments	Source or Reference	Selection agent(s)
SH1000	*Staphylococcus aureus*		(Horsburgh *et al.*, 2002a)	None
UAMS-1	*Staphylococcus aureus*	Proficient biofilm forming strain	(Gillaspy *et al.*, 1995)	None
RP62A	*Staphylococcus epidermidis*	Proficient biofilm forming strain	ATCC35984	None
Newman	*Staphylococcus aureus*		(Clauditz *et al.*, 2006)	None
Newman Δ*crtM*	*Staphylococcus aureus*	Deletion of the staphyloxanthin synthesis gene *crtM*	(Clauditz *et al.*, 2006)	5 µg/ml chloramphenicol
MHK11AM	*Staphylococcus aureus*	*sodA* and *sodM* defective mutant	Gift from Prof S J Foster	5 µg/ml tetracycline and erythromycin
KC043	*Staphylococcus aureus*	*katA* and *ahpC* defective mutant	Gift from Prof S J Foster	5 µg/ml tetracycline and erythromycin
SH1000 *lux*	*Staphylococcus aureus*	Carries *lux ABCDE* Km cassette on chromosome	(Holland *et al.*, 2008)	50 µg/ml kanamycin
SH1000 W1	*Staphylococcus aureus*	Isolated from SH1000 biofilm culture	SH1000	None
SH1000 W2	*Staphylococcus aureus*	Isolated from SH1000 biofilm culture	SH1000	None
SH1000 LP1	*Staphylococcus aureus*	Isolated from SH1000 biofilm culture	SH1000	None
SH1000 LP2	*Staphylococcus aureus*	Isolated from SH1000 biofilm culture	SH1000	None

2.2. Bacterial growth media, chemicals and reagents

All growth media were purchased from Oxoid (Basingstoke, Hampshire, UK). All chemicals and antibiotics were purchased from Sigma-Aldrich (Dorset, UK), with the following exceptions. Mupirocin was donated by GlaxoSmithKline (Brentford, Middlesex, UK) and taurine and vanillin were purchased from MP Biomedicals (Illkirch, France). Rifampicin was solubilised in 50% dimethyl sulphoxide and mupirocin was solubilised in 50% ethanol.

2.3. Microbiological techniques

2.3.1. Standard growth conditions

Bacterial strains (Table 2.1) were grown at 37°C on Mueller-Hinton agar (MHA). Single colonies isolated on MHA plates were inoculated into Mueller Hinton broth (MHB) and incubated at 37°C with aeration to yield stationary phase cultures.

2.3.2. Determination of bacterial susceptibility to antibiotics

Minimum inhibitory concentrations (MICs) of antibiotics were determined by generating 2-fold serial dilution series of the antibiotics (BSAC, 1991). In the case of broth culture MICs, bacterial suspensions (10^6 cfu/ml) were combined with the antibiotic dilutions in 96-well microtitre plates (Nunc, Roskilde, Denmark) and incubated at 37°C for 18 hrs with shaking at 450 rpm (Titramax 1000, Heidolph, Essex, UK). When determining MICs using solid media, antibiotic dilutions were incorporated into MHA. Bacterial suspensions were then spotted onto these plates (10^6 cfu/spot), which were subsequently incubated at 37°C for 18 hrs. The MIC was defined as the lowest concentration of the antibiotic at which no visible bacterial growth was observed.

2.3.3. Biofilm culture

2.3.3.1. Cellulose disk biofilm model

The disk biofilm model utilised mixed cellulose ester membrane filter disks (25 mm diameter, 0.22 µm pore size [Millipore, Billerica, MA, USA]) as a substratum for the propagation of staphylococcal biofilms. Sterile disks were preincubated in 4% (v/v) human plasma (Normal pooled human plasma with sodium citrate [Sera Laboratories International, Bolney, West Sussex, UK]), diluted in 0.05 M carbonate buffer (capsules [Sigma-Aldrich, Dorset, UK]), at 4°C overnight (Griffiths & O'Neill, 2009). The disks were then inoculated with a stationary phase culture of the test organism. Inoculated disks were subsequently placed on brain heart infusion agar (BHA) and incubated at 37°C for a minimum for 48 hrs, to allow adequate growth of at least 10^9 cfu/disk (Figure 2.1). To remove loosely-associated non-adherent cells, the disks were washed in sterile saline with gentle agitation. For *S. aureus* biofilms, adherent cells were then removed from the disks by incubation at 37°C for 30 mins in buffered cellulase (1 mg/ml in 0.05 M citrate buffer [10x stock solution: 0.5 M sodium citrate and 0.5 M citric acid to pH 4.6]), which degrades the biofilm exopolysaccharide (Cescutti *et al.*, 1998). In the case of *S. epidermidis* biofilms, cells were liberated from the disks by incubation in sodium metaperiodate (625 µM sodium metaperiodate and 3.125 mM sodium acetate, diluted in PBS) for 1 hr at 37°C, as this also degrades PIA (Kogan *et al.*, 2006). Following incubation all samples were vigorously vortexed for 5 mins. In both cases, detached cells were subsequently washed in sterile saline and centrifuged for 10 mins at 5000 x *g* to pellet the cells, and then resuspended in sterile saline. These detachment procedures released all visible adherent material; however, the efficiency of release of the adherent cells was not determined due to resource limitation.

Figure 2.1 Image of *S. aureus* SH1000 96 hr biofilms grown on cellulose disks and incubated on BHA.

2.3.3.2. *Sorbarod biofilm model*

This system utilises small cylindrical filters composed of compacted cellulose fibres contained within a sheath, known as sorbarod filters, as a substratum for biofilm formation (Hodgson *et al.*, 1995). The apparatus was assembled with Sorbarod filters (Ilacon, Tonbridge, UK) placed in the compartment at the top of the collection vessel (Figure 2.2). The apparatus was then autoclaved. To promote biofilm formation, 1 ml of 4% (v/v) human plasma was then added aseptically to the sorbarod filters, which were subsequently incubated at 4°C overnight. The apparatus was then incubated at 37°C to prewarm the MHB in the reservoirs prior to inoculation. Filters were inoculated with 1 ml of a stationary phase culture of the test organism (~$1x10^9$ cfu). MHB was then pumped from the reservoirs across the inoculated sorbarod filters at a rate of 1 ml/min using a peristaltic pump (Watson Marlow 205U/CA, Falmouth, England). This not only initiated biofilm formation, but it also provided constant washing of the biofilms, to remove loosely-associated planktonic cells. Waste culture was extracted from the collection vessels every 24 hrs. Sorbarod filters were then harvested from the apparatus and placed in 10 ml saline. To detach the cells from the filter, the biofilms were vigorously vortexed and sonicated for 15 mins. Extended sonication

of up to 30 mins did not yield more cells from the biofilms than those sonicated for 15 mins (data not shown), suggesting that this process was not detrimental to the integrity of the liberated cells.

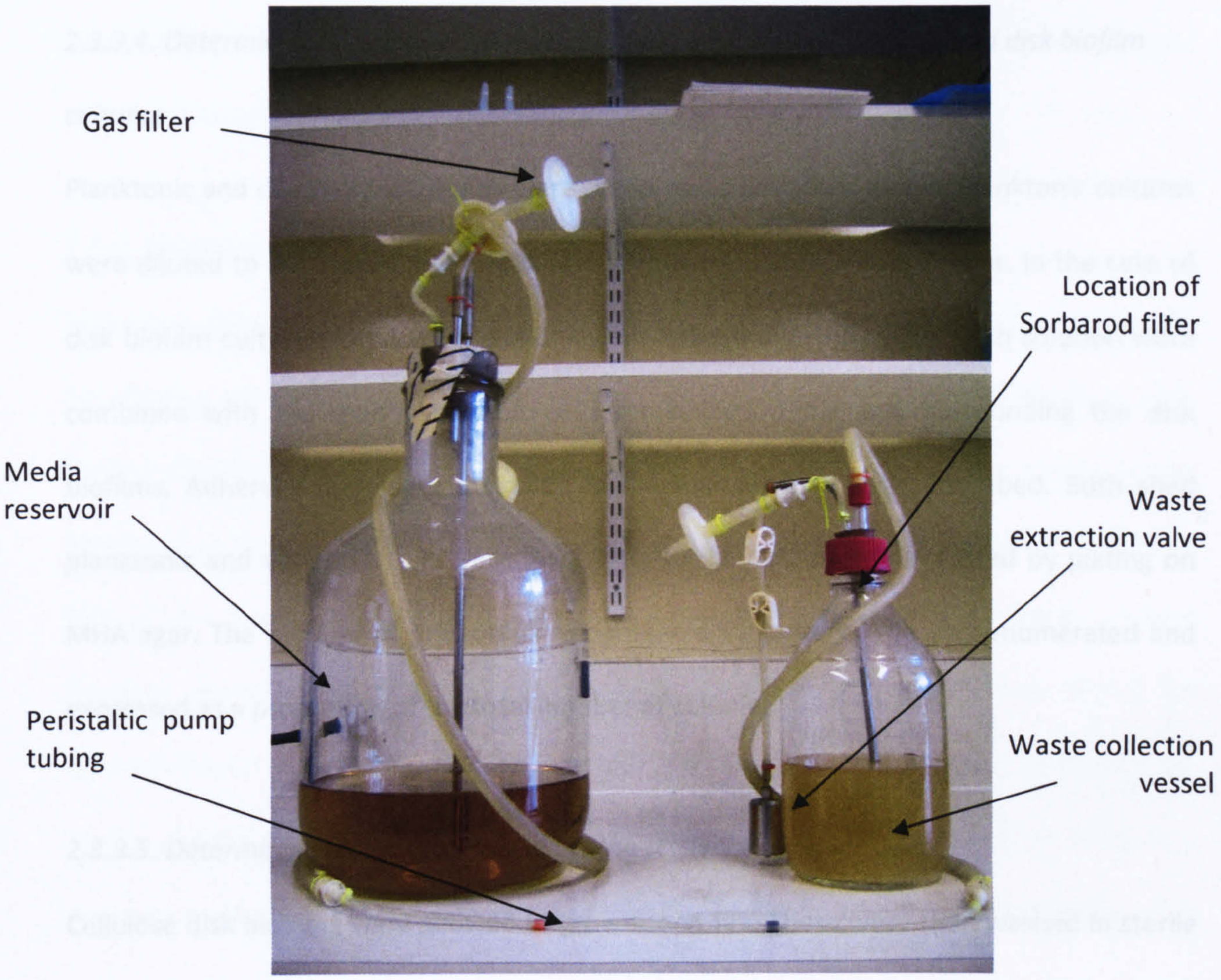

Figure 2.2 Image of the Sorbarod biofilm apparatus.

2.3.3.3. Adherent and planktonic population determination

To determine the proportion of planktonic and adherent cells in cellulose disk biofilms, they were first grown using the method described above. Shed planktonic cells were enumerated by serially diluting the cells washed off during the saline wash step, followed

by plating on MHA agar. Adherent cells were enumerated by serially diluting the resuspended cell pellets after cell detachment, and then plating on MHA agar. The proportion of adherent or planktonic cells was then expressed as a percentage of the sum of both cell counts.

2.3.3.4. Determining the proportion of phenotypic variants in planktonic and disk biofilm cultures

Planktonic and disk biofilm cultures were prepared as described above. Planktonic cultures were diluted to 10^2 cfu/ml and enumerated following plating on MHA agar. In the case of disk biofilm cultures, the shed planktonic cells collected in the saline wash solution were combined with the shed planktonic cells remaining on the agar surrounding the disk biofilms. Adherent cells were detached in the manner previously described. Both shed planktonic and adherent cells were diluted to 10^2 cfu/ml, and enumerated by plating on MHA agar. The number of phenotypic variants in each culture type was enumerated and expressed as a proportion of the total number of colonies.

2.3.3.5. Determining biofilm dissociation in the presence of amino acids

Cellulose disk biofilms were allowed to grow for 48 hrs. These were then washed in sterile saline with agitation for 10 mins to remove loosely-associated planktonic cells. Biofilms were then incubated with either 100 µM L-tyrosine or D-tyrosine (dissolved in PBS), for 30 mins at 37°C with shaking. All biofilms were then vortexed for 1 min, and then incubated with 1 mg cellulase/ml 0.05 M citrate buffer for a further 30 mins at 37°C with shaking. Viable counting was performed on both the amino acid (AA) and cellulase solutions. It was assumed that all cells were dissociated from the cellulose disk following cellulase treatment, as multiple cellulase treatments, or alternative treatments such as proteinase K

(data not shown), do not liberate significantly more organisms from the disks. Dissociation of cells in the presence of AA was then calculated as follows:

$$\% \text{ cells dissociated} = [\text{AA cfu/ml} / (\text{AA cfu/ml} + \text{cellulase cfu/ml})] \times 100$$

Where AA cfu/ml and cellulase cfu/ml represent the viable counts yielded from the AA and cellulase treatments, respectively.

2.3.4. Mutation frequencies

MFs were determined in a similar manner to those described by O'Neill *et al.* (2001). Specifically, planktonic and resuspended biofilm cultures were serially diluted in PBS. Culture dilutions containing between 10^7 and 10^9 cfu/ml (depending on the strain and the antibiotic used for selection) were inoculated onto selection plates (MHA) containing 4x MIC of the selection antibiotic for isolation of spontaneous resistant mutants. Culture dilutions containing ~10^2 cfu/ml were inoculated onto drug free plates (MHA) for enumeration of the total number of viable cells in the culture. Plates were incubated for 48-72 hrs at 37°C and MFs determined as the number of antibiotic-resistant mutants as a proportion of the total cell count. Antibiotic-resistant mutants were subcultured onto selective plates, also containing 4x MIC of the selective agent, to confirm the antibiotic resistant phenotype.

2.3.5. Determining hydrogen peroxide susceptibility

This method was adapted from Clauditz *et al.* (2006). Stationary phase bacteria were resuspended in PBS at a cell density of 10^6 cfu/ml, supplemented with various concentrations of hydrogen peroxide, and incubated at 4°C in the dark for 45 mins. To inactivate the hydrogen peroxide, catalase was added to the samples to a final

concentration of 2 U/ml. Samples were then serially diluted in PBS, plated on MHA and incubated at 37°C overnight. Colonies were then enumerated, and the susceptibility to hydrogen peroxide expressed as the percentage survival of the cells compared with the hydrogen peroxide-free control.

2.3.6. Determination of bacterial doubling times

To determine the doubling times of bacterial strains, their growth was monitored during exponential phase. Specifically, MHB media was inoculated with a 1:200 dilution of a stationary phase culture of the test strain. The OD_{600} value of this culture was then followed until it reached 0.1. OD_{600} measurements were then recorded every 5 mins until the culture reached an OD_{600} value of 0.3. These values were then converted to log_{10} values and plotted against time (mins), and a line of best fit (LBF) calculated. Doubling times for the test strain was then determined using the following formula (Koch, 1994):

Doubling time = $Log_{10}2$/Gradient of LBF

2.3.7. Time-kill determination

Time-kill curves in the presence of 32x MIC rifampicin were determined for *S. aureus* planktonic and biofilm cultures. Planktonic cultures were grown overnight and then diluted 1:100 into fresh BHB and incubated at 37°C with shaking, until an OD_{600} 0.3 was reached. Planktonic cultures were then centrifuged at 5000 x *g* to pellet the cells. Cell pellets were resuspended in $1/10^{th}$ volume of BHB containing 32x MIC rifampicin. Conversely, biofilm cultures were grown using the cellulose disk model for 48 hrs at 37°C. Biofilms were then placed on BHA containing 32x MIC of rifampicin. For both culture types, drug-free control samples were also conducted. For biofilm cultures, individual biofilms were harvested

hourly and viable counts determined. However, for planktonic cultures, samples were taken from the cultures hourly, and the viable counts determined.

2.4. Microscopy

2.4.1. Confocal microscopy

To visualise biofilms grown using the cellulose disk model, *S. aureus* biofilms were stained with FilmTracer™ FM® 1-43 biofilm stain (Invitrogen, Paisley, UK), and then visualised by confocal microscopy according to the manufacturers instructions. Images of *S. aureus* biofilm were captured on a Leica TCS SP2 Confocal Laser Scanning microscope under a 100x oil immersion objective using excitation/emission wavelengths of 472/580 nm.

2.4.2. Atomic force microscopy

S. aureus biofilms were also visualised by atomic force microscopy. Initially, *S. aureus* biofilms were placed on slides and air dried for 20 mins. Samples were then visualised using an Asylum MFP3D microscope (Asylum research, Santa Barbara, CA, USA) in tapping mode under ambient conditions using a silicone probe (nominal resonant frequency ~300kHz [Olympus, Essex, UK]).

2.5. Molecular biology techniques

2.5.1. Genomic DNA purification

Cultures were grown in BHB to achieve maximum cell densities. Chromosomal DNA was then extracted and purified using the PurElute Bacterial Genomic kit (EdgeBio, Gaithersburg, MD, USA). Cell lysis was achieved by supplementing the Spheroblast buffer with 150 µg/ml lysostaphin (Sigma, Dorset, UK) and 25% (w/v) sucrose and incubating for 1 hr at 37°C.

2.5.2. Polymerase Chain Reaction (PCR)

Genomic DNA was amplified using Thermo-start *Taq* DNA polymerase ReddyMix (ABgene, Epsom, UK). All reactions were carried out using 45 µl of ReddyMix, 1.5 µl of oligonucleotide primers (10 µM each), up to 3 µl of genomic DNA and DEPC (diethylpyrocarbonate) treated water to a final volume of 50 µl. DNA was amplified using the conditions detailed in Table 2.2. Oligonucleotide primers (Table 2.3) were synthesised by Eurofins MWG Operon (Ebersburg, Germany). Amplified products were visualised using agarose gel electrophoresis and subjected to DNA sequencing by Beckman Coulter Genomics (Essex, UK).

Table 2.2 PCR cycling conditions used for the amplification of *rsbU* and *sigB* in *S. aureus* SH1000.

Phase	Temperature (°C)	Time (mins)	Cycles
Initial denaturation	94	5	1
Denaturation	94	1	
Annealing	50	1	30
Extension	72	1.5	
Final extension	72	10	1
Hold	4	-	-

Table 2.3 Oligonucleotide primers used in the PCR amplification of *rsbU* and *sigB* in *S. aureus* SH1000.

Primer Name	Sequence (5'-3')
Fwd_SigB_PCR	TCGCGACATTTATGTGGATA
Fwd_SigB_Int	CGCGATCAGATAATATAGGT
Rev_SigB_Int	CGTATTAAAGAAATTGGGCC
Rev_SigB_PCR	TTTAACACTTTCTGGCGTTT
rsbU_Fwd	AGCGCTGTATCCACCATATT
rsbU_Rev	TGTTGCGGCAATAACTGGTA
rsbU_Int_Fwd	CTAGCTTCAGTCACACCATC
rsbU_Int_Rev	GCTTACAACAAACAATGCTT

2.5.3. Agarose gel electrophoresis

To visualise DNA fragments or genomic extracts, samples were subjected to agarose gel electrophoresis. Agarose was dissolved in Tris-acetate-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA [Ethylenediaminetetraacetic acid]) to a final concentration of 1% (w/v). Molten 1% (w/v) agarose was combined with SYBRsafe DNA gel stain (Invitrogen, Paisley, UK) when pouring gels to allow visualisation of DNA. DNA markers, Hyperladder I or II (Bioline, London, UK), were used alongside DNA samples to allow determination of fragment sizes. A potential difference of 100 V was applied across the gel for 20-30 mins. DNA bands were then visualised by exposing the gel to blue light (450-495 nm). If necessary, bands were extracted and purified from the gel using the MinElute (elution in 10 µl) or QIAquick (elution in 30 µl or more) Gel Extraction kit (Qiagen, Crawley, UK) according to the manufacturers instructions.

2.5.4. DNA quantification

The concentration of DNA in samples was elucidated by determining their absorbance at 260 nm. Specifically, a value of 1 represented a DNA concentration of 50 µg/ml (Sambrook *et al.*, 1989). DNA purity was checked by determining the ratio of the absorbance values at 260 nm:280 nm and 260 nm:230 nm. Here, ratios of around 1.8 were considered to indicate a sample free of contaminating protein and other organic compounds.

2.5.5. Concentrating DNA by ethanol precipitation

To increase the concentration of DNA in samples, ethanol precipitation was performed (Sambrook *et al.*, 1989). Initially, sodium acetate (3 M pH 5.2) was added to DNA to a final concentration of 0.3 M. Two volumes of ice cold 100% ethanol were added to the DNA followed by incubation on ice for 15-30 mins. Samples were then centrifuged at 13000 x *g* for 15 mins, followed by careful removal of the supernatant. DNA pellets were washed with 70% ethanol (v/v) and centrifuged again for 15 mins. The supernatant was removed and the DNA pellets air dried. DNA pellets were then resuspended in DEPC treated water or Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) to the desired concentration.

2.5.6. Total RNA extraction

Total RNA was extracted from planktonic cultures and from biofilm cultures, grown using the cellulose disk biofilm model, of *S. aureus* SH1000. Planktonic cultures and resuspended biofilm cultures were centrifuged at 5000 x *g* to pellet cells, and then resuspended in 10 ml of RNAprotect bacterial reagent (Qiagen, Crawley, UK), to protect RNA integrity and prevent further gene expression changes, and then centrifuged again. The supernatant was removed and the pellets stored at -80°C.

Pellets were thawed at room temperature and washed in 10 ml of TE buffer to remove residual RNAprotect. Washed cells were then centrifuged at 5000 x *g* to pellet cells. Cells were resuspended in 1 ml TE buffer containing 200 µg/ml RNase free lysostaphin (Sigma-Aldrich, Dorset, UK) and incubated at 37°C for 60-90 mins. Following this, proteinase K (Sigma-Aldrich, Dorset, UK) was added to the samples, to a final concentration of 40 µg/ml, and incubated for 10 mins at room temperature. Total RNA was then purified from these samples using the RNeasy Midi kit (Qiagen, Crawley, UK) following the manufacturers instructions, including an on-column DNase digestion using the RNase-free DNase kit (Qiagen, Crawley, UK). RNA samples were stored at -80°C for up to 1 year.

The concentration of RNA was calculated by determining the absorbance value at 260 nm. Specifically, an absorbance value of 1 at this wavelength corresponds to 40 µg/ml of RNA (QIAGEN, 2001).

The purity of RNA in samples was determined by elucidating the ratio of absorbance values at 260 nm:280 nm and 260 nm:230 nm. Ratio of around 2 were considered to be free of protein, or other organic, contaminants (QIAGEN, 2001).

2.5.7. Analysis of differential gene expression by microarray analysis

Transcriptional profiling was performed using total RNA extracted from planktonic and cellulose disk grown biofilm cultures. cDNA synthesis, cDNA labelling, hybridisation and quantification of gene expression was performed by Roche Nimblegen (Madison, WI, USA) using 4x72K multiplex microarrays (4 samples at 72,000 probes per sample). Of the 2892 predicted protein coding open reading frames (ORFs), 2887 were represented on the microarrays due to shared probes. Data were analysed using ArrayStar™ (DNASTAR). Here, genes were considered to be differentially regulated if they exhibited 2 or more fold up or downregulation (Beenken *et al.*, 2004; Sauer *et al.*, 2004).

2.6. Internet resources

S. aureus NCTC 8325 genome sequence (Last access: 04/08/2010):

http://www.ncbi.nlm.nih.gov/sites/entrez?db=Genome&itool=toolbar

Genome annotation for *S. aureus* NCTC 8325 (Last access: 06/09/2010):

http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi

S. aureus NCTC8325 gene ontology information (Last access: 06/09/2010):

http://www.uniprot.org/uniprot/?query=nctc8325&by=go

S. aureus microarray meta-database (Last access): 13/09/2010):

http://www.bioinformatics.org/sammd/

CHAPTER 3

3. Development of a cellulose disk biofilm model

3.1. Abstract

The majority of bacterial biofilm models developed to date are complex and low throughput in nature. To address this, a higher throughput system was developed for the generation of staphylococcal biofilms that yielded high cell densities, as was required for subsequent biofilm mutability investigations.

This system was initially optimised for use with *S. aureus* SH1000. Here, human plasma was incorporated to promote cellular adherence to cellulose membrane filter disks which were incubated on solid media. A minimum concentration of 4% (v/v) was sufficient to achieve maximum adherence of *S. aureus* SH1000 after 48 hrs of incubation, with approximately 95% of cells remaining adherent following a saline wash. Subsequently, it was established that addition of human plasma every 48 hrs of incubation was required to achieve similar levels of adherence when older disk cultures of up to 144 hrs were grown. This protocol was also optimised for use with *S. aureus* UAMS-1 and *S. epidermidis* RP62A. For detachment of *S. aureus* from the disks, buffered cellulase was effective. However, for *S. epidermidis*, sodium metaperiodate was used.

Following method optimisation, the system was validated by confirming recalcitrance to killing by rifampicin, dissociation in the presence of D-tyrosine, and transcriptional profiling to compare gene expression with other *S. aureus* biofilm models. These validation procedures demonstrated this system to be an effective tool for the generation of staphylococcal biofilms. Microscopic visualisation using confocal and atomic

force microscopy revealed the biofilms to possess areas of undulation on their surface and a densely packed population of cells.

In summary, a simple high-throughput biofilm model has been successfully developed for use with staphylococci. The high cell numbers achieved with this system make it useful for a broad range of applications.

3.2. Introduction

As previously discussed, biofilms are found ubiquitously throughout the natural environment. The development, and subsequent investigation, of biofilms *in vitro* and *in vivo* has proven useful for the expansion of our understanding of these complex microbial communities. Indeed, the diverse range of available models has broadened our knowledge of both environmentally and medically relevant bacterial biofilms.

3.2.1. Environmental biofilms

Growth as a biofilm is common amongst organisms in the natural environment. Where the number of bacteria found colonising surfaces, such as riverbed rocks, significantly outnumbers those found as planktonic organisms (Zobell, 1943). These range from ubiquitous soil organisms to the bacteria colonising extreme ecological niches such as volcano vents. Numerous techniques have been developed to investigate biofilms from vastly differing environments. The models utilised to investigate biofilms formed by these organisms are also diverse, reflecting the differing environments from which they originate. One of the most straight forward techniques for studying environmental organisms in the field is the suspended slide method. Essentially, a microscope slide is suspended in soil or water, allowing the adherence of surrounding organisms (Marshall, 1985). This subsequently provides a platform for microscopic visualisation and analysis of these organisms directly from their natural habitat. Similarly, techniques utilising slides suspended in inoculated growth medium have also been used to investigate antibiofilm compounds for use against aquatic organisms such as *P. aeruginosa* (Borchardt *et al.*, 2001). Systems have also been developed to investigate other environmental niches. For example, rhizosphere biofilms have been studied using seed adherence and rumen biofilms via extracted rumenal fluid and particulate feed (Cheng *et al.*, 1981; Espinosa-Urgel *et al.*,

2000). Biofilms that colonise animals have also been investigated. Biofilms of *Vibrio fischeri* colonising the *Euprymna scolopes* squid light organ, for example, have been investigated by incubating juvenile squid with seawater containing specific inocula and then monitoring colonisation and biofilm formation (Nyholm *et al.*, 2002).

Techniques have also been developed for the investigation of biofilms found in extreme environments, such as those of hyperthermophiles. Indeed, biofilms of *Archaeoglobus fulgidus* were grown on the sides of culture vessels incubated anaerobically at 83°C, representing the natural environment of this hydrothermal vent and hot spring-dwelling archaeon (Lapaglia & Hartzell, 1997). Furthermore, the influence of microgravity on biofilm formation during spaceflight has also been elucidated. Specifically, biofilm growth on polycarbonate filters was investigated under microgravity conditions for *P. aeruginosa* (McLean *et al.*, 2001).

3.2.2. Medically relevant biofilms

Biofilms are involved in the majority of bacterial infections including, but not limited to, CF, osteomyelitis, cutaneous infections, infective endocarditis, indwelling medical device-related infections and dental caries (Heimberger & Duma, 1989; May *et al.*, 1991; Shani *et al.*, 2000; Akiyama *et al.*, 2003; Brady *et al.*, 2008). Furthermore, the biofilm mode of growth is also prevalent amongst members of the human microflora (Macfarlane, 2008). To investigate the biofilms formed by such bacteria, as well as their antibiotic susceptibility profiles, a broad range of systems have been implemented. These can be categorised into *in vivo* and *in vitro* systems.

3.2.2.1. In vivo systems

To represent biofilms during infection, on both medical devices and biological tissue, many *in vivo* systems have been employed (Table 3.1).

Table 3.1 Examples of *in vivo* biofilm investigations in medically significant organisms.

Infection/condition	Technique	Investigation	Source
Medical device related	Rabbit biofilm model	Testing *E. coli* colonisation of a modified urinary catheter, with microbicidal outlet tube, in a rabbit model	(Nickel *et al.*, 1991)
Plague	*Caenorhabditis elegans* biofilm model	Investigating biofilm formation by *Yersinia pestis* as a mechanism to prevent food intake in *Caenorhabditis elegans*	(Darby *et al.*, 2002)
Infectious endocarditis	Rat biofilm model	Determining the role of a collagen adhesin in the pathogenesis of *E. faecium* during experimental endocarditis	(Nallapareddy *et al.*, 2008)
Urinary stones	Rat biofilm model	Examining the role of biofilm formation by *P. mirabilis* on struvite calculus formation during urinary stone development	(Nickel *et al.*, 1987)
CF pneumonia	CF sputum examination	Determining the presence of *P. aeruginosa* biofilms in the CF lung	(Singh *et al.*, 2000)
Foreign body associated osteomyelitis	Rat biofilm model	Investigating the host immune response after antibiotic treatment for *S. aureus* osteomyelitis	(Garcia-Alvarez *et al.*, 2006)
Dental caries	Rat biofilm model	Elucidating the cariogenic activity of starch in comparison with sugar	(Thurnheer *et al.*, 2008)

Table 3.2 Examples of *in vitro* biofilm models.

Biofilm model	Investigation	Source
Continuous culture		
Sorbarod filters	Investigating the mutability of *P. aeruginosa* when growing as a biofilm	(Driffield *et al.*, 2008)
Flow cell	Using a chemostat flow cell to investigate antiplaque compounds	(Herles *et al.*, 1994)
CDC Biofilm Reactor	Testing the efficacy of the CDC Biofilm Reactor to grow *S. epidermidis* biofilms	(Williams & Bloebaum, 2010)
Chemostat with suspended substrata	Investigating natural genetic transformation in *Streptococcus mutans* biofilms	(Li *et al.*, 2001)
High-throughput flow cell	Assessing antimicrobial susceptibility in *P. aeruginosa* biofilms	(Benoit *et al.*, 2010)
Batch culture		
Calgary biofilm device	Method development for antimicrobial susceptibility testing in biofilms	(Ceri *et al.*, 1999)
Polystyrene petri dishes	Investigating biofilm formation by oral bacterial species	(Kaplan & Fine, 2002)
Polycarbonate membrane filters	Investigations into ciprofloxacin and tobramycin tolerance in *P. aeruginosa* biofilms	(Walters *et al.*, 2003)
Robbins device	Elucidating the efficacy of an antibiofilm compound for use with oral medical devices	(Coenye *et al.*, 2008)
Suspended coverslip	Determining the effect of *luxS* mutations on *S. mutans* biofilm formation	(Merritt *et al.*, 2003)

3.2.2.2. In vitro systems

Many *in vitro* systems have also been developed to model biofilms formed by pathogens and members of the human microflora, some of which have also been used for antibiotic susceptibility testing in biofilms (Ceri *et al.*, 1999) (Table 3.2). Compared to *in vivo* systems, the advantages of using such laboratory based techniques include ease of experimental control and reduced costs. However, care must be taken when developing *in vitro* models to ensure that they represent the infection, or condition, which they mimic.

To date, many biofilm models have become available which allow development of biofilms from a range of bacterial species. These comprise flow cells, constant-depth film fermenters (CDFF), microtitre plate methods, including the Calgary biofilm device (CBD), and numerous variations of these (Kinniment *et al.*, 1996; Ceri *et al.*, 1999; Driffield *et al.*, 2008; Harmsen *et al.*, 2010). These systems can be broadly categorised into continuous or batch culture methods, and flow or static culture methods. Flow cultures, such as flow cells, utilise a constant flow of liquid media to promote biofilm formation, often on the inside of tubing, coupons or Sorbarod filters (Driffield *et al.*, 2008; Kroukamp *et al.*, 2010). These systems can be advantageous over static systems (no flow) as they provide constant washing of the biofilms, and therefore removal of shed planktonic cells. Models that utilise batch cultures also have advantages over continuous cultures. These cultures are essentially the same as standard batch cultures, but with the addition of a surface for biofilm formation. Advantages include reduced risks of contamination, simple experimental design, high-throughput capacity and reduced costs (McLean *et al.*, 2004).

For the work described in this thesis, a simple high-throughput model was required that yielded staphylococcal biofilms of high cell densities for investigation of biofilm mutability, as described in future chapters. Accordingly, I developed a cellulose based system employing membrane disks on solid media.

3.3. Results and Discussion

For the development of a new biofilm model, cellulose disks were chosen as a substratum as they provided a relatively porous material that could easily be manipulated for the purposes of biofilm development.

3.3.1. Optimising adherence

Initially, attempts were made to develop the cellulose disk model for studies with *S. aureus* SH1000. This strain was chosen due to its capacity to form biofilms, as a result of the repair of a mutation in *rsbU*, present in the parental strain *S. aureus* 8325-4 (Horsburgh *et al.*, 2002a). Specifically, this gene encodes a positive regulator of the alternative sigma factor, SigB, which has previously been shown to regulate production of adhesive molecules necessary for biofilm formation (Nicholas *et al.*, 1999). Furthermore, human serum components, such as fibronectin and fibrinogen, promote the adherence of staphylococci to surfaces by interaction with these adhesins, such as ClfA, ClfB (clumping factors A and B), FnBPA and FnBPB (fibronectin binding proteins A and B) (O'Brien *et al.*, 2002; Keane *et al.*, 2007). To exploit this, human plasma was added to the model to promote adhesion (Griffiths & O'Neill, 2009). Firstly, the minimum concentration of human plasma that promoted maximum adherent viable cell numbers was determined. To do this, disk cultures were first washed in saline to remove the non-adherent planktonic population of cells, and then incubated in buffered cellulase for 30 mins to induce detachment from the cellulose disks. Cellulase was used at it had previously been shown to cause inhibition and dissociation of biofilm cultures (Cescutti *et al.*, 1998; Loiselle & Anderson, 2003). These data revealed that 4% (v/v) human plasma was sufficient to achieve a maximum adherent population of *S. aureus* SH1000, of around 2 x 10^{10} cfu/disk, following 48 hrs of incubation

(Figure 3.1). Specifically, addition of human plasma to the system increased the proportion of adherent cells from 10%, in the absence of human plasma, to 93% (Figure 3.2).

The method was then optimised for the development of older adherent cultures, to allow the formation of more mature biofilms. This was necessary as it became apparent that prolonged incubation of the cultures, at 144 hrs for example, resulted in a significant reduction in the amount of adherent cells, presumably due to degradation of plasma components (Figure 3.3). To address this issue, cultures were submerged in 4% (v/v) human plasma every 48 hrs and placed on a fresh BHA plate. This significantly improved the yield of adherent cells from 16% to 83% (Figure 3.3).

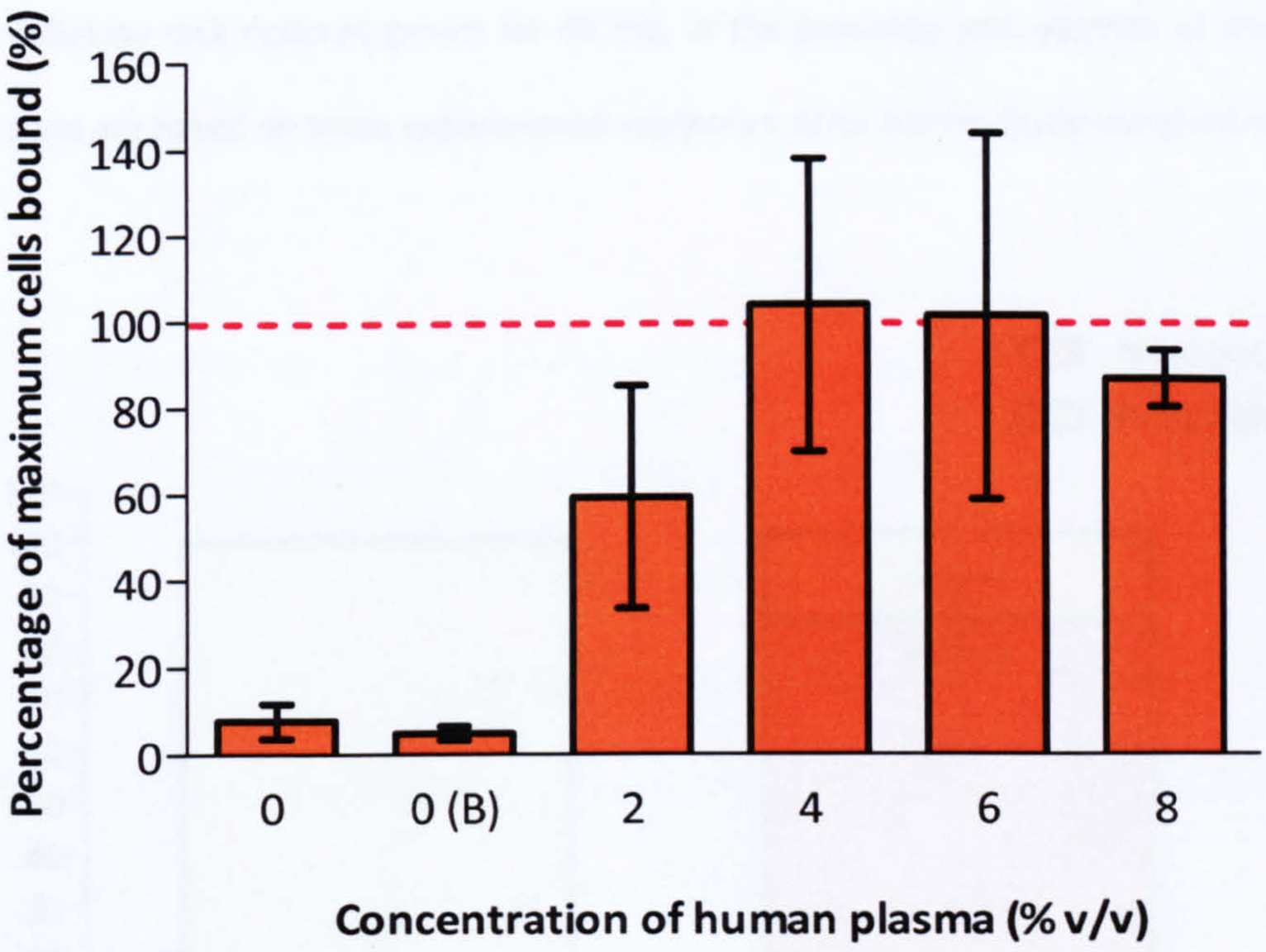

Figure 3.1 Optimisation of the cellulose disk biofilm model for use with *S. aureus* SH1000. Human plasma was diluted in 0.05 M carbonate bicarbonate buffer. The x-axis label 0 represents no human plasma or buffer, and 0(B) is buffer only. Data indicate the proportion of adherent cells, in 48 hr disk cultures, as a percentage of the maximum achieved at 10% (v/v) human plasma (red line). Data are based on three experimental replicates. Error bars indicate standard deviations. Maximum bound cells using 10% (v/v) human plasma was ~1.8×10^{10} cfu/disk.

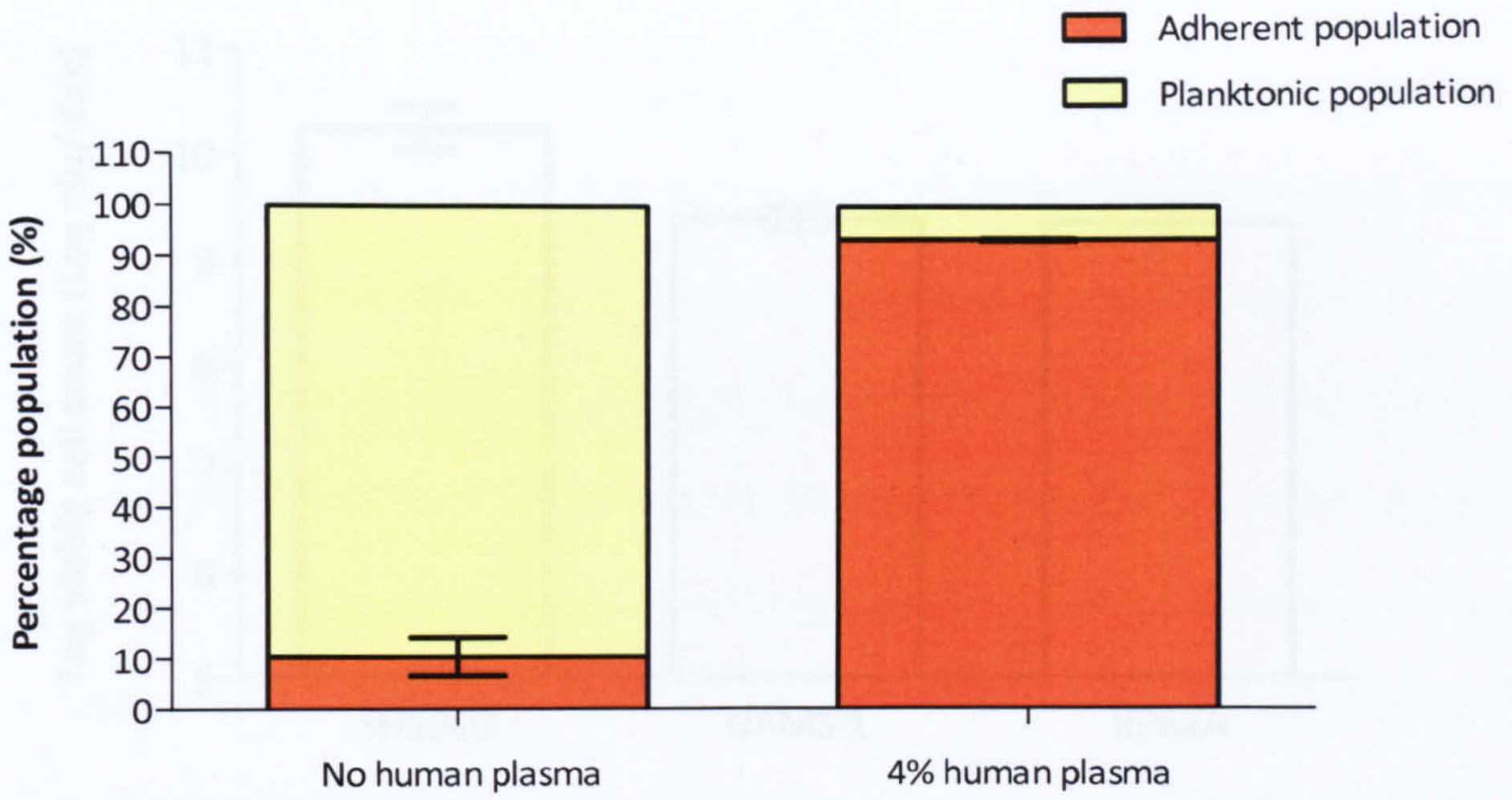

Figure 3.2 Determination of the proportion of adherent and planktonic cells present in *S. aureus* SH1000 cellulose disk cultures grown for 48 hrs, in the presence and absence of 4% (v/v) human plasma. Data are based on three experimental replicates. Error bars indicate standard deviations.

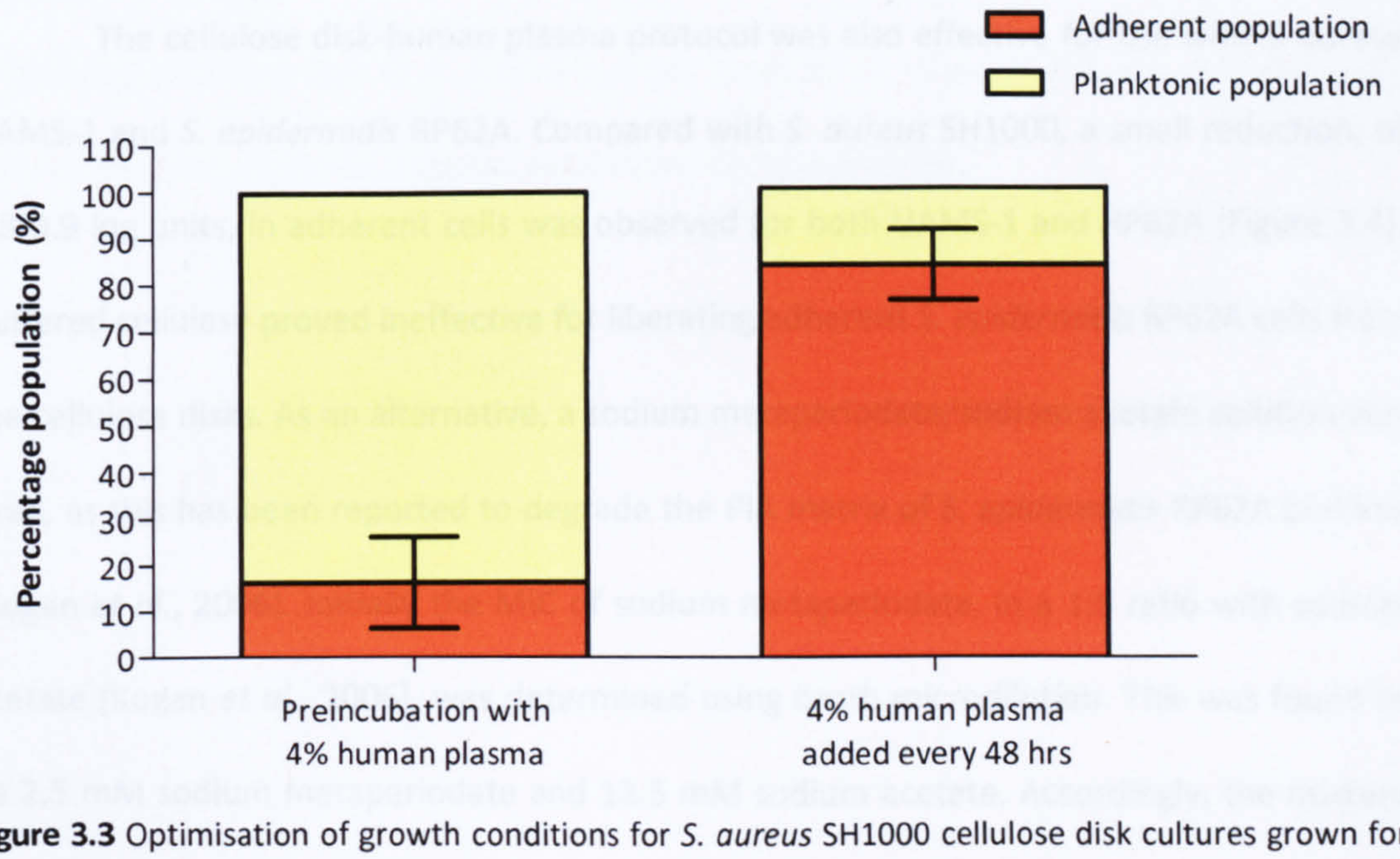

Figure 3.3 Optimisation of growth conditions for *S. aureus* SH1000 cellulose disk cultures grown for longer than 48 hrs. Graph illustrates the effect of human plasma addition every 48 hrs on biofilms grown for 144 hrs, compared with equivalent cultures only provided with human plasma before inoculation. Data are based on three experimental replicates. Error bars indicate standard deviations.

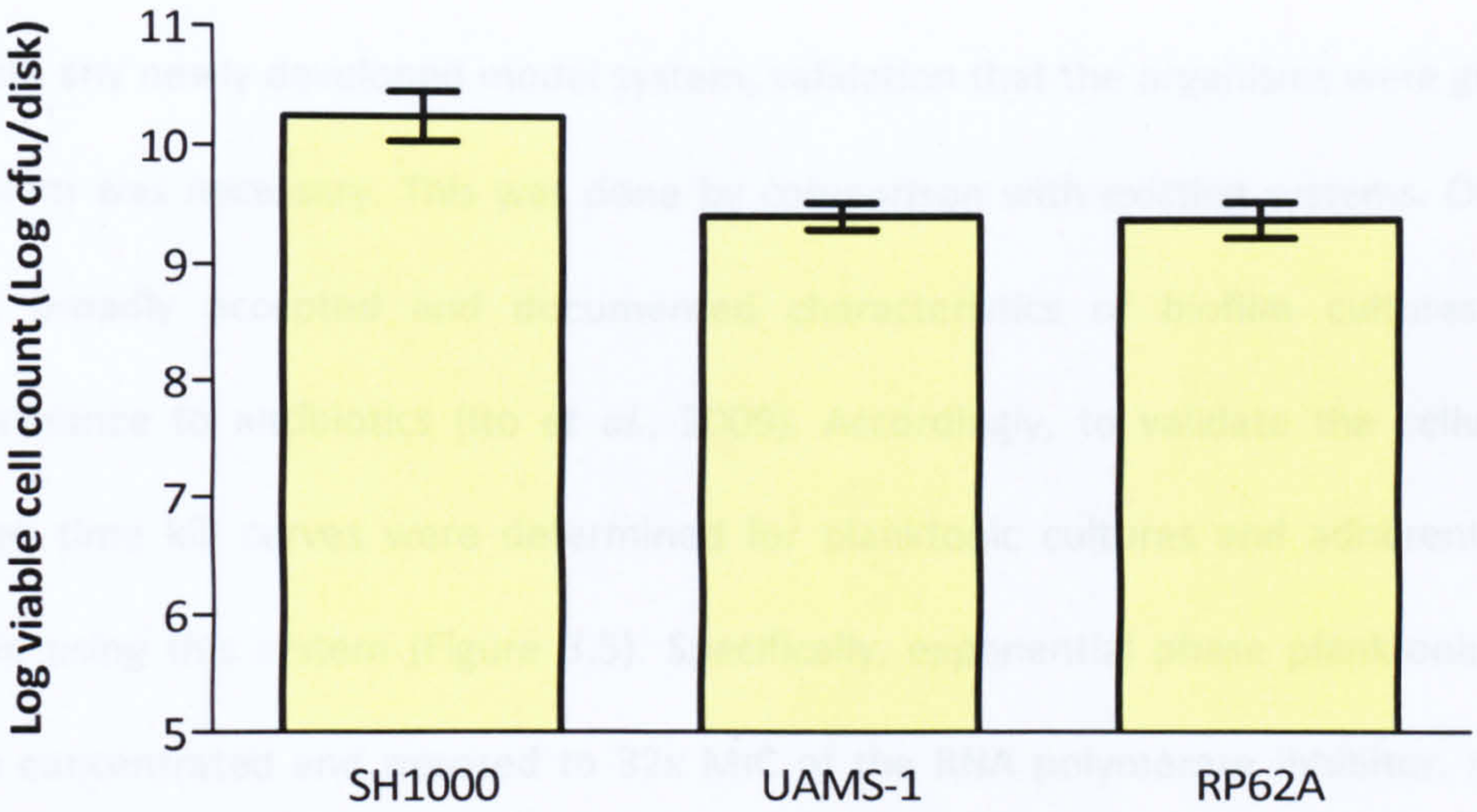

Figure 3.4 Adherent cell numbers of *S. aureus* UAMS-1 and *S. epidermidis* RP62A disk cultures compared with *S. aureus* SH1000. Data based on three experimental replicates. Error bars indicate standard deviations.

The cellulose disk-human plasma protocol was also effective for use with *S. aureus* UAMS-1 and *S. epidermidis* RP62A. Compared with *S. aureus* SH1000, a small reduction, of 0.8-0.9 log units, in adherent cells was observed for both UAMS-1 and RP62A (Figure 3.4). Buffered cellulase proved ineffective for liberating adherent *S. epidermidis* RP62A cells from the cellulose disks. As an alternative, a sodium metaperiodate/sodium acetate solution was used, as this has been reported to degrade the PIA matrix of *S. epidermidis* RP62A biofilms (Kogan *et al.*, 2006). Initially the MIC of sodium metaperiodate, in a 1:5 ratio with sodium acetate (Kogan *et al.*, 2006), was determined using broth microdilution. This was found to be 2.5 mM sodium metaperiodate and 12.5 mM sodium acetate. Accordingly, the mixture was then used at a subinhibitory concentration of ¼ x MIC for 30 min at 37°C, to promote biofilm dissociation.

3.3.2. Model validation

As with any newly developed model system, validation that the organisms were growing as a biofilm was necessary. This was done by comparison with existing systems. One of the most broadly accepted and documented characteristics of biofilm cultures is their recalcitrance to antibiotics (Ito *et al.*, 2009). Accordingly, to validate the cellulose disk model, time kill curves were determined for planktonic cultures and adherent bacteria grown using this system (Figure 3.5). Specifically, exponential phase planktonic cultures were concentrated and exposed to 32x MIC of the RNA polymerase inhibitor, rifampicin (MIC = 0.0078 μg/ml) (Campbell *et al.*, 2001). In a similar manner, adherent cultures were grown for 48 hrs and then placed on agar plates containing 32x MIC rifampicin. Planktonic time kill data revealed that rifampicin reduced the number of viable cells by 2 logs after 5 hrs. In contrast, adherent cultures demonstrated no significant reduction in viable cell number after 5 hrs of exposure. This demonstrates that the cellulose disk model is capable of growing adherent cells of *S. aureus* that are recalcitrant to antibiotics, i.e. with the same phenotypic characteristics as other established biofilm systems.

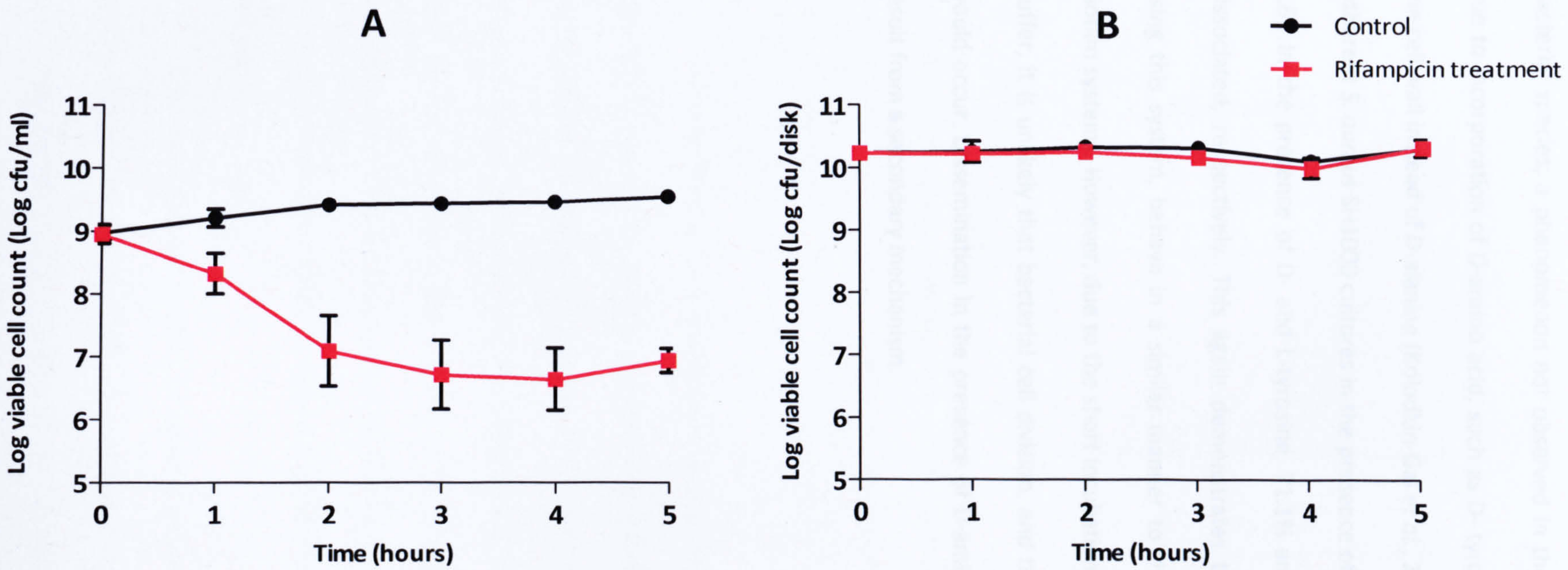

Figure 3.5 Time kill curve of *S. aureus* SH1000 **A** planktonic and **B** 48 hr cellulose disk cultures exposed to 32x MIC rifampicin. Data are based on three experimental replicates. Error bars indicate standard deviations.

D-amino acids cause the breakdown of biofilms formed by staphylococci, and other bacterial species, a phenomenon not observed in the presence of L-enantiomers. This is due to incorporation of D-amino acid, such as D- tyrosine, into the peptide side chains of the cell wall instead of D-alanine (Kolodkin-Gal *et al.*, 2010). Accordingly, the dissociation of adherent *S. aureus* SH1000 cultures in the presence of a D-amino acid was assessed (Figure 3.6). In the presence of D- and L-tyrosine, 71.1% and 7.0% of the total population was dissociated, respectively. This again demonstrates that adherent organisms, generated using this system, behave in a similar manner to those generated in other established biofilm systems. However, due to the short incubation time with D-tyrosine, and the use of buffer, it is unlikely that bacterial cell division, and therefore peptidoglycan biosynthesis, would occur. Dissemination in the presence of D-amino acids in this case must therefore result from a secondary mechanism.

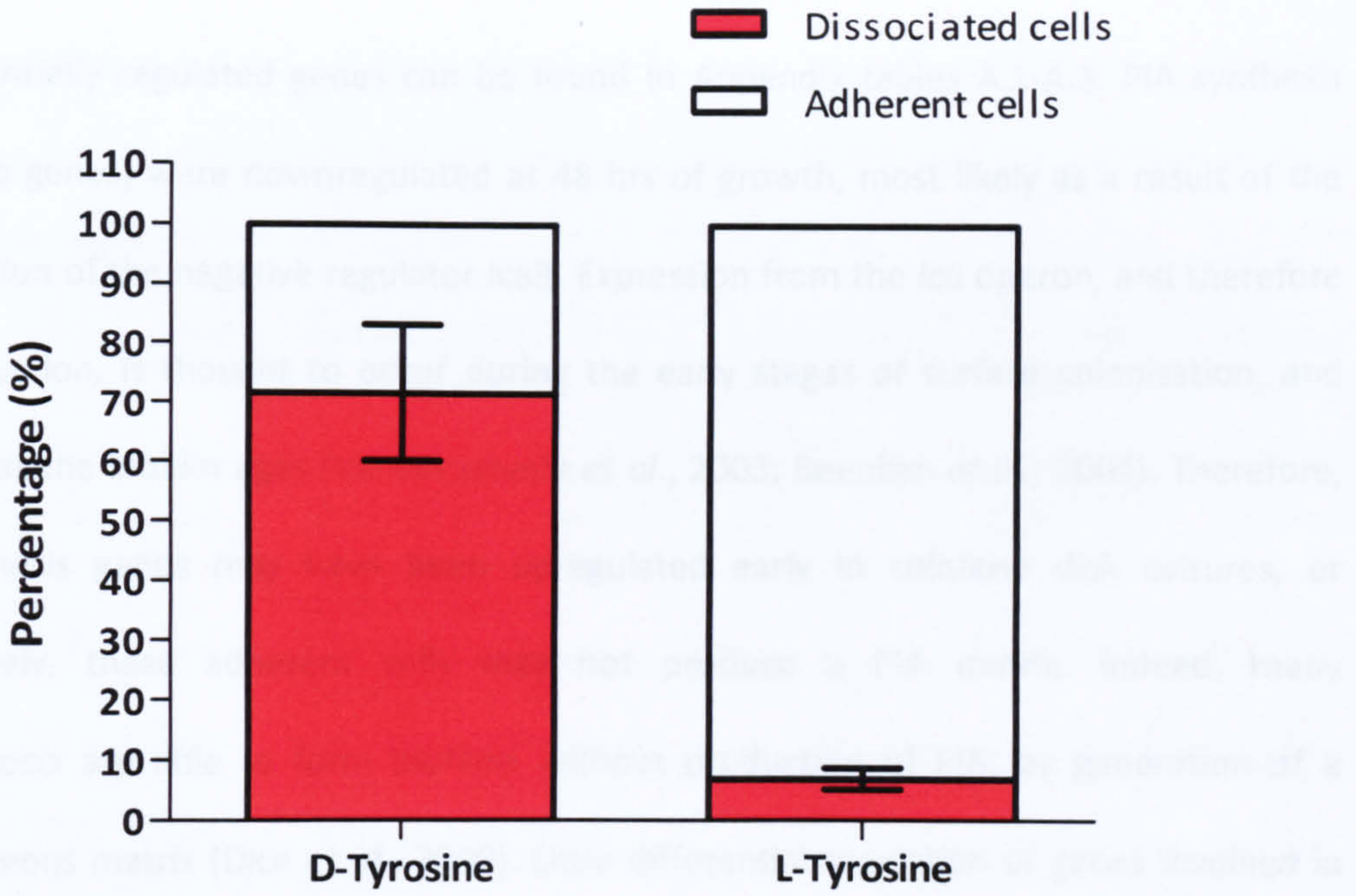

Figure 3.6 The dissociation of adherent cells of *S. aureus* SH1000 on cellulose disks in the presence of D-tyrosine and L-tyrosine. Data are based on three experimental replicates. Error bars indicate standard deviations.

Several studies have generated transcriptional profiles of staphylococcal biofilm cultures, compared with planktonic cultures (Beenken *et al.*, 2004; Resch *et al.*, 2005). It was therefore appropriate to conduct DNA microarray analysis of adherent *S. aureus* SH1000 cultures, generated using the cellulose disk model, and compare these data with those of equivalent planktonic cultures to identify differentially regulated genes (an in depth discussion of the transcriptional profiles generated will be presented in Chapter 5). Differential gene expression profiles could then be compared with the previously published DNA microarray data.

The transcriptional profiles of cultures generated using the cellulose disk model were compared to the available transcriptional profiles of other *S. aureus* biofilms (http://www.bioinformatics.org/sammd/) to identify similarities in gene expression.

Initially, genes involved in biofilm formation were considered (Table 3.3). Statistical analysis of differentially regulated genes can be found in Appendix tables A.1-A.3. PIA synthesis genes (*ica* genes) were downregulated at 48 hrs of growth, most likely as a result of the upregulation of the negative regulator IcaR. Expression from the *ica* operon, and therefore PIA production, is thought to occur during the early stages of surface colonisation, and diminish as the biofilm ages (Vandecasteele *et al.*, 2003; Beenken *et al.*, 2004). Therefore, PIA synthesis genes may have been upregulated early in cellulose disk cultures, or alternatively, these adherent cells may not produce a PIA matrix. Indeed, many staphylococci are able to form biofilms without production of PIA, by generation of a proteinaceous matrix (Dice *et al.*, 2009). Little differential regulation of genes involved in adhesin production was observed in cellulose disk cultures, such as those of clumping factors and fibronectin binding proteins, as observed in other biofilm systems (Resch *et al.*, 2005). In fact, both ClfA, which binds serum components such as fibrinogen (Keane *et al.*, 2007), and SdrC, which promotes nasal epithelial cell colonisation (Corrigan *et al.*, 2009), were downregulated in 144 hr and 48 hr cellulose disk cultures, respectively, when compared with planktonic cultures. However, a putative fibrinogen binding protein was upregulated in 48 hr disk cultures.

Due to the protocol utilised for the collection of biofilm cells, where biofilms were washed in PBS and dispersed with buffered cellulase, significant gene expression changes may have occurred prior to the addition of RNAprotect. Therefore, although these data provide a valuable insight into the biofilm transcriptome, performing RT-PCR to corroborate these results would have been useful, had time permitted.

Table 3.3 Differential gene expression (fold change) of genes involved in biofilm formation and adherence in 48 hr disk (D48h) versus planktonic (P) cultures, 144 hr disk (D144h) versus planktonic cultures and 144 hr versus 48 hr disk cultures of *S. aureus* SH1000. Data are the mean of three experimental replicates.

Locus tag	Gene name	Function	Fold change P/D48h	Fold change P/D144h	Fold change D48h/D144h
SAOUHSC_00620	*sarA*	accessory regulator A	1.231 up	1.339 down	1.648 down
SAOUHSC_03003	*icaD*	poly-beta-1,6-N-acetyl-D-glucosamine synthesis protein IcaD	**2.236 down**	1.356 down	1.648 up
SAOUHSC_03004	*icaB*	biofilm PIA synthesis deacetylase IcaB	**2.019 down**	1.381 down	1.461 up
SAOUHSC_03002	*icaA*	poly-beta-1,6-N-acetyl-D-glucosamine synthase	**2.775 down**	1.550 down	1.790 up
SAOUHSC_03005	*icaC*	biofilm PIA synthesis protein IcaC	1.479 down	1.166 down	1.268 up
SAOUHSC_02798	*sasG*	surface protein G	**2.247 down**	1.468 down	1.531 up
SAOUHSC_00021	*walK*	sensor protein YycG	1.121 down	1.422 down	1.268 down
SAOUHSC_03001	*icaR*	biofilm operon icaabcd hth-type negative transcriptional regulator IcaR	**5.525 up**	**2.089 up**	**2.644 down**
SAOUHSC_00020	*walR*	transcriptional regulatory protein WalR	1.301 down	1.398 down	1.074 down
SAOUHSC_01964	*traP*	signal transduction protein TRAP	1.466 up	1.163 up	1.260 down
SAOUHSC_00505	*clpC*	ATP-dependent Clp protease, ATP binding subunit ClpC	**4.311 up**	**5.157 up**	1.196 up
SAOUHSC_02862	*clpL*	ATP-dependent Clp protease, ATP binding subunit ClpL	**6.058 down**	**5.460 down**	1.109 up
SAOUHSC_01378	*oppD2*	peptide ABC transporter, ATP-binding protein	1.923 up	1.381 up	1.392 down
SAOUHSC_01377	*oppF2*	peptide ABC transporter, ATP-binding protein	1.133 up	1.127 up	1.005 down
SAOUHSC_02299	*rsbW*	sigmaB negative effector RsbW	1.258 up	1.042 up	1.207 down
SAOUHSC_01897	*sigS*	RNA polymerase sigma factor, SigS	1.533 up	1.052 up	1.456 down
SAOUHSC_00502	*ctsR*	transcriptional regulator CtsR	**8.875 up**	**10.829 up**	1.220 up
SAOUHSC_01380	*oppB2*	peptide ABC transporter, permease protein	1.496 up	1.301 up	1.150 down
SAOUHSC_01379	*oppC2*	oligopeptide transporter membrane permease domain	1.747 up	1.366 up	1.278 down
SAOUHSC_01586	*srrA*	transcriptional regulatory protein SrrA	**2.666 up**	1.675 up	1.591 down
SAOUHSC_01585	*srrB*	sensor protein SrrB	1.160 up	1.126 up	1.029 down

Table 3.3 cont.

SAOUHSC_00812	*clfA*	clumping factor A	1.924 down	**2.391 down**	1.242 down
SAOUHSC_02963	*clfB*	clumping factor B	1.248 down	1.050 down	1.188 up
SAOUHSC_02803	*fnbA*	fibronectin-binding protein A	1.835 down	1.048 up	1.923 up
SAOUHSC_00544	*sdrC*	serine-aspartate repeat-containing protein SdrC	**2.322 down**	1.330 down	1.746 up
SAOUHSC_00545	*sdrD*	serine-aspartate repeat-containing protein SdrD	1.735 down	1.249 down	1.389 up
SAOUHSC_02690		zinc-binding lipoprotein AdcA	1.122 up	1.440 up	1.283 up
SAOUHSC_02802		fibronectin binding protein B, putative	1.564 down	1.007 up	1.575 up
SAOUHSC_01175		fibrinogen binding protein	**2.250 up**	1.763 up	1.276 down

Other *S. aureus* biofilm transcriptional profiles have identified urease (*ure* cluster) and arginine demaminase (*arc* cluster) activity as being upregulated, compared with planktonic cultures (Resch *et al.*, 2005). Accordingly, expression from the *ure* and *arc* genes was investigated (Table 3.4). Similar to the findings of Resch *et al.*, expression of the *ure* genes was significantly increased. The *arc* genes, however, were not significantly differentially regulated. Biofilm cultures have also previously been identified as downregulating the expression of toxins and proteases (Resch *et al.*, 2005). In agreement with this, many genes encoding toxins and proteases were also found to be downregulated in the cellulose disk system (Tables 3.5 & 3.6). Furthermore, the majority of downregulated toxin or protease genes were identified in 48 hr disk cultures, not 144 hr cultures, suggesting that this phenomenon may primarily occur in immature adherent populations. Of these proteases, several Ssp and Spl type proteases were found to be downregulated. Both of these protease types are known to influence biofilm formation, where their overexpression, which can result from *sigB* deletion, inhibits *ica*-independent biofilm formation in *S. aureus* (Lauderdale *et al.*, 2009; Marti *et al.*, 2010). This suggests that the maintenance of extracellular protease levels, by SigB, is necessary for biofilm maturation.

Table 3.4 Differential gene expression (fold change) of genes involved in urease (*ure*) and arginine deaminase (*arc*) production in 48 hr disk (D48h) versus planktonic (P) cultures, 144 hr disk (D144h) versus planktonic cultures and 144 hr versus 48 hr disk cultures of *S. aureus* SH1000. Data are the mean of three experimental replicates.

Locus tag	Gene name	Function	Fold change P/D48h	Fold change P/D144h	Fold change D48h/D144h
SAOUHSC_02561	*ureC*	urease, alpha subunit	**2.631 up**	1.665 up	1.579 down
SAOUHSC_02558	*ureA*	urease, gamma subunit	**2.285 up**	1.565 up	1.460 down
SAOUHSC_02559	*ureB*	urease, beta subunit	1.690 up	1.309 up	1.290 down
SAOUHSC_02565	*ureD*	urease accessory protein UreD	**4.712 up**	1.781 up	**2.645 down**
SAOUHSC_02563	*ureF*	urease accessory protein UreF	**3.365 up**	**2.008 up**	1.675 down
SAOUHSC_02564	*ureG*	urease accessory protein UreG	**2.478 up**	1.733 up	1.430 down
SAOUHSC_02562	*ureE*	urease accessory protein UreE	**2.641 up**	1.821 up	1.449 down
SAOUHSC_02964	*arcR*	cyclic nucleotide-binding domain protein	1.694 down	1.290 down	1.312 up
SAOUHSC_02969	*arcA*	arginine deiminase	1.466 down	1.316 down	1.113 up
SAOUHSC_02965	*arcC2*	carbamate kinase	1.884 down	1.361 down	1.383 up
SAOUHSC_01129	*arcC1*	carbamate kinase	**2.320 down**	1.500 down	1.546 up

Table 3.5 Differential gene expression (fold change) of genes involved in protease production in 48 hr disk (D48h) versus planktonic (P) cultures, 144 hr disk (D144h) versus planktonic cultures and 144 hr versus 48 hr disk cultures of *S. aureus* SH1000. Data are the mean of three experimental replicates.

Locus tag	Gene name	Function	Fold change P/D48h	Fold change P/D144h	Fold change D48h/D144h
SAOUHSC_01778	*clpX*	ATP-dependent protease ATP-binding subunit ClpX	**2.952 up**	**2.650 up**	1.114 down
SAOUHSC_01716		peptidase U32	**2.472 up**	**2.003 up**	1.233 down
SAOUHSC_00958		serine protease HtrA-like	1.514 down	1.288 down	1.175 up
SAOUHSC_01717		peptidase U32	**2.811 up**	**2.189 up**	1.283 down
SAOUHSC_00987	*sspB*	staphopain B	**2.682 down**	**2.483 down**	1.079 up
SAOUHSC_00988	*sspA*	glutamyl endopeptidase	**4.128 down**	**2.841 down**	1.453 up
SAOUHSC_01942	*splA*	serine protease SplA	**2.111 down**	1.405 down	1.502 up
SAOUHSC_01941	*splB*	serine protease SplB	**3.700 down**	1.728 down	2.140 up
SAOUHSC_01939	*splC*	serine protease SplC	**2.592 down**	1.529 down	1.695 up
SAOUHSC_01938	*splD*	serine protease SplD	1.888 down	1.258 down	1.501 up

Table 3.6 Differential gene expression (fold change) of genes involved in toxin production in 48 hr disk (D48h) versus planktonic (P) cultures, 144 hr disk (D144h) versus planktonic cultures and 144 hr versus 48 hr disk cultures of *S. aureus* SH1000. Data are the mean of three experimental replicates.

Locus tag	Gene name	Function	Fold change P/D48h	Fold change P/D144h	Fold change D48h/D144h
SAOUHSC_00354		staphylococcal enterotoxin, putative	1.382 down	1.147 down	1.205 up
SAOUHSC_00383		exotoxin 6	1.576 down	1.234 down	1.277 up
SAOUHSC_00384		exotoxin 7	1.153 down	1.126 down	1.024 up
SAOUHSC_00386		exotoxin	**2.473 down**	1.389 down	1.780 up
SAOUHSC_00389		exotoxin	**2.819 down**	1.410 down	1.998 up
SAOUHSC_00390		exotoxin 10	**2.314 down**	1.457 down	1.587 up
SAOUHSC_00391		exotoxin	1.982 down	1.327 down	1.493 up
SAOUHSC_00392		staphylococcal exotoxin 5	1.430 down	1.238 down	1.155 up
SAOUHSC_00393		exotoxin	1.231 down	1.162 down	1.059 up
SAOUHSC_00394		staphylococcal exotoxin 3	1.735 down	1.494 down	1.161 up
SAOUHSC_00395		staphylococcal exotoxin 2	1.384 down	1.257 down	1.100 up
SAOUHSC_00399		staphylococcal exotoxin 1	1.669 down	1.239 down	1.347 up
SAOUHSC_01124		exotoxin	1.653 down	1.261 down	1.310 up
SAOUHSC_01125		exotoxin	1.537 down	1.270 down	1.209 up
SAOUHSC_01127		exotoxin	1.510 down	1.177 down	1.283 up
SAOUHSC_01133		exfoliative toxin A	1.186 down	1.155 down	1.026 up
SAOUHSC_01705		enterotoxin P	1.054 down	1.092 down	1.035 down
SAOUHSC_01954		leukocidin F subunit	**2.301 down**	1.497 down	1.536 up
SAOUHSC_01955		leukotoxin LukE	**2.479 down**	1.422 down	1.742 up
SAOUHSC_02241		bi-component toxin	1.474 down	1.302 down	1.132 up
SAOUHSC_02709		gamma-hemolysin component C	1.293 down	1.102 up	1.425 up
SAOUHSC_01121		alpha-hemolysin	1.152 down	1.049 up	1.208 up
SAOUHSC_02260	*hld*	delta-hemolysin	**5.873 down**	**10.936 down**	1.861 down
SAOUHSC_02243		aerolysin/Leukocidin family protein	1.734 down	1.361 down	1.273 up
SAOUHSC_02163	*hlb*	phospholipase C	1.281 down	1.491 down	1.163 down
SAOUHSC_02240		phospholipase C	1.694 down	1.385 down	1.222 up
SAOUHSC_02708		gamma-hemolysin h-gamma-II subunit, putative	1.197 up	**2.210 up**	1.846 up
SAOUHSC_02710		leukocidin F subunit	1.930 down	1.378 down	1.399 up
SAOUHSC_02709		gamma-hemolysin component C	1.293 down	1.102 up	1.425 up

3.3.3. Microscopic analysis

Following optimisation of the cellulose disk model protocol, both confocal microscopy (CM) and atomic force microscopy (AFM) were used to visualise, and therefore further characterise, bacteria grown in this system. CM was performed using a cell membrane fluorescent stain (FilmTracer™ FM® 1-43). This generated images, showing a rough and undulating surface structure of the adherent cells (Figure 3.7). This type of surface is similar to previously published confocal images of staphylococcal biofilms (Rice *et al.*, 2007). However, these images (Figure 3.7) had poor cellular resolution, possibly due to poor staining as a result of interference by the biofilm extracellular matrix. This is a phenomenon that has previously been attributed to the slow penetration of chemicals into *S. aureus* biofilms (Jefferson *et al.*, 2005). In response to this, disk cultures of *S. aureus* SH1000 grown for 48 and 144 hrs were subjected to AFM (Figure 3.8 and 3.9) (many thanks to Prof Simon Foster's group for assistance). This revealed similar surface structures to those observed using CM, whilst also providing significantly increased cellular resolution. No PIA was observed in these images. This may result from lack of PIA production by this strain in this system, or because this microscopic technique does not allow resolution of such structures, as no matrix is obviously apparent in any other published AFM images of biofilm cultures (Scher *et al.*, 2007; Oh *et al.*, 2009). However, taken into consideration with the transcriptional profiling data, demonstrating downregulation of PIA synthetic genes, it would appear that the organisms growing in this system may not be synthesising a polysaccharide matrix.

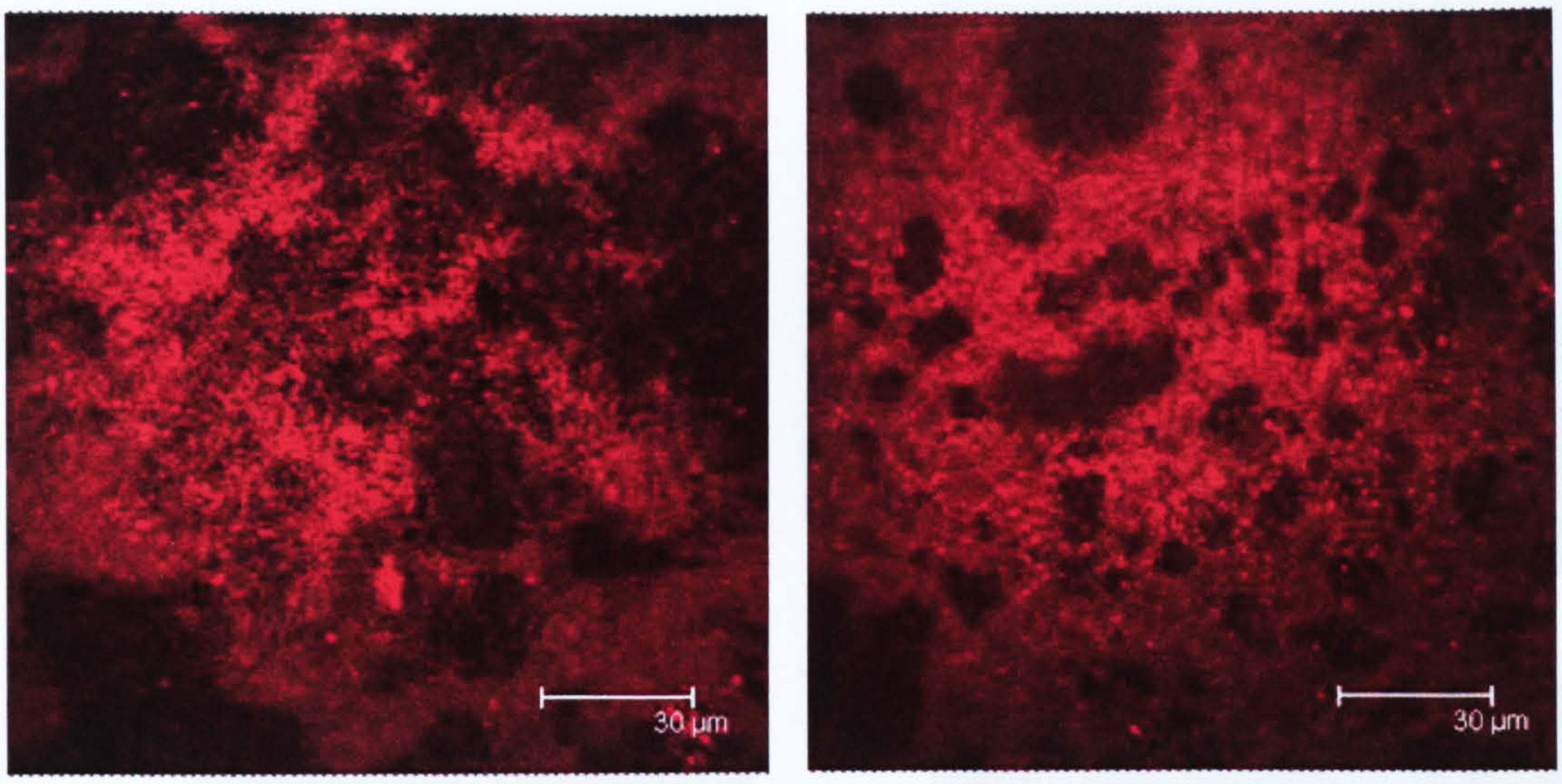

Figure 3.7 Confocal micrographs of cellulose disk biofilms of **A** *S. aureus* SH1000 and **B** *S. aureus* UAMS-1, incubated for 144 hrs. Cells were stained with FilmTracer™ FM® 1-43

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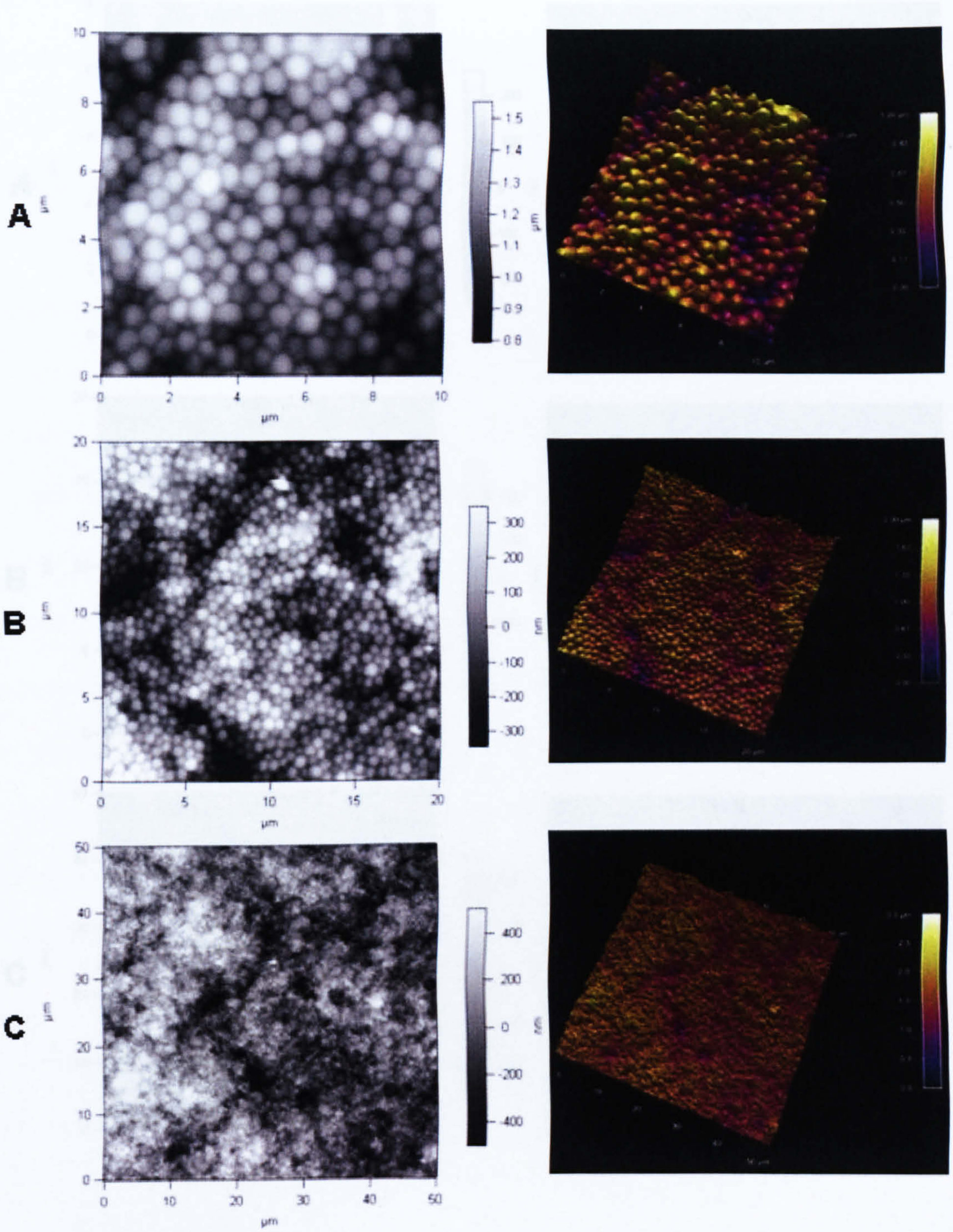

Figure 3.8 Atomic force micrographs (left column) and 3D surface topography (right column) of adherent *S. aureus* SH1000 cultures grown for 48 hrs and generated using the cellulose disk model. Images show areas of **A** 10 μm^2, **B** 20 μm^2 and **C** 50 μm^2. Work performed at the University of Sheffield in collaboration with Professor Simon Foster and Dr Robert Turner.

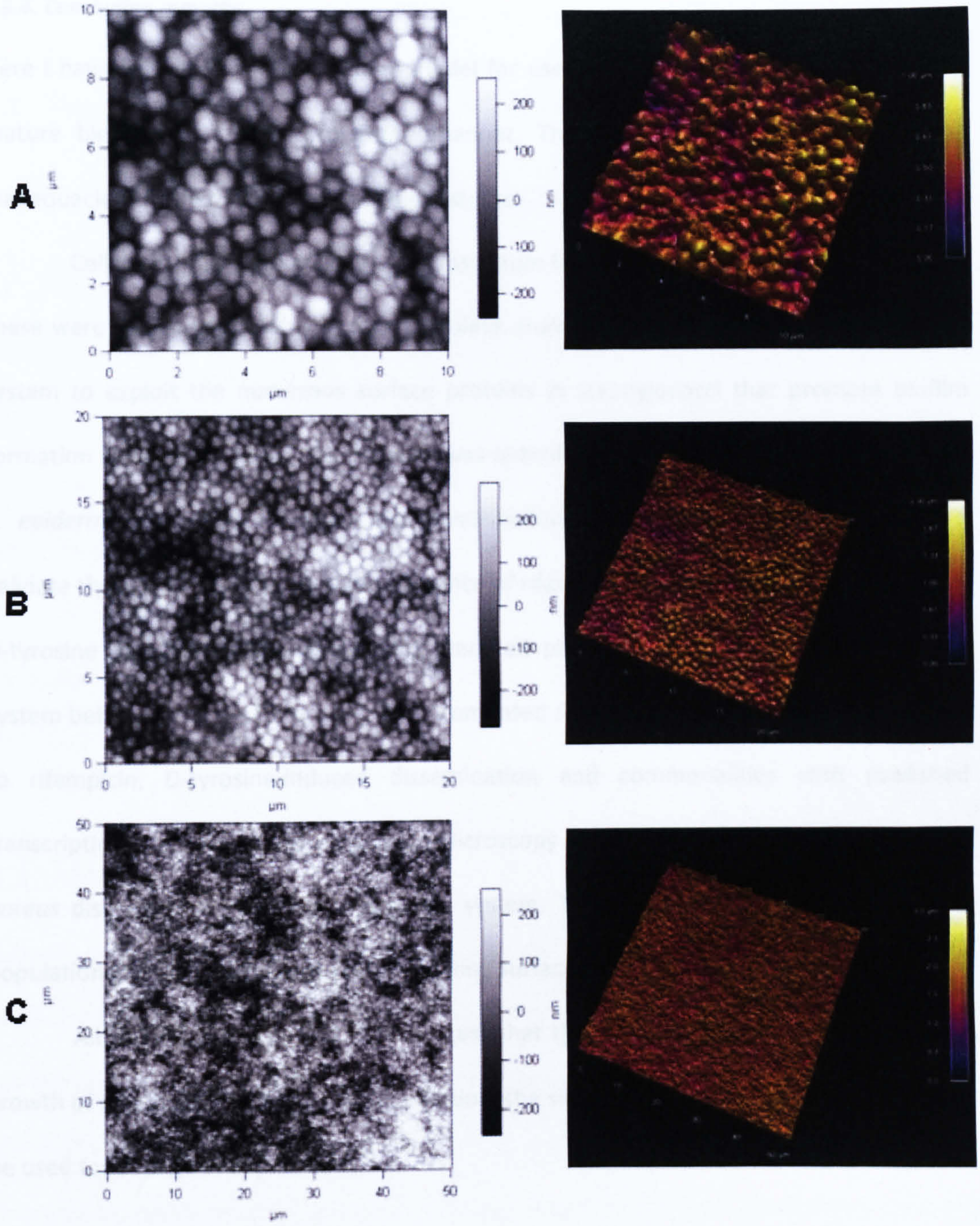

Figure 3.9 Atomic force micrographs (left column) and 3D surface topography (right column) of adherent *S. aureus* SH1000 cultures grown for 144 hrs and generated using the cellulose disk model. Images show areas of **A** 10 μm^2, **B** 20 μm^2 and **C** 50 μm^2. Work performed at the University of Sheffield in collaboration with Professor Simon Foster and Dr Robert Turner.

3.3.4. Concluding remarks

Here I have generated a novel biofilm model for use with staphylococci that can develop mature biofilms in a high-throughput manner. This was necessary to overcome the inadequacies of other currently available systems.

Cellulose disks were chosen as a substratum for biofilm formation on agar plates, as these were inexpensive and easy to manipulate. Human plasma was incorporated into the system to exploit the numerous surface proteins in staphylococci that promote biofilm formation (Patti *et al.*, 1994). The method was optimised for developing both *S. aureus* and *S. epidermidis* biofilms for mutability investigations (presented in later chapters). To validate the system, kill curves in the presence of rifampicin, dissociation in the presence of D-tyrosine and transcriptional profiling were all performed. Bacteria grown using this system behaved in a similar manner to documented *S. aureus* biofilms, where recalcitrance to rifampicin, D-tyrosine-induced dissemination and commonalities with published transcriptional profiles were observed. Microscopy also allowed the visualisation of *S. aureus* disk cultures generated using this system. This demonstrated a densely packed population of cells with a rough and undulating surface.

All the studies reported here suggest that the cellulose disk model supports the growth of staphylococci as biofilms. In addition, the simplicity of the system will enable it to be used for a range of applications.

CHAPTER 4

4. Enhanced mutability of *S. aureus* biofilms and the effects of antioxidants

Work presented in this chapter has also been presented as an oral presentation and as poster:

Ryder, V. J., Miller, K., O'Neill, A. J. & Chopra, I. (2009) Enhanced mutability in staphylococcal biofilms. *ARM,* Birmingham, UK.

Ryder, V. J., Miller, K., O'Neill, A. J. & Chopra, I. (2009) C1-1356 Enhanced mutability of *Staphylococcus aureus* in biofilms results from oxidative damage. *ICAAC,* San Francisco, USA.

4.1. Abstract

Growth as a biofilm renders bacteria recalcitrant to killing by most antibiotic classes. Physiological factors like slow growth and the prevention of antibiotic penetration by the biofilm matrix may contribute to this phenomenon. Also, the role of the biofilm growth phase as a mechanism to maximise gene transfer and genetic mutation is being recognised. This process could result in biofilm populations acting as a source of antibiotic resistant mutants. Previous studies have identified increased MFs in *P. aeruginosa* and *S. pneumoniae* biofilms. However, the contribution of genetic mutation in the emergence of antibiotic resistance in staphylococcal biofilms has not previously been studied. This work sought to investigate the mutability of staphylococcal biofilms, and elucidate mechanisms underlying any increased mutability.

MFs were determined, using rifampicin and mupirocin selection, for planktonic and biofilm cultures. For *S. aureus* SH1000, increases in MF of up to 4-fold and 14-fold were observed in biofilm cultures grown using the cellulose disk model and Sorbarod model, respectively. This increase in MF did not result from stationary phase dormancy, as stationary phase planktonic cultures demonstrated no increase in MF. *S. aureus* UAMS-1 and *S. epidermidis* RP62A, prominent biofilm forming organisms, exhibited MF increases of up to 68-fold and 4-fold, respectively.

Previous studies have suggested a role for oxidative stress in biofilm mutability. Accordingly, antioxidant compounds (taurine, vanillin and ascorbic acid) were incorporated into *S. aureus* biofilms grown using the cellulose disk model. All antioxidants tested reduced *S. aureus* SH1000 biofilm MFs to the level of planktonic cultures. For *S. aureus* UAMS-1, mupirocin selection identified taurine and vanillin as agents that reduced the biofilm MF. Rifampicin selection only identified vanillin as reducing the MF.

Therefore, biofilms appear to represent a bacterial growth phase that is capable of accelerating the emergence of antibiotic resistant organisms. Furthermore, the increases in MF observed are likely to result from endogenous oxidative stress.

4.2. Introduction

Growth as a biofilm is extensively reported to enable bacteria to resist killing by many antibiotic classes (Nickel *et al.*, 1985; Ashby *et al.*, 1994). As previously discussed, antibiotic recalcitrance may be phenotypic in nature and result from physiological features of the biofilm, such as the matrix, or slow growth as a result of starvation or oxygen limitation (Evans *et al.*, 1991; Shigeta *et al.*, 1997; Borriello *et al.*, 2004). Alternatively, increased rates of genetic transfer by transduction or conjugation in biofilms, could also contribute to the spread of genetically inheritable antibiotic resistance (Cvitkovitch, 2004; Blokesch & Schoolnik, 2008). In a similar manner, chromosomal mutation may also have a role in the development of antibiotic resistance in the biofilm. Indeed, increased MFs have previously been identified in *P. aeruginosa* and *S. pneumoniae* biofilms (Allegrucci & Sauer, 2008; Driffield *et al.*, 2008). Accordingly, this work sought to demonstrate whether increased MFs might be a phenomenon that occurs in staphylococcal biofilms. To do so, investigations were undertaken to examine the mutability of planktonic and biofilm cultures of *S. aureus* and *S. epidermidis*.

4.2.1. Bacterial mutation

Genetic mutation in bacteria is a tightly controlled process that directly affects the speed at which a population can adapt to the external environment (Brock *et al.*, 1994). Indeed, the ability to survive in a changing environment, as a result of mutation, has been extensively reported in the characterisation of antibiotic resistant organisms arising in bacterial populations (Ovchinnikov Yu *et al.*, 1981; Antonio *et al.*, 2002). Such mutations are spontaneous in nature and result from the exposure of DNA to intra- or extracellular mutagens, such as reactive oxygen species (ROS) or radiation, or alternatively, the failure of DNA repair systems to correct replicative errors. Increased levels of intracellular ROS may

occur in biofilms (Beenken *et al.*, 2004; Boles & Singh, 2008), and as such their role in mutation will be considered in more detail.

4.2.1.1. Reactive oxygen species (ROS)

ROS arise from normal cellular enzymatic reactions in aerobic conditions. Aerobic respiration, for example, utilises oxygen reduction to release energy, a by-product of which is the formation ROS (Fridovich, 1978). This has been suggested to make the major contribution to the production of ROS. However, subsequent reports have suggested that aerobic respiration contributes little to total ROS production, with the majority coming from other enzymatic reactions (Seaver & Imlay, 2004). These ROS, although important for cell signalling, can lead to the oxidation of vital cellular macromolecules such as DNA, proteins and lipids, if their levels are not maintained (Armel *et al.*, 1977; Kono & Fridovich, 1982).

The reduction of molecular oxygen leads directly to the production of superoxide anion radicals ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH^-$), by the removal of 1, 2 or 3 electrons, respectively (Sies, 1997). These different ROS cause mutations in DNA, often through chemical alteration to the hydrogen bonds of the nucleotide moieties, resulting in modification of their coding specificity. However, mutation is also thought to occur due to conformational changes in the DNA or polymerase as a result of oxidative damage (Feig *et al.*, 1994). Superoxide radicals can lead to C to T, G to T or G to C substitutions or CC to TT transitions (McBride *et al.*, 1991; Tkeshelashvili *et al.*, 1991), and can also lead to strand breaks (Morgan *et al.*, 1976). Hydrogen peroxide results in single stranded and double stranded breaks, although this is thought to be through a Fenton reaction with a variable valency metal ion bound to DNA, yielding hydroxyl radicals (Ananthaswamy & Eisenstark, 1977; Ward *et al.*, 1987).

4.2.2. Antioxidants as antimutagens

An antioxidant is defined as 'any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate' (Halliwell & Gutteridge, 1989). These agents may be enzymatic or non-enzymatic in nature.

Bacteria utilise both enzymatic and non-enzymatic antioxidants to maintain the levels of ROS within the cell. These compounds eradicate various ROS types from the cell, and therefore prevent oxidation and subsequent damage to vital macromolecules such as DNA.

4.2.2.1. Staphylococcal antioxidants

Staphylococci possess numerous antioxidant enzymes that protect cellular macromolecules from ROS. Several major iron-dependent global regulators in *S. aureus* protect against oxidative stress. These are SarA, Fur, PerR, Zur and MntR (Horsburgh *et al.*, 2001; Lindsay & Foster, 2001; Horsburgh *et al.*, 2002b; Ballal & Manna, 2009). The antioxidant enzymes regulated by these proteins can be categorised into the superoxide dismutases (SODs), the catalases and the peroxidases (Harris, 1992).

SODs catalyse the reduction of the superoxide anion into hydrogen peroxide and oxygen (McCord & Fridovich, 1968). These enzymes are classified according to the metal ion cofactor present in their active site; manganese-dependent, iron-dependent, copper/zinc-dependent or nickel-dependent (McCord & Fridovich, 1969; Keele *et al.*, 1970; Lengfelder & Elstner, 1979; Kim *et al.*, 1998). The best studied SOD in staphylococci, SodA, is a manganese-dependent SOD whose regulation is controlled by SarA (Ballal & Manna, 2009). Another SOD gene, *sodM*, has subsequently been identified in *S. aureus*. SodM was shown to possess 75% homology to that of SodA, but is absent in CNS (Valderas & Hart,

2001; Valderas *et al.*, 2002). These enzymes have a significant role in the virulence of *S. aureus* in the vertebrate host. Indeed, isogenic *sodA*, *sodM* and *sodA/sodM* deletion mutants were less virulent, in a mouse abscess model of infection, when compared to the parental strain (Karavolos *et al.*, 2003).

Catalases reduce hydrogen peroxide to water and oxygen. Only one catalase (KatA) has been identified in *S. aureus*. This enzyme is regulated by Fur and PerR, in an Fe(II)-dependent manner (Horsburgh *et al.*, 2001). Although the involvement of catalase in the virulence of staphylococci remains unclear, it may be important for the intracellular proliferation of *S. aureus* in a eukaryotic cell line (Martinez-Pulgarin *et al.*, 2009).

Similar to catalases, peroxidases function to reduce a range of peroxides, including hydrogen peroxide, but with the use of an external electron donor. The alkyl hydroperoxide reductase in *S. aureus*, AhpC, belonging to the family peroxiredoxins, is induced by hyperosmotic shock (Armstrong-Buisseret *et al.*, 1995). This peroxidase has been shown to work in combination with KatA to protect against hydrogen peroxide. Specifically, mutations in either gene resulted in no loss of protection against hydrogen peroxide, due to compensatory expression of the other enzyme (Cosgrove *et al.*, 2007). Furthermore, an atypical two-cysteine peroxidase (gene allocation SAOUHSC_01822) has recently been identified and crystallised from *S. aureus* NCTC8325, although the function of this remains unclear (Bhattacharyya *et al.*, 2009).

Non-enzymatic antioxidants in *S. aureus* include pigments, such as staphyloxanthin. Staphyloxanthin provides protection against hydrogen peroxide. Specifically, a staphyloxanthin-deficient mutant was less able to survive challenge with hydrogen peroxide than the parent strain (Clauditz *et al.*, 2006).

4.2.3. Oxidative stress within biofilms

Differential regulation of antioxidant enzyme gene expression has previously been reported for several organisms growing in biofilm culture. *E. coli,* for example, has been shown to display reduced expression of *sodA* (Schembri et al., 2003). Similarly, transcriptional analysis of *P. aeruginosa* biofilms has also revealed reduced expression of antioxidant enzymes, namely *sodB*, *ahpC* and *katA* (Driffield *et al.*, 2008). *S. aureus,* however, has been shown to up and downregulate *sodA* expression depending on the strain and biofilm model utilised (Resch *et al.*, 2005; Brady *et al.*, 2006). It was therefore hypothesised that bacteria in biofilms may harbour increased levels of intracellular ROS. This may ultimately increase the MFs of these biofilm bacteria. Furthermore, the increased numbers of antibiotic resistant organisms emerging from *P. aeruginosa* biofilms, when compared with planktonic cultures, has been attributed to increased DNA strand breakage due to endogenous oxidative stress (Boles & Singh, 2008; Driffield *et al.*, 2008).

To assess the role of oxidative stress in the mutation of cells within bacterial biofilms, compounds previously demonstrated to possess antioxidant activity and antimutagenic activity in bacteria will be used to supplement cultures (Chopra *et al.*, 2003). These compounds are vanillin, from the vanilla bean, taurine, which is found in most animal tissues, and ascorbic acid (vitamin C), which is found extensively in plants and animals (Figure 4.1) (Aruoma *et al.*, 1988; Kumar *et al.*, 2000; Valpuesta & Botella, 2004). The antioxidant mode of action of ascorbic acid is well documented as being a peroxyl and oxygen radical scavenger (Jacob & Burri, 1996). Similarly, vanillin can reduce a variety of ROS (Lee *et al.*, 2009). Taurine, however, has only been demonstrated to reducing hypochlorous acid, but it may function to prevent ROS generation (Schaffer *et al.*, 2009).

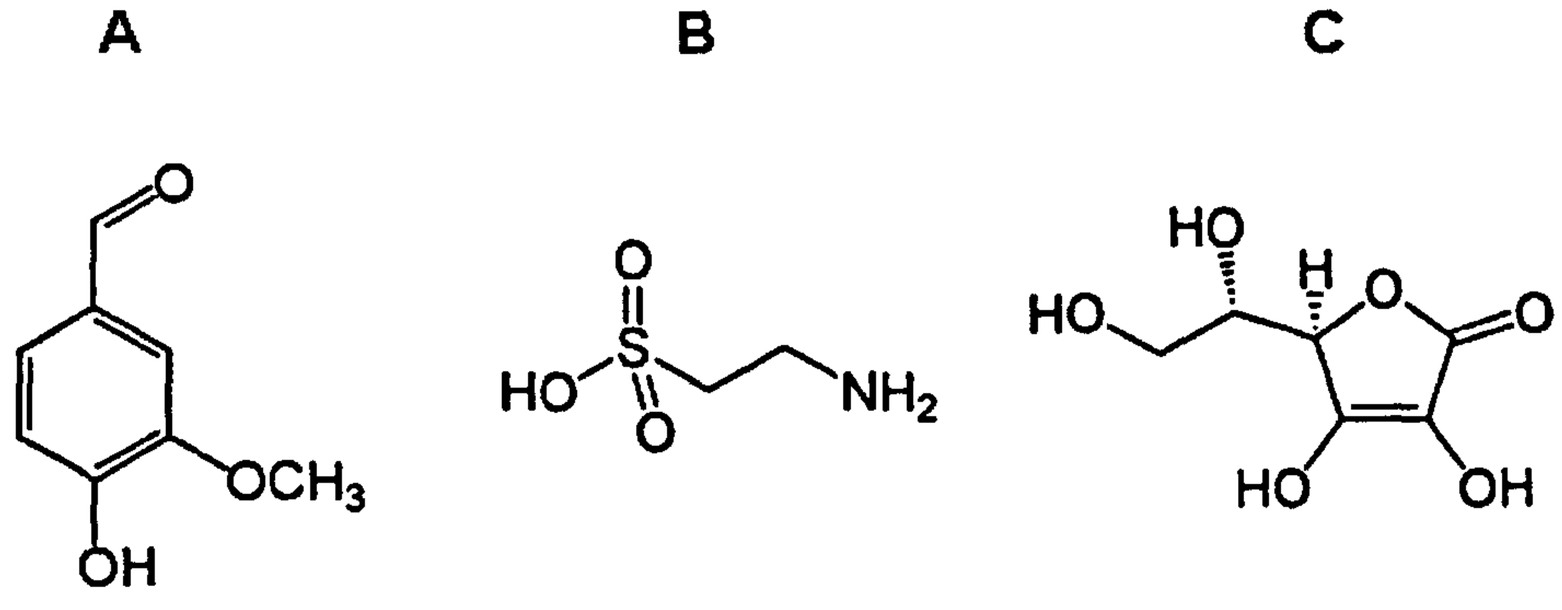

Figure 4.1 The chemical structures of antioxidants used in this work. **A**. Vanillin **B**. Taurine **C**. Ascorbic acid.

4.3. Results and Discussion

4.3.1. Increased mutability of staphylococcal biofilm cultures

MICs were first established for the antibiotics to be used in MF determinations (Table 4.1). Following this, planktonic and biofilm cultures were assessed for their respective MFs by selection of spontaneously arising resistant mutants using the RNA polymerase inhibitor, rifampicin, and the isoleucyl tRNA synthetase inhibitor, mupirocin (Hughes & Mellows, 1980; Campbell *et al.*, 2001). Spontaneous resistance to rifampicin and mupirocin occurs due to mutations in *rpoB* and *ileS*, encoding the RNA polymerase β-subunit and isoleucyl tRNA synthetase enzyme, respectively (Ovchinnikov *et al.*, 1983; Yang *et al.*, 2006). All stated P values were determined using an unpaired two-tailed Student's t-test.

Table 4.1 Minimum inhibitory concentrations of the selections antibiotics for use in determining mutation frequencies of *S. aureus* SH1000, *S. aureus* UAMS-1 and *S. epidermidis* RP62A.

	MIC (mg/L)	
Strain	**Rifampicin**	**Mupirocin**
S. aureus SH1000	0.0078	0.125
S. aureus UAMS-1	0.0156	0.125
S. epidermidis RP62A	0.0156	0.125

4.3.1.1. Mutation frequencies in biofilms of S. aureus SH1000

Initially, MFs were determined for *S. aureus* SH1000 planktonic and biofilm cultures (grown using the cellulose disk model). From these data, significant increases in MF were observed between mature biofilm cultures and planktonic cultures (Figure 4.2). Specifically, statistically significant increases were observed between planktonic and 96 hr biofilm cultures, using rifampicin selection (>95% confidence, $P = 2.57 \times 10^{-6}$), and between planktonic and both 72 hr and 96 hr biofilm culture, using mupirocin selection (>95% confidence, $P = 1.87 \times 10^{-4}$ and 4.27×10^{-3}, respectively). Biofilms incubated for 96 hrs had

increases in MF of 4.1-fold and 4.3-fold for rifampicin and mupirocin selection, respectively. Unexpectedly, a reduction in the MF of biofilms grown for 144 hrs, when compared with those at 96 hrs, was observed. It was postulated that these biofilms were yielding mutants with reduced growth rates, which would therefore not be visible on the selection plates for enumeration after 48 hrs of incubation. To investigate this, antibiotic selection plates were incubated for 72 hrs, instead of 48 hrs (Figure 4.3). This additional incubation time isolated significantly more mutants from the 144 hr biofilm cultures using mupirocin selection (>95% confidence, $P = 2.18x10^{-4}$) and rifampicin selection (>95% confidence, $P = 1.39x10^{-2}$). The number of spontaneous mutants yielded after 72 hrs of incubation of the selection plates was also assessed for 96 hr biofilm cultures. This did not yield more mutants than at 48 hrs of incubation (data not shown), suggesting that the additional mutants generated for the 144 hr biofilms were indeed slow growing, and not just a result of prolonged incubation.

To confirm that the observations made using the cellulose disk model were not specific to a static biofilm model, the experiments were repeated under shear flow using the Sorbarod model (Figure 4.4). Similar to the mature 144 hr biofilms grown using the cellulose disk model, mutants from this system were also slow growing. Therefore, selection plates were once again incubated for 72 hrs. This experiment also revealed significant increases in MF between planktonic and biofilm cultures of 13.8-fold and 6.7-fold using rifampicin (>95% confidence, $P = 2.71x10^{-2}$) and mupirocin (>95% confidence, $P = 6.67x10^{-3}$), respectively. Furthermore, to confirm that human plasma was not mutagenic and artificially increasing the MFs of the static biofilm cultures, 96 hr sorbarod biofilms were grown in the absence of human plasma (Figure 4.4). This confirmed that these cultures had equivalent MFs as those which incorporated human plasma, using both

rifampicin (<95% confidence, P = $5.74x10^{-1}$) and mupirocin selection (<95% confidence, P = $9.74x10^{-1}$).

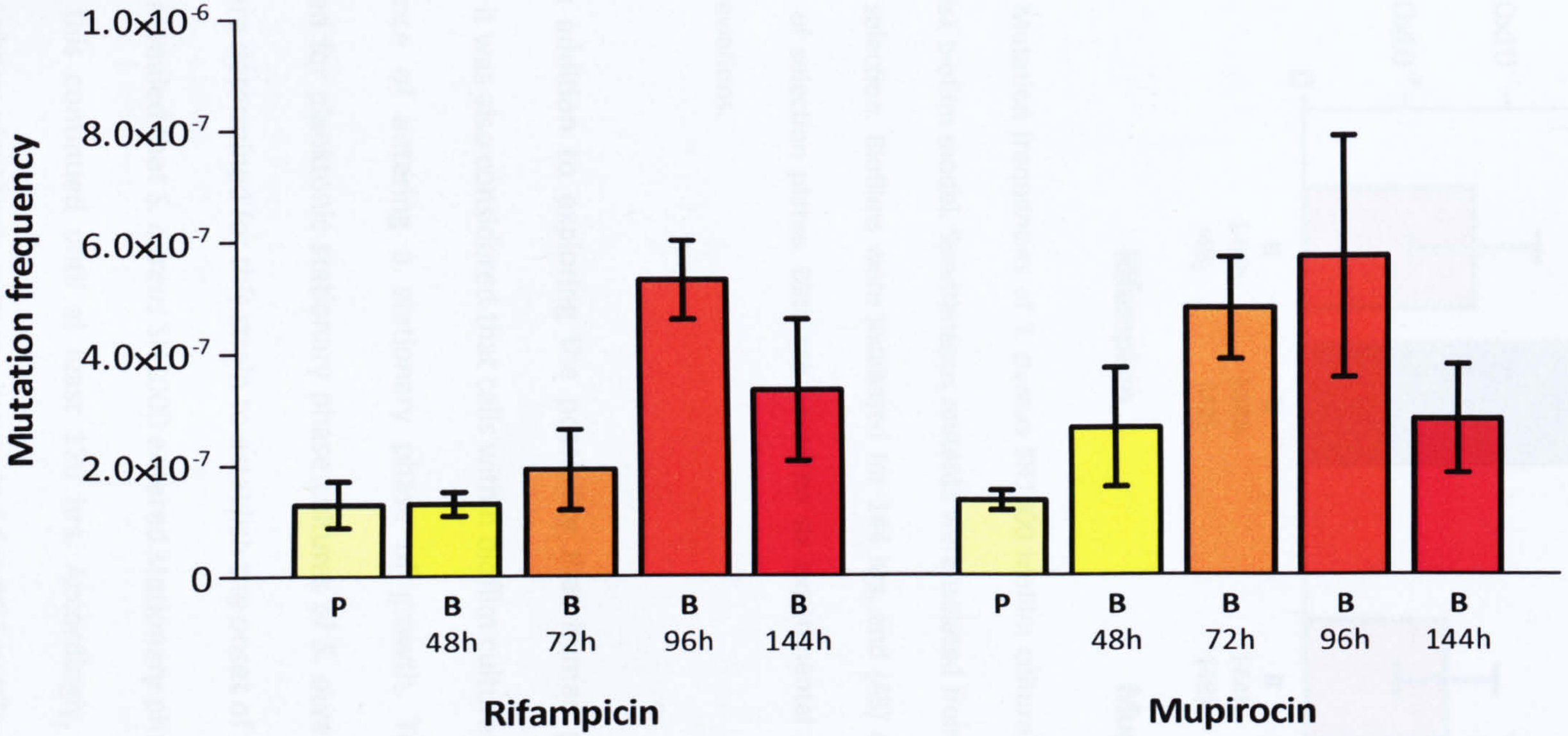

Figure 4.2 Mutation frequencies of *S. aureus* SH1000 planktonic and biofilm cultures grown using the cellulose disk biofilm model. Spontaneous mutants were isolated from cultures using the selection antibiotics rifampicin and mupirocin. P indicates planktonic cultures and 48h, 72h, 96h and 144h indicate biofilm (B) incubation times. Data are based on six experimental replicates. Error bars indicate standard deviations.

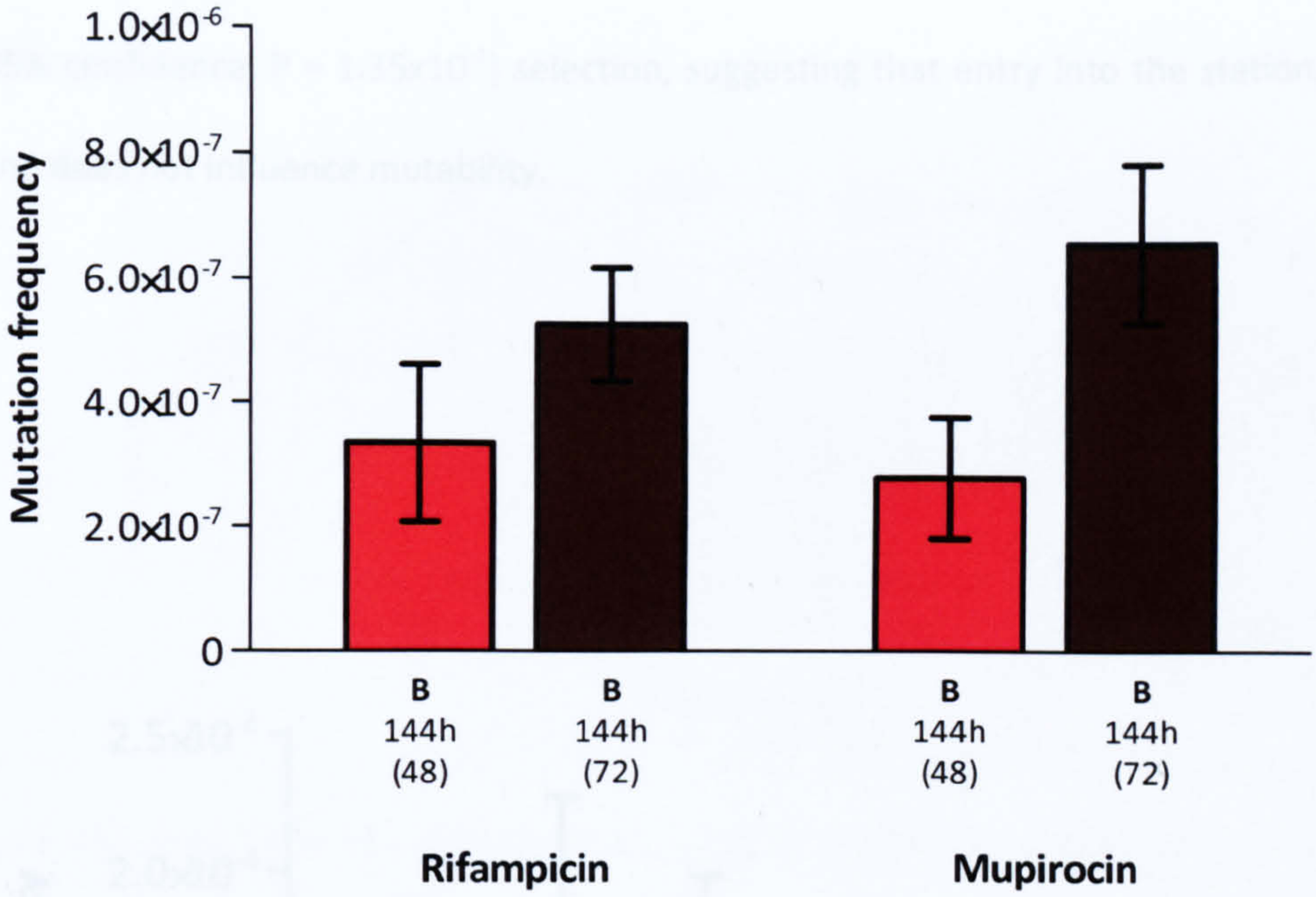

Figure 4.3 Mutation frequencies of *S. aureus* SH1000 biofilm cultures grown for 144 hrs using the cellulose disk biofilm model. Spontaneous mutants were isolated from cultures using rifampicin and mupirocin selection. Biofilms were incubated for 144 hrs, and (48) or (72) indicate a 48 or 72 hr incubation of selection plates. Data are based on six experimental replicates. Error bars indicate standard deviations.

In addition to exploring the possibility that human plasma was behaving as a mutagen, it was also considered that cells within biofilm cultures may be more mutable as a consequence of entering a stationary phase of growth. To analyse this, MFs were determined for planktonic stationary phase cultures of *S. aureus* SH1000. Initially, growth curves were determined for this strain to establish the onset of the stationary phase (Figure 4.5). This revealed that *S. aureus* SH1000 entered stationary phase at approximately 24 hrs, and that this continued until at least 120 hrs. Accordingly, MFs were determined for stationary phase planktonic cultures incubated for 96 hrs (Figure 4.6), as this represented the time point at which the greatest increase in MF occurred in the cellulose disk biofilm cultures. This revealed no significant increase in MF compared with the planktonic cultures

grown for 18 hrs using either rifampicin (<95% confidence, P = $7.72x10^{-1}$) or mupirocin (<95% confidence, P = $1.35x10^{-1}$) selection, suggesting that entry into the stationary phase alone does not influence mutability.

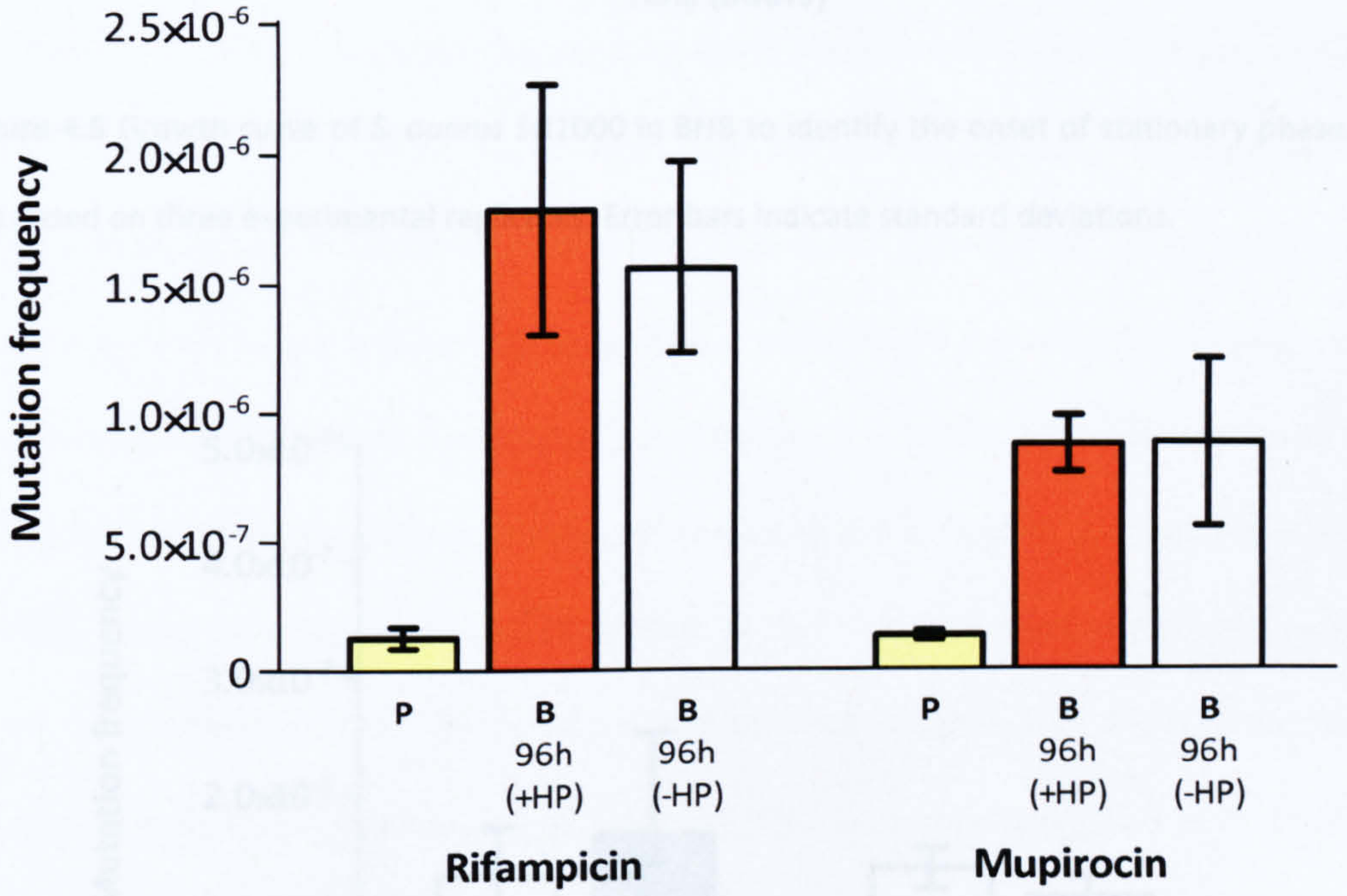

Figure 4.4 Mutation frequencies of planktonic and Sorbarod biofilm cultures of *S. aureus* SH1000. Spontaneous mutants arising from these cultures were isolated using rifampicin and mupirocin selection. P and B96h indicate planktonic and 96 hr biofilm cultures, respectively. Biofilm cultures were incubated in the presence (+HP) and absence (-HP) of 4% human plasma. Data are based on three experimental replicates. Error bars indicate standard deviations.

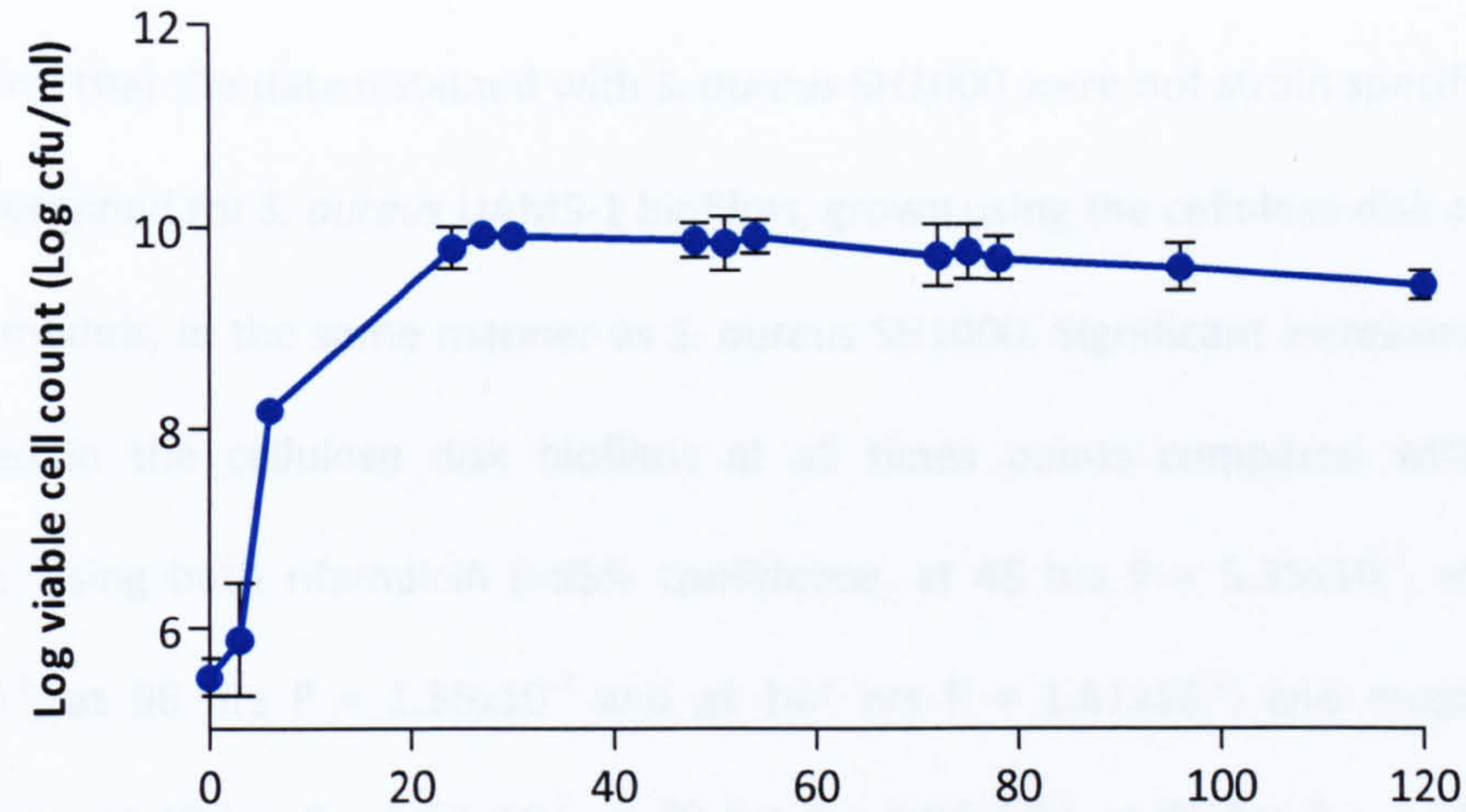

Figure 4.5 Growth curve of *S. aureus* SH1000 in BHB to identify the onset of stationary phase. Data are based on three experimental replicates. Error bars indicate standard deviations.

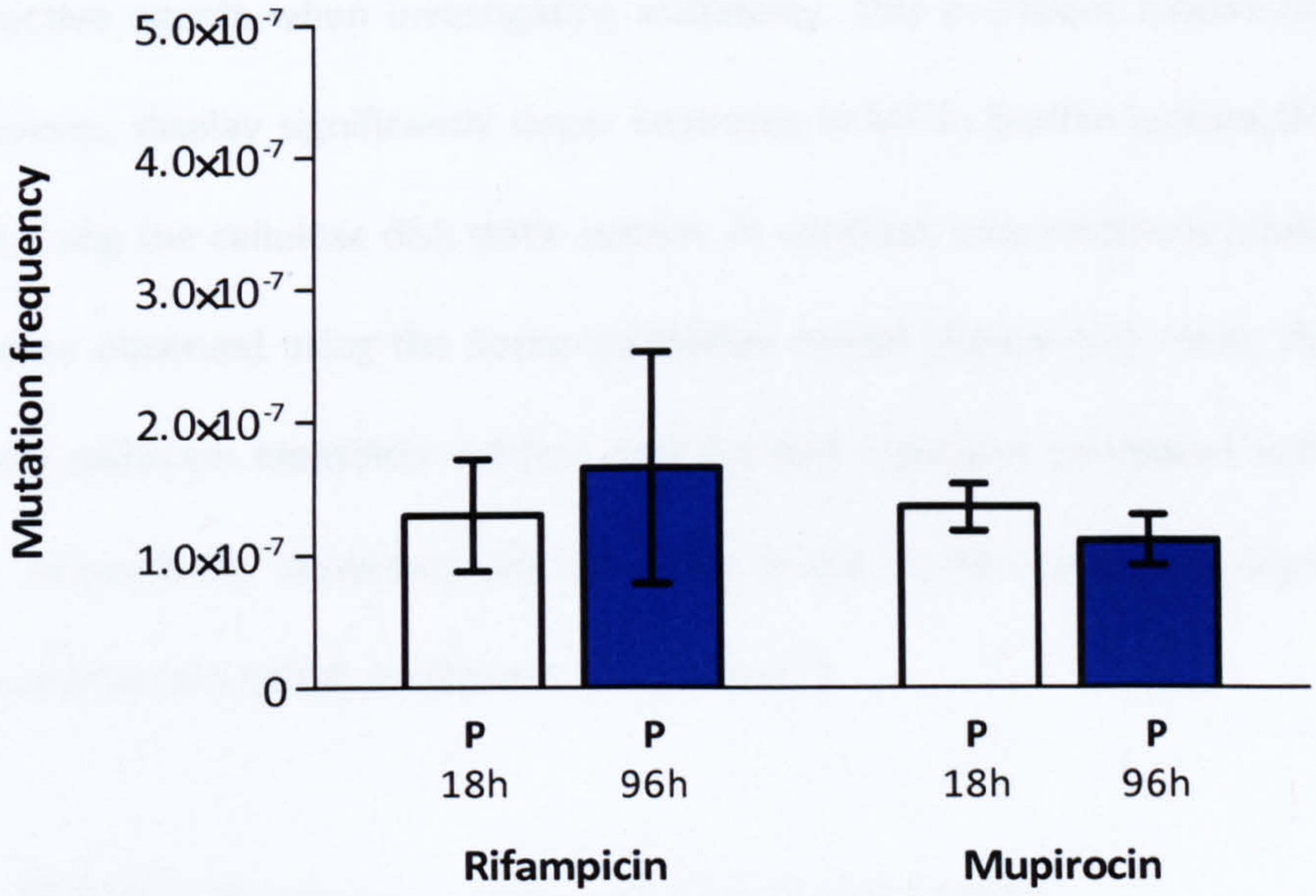

Figure 4.6 Mutation frequencies of planktonic (P) *S. aureus* SH1000 cultures, in BHB, grown for 18 hrs and 96 hrs. Spontaneous mutants were isolated from cultures using the selection antibiotics rifampicin and mupirocin. Data are based on three experimental replicates. Error bars indicate standard deviations.

4.3.1.2. Mutation frequencies in biofilms of S. aureus UAMS-1

To confirm that the data obtained with *S. aureus* SH1000 were not strain specific, MFs were also determined for *S. aureus* UAMS-1 biofilms, grown using the cellulose disk and Sorbarod biofilm models, in the same manner as *S. aureus* SH1000. Significant increases in MF were identified in the cellulose disk biofilms at all times points compared with planktonic cultures, using both rifampicin (>95% confidence, at 48 hrs P = $5.35x10^{-3}$, at 72 hrs P = $3.95x10^{-3}$, at 96 hrs P = $1.56x10^{-2}$ and at 144 hrs P = $1.81x10^{-2}$) and mupirocin (>95% confidence, at 48 hrs P = $1.63x10^{-2}$, at 72 hrs P = $4.90x10^{-3}$, at 96 hrs P = $1.51x10^{-4}$ and at 144 hrs P = $1.79x10^{-3}$) selection (Figure 4.7). Here, the maximum MFs observed at 144 hrs were 67.8-fold and 9.3-fold higher than the planktonic cultures, for rifampicin and mupirocin selection, respectively. The differences in MF determined using rifampicin and mupirocin remains unexplained, although this does highlight the need for utilising at least two selective agents when investigating mutability. This proficient biofilm-forming strain did, however, display significantly larger increases in MF in biofilm culture than *S. aureus* SH1000, using the cellulose disk static system. In contrast, only relatively modest increases in MF were observed using the Sorbarod biofilm model (Figure 4.8). Here, rifampicin and mupirocin selection identified 2.8-fold and 3.1-fold increases compared with planktonic culture, respectively. However, this was only found to be statistically significant using mupirocin selection (>95% confidence, P = $2.76x10^{-2}$).

4.3.1.3. Mutation frequencies in biofilms of S. epidermidis RP62A

To investigate whether the observed phenomena on MFs was specific to *S. aureus*, or whether it might be common amongst staphylococci, MFs were determined for *S. epidermidis* RP62A, also using the cellulose disk biofilm model (Figure 4.9). In this case, statistically significant differences were observed between planktonic cultures and biofilm

cultures at all times points using both rifampicin (>95% confidence, at 48 hrs $P = 3.35x10^{-2}$, at 72 hr $P = 7.01x10^{-3}$, at 96 hrs $P = 6.20x10^{-3}$ and at 144 hrs $P = 1.11x10^{-2}$) and mupirocin (>95% confidence, at 48 hrs $P = 7.27x10^{-3}$, at 72 hrs $P = 3.70x10^{-3}$, at 96 hrs $P = 5.98x10^{-3}$ and at 144 hrs $P = 1.03x10^{-2}$) selection. Maximum MFs were achieved at 72 hrs of incubation for biofilm culture, where 4.1-fold and 3.9-fold increases were observed, compared with the planktonic cultures, using rifampicin and mupirocin selection, respectively. This demonstrates that enhanced mutability in the biofilm mode of growth may therefore be a general occurrence amongst staphylococci.

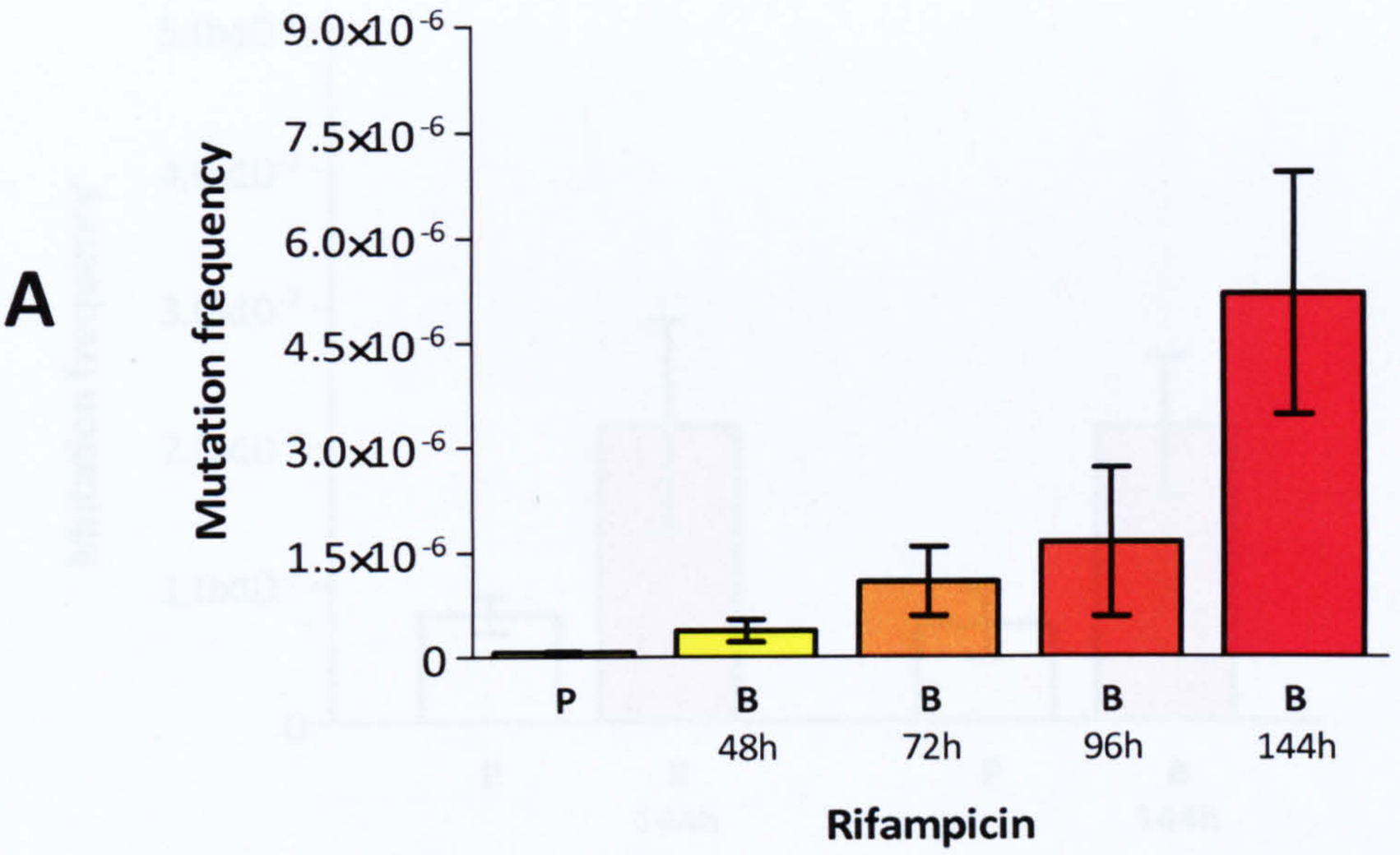

Figure 4.7 Mutation frequencies of planktonic and cellulose disk-grown biofilm cultures of *S. aureus* UAMS-1. Spontaneous mutants were isolated from the cultures using **A** rifampicin and **B** mupirocin selection. P indicates planktonic cultures and 48h, 72h, 96h and 144h indicate biofilm (B) incubation time. Data are based on six experimental replicates. Error bars indicate standard deviations.

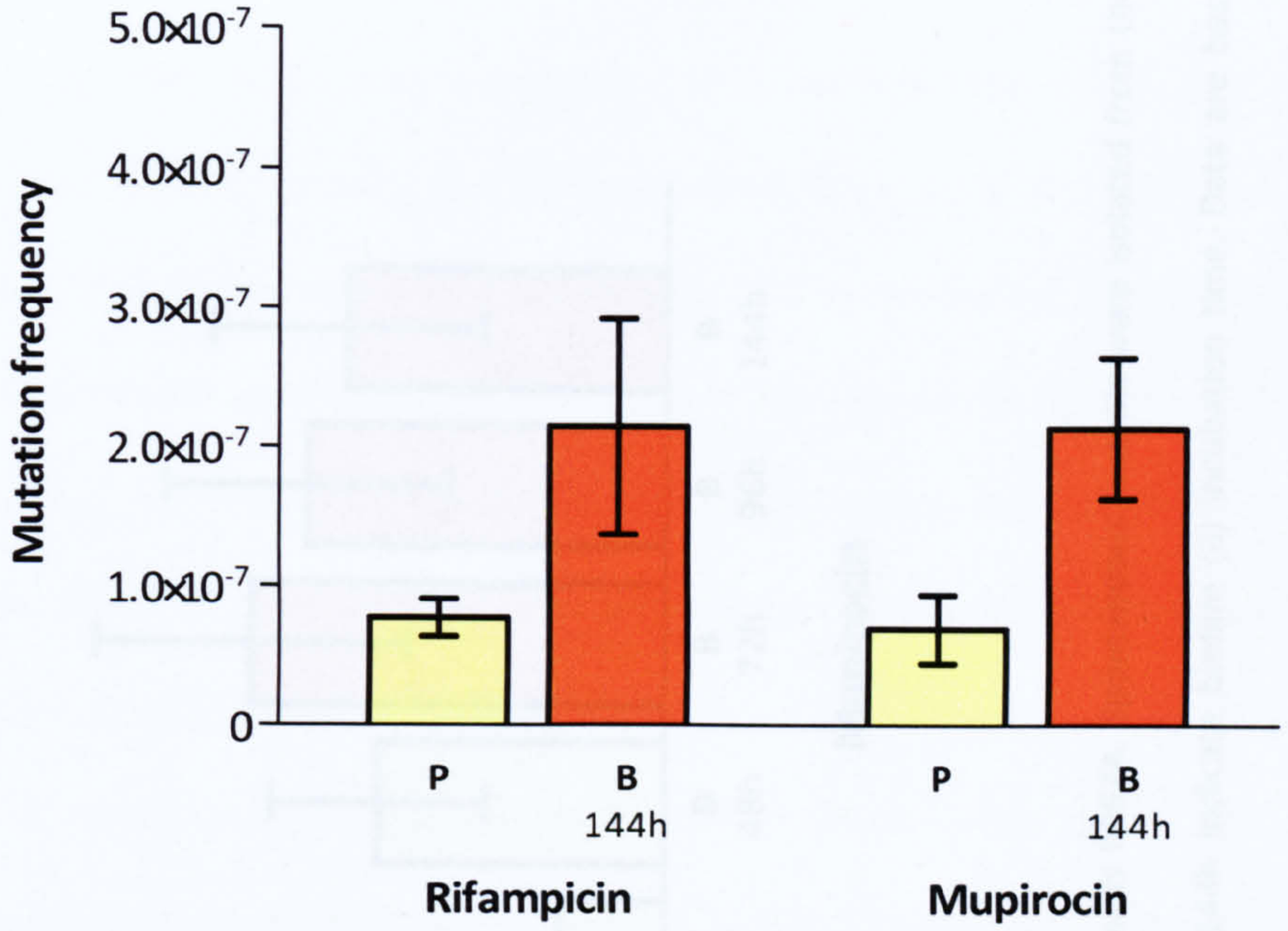

Figure 4.8 Mutation frequencies of planktonic and Sorbarod biofilm cultures of *S. aureus* UAMS-1. Spontaneous mutants arising from these cultures were isolated using rifampicin and mupirocin selection. P indicates planktonic and B144h indicates 144 hr biofilm (B) cultures, respectively. Data are based on three experimental replicates. Error bars indicate standard deviations.

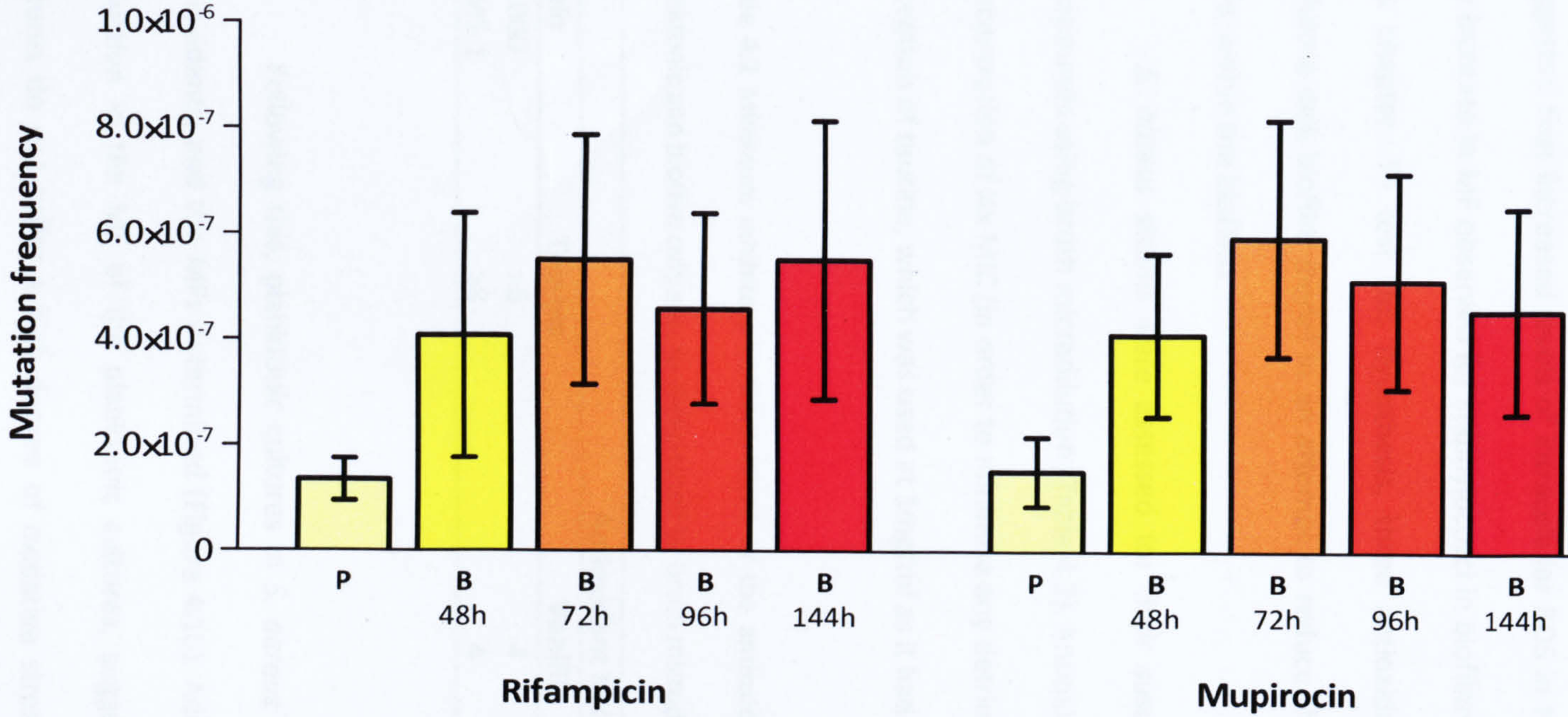

Figure 4.9 Mutation frequencies of planktonic and cellulose disk-grown biofilm cultures of *S. epidermidis* RP62A. Spontaneous mutants were isolated from the cultures using rifampicin and mupirocin selection. P indicates planktonic cultures and 48h, 72h, 96h and 144h indicate biofilm (B) incubation time. Data are based on six experimental replicates. Error bars indicate standard deviations.

4.3.2. S. aureus biofilm mutability and the effects of antioxidants

4.3.2.1. Exogenous antioxidant agents

There have been some reports suggesting that bacteria may suffer from endogenous oxidative stress when growing as a biofilm (Boles & Singh, 2008; Driffield *et al.*, 2008). This suggested that increased levels of intracellular ROS in the biofilm may be responsible for the increase in MF observed for staphylococci in biofilms, as reported in earlier sections of this chapter. To test this hypothesis, three antioxidants were incorporated into the cellulose disk biofilm model in an attempt to reduce the level of ROS, and subsequently MFs, within the biofilms.

S. aureus strains were assessed for their susceptibility to the test antioxidant compounds using broth microdilution (Table 4.2). Antioxidants were subsequently used at a concentration of ¼x MIC (in order to minimise any detrimental effects on growth), with the exception of taurine, which was used at 1mg/ml as it had no recordable MIC.

Table 4.2 Minimum inhibitory concentrations of the antioxidant compounds used to supplement planktonic and biofilm cultures, as determined by broth microdilution.

	Antioxidant MIC (g/L)		
Strain	**Taurine**	**Vanillin**	**Ascorbic acid**
SH1000	>8	4	0.125
UAMS-1	>8	4	0.125

Following this, planktonic cultures of *S. aureus* SH1000 were supplemented with antioxidants, and the MFs determined (Figure 4.10). Addition of antioxidants revealed no reduction in the MF of the planktonic cultures, suggesting that bacteria within these cultures do not suffer a high degree of oxidative stress. However, the antioxidants did significantly increase MFs when rifampicin was used as the selective agent (>95%

confidence, with Taurine P = $5.36x10^{-6}$, with vanillin P = $2.72x10^{-4}$ and with ascorbic acid P = $7.15x10^{-5}$) (Figure 4.10). The basis of this response is unknown. Subsequently, the same antioxidants were used to supplement *S. aureus* SH1000 biofilm cultures grown using the cellulose disk model for 96 hrs (Figure 4.11). Biofilms were incubated for 96 hrs, as cultures of this time point demonstrated the greatest increase in MF compared with planktonic cultures, in the absence of antioxidants. The antioxidants significantly reduced the MFs of the 96 hr biofilm cultures to the level of the planktonic cultures, using both rifampicin (>95% confidence, with taurine P = $2.25x10^{-5}$, with vanillin P = $1.64x10^{-5}$ and with ascorbic acid P = $4.67x10^{-6}$) and mupirocin (>95% confidence, with taurine P = $3.27x10^{-3}$, with vanillin P = $3.66x10^{-3}$ and with ascorbic acid P = $3.80x10^{-3}$) selection (Figure 4.11). These biofilms, therefore, may be suffering from endogenous oxidative stress.

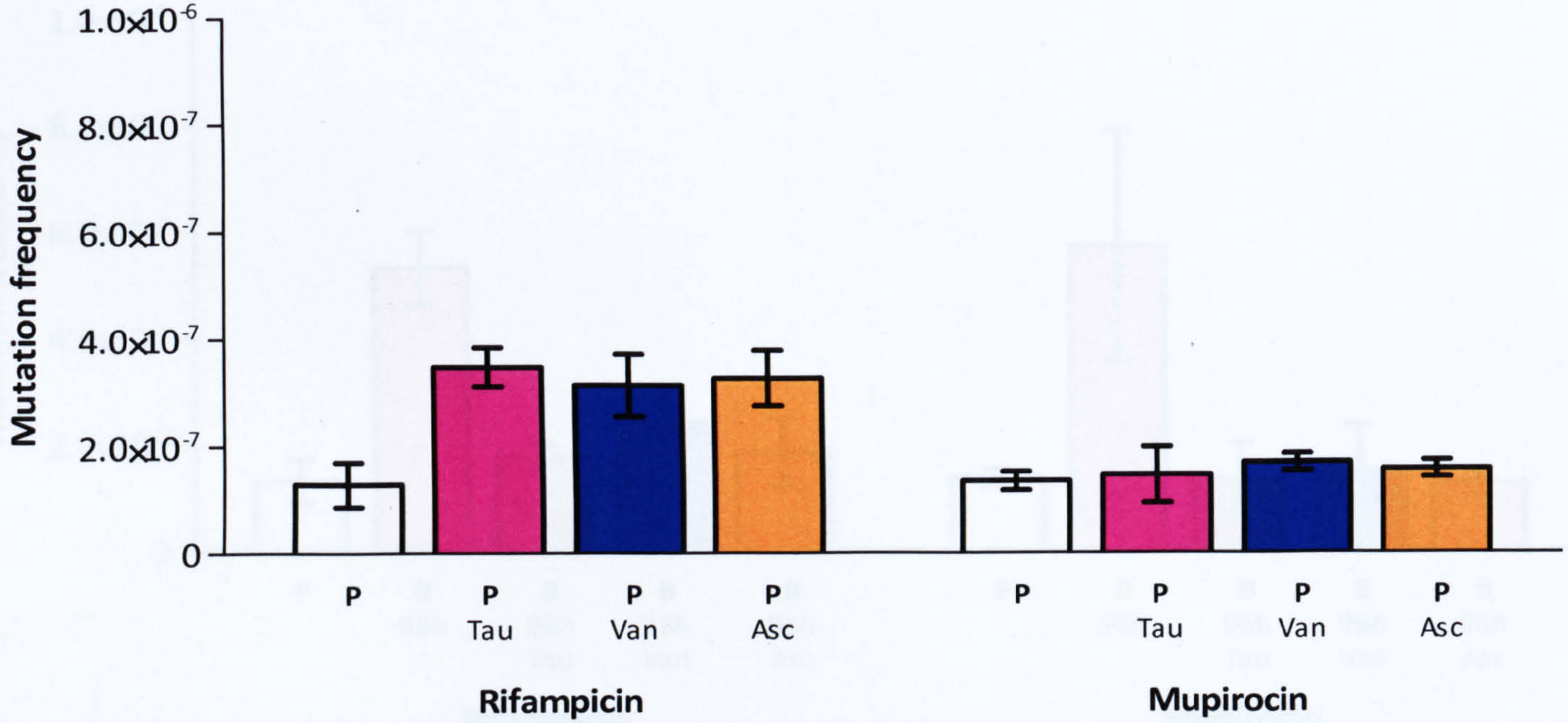

Figure 4.10 Mutation frequencies of *S. aureus* SH1000 planktonic cultures supplemented with antioxidants. Spontaneous mutants were isolated from cultures using the selection antibiotics rifampicin and mupirocin. P indicates planktonic cultures in the absence of antioxidants and Tau, Van and Asc indicate cultures to which taurine, vanillin, and ascorbic acid, were added respectively. Data are based on six experimental replicates. Error bars indicate standard deviations.

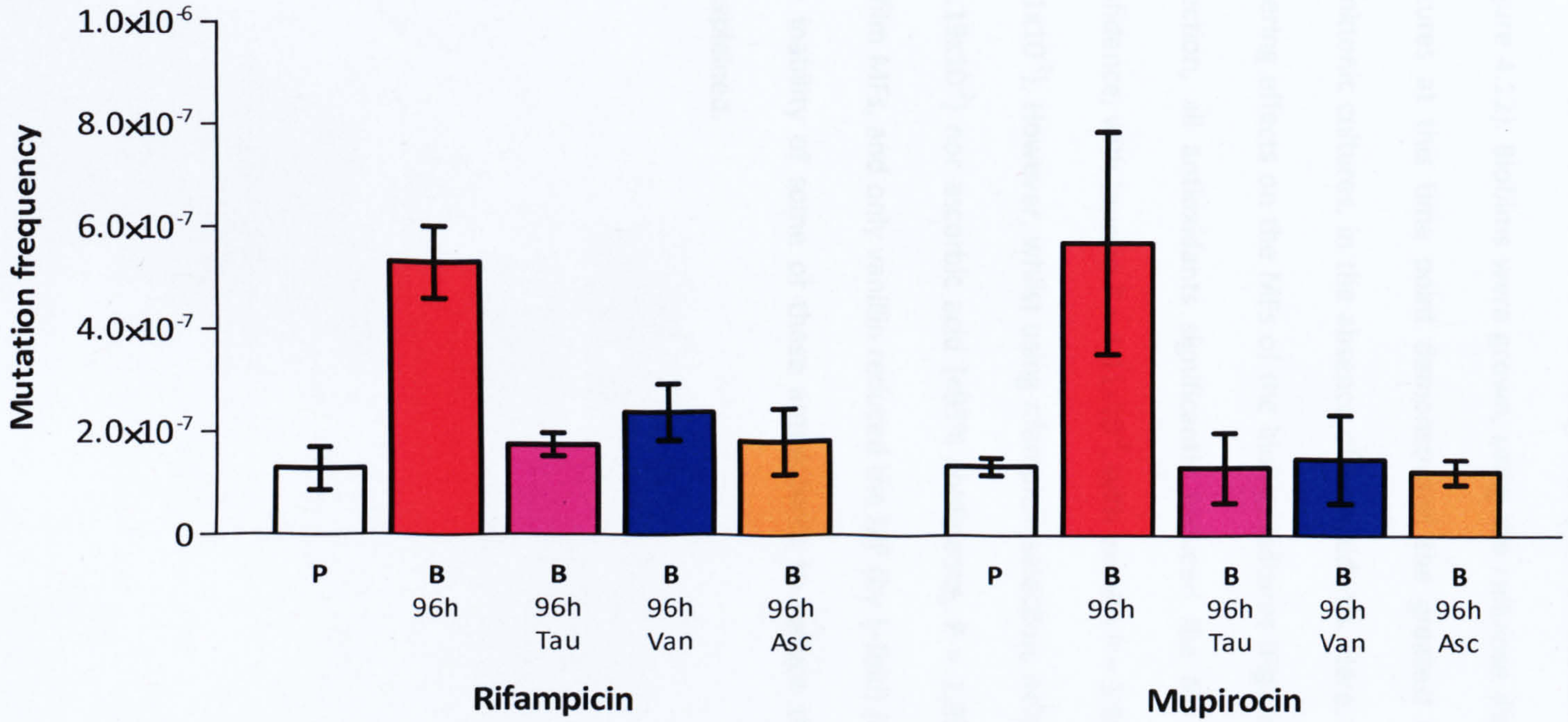

Figure 4.11 Mutation frequencies of *S. aureus* SH1000 planktonic and biofilm cultures supplemented with antioxidants. Spontaneous mutants were isolated from cultures using the selection antibiotics rifampicin and mupirocin. P indicates planktonic cultures in the absence of antioxidants, 96h indicates biofilms (B) incubated for 96hrs and those supplemented with taurine (Tau), vanillin (Van) and ascorbic acid (Asc), respectively. Data are based on six experimental replicates. Error bars indicate standard deviations.

To confirm these data, antioxidants were also added to *S. aureus* UAMS-1 planktonic and biofilm cultures. Antioxidant compounds had no significant effect (<95% confidence) on the MF of UAMS-1 planktonic cultures, except vanillin that marginally, but significantly, increased the MF using rifampicin (>95% confidence, $P = 5.35x10^{-4}$) selection (Figure 4.12). Biofilms were grown, using the cellulose disk model, for 144 hrs, as biofilm cultures at this time point demonstrated the greatest increase in MF compared with planktonic cultures, in the absence of antioxidants. Here, the antioxidant compounds had differing effects on the MFs of the biofilm cultures (Figure 4.13). In the case of mupirocin selection, all antioxidants significantly reduced the MF of the biofilm cultures (>95% confidence, with taurine $P = 4.11x10^{-3}$, with vanillin $P = 5.59x10^{-3}$ and with ascorbic acid $P = 2.91x10^{-2}$). However, whilst using rifampicin selection, neither taurine (<95% confidence, $P = 9.19x10^{-2}$) nor ascorbic acid (<95% confidence, $P = 1.95x10^{-1}$) significantly affected the biofilm MFs, and only vanillin reduced the MF (by 5-fold) (>95% confidence, $P = 2.99x10^{-3}$). The inability of some of these antioxidants to reduce the biofilm MFs of this strain is unexplained.

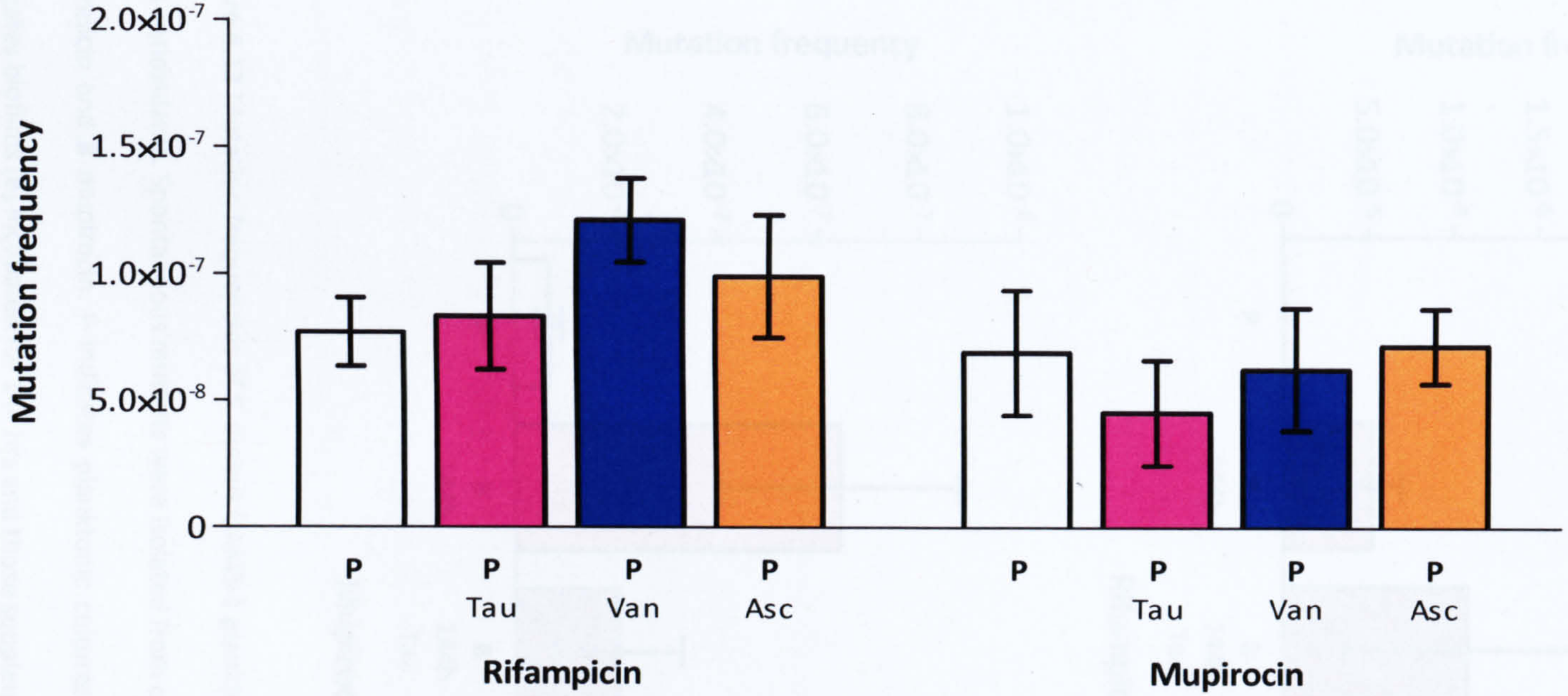

Figure 4.12 Mutation frequencies of *S. aureus* UAMS-1 planktonic cultures supplemented with antioxidants. Spontaneous mutants were isolated from cultures using the selection antibiotics rifampicin and mupirocin. P indicates planktonic cultures in the absence of antioxidants and PTau, PVan, and PAsc indicate planktonic cultures supplemented with taurine, vanillin, and ascorbic acid, respectively. Data are based on six experimental replicates. Error bars indicate standard deviations.

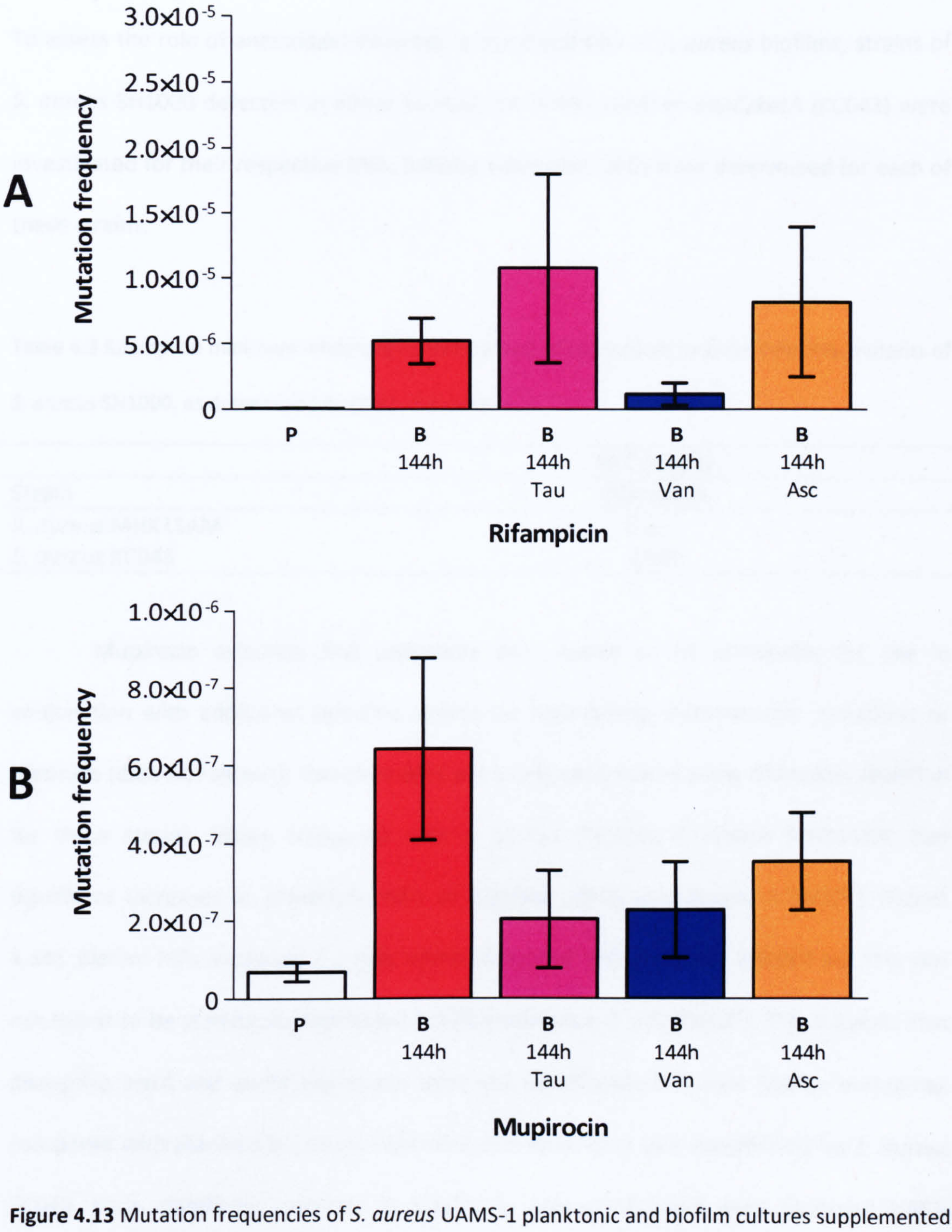

Figure 4.13 Mutation frequencies of *S. aureus* UAMS-1 planktonic and biofilm cultures supplemented with antioxidants. Spontaneous mutants were isolated from cultures using the selection antibiotics **A** rifampicin and **B** mupirocin. P indicates planktonic cultures in the absence of antioxidants, 144h indicates biofilms (B) incubated for 144 hrs and those supplemented with taurine (Tau), vanillin (Van) and ascorbic acid (Asc), respectively. Data are based on six experimental replicates. Error bars indicate standard deviations.

4.3.2.2. Endogenous antioxidant enzymes

To assess the role of antioxidant enzymes in the mutability of *S. aureus* biofilms, strains of *S. aureus* SH1000 defective in either *sodA/sodM* (MHK11AM) or *ahpC/katA* (KC043) were investigated for their respective MFs. Initially, rifampicin MICs were determined for each of these strains.

Table 4.3 Rifampicin minimum inhibitory concentrations of antioxidant enzyme defective mutants of *S. aureus* SH1000, as determined by broth microdilution.

	MIC (mg/L)
Strain	**Rifampicin**
S. aureus MHK11AM	0.031
S. aureus KC043	0.031

Mupirocin selection had previously been found to be unsuitable for use in conjunction with additional selective agents for maintaining chromosomal mutations or plasmids (data not shown); therefore MFs were only determined using rifampicin selection for these strains. When compared with *S. aureus* SH1000, *S. aureus* MHK11AM had significant increases in planktonic MFs of 2.8-fold (>95% confidence, 1.78×10^{-2}) (Figure 4.14). Biofilm MFs increased 2.2-fold, when compared with *S. aureus* SH1000 but this was not found to be statistically significant (<95% confidence, $P = 5.30 \times 10^{-2}$). This suggests that disrupting *sodA* and *sodM* expression does not significantly influence biofilm mutability, compared with planktonic culture. Following this, MFs were also determined for *S. aureus* KC043. Here, significant increases in planktonic MFs of 10.1-fold were observed (>95% confidence, $P = 4.69 \times 10^{-6}$). Furthermore, biofilm MFs were increased 7.9-fold and were statistically significant (>95% confidence, $P = 2.76 \times 10^{-5}$). Therefore, for both of these strains, the fold change in MF that occurs due to the absence of antioxidant enzymes is similar for planktonic and biofilm cultures. This demonstrates that although loss of

antioxidant enzymes causes an increase in mutability, this is not exacerbated by growth as a biofilm, and that loss of these enzymes is not therefore responsible for the increase in mutability observed in biofilms.

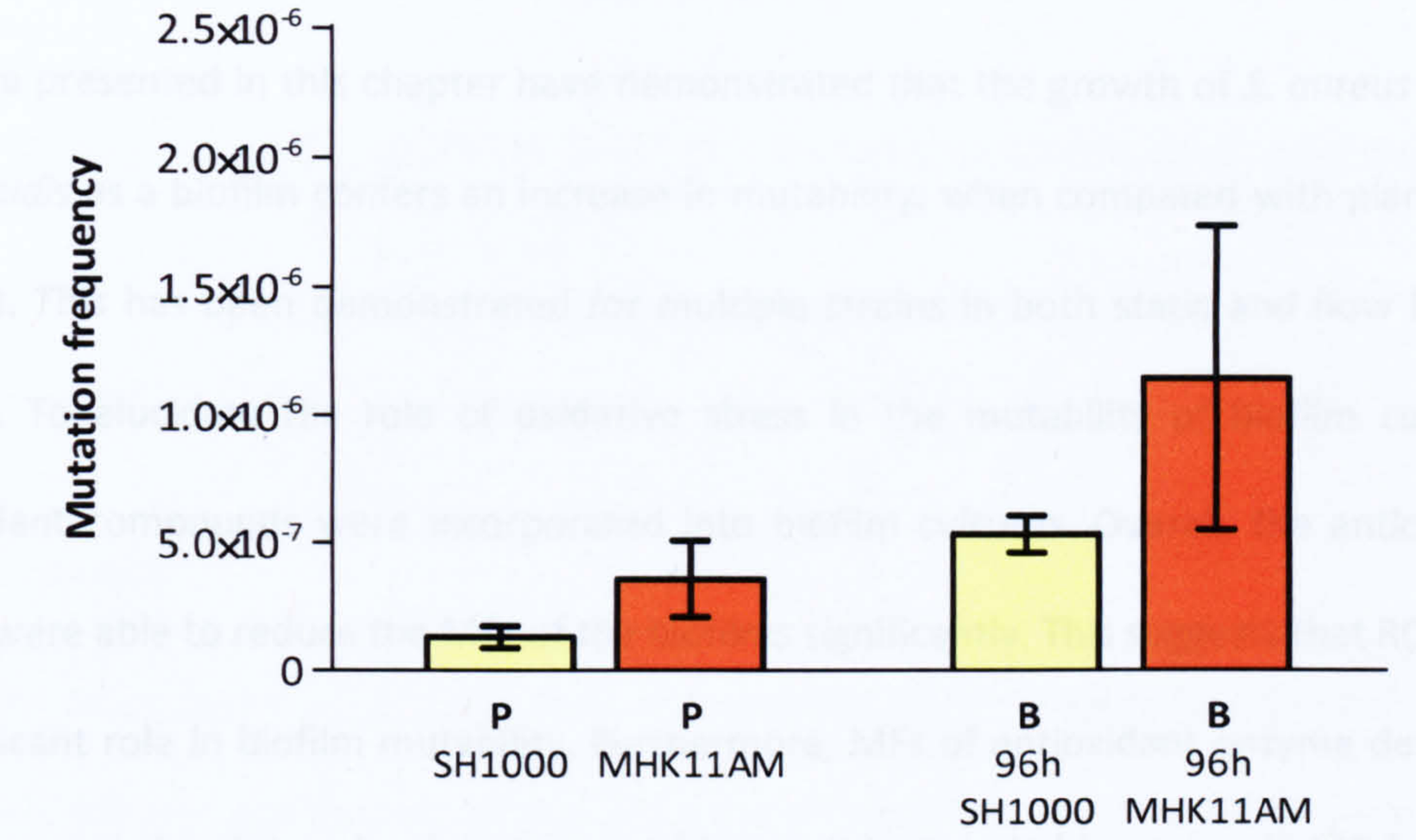

Figure 4.14 Mutation frequencies of *S. aureus* SH1000 and MHK11AM planktonic cultures (P) and biofilm cultures (B) incubated for 96 hrs generated using the cellulose disk biofilm model. Spontaneous mutants were isolated from cultures using rifampicin selection. Data are based on six experimental replicates. Error bars indicate standard deviations.

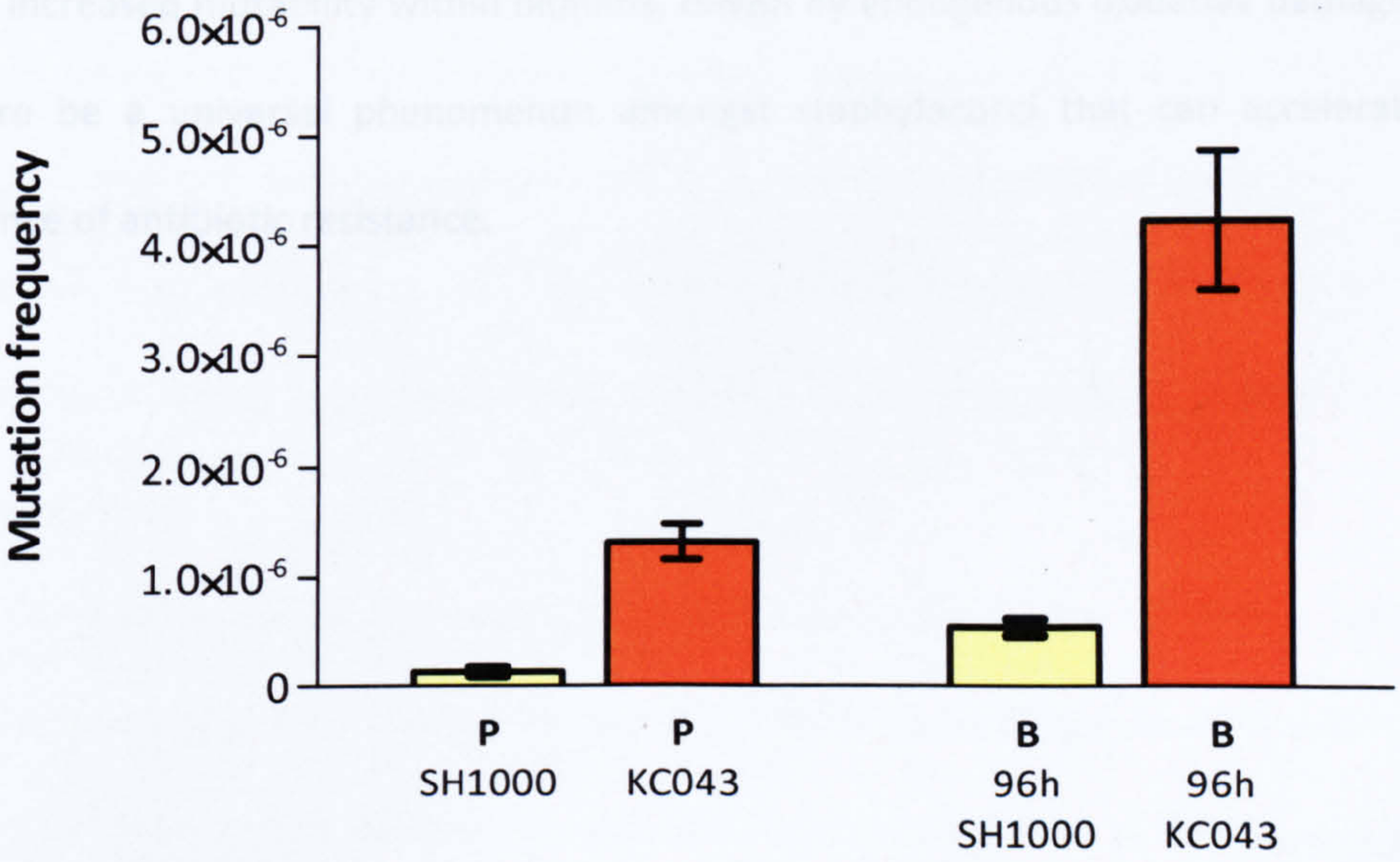

Figure 4.15 Mutation frequencies of *S. aureus* SH1000 and KC043 planktonic cultures (P) and biofilm cultures (B) incubated for 96 hrs generated using the cellulose disk biofilm model. Spontaneous mutants were isolated from cultures using rifampicin selection. Data are based on six experimental replicates. Error bars indicate standard deviations.

4.3.3. Concluding remarks

The data presented in this chapter have demonstrated that the growth of *S. aureus* and *S. epidermidis* as a biofilm confers an increase in mutability, when compared with planktonic cultures. This has been demonstrated for multiple strains in both static and flow biofilm models. To elucidate the role of oxidative stress in the mutability of biofilm cultures, antioxidant compounds were incorporated into biofilm cultures. Overall, the antioxidant agents were able to reduce the MFs of the biofilms significantly. This suggests that ROS play is significant role in biofilm mutability. Furthermore, MFs of antioxidant enzyme defective mutants were also determined. However, although this revealed increases in MF for both planktonic and biofilm cultures compared to the wild-type, the fold increase in MF previously observed between planktonic and biofilm cultures was maintained in these mutants. This demonstrates that the mechanism driving biofilm mutability was distinct from the antioxidant enzymes being investigated.

Increased mutability within biofilms, driven by endogenous oxidative damage, may therefore be a universal phenomenon amongst staphylococci that can accelerate the emergence of antibiotic resistance.

CHAPTER 5

5. Investigating *S. aureus* biofilm development and enhanced mutability by transcriptional profiling

5.1. Abstract

DNA microarray analysis was performed to reveal the transcriptional profiles of *S. aureus* planktonic cultures and biofilms grown using the cellulose disk model. This analysis aimed to identify genes potentially involved in biofilm maturation and elucidate mechanisms underlying the increased mutational status of these biofilm cultures.

When the transcriptional profiles of 48 hr and 144 hr biofilm cultures were compared, several functional groups were highly represented. At 144 hrs amino acid biosynthesis, cell envelope synthesis, regulatory functions, transport and binding groups all contained several differentially regulated genes. Specifically, at 144 hrs, many genes involved in histidine biosynthesis and encoding membrane proteins were found to be upregulated. Furthermore, at 144 hrs, transcriptional regulators that repress biofilm formation were found to be downregulated, whilst those that promote biofilm formation were upregulated.

To investigate biofilm mutability, the expression levels of genes conferring antioxidant activity and encoding DNA repair activity were investigated. Antioxidant genes were found to be largely unaffected, with only *sodA* upregulated in the 48 hr biofilms. Staphyloxanthin expression, however, was significantly reduced. DNA repair genes were significantly upregulated in biofilm culture. The increased mutability previously observed during the biofilm mode of growth in *S. aureus* is therefore associated with an increase in DNA repair activity.

5.2. Introduction

DNA microarray analysis has been used extensively to assess the transcriptional profiles of bacteria grown under different conditions. This technique is particularly useful as it can simultaneously provide information on the expression levels of all genes a given genome. In biofilm studies, this technique has been used to try to identify changes in gene expression between planktonic and biofilm cultures. Biofilm models utilised for such transcriptional profiling have included both static and flow culture systems, and have identified that gene expression in biofilms is distinct from that of both exponential and stationary phase planktonic growth (Beenken *et al.*, 2004; Resch *et al.*, 2005). However, data generated from microarray analysis can be affected by the biofilm model, growth media and strain used, as well as the microarray platform employed (Nagarajan *et al.*, 2009).

This work sought to utilise DNA microarray analysis to identify genes differentially expressed between 144 hr and 48 hr biofilms to investigate biofilm maturation and to elucidate a mechanism by which *S. aureus* becomes more mutable whilst growing as a biofilm. In addition, this was the first transcriptional profiling of *S. aureus* SH1000 biofilms, and will therefore provide a useful comparison with the biofilm transcriptional profiles already compiled on other *S. aureus* strains such as *S. aureus* UAMS-1 and 113 (Beenken *et al.*, 2004; Resch *et al.*, 2005).

5.3. Results and Discussion

To assess the transcriptional status of planktonic and biofilm cultures, total RNA was extracted from planktonic cultures and 48 hr and 144 hr biofilm cultures, grown using the cellulose disk biofilm model, of *S. aureus* SH1000. The transcriptional profiles of these cultures were then determined by DNA microarray analysis, performed by Roche Nimblegen. The arrays contained probes for 2887, out of 2892, predicted coding sequences of the *S. aureus* NCTC 8325 genome due to shared probes. Microarray data were analysed using ArrayStar™ 4 software (DNASTAR), where genes exhibiting ≥2-fold difference in expression were regarded as being significantly differentially expressed (Beenken *et al.*, 2004). Statistical analysis of differentially regulated genes can be found in Appendix tables A.1-A.3,

In 48 hr and 144 hr biofilms compared with planktonic cultures grown overnight, 831 and 262 genes were differentially expressed, respectively (Figure 5.1 A and B). However, when biofilm cultures were compared, only 110 genes were differentially expressed (Figure 5.1C). From this it was concluded that the majority of differential gene expression in biofilms, when compared with planktonic cultures, occurs in the early stages of biofilm formation. For a comprehensive list of differentially expressed genes, please see Appendix tables A.1-A.3.

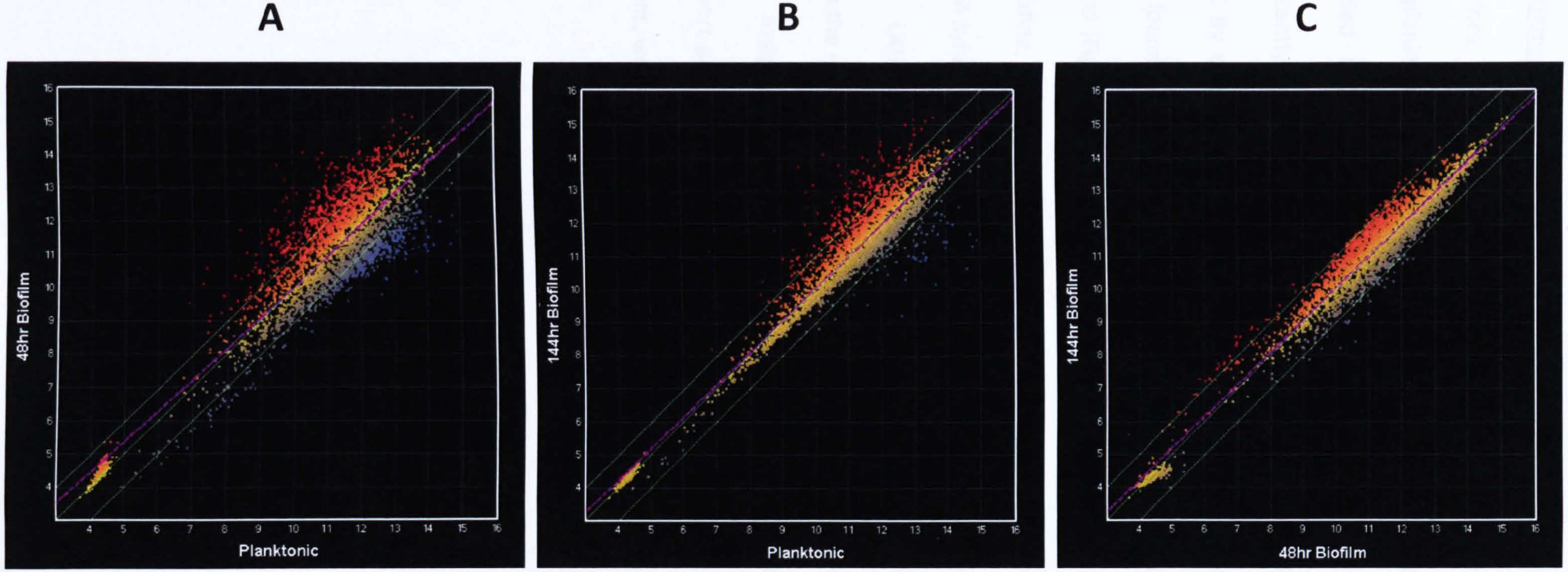

Figure 5.1 Scatter plots of differentially expressed genes in **A** 48 hr biofilm cultures compared with planktonic cultures, **B** 144 hr biofilm cultures compared with planktonic cultures and **C** 144 hr biofilm cultures compared with 48 hr biofilm cultures, of *S. aureus* SH1000. Data analysis and plot generation were performed using ArrayStar™ 4 software (DNASTAR). The green lines represent 2-fold up or down regulation markers, hence genes in the test condition (y-axis) with an average regulation 2-fold higher or lower than the control condition (x axis) will fall outside of these lines. Data are based on the mean of three experimental replicates.

Genes were grouped according to the JCVI gene ontology classification (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntsa05). In both biofilm culture conditions, many cell envelope-associated genes were downregulated, when compared with planktonic culture (Figure 5.2 and 5.3A). This supports previous reports which identified little increase in viable cells following biofilm establishment, suggesting significantly reduced growth rates in the biofilm (Evans & Holmes, 1987; Prosser *et al.*, 1987). By comparison, differentially expressed genes involved in transport and binding were found to be mostly downregulated in 48 hr biofilms, but upregulated in 144 hr biofilms (Figure 5.2 and 5.3A). Furthermore, differentially expressed genes in the purine, pyrimidine, nucleotide and nucleoside groups, those involved in DNA metabolism and protein synthesis were mostly upregulated in both biofilm conditions (Figure 5.2 and 5.3A).

Little difference was observed between the 48 hr and 144 hr biofilm cultures, where the majority of differentially expressed genes encoded hypothetical proteins. Of the genes assigned into ontological groups, differentially expressed genes were mostly transport and binding proteins, cell envelope-associated proteins or amino acid synthesis proteins, where the majority were upregulated.

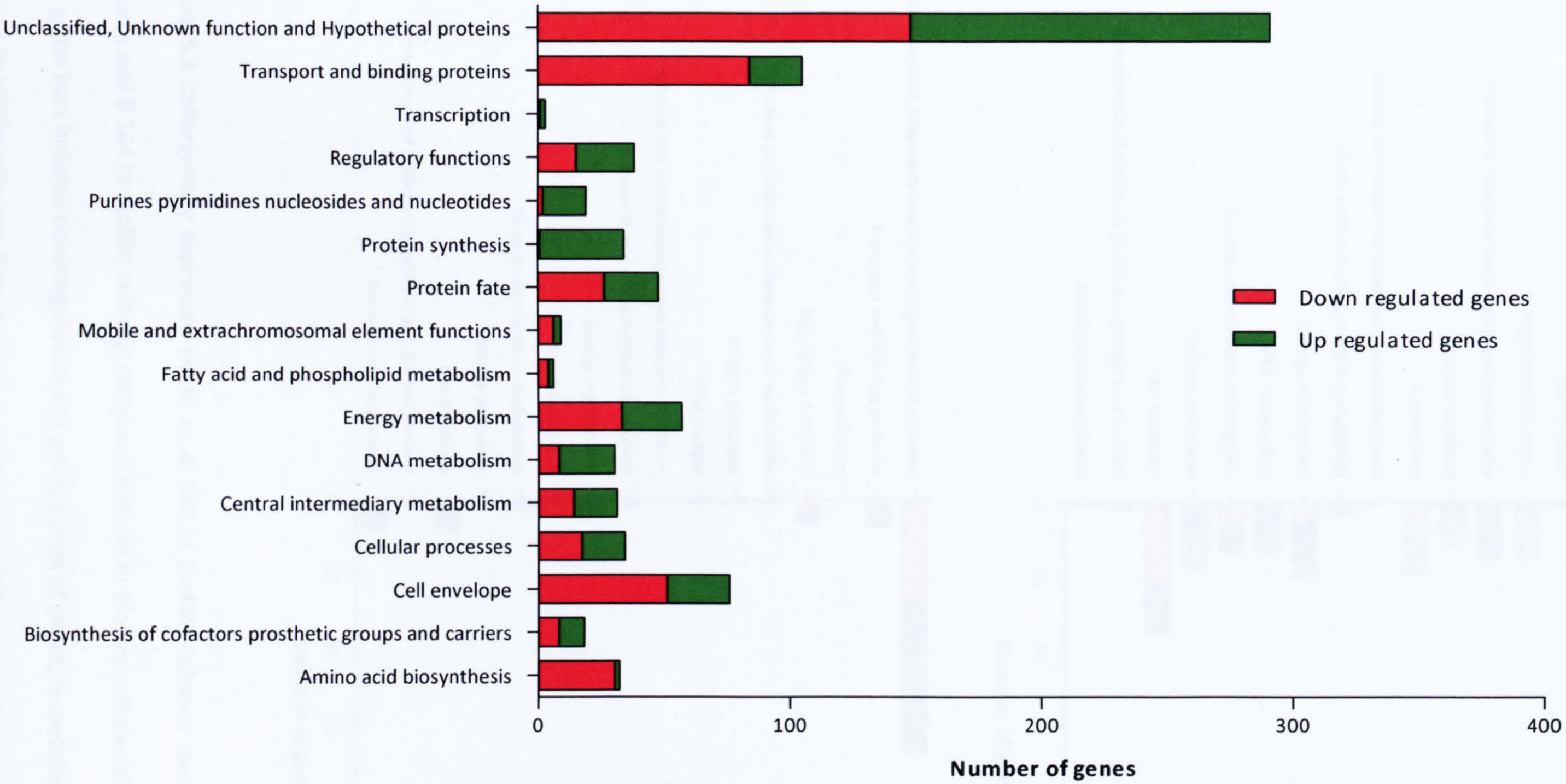

Figure 5.2 Differentially expressed genes in 48 hr biofilm cultures compared with planktonic cultures. Red and green bars indicate downregulation and upregulation of genes, respectively, by 2 or more fold in the biofilm cultures. Data are based on the mean of three experimental replicates.

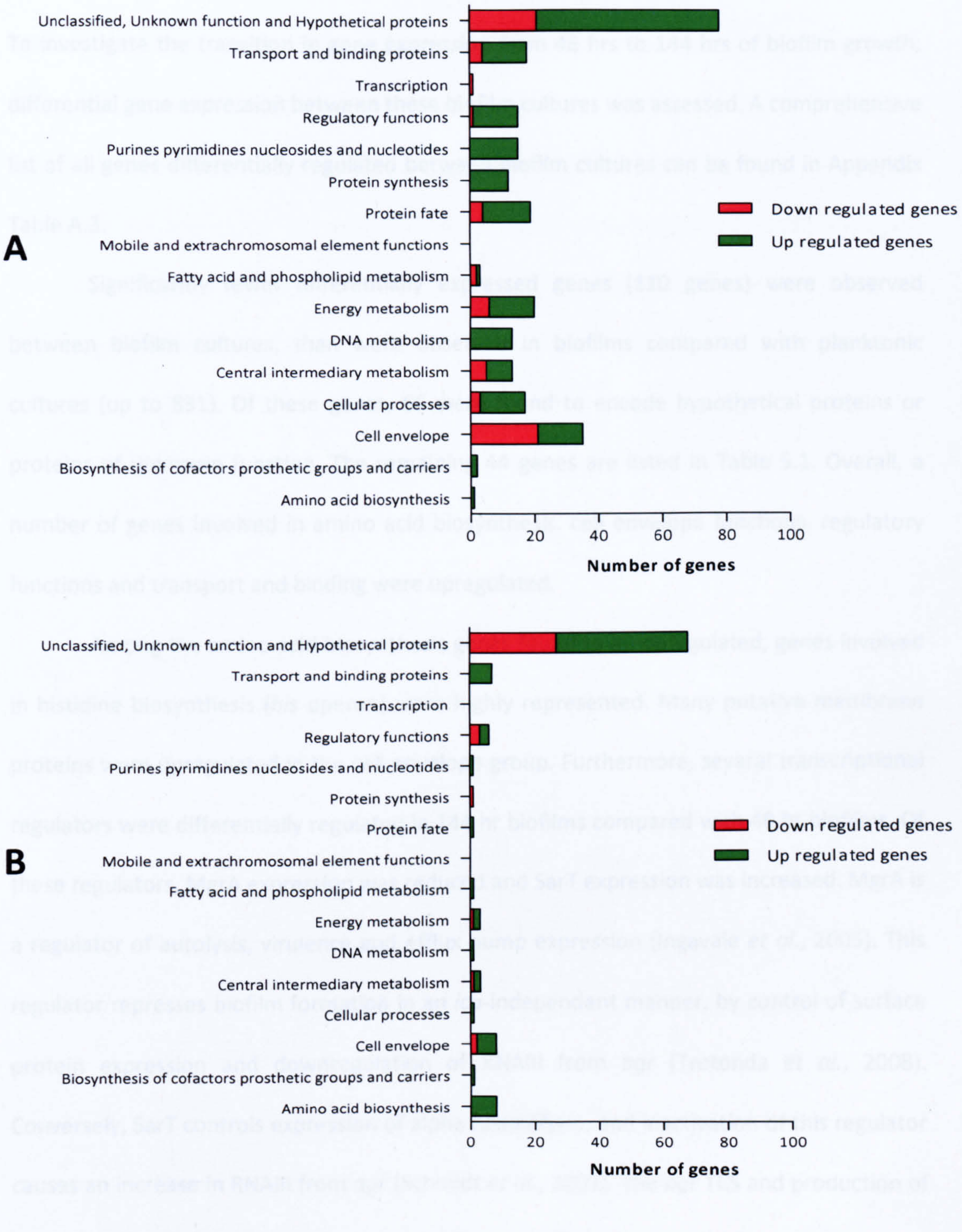

Figure 5.3 Differentially expressed genes in **A** 144 hr biofilm cultures compared with planktonic cultures and **B** 144 hr biofilm cultures compared with 48 hr biofilm cultures of *S. aureus* SH1000. Red and green bars indicate downregulation and upregulation of genes, respectively, by 2 or more fold in the 144 hr biofilm cultures. Data are based on the mean of three experimental replicates.

5.3.1. Investigating biofilm maturation

To investigate the transition in gene expression from 48 hrs to 144 hrs of biofilm growth, differential gene expression between these biofilm cultures was assessed. A comprehensive list of all genes differentially regulated between biofilm cultures can be found in Appendix Table A.3.

Significantly fewer differentially expressed genes (110 genes) were observed between biofilm cultures, than were observed in biofilms compared with planktonic cultures (up to 831). Of these genes, 66 were found to encode hypothetical proteins or proteins of unknown function. The remaining 44 genes are listed in Table 5.1. Overall, a number of genes involved in amino acid biosynthesis, cell envelope functions, regulatory functions and transport and binding were upregulated.

Among the amino acid biosynthesis genes found to be upregulated, genes involved in histidine biosynthesis (*his* operon) were highly represented. Many putative membrane proteins were upregulated in the cell envelope group. Furthermore, several transcriptional regulators were differentially regulated in 144 hr biofilms compared with 48 hr biofilms. Of these regulators, MgrA expression was reduced and SarT expression was increased. MgrA is a regulator of autolysis, virulence and efflux pump expression (Ingavale *et al.*, 2005). This regulator represses biofilm formation in an *ica*-independent manner, by control of surface protein expression and downregulation of RNAIII from *agr* (Trotonda *et al.*, 2008). Conversely, SarT controls expression of alpha haemolysin, and inactivation of this regulator causes an increase in RNAIII from *agr* (Schmidt *et al.*, 2001). The *agr* TCS and production of the regulatory RNAIII, influence the production of biofilms in an *ica*-independent manner, through control of genes such as extracellular proteases (Boles & Horswill, 2008; Lauderdale *et al.*, 2009). Although no significant difference in gene expression was observed for *agr*, significant downregulation of delta haemolysin, encoded by RNAIII

(Janzon *et al.*, 1989), was observed in both 48 hr and 144 hr biofilms (Table 3.6). The differential gene expression observed in these biofilms may therefore be promoting biofilm formation in an *ica*-independent manner (Lauderdale *et al.*, 2009). This type of staphylococcal biofilm has been identified in a number of clinical isolates that do not synthesize PIA, and instead synthesize a proteinaceous matrix (Kogan *et al.*, 2006; Chaignon *et al.*, 2007). Such a matrix could also, therefore, be present in *S. aureus* SH1000 biofilms generated in this system.

Table 5.1 Characterised differentially expressed genes in 144 hr biofilms (B144h) compared with 48 hr biofilms (B48h) of *S. aureus* SH1000. Data are the mean of three experimental replicates.

ORF	Gene name	Function	Fold change B48h/B144h	Functional group
SAOUHSC_00341		cystathionine gamma-synthase (CGS) (O-succinylhomoserine(thiol)-lyase)	**2.096 up**	Amino acid biosynthesis
SAOUHSC_01366		anthranilate synthase component I	**2.062 up**	Amino acid biosynthesis
SAOUHSC_01367		anthranilate synthase component II	**2.249 up**	Amino acid biosynthesis
SAOUHSC_02281	*ilvD*	dihydroxy-acid dehydratase	**2.233 up**	Amino acid biosynthesis
SAOUHSC_03009	*hisA*	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	**2.468 up**	Amino acid biosynthesis
SAOUHSC_03010	*hisH*	imidazole glycerol phosphate synthase, glutamine amidotransferase subunit	**2.304 up**	Amino acid biosynthesis
SAOUHSC_03013		histidinol dehydrogenase	**2.099 up**	Amino acid biosynthesis
SAOUHSC_03014	*hisG*	ATP phosphoribosyltransferase catalytic subunit	**2.009 up**	Amino acid biosynthesis
SAOUHSC_03015	*hisZ*	ATP phosphoribosyltransferase regulatory subunit	**2.328 up**	Amino acid biosynthesis
SAOUHSC_02715		adenosylmethionine-8-amino-7-oxononanoate aminotransferase	**2.024 up**	Biosynthesis of cofactors, prosthetic groups, and carriers
SAOUHSC_00089		sugar transferase	**2.277 up**	Cell envelope
SAOUHSC_00182		membrane protein, putative	**2.112 up**	Cell envelope
SAOUHSC_00254		membrane protein, putative	**2.677 up**	Cell envelope
SAOUHSC_00298	*nanE*	N-acetylmannosamine-6-P epimerase	**2.136 up**	Cell envelope
SAOUHSC_00718		membrane protein, putative	**2.211 down**	Cell envelope
SAOUHSC_00871	*dltC*	D-alanine--poly(phosphoribitol) ligase subunit 2	**2.090 down**	Cell envelope
SAOUHSC_02576		secretory antigen precursor SsaA, putative	**2.076 up**	Cell envelope
SAOUHSC_02888		membrane protein, putative	**2.268 up**	Cell envelope
SAOUHSC_02932	*betA*	choline dehydrogenase	**2.084 up**	Cellular processes
SAOUHSC_02565	*ureD*	urease accessory protein UreD	**2.645 down**	Central intermediary metabolism
SAOUHSC_02924		4-aminobutyrate aminotransferase	**2.252 up**	Central intermediary metabolism
SAOUHSC_03012		histidinol-phosphate aminotransferase, putative	**2.011 up**	Central intermediary metabolism
SAOUHSC_02645		LytTr DNA-binding region	**2.208 up**	DNA metabolism
SAOUHSC_00076		ornithine cyclodeaminase, putative	**2.081 up**	Energy metabolism
SAOUHSC_01452	*ald1*	alanine dehydrogenase 1	**2.093 up**	Energy metabolism
SAOUHSC_02830		D-lactate dehydrogenase	**2.077 down**	Energy metabolism
SAOUHSC_00051		1-phosphatidylinositol phosphodiesterase precursor, putative	**2.106 up**	Fatty acid and phospholipid metabolism

Table 5.1 cont.

SAOUHSC_01941	*splB*	serine protease SplB	**2.140 up**	Protein fate
SAOUHSC_00017	*rplI*	ribosomal protein L9	**2.040 down**	Protein synthesis
SAOUHSC_00694	*mgrA*	HTH-type transcriptional regulator MgrA	**2.391 down**	Regulatory functions
SAOUHSC_01285		regulatory protein, MerR	**2.194 down**	Regulatory functions
SAOUHSC_02461		transcriptional regulator, MerR family	**2.443 down**	Regulatory functions
SAOUHSC_02726		transcription activator, effector binding	**2.239 up**	Regulatory functions
SAOUHSC_02799	*sarT*	HTH-type transcriptional regulator SarT	**2.205 up**	Regulatory functions
SAOUHSC_02908		aminoglycoside phosphotransferase:Fructosamine kinase	**2.073 up**	Regulatory functions
SAOUHSC_03001	*icaR*	biofilm operon icaabcd hth-type negative transcriptional regulator IcaR	**2.644 down**	Regulatory functions
SAOUHSC_00078		general substrate transporter:Major facilitator superfamily MFS_1	**2.003 up**	Transport and binding proteins
SAOUHSC_00136		nitrate transport ATP-binding protein NrtD	**2.103 up**	Transport and binding proteins
SAOUHSC_00138		ABC transporter, permease protein	**2.288 up**	Transport and binding proteins
SAOUHSC_00209		PTS system, glucose-specific IIBC component, putative	**2.319 up**	Transport and binding proteins
SAOUHSC_02662		PTS system, sucrose-specific IIBC component	**2.020 up**	Transport and binding proteins
SAOUHSC_02754		ABC transporter, ATP-binding protein	**2.045 up**	Transport and binding proteins
SAOUHSC_02806		gluconate permease, putative	**2.150 up**	Transport and binding proteins

5.3.2.

5.3.3. Investigating biofilm mutability

5.3.3.1. Genes with antioxidant capacity

To deduce the nature of the increased mutability observed for staphylococcal biofilm cultures, the transcriptional profiling data were analysed for differential regulation of genes that may contribute to genetic mutation. Accordingly, genes encoding proteins with antioxidant activity were investigated for their respective expression levels, as were genes involved in DNA repair.

Previous studies have identified differential regulation of genes encoding antioxidant enzymes in biofilm culture, compared with planktonic culture. In *P. aeruginosa* and *E. coli*, these enzymes have been identified as downregulated (Schembri *et al.*, 2003; Driffield *et al.*, 2008). These results suggest that downregulation of antioxidant enzymes in biofilm cultures may render the cells more sensitive to oxidative stress, and therefore mutation due to ROS. However, in *S. aureus* biofilms, enzymes such as *sodA*, are both upregulated and downregulated in biofilm culture, depending on the biofilm model and strain utilised (Resch *et al.*, 2005; Brady *et al.*, 2006).

Of all the antioxidant enzymes investigated here, only *sodA* was found to be upregulated in 48 hr biofilm culture, compared with planktonic culture (Table 5.2). This supports other transcriptional profiling data for *S. aureus*, identifying *sodA* as being upregulated in static biofilms (Resch *et al.*, 2005). This manganese-dependent SOD is regulated by SarA and provides protection against superoxide radicals (Ballal & Manna, 2009). Upregulation of this enzyme in the biofilm state suggests that cells within these cultures are suffering increased endogenous oxidative stress. No other significant differences in antioxidant enzyme expression were found between the biofilm and planktonic conditions. Furthermore, expression of this enzyme is not upregulated in 144 hr biofilms, which, unlike 48 hr biofilms, display increased MFs compared with planktonic cultures. Therefore, the impact of oxidative stress on the older biofilm cultures may be greater, since *sodA* expression does not increase.

Table 5.2 Differential gene expression (fold change) of antioxidant genes in 48 hr biofilms (B48h) compared with planktonic cultures (P), 144 hr biofilms (B144h) compared with planktonic cultures and 144 hr biofilms compared with 48 hr biofilms of *S. aureus* SH1000. Data are the mean of three experimental replicates.

ORF	Gene name	Function	Fold change P/B48h	Fold change P/B144h	Fold change B48h/B144h
SAOUHSC_00365	*ahpC*	alkyl hydroperoxide reductase subunit C	1.364 up	1.124 up	1.212 down
SAOUHSC_01327	*katA*	catalase	1.247 down	1.533 down	1.229 down
SAOUHSC_01653	*sodA*	superoxide dismutase [Mn]	**2.256 up**	1.577 up	1.430 down
SAOUHSC_00093	*sodM*	superoxide dismutase [Mn]	1.361 down	1.260 down	1.080 up
SAOUHSC_00785	*trxB*	thioredoxin-disulfide reductase	1.263 up	none	1.263 down
SAOUHSC_01999		alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen	1.220 up	1.022 down	1.247 down
SAOUHSC_02949		glutathione peroxidase	1.233 down	1.173 down	1.051 up
SAOUHSC_01282		glutathione peroxidase	1.439 up	1.163 up	1.236 down
SAOUHSC_01822	*tpx*	alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen	1.971 up	1.468 up	1.342 down

The expression of genes involved in the synthesis of carotenoid pigment was also investigated, as such pigments, like staphyloxanthin, are known to possess antioxidant activity (Clauditz *et al.*, 2006) (Table 5.3). These genes were for the most part downregulated. Staphyloxanthin production, from the *crt* operon, was found to be significantly affected, as all the biosynthetic genes in this pathway were downregulated in one or both of the biofilm cultures. However, although loss of this pigment causes an increase in susceptibility to killing by hydrogen peroxide, it does not cause in an increase in mutability (please see Chapter 6 section 6.3.1 for details).

Table 5.3 Differential gene expression (fold change) of genes involved in pigment biosynthesis in 48 hr biofilms (B48h) compared with planktonic cultures (P), 144 hr biofilms (B144h) compared with planktonic cultures and 144 hr biofilms compared with 48 hr biofilms of *S. aureus* SH1000. Data are the mean of three experimental replicates.

ORF	Gene name	Function	Fold change P/B48h	Fold change P/B144h	Fold change B48h/B144h
SAOUHSC_02879	*crtM*	dehydrosqualene synthase	**3.233 down**	**2.717 down**	1.190 up
SAOUHSC_02877	*crtN*	dehydrosqualene desaturase	**3.989 down**	**2.654 down**	1.502 up
SAOUHSC_02882	*crtO*	glycosyl-4,4'-diaponeurosporenoate acyltransferase	**2.569 down**	1.539 down	1.669 up
SAOUHSC_02881	*crtP*	4,4'-diaponeurosporene oxidase	**3.883 down**	**2.022 down**	1.919 up
SAOUHSC_02880	*crtQ*	4,4'-diaponeurosporenoate glycosyltransferase	**4.095 down**	**2.409 down**	1.699 up

5.3.3.2. Genes involved in DNA repair

Of the 47 genes identified as being involved in DNA repair, 14 and 9 were found to be differentially regulated in 48 hr and 144 hr biofilms, respectively, when compared with planktonic cultures (Table 5.4). No differential expression of this group of genes was observed between biofilm conditions. Furthermore, in 48 hr biofilm cultures, 12 of the 14 differentially expressed genes were upregulated, 8 of which were also upregulated in 144 hr biofilm cultures.

In both biofilm conditions the expression of *mutS2* was significantly increased. This gene product is part of the family of proteins that form the methyl-directed mismatch repair (MMR) system. However, unlike the homologue MutS1, which is involved in DNA mismatch recognition, MutS2 is primarily involved in preventing homologous and homeologous recombination (Pinto *et al.*, 2005).

Proteins involved in homologous recombination, such as RecF, RuvA and RecU, were also upregulated in the biofilm state (He *et al.*, 2006; Carrasco *et al.*, 2009). RecF and RuvA, have been implicated in the repair of double-stranded DNA breaks, whilst also

promoting mutation, a phenomenon that is thought to allow the bacterial population to adapt to overcome environmental stress (He *et al.*, 2006).

Expression of the LexA repressor was also upregulated in biofilm cultures. The expression from this ORF was upregulated in 48 hr biofilm cultures, but not 144 hr biofilm cultures, when compared with planktonic cultures. This protein is involved in the bacterial SOS response, where it negatively regulates itself and the production of genes involved in this response (Brent & Ptashne, 1980). Specifically, RecA binds DNA lesions, resulting from stress conditions, resulting in the cleavage of LexA and the production of SOS response genes (Brent & Ptashne, 1980; Horii *et al.*, 1981). This response occurs in populations of bacteria undergoing stress, such as starvation (Taddei *et al.*, 1995). Upregulation of LexA in this case, however, suggests a downregulation of the SOS response due to its negative regulation of SOS response genes.

Table 5.4 Differential gene expression (fold change) of genes involved in DNA repair, in 48 hr biofilms (B48h) compared with planktonic cultures (P), 144 hr biofilms (B144h) compared with planktonic cultures and 144 hr biofilms compared with 48 hr biofilms of *S. aureus* SH1000. Data are the mean of three experimental replicates.

ORF	Gene name	Function	Fold change P/B48h	Fold change P/B144h	Fold change B48h/B144h
SAOUHSC_02621		3-methyladenine DNA glycosylase	**2.230 down**	1.520 down	1.467 up
SAOUHSC_00905	*addA*	recombination helicase AddA	1.671 up	1.475 up	1.132 down
SAOUHSC_00904	*addB*	ATP-dependent nuclease subunit B	1.644 up	1.388 up	1.184 down
SAOUHSC_00505	*clpC*	ATP-dependent Clp protease ATP-binding subunit clpC	**4.311 up**	**5.157 up**	1.196 up
SAOUHSC_02862	*clpL*	ATP-dependent Clp protease, ATP-binding subunit ClpL	**6.058 down**	**5.460 down**	1.109 up
SAOUHSC_01472		DnaQ family exonuclease/DinG family helicase, putative	1.709 up	1.078 up	1.585 down
SAOUHSC_02122	*ligA*	DNA ligase, NAD-dependent	1.648 up	1.236 up	1.332 down
SAOUHSC_01658		endonuclease IV	1.313 up	1.341 up	1.021 up
SAOUHSC_01333	*lexA*	LexA repressor	**2.421 up**	1.804 up	1.341 down
SAOUHSC_00477	*mfd*	transcription-repair coupling factor	1.063 up	1.017 down	1.081 down
SAOUHSC_01273	*mutL*	DNA mismatch repair protein MutL	1.555 down	1.365 down	1.139 up
SAOUHSC_01099	*mutS2*	MutS2 protein	**3.049 up**	**2.440 up**	1.249 down
SAOUHSC_01272	*mutS*	DNA mismatch repair protein MutS	1.224 down	1.195 down	1.023 up
SAOUHSC_02123	*pcrA*	ATP-dependent DNA helicase PcrA	1.322 up	1.111 up	1.189 down
SAOUHSC_00004	*recF*	DNA replication and repair protein RecF	**2.683 up**	**2.233 up**	1.201 down
SAOUHSC_01194	*recG*	ATP-dependent DNA helicase RecG	1.972 up	1.287 up	1.531 down
SAOUHSC_00445	*recR*	recombination protein RecR	1.700 up	1.248 up	1.361 down
SAOUHSC_01751	*ruvA*	Holliday junction DNA helicase RuvA	**2.970 up**	**2.016 up**	1.472 down
SAOUHSC_01750	*ruvB*	Holliday junction DNA helicase RuvB	1.365 up	1.367 up	1.001 up
SAOUHSC_01720		putative Holliday junction resolvase	**2.125 up**	1.369 up	1.552 down
SAOUHSC_01342		exonuclease SbcC	1.786 up	1.112 up	1.606 down
SAOUHSC_01341		exonuclease SbcD	1.042 down	1.058 down	1.015 down
SAOUHSC_00564	*ung*	uracil-DNA glycosylase	1.451 down	1.208 down	1.200 up
SAOUHSC_01102	*uvrC*	excinuclease ABC, C subunit	**2.030 up**	1.579 up	1.284 down
SAOUHSC_00458		excinuclease ABC, C subunit, N-terminal	1.002 up	1.006 down	1.009 down

Table 5.4 cont.

SAOUHSC_01796	*mutM*	formamidopyrimidine-DNA glycosylase	1.856 up	1.439 up	1.289 down
SAOUHSC_02276		DNA mismatch repair protein MutS, C-terminal	1.312 down	1.307 down	1.003 up
SAOUHSC_01615	*recN*	DNA repair protein RecN	1.948 up	1.725 up	1.128 down
SAOUHSC_00507	*radA*	DNA repair protein RadA	1.611 up	1.382 up	1.165 down
SAOUHSC_00730	*recQ*	ATP-dependent DNA helicase RecQ	1.962 up	1.437 up	1.365 down
SAOUHSC_01262	*recA*	protein RecA	1.834 up	1.687 up	1.086 down
SAOUHSC_01098		DNA-dependent DNA polymerase beta chain	**5.763 up**	**4.058 up**	1.420 down
SAOUHSC_01744	*recJ*	single-stranded-DNA-specific exonuclease RecJ	1.119 up	1.031 down	1.154 down
SAOUHSC_01693		competence protein ComEA helix-hairpin-helix region	1.197 down	1.072 down	1.116 up
SAOUHSC_02005	*mutY*	A/G-specific adenine glycosylase	1.591 down	1.337 down	1.190 up
SAOUHSC_00780	*uvrA*	excinuclease ABC, A subunit	1.030 down	1.102 down	1.070 down
SAOUHSC_01768		DNA-3-methyladenine glycosylase 1	1.459 up	1.115 up	1.308 down
SAOUHSC_00503		UvrB/UvrC motif-containing protein	**2.876 up**	**2.982 up**	1.036 up
SAOUHSC_00612		HhH-GPD	1.019 down	1.171 down	1.148 down
SAOUHSC_01466	*recU*	recombination protein U	**5.242 up**	**4.832 up**	1.084 down
SAOUHSC_01469		endonuclease III, putative	**2.979 up**	**2.235 up**	1.332 down
SAOUHSC_01667	*recO*	DNA repair protein RecO	1.512 up	1.164 up	1.299 down
SAOUHSC_01363		ImpB/MucB/SamB family protein	1.587 up	1.653 up	1.041 up
SAOUHSC_02111		DNA polymerase IV	1.381 up	1.402 up	1.015 up
SAOUHSC_02861	*ogt*	methylated-DNA--protein-cysteine methyltransferase	1.034 up	1.043 up	1.008 up
SAOUHSC_00699		DNA photolyase, FAD-binding	1.346 down	1.342 down	1.002 up
SAOUHSC_00779		UvrABC system protein B	1.619 up	1.217 up	1.330 down

5.3.4. Concluding remarks

Initially, the DNA microarray data obtained from transcriptional profiling were used to highlight genes potentially involved in biofilm maturation, by comparing 48 hr and 144 hr *S. aureus* biofilm transcriptional profiles. The amino acid biosynthesis, cell envelope, transport and binding and regulatory function groups were particularly represented in the group of genes found to be differentially regulated between the two cultures. Histidine biosynthetic genes and genes encoding cell membrane proteins were significantly upregulated.

Furthermore, genes encoding transcriptional regulators of virulence and biofilm formation were also found to be differentially expressed.

These data were also used to elucidate the mechanism of increased mutability observed within staphylococcal biofilms, when compared with planktonic cultures. It was hypothesised that differential regulation of genes conferring antioxidant capacity may promote mutation in biofilm cultures. However, little differential regulation of genes encoding antioxidant enzymes was observed in biofilm cultures, with only *sodA* being upregulated in 48 hr biofilms. However, expression of genes involved in carotenoid pigment biosynthesis, which possesses antioxidant activity, was significantly downregulated. Additionally, increased expression of numerous genes involved in DNA repair was observed in the biofilm cultures, some of which promote mutation. However, a definitive mechanism for increased mutability in biofilms remains elusive.

CHAPTER 6

6. Emergence of new *S. aureus* phenotypes from biofilm cultures

Work presented in this chapter has also been presented as a poster:

Ryder, V. J., O'Neill, A. J & Chopra, I. (2010). "Phenotypic variants arising in *Staphylococcus aureus* biofilms may promote dissemination during infection". *Biofilms4*, Southampton, UK.

6.1. Abstract

The biofilm mode of growth promotes the emergence of phenotypic variants, such as small colony variants which have reduced antibiotic sensitivity and increased biofilm forming capacity. Here, I have observed and characterised two other phenotypic variant types emerging from *S. aureus* SH1000 biofilms. These were either white variants (WVs) or large pale variants (LPVs).

The variant types were present in both the adherent population of the biofilm, and the shed population of cells, in both static and flow biofilm systems. In the static system, WVs comprised up to 2.0% and 26.5% of the adherent and shed phases, respectively. LPVs, by comparison, formed up to 14.9% and 51.6% of the adherent and shed phases, respectively. WVs had lost biofilm forming capacity, as adherence to cellulose disks was significantly diminished when compared to the parental strain, *S. aureus* SH1000. LPVs, however, retained biofilm forming capacity. In terms of mutability, WVs displayed up to a 2-fold increase in MF compared with SH1000. LPV MFs remained the same as *S. aureus* SH1000. Additionally, WVs had reduced resistance to hydrogen peroxide, but this did not

result in increased MF in the presence of sublethal concentrations of hydrogen peroxide. Subsequently, mutations in the alternative sigma factor, SigB, were identified in WVs.

Due to their high proportion in the shed planktonic population of cells, and the inability of WVs to form biofilms, these phenotypic variants isolated from *S. aureus* biofilm cultures may represent a novel mechanism by which cells can disseminate from biofilm culture.

6.2. Introduction

Recent studies suggest that biofilms comprise heterogeneous populations of bacteria. The results discussed so far in this thesis have supported this idea, since antibiotic resistant subpopulations exist in the biofilms. Several papers have reported the emergence of phenotypic variants, with altered colony morphology, from biofilm culture. One example is the small colony variant (SCV). Several bacterial species enrich their population with SCVs upon growth as a biofilm. *P. aeruginosa* biofilms generate large numbers of SCVs, the properties of which include autoaggregation in liquid culture and increased expression of genes involved in surface adherence, specifically the *psl* and *pel* loci (Kirisits *et al.*, 2005).

Similarly, some *S. aureus* strains also produce SCVs in biofilm culture. Much like the SCVs isolated from *P. aeruginosa*, *S. aureus* SCVs exhibit enhanced biofilm forming capacity. These SCVs autoaggregate and produce higher amounts of PIA than the wild-type (Singh *et al.*, 2010a). *S. aureus* SCVs have also been isolated from various infections, suggesting that these phenotypic variants may have a role in pathogenicity (Agarwal *et al.*, 2007; Schneider *et al.*, 2008). Such *S. aureus* SCV isolates display an enhanced ability to persist intracellularly, and their transcriptional profiles indicate an upregulation of the fermentation pathway (Moisan *et al.*, 2006). Moreover, these variants display reduced sensitivity to a variety of antimicrobials, such as triclosan, cephalosporins, linezolid, vancomycin and the fluoroquinolones (von Eiff *et al.*, 2005; Bayston *et al.*, 2007; Tsuji *et al.*, 2008).

Colonies exhibiting morphological variation also emerge from *S. pneumoniae* biofilms. These variants include both SCVs and larger mucoid variants. *S. pneumoniae* SCVs autoaggregate in liquid culture and form well-structured biofilms. However, the large mucoid variants form unstructured biofilms and do not aggregate in liquid culture (Allegrucci & Sauer, 2007).

This chapter will discuss the identification, emergence and characterisation of WVs and LPVs from *S. aureus* SH1000 biofilms.

6.3. Results and Discussion

During mutability studies with *S. aureus* SH1000, as described in previous chapters, it became apparent that two distinct colony variants emerged from cellulose disk biofilms. These were WVs and LPVs (Figure 6.1). Steps were taken to confirm that these variants were not merely contaminants of the cultures. To demonstrate this, biofilms of *S. aureus* SH1000 *lux* (carries *lux ABCDE* Km cassette on chromosome) were grown. Two WVs (W1 and W2) and LPVs (LP1 and LP2) were isolated from 144 hr cellulose disk biofilms of *S. aureus* SH1000 and SH1000 *lux* and then assessed for luminescence (Figure 6.2). This demonstrated that all colony types isolated from *S. aureus* SH1000 *lux* biofilms were luminescent, and were therefore not contaminants. Interestingly, the WVs were significantly more luminescent than the wild-type and LPV phenotypes. However, the reasons for this remain unclear.

The emergence of both of these variants from *S. aureus* SH1000 cellulose disk biofilms was also quantified by determining the proportion of each variant in the adherent and the shed planktonic populations (Figure 6.3).

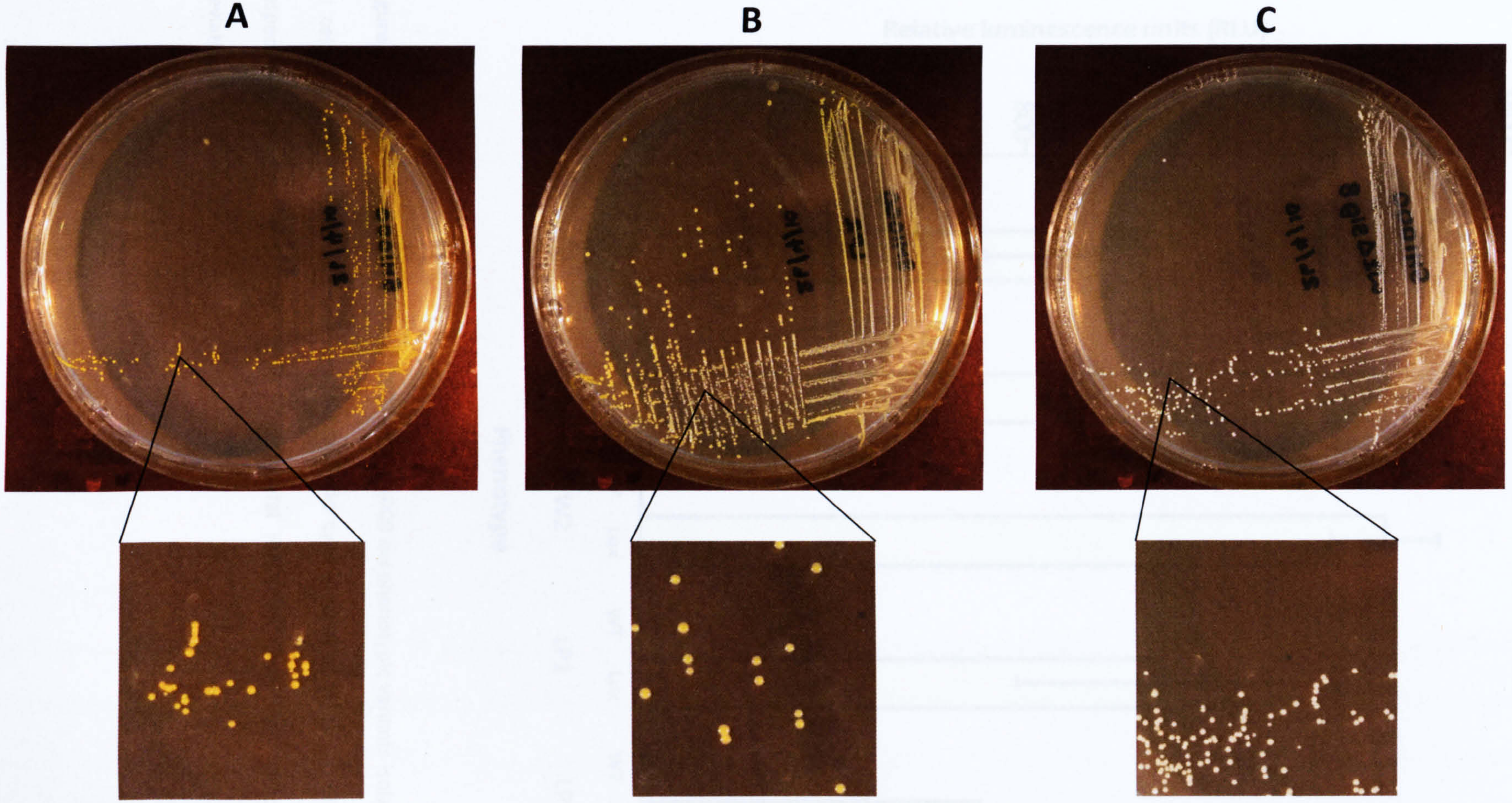

Figure 6.1 Morphological appearance of *S. aureus* SH1000 wild type (**A**), LPVs (**B**) and WVs (**C**).

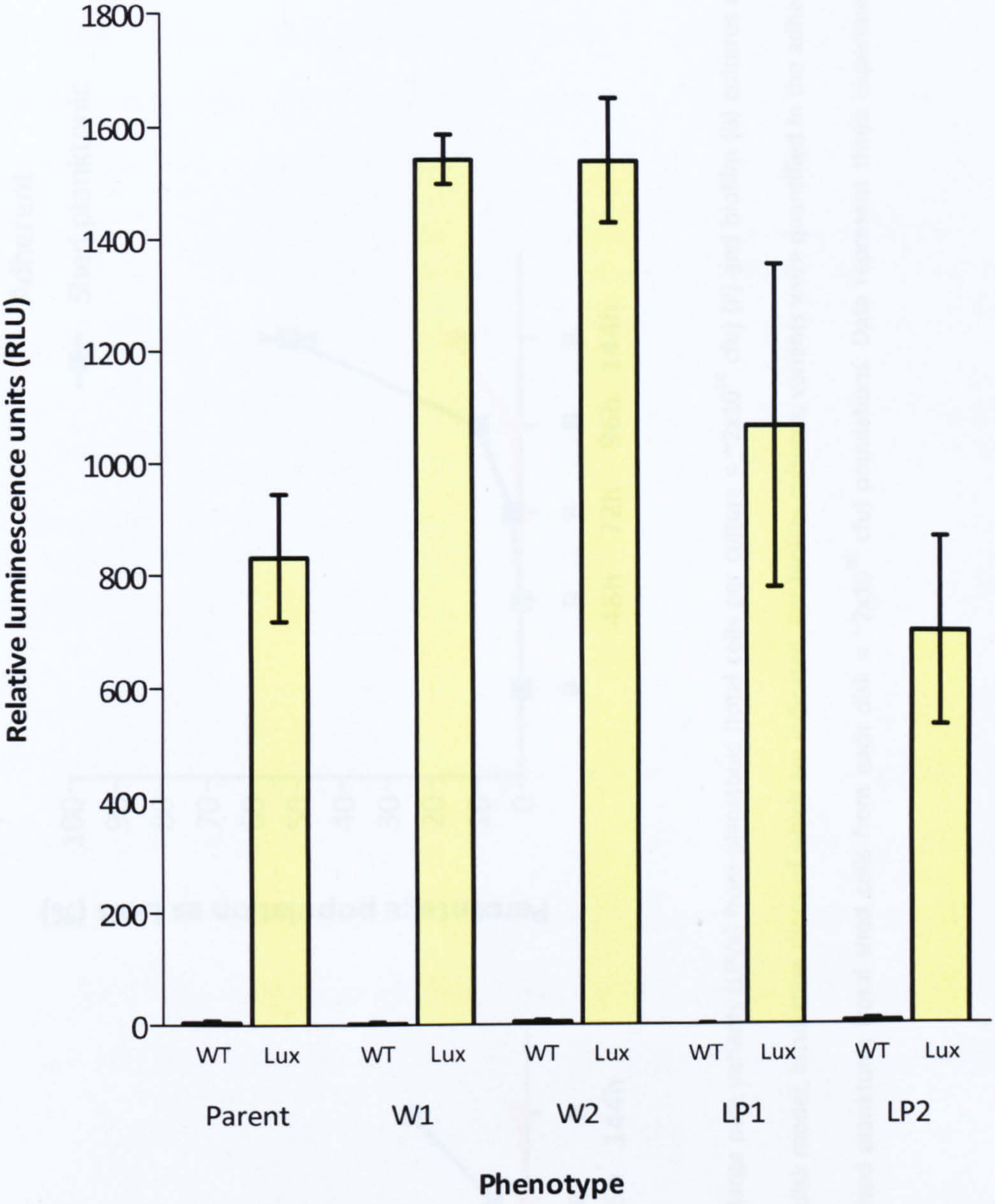

Figure 6.2 Luminescence of *S. aureus* SH1000 and SH1000 *lux* phenotypic variants isolated from 144 hr cellulose disk biofilms. WT and Lux denote wild type *S. aureus* SH1000 and SH1000 *lux*, respectively. Data are based on three experimental replicates. Error bars indicate standard deviations.

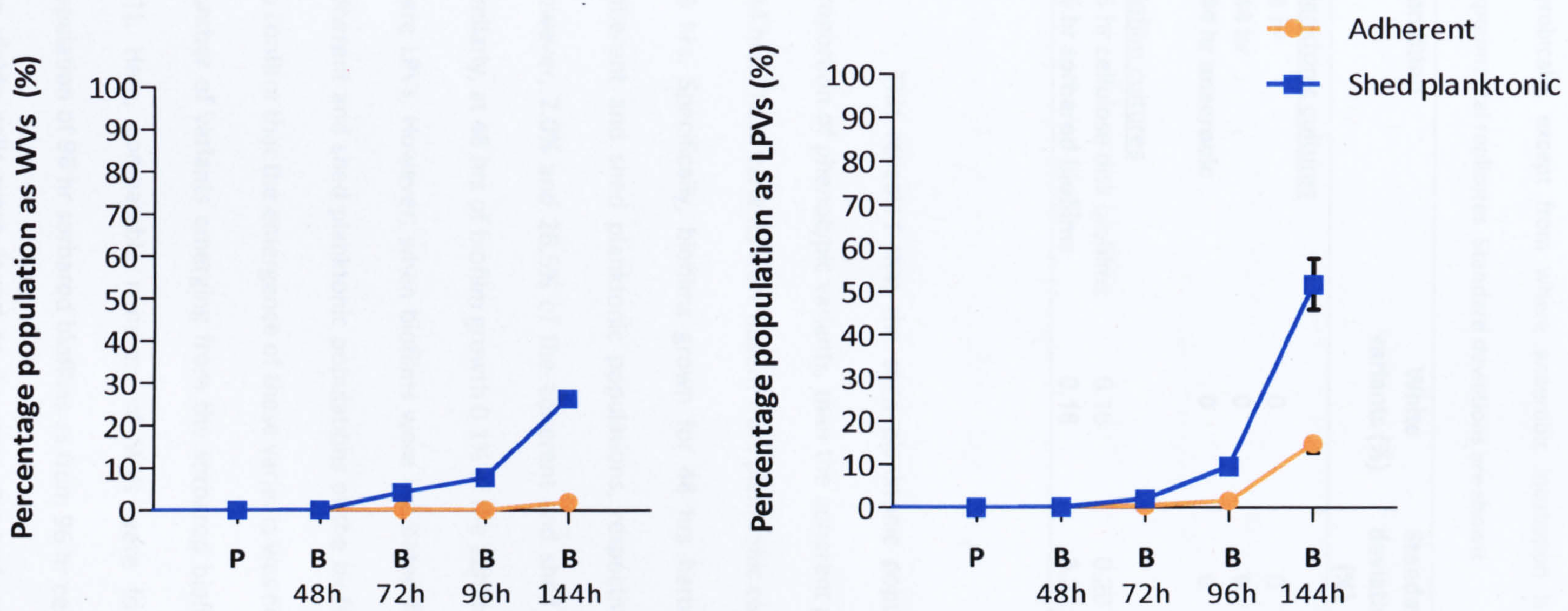

Figure 6.3 The emergence of white variants (WVs) and large pale variants (LPVs) from planktonic (total cells per culture = ~$2x10^{10}$ cfu) (P) and biofilm (B) cultures of *S. aureus* SH1000. Biofilms were grown using the cellulose disk model, incubation times of which are indicated. For biofilm cultures, variants were quantified in the adherent (total adherent cells on each disk = ~$2x10^{10}$ cfu) and shed planktonic (total shed cells from each disk = ~$2x10^{10}$ cfu) populations. Data represent three experimental replicates. Error bars indicate standard deviations.

Table 6.1 The emergence of white variants and large pale variants from additional planktonic and biofilm culture conditions (bold text) than those shown in Figure 6.1. Cultures were incubated aerobically, except from where anaerobic incubation is indicated. Data are based on three experimental replicates. Standard deviations are shown.

Condition	**White variants (%)**	**Standard deviation (%)**	**Large pale variants (%)**	**Standard deviation (%)**
<u>Planktonic cultures</u>				
18 hr	0	0	0	0
144 hr	**0**	**0**	**1.09**	**0.82**
144 hr anaerobic	**0**	**0**	**1.55**	**1.22**
<u>Biofilm cultures</u>				
96 hr cellulose disk biofilms	0.16	0.20	1.62	0.59
96 hr sorbarod biofilms	**0.16**	**0.24**	**2.35**	**2.68**

This revealed that the shed planktonic population of cells harboured a higher proportion of phenotypic variants, than the adherent population of the biofilm cultures at 144 hrs. These variants were absent from planktonic cultures of *S. aureus* SH1000 grown for 18 hrs. Specifically, biofilms grown for 48 hrs harboured 0.1% and 0.2% WVs in the adherent and shed planktonic populations, respectively. At 144 hrs of biofilm growth, however, 2.0% and 26.5% of the adherent and shed planktonic populations were WVs.. Similarly, at 48 hrs of biofilm growth 0.1% of the adherent and shed planktonic populations were LPVs. However, when biofilms were incubated for 144 hrs, 14.9% and 51.6% of the adherent and shed planktonic populations of the biofilm cultures were LPVs, respectively. To confirm that the emergence of these variants was not specific to this biofilm system, the number of variants emerging from the sorbarod biofilm model was also deduced (Table 6.1). Here, comparable numbers of WVs were found emerging from the adherent population of 96 hr sorbarod biofilms as from 96 hr cellulose disk biofilms, where 0.16% of the viable cells were found to be WVs. Similarly, 1.62% and 2.35% of the adherent

population of 96 hr cellulose disk and sorbarod biofilms, respectively, were found to be LPVs.

To elucidate further the mechanism driving the emergence of these variants, planktonic cultures were incubated for 144 hrs in both aerobic and anaerobic conditions. This aimed to determine whether older cultures harboured more WVs and LPVs, and whether anaerobic growth may also promote the emergence of these variants. Under all planktonic conditions tested, no WVs were observed, indicating that these variants only emerge during the biofilm mode of growth. LPVs, however, emerged from 144 hr cultures. Under aerobic and anaerobic conditions, 1.09% and 1.55% were found, respectively. However, these values are significantly lower than the values determined for cellulose disk biofilm cultures. Therefore, these data suggest that the biofilm mode of growth exacerbates the production of both of these variant types. Furthermore, as anaerobic conditions did not affect the respective levels of WVs and LPVs in planktonic cultures, this environment does not promote variant emergence.

After quantifying the total proportions of WVs and LPVs in *S. aureus* SH1000 biofilms, the two representative isolates (W1-2 and LP1-2) for each phenotype, from two distinct biofilms, were characterised further. Initially, growth rates, during exponential phase, were determined for W1, W2, LP1 and LP2 and compared with *S. aureus* SH1000 (Table 6.2). There were no significant changes in the growth rates of these phenotypic variants, suggesting that the genetic basis of their variation does not influence doubling time.

Table 6.2 Growth rates of *S. aureus* SH1000, W1, W2, LP1 and LP2.

***S. aureus* strain**	**Doubling time (mins)**	**Standard deviation**
SH1000	32.30	1.95
W1	32.76	1.26
W2	31.36	2.45
LP1	31.75	1.58
LP2	30.78	2.07

6.3.1. Characterising white variants

Attempts were made to characterise the WVs W1 and W2. Biofilm forming capacity was initially assessed using the cellulose disk biofilm model (Figure 6.4). In this case, biofilms were grown for 48 hrs and then washed to remove loosely associated planktonic cells. Adherent cells were then removed by incubation with cellulase. The viable counts of both the wash solution and cellulase-detached adherent population were then calculated. Compared to the parental strain, the WVs were significantly impaired in biofilm formation, with the vast majority of the cells being washed off the cellulose disks. Specifically, only 6.5% and 3.6% of the total population of cells were adherent in biofilms of W1 and W2, respectively. Following this, the WVs were assessed for their MF (Figure 6.5) using MIC data of the selection antibiotics (Table 6.3). This revealed that WVs exhibited marginal increases in MF of up to 2-fold compared with the parental strain, *S. aureus* SH1000. However, differences in MF between the wild-type and the WVs were not found to be statistically significant, except for W2 using rifampicin selection (>95% confidence, $P = 1.54 \times 10^{-4}$).

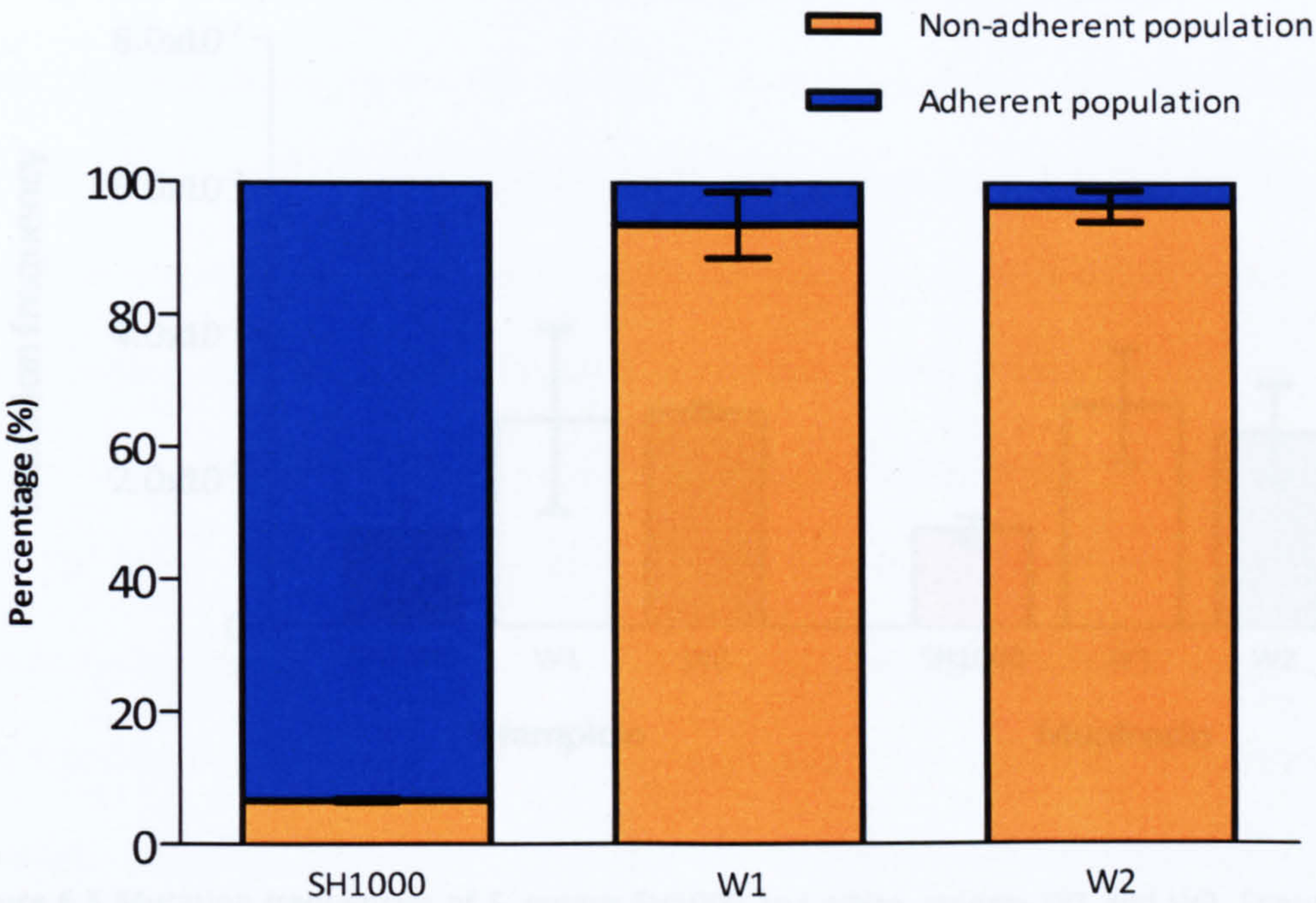

Figure 6.4 The proportion of adherent and non-adherent cells on cellulose disk biofilms of *S. aureus* SH1000, W1 and W2. Data shown represent three experimental replicates. Errors bars indicate standard deviations.

Table 6.3 Minimum inhibitory concentrations of rifampicin and mupirocin for various strains of *S. aureus*, determined by agar dilution.

	MIC (mg/L)				
	SH1000	**W1**	**W2**	**Newman**	**Newman *ΔcrtM***
Rifampicin	0.0078	0.0078	0.0078	0.0078	0.0078
Mupirocin	0.125	0.0625	0.0625	0.125	0.0625

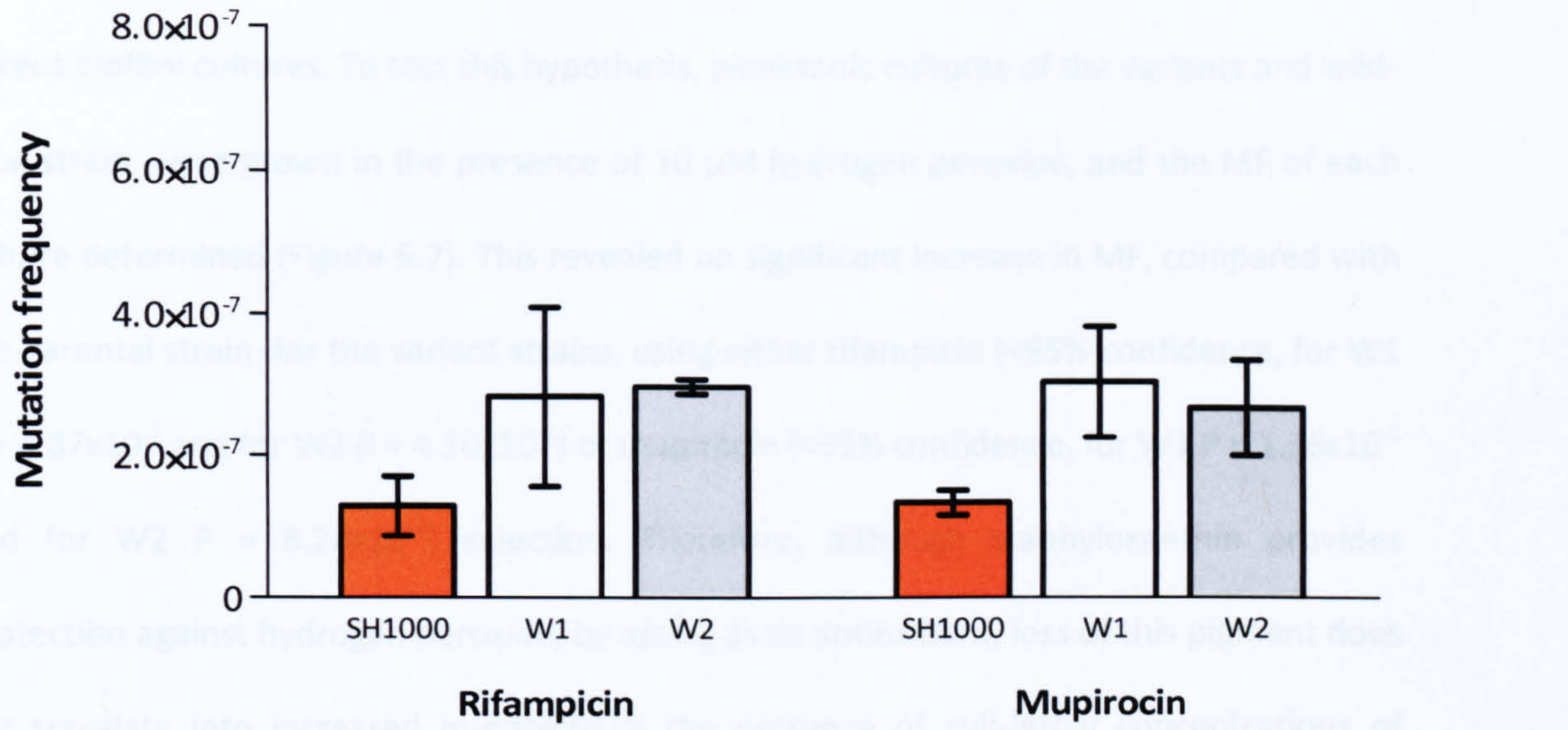

Figure 6.5 Mutation frequencies of *S. aureus* SH1000 and white variants W1 and W2. Spontaneous mutants were isolated from the cultures using the selection antibiotics rifampicin and mupirocin. Data are based on three experimental replicates. Error bars indicate standard deviations.

A previous study demonstrated increased susceptibility to hydrogen peroxide in a staphyloxanthin deficient mutant (Clauditz *et al.*, 2006). Accordingly, the susceptibility of W1 and W2 to hydrogen peroxide was also determined (Figure 6.6). In this case, susceptibility was calculated as the percentage survival following exposure to a range of hydrogen peroxide concentrations. Strains W1 and W2 demonstrated increased susceptibility to hydrogen peroxide, since concentrations as low as 1 mM were sufficient to reduce the population of the variant strains by up to 51%, with no effect on wild-type cells (Figure 6.6). These observations are consistent with loss of staphyloxanthin synthesis in W1 and W2, also explaining their white phenotypes.

Due to the observed increased susceptibility to hydrogen peroxide, and their moderate increase in MF, it was hypothesised that when exposed to subinhibitory concentrations of hydrogen peroxide, the W1 and W2 variants may be more mutable than

wild-type SH1000. This may contribute to the increased mutability previously observed in *S. aureus* biofilm cultures. To test this hypothesis, planktonic cultures of the variants and wild-type strain, were grown in the presence of 10 μM hydrogen peroxide, and the MF of each culture determined (Figure 6.7). This revealed no significant increase in MF, compared with the parental strain, for the variant strains, using either rifampicin (<95% confidence, for W1 $P = 4.87x10^{-1}$ and for W2 $P = 4.10x10^{-1}$) or mupirocin (<95% confidence, for W1 $P = 1.36x10^{-1}$ and for W2 $P = 8.20x10^{-2}$) selection. Therefore, although staphyloxanthin provides protection against hydrogen peroxide, by acting as an antioxidant, loss of this pigment does not translate into increased mutability in the presence of sub-lethal concentrations of hydrogen peroxide. To explore further that loss of staphyloxanthin production does not cause increased mutability, MFs were also determined for *S. aureus* Newman and a staphyloxanthin defective mutant (Δ*crtM)* (MIC data of selection antibiotics shown in Table 6.3). Whilst determining MFs using mupirocin selection, for *S. aureus* Newman Δ*crtM,* it was apparent that chloramphenicol selection, to maintain the *crtM* mutation, was interfering with the experiment, causing the MFs to appear artificially high (data not shown). To overcome this, planktonic cultures of this strain were initially grown in the presence of chloramphenicol, which was then omitted from the mupirocin selection plates during MF determination. To confirm mupirocin resistant mutants had retained the *crtM* mutation, colonies were subcultured onto chloramphenicol selection plates. The MF data revealed that loss of staphyloxanthin caused no statistically significant increase in mutability compared to the wild-type using rifampicin (<95% confidence, $P = 6.08x10^{-2}$) or mupirocin (<95% confidence, $P = 7.06x10^{-1}$) selection.

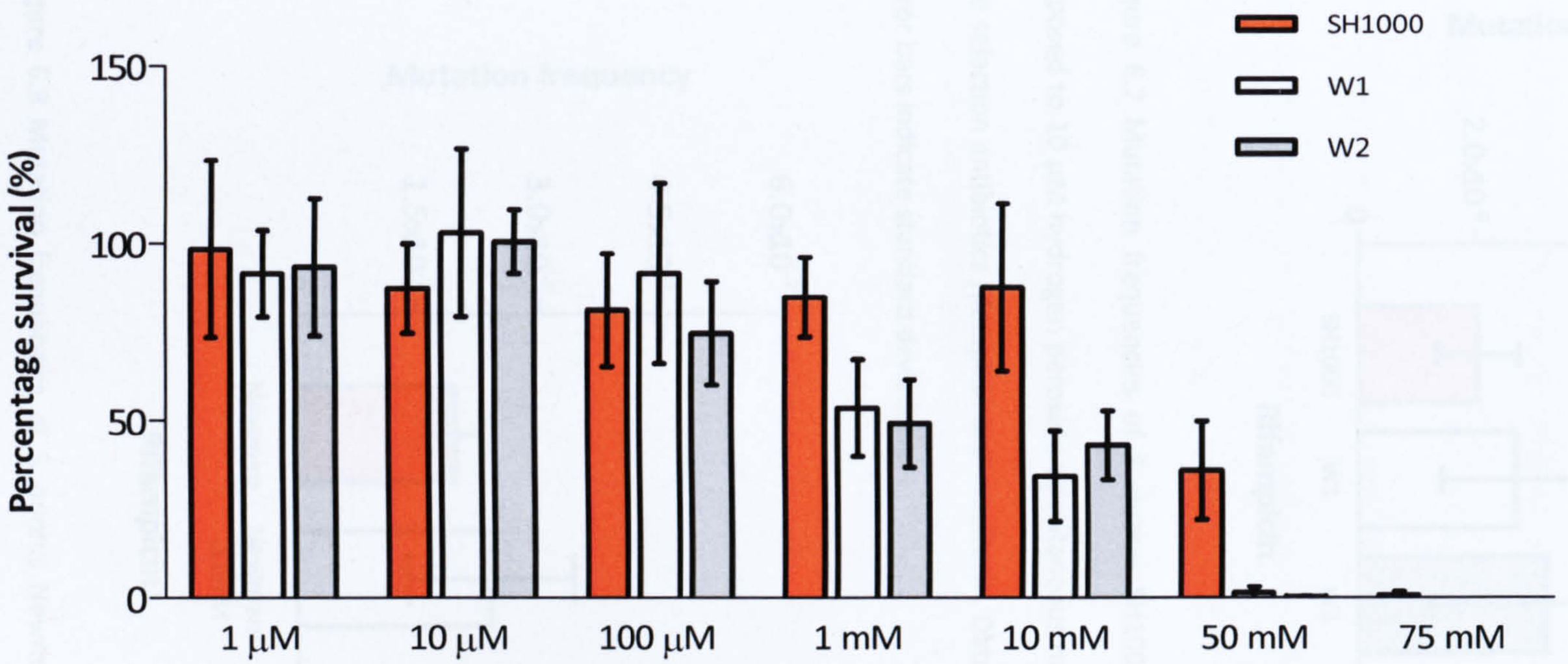

Figure 6.6 Susceptibility of *S. aureus* SH1000 and white variants W1 and W2 to hydrogen peroxide exposure. These data represent three experimental replicates. Error bars indicate standard deviations.

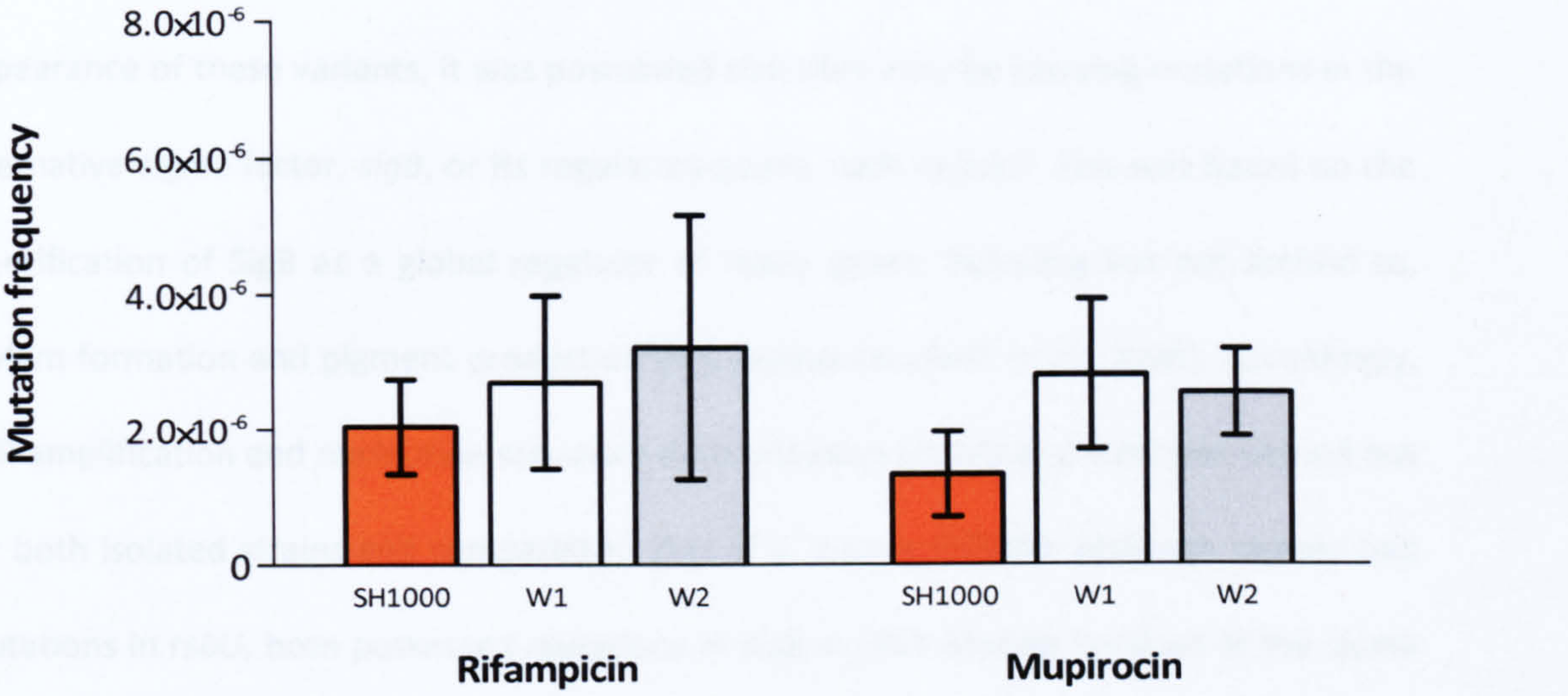

Figure 6.7 Mutation frequencies of *S. aureus* SH1000 and white variants W1 and W2 cultures exposed to 10 µM hydrogen peroxide. Spontaneous mutants were isolated from the cultures using the selection antibiotics rifampicin and mupirocin. Data are based on three experimental replicates. Error bars indicate standard deviations.

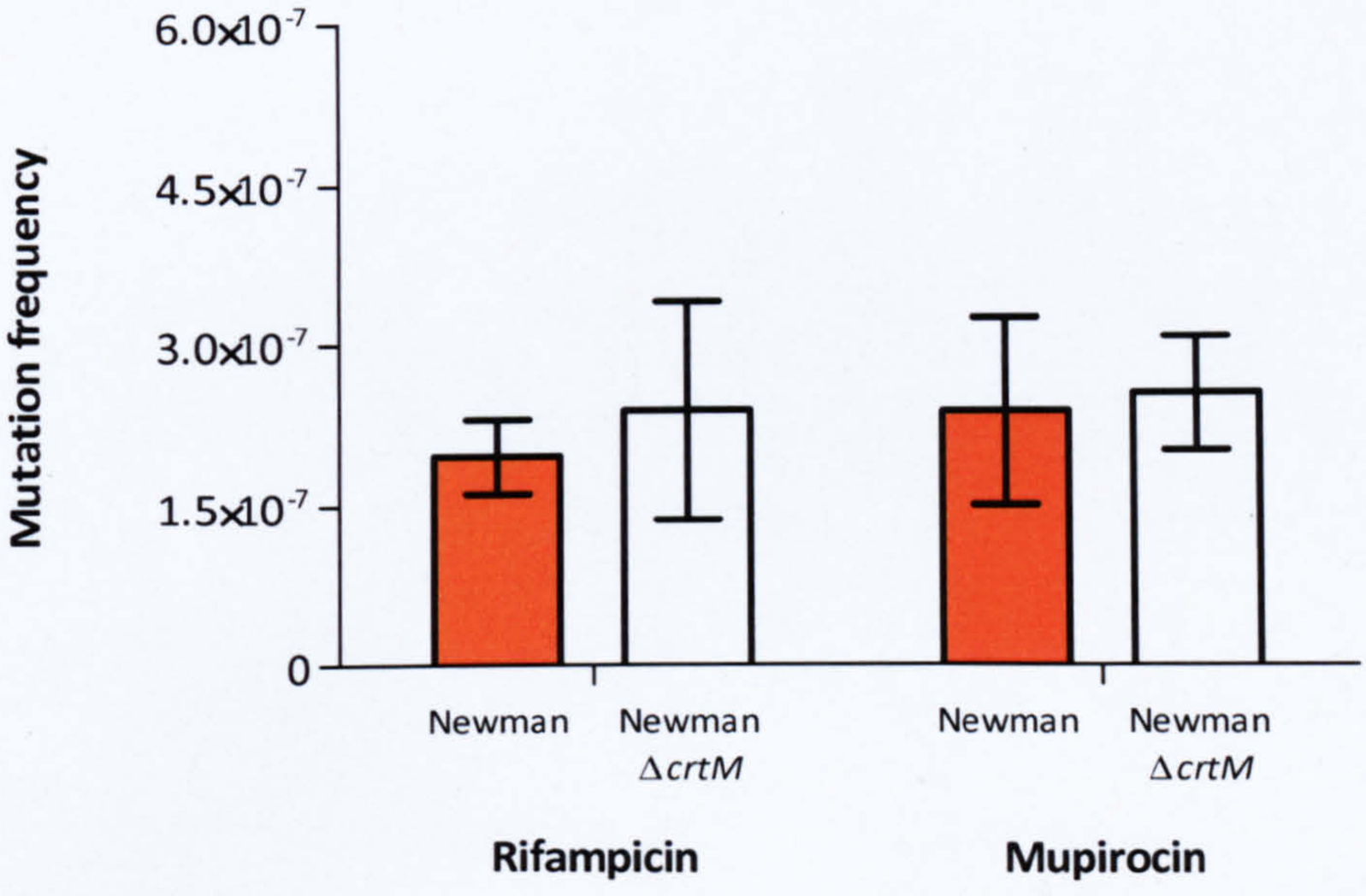

Figure 6.8 Mutation frequencies of *S. aureus* Newman and Newman Δ*crtM* planktonic cultures. Spontaneous mutants were isolated from the cultures using the selection antibiotics rifampicin and mupirocin. Data are based on three experimental replicates. Error bars indicate standard deviations.

Considering both the biofilm-forming deficiency and the non-pigmented appearance of these variants, it was postulated that they may be carrying mutations in the alternative sigma factor, *sigB*, or its regulatory genes, such as *rsbU*. This was based on the identification of SigB as a global regulator of many genes, including but not limited to, biofilm formation and pigment production in *S. aureus* (Bischoff *et al.*, 2004). Accordingly, PCR amplification and nucleotide sequence determination of *sigB* and *rsbU* was carried out for both isolated strains and compared to that of *S. aureus* SH1000. Although neither had mutations in *rsbU*, both possessed mutations in *sigB* at DNA binding residues of the sigma factor, presumably preventing interaction with gene promoter regions. W1 had a 163 base pair deletion in the chromosome from bases 2131888-2132050, resulting in the truncation of the protein from Cys_{217}. W2 had an A to G substitution at chromosome base 2131975, altering the amino acid sequence at Leu_{242}Pro.

6.3.2. Characterising large pale variants

In a similar manner to the WVs, the two LPVs isolated from *S. aureus* SH1000 cellulose disk biofilms were initially assessed for biofilm forming capacity (Figure 6.9). Unlike the WVs, the LPVs formed biofilms comparable to those of the parent strain. Indeed, of the total cells, biofilms of SH1000, LP1 and LP2 were comprised of 93.4%, 84.6% and 83.0% adherent cells, respectively.

Following this, MFs were determined for LP1 and LP2 (Figure 6.10). MIC data revealed that these mutants were slightly less susceptible to rifampicin and mupirocin compared to SH1000 (Table 6.4). Specifically, the MICs were consistently 4-fold higher for rifampicin and 2-fold higher for mupirocin, compared with SH1000. Marginal but significant increases in MF were observed for the LPVs compared with SH1000 (>95% confidence, LP2 using rifampicin selection $P = 2.20x10^{-2}$, LP1 using mupirocin selection $P = 4.00x10^{-2}$ and LP2 using mupirocin selection $P = 3.98x10^{-2}$) except LP1 using rifampicin selection which was not statistically significant (<95% confidence, $P = 1.02x10^{-1}$).

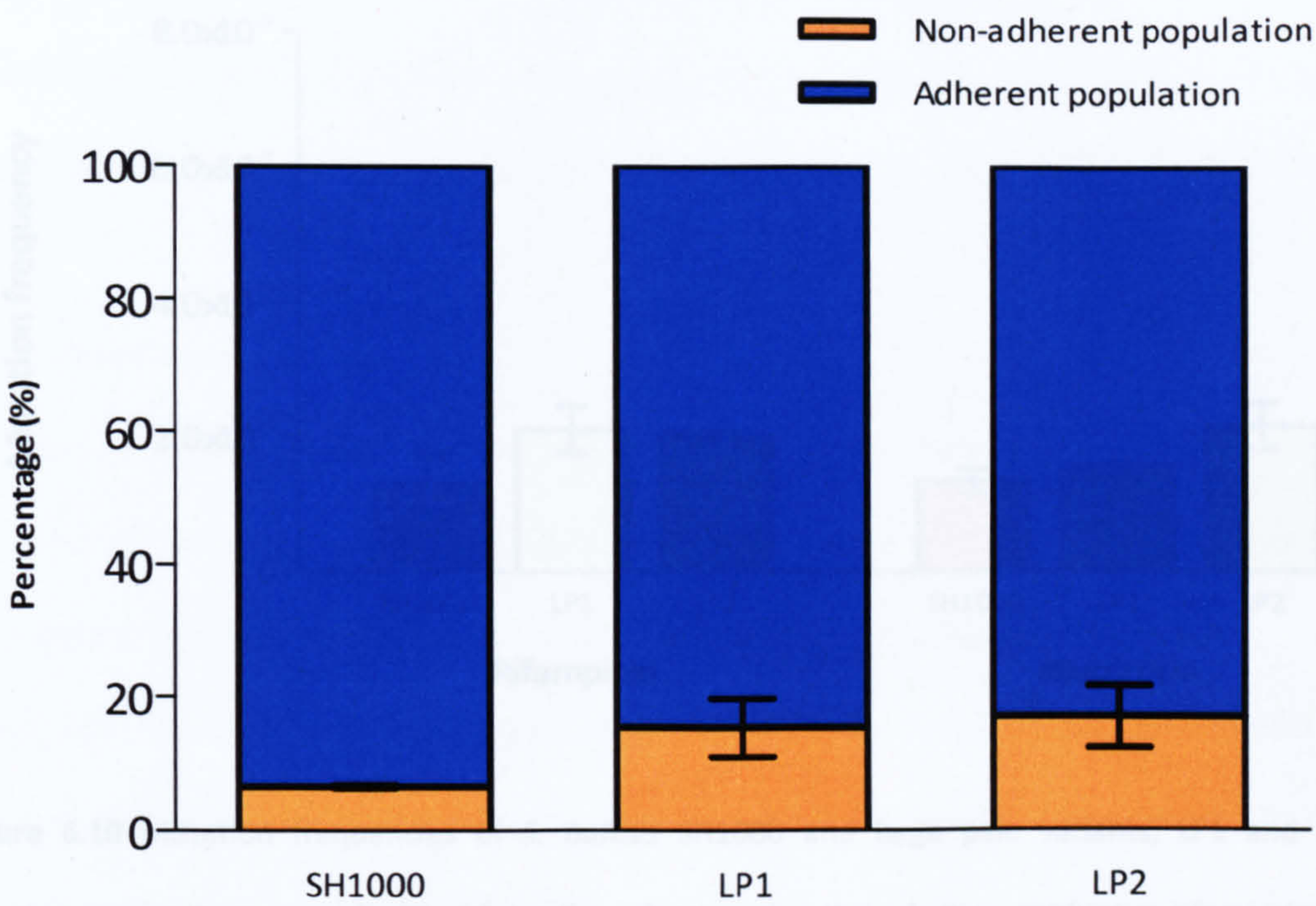

Figure 6.9 The proportion of adherent and non-adherent cells in cellulose disk biofilms of *S. aureus* SH1000, LP1 and LP2. Data represent three experimental replicates. Errors bars indicate standard deviations.

Table 6.4 Minimum inhibitory concentrations of rifampicin and mupirocin for *S. aureus* SH1000, LP1 and LP2, determined by agar dilution.

	MIC (mg/L)		
	SH1000	**LP1**	**LP2**
Rifampicin	0.0078	0.0313	0.0313
Mupirocin	0.1250	0.2500	0.2500

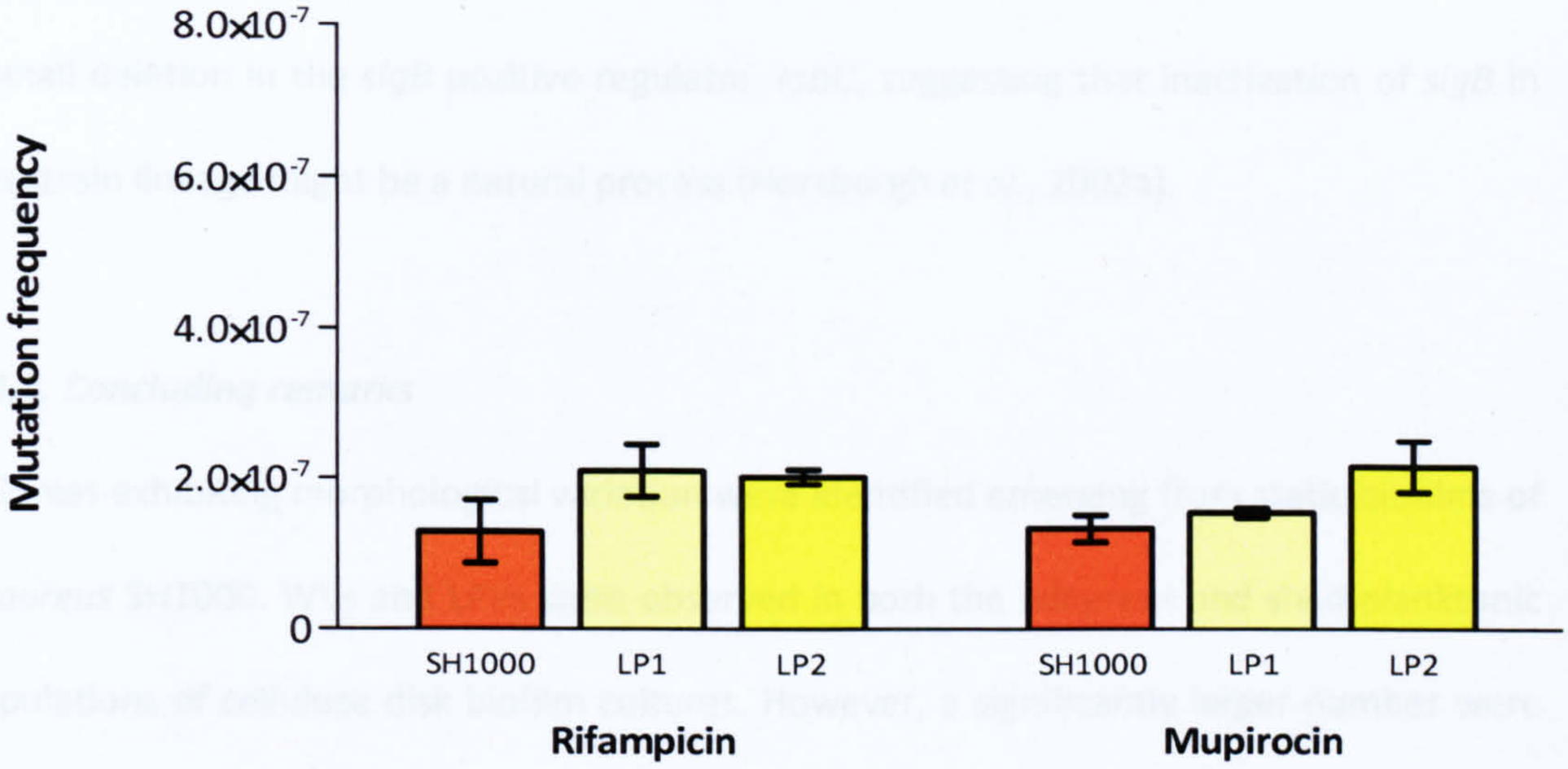

Figure 6.10 Mutation frequencies of *S. aureus* SH1000 and large pale variants, LP1 and LP2. Spontaneous mutants were isolated from the cultures using the selection antibiotics rifampicin and mupirocin. Data are based on three experimental replicates. Error bars indicate standard deviations.

The inability of WVs to form biofilms in the cellulose disk model, and the preferential appearance of WVs and LPVs in biofilm cultures, compared with planktonic culture, where they are found mostly in the shed phase, suggests that they may have a role in dissemination from the biofilm. This process is regarded to be the last step in biofilm formation and is though to enable bacteria to disperse and colonise new ecological niches (Sauer *et al.*, 2002). Although detachment can occur by an increase in shear forces, many organisms have developed active dissemination mechanisms. *P. aeruginosa* and *S. mutans*, for example, synthesise enzymes that degrade the biofilm exopolysaccharide matrix and surface proteins, respectively, liberating the attached cells (Boyd & Chakrabarty, 1994; Lee *et al.*, 1996). *S. aureus* SH1000 may therefore enrich the biofilm population with phenotypic variants that promote dissemination. The *sigB* mutations carried by the WVs, although allowing the release of *S. aureus* from biofilm culture, may also result in virulence attenuation, as this sigma factor controls the production of a variety of virulence factors

(Bischoff *et al.*, 2004). However, the parent strain of *S. aureus* SH1000, 8325-4, also carries a small deletion in the *sigB* positive regulator, *rsbU*, suggesting that inactivation of *sigB* in this strain lineage might be a natural process (Horsburgh *et al.*, 2002a).

6.3.3. Concluding remarks

Colonies exhibiting morphological variation were identified emerging from static biofilms of *S. aureus* SH1000. WVs and LPVs were observed in both the adherent and shed planktonic populations of cellulose disk biofilm cultures. However, a significantly larger number were observed in the shed planktonic populations of the biofilms. Variants were also identified in biofilms grown using a flow system (Sorbarod biofilm model), suggesting that their production is not model specific.

WVs had lost biofilm formation, and possessed minor increases in MF compared to the wild-type. However, this was not observed in a staphyloxanthin defective mutant, suggesting that pigment loss does not equate to an increase in mutability. WVs also had increased susceptibility to killing by hydrogen peroxide. However, this did not translate to an increase in mutability in the presence of sublethal concentrations of hydrogen peroxide, compared with *S. aureus* SH1000. Molecular analysis revealed that WVs harbour mutations in the alternative sigma factor, SigB, a global regulator of both biofilm formation and staphyloxanthin production.

LPVs had similar biofilm forming capacity as the wild-type. No increase in MF was observed for these variants compared to the parental strain. The molecular basis of their variation remains undefined.

Due to preferential emergence of both variant types in biofilm cultures, compared with planktonic culture, their larger numbers in the population of planktonic cells shed

from biofilms and the loss of biofilm formation exhibited by WVs, it appears that these phenotypic variants may promote dissemination of *S. aureus* SH1000 from biofilms.

7. General conclusions and future studies

Growth of bacteria as a biofilm confers broad antibiotic recalcitrance through mechanisms that remain largely undefined. Some studies have demonstrated reduced antibiotic penetration, high levels of persisters and slow growth in biofilms, as mechanisms to promote antibiotic recalcitrance (Ito *et al.*, 2009; Singh *et al.*, 2009; Singh *et al.*, 2010b). In addition, there is limited evidence for increased genetic transfer and mutation in biofilm cultures, when compared with planktonic cultures, to confer genotypic antibiotic resistance (Cvitkovitch, 2004; Allegrucci & Sauer, 2008). However, the role for genetic mutability as a mechanism that may contribute to the emergence antibiotic resistance in biofilms, particularly in Gram positive bacteria, has not been investigated in detail. This work sought to investigate this issue.

Initially, a novel biofilm model was generated to allow high-throughput screening of biofilms for an array of applications. This was necessary as the currently available biofilm models are often low-throughput in nature, or if high-throughput, only yield simplistic biofilms (Ceri *et al.*, 1999; Driffield *et al.*, 2008). Cellulose disks were chosen as a substratum, with the incorporation of human plasma to exploit staphylococcal surface proteins that bind human serum components resulting in surface adherence (MSCRAMMs) (Bozzini *et al.*, 1992; Nicholas *et al.*, 1999; O'Brien *et al.*, 2002). Validation of the model was performed by demonstrating antibiotic recalcitrance and dissemination in the presence of D-amino acids, as both of these phenomena had been previously reported for staphylococcal biofilms (Zheng & Stewart, 2002; Kolodkin-Gal *et al.*, 2010). Transcriptional profiling also demonstrated upregulation of genes encoding urease activity, and downregulation of toxin and protease genes, differential expression of which has previously been demonstrated for *S. aureus* in biofilms (Resch *et al.*, 2005). Visualisation of the

cultures grown on the disks by confocal and atomic force microscopy, revealed a compact population of cells harbouring a rough surface, also consistent with biofilm growth.

This new model is therefore an efficient means of generating staphylococcal biofilms. Furthermore, the simplicity of the system could enable it to be developed for use with other bacterial species, in addition to *S. aureus* and *S. epidermidis,* by exploitation of factors that promote adherence, such as the human plasma that was used in this case.

Mutability studies were then conducted utilising the novel biofilm system generated. *S. aureus* and *S. epidermidis* biofilms were shown to harbour bacteria with increased MFs compared with planktonic cultures, both in this new static biofilm model and also an established flow system. Limited studies with other organisms, such as *S. pneumoniae* and *P. aeruginosa*, have shown increased mutability when these species grow as a biofilm (Allegrucci & Sauer, 2008; Driffield *et al.*, 2008). Biofilms of *E. coli* and *P. aeruginosa* downregulate expression of antioxidant enzymes, whilst *S. aureus* can up and downregulate expression of such enzymes depending on the biofilm model and strain utilised (Schembri *et al.*, 2003; Resch *et al.*, 2005; Brady *et al.*, 2006; Driffield *et al.*, 2008). It was therefore hypothesised, that increased mutability in cellulose disk biofilm cultures of *S. aureus* may be a consequence of endogenous oxidative stress, potentially due to downregulation of antioxidant enzyme gene expression. Accordingly, antioxidant agents were incorporated into *S. aureus* biofilms. This revealed that antioxidant agents significantly reduced biofilm MFs, confirming an involvement for ROS in biofilm mutability. Transcriptional profiling revealed little differential expression of antioxidant enzymes, where only *sodA* was upregulated in the biofilm cultures. However, significant downregulation of genes involved in carotenoid pigment biosynthesis, which provide protection against oxidative stress (Clauditz *et al.*, 2006), was observed. To further validate the observation of downregulation of staphyloxanthin biosynthesis in these biofilms, the

respective levels of this pigment could be directly assayed (Resch *et al.*, 2005). It was hypothesised that reduced pigmentation of cells within biofilm cultures may render them more susceptible to endogenous oxidative damage by ROS. However, non-pigmented mutants (as discussed in Chapter 6), although less able to survive hydrogen peroxide treatment, were not more mutable in the presence of sublethal concentrations of hydrogen peroxide. The DNA microarray analysis data also demonstrated upregulation of numerous genes involved in DNA repair in biofilm cultures, consistent with the observations of increased mutability of *S. aureus* in biofilm culture.

As well as further investigating the nature of increased mutability in biofilms, it may also be useful to assess the levels of recombination. Specifically, recombination frequencies could be deduced and compared with planktonic cultures, to see if these events also contribute to increased mutability. Furthermore, as previously stated, there are limited reports of increased rates of conjugative transfer in biofilms, assumed to result from increased cell-to-cell contact due to the high cell densities in biofilms (Cvitkovitch, 2004). AFM of cellulose disk biofilms revealed that they form a tightly compact population of cells. Therefore, this may be an appropriate system to investigate the impact of conjugative transfer on the emergence of antibiotic resistance in biofilms, in the case of strains with conjugative plasmids encoding an antibiotic resistance marker.

In addition, as several genes responsible for the regulation of *agr* and RNAIII were differentially regulated in the biofilm, further investigation of the effect of expression of these genes on biofilm architecture is required. Specifically, reduced expression of *agr* and RNAIII genes is necessary to maintain the integrity of *ica*-independent biofilms possessing a proteinaceous matrix (Boles & Horswill, 2008; Lauderdale *et al.*, 2009). The data generated here suggest that RNAIII or *agr* expression would be downregulated. No differential regulation of *agr* was observed, however, significant downregulation of the delta

haemolysin was observed in biofilms, which is encoded by RNAIII (Janzon *et al.*, 1989). Furthermore, preliminary experiments showing complete degradation of *S. aureus* biofilms subjected to proteinase K treatment (data not shown) suggest that these cultures rely heavily on proteinaceous components for adherence to the disks.

Biofilms are known to harbour phenotypic variants, such as SCVs. Whilst generating staphylococcal biofilms using the cellulose disk model, two new and distinct colony types displaying morphological variation were identified in the biofilm cultures. These colonies were WVs and LPVs. WVs were shown to have lost biofilm forming capacity in the cellulose disk system, harbour a modestly increased MF and have an increased susceptibility to hydrogen peroxide. This is likely due to the observed mutations in the alternative sigma factor, SigB, which controls the expression of numerous genes involved in staphyloxanthin production and biofilm formation. LPVs, however, maintained biofilm forming capacity and had no significant increase in MF, compared with the parental strain. Both of these variants were mostly found in the population of planktonic cells which is shed from the biofilm, suggesting a role for them in the dissemination of *S. aureus* from biofilms. If the information gathered using DNA microarray analysis is taken into consideration with the observation of mutations in SigB in the WVs, this is highly indicative of *ica*-independent mechanisms of biofilm formation in the disk model. SigB controls expression of the *agr* quorum sensing system which is involved in dissemination of *S. aureus* from biofilms (Boles & Horswill, 2008). Specifically, mutants defective in *sigB* show higher levels of *agr* and RNAIII expression, which results in higher levels of extracellular proteases, presumably resulting in the breakdown of proteinaceous structures providing adherence (Lauderdale *et al.*, 2009). Therefore, if biofilms formed by *S. aureus* SH1000 have a proteinaceous matrix, biofilm-forming capacity of the WVs should be restored in the presence of extracellular protease inhibitors (Lauderdale *et al.*, 2009).

Due to time constraints, the genetic basis of the LPV phenotype was not elucidated. However, whole genome nucleotide sequence determinations of these variants would be useful in determining the nature of their morphological variation. Furthermore, assessing both WVs and LPVs in a model of infection would provide information as to whether they are attenuated in virulence. WVs in particular could be attenuated due to their mutations in SigB (Bischoff *et al.*, 2004). In addition, it will be important to assess other *S. aureus* strains for such variants, to confirm whether this phenomenon is specific to this strain.

In summary, I have demonstrated that staphylococcal biofilms are significantly more mutable than their planktonic counterparts, and that this phenomenon may promote heterogeneity in the biofilm, in the form of both antibiotic resistant mutants and morphological variants.

Appendix

Table A.1 Differentially expressed genes in 48 hr biofilm cultures versus planktonic cultures, of *S. aureus* SH1000. Data are the average of three experimental replicates. P values were determined using a Student's t-test in ArrayStar™ (DNASTAR).

Locus tag	Gene name	Product name/Function	Fold change	P value
Upregulated genes				
SAOUHSC_00001	*dnaA*	chromosomal replication initiator protein DnaA	3.237 up	$3.18x10^{-27}$
SAOUHSC_00003		hypothetical protein	3.673 up	$4.52x10^{-27}$
SAOUHSC_00004	*recF*	DNA replication and repair protein RecF	2.683 up	$4.29x10^{-24}$
SAOUHSC_00005		DNA gyrase, B subunit	2.072 up	$2.32x10^{-19}$
SAOUHSC_00014		membrane protein, putative	2.160 up	$2.46x10^{-22}$
SAOUHSC_00017	*rplI*	ribosomal protein L9	3.055 up	$2.64x10^{-23}$
SAOUHSC_00028		hypothetical protein	2.399 up	$5.14x10^{-9}$
SAOUHSC_00029		hypothetical protein	3.193 up	$1.58x10^{-9}$
SAOUHSC_00032		hypothetical protein	3.880 up	$2.23x10^{-23}$
SAOUHSC_00035		pyridine nucleotide-disulphide oxidoreductase family protein	3.002 up	$1.05x10^{-21}$
SAOUHSC_00036		metallo-beta-lactamase family protein	2.560 up	$8.29x10^{-23}$
SAOUHSC_00037		sulfide:quinone oxidoreductase	3.927 up	$9.56x10^{-22}$
SAOUHSC_00141		hypothetical protein	2.777 up	$6.98x10^{-22}$
SAOUHSC_00173	*azoR*	azoreductase	2.639 up	$6.36x10^{-28}$
SAOUHSC_00245		truncated transposase	2.067 up	$8.32x10^{-15}$
SAOUHSC_00345		hypothetical protein	2.246 up	$7.57x10^{-17}$
SAOUHSC_00348	*rpsF*	ribosomal protein S6	4.889 up	$8.31x10^{-23}$
SAOUHSC_00349		single-stranded DNA-binding protein	2.894 up	$4.45x10^{-19}$
SAOUHSC_00440		hypothetical protein	3.249 up	$6.98x10^{-20}$
SAOUHSC_00441		acetyltransferase family protein	3.389 up	$1.18x10^{-29}$
SAOUHSC_00442		DNA polymerase III gamma/tau subunit	3.386 up	$3.35x10^{-31}$
SAOUHSC_00444		hypothetical protein	2.378 up	$2.41x10^{-23}$
SAOUHSC_00453		hypothetical protein	2.092 up	$2.65x10^{-7}$
SAOUHSC_00465		protein veg	3.055 up	$7.53x10^{-16}$
SAOUHSC_00472	*prs*	ribose-phosphate pyrophosphokinase	2.846 up	$6.93x10^{-22}$
SAOUHSC_00473		hypothetical protein	3.435 up	$3.22x10^{-20}$
SAOUHSC_00474	*rplY*	ribosomal protein L25, Ctc-form	2.581 up	$2.96x10^{-12}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_00491		2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	2.654 up	$3.59x10^{-24}$
SAOUHSC_00492		hypothetical protein	5.062 up	$2.00x10^{-21}$
SAOUHSC_00493	*lysS*	lysyl-tRNA synthetase	2.261 up	$6.68x10^{-26}$
SAOUHSC_00502	*ctsR*	transcriptional regulator CtsR	8.875 up	$9.23x10^{-29}$
SAOUHSC_00503		UvrB/UvrC motif-containing protein	2.876 up	$2.54x10^{-22}$
SAOUHSC_00504		ATP:guanido phosphotransferase	4.200 up	$2.29x10^{-22}$
SAOUHSC_00505	*clpC*	ATP-dependent Clp protease ATP binding subunit ClpC	4.311 up	$6.80x10^{-27}$
SAOUHSC_00508		PIN domain protein	2.022 up	$1.39x10^{-13}$
SAOUHSC_00516	*secE*	preprotein translocase, SecE subunit	2.062 up	$6.52x10^{-11}$
SAOUHSC_00519	*rplA*	ribosomal protein L1	2.096 up	$1.13x10^{-21}$
SAOUHSC_00550		GlcNAc-PI de-N-acetylase family protein	3.452 up	$8.42x10^{-29}$
SAOUHSC_00551		hypothetical protein	2.285 up	$6.11x10^{-24}$
SAOUHSC_00555		HAD-superfamily hydrolase, subfamily IA, variant 1	3.236 up	$1.14x10^{-16}$
SAOUHSC_00556		proline/betaine transporter, putative	4.042 up	$1.29x10^{-31}$
SAOUHSC_00560		hypothetical protein	2.362 up	$1.10x10^{-21}$
SAOUHSC_00561		hypothetical protein	4.170 up	$4.76x10^{-14}$
SAOUHSC_00574	*eutD*	phosphotransacetylase	3.600 up	$2.06x10^{-29}$
SAOUHSC_00575		lipoate-protein ligase A family protein	4.252 up	$2.88x10^{-27}$
SAOUHSC_00604		hypothetical protein	2.648 up	$7.76x10^{-23}$
SAOUHSC_00605		acetyltransferase, gnat family	2.031 up	$9.69x10^{-13}$
SAOUHSC_00617		hypothetical protein	2.012 up	$3.70x10^{-15}$
SAOUHSC_00618		hypothetical protein	2.861 up	$1.71x10^{-15}$
SAOUHSC_00634		manganese ABC transporter substrate-binding lipoprotein	2.671 up	$5.62x10^{-12}$
SAOUHSC_00636		ABC-3	2.068 up	$5.24x10^{-7}$
SAOUHSC_00638		iron dependent repressor:FeoA	2.909 up	$9.41x10^{-21}$
SAOUHSC_00641	*tagH*	teichoic acids export protein ATP-binding subunit	2.618 up	$1.07x10^{-24}$
SAOUHSC_00651		YitT family protein	2.374 up	$1.42x10^{-18}$
SAOUHSC_00652		ferrichrome transport ATP-binding protein FhuC	2.952 up	$2.88x10^{-24}$
SAOUHSC_00659		hypothetical protein	2.876 up	$1.09x10^{-23}$
SAOUHSC_00663		acetyltransferase, gnat family	3.409 up	$1.66x10^{-12}$
SAOUHSC_00668		ABC transporter, permease protein	2.268 up	$5.17x10^{-19}$
SAOUHSC_00669		YkaA	2.713 up	$9.80x10^{-15}$
SAOUHSC_00676		hypothetical protein	2.104 up	$1.73x10^{-16}$
SAOUHSC_00678		hypothetical protein	3.235 up	$5.49x10^{-14}$
SAOUHSC_00680		hypothetical protein	4.355 up	$4.22x10^{-13}$
SAOUHSC_00694	*mgrA*	HTH-type transcriptional regulator MgrA	5.235 up	$2.38x10^{-24}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_00711		CBS domain protein	2.488 up	$1.24x10^{-20}$
SAOUHSC_00714	*saeS*	sensor histidine kinase SaeS	2.328 up	$1.01x10^{-17}$
SAOUHSC_00718		membrane protein, putative	3.091 up	$2.12x10^{-13}$
SAOUHSC_00728	*ltaS*	glycerol phosphate lipoteichoic acid synthase	4.056 up	$3.09x10^{-27}$
SAOUHSC_00729		ABC transporter, ATP-binding protein	2.642 up	$2.01x10^{-21}$
SAOUHSC_00735		hypothetical protein	3.013 up	$5.63x10^{-15}$
SAOUHSC_00741	*nrdI*	ribonucleotide reductase stimulatory protein	6.012 up	$1.54x10^{-31}$
SAOUHSC_00742		ribonucleoside-diphosphate reductase subunit alpha	3.281 up	$1.60x10^{-25}$
SAOUHSC_00743	*nrdF*	ribonucleoside-diphosphate reductase, beta subunit	2.030 up	$1.03x10^{-21}$
SAOUHSC_00790	*clpP*	ATP-dependent Clp protease, proteolytic subunit ClpP	2.617 up	$7.13x10^{-27}$
SAOUHSC_00800		hypothetical protein	2.477 up	$1.87x10^{-16}$
SAOUHSC_00801	*secG*	preprotein translocase, SecG subunit	2.019 up	$2.27x10^{-16}$
SAOUHSC_00819		hypothetical protein	3.352 up	$5.16x10^{-24}$
SAOUHSC_00833		nitroreductase	3.897 up	$1.18x10^{-33}$
SAOUHSC_00835		arsenate reductase	2.535 up	$3.23x10^{-12}$
SAOUHSC_00861	*lipA*	lipoic acid synthetase	2.807 up	$2.60x10^{-21}$
SAOUHSC_00867		hypothetical protein	8.863 up	$1.93x10^{-22}$
SAOUHSC_00868		hypothetical protein	7.379 up	0
SAOUHSC_00869	*dltA*	D-alanine-activating enzyme	2.966 up	$7.47x10^{-25}$
SAOUHSC_00870		DltB protein, putative	4.964 up	$6.72x10^{-32}$
SAOUHSC_00871	*dltC*	D-alanine--poly(phosphoribitol) ligase subunit 2	5.021 up	$4.97x10^{-30}$
SAOUHSC_00872		DltD, C-terminal:DltD, N-terminal:DltD, central region	2.201 up	$2.12x10^{-23}$
SAOUHSC_00880		Na+/H+ antiporter family protein	2.024 up	$3.52x10^{-13}$
SAOUHSC_00892		RNA binding S1	2.505 up	$7.76x10^{-19}$
SAOUHSC_00900	*pgi*	glucose-6-phosphate isomerase	2.906 up	$1.09x10^{-28}$
SAOUHSC_00909		Cof protein:HAD-superfamily hydrolase subfamily IIB	2.004 up	$3.80x10^{-20}$
SAOUHSC_00910		N-6 Adenine-specific DNA methylase YitW	2.379 up	$2.16x10^{-13}$
SAOUHSC_00912		ATP-dependent Clp protease, ATP-binding subunit ClpB	3.924 up	$1.91x10^{-28}$
SAOUHSC_00934	*spxA*	transcriptional regulator Spx	2.947 up	$1.14x10^{-31}$
SAOUHSC_00935	*mecA*	adapter protein	4.675 up	$1.85x10^{-28}$
SAOUHSC_00938		hypothetical protein	2.445 up	$2.15x10^{-13}$
SAOUHSC_00939		protozoan/cyanobacterial globin family protein	2.271 up	$2.45x10^{-15}$
SAOUHSC_00941		hypothetical protein	2.879 up	$5.20x10^{-19}$
SAOUHSC_00942		GTP pyrophosphokinase	2.525 up	$1.77x10^{-27}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_00943	*ppnK*	probable inorganic polyphosphate/ATP-NAD kinase (Poly(P)/ATP NAD kinase)	2.128 up	$5.19x10^{-24}$
SAOUHSC_00944		ribosomal large subunit pseudouridine synthase, RluD subfamily	2.303 up	$1.74x10^{-18}$
SAOUHSC_00947		enoyl-[acyl-carrier-protein] reductase [NADH]	2.375 up	$3.63x10^{-21}$
SAOUHSC_00982		isochorismate synthase family protein	2.233 up	$3.17x10^{-22}$
SAOUHSC_00991		acyltransferase 3	3.084 up	$9.34x10^{-14}$
SAOUHSC_00996		hypothetical protein	2.518 up	$2.95x10^{-20}$
SAOUHSC_00997		transcriptional regulator, putative	2.701 up	$1.71x10^{-26}$
SAOUHSC_00998	*fmtA*	Protein FmtA	3.338 up	$3.36x10^{-18}$
SAOUHSC_01005		hypothetical protein	7.220 up	$3.54x10^{-37}$
SAOUHSC_01007	*folD*	FolD bifunctional protein	2.203 up	$1.76x10^{-13}$
SAOUHSC_01008		phosphoribosylaminoimidazole carboxylase, catalytic subunit	2.911 up	$2.88x10^{-20}$
SAOUHSC_01009		phosphoribosylaminoimidazole carboxylase, ATPase subunit	2.412 up	$1.28x10^{-22}$
SAOUHSC_01010	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase	2.977 up	$2.17x10^{-18}$
SAOUHSC_01011		phosphoribosylformylglycinamidine synthase, PurS protein	2.053 up	$1.15x10^{-22}$
SAOUHSC_01025		membrane protein, putative	4.998 up	$4.25x10^{-21}$
SAOUHSC_01036		hypothetical protein	2.881 up	$1.40x10^{-13}$
SAOUHSC_01037		hypothetical protein	4.314 up	$1.74x10^{-14}$
SAOUHSC_01055		inositol monophosphatase family protein, putative	2.104 up	$1.32x10^{-21}$
SAOUHSC_01060		hypothetical protein	2.251 up	$1.21x10^{-7}$
SAOUHSC_01069		allergen V5/Tpx-1 related	2.254 up	$1.02x10^{-16}$
SAOUHSC_01073		hypothetical protein	2.075 up	$3.95x10^{-17}$
SAOUHSC_01074		hypothetical protein	2.427 up	$1.04x10^{-17}$
SAOUHSC_01077		hypothetical protein	5.903 up	$6.63x10^{-32}$
SAOUHSC_01078	*rpmF*	ribosomal protein L32	3.241 up	$4.03x10^{-23}$
SAOUHSC_01096		hypothetical protein	2.200 up	$3.87x10^{-11}$
SAOUHSC_01097		CvpA family protein	2.506 up	$1.26x10^{-12}$
SAOUHSC_01098		DNA-dependent DNA polymerase beta chain	5.763 up	$7.56x10^{-31}$
SAOUHSC_01099	*mutS2*	MutS2 protein	3.049 up	$9.65x10^{-26}$
SAOUHSC_01100	*trxA*	thioredoxin	3.157 up	$7.73x10^{-23}$
SAOUHSC_01101		hypothetical protein	3.368 up	$8.86x10^{-15}$
SAOUHSC_01102	*uvrC*	excinuclease ABC, C subunit	2.030 up	$1.10x10^{-20}$
SAOUHSC_01108		hypothetical protein	2.078 up	$2.30x10^{-25}$
SAOUHSC_01120		hypothetical protein	2.065 up	$5.27x10^{-7}$
SAOUHSC_01139		hypothetical protein	4.591 up	$1.73x10^{-29}$
SAOUHSC_01140		hypothetical protein	5.289 up	$9.15x10^{-30}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_01141		hypothetical protein	4.130 up	$1.04x10^{-27}$
SAOUHSC_01152		hypothetical protein	2.253 up	$3.73x10^{-23}$
SAOUHSC_01153		hypothetical protein	2.179 up	$1.08x10^{-24}$
SAOUHSC_01154	*sepF*	cell division protein, SepF	2.471 up	$1.19x10^{-22}$
SAOUHSC_01155		hypothetical protein	3.562 up	$7.72x10^{-17}$
SAOUHSC_01158		hypothetical protein	5.127 up	$4.68x10^{-30}$
SAOUHSC_01164	*pyrR*	PyrR bifunctional protein	3.935 up	$1.31x10^{-18}$
SAOUHSC_01165		uracil permease	2.827 up	$3.87x10^{-24}$
SAOUHSC_01166	*pyrB*	aspartate carbamoyltransferase catalytic subunit	5.777 up	$1.27x10^{-31}$
SAOUHSC_01168	*pyrC*	dihydroorotase	2.830 up	$2.02x10^{-24}$
SAOUHSC_01169		carbamoyl phosphate synthase small subunit	3.834 up	$7.57x10^{-30}$
SAOUHSC_01170	*carB*	carbamoyl phosphate synthase large subunit	2.049 up	$7.65x10^{-26}$
SAOUHSC_01172	*pyrE*	orotate phosphoribosyltransferase	2.272 up	$8.07x10^{-19}$
SAOUHSC_01173		hypothetical protein	2.948 up	$4.09x10^{-12}$
SAOUHSC_01175		fibrinogen binding protein	2.250 up	$1.42x10^{-18}$
SAOUHSC_01176	*gmk*	guanylate kinase	2.745 up	$5.16x10^{-27}$
SAOUHSC_01178		phosphopantothenoylcysteine decarboxylase/phosphopantothenate--cysteine ligase	2.168 up	$3.34x10^{-15}$
SAOUHSC_01182		polypeptide deformylase	2.325 up	$1.25x10^{-17}$
SAOUHSC_01185	*rlmN*	Ribosomal RNA large subunit methyltransferase N	2.109 up	$4.07x10^{-20}$
SAOUHSC_01188	*rsgA*	ribosome small subunit-dependent GTPase A	2.419 up	$2.88x10^{-21}$
SAOUHSC_01191	*rpmB*	ribosomal protein L28	2.213 up	$7.85x10^{-12}$
SAOUHSC_01192		hypothetical protein	3.726 up	$2.11x10^{-22}$
SAOUHSC_01201	*acpP*	acyl carrier protein	2.969 up	$5.13x10^{-14}$
SAOUHSC_01211	*rplS*	ribosomal protein L19	2.174 up	$1.92x10^{-10}$
SAOUHSC_01216	*sucC*	succinyl-CoA synthetase subunit beta	2.575 up	$1.85x10^{-11}$
SAOUHSC_01232	*rpsB*	ribosomal protein S2	2.839 up	$2.35x10^{-18}$
SAOUHSC_01233		hypothetical protein	4.368 up	$1.68x10^{-18}$
SAOUHSC_01234	*tsf*	translation elongation factor Ts	2.292 up	$2.51x10^{-18}$
SAOUHSC_01242	*rimP*	ribosome maturation factor RimP	3.885 up	$5.78x10^{-24}$
SAOUHSC_01250	*rpsO*	ribosomal protein S15	2.484 up	$3.94x10^{-15}$
SAOUHSC_01261		competence/damage-inducible protein CinA N-terminal domain, putative	2.284 up	$9.62x10^{-22}$
SAOUHSC_01283		GTP-binding protein	2.242 up	$1.16x10^{-19}$
SAOUHSC_01285		regulatory protein, MerR	2.991 up	$4.84x10^{-11}$
SAOUHSC_01309		hypothetical protein	3.602 up	$1.81x10^{-12}$
SAOUHSC_01313		sensor kinase protein	2.140 up	$1.34x10^{-12}$
SAOUHSC_01317		hypothetical protein	2.790 up	$4.83x10^{-21}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_01330	*guaC*	guanosine 5'-monophosphate oxidoreductase	2.507 up	$5.06x10^{-27}$
SAOUHSC_01331		hypothetical protein	5.471 up	$5.13x10^{-26}$
SAOUHSC_01332		hypothetical protein	2.848 up	$2.80x10^{-17}$
SAOUHSC_01333	*lexA*	LexA repressor	2.421 up	$4.84x10^{-20}$
SAOUHSC_01336		protein Stu0508	3.395 up	$1.34x10^{-15}$
SAOUHSC_01337		transketolase	2.566 up	$4.58x10^{-18}$
SAOUHSC_01347		aconitate hydratase 1	2.577 up	$1.90x10^{-17}$
SAOUHSC_01349		hypothetical protein	2.167 up	$6.66x10^{-13}$
SAOUHSC_01359	*mprF*	phosphatidylglycerol lysyltransferase	2.549 up	$1.84x10^{-20}$
SAOUHSC_01360	*msrA1*	methionine-S-sulfoxide reductase	2.171 up	$1.14x10^{-22}$
SAOUHSC_01361	*msrR*	regulatory protein MsrR	2.334 up	$7.66x10^{-12}$
SAOUHSC_01362		hypothetical protein	2.182 up	$1.22x10^{-9}$
SAOUHSC_01373	*femA*	aminoacyltransferase FemA	2.639 up	$5.22x10^{-14}$
SAOUHSC_01392		ABC transporter, ATP-binding protein, putative	2.411 up	$1.18x10^{-15}$
SAOUHSC_01402	*msa*	protein Msa	2.004 up	$8.76x10^{-11}$
SAOUHSC_01403	*cspA*	cold shock protein CspA	4.560 up	$8.35x10^{-22}$
SAOUHSC_01404		hypothetical protein	6.375 up	$8.63x10^{-13}$
SAOUHSC_01405		hypothetical protein	3.603 up	$4.94x10^{-19}$
SAOUHSC_01406	*acyP*	acylphosphatase	2.995 up	$6.57x10^{-12}$
SAOUHSC_01407		5-bromo-4-chloroindolyl phosphate hydrolysis protein XpaC	3.011 up	$4.55x10^{-10}$
SAOUHSC_01408		hypothetical protein	3.091 up	$4.47x10^{-10}$
SAOUHSC_01430		glucose-specific phosphotransferase enzyme IIA component	2.303 up	$1.47x10^{-16}$
SAOUHSC_01431	*msrB*	methionine-R-sulfoxide reductase	2.273 up	$6.88x10^{-17}$
SAOUHSC_01432	*msrA2*	methionine-S-sulfoxide reductase	2.107 up	$2.28x10^{-12}$
SAOUHSC_01436		YphP	2.018 up	$3.05x10^{-16}$
SAOUHSC_01437	*cvfC*	conserved virulence factor C	2.084 up	$1.03x10^{-19}$
SAOUHSC_01439		hypothetical protein	2.461 up	$3.45x10^{-26}$
SAOUHSC_01462	*gpsB*	cell cycle protein GpsB	3.483 up	$9.45x10^{-12}$
SAOUHSC_01463		hypothetical protein	2.974 up	$1.45x10^{-12}$
SAOUHSC_01464		hypothetical protein	5.377 up	$1.01x10^{-18}$
SAOUHSC_01466	*recU*	recombination protein U	5.242 up	$1.73x10^{-19}$
SAOUHSC_01467		penicillin-binding protein 2	3.889 up	$3.86x10^{-23}$
SAOUHSC_01468		hypothetical protein	3.990 up	$2.46x10^{-16}$
SAOUHSC_01469		endonuclease III, putative	2.979 up	$4.82x10^{-22}$
SAOUHSC_01470		DnaD and phage-associated region	2.508 up	$7.06x10^{-20}$
SAOUHSC_01476		MazG nucleotide pyrophosphohydrolase domain superfamily	2.594 up	$2.43x10^{-15}$
SAOUHSC_01477		Zn-dependent protease	3.506 up	$1.55x10^{-10}$
SAOUHSC_01478		YpjA	2.243 up	$9.25x10^{-26}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_01479		hypothetical protein	2.156 up	$2.46x10^{-20}$
SAOUHSC_01480		TPR repeat	2.622 up	$2.89x10^{-18}$
SAOUHSC_01490		DNA-binding protein HU 1	2.520 up	$1.54x10^{-7}$
SAOUHSC_01504		ferredoxin	6.732 up	$3.78x10^{-29}$
SAOUHSC_01505		riboflavin transporter	4.083 up	$2.16x10^{-24}$
SAOUHSC_01586	*srrA*	transcriptional regulatory protein SrrA	2.666 up	$1.72x10^{-14}$
SAOUHSC_01588	*scpB*	segregation and condensation protein B	2.036 up	$1.06x10^{-16}$
SAOUHSC_01589	*scpA*	segregation and condensation protein A	2.694 up	$2.73x10^{-14}$
SAOUHSC_01599		glucose-6-phosphate 1-dehydrogenase	4.299 up	$3.80x10^{-32}$
SAOUHSC_01605		6-phosphogluconate dehydrogenase, decarboxylating	2.014 up	$1.95x10^{-13}$
SAOUHSC_01607		hypothetical protein	2.601 up	$1.58x10^{-11}$
SAOUHSC_01608		hypothetical protein	2.413 up	$9.58x10^{-7}$
SAOUHSC_01610		YqiW	2.224 up	$5.97x10^{-22}$
SAOUHSC_01621	*nusB*	transcription antitermination factor NusB	2.658 up	$2.25x10^{-13}$
SAOUHSC_01622		YqhY	2.237 up	$7.58x10^{-16}$
SAOUHSC_01627		lipoprotein, putative	2.055 up	$2.98x10^{-8}$
SAOUHSC_01647		hypothetical protein	2.507 up	$3.09x10^{-8}$
SAOUHSC_01648		tetratricopeptide repeat domain protein	2.823 up	$2.59x10^{-9}$
SAOUHSC_01653	*sodA*	superoxide dismutase [Mn]	2.256 up	$2.11x10^{-5}$
SAOUHSC_01655	*fur*	ferric uptake regulation protein	2.140 up	$6.88x10^{-12}$
SAOUHSC_01656		hydrophobic membrane protein ZurM	2.319 up	$4.31x10^{-22}$
SAOUHSC_01657		ABC transporter, putative	2.680 up	$3.16x10^{-22}$
SAOUHSC_01662	*rpoD*	RNA polymerase sigma factor RpoD	2.837 up	$7.20x10^{-24}$
SAOUHSC_01663		DNA primase	4.080 up	$1.39x10^{-26}$
SAOUHSC_01665		CBS domain protein	2.345 up	$2.86x10^{-10}$
SAOUHSC_01673		PhoH family protein	2.037 up	$7.43x10^{-19}$
SAOUHSC_01678	*rpsU*	ribosomal protein S21	2.722 up	$6.38x10^{-17}$
SAOUHSC_01679		hypothetical protein	2.230 up	$3.52x10^{-17}$
SAOUHSC_01681	*prmA*	ribosomal protein L11 methyltransferase	3.312 up	$9.45x10^{-35}$
SAOUHSC_01682	*dnaJ*	DnaJ protein	2.396 up	$1.35x10^{-27}$
SAOUHSC_01683	*dnaK*	molecular chaperone DnaK	3.575 up	$5.92x10^{-25}$
SAOUHSC_01684	*grpE*	co-chaperone GrpE	13.738 up	$6.67x10^{-28}$
SAOUHSC_01685	*hrcA*	heat-inducible transcription repressor HrcA	18.090 up	$3.94x10^{-30}$
SAOUHSC_01689	*rpsT*	ribosomal protein S20	2.764 up	$6.85x10^{-15}$
SAOUHSC_01698		hypothetical protein	2.216 up	$4.19x10^{-13}$
SAOUHSC_01701		had superfamily (subfamily iiia) phosphatase	2.231 up	$1.40x10^{-21}$
SAOUHSC_01702	*mtnN*	MTA/SAH nucleosidase	2.383 up	$5.71x10^{-18}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_01715	*udk*	uridine kinase	2.453 up	$6.40x10^{-26}$
SAOUHSC_01716		peptidase U32	2.472 up	$6.55x10^{-27}$
SAOUHSC_01717		peptidase U32	2.811 up	$3.47x10^{-27}$
SAOUHSC_01718		O-methyltransferase, family 3	2.462 up	$3.72x10^{-10}$
SAOUHSC_01720		putative Holliday junction resolvase	2.125 up	$1.29x10^{-25}$
SAOUHSC_01721		protein Stu1959	2.216 up	$4.24x10^{-23}$
SAOUHSC_01725	*mnmA*	tRNA-specific 2-thiouridylase MnmA	3.004 up	$9.27x10^{-29}$
SAOUHSC_01726	*mnmA*	tRNA-specific 2-thiouridylase MnmA	2.806 up	$2.89x10^{-23}$
SAOUHSC_01727		aminotransferase, class V	2.650 up	$1.28x10^{-24}$
SAOUHSC_01736		hypothetical protein	3.554 up	$5.98x10^{-13}$
SAOUHSC_01741	*dtd*	D-tyrosyl-tRNA(Tyr) deacylase	2.586 up	$3.70x10^{-28}$
SAOUHSC_01751	*ruvA*	Holliday junction DNA helicase RuvA	2.970 up	$2.09x10^{-18}$
SAOUHSC_01752		ACT domain protein PheB	3.036 up	$1.76x10^{-23}$
SAOUHSC_01753	*obgE*	Spo0B-associated GTP-binding protein	2.120 up	$1.86x10^{-23}$
SAOUHSC_01755	*rpmA*	ribosomal protein L27	3.096 up	$1.84x10^{-27}$
SAOUHSC_01756		hypothetical protein	11.287 up	$1.17x10^{-32}$
SAOUHSC_01757	*rplU*	ribosomal protein L21	8.655 up	$5.20x10^{-37}$
SAOUHSC_01758		rod shape-determining protein MreD	3.599 up	$1.01x10^{-24}$
SAOUHSC_01759		rod shape-determining protein MreC	3.964 up	$6.97x10^{-20}$
SAOUHSC_01760		hypothetical protein	4.448 up	$4.02x10^{-28}$
SAOUHSC_01761		hypothetical protein	5.602 up	$1.10x10^{-34}$
SAOUHSC_01762		hypothetical protein	2.662 up	$3.60x10^{-22}$
SAOUHSC_01778	*clpX*	ATP-dependent protease ATP-binding subunit ClpX	2.952 up	$1.17x10^{-18}$
SAOUHSC_01779	*tig*	trigger factor	4.831 up	$6.02x10^{-33}$
SAOUHSC_01780		hypothetical protein	3.626 up	$1.18x10^{-12}$
SAOUHSC_01784	*rplT*	ribosomal protein L20	2.204 up	$1.58x10^{-12}$
SAOUHSC_01785	*rpmI*	ribosomal protein L35	3.393 up	$3.25x10^{-25}$
SAOUHSC_01786	*infC*	translation initiation factor IF-3	4.049 up	$9.74x10^{-25}$
SAOUHSC_01789		hypothetical protein	3.157 up	$4.82x10^{-22}$
SAOUHSC_01792		replication initiation and membrane attachment protein	2.512 up	$4.22x10^{-21}$
SAOUHSC_01793	*nrdR*	transcriptional regulator NrdR	3.033 up	$2.53x10^{-21}$
SAOUHSC_01797		DNA polymerase I	2.297 up	$2.73x10^{-14}$
SAOUHSC_01801		isocitrate dehydrogenase	3.216 up	$2.13x10^{-15}$
SAOUHSC_01802		citrate synthase 2	2.412 up	$2.28x10^{-19}$
SAOUHSC_01829	*rpsD*	ribosomal protein S4	2.026 up	$4.25x10^{-14}$
SAOUHSC_01850		catabolite control protein A	3.620 up	$5.01x10^{-20}$
SAOUHSC_01851		hypothetical protein	2.301 up	$2.18x10^{-6}$
SAOUHSC_01865	*trmB*	tRNA (guanine-N(7)-)-methyltransferase	3.914 up	$2.18x10^{-24}$
SAOUHSC_01866		aminoglycoside phosphotransferase	2.269 up	$1.39x10^{-23}$
SAOUHSC_01870		RNA pseudouridine synthase family protein	2.219 up	$1.85x10^{-17}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_01896		hypothetical protein	2.678 up	$4.08x10^{-12}$
SAOUHSC_01927		transposase, IS3 family, truncation-related protein, putative	2.539 up	$4.09x10^{-13}$
SAOUHSC_01928		transposase	2.662 up	$1.27x10^{-12}$
SAOUHSC_01957		hypothetical protein	5.337 up	$2.44x10^{-19}$
SAOUHSC_01962	*hemE*	uroporphyrinogen decarboxylase	2.169 up	$2.36x10^{-17}$
SAOUHSC_01963		hypothetical protein	2.090 up	$4.84x10^{-11}$
SAOUHSC_01972	*prsA*	foldase protein PrsA	4.742 up	$6.66x10^{-25}$
SAOUHSC_01997	*perR*	peroxide-responsive repressor PerR	5.120 up	$1.34x10^{-24}$
SAOUHSC_02001		hypothetical protein	3.042 up	$1.35x10^{-23}$
SAOUHSC_02012	*mgt*	monofunctional glycosyltransferase	4.316 up	$5.01x10^{-32}$
SAOUHSC_02098		DNA-binding response regulator VraR, putative	3.807 up	$8.23x10^{-29}$
SAOUHSC_02099		sensor protein VraS	5.584 up	$6.29x10^{-25}$
SAOUHSC_02100		hypothetical protein	10.020 up	$1.24x10^{-26}$
SAOUHSC_02101		hypothetical protein	7.618 up	$1.16x10^{-26}$
SAOUHSC_02107		Mur ligase family protein	2.429 up	$7.97x10^{-21}$
SAOUHSC_02108	*ftnA*	ferritin	4.046 up	$1.97x10^{-24}$
SAOUHSC_02109		hypothetical protein	5.468 up	$5.70x10^{-16}$
SAOUHSC_02110		exonuclease	3.475 up	$9.40x10^{-23}$
SAOUHSC_02112		hypothetical protein	3.179 up	$4.97x10^{-24}$
SAOUHSC_02124	*pcrB*	protein PcrB	2.390 up	$3.94x10^{-17}$
SAOUHSC_02150		: Thiol-disulfide isomerase and thioredoxins	2.732 up	$5.24x10^{-18}$
SAOUHSC_02151		ABC-2 type transport system permease protein	2.134 up	$2.62x10^{-6}$
SAOUHSC_02153		membrane protein, putative	2.117 up	$4.26x10^{-8}$
SAOUHSC_02175		hypothetical phage protein	2.701 up	$6.04x10^{-13}$
SAOUHSC_02176		hypothetical protein	2.910 up	$5.39x10^{-21}$
SAOUHSC_02254	*groEL*	chaperonin GroEL	3.080 up	$1.82x10^{-23}$
SAOUHSC_02255	*groES*	co-chaperonin GroES	8.246 up	$8.90x10^{-32}$
SAOUHSC_02258		nitroreductase	3.747 up	$2.62x10^{-18}$
SAOUHSC_02280		hypothetical protein	2.183 up	$2.45x10^{-13}$
SAOUHSC_02294		hypothetical protein	10.127 up	$6.27x10^{-19}$
SAOUHSC_02301		phosphoserine phosphatase RsbU	2.363 up	$7.77x10^{-19}$
SAOUHSC_02302		sigmaB regulation protein RsbU, putative	3.465 up	$7.75x10^{-19}$
SAOUHSC_02303	*mazF*	Toxin MazF	2.966 up	$1.46x10^{-17}$
SAOUHSC_02316		DEAD-box ATP dependent DNA helicase	3.897 up	$1.55x10^{-27}$
SAOUHSC_02318	*ddl*	D-alanine--D-alanine ligase	2.095 up	$4.41x10^{-18}$
SAOUHSC_02343		ATP synthase F1, gamma subunit	2.072 up	$2.07x10^{-9}$
SAOUHSC_02346	*atpH*	ATP synthase F1, delta subunit	2.659 up	$8.46x10^{-12}$
SAOUHSC_02350	*atpB*	ATP synthase F0, A subunit	2.889 up	$5.47x10^{-11}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_02351		hypothetical protein	3.197 up	$4.77x10^{-13}$
SAOUHSC_02364		hypothetical protein	3.507 up	$1.37x10^{-27}$
SAOUHSC_02365		UDP-N-acetylglucosamine 1-carboxyvinyltransferase	3.545 up	$3.59x10^{-25}$
SAOUHSC_02381		general stress protein 20U	3.594 up	$1.69x10^{-11}$
SAOUHSC_02391		hypothetical protein	2.481 up	$8.10x10^{-17}$
SAOUHSC_02406		hypothetical protein	2.546 up	$8.34x10^{-15}$
SAOUHSC_02409		arginase	2.007 up	$1.91x10^{-17}$
SAOUHSC_02416		hypothetical protein	7.246 up	$3.49x10^{-16}$
SAOUHSC_02423		UTP-glucose-1-phosphate uridylyltransferase family protein	2.056 up	$1.45x10^{-23}$
SAOUHSC_02430		ABC transporter periplasmic binding protein, putative	2.936 up	$1.44x10^{-16}$
SAOUHSC_02448		cell surface hydrolase	2.599 up	$2.67x10^{-27}$
SAOUHSC_02460		aldo/keto reductase	2.078 up	$2.51x10^{-25}$
SAOUHSC_02461		transcriptional regulator, MerR family	3.685 up	$1.41x10^{-23}$
SAOUHSC_02471		hypothetical protein	2.678 up	$2.74x10^{-8}$
SAOUHSC_02473		hypothetical protein	2.262 up	$3.73x10^{-12}$
SAOUHSC_02478	*rplM*	ribosomal protein L13	2.704 up	$4.03x10^{-29}$
SAOUHSC_02486	*rpsK*	ribosomal protein S11	2.135 up	$1.28x10^{-12}$
SAOUHSC_02488	*rpmJ*	ribosomal protein L36	2.181 up	$1.13x10^{-16}$
SAOUHSC_02503	*rpsQ*	ribosomal protein S17	2.321 up	$2.48x10^{-15}$
SAOUHSC_02515		hypothetical protein	2.643 up	$2.51x10^{-15}$
SAOUHSC_02516		permease family protein	2.085 up	$3.86x10^{-17}$
SAOUHSC_02536	*moaA*	molybdenum cofactor biosynthesis protein A	2.061 up	$2.92x10^{-23}$
SAOUHSC_02538		molybdopterin converting factor, subunit 1	2.264 up	$1.09x10^{-26}$
SAOUHSC_02540		molybdopterin converting factor, subunit 2	2.320 up	$2.81x10^{-22}$
SAOUHSC_02558	*ureA*	urease, gamma subunit	2.285 up	$1.16x10^{-22}$
SAOUHSC_02561	*ureC*	urease, alpha subunit	2.631 up	$9.55x10^{-31}$
SAOUHSC_02562	*ureE*	urease accessory protein UreE	2.641 up	$4.99x10^{-30}$
SAOUHSC_02563	*ureF*	urease accessory protein UreF	3.365 up	$1.36x10^{-27}$
SAOUHSC_02564	*ureG*	urease accessory protein UreG	2.478 up	$1.36x10^{-28}$
SAOUHSC_02565	*ureD*	urease accessory protein UreD	4.712 up	$3.00x10^{-22}$
SAOUHSC_02566	*sarR*	accessory regulator	2.510 up	$3.01x10^{-9}$
SAOUHSC_02583		transcriptional regulator, putative	3.278 up	$2.36x10^{-22}$
SAOUHSC_02589		phosphosugar-binding transcriptional regulator, RpiR family	3.503 up	$4.95x10^{-28}$
SAOUHSC_02645		LytTr DNA-binding region	2.223 up	$6.55x10^{-27}$
SAOUHSC_02646		hypothetical protein	2.177 up	$6.07x10^{-24}$
SAOUHSC_02659		regulatory protein, TetR	4.229 up	$4.81x10^{-24}$
SAOUHSC_02660		cationic transporter	2.685 up	$4.97x10^{-22}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_02691		addiction module toxin, Txe/YoeB family	2.976 up	$1.80x10^{-23}$
SAOUHSC_02692		prevent-host-death protein	2.695 up	$5.30x10^{-29}$
SAOUHSC_02694		lipoprotein, putative	2.769 up	$7.87x10^{-12}$
SAOUHSC_02695		lipoprotein, putative	3.356 up	$3.14x10^{-12}$
SAOUHSC_02698		probable amino-acid ABC transporter permease protein YckJ	2.239 up	$2.45x10^{-15}$
SAOUHSC_02724		hypothetical protein	2.926 up	$6.85x10^{-23}$
SAOUHSC_02747		hypothetical protein	2.889 up	$1.04x10^{-25}$
SAOUHSC_02756		addiction module toxin, Txe/YoeB family	2.189 up	$2.62x10^{-9}$
SAOUHSC_02757		prevent-host-death protein	3.747 up	$5.68x10^{-12}$
SAOUHSC_02830		D-lactate dehydrogenase	2.123 up	$5.69x10^{-15}$
SAOUHSC_02839		L-serine dehydratase, iron-sulfur-dependent, alpha subunit	2.419 up	$4.26x10^{-23}$
SAOUHSC_02840		L-serine dehydratase, iron-sulfur-dependent, beta subunit	2.320 up	$2.05x10^{-18}$
SAOUHSC_02841		regulatory protein PfoR	3.152 up	$1.11x10^{-19}$
SAOUHSC_02845		thioredoxin	2.801 up	$4.32x10^{-33}$
SAOUHSC_02846		hypothetical protein	3.151 up	$1.97x10^{-26}$
SAOUHSC_02848	*glcB*	pts system, glucose-specific iibc component	2.343 up	$6.36x10^{-17}$
SAOUHSC_02872		hypothetical protein	3.604 up	$3.50x10^{-25}$
SAOUHSC_03001	*icaR*	biofilm operon icaabcd hth-type negative transcriptional regulator IcaR	5.525 up	$3.22x10^{-20}$
SAOUHSC_03055	*rpmH*	ribosomal protein L34	2.931 up	$9.22x10^{-17}$
SAOUHSC_A00332		hypothetical protein	2.604 up	$1.02x10^{-21}$
SAOUHSC_A01041		hypothetical protein	7.603 up	$1.38x10^{-31}$
SAOUHSC_A01081		hypothetical protein	2.138 up	$1.32x10^{-10}$
SAOUHSC_A01910		hypothetical protein	2.344 up	$7.87x10^{-16}$
SAOUHSC_A01912		hypothetical protein	4.034 up	$1.99x10^{-26}$
SAOUHSC_A02189		hypothetical protein	4.253 up	$2.42x10^{-16}$

Downregulated genes

SAOUHSC_00009	*serS*	seryl-tRNA synthetase	2.311 down	$3.97x10^{-12}$
SAOUHSC_00010		AzlC family protein	2.702 down	$3.22x10^{-10}$
SAOUHSC_00012		branched-chain amino acid transport protein (AzlD)	2.232 down	$8.12x10^{-9}$
SAOUHSC_00025		Ser/Thr protein phosphatase family protein	3.637 down	$9.80x10^{-10}$
SAOUHSC_00049		hypothetical protein	2.670 down	$9.11x10^{-12}$
SAOUHSC_00051		1-phosphatidylinositol phosphodiesterase precursor, putative	3.120 down	$4.77x10^{-9}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_00057		aminoacylase	2.054 down	0
SAOUHSC_00058		major facilitator superfamily MFS_1	2.730 down	$4.96x10^{-12}$
SAOUHSC_00060		Na/Pi cotransporter family protein	2.295 down	0
SAOUHSC_00062		integral membrane domain protein	2.285 down	$1.11x10^{-9}$
SAOUHSC_00064	*norG*	HTH-type transcriptional regulator NorG	2.318 down	$5.19x10^{-7}$
SAOUHSC_00071		iron compound ABC transporter, permease protein SirC	2.462 down	$1.75x10^{-10}$
SAOUHSC_00072		iron compound ABC transporter, permease protein SirB	2.745 down	$1.54x10^{-13}$
SAOUHSC_00074		lipoprotein	2.472 down	$2.90x10^{-11}$
SAOUHSC_00075	*sbnA*	Probable siderophore biosynthesis protein SbnA	2.324 down	$1.59x10^{-9}$
SAOUHSC_00076		ornithine cyclodeaminase, putative	3.087 down	$8.40x10^{-10}$
SAOUHSC_00077		lucA/lucC	3.113 down	$6.17x10^{-10}$
SAOUHSC_00078		general substrate transporter:Major facilitator superfamily MFS_1	3.066 down	$2.03x10^{-10}$
SAOUHSC_00079		lucA/lucC	2.466 down	$1.23x10^{-10}$
SAOUHSC_00080		lucA/lucC	2.118 down	$1.73x10^{-11}$
SAOUHSC_00081		hpch/hpai aldolase family protein	2.353 down	$2.48x10^{-12}$
SAOUHSC_00082		pyridoxal-dependent decarboxylase	2.634 down	$1.31x10^{-12}$
SAOUHSC_00083		hypothetical protein	2.540 down	$3.48x10^{-10}$
SAOUHSC_00084		hypothetical protein	2.278 down	$4.96x10^{-8}$
SAOUHSC_00087		hypothetical protein	2.034 down	$1.05x10^{-2}$
SAOUHSC_00088		NAD-dependent epimerase/dehydratase family protein	2.515 down	$5.45x10^{-9}$
SAOUHSC_00089		sugar transferase	3.632 down	$5.57x10^{-10}$
SAOUHSC_00090		glycosyl transferase, group 1 family protein	2.450 down	$5.81x10^{-10}$
SAOUHSC_00091		O-antigen polymerase	2.419 down	$9.42x10^{-8}$
SAOUHSC_00092		polysaccharide extrusion protein	2.267 down	$2.46x10^{-11}$
SAOUHSC_00103		phosphonate ABC transporter, permease protein	2.454 down	$2.67x10^{-12}$
SAOUHSC_00104	*phnC*	phosphonate ABC transporter, ATP-binding protein	2.488 down	$1.09x10^{-12}$
SAOUHSC_00106		: PPE-repeat proteins	3.139 down	$6.53x10^{-12}$
SAOUHSC_00108		hypothetical protein	2.269 down	$2.31x10^{-3}$
SAOUHSC_00113		alcohol dehydrogenase, iron-containing	2.098 down	$6.98x10^{-13}$
SAOUHSC_00114		capsular polysaccharide biosynthesis protein, putative	5.352 down	0
SAOUHSC_00115		capsular polysaccharide synthesis enzyme Cap5B	7.641 down	$1.33x10^{-15}$
SAOUHSC_00116		capsular polysaccharide synthesis enzyme Cap8C	5.626 down	0

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_00117		capsular polysaccharide biosynthesis protein Cap5D, putative	7.224 down	$2.51x10^{-15}$
SAOUHSC_00118		capsular polysaccharide biosynthesis protein Cap5E, putative	5.721 down	0
SAOUHSC_00119		capsular polysaccharide synthesis enzyme Cap8F	5.759 down	0
SAOUHSC_00120		UDP-N-acetylglucosamine 2-epimerase	4.880 down	0
SAOUHSC_00121		capsular polysaccharide synthesis enzyme O-acetyl transferase Cap5H, putative	3.955 down	$7.03x10^{-11}$
SAOUHSC_00122		capsular polysaccharide biosynthesis protein Cap5I, putative	3.538 down	$8.40x10^{-11}$
SAOUHSC_00123		capsular polysaccharide biosynthesis protein Cap5J, putative	2.448 down	$5.42x10^{-6}$
SAOUHSC_00124		capsular polysaccharide biosynthesis protein Cap5K, putative	2.398 down	$8.91x10^{-6}$
SAOUHSC_00125		Cap5L protein/glycosyltransferase, putative	4.082 down	$2.83x10^{-12}$
SAOUHSC_00126		capsular polysaccharide biosynthesis protein Cap8M	4.296 down	$3.52x10^{-15}$
SAOUHSC_00127		Cap5N protein/UDP-glucose 4-epimerase, putative	3.903 down	$2.54x10^{-15}$
SAOUHSC_00128		Cap5O protein/UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase	4.606 down	0
SAOUHSC_00129		UDP-N-acetylglucosamine 2-epimerase	2.152 down	0
SAOUHSC_00134		hypothetical protein	2.172 down	$4.59x10^{-15}$
SAOUHSC_00135		pANL51	2.576 down	0
SAOUHSC_00136		nitrate transport ATP-binding protein NrtD	3.274 down	$5.18x10^{-12}$
SAOUHSC_00137		lipoprotein, putative	2.611 down	$2.53x10^{-15}$
SAOUHSC_00138		ABC transporter, permease protein	3.849 down	$1.94x10^{-11}$
SAOUHSC_00139		hypothetical protein	2.771 down	$2.81x10^{-12}$
SAOUHSC_00144		surfactin synthetase subunit 2	2.404 down	$8.02x10^{-11}$
SAOUHSC_00147	*argB*	acetylglutamate kinase	2.148 down	$1.32x10^{-11}$
SAOUHSC_00148	*argJ*	arginine biosynthesis bifunctional protein ArgJ	2.484 down	$8.53x10^{-14}$
SAOUHSC_00150		ornithine--oxo-acid transaminase	2.471 down	$7.47x10^{-13}$
SAOUHSC_00154		hypothetical protein	2.439 down	$7.20x10^{-8}$
SAOUHSC_00162		type I restriction-modification system, R subunit	2.030 down	$2.76x10^{-11}$
SAOUHSC_00167		peptide ABC transporter, ATP-binding protein	2.280 down	$1.32x10^{-15}$
SAOUHSC_00169		peptide ABC transporter, permease protein	3.073 down	$1.47x10^{-9}$
SAOUHSC_00170		RGD-containing lipoprotein	2.750 down	$2.32x10^{-11}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_00175		multiple sugar-binding transport ATP-binding protein	2.855 down	$2.85x10^{-10}$
SAOUHSC_00176		extracellular solute-binding protein, family 1	2.524 down	$7.04x10^{-13}$
SAOUHSC_00177		binding-protein-dependent transport systems inner membrane component:TM2	2.347 down	$6.06x10^{-11}$
SAOUHSC_00179		oxidoreductase, Gfo/Idh/MocA family	2.631 down	$1.86x10^{-12}$
SAOUHSC_00180		oxidoreductase, N-terminal:Oxidoreductase, C-terminal	2.201 down	$1.35x10^{-11}$
SAOUHSC_00181		sugar phosphate isomerase/epimerase	2.129 down	$1.36x10^{-12}$
SAOUHSC_00182		membrane protein, putative	2.796 down	$2.99x10^{-7}$
SAOUHSC_00183	*uhpT*	major facilitator superfamily MFS_1	2.130 down	$7.80x10^{-11}$
SAOUHSC_00185		sensor histidine kinase family protein	2.689 down	$3.08x10^{-13}$
SAOUHSC_00186		periplasmic iron binding protein	2.315 down	$8.64x10^{-13}$
SAOUHSC_00188	*pflA*	pyruvate formate-lyase 1-activating enzyme	2.661 down	$2.77x10^{-13}$
SAOUHSC_00189		hypothetical protein	2.288 down	$7.86x10^{-9}$
SAOUHSC_00190		glycerophosphoryl diester phosphodiesterase	2.003 down	$1.46x10^{-10}$
SAOUHSC_00200		membrane protein, putative	2.922 down	$6.66x10^{-11}$
SAOUHSC_00205		hypothetical protein	2.245 down	$1.76x10^{-5}$
SAOUHSC_00208		hypothetical protein	2.036 down	$7.09x10^{-8}$
SAOUHSC_00209		PTS system, glucose-specific IIBC component, putative	3.873 down	$1.03x10^{-10}$
SAOUHSC_00211		pyrimidine-specific ribonucleoside hydrolase RihA	2.346 down	$4.67x10^{-13}$
SAOUHSC_00213		phosphoenolpyruvate-dependent sugar phosphotransferase system, eiia 2:prd	2.012 down	$6.20x10^{-11}$
SAOUHSC_00214		phosphoenolpyruvate-dependent sugar phosphotransferase system, eiia 2	2.253 down	$1.49x10^{-12}$
SAOUHSC_00216		PTS system, sorbitol-specific IIC component	3.021 down	$1.26x10^{-12}$
SAOUHSC_00217		arabitol-phosphate dehydrogenase	2.448 down	$7.86x10^{-12}$
SAOUHSC_00219		hexitol dehydrogenase	2.645 down	$1.77x10^{-13}$
SAOUHSC_00221		alcohol dehydrogenase, zinc-dependent	2.199 down	$1.99x10^{-11}$
SAOUHSC_00233	*lrgB*	Antiholin-like protein lrgB	2.692 down	$2.65x10^{-12}$
SAOUHSC_00242		regulatory protein, LacI:Periplasmic binding protein/LacI transcriptional regulator	2.470 down	$1.35x10^{-15}$
SAOUHSC_00251		membrane protein, putative	2.220 down	$5.23x10^{-10}$
SAOUHSC_00254		membrane protein, putative	3.522 down	$4.68x10^{-5}$
SAOUHSC_00256		chap	3.095 down	$6.25x10^{-11}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_00257		virulence factor EsxA	2.358 down	$1.48x10^{-14}$
SAOUHSC_00264		protein EsaC	2.607 down	$3.34x10^{-11}$
SAOUHSC_00265		virulence factor EsxB	2.604 down	$3.43x10^{-13}$
SAOUHSC_00267		hypothetical protein	3.086 down	$2.46x10^{-15}$
SAOUHSC_00284		5-nucleotidase, lipoprotein e(P4) family	2.633 down	$1.67x10^{-9}$
SAOUHSC_00285		ABC transporter, permease protein	2.885 down	0
SAOUHSC_00287		ABC transporter, ATP-binding protein, putative	2.410 down	$2.43x10^{-15}$
SAOUHSC_00289		hypothetical protein	3.586 down	$4.38x10^{-4}$
SAOUHSC_00290		regulatory protein PfoR	2.857 down	$1.78x10^{-12}$
SAOUHSC_00294		sodium:solute symporter family protein	2.578 down	$2.40x10^{-11}$
SAOUHSC_00295	*nanA*	sialic acid lyase	3.014 down	$9.82x10^{-15}$
SAOUHSC_00296		ROK family protein	2.871 down	$8.46x10^{-12}$
SAOUHSC_00298	*nanE*	N-acetylmannosamine-6-phosphate 2-epimerase	3.308 down	$8.81x10^{-12}$
SAOUHSC_00299		nucleoside recognition	3.107 down	$4.00x10^{-11}$
SAOUHSC_00300		lipase precursor	5.127 down	0
SAOUHSC_00302		NADH-dependent flavin oxidoreductase, oye family	2.743 down	0
SAOUHSC_00310	*ulaA*	ascorbate-specific PTS system enzyme IIC	2.630 down	$8.99x10^{-12}$
SAOUHSC_00311		phosphotransferase system, lactose/cellobiose-specific IIB subunit	2.638 down	$5.27x10^{-13}$
SAOUHSC_00312		phosphoenolpyruvate-dependent sugar phosphotransferase system, eiia 2	2.365 down	$3.04x10^{-13}$
SAOUHSC_00316		hypothetical protein	2.162 down	$7.80x10^{-10}$
SAOUHSC_00317		glycerol-3-phosphate transporter	2.424 down	$3.29x10^{-10}$
SAOUHSC_00324		YdaF	2.342 down	$1.60x10^{-11}$
SAOUHSC_00326		Tat-translocated enzyme	2.192 down	$5.35x10^{-11}$
SAOUHSC_00334		hypothetical protein	2.161 down	$6.00x10^{-12}$
SAOUHSC_00335		low temperature requirement protein LtrA	2.187 down	$1.27x10^{-10}$
SAOUHSC_00338	*metE*	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	2.008 down	$4.16x10^{-14}$
SAOUHSC_00339		methylenetetrahydrofolate reductase:Homocysteine S-methyltransferase	2.662 down	$8.54x10^{-14}$
SAOUHSC_00340		trans-sulfuration enzyme family protein	3.115 down	$2.16x10^{-12}$
SAOUHSC_00341		cystathionine gamma-synthase (CGS) (O-succinylhomoserine(thiol)-lyase)	3.340 down	$2.22x10^{-10}$
SAOUHSC_00356		propeptide, PepSY amd peptidase M4	2.604 down	0

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SAOUHSC_00358		transglycosylase-associated protein	3.376 down	0
SAOUHSC_00386		exotoxin	2.473 down	$4.33x10^{-12}$
SAOUHSC_00389		exotoxin	2.819 down	$1.04x10^{-10}$
SAOUHSC_00390		exotoxin 10	2.314 down	$2.19x10^{-12}$
SAOUHSC_00396		hypothetical protein	3.461 down	$1.74x10^{-10}$
SAOUHSC_00408		hypothetical protein	2.082 down	$4.33x10^{-13}$
SAOUHSC_00409		hypothetical protein	2.084 down	$9.80x10^{-9}$
SAOUHSC_00411		hypothetical protein	2.882 down	0
SAOUHSC_00418		hypothetical protein	2.912 down	$2.11x10^{-10}$
SAOUHSC_00420		sodium:neurotransmitter symporter	2.589 down	$1.28x10^{-9}$
SAOUHSC_00423	*metN1*	ABC transporter, ATP-binding protein	2.246 down	$1.17x10^{-14}$
SAOUHSC_00424		binding-protein-dependent transport systems inner membrane component	2.018 down	$2.49x10^{-13}$
SAOUHSC_00427	*sle1*	N-acetylmuramoyl-L-alanine amidase Sle1	2.635 down	0
SAOUHSC_00434		regulatory protein, LysR	2.575 down	$1.44x10^{-11}$
SAOUHSC_00437		pts system, trehalose-specific iibc component	2.018 down	$1.36x10^{-10}$
SAOUHSC_00450		Orn/Lys/Arg decarboxylase	2.241 down	$5.53x10^{-11}$
SAOUHSC_00468		endoribonuclease L-PSP, putative	2.905 down	0
SAOUHSC_00469	*spoVG*	SpoVG superfamily	2.426 down	$9.57x10^{-13}$
SAOUHSC_00541		cytidine/deoxycytidylate deaminase, zinc-binding region	2.010 down	0
SAOUHSC_00544	*sdrC*	serine-aspartate repeat-containing protein SdrC	2.322 down	$7.59x10^{-9}$
SAOUHSC_00583		hypothetical protein	2.291 down	$6.09x10^{-8}$
SAOUHSC_00596		staphylococcus aureus paralogous family	2.017 down	$9.59x10^{-7}$
SAOUHSC_00608	*adh*	alcohol dehydrogenase, propanol-preferring	2.577 down	$9.41x10^{-13}$
SAOUHSC_00622		hypothetical protein	2.059 down	$1.90x10^{-11}$
SAOUHSC_00625	*mnhA2*	putative antiporter subunit MnhA2	2.349 down	$6.77x10^{-14}$
SAOUHSC_00628	*mnhD2*	putative antiporter subunit MnhD2	2.356 down	$1.13x10^{-13}$
SAOUHSC_00630		hypothetical protein	2.041 down	$1.18x10^{-14}$
SAOUHSC_00632	*mnhG2*	putative antiporter subunit MnhG2	2.097 down	0
SAOUHSC_00687		acetyltransferase, gnat family	2.167 down	$3.73x10^{-10}$
SAOUHSC_00688		hypothetical protein	2.024 down	$1.56x10^{-13}$
SAOUHSC_00709		hypothetical protein	2.221 down	$1.57x10^{-3}$
SAOUHSC_00737		hypothetical protein	2.173 down	$8.05x10^{-5}$
SAOUHSC_00773		LysM domain protein	2.045 down	$4.32x10^{-11}$
SAOUHSC_00788		hypothetical protein	2.627 down	$2.63x10^{-14}$
SAOUHSC_00789	*whiA*	putative sporulation transcription regulator WhiA	2.337 down	$2.37x10^{-10}$
SAOUHSC_00811		acetyltransferase, gnat family	2.070 down	$2.49x10^{-15}$

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SAOUHSC_00814		truncated secreted von Willebrand factor-binding protein	2.279 down	$5.07x10^{-12}$
SAOUHSC_00818		thermonuclease (TNase) (Micrococcal nuclease)(Staphylococcal nuclease)	2.178 down	$1.21x10^{-8}$
SAOUHSC_00827		phosphoglycerate/bisphosphoglycerate mutase	2.109 down	$2.41x10^{-11}$
SAOUHSC_00831		general stress protein 170	2.578 down	0
SAOUHSC_00845		hypothetical protein	7.125 down	$1.37x10^{-15}$
SAOUHSC_00846		integral membrane protein	3.492 down	0
SAOUHSC_00855		2-nitropropane dioxygenase, NPD	2.357 down	$1.38x10^{-13}$
SAOUHSC_00857		hypothetical protein	2.157 down	$2.67x10^{-12}$
SAOUHSC_00917		hypothetical protein	2.082 down	$7.42x10^{-12}$
SAOUHSC_00918		map protein, programmed	2.274 down	0
SAOUHSC_00923		oligopeptide transport system permease protein OppB	2.603 down	$1.88x10^{-10}$
SAOUHSC_00924		dipeptide transport system permease protein DppC	2.120 down	$1.09x10^{-10}$
SAOUHSC_00926		oligopeptide transport ATP-binding protein OppF	2.130 down	$1.79x10^{-11}$
SAOUHSC_00928		oligopeptide ABC transporter, oligopeptide-binding protein	2.645 down	$4.36x10^{-11}$
SAOUHSC_00929		oligopeptide ABC transporter, ATP-binding protein	2.096 down	$2.25x10^{-14}$
SAOUHSC_00949		sodium:alanine symporter	2.395 down	$1.87x10^{-11}$
SAOUHSC_00957		integral membrane protein TerC	2.798 down	$7.74x10^{-13}$
SAOUHSC_00967		hypothetical protein	2.694 down	$1.26x10^{-3}$
SAOUHSC_00976		periplasmic binding protein	2.085 down	$2.16x10^{-11}$
SAOUHSC_00986	*sspC*	staphostatin B	2.622 down	$6.65x10^{-15}$
SAOUHSC_00987	*sspB*	staphopain B	2.682 down	$1.49x10^{-9}$
SAOUHSC_00988	*sspA*	glutamyl endopeptidase	4.128 down	$4.59x10^{-13}$
SAOUHSC_00994	*atl*	bifunctional autolysin	3.905 down	0
SAOUHSC_01081	*isdA*	iron-regulated surface determinant protein A	2.505 down	$6.02x10^{-11}$
SAOUHSC_01086	*isdF*	transport system permease protein	2.541 down	$1.11x10^{-12}$
SAOUHSC_01087		iron compound ABC transporter, permease protein	2.660 down	$4.58x10^{-12}$
SAOUHSC_01090		hypothetical protein	2.439 down	$1.24x10^{-6}$
SAOUHSC_01109		hypothetical protein	3.035 down	$3.01x10^{-4}$
SAOUHSC_01128		ornithine carbamoyltransferase	2.257 down	$1.66x10^{-10}$
SAOUHSC_01129	*arcC1*	carbamate kinase	2.320 down	$1.02x10^{-12}$
SAOUHSC_01130		C4-dicarboxylate anaerobic carrier	2.604 down	$1.23x10^{-13}$
SAOUHSC_01135		hypothetical protein	6.725 down	$1.33x10^{-15}$
SAOUHSC_01136		hypothetical protein	4.191 down	$1.24x10^{-9}$
SAOUHSC_01254		UbiC transcription regulator-associated	2.064 down	$6.74x10^{-13}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_01257		NAD-dependent epimerase/dehydratase:Short-chain dehydrogenase/reductase SDR:3-beta hydroxysteroid dehydrogenase/isomerase	2.093 down	$4.58x10^{-15}$
SAOUHSC_01297		hypothetical protein	2.215 down	$8.00x10^{-4}$
SAOUHSC_01299		hypothetical protein	3.203 down	$3.96x10^{-4}$
SAOUHSC_01300		hypothetical protein	2.638 down	$1.06x10^{-5}$
SAOUHSC_01302		: NAD+--asparagine ADP-ribosyltransferase	2.228 down	0
SAOUHSC_01339		hypothetical protein	2.485 down	$5.42x10^{-7}$
SAOUHSC_01366		anthranilate synthase component I	2.929 down	$9.20x10^{-9}$
SAOUHSC_01367		anthranilate synthase component II	3.503 down	$1.25x10^{-9}$
SAOUHSC_01369	*trpC*	indole-3-glycerol phosphate synthase	2.524 down	$4.91x10^{-8}$
SAOUHSC_01370	*trpF*	N-(5phosphoribosyl)anthranilate isomerase	2.112 down	$2.87x10^{-7}$
SAOUHSC_01371	*trpB*	tryptophan synthase, beta subunit	2.751 down	$2.98x10^{-10}$
SAOUHSC_01372	*trpA*	tryptophan synthase, alpha subunit	2.159 down	$3.66x10^{-13}$
SAOUHSC_01387		phosphate ABC transporter, permease protein PstC	2.783 down	$3.33x10^{-10}$
SAOUHSC_01389	*pstS*	phosphate-binding protein PstS	2.373 down	$9.50x10^{-13}$
SAOUHSC_01394		aspartokinase 2 (Aspartokinase II) (Aspartate kinase 2)	2.011 down	$9.92x10^{-12}$
SAOUHSC_01443		cell wall enzyme EbsB	2.086 down	$2.47x10^{-11}$
SAOUHSC_01450		amino acid permease-associated region	2.233 down	$5.29x10^{-8}$
SAOUHSC_01451	*tdcB*	threonine dehydratase	2.719 down	$4.99x10^{-8}$
SAOUHSC_01452	*ald1*	alanine dehydrogenase 1	3.023 down	$3.03x10^{-9}$
SAOUHSC_01458		hypothetical protein	2.557 down	$4.67x10^{-9}$
SAOUHSC_01494		hypothetical protein	2.076 down	$5.72x10^{-4}$
SAOUHSC_01643		type II secretion system protein E	2.355 down	$2.88x10^{-10}$
SAOUHSC_01794		glyceraldehyde-3-phosphate dehydrogenase 2	2.093 down	0
SAOUHSC_01804		transposase (IS4 family)	2.128 down	$1.52x10^{-7}$
SAOUHSC_01854		hypothetical protein	2.189 down	$2.48x10^{-15}$
SAOUHSC_01855		hypothetical protein	2.838 down	$2.58x10^{-12}$
SAOUHSC_01880		transposase	2.290 down	$1.13x10^{-13}$
SAOUHSC_01905		transposase	2.569 down	$8.55x10^{-11}$
SAOUHSC_01906		transposase (IS4 family)	2.027 down	$1.51x10^{-7}$
SAOUHSC_01912		hypothetical protein	2.715 down	$5.48x10^{-12}$
SAOUHSC_01913		nucleoside triphosphatase YtkD	2.223 down	$2.48x10^{-8}$
SAOUHSC_01914		hypothetical protein	2.517 down	0
SAOUHSC_01917		lipoprotein, putative	2.191 down	$3.73x10^{-9}$
SAOUHSC_01922		hypothetical protein	2.055 down	$1.35x10^{-15}$

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SAOUHSC_01933		type I restriction-modification system, M subunit	2.042 down	$3.24x10^{-7}$
SAOUHSC_01934		hypothetical protein	2.504 down	$1.14x10^{-4}$
SAOUHSC_01935	*splF*	serine protease SplF	2.866 down	$3.28x10^{-11}$
SAOUHSC_01936	*splE*	serine protease	2.637 down	$1.08x10^{-10}$
SAOUHSC_01937		hypothetical protein	2.020 down	$2.71x10^{-3}$
SAOUHSC_01939	*splC*	serine protease SplC	2.592 down	$5.73x10^{-12}$
SAOUHSC_01941	*splB*	serine protease SplB	3.700 down	$8.94x10^{-12}$
SAOUHSC_01942	*splA*	serine protease SplA	2.111 down	$2.67x10^{-12}$
SAOUHSC_01945		putative lantibiotic ABC transporter protein	4.058 down	0
SAOUHSC_01947		membrane protein, putative	5.114 down	0
SAOUHSC_01948		ABC transporter	5.500 down	$1.35x10^{-15}$
SAOUHSC_01949		epidermin leader peptide-processing serine protease EpiP	2.061 down	$5.50x10^{-13}$
SAOUHSC_01954		leukocidin F subunit	2.301 down	$1.52x10^{-11}$
SAOUHSC_01955		leukotoxin LukE	2.479 down	$1.66x10^{-9}$
SAOUHSC_02000	*hemL2*	glutamate-1-semialdehyde-2,1-aminomutase	2.008 down	$1.37x10^{-14}$
SAOUHSC_02105		hypothetical protein	2.539 down	$5.24x10^{-4}$
SAOUHSC_02138		parallel beta-helix repeat	2.675 down	0
SAOUHSC_02144		hypothetical protein	2.182 down	$2.41x10^{-15}$
SAOUHSC_02146		acyl-coenzyme A:6-aminopenicillanic acid acyl-transferase	2.017 down	$4.75x10^{-14}$
SAOUHSC_02244		succinyl-diaminopimelate desuccinylase	2.932 down	$4.33x10^{-14}$
SAOUHSC_02260	*hld*	delta-hemolysin	5.873 down	0
SAOUHSC_02270		ammonium transporter	2.156 down	$6.70x10^{-11}$
SAOUHSC_02271		hypothetical protein	2.804 down	$3.76x10^{-12}$
SAOUHSC_02272		inner membrane protein YeeE	2.405 down	$2.54x10^{-14}$
SAOUHSC_02281	*ilvD*	dihydroxy-acid dehydratase	3.967 down	$4.10x10^{-12}$
SAOUHSC_02282		acetolactate synthase, large subunit, biosynthetic type	3.102 down	$1.10x10^{-11}$
SAOUHSC_02283		amino acid-binding ACT	2.826 down	$2.38x10^{-9}$
SAOUHSC_02284	*ilvC*	ketol-acid reductoisomerase	2.597 down	$2.57x10^{-10}$
SAOUHSC_02285	*leuA*	2-isopropylmalate synthase	2.616 down	$2.40x10^{-10}$
SAOUHSC_02286	*leuB*	3-isopropylmalate dehydrogenase	2.512 down	$1.09x10^{-10}$
SAOUHSC_02287	*leuC*	3-isopropylmalate dehydratase, large subunit	2.699 down	$2.11x10^{-11}$
SAOUHSC_02288	*leuD*	3-isopropylmalate dehydratase, small subunit	2.763 down	$2.56x10^{-11}$
SAOUHSC_02290		hypothetical protein	2.244 down	$1.96x10^{-5}$
SAOUHSC_02310	*kdpC*	K+-transporting ATPase, C subunit	2.009 down	$2.44x10^{-15}$

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SAOUHSC_02315		response regulator receiver:Transcriptional regulatory protein, C-terminal	2.022 down	$1.96x10^{-14}$
SAOUHSC_02324		HD domain protein	2.187 down	$2.22x10^{-12}$
SAOUHSC_02325		hypothetical protein	3.178 down	$2.41x10^{-11}$
SAOUHSC_02326		hypothetical protein	3.059 down	$1.60x10^{-10}$
SAOUHSC_02329	*thiM*	hydroxyethylthiazole kinase	2.371 down	$3.54x10^{-15}$
SAOUHSC_02339		hypothetical protein	2.092 down	$9.42x10^{-4}$
SAOUHSC_02357		Sua5/YciO/YrdC/YwlC family protein	2.276 down	$1.32x10^{-12}$
SAOUHSC_02387		NAD-dependent epimerase/dehydratase	3.552 down	$4.57x10^{-15}$
SAOUHSC_02402		mannitol-specific phosphotransferase enzyme IIA component	2.792 down	$2.42x10^{-15}$
SAOUHSC_02403	*mtlD*	mannitol-1-phosphate 5-dehydrogenase	2.201 down	0
SAOUHSC_02404		hypothetical protein	2.095 down	$9.50x10^{-13}$
SAOUHSC_02438		transposase	2.373 down	$2.28x10^{-9}$
SAOUHSC_02440		transposase	2.018 down	$5.22x10^{-7}$
SAOUHSC_02441	*asp23*	alkaline shock protein 23	10.312 down	0
SAOUHSC_02442		hypothetical protein	8.834 down	$2.41x10^{-15}$
SAOUHSC_02443		hypothetical protein	13.744 down	0
SAOUHSC_02444		glycine betaine transporter OpuD	5.240 down	0
SAOUHSC_02450		PTS system, lactose-specific IIC component	2.736 down	0
SAOUHSC_02451		lactose-specific phosphotransferase enzyme IIA component	2.601 down	$9.26x10^{-14}$
SAOUHSC_02452	*lacD*	tagatose 1,6-diphosphate aldolase	2.991 down	$1.29x10^{-12}$
SAOUHSC_02453	*lacC*	tagatose-6-phosphate kinase	2.378 down	0
SAOUHSC_02454	*lacB*	galactose-6-phosphate isomerase, LacB subunit	2.010 down	$1.07x10^{-14}$
SAOUHSC_02466		truncated MHC class II analog protein	9.104 down	0
SAOUHSC_02551		acetyltransferase, gnat family	2.331 down	$7.34x10^{-13}$
SAOUHSC_02552		BioY family protein	2.525 down	0
SAOUHSC_02567		hypothetical protein	2.475 down	$4.98x10^{-7}$
SAOUHSC_02572		hypothetical protein	2.778 down	$2.59x10^{-5}$
SAOUHSC_02573		Na+/H+ antiporter NhaC	2.102 down	$8.05x10^{-14}$
SAOUHSC_02575		putative membrane protein	2.175 down	$3.91x10^{-14}$
SAOUHSC_02576		secretory antigen precursor SsaA, putative	3.668 down	$2.79x10^{-12}$
SAOUHSC_02581		hypothetical protein	2.643 down	0
SAOUHSC_02582		formate dehydrogenase, alpha subunit, putative	2.978 down	$2.03x10^{-13}$
SAOUHSC_02595		sodium/bile acid symporter family protein	2.790 down	$2.81x10^{-11}$
SAOUHSC_02596		conserved hypothetical protein	2.356 down	$4.64x10^{-8}$

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SAOUHSC_02597		pts system, alpha-glucoside-specific iibc component subfamily	2.021 down	$4.30x10^{-12}$
SAOUHSC_02599		phosphosugar-binding transcriptional regulator	2.251 down	$9.33x10^{-14}$
SAOUHSC_02604		oxidoreductase	3.587 down	$3.55x10^{-15}$
SAOUHSC_02605		amidohydrolase subfamily	2.738 down	$6.38x10^{-12}$
SAOUHSC_02607		urocanate hydratase	2.198 down	$7.02x10^{-14}$
SAOUHSC_02609	*fosB*	metallothiol transferase FosB	2.128 down	$1.70x10^{-8}$
SAOUHSC_02610	*hutG*	formimidoylglutamase	2.263 down	$9.04x10^{-14}$
SAOUHSC_02616		hypothetical protein	2.656 down	$3.29x10^{-4}$
SAOUHSC_02618		ABC-type Na+ efflux pump permease component	2.809 down	$6.32x10^{-12}$
SAOUHSC_02619		ABC transporter, ATP-binding protein	2.332 down	$2.81x10^{-14}$
SAOUHSC_02621		3-methyladenine DNA glycosylase	2.230 down	$2.91x10^{-14}$
SAOUHSC_02622		sodium/glutamate symporter	2.082 down	$1.12x10^{-12}$
SAOUHSC_02650		TpgX protein	2.006 down	$1.34x10^{-15}$
SAOUHSC_02657		hypothetical protein	2.245 down	$1.09x10^{-9}$
SAOUHSC_02661		pts system, sucrose-specific iibc component	2.103 down	$1.11x10^{-12}$
SAOUHSC_02662		PTS system sucrose-specific IIBC component	2.639 down	$1.27x10^{-12}$
SAOUHSC_02671	*narT*	nitrite extrusion protein	2.098 down	$1.20x10^{-11}$
SAOUHSC_02679		nitrate reductase molybdenum cofactor assembly chaperone	2.142 down	$5.22x10^{-14}$
SAOUHSC_02680		nitrate reductase, beta subunit	2.234 down	$9.44x10^{-13}$
SAOUHSC_02681		nitrate reductase, alpha subunit	2.670 down	$1.28x10^{-11}$
SAOUHSC_02682		uroporphyrin-III C-methyltransferase	2.014 down	$1.21x10^{-11}$
SAOUHSC_02684		nitrite reductase [NAD(P)H], large subunit	2.175 down	$6.73x10^{-13}$
SAOUHSC_02685		transcriptional regulator NirR	2.050 down	$1.18x10^{-14}$
SAOUHSC_02687		formate/nitrite transporter	2.250 down	$2.08x10^{-13}$
SAOUHSC_02688		hypothetical protein	3.339 down	$5.75x10^{-6}$
SAOUHSC_02696		aminoacyltransferase FemA	2.089 down	$1.17x10^{-13}$
SAOUHSC_02711		hypothetical protein	2.172 down	$7.59x10^{-11}$
SAOUHSC_02712	*bioW*	6-carboxyhexanoate--CoA ligase	2.317 down	$1.34x10^{-15}$
SAOUHSC_02715		adenosylmethionine-8-amino-7-oxononanoate aminotransferase	2.835 down	$2.43x10^{-11}$
SAOUHSC_02717		hypothetical protein	2.256 down	$3.35x10^{-7}$
SAOUHSC_02718		ABC transporter, ATP-binding protein	2.340 down	$3.51x10^{-15}$
SAOUHSC_02719		ABC transporter ATP-binding protein	2.636 down	$2.77x10^{-9}$
SAOUHSC_02720		hypothetical protein	3.665 down	$3.02x10^{-4}$
SAOUHSC_02726		transcription activator, effector binding	3.127 down	$1.74x10^{-9}$
SAOUHSC_02728		metallophosphoesterase	2.005 down	$4.58x10^{-13}$
SAOUHSC_02751		para-nitrobenzyl esterase	2.283 down	0

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_02752		general substrate transporter:Major facilitator superfamily MFS_1	2.057 down	0
SAOUHSC_02753		membrane protein, putative	2.668 down	$3.73x10^{-13}$
SAOUHSC_02754		ABC transporter, ATP-binding protein, putative	3.078 down	$2.13x10^{-11}$
SAOUHSC_02763		peptide ABC transporter, ATP-binding protein	2.042 down	$5.71x10^{-14}$
SAOUHSC_02764		peptide ABC transporter, ATP-binding protein	2.679 down	$5.77x10^{-12}$
SAOUHSC_02765		binding-protein-dependent transport systems inner membrane component	2.891 down	$3.75x10^{-11}$
SAOUHSC_02766		binding-protein-dependent transport systems inner membrane component	2.691 down	$1.06x10^{-9}$
SAOUHSC_02767		nickel ABC transporter, periplasmic nickel-binding protein	2.363 down	$5.18x10^{-13}$
SAOUHSC_02772		IMP dehydrogenase/GMP reductase:Short-chain dehydrogenase/reductase SDR	3.585 down	0
SAOUHSC_02773		aminobenzoyl-glutamate transport protein	2.226 down	0
SAOUHSC_02774		alkylhydroperoxidase AhpD core	2.379 down	$2.25x10^{-7}$
SAOUHSC_02787		hypothetical protein	2.786 down	$1.02x10^{-12}$
SAOUHSC_02796		hypothetical protein	2.903 down	$2.95x10^{-4}$
SAOUHSC_02797		major facilitator superfamily MFS_1	2.179 down	$3.10x10^{-10}$
SAOUHSC_02798	*sasG*	surface protein G	2.247 down	$1.45x10^{-11}$
SAOUHSC_02799	*sarT*	HTH-type transcriptional regulator SarT	2.571 down	$4.64x10^{-4}$
SAOUHSC_02800	*sarU*	HTH-type transcriptional regulator SarU	2.212 down	$1.02x10^{-5}$
SAOUHSC_02805		hypothetical protein	2.700 down	$1.43x10^{-6}$
SAOUHSC_02806		gluconate permease, putative	2.919 down	$5.38x10^{-13}$
SAOUHSC_02808		gluconate kinase	2.201 down	$7.48x10^{-13}$
SAOUHSC_02809		gluconate operon transcriptional repressor	2.303 down	$4.43x10^{-12}$
SAOUHSC_02817		hypothetical protein	2.222 down	$1.09x10^{-4}$
SAOUHSC_02824		phospholipase/carboxylesterase family protein	2.319 down	$1.01x10^{-10}$
SAOUHSC_02849		pyruvate oxidase	3.232 down	0
SAOUHSC_02850	*cidB*	holin-like protein CidB	2.977 down	$2.44x10^{-14}$
SAOUHSC_02851	*cidA*	holin-like protein CidA	2.002 down	$1.98x10^{-11}$
SAOUHSC_02852		regulatory protein, LysR:LysR, substrate-binding	2.398 down	$2.90x10^{-14}$
SAOUHSC_02855		chap	2.690 down	$2.46x10^{-13}$
SAOUHSC_02862	*clpL*	ATP-dependent Clp protease, ATP-binding subunit ClpL	6.058 down	0
SAOUHSC_02866		mmpl family	2.244 down	$2.81x10^{-12}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_02871		maltose O-acetyltransferase	2.270 down	0
SAOUHSC_02877	*crtN*	dehydrosqualene desaturase	3.989 down	$1.37x10^{-15}$
SAOUHSC_02879	*crtM*	dehydrosqualene synthase	3.233 down	$2.41x10^{-15}$
SAOUHSC_02880	*crtQ*	4,4'-diaponeurosporenoate glycosyltransferase	4.095 down	$2.50x10^{-13}$
SAOUHSC_02881	*crtP*	4,4'-diaponeurosporene oxidase	3.883 down	$6.76x10^{-12}$
SAOUHSC_02882	*crtO*	glycosyl-4,4'-diaponeurosporenoate acyltransferase	2.569 down	$3.95x10^{-11}$
SAOUHSC_02883	*ssaA*	staphylococcal secretory antigen SsaA	2.741 down	$2.74x10^{-12}$
SAOUHSC_02887	*isaA*	probable transglycosylase IsaA	2.183 down	$1.30x10^{-15}$
SAOUHSC_02888		membrane protein, putative	3.550 down	$4.06x10^{-12}$
SAOUHSC_02889		hypothetical protein	3.194 down	$1.38x10^{-3}$
SAOUHSC_02890		hypothetical protein	2.925 down	$4.28x10^{-5}$
SAOUHSC_02894		glyoxalase/bleomycin resistance protein/dioxygenase	2.623 down	$4.54x10^{-10}$
SAOUHSC_02895		hypothetical protein	2.107 down	$1.27x10^{-14}$
SAOUHSC_02896		hypothetical protein	2.496 down	$2.86x10^{-13}$
SAOUHSC_02900		hydrolase, alpha/beta hydrolase fold family	3.799 down	0
SAOUHSC_02901		cobalamin synthesis protein/P47K:Cobalamin synthesis CobW, C-terminal	2.392 down	$1.06x10^{-11}$
SAOUHSC_02902		nucleoside recognition	2.668 down	$1.13x10^{-10}$
SAOUHSC_02903		nucleoside recognition	2.260 down	$4.71x10^{-13}$
SAOUHSC_02904		flavin-containing monooxygenase FMO:FAD-dependent pyridine nucleotide-disulphide oxidoreductase	2.615 down	$7.45x10^{-14}$
SAOUHSC_02906		hypothetical protein	2.364 down	0
SAOUHSC_02908		aminoglycoside phosphotransferase:Fructosamine kinase	3.650 down	$1.56x10^{-12}$
SAOUHSC_02911		hypothetical protein	2.113 down	$1.87x10^{-11}$
SAOUHSC_02915		peptidase S15	2.099 down	$4.33x10^{-11}$
SAOUHSC_02921		alpha-acetolactate decarboxylase	2.397 down	$1.66x10^{-12}$
SAOUHSC_02923		amino acid permease-associated region	2.074 down	$4.06x10^{-12}$
SAOUHSC_02924		4-aminobutyrate aminotransferase	3.057 down	$4.75x10^{-10}$
SAOUHSC_02925		hypothetical protein	2.069 down	$1.73x10^{-12}$
SAOUHSC_02930		antibiotic biosynthesis monooxygenase	2.075 down	$1.37x10^{-15}$
SAOUHSC_02932	*betA*	choline dehydrogenase	3.335 down	$1.31x10^{-10}$
SAOUHSC_02933		betaine aldehyde dehydrogenase	2.301 down	$1.79x10^{-12}$
SAOUHSC_02934		hypothetical protein	3.192 down	$1.82x10^{-6}$
SAOUHSC_02936		hypothetical protein	2.571 down	$3.71x10^{-9}$
SAOUHSC_02937		bcct transporter	2.525 down	$8.34x10^{-12}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_02939		hypothetical protein	2.736 down	$4.39x10^{-4}$
SAOUHSC_02940		hypothetical protein	2.421 down	$2.52x10^{-4}$
SAOUHSC_02941		anaerobic ribonucleoside-triphosphate reductase activating protein	2.158 down	$2.07x10^{-11}$
SAOUHSC_02943		citrate transporter	2.047 down	$9.13x10^{-11}$
SAOUHSC_02948		hypothetical protein	2.424 down	$1.23x10^{-3}$
SAOUHSC_02950		hypothetical protein	2.943 down	$1.22x10^{-4}$
SAOUHSC_02958		alkaline phosphatase 3	2.326 down	$8.17x10^{-11}$
SAOUHSC_02971		zinc metalloproteinase aureolysin	3.052 down	$5.20x10^{-11}$
SAOUHSC_02973		hypothetical protein	3.008 down	$2.03x10^{-10}$
SAOUHSC_02983		hypothetical protein	2.519 down	$2.30x10^{-11}$
SAOUHSC_02984		hypothetical protein	3.000 down	$2.47x10^{-15}$
SAOUHSC_02985	*secA2*	protein translocase subunit SecA2	2.671 down	$3.17x10^{-14}$
SAOUHSC_02986		accessory secretory protein Asp3, putative	2.099 down	$1.30x10^{-12}$
SAOUHSC_02987		accessory secretory protein Asp2	2.414 down	$2.81x10^{-12}$
SAOUHSC_02988		hypothetical protein	2.670 down	$6.23x10^{-11}$
SAOUHSC_02994		hypothetical protein	2.300 down	0
SAOUHSC_02995		YwrF	2.466 down	$1.02x10^{-13}$
SAOUHSC_02996		peptide methionine sulfoxide reductase MsrA/msrB	2.703 down	$6.83x10^{-11}$
SAOUHSC_02997		acetyltransferase	2.522 down	$1.03x10^{-8}$
SAOUHSC_02998		putative tyrosine-protein phosphatase CapC	2.402 down	$4.89x10^{-9}$
SAOUHSC_02999		exopolysaccharide synthesis protein	2.595 down	$3.33x10^{-10}$
SAOUHSC_03002	*icaA*	poly-beta-1,6-N-acetyl-D-glucosamine synthase	2.775 down	$5.23x10^{-11}$
SAOUHSC_03003	*icaD*	poly-beta-1,6-N-acetyl-D-glucosamine synthesis protein IcaD	2.236 down	$4.80x10^{-8}$
SAOUHSC_03004	*icaB*	biofilm PIA synthesis deacetylase IcaB	2.019 down	$8.94x10^{-11}$
SAOUHSC_03008		imidazole glycerol phosphate synthase subunit HisF, putative	2.971 down	$1.17x10^{-11}$
SAOUHSC_03009	*hisA*	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	3.831 down	$3.39x10^{-10}$
SAOUHSC_03010	*hisH*	imidazole glycerol phosphate synthase, glutamine amidotransferase subunit	3.393 down	$9.20x10^{-10}$
SAOUHSC_03011	*hisB*	imidazoleglycerol-phosphate dehydratase	3.178 down	$2.19x10^{-10}$
SAOUHSC_03012		histidinol-phosphate aminotransferase, putative	2.914 down	$2.73x10^{-9}$
SAOUHSC_03013		histidinol dehydrogenase	3.271 down	$1.58x10^{-9}$
SAOUHSC_03014	*hisG*	ATP phosphoribosyltransferase catalytic subunit	3.043 down	$7.97x10^{-10}$

Locus tag	Gene name	Product name/Function	Fold change	P value
SAOUHSC_03015	*hisZ*	ATP phosphoribosyltransferase regulatory subunit	3.478 down	$7.04x10^{-9}$
SAOUHSC_03019		ATP-binding ABC transporter	2.604 down	$4.04x10^{-11}$
SAOUHSC_03020		integral membrane protein	2.511 down	$6.67x10^{-10}$
SAOUHSC_03021		hypothetical protein	2.519 down	$4.17x10^{-10}$
SAOUHSC_03025	*pcp*	pyrrolidone-carboxylate peptidase	2.211 down	$8.72x10^{-13}$
SAOUHSC_03030		anion transporter family protein	2.038 down	$5.62x10^{-15}$
SAOUHSC_03032		hypothetical protein	2.917 down	$4.04x10^{-10}$
SAOUHSC_03036		bacitracin export ATP-binding protein BceA	2.246 down	0
SAOUHSC_03047		hypothetical protein	3.248 down	$6.07x10^{-13}$
SAOUHSC_03048		hypothetical protein	2.380 down	$2.18x10^{-12}$
SAOUHSC_A00084		hypothetical protein	3.189 down	$1.50x10^{-9}$
SAOUHSC_A00283		hypothetical protein	2.035 down	$2.44x10^{-9}$
SAOUHSC_A00354		hypothetical protein	3.104 down	$1.49x10^{-5}$
SAOUHSC_A00379		hypothetical protein	3.288 down	$3.11x10^{-4}$
SAOUHSC_A00526		hypothetical protein	2.287 down	$2.49x10^{-5}$
SAOUHSC_A02169		hypothetical protein	2.586 down	$4.91x10^{-8}$
SAOUHSC_A02445		hypothetical protein	2.471 down	$1.14x10^{-9}$
SAOUHSC_A02577		hypothetical protein	2.769 down	$2.63x10^{-6}$
SAOUHSC_A02635		hypothetical protein	2.211 down	$1.30x10^{-10}$
SAOUHSC_A02795		hypothetical protein	2.830 down	$2.06x10^{-5}$
SAOUHSC_A02811		hypothetical protein	2.624 down	$2.28x10^{-12}$

Table A.2 Differentially expressed genes in 144 hr biofilm cultures versus planktonic cultures of *S. aureus* SH1000. Data are the average of three experimental replicates. P values were determined using a Student's t-test in ArrayStar™ (DNASTAR).

Locus tag	Gene name	Product name/function	Fold change	P value
Up-regulated genes				
SAOUHSC_00001	*dnaA*	chromosomal replication initiator protein DnaA	2.686 up	$6.24x10^{-22}$
SAOUHSC_00003		hypothetical protein	2.857 up	$7.31x10^{-24}$
SAOUHSC_00004	*recF*	DNA replication and repair protein RecF	2.233 up	$9.04x10^{-20}$
SAOUHSC_00032		hypothetical protein	2.605 up	$1.30x10^{-8}$
SAOUHSC_00035		pyridine nucleotide-disulphide oxidoreductase family protein	2.210 up	$5.06x10^{-12}$
SAOUHSC_00036		metallo-beta-lactamase family protein	2.290 up	$1.99x10^{-15}$
SAOUHSC_00037		sulfide:quinone oxidoreductase	2.221 up	$2.35x10^{-10}$
SAOUHSC_00094		hypothetical protein	2.154 up	$3.52x10^{-10}$
SAOUHSC_00141		hypothetical protein	2.612 up	$6.88x10^{-21}$
SAOUHSC_00173	*azoR*	azoreductase	2.378 up	$1.58x10^{-24}$
SAOUHSC_00345		hypothetical protein	2.216 up	$7.72x10^{-19}$
SAOUHSC_00348	*rpsF*	ribosomal protein S6	4.904 up	$4.11x10^{-21}$
SAOUHSC_00349		single-stranded DNA-binding protein	3.045 up	$3.85x10^{-19}$
SAOUHSC_00472	*prs*	ribose-phosphate pyrophosphokinase	2.271 up	$7.33x10^{-21}$
SAOUHSC_00473		hypothetical protein	2.453 up	$4.46x10^{-25}$
SAOUHSC_00491		2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	2.044 up	$2.97x10^{-10}$
SAOUHSC_00492		hypothetical protein	2.678 up	$1.80x10^{-12}$
SAOUHSC_00502	*ctsR*	transcriptional regulator CtsR	10.829 up	$5.00x10^{-32}$
SAOUHSC_00503		UvrB/UvrC motif-containing protein	2.982 up	$1.32x10^{-22}$
SAOUHSC_00504		ATP:guanido phosphotransferase	4.651 up	$2.05x10^{-23}$
SAOUHSC_00505	*clpC*	ATP-dependent Clp protease ATP-binding subunit ClpC	5.157 up	$6.20x10^{-28}$
SAOUHSC_00535		hypothetical protein	2.290 up	$3.57x10^{-17}$
SAOUHSC_00550		GlcNAc-PI de-N-acetylase family protein	2.048 up	$5.84x10^{-12}$
SAOUHSC_00555		HAD-superfamily hydrolase, subfamily IA, variant 1	2.234 up	$3.96x10^{-12}$
SAOUHSC_00556		proline/betaine transporter, putative	3.393 up	$8.60x10^{-23}$
SAOUHSC_00559		hypothetical protein	2.436 up	$2.15x10^{-17}$
SAOUHSC_00560		hypothetical protein	3.455 up	$4.38x10^{-19}$
SAOUHSC_00561		hypothetical protein	4.542 up	$1.42x10^{-14}$
SAOUHSC_00574	*eutD*	phosphotransacetylase	2.834 up	$1.73x10^{-24}$
SAOUHSC_00575		lipoate-protein ligase A family protein	2.960 up	$2.47x10^{-25}$

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_00604		hypothetical protein	2.088 up	$2.62x10^{-20}$
SAOUHSC_00641	*tagH*	teichoic acids export protein ATP-binding subunit	2.145 up	$1.46x10^{-23}$
SAOUHSC_00694	*mgrA*	HTH-type transcriptional regulator MgrA	2.189 up	$1.47x10^{-22}$
SAOUHSC_00714	*saeS*	sensor histidine kinase SaeS	2.750 up	$1.14x10^{-14}$
SAOUHSC_00715	*saeR*	response regulator SaeR	2.361 up	$2.06x10^{-8}$
SAOUHSC_00717		hypothetical protein	2.836 up	$1.34x10^{-8}$
SAOUHSC_00728	*ltaS*	glycerol phosphate lipoteichoic acid synthase	2.989 up	$2.62x10^{-21}$
SAOUHSC_00741	*nrdI*	ribonucleotide reductase stimulatory protein	5.433 up	$1.12x10^{-19}$
SAOUHSC_00742		ribonucleotide-diphosphate reductase subunit alpha	3.308 up	$4.62x10^{-21}$
SAOUHSC_00790	*clpP*	ATP-dependent Clp protease, proteolytic subunit ClpP	2.369 up	$6.66x10^{-28}$
SAOUHSC_00819		hypothetical protein	2.043 up	$8.18x10^{-12}$
SAOUHSC_00833		nitroreductase	2.710 up	$2.67x10^{-22}$
SAOUHSC_00867		hypothetical protein	2.002 up	$8.81x10^{-13}$
SAOUHSC_00868		hypothetical protein	2.765 up	$1.96x10^{-17}$
SAOUHSC_00869	*dltA*	D-alanine activating enzyme	2.032 up	$8.13x10^{-15}$
SAOUHSC_00870		DltB protein, putative	2.720 up	$1.23x10^{-16}$
SAOUHSC_00871	*dltC*	D-alanyl carrier protein	2.401 up	$2.38x10^{-15}$
SAOUHSC_00900	*pgi*	glucose-6-phosphate isomerase	2.055 up	$1.63x10^{-27}$
SAOUHSC_00912		ATP-dependent Clp protease, ATP-binding subunit ClpB	4.351 up	$7.00x10^{-32}$
SAOUHSC_00934	*spxA*	transcriptional regulator Spx	2.226 up	$3.53x10^{-26}$
SAOUHSC_00935	*mecA*	adaptor protein	3.007 up	$9.52x10^{-24}$
SAOUHSC_00942		GTP pyrophosphokinase	2.099 up	$6.29x10^{-23}$
SAOUHSC_00997		transcriptional regulator, putative	2.362 up	$3.26x10^{-18}$
SAOUHSC_00998	*fmtA*	Protein FmtA	2.005 up	$6.84x10^{-13}$
SAOUHSC_01005		hypothetical protein	6.356 up	$9.14x10^{-36}$
SAOUHSC_01007	*folD*	FolD bifunctional protein	2.025 up	$1.43x10^{-11}$
SAOUHSC_01008		phosphoribosylaminoimidazole carboxylase, catalytic subunit	3.228 up	$1.20x10^{-27}$
SAOUHSC_01009		phosphoribosylaminoimidazole carboxylase ATPase subunit	2.639 up	$8.43x10^{-24}$
SAOUHSC_01010	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase	2.389 up	0
SAOUHSC_01011		phosphoribosylformylglycinamidine synthase, PurS protein	2.075 up	$2.95x10^{-26}$
SAOUHSC_01025		membrane protein, putative	3.621 up	$7.19x10^{-22}$
SAOUHSC_01055		inositol monophosphatase family protein, putative	2.168 up	$1.24x10^{-27}$
SAOUHSC_01077		hypothetical protein	5.286 up	$2.17x10^{-27}$
SAOUHSC_01078	*rpmF*	ribosomal protein L32	2.519 up	$6.68x10^{-27}$

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_01098		DNA-dependent DNA polymerase beta chain	4.058 up	$6.18x10^{-24}$
SAOUHSC_01099	*mutS2*	MutS2 protein	2.440 up	$4.46x10^{-21}$
SAOUHSC_01100	*trxA*	thioredoxin	2.124 up	$8.65x10^{-16}$
SAOUHSC_01139		hypothetical protein	2.583 up	$6.33x10^{-16}$
SAOUHSC_01140		hypothetical protein	3.042 up	$3.17x10^{-16}$
SAOUHSC_01141		hypothetical protein	2.930 up	$9.59x10^{-21}$
SAOUHSC_01142	*mraZ*	cell division protein MraZ	2.206 up	$3.34x10^{-16}$
SAOUHSC_01158		hypothetical protein	3.655 up	$1.66x10^{-26}$
SAOUHSC_01164	*pyrR*	PyrR bifunctional protein	2.608 up	$9.63x10^{-19}$
SAOUHSC_01165		uracil permease	2.252 up	$5.25x10^{-16}$
SAOUHSC_01166	*pyrB*	aspartate carbamoyltransferase catalytic subunit	4.406 up	$7.24x10^{-28}$
SAOUHSC_01168	*pyrC*	dihydroorotase	2.511 up	$3.08x10^{-19}$
SAOUHSC_01169		carbamoyl phosphate synthase small subunit	3.188 up	$5.18x10^{-25}$
SAOUHSC_01176	*gmk*	guanylate kinase	2.434 up	$6.28x10^{-23}$
SAOUHSC_01192		hypothetical protein	2.515 up	$2.02x10^{-20}$
SAOUHSC_01216	*sucC*	succinyl-CoA synthetase subunit beta	2.112 up	$7.00x10^{-9}$
SAOUHSC_01232	*rpsB*	ribosomal protein S2	2.571 up	$2.78x10^{-19}$
SAOUHSC_01233		hypothetical protein	2.348 up	$2.29x10^{-17}$
SAOUHSC_01242	*rimP*	ribosome maturation factor RimP	2.760 up	$9.59x10^{-24}$
SAOUHSC_01330	*guaC*	guanosine 5'-monophosphate oxidoreductase	2.412 up	$6.52x10^{-19}$
SAOUHSC_01331		hypothetical protein	3.568 up	$4.66x10^{-19}$
SAOUHSC_01332		hypothetical protein	2.132 up	$2.90x10^{-14}$
SAOUHSC_01337		transketolase	2.260 up	$2.66x10^{-16}$
SAOUHSC_01392		ABC transporter, ATP-binding protein, putative	2.238 up	$2.29x10^{-16}$
SAOUHSC_01403	*cspA*	cold shock protein CspA	3.003 up	$1.04x10^{-31}$
SAOUHSC_01404		hypothetical protein	2.519 up	$9.24x10^{-28}$
SAOUHSC_01405		hypothetical protein	2.343 up	$1.43x10^{-24}$
SAOUHSC_01406	*acyP*	acylphosphatase	2.283 up	$3.49x10^{-8}$
SAOUHSC_01407		5-bromo-4-chloroindolyl phosphate hydrolysis protein XpaC	2.586 up	$2.12x10^{-8}$
SAOUHSC_01408		hypothetical protein	2.392 up	$2.60x10^{-7}$
SAOUHSC_01462	*gpsB*	cell cycle protein GpsB	2.126 up	$8.34x10^{-7}$
SAOUHSC_01463		hypothetical protein	2.249 up	$4.06x10^{-9}$
SAOUHSC_01464		hypothetical protein	2.702 up	$3.69x10^{-15}$
SAOUHSC_01466	*recU*	recombination protein U	4.832 up	$1.62x10^{-18}$
SAOUHSC_01467		penicillin-binding protein 2	3.423 up	$5.72x10^{-22}$
SAOUHSC_01469		endonuclease III, putative	2.235 up	$7.15x10^{-22}$
SAOUHSC_01477		Zn-dependent protease	3.227 up	$1.29x10^{-9}$
SAOUHSC_01504		ferredoxin	4.754 up	$2.64x10^{-18}$

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_01505		riboflavin transporter	2.272 up	$3.18x10^{-18}$
SAOUHSC_01599		glucose-6-phosphate 1-dehydrogenase	2.609 up	$2.98x10^{-26}$
SAOUHSC_01656		hydrophobic membrane protein ZurM	2.502 up	$5.60x10^{-18}$
SAOUHSC_01657		ABC transporter, putative	2.387 up	$5.81x10^{-16}$
SAOUHSC_01663		DNA primase	2.245 up	$4.31x10^{-14}$
SAOUHSC_01681	*prmA*	ribosomal protein L11 methyltransferase	3.734 up	$6.76x10^{-32}$
SAOUHSC_01682	*dnaJ*	DnaJ protein	2.760 up	$2.75x10^{-28}$
SAOUHSC_01683	*dnaK*	molecular chaperone DnaK	3.320 up	$2.59x10^{-24}$
SAOUHSC_01684	*grpE*	co-chaperone GrpE	13.687 up	$3.09x10^{-27}$
SAOUHSC_01685	*hrcA*	heat-inducible transcription repressor HrcA	18.112 up	$5.24x10^{-31}$
SAOUHSC_01715	*udk*	uridine kinase	2.115 up	$7.01x10^{-20}$
SAOUHSC_01716		peptidase U32	2.003 up	$2.53x10^{-15}$
SAOUHSC_01717		peptidase U32	2.189 up	$6.43x10^{-17}$
SAOUHSC_01725	*mnmA*	tRNA-specific 2-thiouridylase MnmA	2.022 up	$1.05x10^{-17}$
SAOUHSC_01726	*mnmA*	tRNA-specific 2-thiouridylase MnmA	2.143 up	$4.08x10^{-15}$
SAOUHSC_01727		aminotransferase, class V	2.176 up	$1.36x10^{-19}$
SAOUHSC_01751	*ruvA*	Holliday junction DNA helicase RuvA	2.016 up	$4.87x10^{-10}$
SAOUHSC_01752		ACT domain protein PheB	2.523 up	$2.79x10^{-17}$
SAOUHSC_01755	*rpmA*	ribosomal protein L27	2.228 up	$7.47x10^{-19}$
SAOUHSC_01756		hypothetical protein	9.109 up	$7.02x10^{-29}$
SAOUHSC_01757	*rplU*	ribosomal protein L21	8.042 up	$9.86x10^{-34}$
SAOUHSC_01758		rod shape-determining protein MreD	3.195 up	$5.78x10^{-20}$
SAOUHSC_01759		rod shape-determining protein MreC	3.171 up	$1.11x10^{-15}$
SAOUHSC_01760		hypothetical protein	4.878 up	$1.06x10^{-28}$
SAOUHSC_01761		hypothetical protein	5.525 up	0
SAOUHSC_01762		hypothetical protein	2.610 up	$5.94x10^{-13}$
SAOUHSC_01778	*clpX*	ATP-dependent protease ATP-binding subunit ClpX	2.650 up	$4.48x10^{-17}$
SAOUHSC_01779	*tig*	trigger factor	3.431 up	$2.93x10^{-27}$
SAOUHSC_01785	*rpmI*	ribosomal protein L35	2.933 up	$2.58x10^{-25}$
SAOUHSC_01786	*infC*	translation initiation factor IF-3	3.398 up	$1.97x10^{-22}$
SAOUHSC_01792		replication initiation and membrane attachment protein	2.369 up	$3.61x10^{-17}$
SAOUHSC_01793	*nrdR*	transcriptional regulator NrdR	2.429 up	$2.88x10^{-19}$
SAOUHSC_01801		isocitrate dehydrogenase	2.316 up	$3.83x10^{-11}$
SAOUHSC_01850		catabolite control protein A	2.703 up	$7.72x10^{-15}$
SAOUHSC_01865	*trmB*	tRNA (guanine-N(7)-)-methyltransferase	2.491 up	$2.61x10^{-15}$
SAOUHSC_01957		hypothetical protein	2.937 up	$1.50x10^{-22}$
SAOUHSC_01972	*prsA*	foldase protein PrsA	2.782 up	$2.22x10^{-24}$
SAOUHSC_01997	*perR*	peroxide-responsive repressor PerR	3.763 up	$2.70x10^{-28}$
SAOUHSC_02001		hypothetical protein	2.631 up	$1.07x10^{-24}$
SAOUHSC_02012	*mgt*	monofunctional glycosyltransferase	3.802 up	$1.82x10^{-32}$

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_02098		DNA-binding response regulator VraR, putative	3.578 up	$1.67x10^{-25}$
SAOUHSC_02099		sensor protein VraS	5.797 up	$6.66x10^{-26}$
SAOUHSC_02100		hypothetical protein	8.010 up	$1.16x10^{-25}$
SAOUHSC_02101		hypothetical protein	6.499 up	$1.48x10^{-27}$
SAOUHSC_02108	*ftnA*	ferritin	4.142 up	$7.52x10^{-25}$
SAOUHSC_02109		hypothetical protein	2.911 up	$2.53x10^{-14}$
SAOUHSC_02110		exonuclease	2.368 up	$1.12x10^{-11}$
SAOUHSC_02112		hypothetical protein	3.815 up	$6.80x10^{-26}$
SAOUHSC_02152		ABC transporter, ATP-binding protein, putative	2.107 up	$1.66x10^{-8}$
SAOUHSC_02153		membrane protein, putative	2.678 up	$2.04x10^{-7}$
SAOUHSC_02154		ABC transporter, ATP-binding protein, putative	2.644 up	$1.25x10^{-7}$
SAOUHSC_02254	*groEL*	chaperonin GroEL	3.223 up	$1.93x10^{-23}$
SAOUHSC_02255	*groES*	co-chaperonin GroES	9.288 up	$6.68x10^{-31}$
SAOUHSC_02258		nitroreductase	3.350 up	$7.59x10^{-12}$
SAOUHSC_02294		hypothetical protein	3.556 up	$4.92x10^{-18}$
SAOUHSC_02302		sigmaB regulation protein RsbU, putative	2.232 up	$2.28x10^{-15}$
SAOUHSC_02316		DEAD-box ATP dependent DNA helicase	2.904 up	$1.44x10^{-25}$
SAOUHSC_02346	*atpH*	ATP synthase F1, delta subunit	2.345 up	$7.95x10^{-8}$
SAOUHSC_02350	*atpB*	ATP synthase F0, A subunit	2.779 up	$4.75x10^{-9}$
SAOUHSC_02351		hypothetical protein	2.596 up	$2.59x10^{-9}$
SAOUHSC_02364		hypothetical protein	3.772 up	$8.13x10^{-26}$
SAOUHSC_02365		UDP-N-acetylglucosamine 1-carboxyvinyltransferase	4.018 up	$3.24x10^{-25}$
SAOUHSC_02368	*pyrG*	CTP synthetase	2.014 up	$9.37x10^{-9}$
SAOUHSC_02381		general stress protein 20U	3.464 up	$6.01x10^{-11}$
SAOUHSC_02430		ABC transporter periplasmic binding protein, putative	2.224 up	$1.49x10^{-23}$
SAOUHSC_02478	*rplM*	ribosomal protein L13	2.690 up	$9.92x10^{-26}$
SAOUHSC_02563	*ureF*	urease accessory protein UreF	2.008 up	$1.51x10^{-14}$
SAOUHSC_02583		transcriptional regulator, putative	3.135 up	$2.77x10^{-23}$
SAOUHSC_02589		phosphosugar-binding transcriptional regulator, RpiR family	2.830 up	$9.23x10^{-31}$
SAOUHSC_02629		EmrB/QacA family drug resistance transporter	2.651 up	$7.35x10^{-11}$
SAOUHSC_02630		hypothetical protein	2.303 up	$2.10x10^{-10}$
SAOUHSC_02645		LytTr DNA-binding region	4.910 up	$4.01x10^{-31}$
SAOUHSC_02646		hypothetical protein	4.464 up	$6.40x10^{-28}$
SAOUHSC_02659		regulatory protein, TetR	4.433 up	$2.75x10^{-18}$
SAOUHSC_02660		cationic transporter	2.902 up	$3.53x10^{-22}$
SAOUHSC_02691		addiction module toxin, Txe/YoeB family	2.972 up	$1.70x10^{-30}$
SAOUHSC_02692		prevent-host-death protein	3.058 up	$1.99x10^{-27}$

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_02708		gamma-hemolysin h-gamma-II subunit, putative	2.210 up	$1.07x10^{-14}$
SAOUHSC_02724		hypothetical protein	3.053 up	$2.78x10^{-23}$
SAOUHSC_02747		hypothetical protein	2.125 up	$2.89x10^{-20}$
SAOUHSC_02756		addiction module toxin, Txe/YoeB family	2.019 up	$9.01x10^{-15}$
SAOUHSC_02757		prevent-host-death protein	2.737 up	$6.89x10^{-17}$
SAOUHSC_02841		regulatory protein PfoR	2.338 up	$1.34x10^{-13}$
SAOUHSC_02845		thioredoxin	2.134 up	$1.16x10^{-29}$
SAOUHSC_02846		hypothetical protein	2.252 up	$9.63x10^{-23}$
SAOUHSC_02848	*glcB*	pts system, glucose-specific iibc component	2.760 up	$3.67x10^{-22}$
SAOUHSC_02872		hypothetical protein	3.406 up	$5.21x10^{-21}$
SAOUHSC_02873	*copA*	cation transporter E1-E2 family ATPase	2.278 up	$1.61x10^{-12}$
SAOUHSC_03001	*icaR*	biofilm operon icaabcd hth-type negative transcriptional regulator IcaR	2.089 up	$1.41x10^{-23}$
SAOUHSC_A01041		hypothetical protein	4.826 up	$8.05x10^{-24}$
SAOUHSC_A01912		hypothetical protein	3.558 up	$6.65x10^{-26}$
SAOUHSC_A02189		hypothetical protein	2.089 up	$6.97x10^{-12}$

Down-regulated genes

SAOUHSC_00114		capsular polysaccharide biosynthesis protein, putative	5.249 down	$4.43x10^{-15}$
SAOUHSC_00115		capsular polysaccharide synthesis enzyme Cap5B	5.894 down	0
SAOUHSC_00116		capsular polysaccharide synthesis enzyme Cap8C	4.645 down	0
SAOUHSC_00117		capsular polysaccharide biosynthesis protein Cap5D, putative	4.641 down	0
SAOUHSC_00118		capsular polysaccharide biosynthesis protein Cap5E, putative	4.190 down	0
SAOUHSC_00119		capsular polysaccharide synthesis enzyme Cap8F	4.032 down	0
SAOUHSC_00120		UDP-N-acetylglucosamine 2-epimerase	3.680 down	$4.35x10^{-15}$
SAOUHSC_00121		capsular polysaccharide synthesis enzyme O-acetyl transferase Cap5H, putative	4.126 down	$5.28x10^{-11}$
SAOUHSC_00122		capsular polysaccharide biosynthesis protein Cap5I, putative	3.323 down	$4.43x10^{-10}$
SAOUHSC_00123		capsular polysaccharide biosynthesis protein Cap5J, putative	2.648 down	$1.60x10^{-6}$
SAOUHSC_00124		capsular polysaccharide biosynthesis protein Cap5K, putative	2.788 down	$4.88x10^{-7}$
SAOUHSC_00125		Cap5L protein/glycosyltransferase, putative	3.508 down	$7.01x10^{-11}$

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_00126		capsular polysaccharide biosynthesis protein Cap8M	3.235 down	$2.38x10^{-13}$
SAOUHSC_00127		Cap5N protein/UDP-glucose 4-epimerase, putative	3.349 down	$3.30x10^{-13}$
SAOUHSC_00128		Cap5O protein/UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase	3.277 down	0
SAOUHSC_00144		surfactin synthetase subunit 2	2.008 down	$1.41x10^{-8}$
SAOUHSC_00285		ABC transporter, permease protein	2.734 down	0
SAOUHSC_00287		ABC transporter, ATP-binding protein, putative	2.314 down	$2.28x10^{-15}$
SAOUHSC_00300		lipase precursor	3.584 down	$2.34x10^{-13}$
SAOUHSC_00358		transglycosylase-associated protein	2.007 down	0
SAOUHSC_00411		hypothetical protein	2.015 down	$8.52x10^{-14}$
SAOUHSC_00468		endoribonuclease L-PSP, putative	3.313 down	0
SAOUHSC_00469	*spoVG*	SpoVG superfamily	2.677 down	$1.07x10^{-13}$
SAOUHSC_00788		hypothetical protein	2.574 down	$2.39x10^{-13}$
SAOUHSC_00789	*whiA*	putative sporulation transcription regulator WhiA	2.314 down	$7.70x10^{-10}$
SAOUHSC_00812	*clfA*	clumping factor A	2.391 down	0
SAOUHSC_00825		hypothetical protein	2.421 down	$2.62x10^{-10}$
SAOUHSC_00845		hypothetical protein	10.601 down	$2.34x10^{-15}$
SAOUHSC_00846		integral membrane protein	2.003 down	$4.21x10^{-15}$
SAOUHSC_00987	*sspB*	staphopain B	2.483 down	$1.28x10^{-8}$
SAOUHSC_00988	*sspA*	glutamyl endopeptidase	2.841 down	$1.89x10^{-10}$
SAOUHSC_00994	*atl*	bifunctional autolysin	5.048 down	0
SAOUHSC_01135		hypothetical protein	6.344 down	0
SAOUHSC_01136		hypothetical protein	3.965 down	$4.41x10^{-9}$
SAOUHSC_01794		glyceraldehyde 3-phosphate dehydrogenase 2	2.050 down	0
SAOUHSC_01854		hypothetical protein	2.170 down	$2.84x10^{-14}$
SAOUHSC_01855		hypothetical protein	4.553 down	0
SAOUHSC_01945		putative lantibiotic ABC transporter protein	4.396 down	0
SAOUHSC_01947		membrane protein, putative	5.253 down	0
SAOUHSC_01948		ABC transporter	5.350 down	0
SAOUHSC_02244		succinyl-diaminopimelate desuccinylase	2.047 down	$7.70x10^{-12}$
SAOUHSC_02260	*hld*	delta-hemolysin	10.936 down	0
SAOUHSC_02387		NAD-dependent epimerase/dehydratase	2.116 down	0
SAOUHSC_02402		mannitol-specific phosphotransferase enzyme IIA component	2.421 down	0
SAOUHSC_02403	*mtlD*	mannitol-1-phosphate 5-dehydrogenase	2.152 down	$2.32x10^{-15}$
SAOUHSC_02441	*asp23*	alkaline shock protein 23	7.979 down	$2.36x10^{-15}$
SAOUHSC_02442		hypothetical protein	11.232 down	0
SAOUHSC_02443		hypothetical protein	15.691 down	0
SAOUHSC_02444		glycine betaine transporter OpuD	3.402 down	0

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_02466		truncated MHC class II analog protein	10.464 down	0
SAOUHSC_02582		formate dehydrogenase, alpha subunit, putative	3.210 down	$4.73x10^{-14}$
SAOUHSC_02604		oxidoreductase	2.160 down	0
SAOUHSC_02650		TpgX protein	2.430 down	0
SAOUHSC_02751		para-nitrobenzyl esterase	2.051 down	$5.65x10^{-14}$
SAOUHSC_02772		IMP dehydrogenase/GMP reductase:Short-chain dehydrogenase/reductase SDR	2.207 down	0
SAOUHSC_02774		alkylhydroperoxidase AhpD core	2.488 down	$6.15x10^{-8}$
SAOUHSC_02849		pyruvate oxidase	3.272 down	$7.80x10^{-15}$
SAOUHSC_02850	*cidB*	holin-like protein CidB	2.813 down	$2.29x10^{-12}$
SAOUHSC_02862	*clpL*	ATP-dependent Clp protease, ATP-binding subunit ClpL	5.460 down	0
SAOUHSC_02877	*crtN*	dehydrosqualene desaturase	2.654 down	$2.01x10^{-13}$
SAOUHSC_02879	*crtM*	dehydrosqualene synthase	2.717 down	$6.28x10^{-13}$
SAOUHSC_02880	*crtQ*	4,4'-diaponeurosporenoate glycosyltransferase	2.409 down	$7.40x10^{-12}$
SAOUHSC_02881	*crtP*	4,4'-diaponeurosporene oxidase	2.022 down	0
SAOUHSC_02887	*isaA*	probable transglycosylase IsaA	2.275 down	0
SAOUHSC_02900		hydrolase, alpha/beta hydrolase fold family	2.933 down	0
SAOUHSC_02983		hypothetical protein	3.086 down	$4.87x10^{-14}$
SAOUHSC_02984		hypothetical protein	2.578 down	$2.34x10^{-15}$
SAOUHSC_02985	*secA2*	protein translocase subunit SecA2	2.352 down	$3.39x10^{-12}$

Table A.3 Differentially expressed genes in 144 hr versus 48 hr biofilm cultures of *S. aureus* SH1000. Data are the average of three experimental replicates. P values were determined using a Student's t-test in ArrayStar™ (DNASTAR).

Locus tag	Gene name	Product name/function	Fold change	P value
Up-regulated genes				
SAOUHSC_00025		Ser/Thr protein phosphatase family protein	2.298 up	$1.16x10^{-5}$
SAOUHSC_00051		1-phosphatidylinositol phosphodiesterase precursor, putative	2.106 up	$1.64x10^{-5}$
SAOUHSC_00076		ornithine cyclodeaminase, putative	2.081 up	$1.05x10^{-5}$
SAOUHSC_00078		general substrate transporter:Major facilitator superfamily MFS_1	2.003 up	$5.17x10^{-6}$
SAOUHSC_00089		sugar transferase	2.277 up	$7.46x10^{-6}$
SAOUHSC_00106		: PPE-repeat proteins	2.041 up	$8.71x10^{-7}$
SAOUHSC_00136		nitrate transport ATP-binding protein NrtD	2.103 up	$2.85x10^{-7}$
SAOUHSC_00138		ABC transporter, permease protein	2.288 up	$1.70x10^{-6}$
SAOUHSC_00154		hypothetical protein	2.086 up	$4.87x10^{-6}$
SAOUHSC_00182		membrane protein, putative	2.112 up	$6.67x10^{-5}$
SAOUHSC_00209		PTS system, glucose-specific IIBC component, putative	2.319 up	$3.25x10^{-6}$
SAOUHSC_00254		membrane protein, putative	2.677 up	$1.12x10^{-3}$
SAOUHSC_00289		hypothetical protein	2.875 up	$3.42x10^{-3}$
SAOUHSC_00296		ROK family protein	2.029 up	$1.26x10^{-7}$
SAOUHSC_00298	*nanE*	N-acetylmannosamine-6-phosphate 2-epimerase	2.136 up	$5.16x10^{-7}$
SAOUHSC_00341		cystathionine gamma-synthase (CGS) (O-succinylhomoserine(thiol)-lyase)	2.096 up	$6.14x10^{-6}$
SAOUHSC_00396		hypothetical protein	2.158 up	$6.71x10^{-6}$
SAOUHSC_00418		hypothetical protein	2.189 up	$5.84x10^{-7}$
SAOUHSC_00709		hypothetical protein	2.001 up	$6.15x10^{-3}$
SAOUHSC_00967		hypothetical protein	2.310 up	$6.46x10^{-3}$
SAOUHSC_01090		hypothetical protein	2.092 up	$4.01x10^{-5}$
SAOUHSC_01109		hypothetical protein	2.595 up	$1.81x10^{-3}$
SAOUHSC_01299		hypothetical protein	2.651 up	$2.84x10^{-3}$
SAOUHSC_01300		hypothetical protein	2.247 up	$1.84x10^{-4}$
SAOUHSC_01339		hypothetical protein	2.164 up	$1.74x10^{-5}$
SAOUHSC_01366		anthranilate synthase component I	2.062 up	$2.20x10^{-5}$
SAOUHSC_01367		anthranilate synthase component II	2.249 up	$1.02x10^{-5}$
SAOUHSC_01452	*ald1*	alanine dehydrogenase 1	2.093 up	$1.32x10^{-5}$
SAOUHSC_01458		hypothetical protein	2.005 up	$7.32x10^{-6}$

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_01934		hypothetical protein	2.325 up	$4.37x10^{-4}$
SAOUHSC_01941	*splB*	serine protease SplB	2.140 up	$2.72x10^{-6}$
SAOUHSC_02105		hypothetical protein	2.318 up	$1.86x10^{-3}$
SAOUHSC_02281	*ilvD*	dihydroxy-acid dehydratase	2.233 up	$9.57x10^{-7}$
SAOUHSC_02325		hypothetical protein	2.082 up	$3.05x10^{-7}$
SAOUHSC_02326		hypothetical protein	2.181 up	$6.03x10^{-7}$
SAOUHSC_02567		hypothetical protein	2.241 up	$8.57x10^{-6}$
SAOUHSC_02572		hypothetical protein	2.268 up	$5.70x10^{-4}$
SAOUHSC_02576		secretory antigen precursor SsaA, putative	2.076 up	$2.92x10^{-6}$
SAOUHSC_02616		hypothetical protein	2.355 up	$1.61x10^{-3}$
SAOUHSC_02645		LytTr DNA-binding region	2.208 up	$9.89x10^{-21}$
SAOUHSC_02646		hypothetical protein	2.050 up	$2.70x10^{-16}$
SAOUHSC_02662		PTS system sucrose-specific IIBC component	2.020 up	$1.25x10^{-8}$
SAOUHSC_02688		hypothetical protein	2.739 up	$1.02x10^{-4}$
SAOUHSC_02715		adenosylmethionine-8-amino-7-oxononanoate aminotransferase	2.024 up	$3.34x10^{-7}$
SAOUHSC_02720		hypothetical protein	3.085 up	$1.67x10^{-3}$
SAOUHSC_02726		transcription activator, effector binding	2.239 up	$4.12x10^{-6}$
SAOUHSC_02754		ABC transporter, ATP-binding protein, putative	2.045 up	$6.64x10^{-7}$
SAOUHSC_02796		hypothetical protein	2.327 up	$3.55x10^{-3}$
SAOUHSC_02799	*sarT*	HTH-type transcriptional regulator SarT	2.205 up	$3.22x10^{-3}$
SAOUHSC_02805		hypothetical protein	2.136 up	$1.18x10^{-4}$
SAOUHSC_02806		gluconate permease, putative	2.150 up	$2.82x10^{-9}$
SAOUHSC_02888		membrane protein, putative	2.268 up	$2.49x10^{-7}$
SAOUHSC_02889		hypothetical protein	2.784 up	$4.93x10^{-3}$
SAOUHSC_02890		hypothetical protein	2.264 up	$1.28x10^{-3}$
SAOUHSC_02908		aminoglycoside phosphotransferase:Fructosamine kinase	2.073 up	$2.03x10^{-6}$
SAOUHSC_02924		4-aminobutyrate aminotransferase	2.252 up	$7.82x10^{-7}$
SAOUHSC_02932	*betA*	choline dehydrogenase	2.084 up	$6.02x10^{-6}$
SAOUHSC_02934		hypothetical protein	2.168 up	$1.34x10^{-3}$
SAOUHSC_02939		hypothetical protein	2.118 up	$7.31x10^{-3}$
SAOUHSC_02940		hypothetical protein	2.161 up	$1.27x10^{-3}$
SAOUHSC_02950		hypothetical protein	2.321 up	$2.23x10^{-3}$
SAOUHSC_02973		hypothetical protein	2.075 up	$1.94x10^{-6}$
SAOUHSC_03009	*hisA*	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	2.468 up	$3.36x10^{-6}$
SAOUHSC_03010	*hisH*	imidazole glycerol phosphate synthase, glutamine amidotransferase subunit	2.304 up	$4.74x10^{-6}$

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_03012		histidinol-phosphate aminotransferase, putative	2.011 up	$9.14x10^{-6}$
SAOUHSC_03013		histidinol dehydrogenase	2.099 up	$2.03x10^{-5}$
SAOUHSC_03014	*hisG*	ATP phosphoribosyltransferase catalytic subunit	2.009 up	$1.33x10^{-5}$
SAOUHSC_03015	*hisZ*	ATP phosphoribosyltransferase regulatory subunit	2.328 up	$1.74x10^{-5}$
SAOUHSC_03047		hypothetical protein	2.055 up	$1.63x10^{-7}$
SAOUHSC_A00084		hypothetical protein	2.159 up	$8.17x10^{-6}$
SAOUHSC_A00354		hypothetical protein	2.608 up	$1.97x10^{-4}$
SAOUHSC_A00379		hypothetical protein	2.637 up	$3.00x10^{-3}$
SAOUHSC_A02169		hypothetical protein	2.070 up	$1.01x10^{-5}$
SAOUHSC_A02577		hypothetical protein	2.124 up	$2.74x10^{-4}$
SAOUHSC_A02795		hypothetical protein	2.308 up	$4.60x10^{-4}$
Downregulated genes				
SAOUHSC_00017	*rplI*	ribosomal protein L9	2.040 down	$1.17x10^{-12}$
SAOUHSC_00029		hypothetical protein	2.472 down	$1.04x10^{-6}$
SAOUHSC_00440		hypothetical protein	2.281 down	$1.16x10^{-13}$
SAOUHSC_00441		acetyltransferase family protein	2.008 down	$9.66x10^{-12}$
SAOUHSC_00663		acetyltransferase, gnat family	2.575 down	$6.28x10^{-9}$
SAOUHSC_00680		hypothetical protein	2.622 down	$2.48x10^{-8}$
SAOUHSC_00694	*mgrA*	HTH-type transcriptional regulator MgrA	2.391 down	$3.06x10^{-14}$
SAOUHSC_00718		membrane protein, putative	2.211 down	$4.46x10^{-9}$
SAOUHSC_00735		hypothetical protein	2.402 down	$7.19x10^{-8}$
SAOUHSC_00867		hypothetical protein	4.425 down	$2.14x10^{-14}$
SAOUHSC_00868		hypothetical protein	2.668 down	$2.57x10^{-14}$
SAOUHSC_00871	*dltC*	D-alanine--poly(phosphoribitol) ligase subunit 2	2.090 down	$2.79x10^{-14}$
SAOUHSC_00938		hypothetical protein	2.083 down	$8.16x10^{-14}$
SAOUHSC_01036		hypothetical protein	2.400 down	$2.36x10^{-13}$
SAOUHSC_01037		hypothetical protein	3.265 down	$4.95x10^{-11}$
SAOUHSC_01155		hypothetical protein	2.124 down	$2.92x10^{-9}$
SAOUHSC_01173		hypothetical protein	2.017 down	$2.85x10^{-7}$
SAOUHSC_01285		regulatory protein, MerR	2.194 down	$5.13x10^{-14}$
SAOUHSC_01309		hypothetical protein	2.420 down	$4.67x10^{-8}$
SAOUHSC_01404		hypothetical protein	2.530 down	$4.81x10^{-6}$
SAOUHSC_01468		hypothetical protein	2.224 down	$9.99x10^{-10}$
SAOUHSC_01736		hypothetical protein	2.240 down	$2.18x10^{-7}$
SAOUHSC_01780		hypothetical protein	2.108 down	$1.08x10^{-6}$
SAOUHSC_01851		hypothetical protein	2.354 down	$2.64x10^{-6}$
SAOUHSC_01927		transposase, IS3 family, truncation-related protein, putative	2.044 down	$3.06x10^{-9}$

Locus tag	Gene name	Product name/function	Fold change	P value
SAOUHSC_02176		hypothetical protein	2.105 down	$1.25x10^{-12}$
SAOUHSC_02294		hypothetical protein	2.847 down	$3.70x10^{-8}$
SAOUHSC_02416		hypothetical protein	3.732 down	$3.74x10^{-10}$
SAOUHSC_02461		transcriptional regulator, MerR family	2.443 down	0
SAOUHSC_02471		hypothetical protein	2.072 down	$1.89x10^{-5}$
SAOUHSC_02565	*ureD*	urease accessory protein UreD	2.645 down	$3.21x10^{-14}$
SAOUHSC_02781		hypothetical protein	2.240 down	$9.90x10^{-12}$
SAOUHSC_02830		D-lactate dehydrogenase	2.077 down	$2.79x10^{-14}$
SAOUHSC_03001	*icaR*	biofilm operon icaabcd hth-type negative transcriptional regulator IcaR	2.644 down	$4.11x10^{-12}$
SAOUHSC_A02189		hypothetical protein	2.036 down	$2.88x10^{-7}$

References

Agarwal, H., Verrall, R., Singh, S. P., Tang, Y. W. & Wilson, G. (2007) Small colony variant *Staphylococcus aureus* multiorgan infection. *Pediatr Infect Dis J* **26**: 269-271.

Aizenman, E., Engelberg-Kulka, H. & Glaser, G. (1996) An *Escherichia coli* chromosomal "addiction module" regulated by guanosine 3',5'-bispyrophosphate: a model for programmed bacterial cell death. *Proc Natl Acad Sci U S A* **93**: 6059-6063.

Akiyama, H., Hamada, T., Huh, W. K., Yamasaki, O., Oono, T., Fujimoto, W., *et al.* (2003) Confocal laser scanning microscopic observation of glycocalyx production by *Staphylococcus aureus* in skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus. *Br J Dermatol* **148**: 526-532.

Akiyama, H., Huh, W. K., Yamasaki, O., Oono, T. & Iwatsuki, K. (2002) Confocal laser scanning microscopic observation of glycocalyx production by *Staphylococcus aureus* in mouse skin: does *S. aureus* generally produce a biofilm on damaged skin? *Br J Dermatol* **147**: 879-885.

Allegrucci, M. & Sauer, K. (2007) Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. *J Bacteriol* **189**: 2030-2038.

Allegrucci, M. & Sauer, K. (2008) Formation of *Streptococcus pneumoniae* non-phase-variable colony variants is due to increased mutation frequency present under biofilm growth conditions. *J Bacteriol* **190**: 6330-6339.

Allison, D. G., Brown, M. R., Evans, D. E. & Gilbert, P. (1990a) Surface hydrophobicity and dispersal of *Pseudomonas aeruginosa* from biofilms. *FEMS Microbiol Lett* **59**: 101-104.

Allison, D. G., Evans, D. J., Brown, M. R. & Gilbert, P. (1990b) Possible involvement of the division cycle in dispersal of *Escherichia coli* from biofilms. *J Bacteriol* **172**: 1667-1669.

Ananthaswamy, H. N. & Eisenstark, A. (1977) Repair of hydrogen peroxide-induced single-strand breaks in *Escherichia coli* deoxyribonucleic acid. *J Bacteriol* **130**: 187-191.

Antonio, M., McFerran, N. & Pallen, M. J. (2002) Mutations affecting the Rossman fold of isoleucyl-tRNA synthetase are correlated with low-level mupirocin resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **46**: 438-442.

Armel, P. R., Strniste, G. F. & Wallace, S. S. (1977) Studies on *Escherichia coli* X-ray endonuclease specificity. Roles of hydroxyl and reducing radicals in the production of DNA lesions. *Radiat Res* **69**: 328-338.

Armstrong-Buisseret, L., Cole, M. B. & Stewart, G. S. (1995) A homologue to the *Escherichia coli* alkyl hydroperoxide reductase AhpC is induced by osmotic upshock in *Staphylococcus aureus*. *Microbiology* **141 (Pt 7)**: 1655-1661.

Arrecubieta, C., Lee, M. H., Macey, A., Foster, T. J. & Lowy, F. D. (2007) SdrF, a *Staphylococcus epidermidis* surface protein, binds type I collagen. *J Biol Chem* **282**: 18767-18776.

Aruoma, O. I., Halliwell, B., Hoey, B. M. & Butler, J. (1988) The antioxidant action of taurine, hypotaurine and their metabolic precursors. *Biochem J* **256**: 251-255.

Ashby, M. J., Neale, J. E., Knott, S. J. & Critchley, I. A. (1994) Effect of antibiotics on non-growing planktonic cells and biofilms of *Escherichia coli*. *J Antimicrob Chemother* **33**: 443-452.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., *et al.* (2002) Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* **359**: 1819-1827.

Baddiley, J., Buchanan, J. G., Hardy, F. E., Martin, R. O., Rajbhandary, U. L. & Sanderson, A. R. (1961) The structure of the ribitol teichoic acid of *Staphylococcus aureus* H. *Biochim Biophys Acta* **52**: 406-407.

Baddiley, J., Buchanan, J. G., Rajbhandary, U. L. & Sanderson, A. R. (1962) Teichoic acid from the walls of *Staphylococcus aureus* H. Structure of the N-acetylglucosaminyl-ribitol residues. *Biochem J* **82**: 439-448.

Balaban, N., Giacometti, A., Cirioni, O., Gov, Y., Ghiselli, R., Mocchegiani, F., *et al.* (2003a) Use of the quorum-sensing inhibitor RNAIII-inhibiting peptide to prevent biofilm formation *in vivo* by drug-resistant *Staphylococcus epidermidis*. *J Infect Dis* **187**: 625-630.

Balaban, N., Goldkorn, T., Gov, Y., Hirshberg, M., Koyfman, N., Matthews, H. R., *et al.* (2001) Regulation of *Staphylococcus aureus* pathogenesis via target of RNAIII-activating Protein (TRAP). *J Biol Chem* **276**: 2658-2667.

Balaban, N., Gov, Y., Bitler, A. & Boelaert, J. R. (2003b) Prevention of *Staphylococcus aureus* biofilm on dialysis catheters and adherence to human cells. *Kidney Int* **63**: 340-345.

Baldassarri, L., Donnelli, G., Gelosia, A., Voglino, M. C., Simpson, A. W. & Christensen, G. D. (1996) Purification and characterization of the staphylococcal slime-associated antigen and its occurrence among *Staphylococcus epidermis* clinical isolates. *Infect Immun* **64**: 3410-3415.

Ballal, A. & Manna, A. C. (2009) Regulation of superoxide dismutase (*sod*) genes by SarA in *Staphylococcus aureus*. *J Bacteriol* **191**: 3301-3310.

Bayston, R., Ashraf, W. & Smith, T. (2007) Triclosan resistance in methicillin-resistant *Staphylococcus aureus* expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics. *J Antimicrob Chemother* **59**: 848-853.

Beenken, K. E., Dunman, P. M., McAleese, F., Macapagal, D., Murphy, E., Projan, S. J., *et al.* (2004) Global Gene Expression in *Staphylococcus aureus* Biofilms. *J Bacteriol* **186**: 4665-4684.

Beenken, K. E., Mrak, L. N., Griffin, L. M., Zielinska, A. K., Shaw, L. N., Rice, K. C., *et al.* (2010) Epistatic relationships between *sarA* and *agr* in *Staphylococcus aureus* biofilm formation. *PLoS One* **5**: e10790.

Benoit, M. R., Conant, C. G., Ionescu-Zanetti, C., Schwartz, M. & Matin, A. (2010) New device for high-throughput viability screening of flow biofilms. *Appl Environ Microbiol* **76**: 4136-4142.

Berg, T., Firth, N., Apisiridej, S., Hettiaratchi, A., Leelaporn, A. & Skurray, R. A. (1998) Complete nucleotide sequence of pSK41: evolution of staphylococcal conjugative multiresistance plasmids. *J Bacteriol* **180**: 4350-4359.

Bhattacharyya, S., Dutta, D., Ghosh, A. K. & Das, A. K. (2009) Cloning, overexpression, purification, crystallization and preliminary X-ray diffraction analysis of an atypical two-cysteine peroxiredoxin (SAOUHSC_01822) from *Staphylococcus aureus* NCTC 8325. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **65**: 1113-1115.

Bigger, J. (1944) Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *Lancet* **244**: 497-500.

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., *et al.* (2004) Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon. *J Bacteriol* **186**: 4085-4099.

Biswas, R., Voggu, L., Simon, U. K., Hentschel, P., Thumm, G. & Gotz, F. (2006) Activity of the major staphylococcal autolysin Atl. *FEMS Microbiol Lett* **259**: 260-268.

Blokesch, M. & Schoolnik, G. K. (2008) The extracellular nuclease Dns and its role in natural transformation of *Vibrio cholerae*. *J Bacteriol* **190**: 7232-7240.

Boles, B. R. & Horswill, A. R. (2008) *agr*-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog* **4**: e1000052.

Boles, B. R. & Singh, P. K. (2008) Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *Proc Natl Acad Sci U S A* **105**: 12503-12508.

Borchardt, S. A., Allain, E. J., Michels, J. J., Stearns, G. W., Kelly, R. F. & McCoy, W. F. (2001) Reaction of acylated homoserine lactone bacterial signaling molecules with oxidized halogen antimicrobials. *Appl Environ Microbiol* **67**: 3174-3179.

Borriello, G., Werner, E., Roe, F., Kim, A. M., Ehrlich, G. D. & Stewart, P. S. (2004) Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother* **48**: 2659-2664.

Boyd, A. & Chakrabarty, A. M. (1994) Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **60**: 2355-2359.

Bozzini, S., Visai, L., Pignatti, P., Petersen, T. E. & Speziale, P. (1992) Multiple binding sites in fibronectin and the staphylococcal fibronectin receptor. *Eur J Biochem* **207**: 327-333.

Brady, R. A., Leid, J. G., Calhoun, J. H., Costerton, J. W. & Shirtliff, M. E. (2008) Osteomyelitis and the role of biofilms in chronic infection. *FEMS Immunol Med Microbiol* **52**: 13-22.

Brady, R. A., Leid, J. G., Camper, A. K., Costerton, J. W. & Shirtliff, M. E. (2006) Identification of *Staphylococcus aureus* proteins recognized by the antibody-mediated immune response to a biofilm infection. *Infect Immun* **74**: 3415-3426.

Brennan, M. P., Loughman, A., Devocelle, M., Arasu, S., Chubb, A. J., Foster, T. J., *et al.* (2009) Elucidating the role of *Staphylococcus epidermidis* serine-aspartate repeat protein G in platelet activation. *J Thromb Haemost* **7**: 1364-1372.

Brent, R. & Ptashne, M. (1980) The *lexA* gene product represses its own promoter. *Proc Natl Acad Sci U S A* **77**: 1932-1936.

Brock, T. D., Madigan, M. T., Martinko, J. M. & Parker, J. (1994) Introduction: An overview of microbiology and cell biology. In: *Biology of Microorganisms.* Englewood Cliffs, New Jersery: Prentice-Hall Inc, pp. 1-22.

BSAC (1991) A guide to sensitivity testing. Report of the Working Party on Antibiotic Sensitivity Testing of the British Society for Antimicrobial Chemotherapy. *J Antimicrob Chemother* **27 Suppl D**: 1-50.

Burmolle, M., Bahl, M. I., Jensen, L. B., Sorensen, S. J. & Hansen, L. H. (2008) Type 3 fimbriae, encoded by the conjugative plasmid pOLA52, enhance biofilm formation and transfer frequencies in *Enterobacteriaceae* strains. *Microbiology* **154**: 187-195.

Campbell, E. A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A., *et al.* (2001) Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* **104**: 901-912.

Carrasco, B., Canas, C., Sharples, G. J., Alonso, J. C. & Ayora, S. (2009) The N-terminal region of the RecU holliday junction resolvase is essential for homologous recombination. *J Mol Biol* **390**: 1-9.

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D. & Buret, A. (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol* **37**: 1771-1776.

Cescutti, P., Toffanin, R., Fett, W. F., Osman, S. F., Pollesello, P. & Paoletti, S. (1998) Structural investigation of the exopolysaccharide produced by *Pseudomonas flavescens* strain B62 - degradation by a fungal cellulase and isolation of the oligosaccharide repeating unit. *Eur J Biochem* **251**: 971-979.

Chaignon, P., Sadovskaya, I., Ragunah, C., Ramasubbu, N., Kaplan, J. B. & Jabbouri, S. (2007) Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Appl Microbiol Biotechnol* **75**: 125-132.

Chang, Y. M., Jeng, W. Y., Ko, T. P., Yeh, Y. J., Chen, C. K. & Wang, A. H. (2010) Structural study of TcaR and its complexes with multiple antibiotics from *Staphylococcus epidermidis*. *Proc Natl Acad Sci U S A* **107**: 8617-8622.

Cheng, K. J., Fay, J. P., Coleman, R. N., Milligan, L. P. & Costerton, J. W. (1981) Formation of bacterial microcolonies on feed particles in the rumen. *Appl Environ Microbiol* **41**: 298-305.

Chopra, I., O'Neill, A. J. & Miller, K. (2003) The role of mutators in the emergence of antibiotic-resistant bacteria. *Drug Resistance Updates* **6**: 137-145.

Christensen, S. K., Mikkelsen, M., Pedersen, K. & Gerdes, K. (2001) RelE, a global inhibitor of translation, is activated during nutritional stress. *Proc Natl Acad Sci U S A* **98**: 14328-14333.

Christie, G. E., Matthews, A. M., King, D. G., Lane, K. D., Olivarez, N. P., Tallent, S. M., *et al.* (2010) The complete genomes of *Staphylococcus aureus* bacteriophages 80 and 80alpha--implications for the specificity of SaPI mobilization. *Virology* **407**: 381-390.

Clarke, S. R., Harris, L. G., Richards, R. G. & Foster, S. J. (2002) Analysis of Ebh, a 1.1-megadalton cell wall-associated fibronectin-binding protein of *Staphylococcus aureus*. *Infect Immun* **70**: 6680-6687.

Clauditz, A., Resch, A., Wieland, K. P., Peschel, A. & Gotz, F. (2006) Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. *Infect Immun* **74**: 4950-4953.

Cochran, W. L., Suh, S. J., McFeters, G. A. & Stewart, P. S. (2000) Role of RpoS and AlgT in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide and monochloramine. *J Appl Microbiol* **88**: 546-553.

Coenye, T., De Prijck, K., De Wever, B. & Nelis, H. J. (2008) Use of the modified Robbins device to study the *in vitro* biofilm removal efficacy of NitrAdine, a novel disinfecting formula for the maintenance of oral medical devices. *J Appl Microbiol* **105**: 733-740.

Cohen, S., Sweeney, H. M. & Basu, S. K. (1977) Mutations in prophage phi11 that impair the transducibility of their *Staphylococcus aureus* lysogens for methicillin resistance. *J Bacteriol* **129**: 237-245.

Conen, A., Walti, L. N., Merlo, A., Fluckiger, U., Battegay, M. & Trampuz, A. (2008) Characteristics and treatment outcome of cerebrospinal fluid shunt-associated infections in adults: a retrospective analysis over an 11-year period. *Clin Infect Dis* **47**: 73-82.

Conlon, K. M., Humphreys, H. & O'Gara, J. P. (2002a) *icaR* encodes a transcriptional repressor involved in environmental regulation of *ica* operon expression and biofilm formation in *Staphylococcus epidermidis*. *J Bacteriol* **184**: 4400-4408.

Conlon, K. M., Humphreys, H. & O'Gara, J. P. (2002b) Regulation of *icaR* gene expression in *Staphylococcus epidermidis*. *FEMS Microbiol Lett* **216**: 171-177.

Conlon, K. M., Humphreys, H. & O'Gara, J. P. (2004) Inactivations of *rsbU* and *sarA* by IS*256* represent novel mechanisms of biofilm phenotypic variation in *Staphylococcus epidermidis*. *J Bacteriol* **186**: 6208-6219.

Corrigan, R. M., Miajlovic, H. & Foster, T. J. (2009) Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. *BMC Microbiol* **9**: 22.

Corrigan, R. M., Rigby, D., Handley, P. & Foster, T. J. (2007) The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. *Microbiology* **153**: 2435-2446.

Cosgrove, K., Coutts, G., Jonsson, I. M., Tarkowski, A., Kokai-Kun, J. F., Mond, J. J., *et al.* (2007) Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *J Bacteriol* **189**: 1025-1035.

Costerton, J. W., Geesey, G. G. & Cheng, K. J. (1978) How bacteria stick. *Sci Am* **238**: 86-95.

Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W. & Gotz, F. (1999) The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect Immun* **67**: 5427-5433.

Cramton, S. E. & Götz, F. (2004) Biofilm development in *Staphylococcus*. In: *Microbial Biofilms*. Ghannoum, M. & O'Toole, G. A. (eds). Washington: ASM Press, pp. 64-84.

Cramton, S. E., Ulrich, M., Gotz, F. & Doring, G. (2001) Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infect Immun* **69**: 4079-4085.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I. & Penades, J. R. (2001) Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol* **183**: 2888-2896.

Cucarella, C., Tormo, M. A., Ubeda, C., Trotonda, M. P., Monzon, M., Peris, C., *et al.* (2004) Role of biofilm-associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*. *Infect Immun* **72**: 2177-2185.

Cvitkovitch, D. G. (2004) Genetic Exchange in Biofilms. In: *Microbial Biofilms*. Ghannoum, M. & O'Toole, G. A. (eds). Washington DC: ASM Press, pp. 192-205.

Darby, C., Hsu, J. W., Ghori, N. & Falkow, S. (2002) *Caenorhabditis elegans*: plague bacteria biofilm blocks food intake. *Nature* **417**: 243-244.

David, M. Z. & Daum, R. S. (2010) Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. *Clin Microbiol Rev* **23**: 616-687.

Davies, D. G. & Geesey, G. G. (1995) Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *Appl Environ Microbiol* **61**: 860-867.

Debeer, D., Stoodley, P., Roe, F. & Lewandowski, Z. (1994) Effects of biofilm structures on oxygen distribution and mass-transport. *Biotechnol Bioeng* **43**: 1131-1138.

Deighton, M. & Borland, R. (1993) Regulation of slime production in *Staphylococcus epidermidis* by iron limitation. *Infect Immun* **61**: 4473-4479.

Dice, B., Stoodley, P., Buchinsky, F., Metha, N., Ehrlich, G. D. & Hu, F. Z. (2009) Biofilm formation by *ica*-positive and *ica*-negative strains of *Staphylococcus epidermidis in vitro*. *Biofouling* **25**: 367-375.

Donlan, R. M. (2001a) Biofilm formation: a clinically relevant microbiological process. *Clin Infect Dis* **33**: 1387-1392.

Donlan, R. M. (2001b) Biofilms and device-associated infections. *Emerg Infect Dis* **7**: 277-281.

Donlan, R. M. (2002) Biofilms: microbial life on surfaces. *Emerg Infect Dis* **8**: 881-890.

Dougherty, B. A., Hill, C., Weidman, J. F., Richardson, D. R., Venter, J. C. & Ross, R. P. (1998) Sequence and analysis of the 60 kb conjugative, bacteriocin-producing plasmid pMRC01 from *Lactococcus lactis* DPC3147. *Mol Microbiol* **29**: 1029-1038.

Dow, J. M., Crossman, L., Findlay, K., He, Y. Q., Feng, J. X. & Tang, J. L. (2003) Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc Natl Acad Sci U S A* **100**: 10995-11000.

Driffield, K., Miller, K., Bostock, J. M., O'Neill, A. J. & Chopra, I. (2008) Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J Antimicrob Chemother* **61**: 1053-1056.

Dunne, W. M., Jr. & Burd, E. M. (1992) The effects of magnesium, calcium, EDTA, and pH on the *in vitro* adhesion of *Staphylococcus epidermidis* to plastic. *Microbiol Immunol* **36**: 1019-1027.

Elasri, M. O., Thomas, J. R., Skinner, R. A., Blevins, J. S., Beenken, K. E., Nelson, C. L., *et al.* (2002) *Staphylococcus aureus* collagen adhesin contributes to the pathogenesis of osteomyelitis. *Bone* **30**: 275-280.

Espinosa-Urgel, M., Salido, A. & Ramos, J. L. (2000) Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *J Bacteriol* **182**: 2363-2369.

Estes, R. J. & Meduri, G. U. (1995) The pathogenesis of ventilator-associated pneumonia: I. Mechanisms of bacterial transcolonization and airway inoculation. *Intensive Care Med* **21**: 365-383.

Esther, C. R., Jr., Esserman, D. A., Gilligan, P., Kerr, A. & Noone, P. G. (2010) Chronic *Mycobacterium abscessus* infection and lung function decline in cystic fibrosis. *J Cyst Fibros* **9**: 117-123.

Evans, D. J., Allison, D. G., Brown, M. R. & Gilbert, P. (1991) Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. *J Antimicrob Chemother* **27**: 177-184.

Evans, R. C. & Holmes, C. J. (1987) Effect of vancomycin hydrochloride on *Staphylococcus epidermidis* biofilm associated with silicone elastomer. *Antimicrob Agents Chemother* **31**: 889-894.

Faridani, O. R., Nikravesh, A., Pandey, D. P., Gerdes, K. & Good, L. (2006) Competitive inhibition of natural antisense Sok-RNA interactions activates Hok-mediated cell killing in *Escherichia coli*. *Nucleic Acids Res* **34**: 5915-5922.

Feig, D. I., Reid, T. M. & Loeb, L. A. (1994) Reactive oxygen species in tumorigenesis. *Cancer Res* **54**: 1890s-1894s.

Fitzpatrick, F., Humphreys, H. & O'Gara, J. P. (2005) Evidence for *icaADBC*-independent biofilm development mechanism in methicillin-resistant *Staphylococcus aureus* clinical isolates. *J Clin Microbiol* **43**: 1973-1976.

Fitzpatrick, F., Humphreys, H., Smyth, E., Kennedy, C. A. & O'Gara, J. P. (2002) Environmental regulation of biofilm formation in intensive care unit isolates of *Staphylococcus epidermidis*. *J Hosp Infect* **52**: 212-218.

Foley, I., Marsh, P., Wellington, E. M. H., Smith, A. W. & Brown, M. R. W. (1999) General stress response master regulator *rpoS* is expressed in human infection: a possible role in chronicity. *J Antimicrob Chemother* **43**: 164-165.

Foster, T. J. & Hook, M. (1998) Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol* **6**: 484-488.

Fournier, B. & Hooper, D. C. (2000) A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. *J Bacteriol* **182**: 3955-3964.

Fridkin, S. K., Hageman, J. C., Morrison, M., Sanza, L. T., Como-Sabetti, K., Jernigan, J. A., *et al.* (2005) Methicillin-resistant *Staphylococcus aureus* disease in three communities. *N Engl J Med* **352**: 1436-1444.

Fridovich, I. (1978) The biology of oxygen radicals. *Science* **201**: 875-880.

Gander, S., Kinnaird, A. & Finch, R. (2005) Telavancin: *in vitro* activity against staphylococci in a biofilm model. *J Antimicrob Chemother* **56**: 337-343.

Garcia-Alvarez, F., Monzon, M., Grasa, J. M., Lacleriga, A., Amorena, B., Garcia-Alvarez, I., *et al.* (2006) Interleukin-1, interleukin-6, and interleukin-10 responses after antibiotic treatment in experimental chronic *Staphylococcus aureus* osteomyelitis. *J Orthop Sci* **11**: 370-374.

Gerdes, K., Christensen, S. K. & Lobner-Olesen, A. (2005) Prokaryotic toxin-antitoxin stress response loci. *Nat Rev Microbiol* **3**: 371-382.

Gerke, C., Kraft, A., Sussmuth, R., Schweitzer, O. & Gotz, F. (1998) Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. *J Biol Chem* **273**: 18586-18593.

Gillaspy, A. F., Hickmon, S. G., Skinner, R. A., Thomas, J. R., Nelson, C. L. & Smeltzer, M. S. (1995) Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal osteomyelitis. *Infect Immun* **63**: 3373-3380.

Gordon, C. A., Hodges, N. A. & Marriott, C. (1988) Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived *Pseudomonas aeruginosa*. *J Antimicrob Chemother* **22**: 667-674.

Gov, Y., Bitler, A., Dell'Acqua, G., Torres, J. V. & Balaban, N. (2001) RNAIII inhibiting peptide (RIP), a global inhibitor of *Staphylococcus aureus* pathogenesis: structure and function analysis. *Peptides* **22**: 1609-1620.

Green, M., Apel, A. & Stapleton, F. (2008) Risk factors and causative organisms in microbial keratitis. *Cornea* **27**: 22-27.

Greene, C., McDevitt, D., Francois, P., Vaudaux, P. E., Lew, D. P. & Foster, T. J. (1995) Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes. *Mol Microbiol* **17**: 1143-1152.

Griffiths, J. & O'Neill, A. J. (2009) Identification and preliminary characterisation of *Staphylococcus aureus* mutants showing reduced tolerance to antibiotics during biofilm growth. *ASM Conference on Biofilms,* Cancun, Mexico.

Grohmann, E., Muth, G. & Espinosa, M. (2003) Conjugative plasmid transfer in Gram-positive bacteria. *Microbiol Mol Biol Rev* **67**: 277-301.

Gross, M., Cramton, S. E., Gotz, F. & Peschel, A. (2001) Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infect Immun* **69**: 3423-3426.

Hall, A. E., Patel, P. R., Domanski, P. J., Prater, B. D., Gorovits, E. L., Syribeys, P. J., *et al.* (2007) A panel of monoclonal antibodies recognizing the *Staphylococcus epidermidis* fibrinogen-binding MSCRAMM SdrG. *Hybridoma (Larchmt)* **26**: 28-34.

Halliwell, B. & Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford, UK.

Harmsen, M., Lappann, M., Knochel, S. & Molin, S. (2010) Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. *Appl Environ Microbiol* **76**: 2271-2279.

Harris, E. D. (1992) Regulation of antioxidant enzymes. *FASEB J.* **6**: 2675-2683.

Harrison, J. J., Wade, W. D., Akierman, S., Vacchi-Suzzi, C., Stremick, C. A., Turner, R. J., *et al.* (2009) The chromosomal toxin gene *yafQ* is a determinant of multidrug tolerance for *Escherichia coli* growing in a biofilm. *Antimicrob Agents Chemother* **53**: 2253-2258.

He, A. S., Rohatgi, P. R., Hersh, M. N. & Rosenberg, S. M. (2006) Roles of *E. coli* double-strand-break-repair proteins in stress-induced mutation. *DNA Repair (Amst)* **5**: 258-273.

Heilmann, C., Gerke, C., Perdreau-Remington, F. & Gotz, F. (1996a) Characterization of Tn*917* insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infect Immun* **64**: 277-282.

Heilmann, C., Hussain, M., Peters, G. & Gotz, F. (1997) Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Mol Microbiol* **24**: 1013-1024.

Heilmann, C. & Peters, G. (2006) Biology and pathogenicity of *Staphylococcus epidermidis*. In: *Gram-Positive Pathogens.* Fischetti, V. A., Novick, R. P., Ferretti, J. J., Portnoy, D. A. & Rood, J. I. (eds). Washington DC: ASM Press, pp.

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D. & Gotz, F. (1996b) Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol Microbiol* **20**: 1083-1091.

Heimberger, T. S. & Duma, R. J. (1989) Infections of prosthetic heart valves and cardiac pacemakers. *Infect Dis Clin North Am* **3**: 221-245.

Herles, S., Olsen, S., Afflitto, J. & Gaffar, A. (1994) Chemostat flow cell system: an *in vitro* model for the evaluation of antiplaque agents. *J Dent Res* **73**: 1748-1755.

Hodgson, A. E., Nelson, S. M., Brown, M. R. & Gilbert, P. (1995) A simple *in vitro* model for growth control of bacterial biofilms. *J Appl Bacteriol* **79**: 87-93.

Holland, D. B., Bojar, R. A., Jeremy, A. H. T., Ingham, E. & Holland, K. T. (2008) Microbial colonization of an *in vitro* model of a tissue engineered human skin equivalent - a novel approach. *FEMS Microbiol Lett* **279**: 110-115.

Horii, T., Ogawa, T., Nakatani, T., Hase, T., Matsubara, H. & Ogawa, H. (1981) Regulation of SOS functions: purification of *E. coli* LexA protein and determination of its specific site cleaved by the RecA protein. *Cell* **27**: 515-522.

Horsburgh, M. J., Aish, J. L., White, I. J., Shaw, L., Lithgow, J. K. & Foster, S. J. (2002a) σ^B modulates virulence determinant expression and stress resistance: Characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol* **184**: 5457-5467.

Horsburgh, M. J., Ingham, E. & Foster, S. J. (2001) In *Staphylococcus aureus*, Fur is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J Bacteriol* **183**: 468-475.

Horsburgh, M. J., Wharton, S. J., Cox, A. G., Ingham, E., Peacock, S. & Foster, S. J. (2002b) MntR modulates expression of the PerR regulon and superoxide resistance in *Staphylococcus aureus* through control of manganese uptake. *Mol Microbiol* **44**: 1269-1286.

Huesca, M., Peralta, R., Sauder, D. N., Simor, A. E. & McGavin, M. J. (2002) Adhesion and virulence properties of epidemic Canadian methicillin-resistant *Staphylococcus aureus* strain 1: identification of novel adhesion functions associated with plasmin-sensitive surface protein. *J Infect Dis* **185**: 1285-1296.

Hughes, J. & Mellows, G. (1980) Interaction of pseudomonic acid A with *Escherichia coli* B isoleucyl-tRNA synthetase. *Biochem J* **191**: 209-219.

Huseby, M. J., Kruse, A. C., Digre, J., Kohler, P. L., Vocke, J. A., Mann, E. E., *et al.* (2010) Beta toxin catalyzes formation of nucleoprotein matrix in staphylococcal biofilms. *Proc Natl Acad Sci U S A* **107**: 14407-14412.

Hussain, M., Becker, K., von Eiff, C., Schrenzel, J., Peters, G. & Herrmann, M. (2001a) Identification and characterization of a novel 38.5-kilodalton cell surface protein of *Staphylococcus aureus* with extended-spectrum binding activity for extracellular matrix and plasma proteins. *J Bacteriol* **183**: 6778-6786.

Hussain, M., Heilmann, C., Peters, G. & Herrmann, M. (2001b) Teichoic acid enhances adhesion of *Staphylococcus epidermidis* to immobilized fibronectin. *Microb Pathog* **31**: 261-270.

Hussain, M., Herrmann, M., von Eiff, C., Perdreau-Remington, F. & Peters, G. (1997) A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. *Infect Immun* **65**: 519-524.

Ingavale, S., van Wamel, W., Luong, T. T., Lee, C. Y. & Cheung, A. L. (2005) Rat/MgrA, a regulator of autolysis, is a regulator of virulence genes in *Staphylococcus aureus*. *Infect Immun* **73**: 1423-1431.

Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P., *et al.* (1984) *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *J Pediatr* **104**: 206-210.

Ito, A., May, T., Kawata, K. & Okabe, S. (2007) Significance of *rpoS* during maturation of *Escherichia coli* biofilms. *Biotechnol Bioeng* **99**: 1462-1471.

Ito, A., Taniuchi, A., May, T., Kawata, K. & Okabe, S. (2009) Increased antibiotic resistance of *Escherichia coli* in mature biofilms. *Appl Environ Microbiol* **75**: 4093-4100.

Jacob, R. A. & Burri, B. J. (1996) Oxidative damage and defense. *Am J Clin Nutr* **63**: 985S-990S.

Janzon, L., Lofdahl, S. & Arvidson, S. (1989) Identification and nucleotide sequence of the delta-lysin gene, *hld*, adjacent to the accessory gene regulator (*agr*) of *Staphylococcus aureus*. *Mol Gen Genet* **219**: 480-485.

Jayaraman, R. (2008) Bacterial persistence: some new insights into an old phenomenon. *J Biosci* **33**: 795-805.

Jefferson, K. K., Goldmann, D. A. & Pier, G. B. (2005) Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. *Antimicrob Agents Chemother* **49**: 2467-2473.

Jefferson, K. K., Pier, D. B., Goldmann, D. A. & Pier, G. B. (2004) The teicoplanin-associated locus regulator (TcaR) and the intercellular adhesin locus regulator (IcaR) are transcriptional inhibitors of the *ica* locus in *Staphylococcus aureus*. *J Bacteriol* **186**: 2449-2456.

Ji, G., Beavis, R. C. & Novick, R. P. (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc Natl Acad Sci U S A* **92**: 12055-12059.

Jones, H. C., Roth, I. L. & Sanders, W. M., 3rd (1969) Electron microscopic study of a slime layer. *J Bacteriol* **99**: 316-325.

Josefsson, E., McCrea, K. W., Ni Eidhin, D., O'Connell, D., Cox, J., Hook, M., *et al.* (1998) Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus*. *Microbiology* **144**: 3387-3395.

Kaplan, J. B. & Fine, D. H. (2002) Biofilm dispersal of *Neisseria subflava* and other phylogenetically diverse oral bacteria. *Appl Environ Microbiol* **68**: 4943-4950.

Kaplan, S. L., Hulten, K. G., Gonzalez, B. E., Hammerman, W. A., Lamberth, L., Versalovic, J., *et al.* (2005) Three-year surveillance of community-acquired *Staphylococcus aureus* infections in children. *Clin Infect Dis* **40**: 1785-1791.

Karavolos, M. H., Horsburgh, M. J., Ingham, E. & Foster, S. J. (2003) Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. *Microbiology* **149**: 2749-2758.

Karpati, F., Malmborg, A. S., Alfredsson, H., Hjelte, L. & Strandvik, B. (1994) Bacterial colonisation with *Xanthomonas maltophilia*--a retrospective study in a cystic fibrosis patient population. *Infection* **22**: 258-263.

Keane, F. M., Loughman, A., Valtulina, V., Brennan, M., Speziale, P. & Foster, T. J. (2007) Fibrinogen and elastin bind to the same region within the A domain of fibronectin binding protein A, an MSCRAMM of *Staphylococcus aureus*. *Mol Microbiol* **63**: 711-723.

Keele, B. B., Jr., McCord, J. M. & Fridovich, I. (1970) Superoxide dismutase from *Escherichia coli* B. A new manganese-containing enzyme. *J Biol Chem* **245**: 6176-6181.

Keren, I., Shah, D., Spoering, A., Kaldalu, N. & Lewis, K. (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bacteriol* **186**: 8172-8180.

Khardori, N. & Yassien, M. (1995) Biofilms in device-related infections. *J Ind Microbiol* **15**: 141-147.

Kim, E. J., Chung, H. J., Suh, B., Hah, Y. C. & Roe, J. H. (1998) Transcriptional and post-transcriptional regulation by nickel of *sodN* gene encoding nickel-containing superoxide dismutase from *Streptomyces coelicolor* Muller. *Mol Microbiol* **27**: 187-195.

Kinniment, S. L., Wimpenny, J. W., Adams, D. & Marsh, P. D. (1996) Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. *Microbiology* **142 (Pt 3)**: 631-638.

Kirisits, M. J., Prost, L., Starkey, M. & Parsek, M. R. (2005) Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **71**: 4809-4821.

Kluytmans, J., van Belkum, A. & Verbrugh, H. (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* **10**: 505-520.

Knobloch, J. K., Bartscht, K., Sabottke, A., Rohde, H., Feucht, H. H. & Mack, D. (2001) Biofilm formation by *Staphylococcus epidermidis* depends on functional RsbU, an activator of the *sigB* operon: differential activation mechanisms due to ethanol and salt stress. *J Bacteriol* **183**: 2624-2633.

Knobloch, J. K., Horstkotte, M. A., Rohde, H., Kaulfers, P. M. & Mack, D. (2002) Alcoholic ingredients in skin disinfectants increase biofilm expression of *Staphylococcus epidermidis*. *J Antimicrob Chemother* **49**: 683-687.

Knobloch, J. K., Jager, S., Horstkotte, M. A., Rohde, H. & Mack, D. (2004) RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor σB by repression of the negative regulator gene *icaR*. *Infect Immun* **72**: 3838-3848.

Koch, A. L. (1994) Growth Measurement. In: *Methods for General and Molecular Bacteriology.* Gerhardt, P., Murray, R. G. E., Wood, W. A. & Kreig, N. R. (eds). Washington: American Society for Microbiology, pp. 248-292.

Koch, C. & Høiby, N. (1993) Pathogenesis of cystic fibrosis. *Lancet* **341**: 1065-1069.

Kogan, G., Sadovskaya, I., Chaignon, P., Chokr, A. & Jabbouri, S. (2006) Biofilms of clinical strains of *Staphylococcus* that do not contain polysaccharide intercellular adhesin. *FEMS Microbiol Lett* **255**: 11-16.

Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R. & Losick, R. (2010) D-amino acids trigger biofilm disassembly. *Science* **328**: 627-629.

Kono, Y. & Fridovich, I. (1982) Superoxide radical inhibits catalase. *J Biol Chem* **257**: 5751-5754.

Kroukamp, O., Dumitrache, R. G. & Wolfaardt, G. M. (2010) Nature of inoculum has a pronounced effect on biofilm development in flow systems. *Appl Environ Microbiol* **76**: 6025-6031.

Kumar, S. S., Ghosh, A., Devasagayam, T. P. A. & Chauhan, P. S. (2000) Effect of vanillin on methylene blue plus light-induced single-strand breaks in plasmid pBR322 DNA. *Mutat Res* **469**: 207-214.

Landry, R. M., An, D. D., Hupp, J. T., Singh, P. K. & Parsek, M. R. (2006) Mucin-*Pseudomonas aeruginosa* interactions promote biofilm formation and antibiotic resistance. *Mol Microbiol* **59**: 142-151.

Lapaglia, C. & Hartzell, P. L. (1997) Stress-induced production of biofilm in the hyperthermophile *Archaeoglobus fulgidus*. *Appl Environ Microbiol* **63**: 3158-3163.

Lasa, I. & Penades, J. R. (2006) Bap: a family of surface proteins involved in biofilm formation. *Res Microbiol* **157**: 99-107.

Lauderdale, K. J., Boles, B. R., Cheung, A. L. & Horswill, A. R. (2009) Interconnections between Sigma B, *agr*, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. *Infect Immun* **77**: 1623-1635.

Lawrence, J. R., Korber, D. R., Hoyle, B. D., Costerton, J. W. & Caldwell, D. E. (1991) Optical sectioning of microbial biofilms. *J Bacteriol* **173**: 6558-6567.

Lee, C. Y., Sharma, A., Cheong, J. E. & Nelson, J. L. (2009) Synthesis and antioxidant properties of dendritic polyphenols. *Bioorg Med Chem Lett* **19**: 6326-6330.

Lee, S. F., Li, Y. H. & Bowden, G. H. (1996) Detachment of *Streptococcus mutans* biofilm cells by an endogenous enzymatic activity. *Infect Immun* **64**: 1035-1038.

Lee, Y. K., Lim, C. Y., Teng, W. L., Ouwehand, A. C., Tuomola, E. M. & Salminen, S. (2000) Quantitative approach in the study of adhesion of lactic acid bacteria to intestinal cells and their competition with enterobacteria. *Appl Environ Microbiol* **66**: 3692-3697.

Lengfelder, E. & Elstner, E. F. (1979) Cyanide insensitive iron superoxide dismutase in *Euglena gracilis*. Comparison of the reliabilities of different test systems for superoxide dismutases. *Z Naturforsch C* **34C**: 374-380.

Lew, D. P. & Waldvogel, F. A. (1997) Osteomyelitis. *N Engl J Med* **336**: 999-1007.

Lew, D. P. & Waldvogel, F. A. (2004) Osteomyelitis. *Lancet* **364**: 369-379.

Li, Y. H., Lau, P. C., Lee, J. H., Ellen, R. P. & Cvitkovitch, D. G. (2001) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J Bacteriol* **183**: 897-908.

Lina, G., Jarraud, S., Ji, G., Greenland, T., Pedraza, A., Etienne, J., *et al.* (1998) Transmembrane topology and histidine protein kinase activity of AgrC, the *agr* signal receptor in *Staphylococcus aureus*. *Mol Microbiol* **28**: 655-662.

Lindsay, D. & von Holy, A. (2006) Bacterial biofilms within the clinical setting: what healthcare professionals should know. *J Hosp Infect* **64**: 313-325.

Lindsay, J. A. (2010) Genomic variation and evolution of *Staphylococcus aureus*. *Int J Med Microbiol* **300**: 98-103.

Lindsay, J. A. & Foster, S. J. (2001) *zur*: a Zn^{2+}-responsive regulatory element of *Staphylococcus aureus*. *Microbiology* **147**: 1259-1266.

Liu, X., Ng, C. & Ferenci, T. (2000) Global adaptations resulting from high population densities in *Escherichia coli* cultures. *J Bacteriol* **182**: 4158-4164.

Loiselle, M. & Anderson, K. W. (2003) The use of cellulase in inhibiting biofilm formation from organisms commonly found on medical implants. *Biofouling* **19**: 77-85.

Lowy, F. D. (1998) *Staphylococcus aureus* infections. *N Engl J Med* **339**: 520-532.

Macfarlane, S. (2008) Microbial biofilm communities in the gastrointestinal tract. *J Clin Gastroenterol* **42**: S142-143.

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., *et al.* (1996) The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J Bacteriol* **178**: 175-183.

Mack, D., Nedelmann, M., Krokotsch, A., Schwarzkopf, A., Heesemann, J. & Laufs, R. (1994) Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. *Infect Immun* **62**: 3244-3253.

Mack, D., Siemssen, N. & Laufs, R. (1992) Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. *Infect Immun* **60**: 2048-2057.

MacKintosh, E. E., Patel, J. D., Marchant, R. E. & Anderson, J. M. (2006) Effects of biomaterial surface chemistry on the adhesion and biofilm formation of *Staphylococcus epidermidis in vitro*. *J Biomed Mater Res A* **78**: 836-842.

Maeda, S., Ito, M., Ando, T., Ishimoto, Y., Fujisawa, Y., Takahashi, H., *et al.* (2006) Horizontal transfer of nonconjugative plasmids in a colony biofilm of *Escherichia coli*. *FEMS Microbiol Lett* **255**: 115-120.

Mah, T. F. & O'Toole, G. A. (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**: 34-39.

Mann, E. E., Rice, K. C., Boles, B. R., Endres, J. L., Ranjit, D., Chandramohan, L., *et al.* (2009) Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. *PLoS One* **4**: e5822.

Marrie, T. J., Nelligan, J. & Costerton, J. W. (1982) A scanning and transmission electron microscopic study of an infected endocardial pacemaker lead. *Circulation* **66**: 1339-1341.

Marshall, K. C. (1985) Bacterial adhesion in oligotrophic habitats. *Microbiol Sci* **2**: 321-322, 325-326.

Marti, M., Trotonda, M. P., Tormo-Mas, M. A., Vergara-Irigaray, M., Cheung, A. L., Lasa, I., *et al.* (2010) Extracellular proteases inhibit protein-dependent biofilm formation in *Staphylococcus aureus*. *Microbes Infect* **12**: 55-64.

Martinez-Pulgarin, S., Dominguez-Bernal, G., Orden, J. A. & de la Fuente, R. (2009) Simultaneous lack of catalase and beta-toxin in *Staphylococcus aureus* leads to increased intracellular survival in macrophages and epithelial cells and to attenuated virulence in murine and ovine models. *Microbiology* **155**: 1505-1515.

Massol-Deya, A. A., Whallon, J., Hickey, R. F. & Tiedje, J. M. (1995) Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater. *Appl Environ Microbiol* **61**: 769-777.

Matsui, H., Grubb, B. R., Tarran, R., Randell, S. H., Gatzy, J. T., Davis, C. W., *et al.* (1998) Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* **95**: 1005-1015.

May, T. B., Shinabarger, D., Maharaj, R., Kato, J., Chu, L., DeVault, J. D., *et al.* (1991) Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. *Clin Microbiol Rev* **4**: 191-206.

Mazmanian, S. K., Skaar, E. P., Gaspar, A. H., Humayun, M., Gornicki, P., Jelenska, J., *et al.* (2003) Passage of heme-iron across the envelope of *Staphylococcus aureus*. *Science* **299**: 906-909.

McBride, T. J., Preston, B. D. & Loeb, L. A. (1991) Mutagenic spectrum resulting from DNA damage by oxygen radicals. *Biochemistry* **30**: 207-213.

McCord, J. M. & Fridovich, I. (1968) The reduction of cytochrome c by milk xanthine oxidase. *J Biol Chem* **243**: 5753-5760.

McCord, J. M. & Fridovich, I. (1969) Superoxide dismutase. *J Biol Chem* **244**: 6049-6055.

McDevitt, D., Francois, P., Vaudaux, P. & Foster, T. J. (1995) Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus*. *Mol Microbiol* **16**: 895-907.

McKenney, D., Pouliot, K., Wang, Y., Murthy, V., Ulrich, M., Doring, G., *et al.* (2000) Vaccine potential of poly-1-6 beta-D-N-succinylglucosamine, an immunoprotective surface polysaccharide of *Staphylococcus aureus* and *Staphylococcus epidermidis*. *J Biotechnol* **83**: 37-44.

McLean, R. J., Cassanto, J. M., Barnes, M. B. & Koo, J. H. (2001) Bacterial biofilm formation under microgravity conditions. *FEMS Microbiol Lett* **195**: 115-119.

McLean, R. J. C., Bates, C. L., Barnes, M. B., McGowin, C. L. & Aron, G. M. (2004) Methods of studying biofilms. In: *Microbial Biofilms.* Ghannoum, M. & O'Toole, G. A. (eds). Washington: ASM Press, pp.

Merritt, J., Qi, F., Goodman, S. D., Anderson, M. H. & Shi, W. (2003) Mutation of *luxS* affects biofilm formation in *Streptococcus mutans*. *Infect Immun* **71**: 1972-1979.

Moisan, H., Brouillette, E., Jacob, C. L., Langlois-Begin, P., Michaud, S. & Malouin, F. (2006) Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. *J Bacteriol* **188**: 64-76.

Morgan, A. R., Cone, R. L. & Elgert, T. M. (1976) The mechanism of DNA strand breakage by vitamin C and superoxide and the protective roles of catalase and superoxide dismutase. *Nucl Acids Res* **3**: 1139-1150.

Moyed, H. S. & Bertrand, K. P. (1983) *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J Bacteriol* **155**: 768-775.

Nagarajan, V., Smeltzer, M. S. & Elasri, M. O. (2009) Genome-scale transcriptional profiling in *Staphylococcus aureus*: bringing order out of chaos. *FEMS Microbiol Lett* **295**: 204-210.

Nallapareddy, S. R., Singh, K. V. & Murray, B. E. (2008) Contribution of the collagen adhesin Acm to pathogenesis of *Enterococcus faecium* in experimental endocarditis. *Infect Immun* **76**: 4120-4128.

Navarre, W. W. & Schneewind, O. (1994) Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in Gram-positive bacteria. *Mol Microbiol* **14**: 115-121.

Netherwood, T., Bowden, R., Harrison, P., O'Donnell, A. G., Parker, D. S. & Gilbert, H. J. (1999) Gene transfer in the gastrointestinal tract. *Appl Environ Microbiol* **65**: 5139-5141.

Nicholas, R. O., Li, T., McDevitt, D., Marra, A., Sucoloski, S., Demarsh, P. L., *et al.* (1999) Isolation and characterization of a σ^B deletion mutant of *Staphylococcus aureus*. *Infect Immun* **67**: 3667-3669.

Nickel, J. C., Grant, S. K., Lam, K., Olson, M. E. & Costerton, J. W. (1991) Bacteriologically stressed animal model of new closed catheter drainage system with microbicidal outlet tube. *Urology* **38**: 280-289.

Nickel, J. C., Olson, M., McLean, R. J., Grant, S. K. & Costerton, J. W. (1987) An ecological study of infected urinary stone genesis in an animal model. *Br J Urol* **59**: 21-30.

Nickel, J. C., Ruseska, I., Wright, J. B. & Costerton, J. W. (1985) Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrob Agents Chemother* **27**: 619-624.

NIH (2002) Research on microbial biofilms. http://grants.nih.gov/grants/guide/pa-files/PA-03-047.html Last Access: 05/09/2010

Nyholm, S. V., Deplancke, B., Gaskins, H. R., Apicella, M. A. & McFall-Ngai, M. J. (2002) Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl Environ Microbiol* **68**: 5113-5122.

O'Brien, L., Kerrigan, S. W., Kaw, G., Hogan, M., Penades, J., Litt, D., *et al.* (2002) Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. *Mol Microbiol* **44**: 1033-1044.

O'Gara, J. P. (2007) *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. *FEMS Microbiol Lett* **270**: 179-188.

O'Gara, J. P. & Humphreys, H. (2001) *Staphylococcus epidermidis* biofilms: importance and implications. *J Med Microbiol* **50**: 582-587.

O'Neill, A. J., Cove, J. H. & Chopra, I. (2001) Mutation frequencies for resistance to fusidic acid and rifampicin in *Staphylococcus aureus*. *J Antimicrob Chemother* **47**: 647-650.

O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, D. A., Loughman, A., *et al.* (2008) A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *J Bacteriol* **190**: 3835-3850.

O'Sullivan, B. P. & Freedman, S. D. (2009) Cystic fibrosis. *Lancet* **373**: 1891-1904.

O'Toole, G. A. & Kolter, R. (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**: 295-304.

Oh, Y. J., Lee, N. R., Jo, W., Jung, W. K. & Lim, J. S. (2009) Effects of substrates on biofilm formation observed by atomic force microscopy. *Ultramicroscopy* **109**: 874-880.

Otto, M., Sussmuth, R., Jung, G. & Gotz, F. (1998) Structure of the pheromone peptide of the *Staphylococcus epidermidis agr* system. *FEBS Lett* **424**: 89-94.

Ovchinnikov, Y. A., Monastyrskaya, G. S., Guriev, S. O., Kalinina, N. F., Sverdlov, E. D., Gragerov, A. I., *et al.* (1983) RNA polymerase rifampicin resistance mutations in *Escherichia coli*: sequence changes and dominance. *Mol Gen Genet* **190**: 344-348.

Ovchinnikov Yu, A., Monastyrskaya, G. S., Gubanov, V. V., Lipkin, V. M., Sverdlov, E. D., Kiver, I. F., *et al.* (1981) Primary structure of *Escherichia coli* RNA polymerase nucleotide substitution in the beta subunit gene of the rifampicin resistant *rpoB*255 mutant. *Mol Gen Genet* **184**: 536-538.

Parsek, M. R. & Fuqua, C. (2004) Biofilms 2003: Emerging themes and challenges in studies of surface-associated microbial life. *J Bacteriol* **186**: 4427-4440.

Patel, J. D., Ebert, M., Ward, R. & Anderson, J. M. (2007) *S. epidermidis* biofilm formation: effects of biomaterial surface chemistry and serum proteins. *J Biomed Mater Res A* **80**: 742-751.

Patti, J. M., Allen, B. L., McGavin, M. J. & Hook, M. (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu Rev Microbiol* **48**: 585-617.

Patti, J. M., Jonsson, H., Guss, B., Switalski, L. M., Wiberg, K., Lindberg, M., *et al.* (1992) Molecular characterization and expression of a gene encoding a *Staphylococcus aureus* collagen adhesin. *J Biol Chem* **267**: 4766-4772.

Perry, A. M., Ton-That, H., Mazmanian, S. K. & Schneewind, O. (2002) Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. III. Lipid II is an in vivo peptidoglycan substrate for sortase-catalyzed surface protein anchoring. *J Biol Chem* **277**: 16241-16248.

Petersen, F. C., Tao, L. & Scheie, A. A. (2005) DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation. *J Bacteriol* **187**: 4392-4400.

Pinto, A. V., Mathieu, A., Marsin, S., Veaute, X., Ielpi, L., Labigne, A., *et al.* (2005) Suppression of homologous and homeologous recombination by the bacterial MutS2 protein. *Mol Cell* **17**: 113-120.

Prosser, B. L., Taylor, D., Dix, B. A. & Cleeland, R. (1987) Method of evaluating effects of antibiotics on bacterial biofilm. *Antimicrob Agents Chemother* **31**: 1502-1506.

Purcell, K. & Fergie, J. (2005) Epidemic of community-acquired methicillin-resistant *Staphylococcus aureus* infections: a 14-year study at Driscoll Children's Hospital. *Arch Pediatr Adolesc Med* **159**: 980-985.

QIAGEN (2001) Appendix B: Storage, quantitation, and determination of quality of total RNA. In: *RNeasy Midi/Maxi Handbook*. pp. 79-81.

Que, Y.-A., Haefliger, J.-A., Piroth, L., Francois, P., Widmer, E., Entenza, J. M., *et al.* (2005) Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aureus* experimental endocarditis. *J Exp Med* **201**: 1627-1635.

Rachid, S., Ohlsen, K., Wallner, U., Hacker, J., Hecker, M. & Ziebuhr, W. (2000a) Alternative transcription factor sigma(B) is involved in regulation of biofilm expression in a *Staphylococcus aureus* mucosal isolate. *J Bacteriol* **182**: 6824-6826.

Rachid, S., Ohlsen, K., Witte, W., Hacker, J. & Ziebuhr, W. (2000b) Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis*. *Antimicrob Agents Chemother* **44**: 3357-3363.

Raimundo, O., Heussler, H., Bruhn, J. B., Suntrarachun, S., Kelly, N., Deighton, M. A., *et al.* (2002) Molecular epidemiology of coagulase-negative staphylococcal bacteraemia in a newborn intensive care unit. *J Hosp Infect* **51**: 33-42.

Rasmussen, B. (2000) Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit. *Nature* **405**: 676-679.

Rather, P. N. (2005) Swarmer cell differentiation in *Proteus mirabilis*. *Environ Microbiol* **7**: 1065-1073.

Recsei, P., Kreiswirth, B., O'Reilly, M., Schlievert, P., Gruss, A. & Novick, R. P. (1986) Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. *Mol Gen Genet* **202**: 58-61.

Reisin, I. L., Prat, A. G., Abraham, E. H., Amara, J. F., Gregory, R. J., Ausiello, D. A., *et al.* (1994) The cystic fibrosis transmembrane conductance regulator is a dual ATP and chloride channel. *J Biol Chem* **269**: 20584-20591.

Reisner, A., Holler, B. M., Molin, S. & Zechner, E. L. (2006) Synergistic effects in mixed *Escherichia coli* biofilms: conjugative plasmid transfer drives biofilm expansion. *J Bacteriol* **188**: 3582-3588.

Resch, A., Rosenstein, R., Nerz, C. & Gotz, F. (2005) Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. *Appl Environ Microbiol* **71**: 2663-2676.

Rice, K. C., Mann, E. E., Endres, J. L., Weiss, E. C., Cassat, J. E., Smeltzer, M. S., *et al.* (2007) The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **104**: 8113-8118.

Riddle, J. M., Wang, C. H., Magilligan, D. J., Jr. & Stein, P. D. (1989) Scanning electron microscopy of surgically excised human mitral valves in patients over 45 years of age. *Am J Cardiol* **63**: 471-477.

Rindi, S., Cicalini, S., Pietrocola, G., Venditti, M., Festa, A., Foster, T. J., *et al.* (2006) Antibody response in patients with endocarditis caused by *Staphylococcus aureus*. *Eur J Clin Invest* **36**: 536-543.

Robbins, M. J., Soeiro, R., Frishman, W. H. & Strom, J. A. (1986) Right-sided valvular endocarditis: etiology, diagnosis, and an approach to therapy. *Am Heart J* **111**: 128-135.

Roche, F. M., Downer, R., Keane, F., Speziale, P., Park, P. W. & Foster, T. J. (2004) The N-terminal A domain of fibronectin-binding proteins A and B promotes adhesion of *Staphylococcus aureus* to elastin. *J Biol Chem* **279**: 38433-38440.

Rupp, M. E., Fey, P. D., Heilmann, C. & Gotz, F. (2001) Characterization of the importance of *Staphylococcus epidermidis* autolysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter-associated infection in a rat model. *J Infect Dis* **183**: 1038-1042.

Ryder, V. J., Miller, K., O'Neill, A. J. & Chopra, I. (2009a) Enhanced mutability in staphylococcal biofilms. *ARM,* Birmingham, UK.

Ryder, V. J., Miller, K., O'Neill, A. J. & Chopra, I. (2009b) C1-1356 Enhanced mutability of *Staphylococcus aureus* in biofilms results from oxidative damage. *ICAAC,* San Francisco, USA.

Ryder, V. J., O'Neill, A. J. & Chopra, I. (2010) Phenotypic variants arising in *Staphylococcus aureus* biofilms may promote dissemination during infection *Biofilms4,* Winchester, UK.

Sabbuba, N., Hughes, G. & Stickler, D. J. (2002) The migration of *Proteus mirabilis* and other urinary tract pathogens over Foley catheters. *BJU Int* **89**: 55-60.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbour Laboratory Press, New York.

Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W. & Davies, D. G. (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* **184**: 1140-1154.

Sauer, K., Cullen, M. C., Rickard, A. H., Zeef, L. A., Davies, D. G. & Gilbert, P. (2004) Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J Bacteriol* **186**: 7312-7326.

Schaffer, S. W., Azuma, J. & Mozaffari, M. (2009) Role of antioxidant activity of taurine in diabetes. *Can J Physiol Pharmacol* **87**: 91-99.

Scheld, W. M., Valone, J. A. & Sande, M. A. (1978) Bacterial adherence in the pathogenesis of endocarditis. Interaction of bacterial dextran, platelets, and fibrin. *J Clin Invest* **61**: 1394-1404.

Schembri, M. A., Kjaergaard, K. & Klemm, P. (2003) Global gene expression in *Escherichia coli* biofilms. *Mol Microbiol* **48**: 253-267.

Scher, K., Kesselman, E., Shimoni, E. & Yaron, S. (2007) Morphological analysis of young and old pellicles of *Salmonella* Typhimurium. *Biofouling* **23**: 385-394.

Schierle, C. F., De la Garza, M., Mustoe, T. A. & Galiano, R. D. (2009) Staphylococcal biofilms impair wound healing by delaying reepithelialization in a murine cutaneous wound model. *Wound Repair Regen* **17**: 354-359.

Schmidt, K. A., Manna, A. C., Gill, S. & Cheung, A. L. (2001) SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus*. *Infect Immun* **69**: 4749-4758.

Schneider, M., Muhlemann, K., Droz, S., Couzinet, S., Casaulta, C. & Zimmerli, S. (2008) Clinical characteristics associated with isolation of small-colony variants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from respiratory secretions of patients with cystic fibrosis. *J Clin Microbiol* **46**: 1832-1834.

Schumacher-Perdreau, F., Heilmann, C., Peters, G., Gotz, F. & Pulverer, G. (1994) Comparative analysis of a biofilm-forming *Staphylococcus epidermidis* strain and its adhesion-positive, accumulation-negative mutant M7. *FEMS Microbiol Lett* **117**: 71-78.

Seaver, L. C. & Imlay, J. A. (2004) Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? *J Biol Chem* **279**: 48742-48750.

Shani, S., Friedman, M. & Steinberg, D. (2000) The anticariogenic effect of amine fluorides on *Streptococcus sobrinus* and glucosyltransferase in biofilms. *Caries Res* **34**: 260-267.

Sharma-Kuinkel, B. K., Mann, E. E., Ahn, J. S., Kuechenmeister, L. J., Dunman, P. M. & Bayles, K. W. (2009) The *Staphylococcus aureus* LytSR two-component regulatory system affects biofilm formation. *J Bacteriol* **191**: 4767-4775.

Shigeta, M., Tanaka, G., Komatsuzawa, H., Sugai, M., Suginaka, H. & Usui, T. (1997) Permeation of antimicrobial agents through *Pseudomonas aeruginosa* biofilms: a simple method. *Chemotherapy* **43**: 340-345.

Sies, H. (1997) Oxidative stress: oxidants and antioxidants. *Exp Physiol* **82**: 291-295.

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J. & Greenberg, E. P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**: 762-764.

Singh, R., Ray, P., Das, A. & Sharma, M. (2009) Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study. *J Med Microbiol* **58**: 1067-1073.

Singh, R., Ray, P., Das, A. & Sharma, M. (2010a) Enhanced production of exopolysaccharide matrix and biofilm by a menadione-auxotrophic *Staphylococcus aureus* small-colony variant. *J Med Microbiol* **59**: 521-527.

Singh, R., Ray, P., Das, A. & Sharma, M. (2010b) Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *J Antimicrob Chemother* **65**: 1955-1958.

Sinha, B., Francois, P. P., Nusse, O., Foti, M., Hartford, O. M., Vaudaux, P., *et al.* (1999) Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin a5b1. *Cell Microbiol* **1**: 101-117.

Sitges-Serra, A. & Girvent, M. (1999) Catheter-related bloodstream infections. *World J Surg* **23**: 589-595.

Stickler, D. J. (1996) Bacterial biofilms and the encrustation of urethral catheters. *Biofouling* **94**: 293-305.

Taddei, F., Matic, I. & Radman, M. (1995) cAMP-dependent SOS induction and mutagenesis in resting bacterial populations. *Proc Natl Acad Sci U S A* **92**: 11736-11740.

Taylor, C. D., Wirsen, C. O. & Gaill, F. (1999) Rapid microbial production of filamentous sulfur mats at hydrothermal vents. *Appl Environ Microbiol* **65**: 2253-2255.

Tenover, F. C. & Gorwitz, R. J. (2006) The epidemiology of *Staphylococcus* Infections. In: *Gram-Positive Pathogens.* Fischetti, V. A., Novick, R. P., Ferretti, J. J., Portnoy, D. A. & Rood, J. I. (eds). Washington DC: ASM Press, pp.

Tetz, G. V., Artemenko, N. K. & Tetz, V. V. (2009) Effect of DNase and antibiotics on biofilm characteristics. *Antimicrob Agents Chemother* **53**: 1204-1209.

Thomas, R., Ferguson, J., Coombs, G. & Gibson, P. G. (2011) Community acquired methicillin resistant *Staphylococcus aureus* pneumonia - A clinical audit. *Respirology*.

Thurnheer, T., Giertsen, E., Gmur, R. & Guggenheim, B. (2008) Cariogenicity of soluble starch in oral *in vitro* biofilm and experimental rat caries studies: a comparison. *J Appl Microbiol* **105**: 829-836.

Timmerman, C. P., Fleer, A., Besnier, J. M., De Graaf, L., Cremers, F. & Verhoef, J. (1991) Characterization of a proteinaceous adhesin of *Staphylococcus epidermidis* which mediates attachment to polystyrene. *Infect Immun* **59**: 4187-4192.

Tkeshelashvili, L. K., McBride, T., Spence, K. & Loeb, L. A. (1991) Mutation spectrum of copper-induced DNA damage. *J Biol Chem* **266**: 6401-6406.

Tojo, M., Yamashita, N., Goldmann, D. A. & Pier, G. B. (1988) Isolation and characterization of a capsular polysaccharide adhesin from *Staphylococcus epidermidis*. *J Infect Dis* **157**: 713-722.

Tormo, M. A., Knecht, E., Gotz, F., Lasa, I. & Penades, J. R. (2005) Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? *Microbiology* **151**: 2465-2475.

Trotonda, M. P., Tamber, S., Memmi, G. & Cheung, A. L. (2008) MgrA represses biofilm formation in *Staphylococcus aureus*. *Infect Immun* **76**: 5645-5654.

Tsang, L. H., Cassat, J. E., Shaw, L. N., Beenken, K. E. & Smeltzer, M. S. (2008) Factors contributing to the biofilm-deficient phenotype of *Staphylococcus aureus sarA* mutants. *PLoS One* **3**: e3361.

Tsuji, B. T., von Eiff, C., Kelchlin, P. A., Forrest, A. & Smith, P. F. (2008) Attenuated vancomycin bactericidal activity against *Staphylococcus aureus hemB* mutants expressing the small-colony-variant phenotype. *Antimicrob Agents Chemother* **52**: 1533-1537.

Tunkel, A. R. & Mandell, G. L. (1992) Infecting microorganisms. In: *Infective endocarditis*. Kaye, D. (ed). New York: Raven Press, pp. 85-97.

Uhlen, M. & Abrahmsen, L. (1989) Secretion of recombinant proteins into the culture medium by *Escherichia coli* and *Staphylococcus aureus*. *Biochem Soc Trans* **17**: 340-341.

Valderas, M. W., Gatson, J. W., Wreyford, N. & Hart, M. E. (2002) The superoxide dismutase gene *sodM* is unique to *Staphylococcus aureus*: Absence of *sodM* in coagulase-negative staphylococci. *J Bacteriol* **184**: 2465-2472.

Valderas, M. W. & Hart, M. E. (2001) Identification and characterization of a second superoxide dismutase gene (*sodM*) from *Staphylococcus aureus*. *J Bacteriol* **183**: 3399-3407.

Valle, J., Vergara-Irigaray, M., Merino, N., Penades, J. R. & Lasa, I. (2007) σB regulates IS*256*-mediated *Staphylococcus aureus* biofilm phenotypic variation. *J Bacteriol* **189**: 2886-2896.

Valpuesta, V. & Botella, M. A. (2004) Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. *Trends Plant Sci* **9**: 573-577.

van Loosdrecht, M. C., Lyklema, J., Norde, W. & Zehnder, A. J. (1990) Influence of interfaces on microbial activity. *Microbiol Rev* **54**: 75-87.

Vandecasteele, S. J., Peetermans, W. E., R, R. M., Rijnders, B. J. & Van Eldere, J. (2003) Reliability of the *ica*, *aap* and *atlE* genes in the discrimination between invasive, colonizing and contaminant *Staphylococcus epidermidis* isolates in the diagnosis of catheter-related infections. *Clin Microbiol Infect* **9**: 114-119.

Vandenesch, F., Naimi, T., Enright, M. C., Lina, G., Nimmo, G. R., Heffernan, H., *et al.* (2003) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis* **9**: 978-984.

von Eiff, C., Friedrich, A. W., Becker, K. & Peters, G. (2005) Comparative *in vitro* activity of ceftobiprole against staphylococci displaying normal and small-colony variant phenotypes. *Antimicrob Agents Chemother* **49**: 4372-4374.

Vuong, C., Kocianova, S., Voyich, J. M., Yao, Y., Fischer, E. R., DeLeo, F. R., *et al.* (2004) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem* **279**: 54881-54886.

Vuong, C., Saenz, H. L., Gotz, F. & Otto, M. (2000) Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J Infect Dis* **182**: 1688-1693.

Walters, M. C., 3rd, Roe, F., Bugnicourt, A., Franklin, M. J. & Stewart, P. S. (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* **47**: 317-323.

Wang, I. W., Anderson, J. M. & Marchant, R. E. (1993) *Staphylococcus epidermidis* adhesion to hydrophobic biomedical polymer is mediated by platelets. *J Infect Dis* **167**: 329-336.

Wang, R., Braughton, K. R., Kretschmer, D., Bach, T. H., Queck, S. Y., Li, M., *et al.* (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* **13**: 1510-1514.

Wann, E. R., Gurusiddappa, S. & Hook, M. (2000) The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. *J Biol Chem* **275**: 13863-13871.

Ward, J. F., Evans, J. W., Limoli, C. L. & Calabro-Jones, P. M. (1987) Radiation and hydrogen peroxide induced free radical damage to DNA. *Br J Cancer Suppl* **8**: 105-112.

Watnick, P. I., Fullner, K. J. & Kolter, R. (1999) A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. *J Bacteriol* **181**: 3606-3609.

Weidenmaier, C., Peschel, A., Xiong, Y. Q., Kristian, S. A., Dietz, K., Yeaman, M. R., *et al.* (2005) Lack of wall teichoic acids in *Staphylococcus aureus* leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis. *J Infect Dis* **191**: 1771-1777.

Weigel, L. M., Donlan, R. M., Shin, D. H., Jensen, B., Clark, N. C., McDougal, L. K., *et al.* (2007) High-level vancomycin-resistant *Staphylococcus aureus* isolates associated with a polymicrobial biofilm. *Antimicrob Agents Chemother* **51**: 231-238.

Weinstein, L. & Schlesinger, J. J. (1974) Pathoanatomic, pathophysiologic and clinical correlations in endocarditis (second of two parts). *N Engl J Med* **291**: 1122-1126.

Wentland, E. J., Stewart, P. S., Huang, C. T. & McFeters, G. A. (1996) Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. *Biotechnol Prog* **12**: 316-321.

Williams, D. L. & Bloebaum, R. D. (2010) Observing the biofilm matrix of *Staphylococcus epidermidis* ATCC 35984 grown using the CDC biofilm reactor. *Microsc Microanal* **16**: 143-152.

Wolgemuth, C., Hoiczyk, E., Kaiser, D. & Oster, G. (2002) How myxobacteria glide. *Curr Biol* **12**: 369-377.

Xia, G., Kohler, T. & Peschel, A. (2010) The wall teichoic acid and lipoteichoic acid polymers of *Staphylococcus aureus*. *Int J Med Microbiol* **300**: 148-154.

Yamada, K. M. & Olden, K. (1978) Fibronectins--adhesive glycoproteins of cell surface and blood. *Nature* **275**: 179-184.

Yamada, S., Sugai, M., Komatsuzawa, H., Nakashima, S., Oshida, T., Matsumoto, A., *et al.* (1996) An autolysin ring associated with cell separation of *Staphylococcus aureus*. *J Bacteriol* **178**: 1565-1571.

Yang, J. A., Park, D. W., Sohn, J. W., Yang, I. S., Kim, K. H. & Kim, M. J. (2006) Molecular analysis of isoleucyl-tRNA synthetase mutations in clinical isolates of methicillin-resistant *Staphylococcus aureus* with low-level mupirocin resistance. *J Korean Med Sci* **21**: 827-832.

Yang, S. J., Rice, K. C., Brown, R. J., Patton, T. G., Liou, L. E., Park, Y. H., *et al.* (2005) A LysR-type regulator, CidR, is required for induction of the *Staphylococcus aureus cidABC* operon. *J Bacteriol* **187**: 5893-5900.

Yim, L. C., Hongmei, J., Aitchison, J. C. & Pointing, S. B. (2006) Highly diverse community structure in a remote central Tibetan geothermal spring does not display monotonic variation to thermal stress. *FEMS Microbiol Ecol* **57**: 80-91.

Zheng, Z. & Stewart, P. S. (2002) Penetration of rifampin through *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother* **46**: 900-903.

Ziebuhr, W., Heilmann, C., Gotz, F., Meyer, P., Wilms, K., Straube, E., *et al.* (1997) Detection of the intercellular adhesion gene cluster (*ica*) and phase variation in *Staphylococcus epidermidis* blood culture strains and mucosal isolates. *Infect Immun* **65**: 890-896.

Zobell, C. E. (1943) The effect of solid surfaces upon bacterial activity. *J Bacteriol* **46**: 39-56.

Zong, Y., Xu, Y., Liang, X., Keene, D. R., Hook, A., Gurusiddappa, S., *et al.* (2005) A 'Collagen Hug' model for *Staphylococcus aureus* CNA binding to collagen. *EMBO J* **24**: 4224-4236.