Regulation of *S. aureus* biofilm formation.

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by

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Abstract

Regulation of *S. aureus* biofilm formation, by Miranda Johnson.

S. aureus is a natural commensal of the human host and an opportunistic pathogen that causes a wide range of diseases. Biofilm formation is an important *S. aureus* virulence determinant. *S. aureus* colonisation of medical devices and host tissues as a biofilm can impede treatment as genetically and metabolically diverse cells in the different layers of the film can prevent the penetration or activity of the therapeutic agent used. Biofilm formation is a multi-factorial process which can be influenced by many environmental factors. A major environmental stress encountered by bacteria *in vivo* is severe iron-restriction. However, pathogenic bacteria can use low iron concentrations as a signal to up regulate factors responsible for virulence.

This work demonstrates for the first time that *S. aureus* biofilm formation is iron regulated. It demonstrates that biofilms formed in low iron are dependant on the proteins Eap and Emp which are also iron regulated and are positively regulated by the ferric uptake regulator, Fur. This work has also identified that PNAG, which currently has a controversial role in biofilm formation, was capable of compensating for the loss of Eap and Emp in low iron when it was over expressed, but that wild type levels of PNAG appeared to have a limited role. Nevertheless, the *ica* operon responsible for the production of PNAG was essential for the expression of Eap and Emp. In addition, Sae, Fur, Agr, SarA and Hfq were shown to all have interlinking roles in the expression of these and other proteins associated with virulence and low iron biofilm formation. The identification of the importance of Eap and Emp in low iron biofilm formation and the regulatory network controlling their expression may have implications on the development of new therapeutic agents essential for the prevention and treatment of *S. aureus* infection.

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Abbreviations

µg	Microgram
µM	Micromolar
AIP	Auto inducing peptide
bp	Base pair
CA-MRSA	Community acquired methicillin resistant *Staphylococcus aureus*
CRPMI	Tissue culture media RPMI treated with metal ions binding Chelex resin
DEPC	Di-ethyl-pyrocarbonate
DNA	Deoxyribonucleic acid
eDNA	Extracellular deoxyribonucleic acid
Fe	Iron ($FeSO_4$)
g	Gram
HA-MRSA	Hospital acquired methicillin resistant *Staphylococcus aureus*
hVISA	Heterogeneous Vancomycin intermediate resistance *Staphylococcus aureus*
IgG	Immunoglobulin G
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Kb	Kilobase pair
l	Litre
M	Molar
MIC	Minimum inhibitory concentration
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistance *Staphylococcus aureus*
NHS	National Health service
OD	optical density

PBPs	Penicillin binding proteins
PCR	Polymerase chain reaction
PNAG	poly-N-acetylglucosamine
PVL	Panton-Valentine leukocidin
RNA	Ribonucleic acid
SCC*mec*	Staphylococcal chromosome cassette *mec*
SDS	Sodium dodecyl sulphate
sRNA	Small ribonucleic acid
SSI	Surgical site infections
ST	Sequence types
TAE	Tris acetate electrophoresis buffer
TSB	Trypticase(triptic) Soy Broth
v\v	Volume by volume
VISA	Vancomycin intermediate resistance *Staphylococcus aureus*
VRE	Vancomycin resistant *Enterococci*
VRSA	Vancomycin resistant *Staphylococcus aureus*
w\v	Weight by volume
X Gal	bromo-chloro-indolyl-galactopyranoside

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Chapter 1

Introduction

1.1 *S. aureus,* a general introduction and its pathogenicity.

Staphylococci are Gram positive, facultative anaerobes, found associated with the skin and mucosal membranes of mammals. There are 32 recognised Staphylococcal species of which 12 are commonly associated with humans (Crossley and Archer, 1997). Of these, *S. epidermidis* and *S. aureus* are most commonly isolated from staphylococcal infections. *S. aureus* is the most widely studied of the staphylococci, and is an opportunistic pathogen, responsible for a wide range of diseases, ranging in severity from superficial skin infections to life threatening bacteraemia, lung, heart and bone infections. In the UK in 2008, the Health Protection Agency (HPA) reported that *S. aureus* was the leading cause of surgical site infections (SSI), accounting for 38 % of all SSIs reported (Figure 1.1) (Surveillance of Healthcare Associated Infections Report: 2008). The increase in *S. aureus* infections on the whole is largely due to advances in surgical techniques which have lead to the increased use of prosthetic devices that *S. aureus* is able to colonise. Whilst *S. epidermidis* causes similar diseases, the frequency and severity of *S. aureus* infections, due in part to increased toxin production, make *S. aureus* a more serious pathogen. *S. aureus* is commonly isolated from the human host, although it can also be found associated with processed meat, dairy products, soil, dust, air and water (Crossley and Archer, 1997). Isolation from food

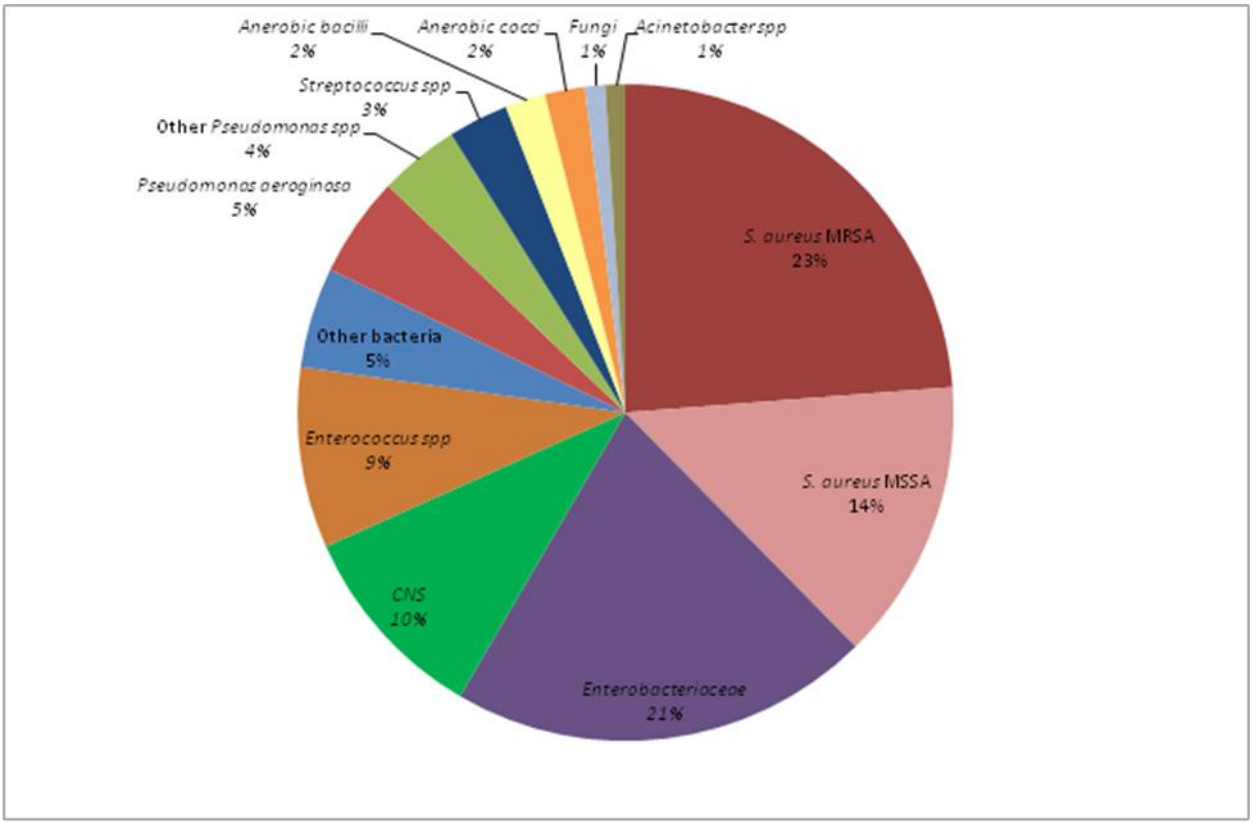


Figure 1.1. The causative agents of all surgical site infections from January 2005 – December 2007. Pie chart representing the proportion of surgical site infections caused by MRSA and MSSA isolates. CNS represents coagulase negative species of *Staphylococcus*, such as *S. epidermidis*. Data was obtained from the Health Protection Agency website, Surveillance of Healthcare Associated Infections report: 2008 (www.hpa.org.uk).

stuffs is often associated with food poisoning which results from the accumulation of toxins excreted by the bacteria, which poison the recipient if the contaminated food is ingested. The toxins involved in food poisoning are described in section 1.6.4. To date there are 15 fully sequenced strains of *S. aureus* and two further partially sequenced strains, which include clinical isolates, laboratory strains and strains with multiple antibiotic resistances, including *S. aureus* strains resistant to the antibiotic methicillin, referred to as MRSA (methicillin resistant *S. aureus*; ncib.gov). The average size of the *S. aureus* genome is ~ 2.8 Mb, with 32 % GC content, and 2,500 -2,900 protein coding genes. Some of the sequenced isolates harbour plasmids which have also been sequenced (ncib.gov). The genome is made up of the 'core genome' which is highly conserved between strains, and accounts for approximately 75 % of the sequence (Lindsay and Holden, 2004). However, there is considerable variation due to point mutations and additional insertions of repeat sequences that affect the expression and regulation of many genes. The remaining 25 % of the sequence makes up the 'accessory genome' and consists of mobile genetic elements (MGEs), such as plasmids, transposons, pathogenicity islands, staphylococcal cassette chromosome (SCC) and bacteriophages. These regions of DNA typically contain genes pertaining to virulence and antibiotic resistance (Lindsay and Holden, 2004).

As a gram positive organism, staphylococci lack an outer cell membrane, and instead have a thick cell wall made up of peptidoglycan and teichoic acids, interspaced with various cell wall proteins. Peptidoglycan is a polymer of multiple N-aceytylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues (Fig 1.2) (Crossley and Archer, 1997). Teichoic acids are polysaccharides made up of ribitol

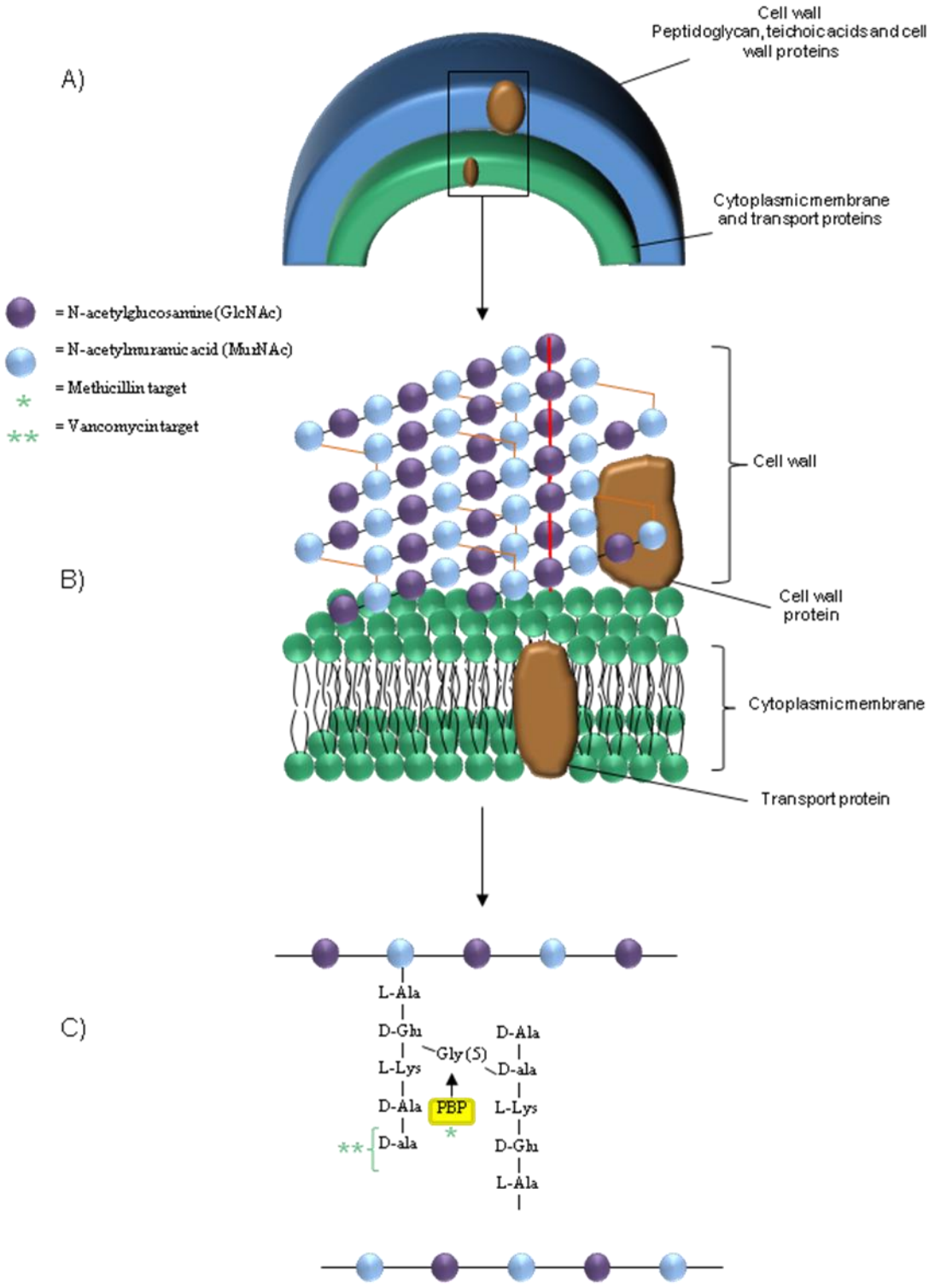


Figure 1.2. A schematic representation of the gram positive cell wall.
A) An illustration of the cell wall in cross section. B) A diagram illustrating the cell membrane including trans-membrane spanning and cell wall proteins, and the constituents of the peptidoglycan cell wall. C) An illustration of transpeptidation reaction joining GlcNAc and MurNAc residues of peptidoglycan facilitated by the penicillin binding proteins PBP.

phosphates linked by phosphodiester bonds, and include lipoteichoic acids which are anchored to the cell membrane, and cell wall teichoic acids which are covalently linked to the peptidoglycan (Crossley and Archer, 1997). GlcNAc and MurNAc residues are cross linked producing an intricate mesh which forms the structure of the cell wall via transpeptidation reactions (Blumberg and Strominger, 1974). These reactions are facilitated by the penicillin binding proteins (PBPs), so called as they are the targets of penicillin type antibiotics (Fig 1.2) (Blumberg and Strominger, 1974).

1.2 *S. aureus* Carriage

As *S. aureus* is a natural human commensal it usually resides on the skin or mucosal membranes of its host with no ill effect; however, when introduced internally, through a breach in the skin's integrity, be it an accidental breach, for example due to a burn, or an intentional breach via surgery, they then have the potential to become pathogenic (Crossley and Archer, 1997). Carriage of *S. aureus* is estimated to occur in 15 – 100 % of individuals depending on the age, demography and health of the population being sampled, and is categorised as either transient or persistent carriage (Coates *et al.*, 2009). Persistent carriage exists in 20 – 35 % of a healthy population, with the remaining 65 - 80% of the population experiencing transient carriage, which can last from as little as a few days, up to a few weeks (VandenBergh *et al.*, 1999). The anterior nares (nostrils) are the site most frequently associated with *S. aureus* carriage (Coates *et al.*, 2009); however it can also be isolated from the perineum (the space between the vagina/scrotum and the rectum), the pharynx (throat) and the axilla (armpit) (Dancer and Noble, 1991). General colonisation of the skin, such as the arms, toes and forehead

also occurs, but this is usually considered as transient carriage (Crossley and Archer, 1997). Colonisation distribution can vary considerably if the population being sampled is somehow compromised. For example HIV/AIDS patients have been shown to have increased persistent nasal carriage (Padoveze *et al.*, 2008), and eczema sufferers tend to have increased persistent generalised skin colonisation (Crossley and Archer, 1997). Nasal colonisation has been found to have a strong link with infection (Kluytmans *et al.*, 1997; Kalmeijer *et al.*, 2000). Patients who tested positive for nasal carriage were three times more likely to develop infection than non-colonised patients (Wertheim *et al.*, 2004). This poses a risk for patients admitted for surgical procedures which may provide a route of entry for the bacteria.

1.3 Antibiotics and resistance

Staphylococci, like many pathogenic bacteria, have developed resistance to certain antibiotic treatments, causing problems for treatment. In the UK 2008 HPA report *S. aureus* was the leading cause of SSIs, 24 % of which were resistant to the antibiotic methicillin (MRSA) (Fig 1.1); in addition *S. aureus* was reported as the third most likely cause of bacteraemia, making *S. aureus* very dangerous in the hospital setting. (Surveillance of Healthcare Associated infections report: 2008). Existing antibiotics have a variety of different modes of action, and those used to treat *S. aureus* infections vary depending on the site, and type of infection. However, antibiotics commonly used to treat *S. aureus* infections have traditionally been the β-lactam group of antibiotics (Lindsay, 2008). This group includes natural antibiotics derived from other microorganisms such as the penicillins; penicillin and methicillin (meticillin/oxacillin/fluoxacillin) and synthetic antibiotics such as cephalosporins (Neu,

1982). β-lactam antibiotics inhibit peptidoglycan synthesis, a major component of the cell wall, and are therefore bactericidal (Lu *et al.*, 2006). They bind to the penicillin binding proteins (PBPs) (Fig 1.2), responsible for the final step in peptidoglycan synthesis, therefore preventing cell wall production and resulting in cell death (Thumanu *et al.*, 2006). *S. aureus* has four penicillin binding proteins which have different roles in peptidoglycan synthesis (Waxman and Strominger, 1983), and PBP2 is the target of methicillin (Mimica *et al.*, 2007).

Resistance to β-lactam antibiotics can arise in two ways. The first mechanism is by the production of β-lactamase enzymes, products of the *blaZ* gene, which hydrolyse the β-lactam ring, a structural component of the antibiotic, rendering it ineffective (Thumanu *et al.*, 2006). Production of β-lactamase by bacteria can be overcome by co-administration of β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam, which bind the β-lactamase enzymes preventing deactivation of the antibiotic, but this is not always effective (Thomson *et al.*, 2007; Paukner *et al.*, 2009). Alternatively, resistance can be via the acquisition of the *mecA* gene responsible for the production of an additional penicillin binding protein PBP2' or PBP2a, which has a different conformation to the normal PBP (Hartman and Tomasz, 1984). These changes prevent methicillin and other β-lactams from binding to the PBP, resulting in continued peptidoglycan synthesis and redundancy of the antibiotic family (Lim and Strynadka, 2002), and are either acquired on a plasmid or encoded in the chromosome (Voladri and Kernodle, 1998).

Methicillin resistant *S. aureus* (MRSA)

Increasing antibiotic resistance in bacteria is a constant concern. Not only does it affect the treatment of the bacteria in which the resistance originated, but it can also be transferred to other bacteria (Sievert *et al.*, 2008). Methicillin resistance in *S. aureus* (MRSA) was first reported in the UK in 1961 (Barber, 1961). It is now found world wide and with increasing incidence (World Health Organisation, Monitoring of Antimicrobial Resistance, 2003). Since 1990 hospitals in the UK have been voluntarily monitoring infection rates of many organisms including *S. aureus* (and MRSA). In 2000 it became mandatory to report and monitor *S. aureus* bacteraemia (positive blood cultures) infections within UK NHS Trust hospitals. In 2006 the Health Protection Agency (HPA) released a report which compiled all data recorded for national *S. aureus* bacteraemia, which included voluntary recordings from 1990, and mandatory recordings from 2001. This report showed that from 1990 to 2001 the number of MRSA cases as a proportion of total *S. aureus* bacteraemia infections rose from 5 % to 40 %. Numbers of MRSA cases peaked in 2003 at around 7500 (HPA – Mandatory Surveillance of Healthcare Associated Infections Report 2006). The current report finds that between 2003 and 2007 the numbers of MRSA bacteraemia has fallen to approximately 5000 (Surveillance of Healthcare Associated Infections Report: 2008). Presumably this is due to the implementation of improved hygiene regimes and increased awareness of cross contamination by hospital staff.

Although these findings are encouraging as the numbers of reported MRSA cases have been reduced, the numbers need to be dramatically reduced before we can say that

MRSA is under control. Why there has been such an increase in methicillin resistant strains is not known. It is possible that they carry additional virulence factors responsible for the increased incidence within the general *S. aureus* population. Particular clonal types of MRSA strains have been identified as being responsible for the majority of all MRSA infections depending on the locality. In the US strain USA300 predominates (Maree *et al.*, 2007; Gorwitz, 2008), whereas in the UK strains EMRSA-15 & EMRSA-16 cause the majority of infections (Sabersheikh and Saunders, 2004). To date no direct link has been found between increased virulence and methicillin resistance. Asymptomatic carriage of MRSA further confuses the situation, suggesting that they are not necessarily more virulent (Mainous *et al.*, 2006).

Community acquired MRSA (CA-MRSA)

S. aureus has been thought of historically as an opportunistic pathogen, only capable of causing infection in people already compromised due to illness, social circumstances or drug abuse, and were therefore generally associated with the hospital setting, this was also the case for MRSA (Groom *et al.*, 2001). Community acquired *S. aureus* infections in otherwise healthy individuals has been generally regarded as a rarity and was usually caused by methicillin sensitive *S. aureus* (MSSA) (Boyle-Vavra and Daum, 2007). However MRSA are now being more frequently isolated from the community. This is of concern, suggesting that these isolates might have acquired additional virulence genes. Additionally, the number of community acquired MRSA (CA-MRSA) isolates being transferred to the hospital setting is on the increase (Gorwitz, 2008). Initially it seemed likely that the origin of CA-MRSA was via transfer of hospital accuired MRSA (HA-MRSA) to the community. However CA-MRSA has been

isolated from patients with no previous exposure routes to explain the infection, for example patients with no previous history of hospital admission or contact with hospital personnel (Daum *et al.*, 2002; Boyle-Vavra and Daum, 2007). It is now therefore accepted that CA-MRSA did not arise from transmission of HA-MRSA (Naimi *et al.*, 2003). The transfer of CA-MRSA to the hospital setting is also concerning; if CA-MRSA strains are more virulent, in a hospital setting this could have grave effects on a large scale as patients may already be compromised. CA-MRSA is a major cause of skin and soft tissue infections (SSTIs) (Moran *et al.*, 2006), in particular boils associated with hair follicles. However it is the more serious invasive infections in healthy members of the community which are of particular concern. Symptoms include necrotising fasciitis (deep tissue infections) and necrotising pneumonia (breakdown of lung tissue) which can be fatal and are not associated generally associated with HA-MRSA (Ebert *et al.*, 2009)).

For some countries clonal types responsible for CA-MRSA infections have been identified. Clonal typing achieved by multi locus sequence typing or MLST, is based on the sequence of seven different housekeeping genes which are reasonably well conserved between isolates. The seven sequences are then compared to previously typed isolates, referred to as sequence types or STs, to determine if the isolates are related. Clonal type ST30 has been identified in Singapore as the predominating isolate, accounting for 64 % of all CA-MRSA over a two year period (Hsu *et al.*, 2006). ST8 (USA300) has been identified in the USA as the prevalent strain (King *et al.*, 2006). In Kuwait a study of 26 CA-MRSA showed that two STs predominated which were ST80 and ST30, however isolates were also found that also belonged to ST6, ST5, ST8, ST28

and ST35 (Udo *et al.*, 2008). Although HA-MRSA isolates also belong to some of the same STs as the CA-MRSA, suggesting that there may not be a clear distinction between ST and CA-MRSA. Although this could also be due to the cross over of CA-MRSA into the hospital setting resulting in the point of isolation not necessarily being the point of origin (Campanile *et al.*, 2009). As yet in the UK no clonal type for CA-MRSA has been identified. This may be due to the small number of reported cases of CA-MRSA compared to the US and other countries, which may be in part due to the ineffective screening methods available in the UK (Maeda *et al.*, 2008). Whilst the ST alone cannot differentiate between HA and CA-MRSA isolates it may be important in the future characterisation if other markers are identified.

In an attempt to characterise factors which lead to the proposed increased virulence of CA-MRSA isolates, studies have identified factors which are associated with the majority of predominant strains. One feature distinguishing CA-MRSA is that they are usually resistant to β-lactam (methicillin) and macrolide antibiotics (erythromycin), but are generally susceptible to other antibiotics used to treat *S. aureus* infections. This is in contrast to HA-MRSA which often have more widespread resistance, although the resistance and sensitivity patterns do vary amongst isolates (Herold *et al.*, 1998; Estivariz *et al.*, 2007; Cunha, 2008). This further supports the theory that CA-MRSA did not result from hospital transfer. The PVL toxin (Panton-Valentine leukocidin) is associated with the majority of predominating CA-MRSA isolates, and is proposed to be responsible for the deep tissue infections and necrotising pneumonia. PVL is phage encoded, and has also been implicated as an identifying feature which could help distinguish CA-MRSA from HA-MRSA (Naimi *et al.*, 2003; Holmes *et al.*, 2005;

Bocchini *et al.*, 2006). The methicillin resistance gene *mecA* is situated within a mobile genetic element in the chromosome referred to as SCC*mec* (Staphylococcal chromosome cassette *mec*) (Katayama *et al.*, 2000; Wielders *et al.*, 2001). To date six different sub types of the SCC*mec* locus have been identified which vary in size and can contain additional antibiotic resistance genes (Ito *et al.*, 1999; Daum *et al.*, 2002; Ma *et al.*, 2002; Ito *et al.*, 2004); it appears that CA-MRSA are associated predominantly with SCC*mec* type IV (Ma *et al.*, 2002; Campana *et al.*, 2007).

However these identifying features are not so clear cut as some HA-MRSA also produce PVL (Naimi *et al.*, 2003; Ramdani-Bouguessa *et al.*, 2006), and some *S. aureus* infections acquired in the community do not produce the PVL toxin (O'Brien *et al.*, 2004; Campana *et al.*, 2007; Cunha, 2008). Furthermore, a recent study found that in two MRSA PVL positive isolates, the types of their SSC*mec* loci could not be determined using the techniques employed, suggesting that a novel subset of SCC*mec* loci may exist (Maeda *et al.*, 2008). In addition some SCC*mec* types other than type IV have been associated with PVL positive CA-MRSA isolates (Deurenberg *et al.*, 2007; Tsai *et al.*, 2008), and there is evidence that CA-MRSA strains defective in PVL toxin production were as virulent in a mouse model as PVL positive strains (Voyich *et al.*, 2006). Therefore the link between methicillin resistance, PVL, and SCC*mec* type is insufficient to confirm the identity of MRSA isolates acquired in the community.

The lack of clarity in distinguishing between CA-MRSA and HA-MRSA is of some concern. Whilst PVL negative CA-MRSA infections present as less pathogenic isolates and are currently treatable with antibiotics ineffective against HA-MRSA, it is worth

considering that both methicillin sensitive and methicillin resistant isolates of *S. aureus*, once an opportunistic pathogen, are now able to cause infection in healthy individuals in the general community. This is of particular concern considering the notorious adaptability and rapid acquisition of multiple antibiotic resistance genes from other staphylococcal species and from other genera, associated with *S. aureus* (Ito *et al.*, 2003; Sievert *et al.*, 2008).

Vancomycin resistant *S. aureus* (VRSA)

Vancomycin is the preferential treatment used to combat strains of *S. aureus* with resistance to multiple antibiotics, in particular MRSA, as it is the only antibiotic which provides uniform coverage of these infections (Chang *et al.*, 2003). Vancomycin acts in a different way to methicillin, binding to the D-alanine-D-alanine residues of peptidoglycan monomers on the cytoplasmic membrane rather than to the PBP (Fig 1.2), but both act to prevent cell wall synthesis (Pootoolal *et al.*, 2002). However, vancomycin resistant *S. aureus* isolates have also been identified. Vancomycin resistance is determined using minimum inhibitory concentration (MIC) assays. Isolates that are sensitive to vancomycin (VSSA) have MICs between 0.5 and 2 µg/ml (Tiwari and Sen, 2006). Vancomycin resistant isolates are classified as either vancomycin intermediate resistant *S. aureus* (VISA), heterogeneous VISA (hVISA or hVRSA) or vancomycin resistant *S. aureus* (VRSA). VISA isolates have an increased tolerance to vancomycin having MICs between 4-8 µg/ml, and strains are described as fully vancomycin resistant (VRSA) if the MIC is in excess of 16 µg/ml (Tiwari and Sen, 2006) (http://www.cdc.gov/ncphi/disss/nndss/casedef/vancomycincurrent.htm). Isolates described as hVISA are mixed populations containing VSSA and VISA, these have an

MIC the same as MSSA (Sun *et al.*, 2009). It has been suggested that preferential treatment of MRSA infections with vancomycin has favoured the survival of cells with increased cell wall thickness, which leads to increased tolerance of the drug (Hiramatsu *et al.*, 1997; Sieradzki and Tomasz, 1999; Cui *et al.*, 2003; Fridkin *et al.*, 2003). It is likely that VISA and hVISA are derived from vancomycin susceptible strains in which clones containing multiple mutations leading to thickening of the cell wall have been selected for by exposure to vancomycin or β-lactam antibiotics, which have also been shown to induce vancomycin tolerance (Cui *et al.*, 2003). VISA isolates have increased tolerance to vancomycin with the potential to become fully resistant via further thickening of the cell wall (Domaracki *et al.*, 2000; Cui *et al.*, 2003). It is probable that the increased cell wall thickness is due to increased peptidoglycan production or decreased peptidoglycan turnover. Because vancomycin binds to the D-alanine-D-alanine residues of peptidoglycan monomers on the cytoplasmic membrane (Fig 1.2), thickened peptidoglycan layers outside the cytoplasmic membrane may inhibit the access of vancomycin to the D-alanine D-alanine targets in the cell wall, effectively mopping up the antibiotic (Hiramatsu, 2001; Cui et al., 2003). Defective Agr and SarA regulation has also been linked to cell wall thickening (Sakoulas *et al.*, 2002; Lamichhane-Khadka *et al.*, 2009), although the precise mechanism of increased resistance is not fully understood in these cases. The regulators SarA and Agr are discussed in more detail in the following Section 1.8.

True or full vancomycin resistance is conferred by the *vanA* gene which is expressed on a conjugative plasmid acquired from vancomycin resistant Enterococci (VRE) (Sievert *et al.*, 2008). *van*A encodes an enzyme which produces D-alanine-D-lactate residues in

the peptidoglycan layers instead of the usual D-alanine-D-alanine residues to which vancomycin binds (Fig 1.2). This D-lactate substitution prevents the binding of vancomycin rendering the antibiotic ineffective and the bacteria resistant. Vancomycin resistance as a result of increased cell wall thickness is deemed as a non-transmissible mechanism of resistance (http://www.dhh.louisiana.gov/offices/miscdocs/docs-249/Manual/StaphVRSA.pdf.), in contrast to *vanA* conferred resistance which can be transferred. VISA isolates have been identified world wide (Tenover *et al.*, 2001), whereas VRSA isolates, resistant to vancomycin via *vanA* expression, have currently only been identified in three localities, 7 isolates from the USA, 1 from India and 1 from Iran (Moubareck *et al.*, 2009). Although there are no current reports of VRSA isolates via acquisition of the *vanA* gene in the UK, UK incidence of VRE is the second highest in Europe (Goossens *et al.*, 2003), and so transfer of the conjugative plasmid is of concern. Also, whilst it is interesting to determine whether a vancomycin resistant strain is VISA (hVISA/hVRSA), resistant via thickening of the cell wall, or VRSA, resistant via acquisition of the *vanA* gene, clinically the only important fact is that these isolates do not respond sufficiently to vancomycin treatment to eradicate the infection.

1.4 Current antibiotic treatments.

There are still some antibiotics available to treat infections resistant to antimicrobials such as vancomycin, and methicillin, which are effective alone or when administered in combinations. Some of the most commonly used antibiotics against multi-resistant *S. aureus* infections are linezolid, quinapristin, trimethroprim and daptomycin (Sievert *et al.*, 2008). However, relying on existing treatments is becoming risky as resistance to Linezolid has also recently emerged (Tsiodras *et al.*, 2001). Standard cleanliness has

been highlighted as a general infection control measure and additional novel antimicrobial therapies have been investigated. In an attempt to control infection the use of heavy metals in coatings for hospital equipment and fixtures has been suggested, such as silver and copper (Bright *et al.*, 2002; Mehtar *et al.*, 2008). Various heavy metals have also been included in coatings for catheters including silver, zinc and copper, which have proved successful to varying degrees (Cowan *et al.*, 2003; Gollwitzer *et al.*, 2003; Rupp *et al.*, 2004). However the toxicity of these substances on human cells, and the possibility of metal resistance has caused some concern (Hollinger, 1996; Chopra, 2007; AshaRani *et al.*, 2009). Antibiotics have also been used to impregnate IV catheters with some success (Darouiche *et al.*, 1999; Aumsuwan *et al.*, 2007; Ramritu *et al.*, 2008), but this does not prevent colonisation by antibiotic resistant isolates. A recommendation by the Centers for Disease Control and Prevention suggests that catheters are removed and replaced every 72-96 hours to prevent colonisation and infection of the device, which is practised in some NHS Trust hospitals (O'Grady *et al.*, 2002; Webster *et al.*, 2008). However this can be impractical in patients who are difficult to catheterise, particularly if they require continuous IV therapies, and has been questioned by some practitioners (Webster *et al.*, 2008). Therefore the search for new preventive therapies and antimicrobials is ongoing (Nagayama *et al.*, 2002; Piper *et al.*, 2009; Smith and Drew, 2009).

1.5 Current methods for the prevention of *S. aureus* infections.

Antibiotic sensitive *S. aureus* isolates also cause serious infections which can be treated with the current range of antimicrobial therapies. However, treatment of *S. aureus* infection is also complicated as *S. aureus* infections are often in the form of a biofilm.

Biofilm formation is discussed in more detail in section 1.6.5 & 1.6.6, and are notoriously difficult to treat as they are multi-layered structures made up of bacterial cells, host and bacterial proteins and polysaccharide. They also contain cells in different physical and biochemical states, due to the nutrient and oxygen status of the different layers of the biofilm which often becomes anaerobic, and they can also be widely heterogeneous (Stewart and Franklin, 2008), and as a result antibiotic treatments often fail. This is due to either antibiotic resistance or tolerance as described previously, or to the inability of the antimicrobial agents to penetrate the biofilm sufficiently to eradicate the infection. Even where treatments do appear successful, the infection often recurs. This is believed to be due to the ability of staphylococci to lie dormant, singly or as part of the biofilm, for long periods of time (Williams *et al.*, 1997). Dormancy can also have a bearing on the effectiveness of antibiotics used, particularly if their target is an active part of cell growth, such as cell wall synthesis. This phenomenon is not true resistance but is antibiotic tolerance caused by the metabolic state of the bacteria within the biofilm and not due to genetic changes which result in antibiotic resistance (Williams *et al.*, 1997). However, dormancy itself has been disputed, and the actual reason behind tolerance is not known (Lewis, 2001).

Infection prevention.

As discussed earlier, *S. aureus* is a human commensal commonly isolated from normal healthy individuals, most frequently carried in the anterior nares (nostrils), which has been linked to increased risk of infection (Kluytmans *et al.*, 1997; Kalmeijer *et al.*, 2000). To address this, UK hospitals are advised to screen all patients for *S. aureus* carriage (Coia *et al.*, 2006), particularly those most likely to be at risk of developing an

infection, such as those who are frequently admitted. Patients may then be decolonised using antibiotic lotions depending on the site of colonisation and the procedure the patient is admitted for. This successfully reduces colonisation and infection for short periods of time; however after a few months colonisation recurs (Coates *et al.*, 2009).

Vaccines

In addition to the search for novel antimicrobial therapies and preventative technologies, recently many efforts have been made to develop a protective staphylococcal vaccine. There is currently no vaccine which prevents *S. aureus* infection in humans. There is a commercially available vaccine called Lysigrin® (Boehringer Ingelheim Vetmedica Inc) which has been used to vaccinate cattle against staphylococcal infection since the 1970s, to reduce *S. aureus* mastitis. In dairy cattle mastitis is a major concern as it is highly contagious and affects the quality of the milk produced by the herd (Middleton, 2008). The vaccine is based on five different types of *S. aureus* belonging to capsule types 5, 8 and 366 (Ma *et al.*, 2004). These capsule types are discussed in more detail in section 1.6.2. The use of the vaccine promotes passive immunity, reducing the severity of the infection (Middleton, 2008), and aids recovery but does not routinely prevent infection (Pankey *et al.*, 1985; Middleton, 2008).

In recent years there have been many attempts to design a human vaccine to immunise at-risk populations against staphylococcal infection. These include Staphvax® (Nabi Biopharmaceuticals), Aurexis® (Inhibitex) and Aurograb® (Novartis formerly NeuTec). These vaccines were developed against staphylococcal virulence factors, such

as cell wall proteins and polysaccharides (described in more detail in section 1.6), and all have subsequently failed to reach approval. Despite providing promising results from early studies, the vaccines could not provide adequate protection in more in-depth clinical trials. There are currently several other vaccines under development such as Pentastaph™ (Nabi Biopharmaceuticals), which as the name suggests, combines 5 different *S. aureus* virulence factors, three polysaccharides, capsular polysaccharides Types 5 and 8 and the cell wall antigen Type 336, plus 2 attenuated toxins, alpha toxin and PVL (www.nabi.com). The majority of *S. aureus* strains produce capsular polysaccharides 5 and 8, but 10 – 20 % of clinical isolates produce polysaccharide 336 instead of capsule (Verdier *et al.*, 2007). Polysaccharide 336 is not true capsule, but it has some similarities to cell wall teichoic acid and has not been fully characterised (Ma *et al.*, 2004). A recent evaluation of 234 clinical isolates in the US revealed that 50 % of isolates expressed capsular polysaccharide type 5, 42 % expressed type 8 and the remaining 8 % expressed polysaccharide 336 (Roghmann *et al.*, 2005). Another study in 2007 identified that, out of a cohort of 195 clinical isolates, 13 % tested negative for capsule type 5 or 8 and positive for type 336 despite carrying the gene for either *cap5* or *cap8* (Verdier *et al.*, 2007). It is possible that these strains contain mutations in their *cap* genes or the *cap* promoter regions, or that the strains have regulatory differences which prevent Cap5 or Cap8 production.

The inclusion of polysaccharide 336 in recent vaccine development may increase the protection offered by the vaccine, although the high adaptability of *S. aureus* and the wealth of virulence determinants it displays may cause limitations in any vaccine (Middleton, 2008). Pentastaph™ vaccine was due to undergo phase I clinical trials

early this year (2009), but as yet no details of the progress of the trial have been released (www.nabi.com). Pagibaximab®, formerly BSYX-A110 (Biosynexus Incorporated), is a broad spectrum Gram positive vaccine based on lipoteichoic acids, a common component of the bacterial cell wall in Gram positive bacteria. This vaccine has performed well in early clinical trials and is currently undergoing phase III trials, estimated to be complete in 2011 (www.biosynexus.com/clinicaltrials.html).

Why do vaccines fail?

Despite ongoing attempts to develop a protective vaccine for *S. aureus* infections the adaptability of the organism and the types of virulence determinants targeted by the vaccines have been speculated to be too narrow, and the cause of failure (Middleton, 2008). Many of the vaccines have targeted similar *S. aureus* antigens and the wide diversity within *S. aureus* populations may require multiple combinations of antigens to provide adequate protection. The fact that the bovine vaccine provides only limited protection despite containing capsular polysaccharides 5, 8 and 336 suggests that the human vaccine Pentastaph™ may encounter similar problems even though it contains extra antigens PVL and α-haemolysin. Only a small proportion of strains produce PVL, albeit the strains causing more serious infections, which also raises questions about the efficacy of the proposed vaccines. Moreover, not all strains produce α-haemolysin, although *in vivo* studies do show that strains that carry *hla* express more α-haemolysin *in vivo* than *in vitro*. The fact that not all *S. aureus* strains express the same array of virulence factors in all types of infection makes the choice of target for novel therapeutics difficult.

S. aureus also produces proteins that compromise humoral (antibody:antigen interactions mediated by B-lymphocytes) and cell mediated immunity (T-lymphocytes), which is discussed in section 1.6.3. This may explain why individuals suffer recurrent infections and may explain why attempts to develop a protective vaccine have so far failed (Foster, 2005; Middleton, 2008).

1.6 *S. aureus* virulence.

The human host is an inhospitable environment for bacteria to survive in. Not only do we have a remarkable immune system that for the majority of the time is successful in preventing infection, but inside the host certain nutrients essential for bacterial growth, such as iron, are very low. How the human commensal *S. aureus* is able to cause disease in the first place, regardless of antibiotic resistance, is a complex multi-factorial process involving many virulence factors such as proteins, polysaccharides and peptides. In addition to the range of factors discussed below, *S. aureus* is also capable of biofilm formation, a major virulence factor. During an infection biofilms help to protect the bacteria from eradication from the host via antibiotic treatments and the host immune response (Vuong *et al.*, 2004a). In addition, factors associated with biofilm formation specifically aid colonisation and interfere with the immune response aiding survival in the host. Components of the multifaceted virulence of *S. aureus* are discussed in more detail in the subsequent sections. Firstly, the major virulence factors are covered which are divided into three functional classes based on their biological action; those that protect the bacteria from the host immune system; those that mediate attachment of bacteria to cells or tissues; and those that promote tissue damage and spread of infection. Next, biofilm formation is discussed as a separate virulence factor,

as a general phenomenon, and then in more detail specifically relating to *S. aureus* disease, and is followed by virulence factor regulation. Finally, iron acquisition and its regulation are discussed, a major virulence factor, by which survival in the host is facilitated via the production of iron scavenging mechanisms which support bacterial growth in the low iron environment of the host. This arsenal of virulence factors makes *S. aureus* a highly adaptable organism. Furthermore, not all *S. aureus* strains express the same array of virulence factors in all types of infection, complicating target selection for novel therapeutics.

1.6.1 The human immune response to infection and *S. aureus* colonisation and immune evasion.

The human immune response is a complex system that comprises a multitude of biological processes. In simple terms we have two main responses to microbial invasion coordinated by our innate or non-specific immunity and by our acquired, specific immunity. The latter of these takes time to mobilise (Playfair and Bancroft, 2004) and involves the recognition of and reaction to specific antigens displayed by the invading microorganism. This is mediated by antibodies which bind the microbial antigen, targeting the organism for destruction (Parham, 2000). This response is generally activated if the innate immune response does not succeed in clearing the infection, and results in reactive protection mediated by antibody production, which protects against recurrent attacks. However, this is generally unsuccessful in protecting against recurrent *S. aureus* infection, as will be described in more detail later in this chapter (Parham, 2000). Innate immunity is primed and ready to respond to infection

at any time, and is the first threat to invading microorganisms (Parham, 2000). Innate immunity can be further sub divided into neutrophil recruitment and complement activation (Foster, 2005). Breach of the skin or mucosal membrane stimulates an immediate nonspecific inflammatory response which results in the recruitment of phagocytic cells to the site of infection, and the activation of complement (Rooijakkers *et al.*, 2005b).

Complement is the term applied to a group of immune system proteins that are involved in both the innate immune response and the acquired, adaptive response and can be activated in three ways. Two of these are dependant on the innate response, the alternative and lectin pathways, and the third, classical pathway, requires products of the adaptive immune response (Foster, 2005). Activation of complement results from the enzymatic cleavage of the main complement protein resulting in both the release of small peptides which act as chemo-attractants (Foster, 2005), and the attachment of an enzyme to the microbial surface called the membrane attack complex which forms a channel in the cell resulting in cell lysis (Roitt and Delves, 1995). Phagocytes recognise chemo-attractants secreted by the invading microbe and produced by the cleavage of complement which attract the phagocytes to the microbe (Foster, 2005). Complement proteins then bind to specific peptides on the microbial surface, a process known as opsonisation, which is then used to target the cell for phagocytosis and destruction (Playfair and Bancroft, 2004).

Activated complement peptides also stimulate the migration of neutrophils, phagocytic white blood cells, from blood vessels to the site of the infection, known as neutrophil

recruitment (Roitt and Delves, 1995). The neutrophils then recognise the opsonised microbes and phagocytose them (Roitt and Delves, 1995). Phagocytosed microbes are then exposed to antimicrobial peptides called α-defensins and the respiratory or oxidative burst, which is a release of deleterious hydrogen peroxide molecules (Playfair and Bancroft, 2004). These antimicrobial secretions are confined to a region of the neutrophil called the lysosome, protecting the host cell from any damaging effects. The engulfed microbe is partitioned off into the phagosome, which fuses with the lysosome to form the phagolysosome (Playfair and Bancroft, 2004). In the phagolysosome the α-defensins form pores in the bacterial cells causing lysis (Ganz and Lehrer, 1995), and peroxide exerts its toxic effects without causing damage to the host cell (Cunliffe, 2003).

1.6.2 *S. aureus* polysaccharides.

The main polysaccharides associated with *S. aureus* virulence are the capsular polysaccharides and polysaccharide intracellular adhesin, PIA.

Capsule

Bacterial capsules consist of high molecular weight polymers (Tzianabos *et al.*, 2001), which provide a protective layer around the cell, protecting them from desiccation. In *S. aureus*, capsules have been linked to evasion of the host immune system, inhibiting phagocytosis and reducing the efficiency of killing by phagocytes (Karakawa *et al.*, 1988; Thakker *et al.*, 1998; Watts *et al.*, 2005). Moreover, capsule production has been shown to be induced in intracellular *S. aureus* cells, further protecting the pathogen from the host defences and enabling persistence (Voyich *et al.*, 2005). There are 11

types of capsule associated with staphylococci, of which types 5 and 8 and polysaccharide type 336 are most commonly found in clinical isolates of *S. aureus* (Arbeit *et al.*, 1984; Sompolinsky *et al.*, 1985; Hochkeppel *et al.*, 1987; Guidry *et al.*, 1998). These have been used as targets for vaccine production as discussed earlier in section 1.5. *S. aureus* is generally considered to produce a microcapsule (Crossley and Archer, 1997), however rarely strains such as Smith Diffuse (type 2) and M (type 1) are heavily encapsulated (Ma *et al.*, 2004).

PIA/PNAG

PIA, polysaccharide intracellular adhesin, is now more commonly referred to as PNAG (Sadovskaya *et al.*, 2005). PNAG (poly-N-acetylglucosamine) is produced via the *ica* operon, under the negative control of the DNA binding protein encoded by *icaR* (Jefferson *et al.*, 2003). The *ica* operon was first described in *S. aureus* in 1999 by Cramton *et al*., (1999) and is made up of four co-transcribed genes organised *icaADBC,* with *icaR* being divergently transcribed, situated upstream of *icaA* (Fig 1.3). *icaA* encodes a trans-membrane protein which has N-acetylglucosaminyltransferase activity (Gerke *et al.*, 1998) but is only fully active in the presence of the product of *icaD*. IcaAD produces short oligomers, but oligomers of optimal lengths representing fully active polysaccharide can only be achieved in the presence of IcaC, a membrane protein (Mack *et al.*, 1996; Gerke *et al.*, 1998). It is believed that IcaC also aids mobilisation of the polysaccharide produced by *icaADC* to the cell surface (Gerke *et al.*, 1998). IcaB is responsible for deacetylation of the polysaccharide increasing its positive charge and is thought to enable attachment of the polysaccharide to the cell surface (Vuong *et al.*, 2004a; Cerca *et al.*, 2007). PNAG produced by *S. epidermidis* provides protection

Figure 1.3. The *ica* operon. Schematic diagram of the *S. aureus ica* operon, showing the four *ica* genes and the divergently transcribed transcriptional regulator of *ica, icaR. icaA* encodes a N-acetly-glucosaminyltransferase transmembrane protein which requires the product of *icaD* for activity. IcaADC is required for the production of fully active polysaccharide PNAG and IcaC is also thought to aid the mobilisation of the polysaccharide produced by *icaADC* to the cell surface. IcaB is responsible for deacetylation of the polysaccharide, increasing its positive charge and enabling attachment of the polysaccharide to the cell surface.

against antimicrobial peptides and increases resistance to phagocytes. In addition, increased resistance to phagocytes due to PNAG expression has been observed in *S. aureus* (Cerca *et al.*, 2007). PNAG has also been implicated with biofilm formation and virulence, but more recently its role has been questioned, which is discussed in more detail in section 1.7.

1.6.3 SERAMs and MSCRAMMs

S. aureus expresses many proteins which bind to host proteins and peptides, some of which are secreted and non-covalently reattached to the cell surface and referred to as secretable expanded repertoire adhesive molecules (SERAMs). Others are covalently attached to the cell wall by the enzyme sortase, referred to as MSCRAMMs, (microbial surface components recognising adhesive matrix molecules, (Patti *et al.*, 1994)). Typically these cell surface proteins specifically bind one or more host proteins as indicated in table 1.1, and some also have a role in immune evasion. Most host ligands are usually unavailable for *S. aureus* to bind as they are contained within tissues, and are only exposed after trauma, as such these bacterial proteins may be important in establishing initial infections.

Plasma contains high levels of fibrinogen, likely to be at the site of most traumas, which *S. aureus* can bind via the surface expression of many proteins including ClfA\B, FnbA\B, Efb, Eap and Emp. Fibrinogen binding by ClfA has also been shown to prevent phagocyte binding by coating the bacterial cell in host protein (Palmqvist *et al.*, 2004), and it was hypothesised that binding of fibrinogen by ClfB may assist protection of *S. aureus* in a similar way (Foster, 2005). Fibronectin is an abundant protein found

in body fluids and the extracellular matrix and is bound by the *S. aureus* surface proteins FnbA\B, Eap and Emp. Binding of *S. aureus* cells to fibronectin has been shown to; aid internalisation of *S. aureus* into endothelial and epithelial cells; mask the cells from immune attack; promote persistence (Peacock *et al.*, 1999; Sinha *et al.*, 1999; Massey *et al.*, 2001), and to facilitate platelet aggregation, which if occurs on damaged heart tissue, can lead to potentially life threatening infective endocarditis (Bayer *et al.*, 1995).

Collagen, found in cartilage and other tissues, is bound by the *S. aureus* cell surface protein Cna (Patti *et al.*, 1992), the only known collagen binding protein of *S. aureus*, and has been shown to facilitate osteomyelitis, a severe *S. aureus* infection of bone (Smeltzer and Gillaspy, 2000; Elasri *et al.*, 2002). Cna has also been implicated in binding to the collagen rich cornea, causing keratitis, a painful inflammation of the cornea which can have a long term implication on sight (Rhem *et al.*, 2000).

Almost all *S. aureus* strains produce the protein coagulase, where as most other staphylococcal species do not. This separates most *S. aureus* isolates from other pathogenic staphylococcal species such as *S. epidermidis* (Crossley and Archer, 1997). Coagulase is covalently linked to the cell wall and considered an important virulence factor as it binds to prothrombin and converts cell bound fibrinogen to fibrin, this causes the cell surface to be coated in fibrin, masking the bacteria from host defences and stimulates clot formation (McDevitt *et al.*, 1992).

The *S. aureus* SERAM Eap, in addition to binding a wide range of host proteins (see table 1.1), also binds to itself (Palma *et al.*, 1999; Hussain *et al.*, 2002). Self binding promotes cell aggregations, and binding host proteins facilitates *S. aureus* attachment to host cells such as fibroblasts and epithelial cells, and also promotes internalisation,

Table 1.1. *S. aureus* surface proteins that bind to host proteins

***S. aureus* cell surface protein**	**Host protein/cell bound**	**Reference**
MSCRAMMs		
Fibronectin binding protein A (FnbA)	Fibronectin Fibrinogen Elastin	(Froman *et al.*, 1987) (Heilmann *et al.*, 2004) (Roche *et al.*, 2004)
Fibronectin binding protein B (FnbB)	Fibronectin Fibrinogen Elastin	(Jonsson *et al.*, 1991) (Heilmann *et al.*, 2004) (Roche *et al.*, 2004)
Clumping factor A (ClfA)	Fibrinogen	(McDevitt *et al.*, 1994)
Clumping factor B (ClfB)	Fibrinogen	(Ni Eidhin *et al.*, 1998)
Collagen binding protein (Cna)	Collagen	(Patti *et al.*, 1992)
Protein A (SpA)	Von Willebrand factor IgG Platelets	(Hartleib *et al.*, 2000), (Forsgren and Sjoquist, 1966) (O'Brien *et al.*, 2002)
Coagulase (Coa)	Prothrombin	(McDevitt *et al.*, 1992)
SERAMs		
Extracellular adherence protein (Eap/Map)	Fibronectin Prothrombin Vitronectin Collagen Fibrinogen Eap Fibroblasts Epithelial cells	(Boden and Flock, 1992) (Palma *et al.*, 1999) (Hussain *et al.*, 2002) (Palma *et al.*, 1999)
Extracellular matrix binding protein (Emp/Ssp)	Fibronectin Fibrinogen Collagen Vitronectin	(Hussain *et al.*, 2001b)
Extracellular fibrinogen binding protein (Efb/Fib)	Fibrinogen	(Boden and Flock, 1994)

aiding persistence as the bacteria are hidden from the general immune response (Garzoni and Kelley, 2009). In addition to binding a wide range of ligands, Eap can also suppress neutrophil migration, and interfere with antibody generation, reducing proliferation and subsequent effectiveness of the antibodies (Chavakis *et al.*, 2002; Lee *et al.*, 2002). Most *S. aureus* strains tested contain *eap* (Hussain *et al.*, 2001a). This may in part explain why *S. aureus* infections are often recurrent, and why vaccines fail.

Emp and Efb also bind host proteins as listed in Table 1, and have both been shown to be important in virulence. The role of Emp in virulence was recently established *in vivo* as it was required for abscess formation in a murine model of infection (Cheng *et al.*, 2009). Also along with other virulence factors, the expression of Emp was shown to be increased in *S. aureus* cells that had been phagocytosed by neutrophils, implicating the surface protein in immune evasion (Voyich *et al.*, 2005). The importance of Efb in virulence was demonstrated in a murine wound infection model, where it was proposed to impede wound healing by preventing the formation of platelet aggregations (Palma *et al.*, 1996; Palma *et al.*, 2001).

Protein A, encoded by the *spa* gene, is a well known surface protein that binds to human immunoglobulin G (IgG). The purpose of IgG antibodies is to bind antigens presented by a range of invading microbes, which then target the microbe for phagocytosis (opsonisation) (Playfair and Bancroft, 2004). Protein A on the surface of *S. aureus* cells binds to the IgG molecule away from the antigen binding site, causing the IgG molecule to be in the wrong orientation, preventing opsonisation and phagocytosis (Crossley and

Archer, 1997). In pneumonia, *S. aureus* causes infection of the airway epithelium, here protein A has been shown to bind and activate TNFR1 cells, part of the immune response, which cause inflammation and influx of neutrophils to the site of infection, causing epithelial damage, and breathing difficulties that present as pneumonia (Gomez *et al.*, 2004). Protein A has also been implicated in thwarting antibody recognition of *S. aureus* by interacting with B cells, stimulating non-specific antibody release in a similar way to the superantigen toxin family discussed in section 1.6.4 (Bekeredjian-Ding *et al.*, 2007).

In addition to promoting bacterial adhesion to host proteins, the expression of some stapylococcal proteins have been shown to prevent bacterial attachment. SasG is a cell surface protein that has previously been shown to promote binding of *S. aureus* to nasal epithelial cells (Roche *et al.*, 2003). However, surface expression of SasG also blocks the recognition of matrix binding proteins by other *S. aureus* cell surface proteins such as ClfB (Roche *et al.*, 2003). This seems contrary as these proteins have an established role in *S. aureus* colonisation. However, recently SasG has been shown to form fimbrils on the bacterial surface which themselves promote attachment to host cells (Corrigan *et al.*, 2007). This may be an effect required for attachment to specific cells such as dead epithelial cells where other host proteins may be unavailable.

Pls is a plasmin sensitive surface protein, the gene for which is located within the SCC*mec* locus which also harbours the methicillin resistance gene *mecA*, and is therefore only found in methicillin resistance strains of *S. aureus* (Ito *et al.*, 2001; Savolainen *et al.*, 2001; Werbick *et al.*, 2007). Pls is a homologue of SasG and the

S. epidermidis surface protein Aap which is essential for biofilm formation in *S. epidermidis*. Like SasG, Aap also forms fibrils which protrude from the cell surface facilitating biofilm formation (Hussain *et al.*, 1997; Banner *et al.*, 2007). Pls has not been assayed for biofilm formation, but it has been shown to be involved in adhesion to nasal epithelial cells which may aid host colonisation (Roche *et al.*, 2003). Furthermore, like SasG, Pls also blocks the recognition of host binding proteins (Savolainen *et al.*, 2001).

1.6.4 Toxins

S. aureus produces a wide range of exotoxins such as cytotoxins and the superantigens. These co-ordinate to cause host cell damage, promote invasion, release nutrients and aid avoidance of the host immune system. Some staphylococcal toxins as well as causing host cell damage directly can also act as superantigens.

Superantigens

Superantigens interfere with the immune response by inerfearing with T-cells. Normally on the detection of an infectious substance T-cells are activated stimulating an immune response (Kohge *et al.*, 1998). The interaction of superantigens with T-cells leads to the release of non-specific antibodies creating an abnormal response that is ineffective (Balaban and Rasooly, 2000). Superantigens include the staphylococcal enterotoxins (SEs, also referred to as endotoxins) SEA-E and SEG-Q, and two toxic shock syndrome toxins, TSST1 and TSST2 (Bradford *et al.*, 1997; Orwin *et al.*, 2003; Schlievert *et al.*, 2008). SEA-E and SEI have been fully characterised as superantigens, but the effects of the remaining SEs have yet to be established *in vivo*, and may be

better described as '*S. aureus* exotoxin like' (SEl) (Lina *et al.*, 2004; Schlievert *et al.*, 2008). TSST1 & 2 cause disease most commonly associated with tampon use, but they can also cause skin infections. Other SEs are also responsible for causing toxic shock syndrome but TSST1 is the most common cause (McCormick *et al.*, 2001).

Enterotoxins are found encoded by various different loci in the *S. aureus* genome, on the chromosome, on phages, pathogenicity islands or on plasmids, and their location can vary between strains (Shalita *et al.*, 1977; Shafer and Iandolo, 1978; Betley and Mekalanos, 1985). In addition to their superantigen activity, SEs are also highly heat stable proteins that resist enzymatic digestion in the gut, and as a result are responsible for *S. aureus* food poisoning outbreaks (Le Loir *et al.*, 2003). Effects of ingestion of toxin are often worst in the elderly, but not usually fatal (Balaban and Rasooly, 2000).

An additional plasmid based toxin, SER, has also been identified which stimulates antibody release like other enterotoxins (Omoe *et al.*, 2003). Five other putative superantigen like toxins have also been discovered called SETs which resemble the SEs but represented a new family of exotoxins (Williams *et al.*, 2000). One of these, SET1, was cloned and expressed and was able to stimulate cells *in vitro* in a superantigen like response. However, the remaining four have not yet been further characterised (Williams *et al.*, 2000). However, to avoid confusuion these toxins have now been renamed to standardise nomenclature of the staphylococcal toxins to staphylococcal superantigen-like proteins SSL (Lina *et al.*, 2004). As previously mentioned in this section Eap can cause an inflammatory response similar to that seen by superantigens (Scriba *et al.*, 2008). The structure of the Eap is similar in some aspects to that of a

superantigen (Geisbrecht *et al.*, 2005). However it has since been determined that Eap is not a superantigen due to its non-specific interactions with a variety of ligands (Massey *et al.*, 2007).

In addition to the above functions, the expression of TSST1 or SEB has been shown to have negative regulatory effects on the expression of themselves and of most other exotoxins, possibly via direct binding of the precursor protein to target promoter sequences (Vojtov *et al.*, 2002). The fact that these toxins can exert a regulatory effect on other virulence factors may suggest that they are preferentially expressed in some types of infection, and further *in vivo* expression studies may help to clarify this.

Cytotoxins

S. aureus also produces an array of cytotoxins such as the haemolytic toxins, α-toxin or α-haemolysin, β-haemolysin, δ-haemolysin, γ-haemolysin and the less common, panton-valentine leukocidin (PVL). These toxins attack red blood cells, platelets, other immune cells, epithelia and endothelial cells releasing nutrients and promoting tissue invasion (Colin *et al.*, 1994; Lindsay, 2008). α-haemolysin is a pore-forming toxin produced by a large proportion of *S. aureus* strains, forming an oligomeric pore in the cell membrane of cells such as erythrocytes, platelets, endothelial cells, fibroblasts, T-cells and keratinocytes (Bhakdi and Tranum-Jensen, 1991). Pore formation leads to diffusion of ions and molecules out of the targeted host cell which results in cell death and can also interfer with various aspects of the immune response, such as platelet activation and cytokine release (Bhakdi and Tranum-Jensen, 1991). Also *hla*, the gene which encodes α-haemolysin has been implicated in virulence in an *in vivo* guinea pig

model of device related infection; levels of *hla* transcription were increased compared to levels of expression observed *in vitro* (Goerke *et al.*, 2001).

β-haemolysin is also able to induce lysis of erythrocytes by cleaving molecules in the eukaryotic cell membranes, allowing small molecules to leak out of the cell leading to the release of nutrients and host cell death. γ-haemolysin has been studied in less detail but also causes pore formation which leads to an ion efflux that damages the host cell and can activate an immune response.

PVL and its association with community acquired *S. aureus* infection has been discussed in section 1.3. PVL is a phage encoded cytotoxin, formed from the combination of two proteins, LukS-PV and LukF-PV (Colin *et al.*, 1994; Miles *et al.*, 2002) . The resulting protein acts as an octamer which forms pores in the cell membrane of leukocytes, resulting in necrosis and host cell death (Miles *et al.*, 2002; Jayasinghe and Bayley, 2005; O'Hara *et al.*, 2008). PVL is mainly associated with necrotic skin lesions, but is also linked to patients with severe necrotising pneumonia, which has a high mortality rate (Kravitz *et al.*, 2005). It has also been shown to be responsible for changes in transcriptional expression of a wide range of genes, including *spa* (protein A) and *clfA* (clumping factor A) (Labandeira-Rey *et al.*, 2007). Suggesting that, like the regulation observed by SEB and TSST1, the expression of certain toxins in certain infection sites may be preferentially expressed over others.

Other virulence factors and immune modulators

Some *S. aureus* strains also express other immune modulating factors, the genes for which are located on pathogenicity islands, regions of hyper-mobile DNA which can be transferred between cells via phage mobilisation, or transposon insertion or via the acquisition of plasmids (Lindsay, 2008). The gene for immune modulation protein CHIPS (chemotaxis inhibitory protein of staphylococci) is located on a mobile region of DNA called the immune evasion cluster (IEC), and is part of a β-heamolysin converting phage (van Wamel *et al.*, 2006), a region of phage inserted DNA which interrupts the β-heamolysin gene. Also located on the IEC is the gene for staphylococcal complement inhibitor SCIN and the toxins staphylokinase (Sak) and enterotoxin A (Sea) (van Wamel *et al.*, 2006). CHIPS is a small excreted protein that inhibits the chemotactic mobilisation of neutrophils by blocking the signals to which they respond (Veldkamp *et al.*, 2000; de Haas *et al.*, 2004), possibly interacting with Eap to suppress neutrophil migration (Foster, 2005). SCIN is able to inhibit all three complement activation pathways (Rooijakkers *et al.*, 2005a).

S. aureus also secrete a range of extracellular proteases, which function as degradative compounds for redundant staphylococcal proteins; however they have also been linked to degradation of molecules involved in the host immune response (Lindsay, 2008). Lipases secreted by *S. aureus* have also been observed to increase resistance to phagocytotic killing (Rollof *et al.*, 1988).

S. aureus was named because of its characteristic golden pigment produced by caroteniods. Recently it has been observed that these pigments protect *S. aureus* from

the oxidative burst encountered by engulfed cells by neutralising oxygen free radicals (Liu *et al.*, 2005); yet another method *S. aureus* have to avoid phagocytosis and clearance from the host.

1.6.5 Biofilm formation, a general introduction.

Biofilm formation is ubiquitous in nature, and is achieved by almost all microorganisms. It is typically associated with wet or damp surfaces, although biofilm formation on dry surfaces does occur on rocks in desert locations (Azua-Bustos *et al.*, 2009). Biofilms are often considered detrimental as they can cause damage, or increase costs. Adhesion of microorganisms to the submerged surface of a boat can pave the way for attachment of higher organisms such as barnacles (Roberts and Tsamenyi, 2008); this has been shown to slow down ships and increase their fuel consumption, a process known as bio-fouling (Roberts and Tsamenyi, 2008). Biofilms are also responsible for the plaque found on our teeth causing gum disease and tooth decay (Marsh, 2006). Biofilms can however, be beneficial. Biofilms can be found inside the roots of plants where the bacteria aid nitrogen fixing, important for plant growth (Fujishige *et al.*, 2006). Also in sewage and water treatment facilities, microorganisms form the major 'active' components of activated sludge and biofilms are encouraged to form on the surfaces of filters over which fouled water is passed (Fouad and Bhargava, 2005). Bacteria within these biofilms breakdown the organic matter and protozoan species remove pathogenic material aiding water purification. These are examples of heterogeneous biofilms, as they consist of multiple species and different genera of microorganisms, typically bacteria and protozoa (Fouad and Bhargava, 2005).

It is believed that all modern-day bacteria have inherited the ability to form biofilms from early waterborne bacteria, that evolved ways of adhering to submerged surfaces in fast flowing rivers and in the sea, to survive the hostile conditions (Lappin-Scott and Costerton, 1995). Biofilms are an advantageous state for bacteria to exist in, as in a biofilm microorganisms are usually encased within a polysaccharide layer providing a protective micro-environment for the enclosed cells, shielding them from fluctuations in temperature, chemical changes, and biological attack (Costerton *et al.*, 1994; Cernohorska and Votava, 2002). The formation of a biofilm is recognised to occur in distinct phases, initial attachment of free living cells, further cohesion and adhesion of proliferating cells, followed by maturation of a multi-layered cohort encased in polysaccharide (Fig 1.4) (Palmer and White, 1997; Vuong *et al* 2002). This is also followed, if conditions are permissive, by detachment of cells from the biofilm, facilitating colonisation of new sites (Romeo, 2008). Detachment is also believed by some researchers to be a programmed evolutionary step, which allows the dispersal of cells which favoured biofilm formation to inhabit new sites (Lappin-Scott and Costerton, 1995).

1.6.6 Biofilm formation and disease.

From a medical standpoint biofilm formation is associated with disease, often via colonisation of artificial surfaces such as replacement joints, heart valves and catheters, collectively referred to as indwelling devices. This is highly detrimental increasing not only morbidity and mortality but also hospital costs due to lengthy hospital stays; often complicated antimicrobial therapies and sometimes further surgery to remove and replace the infected device are required. In the US biofilm related disease has been

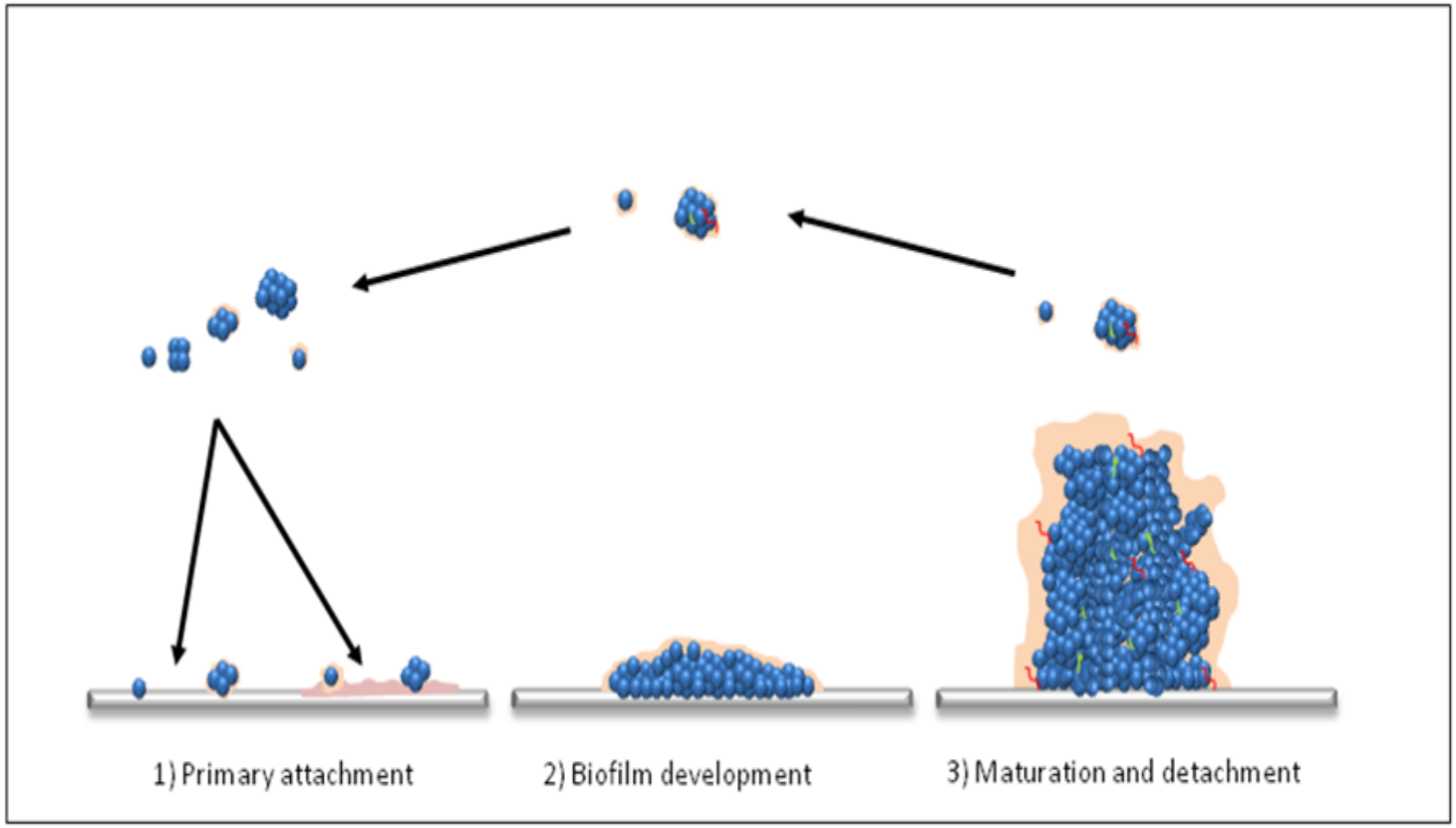


Figure 1.4. Schematic representation of biofilm formation.

1) Single or clumps of cells, from primary infection sites or from a new infection, attach to a prosthetic device directly or to host proteins (pink) coating the device, or directly to host tissue.
2) Attached cells then proliferate and produce polysaccharide and other cohesive factors such as proteins.
3) Mature biofilm develops. These are multi-layerd, multi-factorial entities consisting of protein (green), polysaccharide (buff) and extracellular DNA (red). The cells within a mature biofilm can be in various metabolic states and can also be heterogeneous. These cells can evade the host immune system either due to phase variation or due to the expression of host evasion factors. Individual or clumps of cells detach from the biofilm and circulate to colonise other sites.

reported toaccount for 65 % of all hospital acquired infections (Potera, 1999). Although the majority of biofilms form on artificial implants, some natural host surfaces are also colonised by biofilms, as is the case in native valve endocarditis and osteomyelitis caused by *S. aureus* (Lowy, 1998; Lew and Waldvogel, 2004), and biofilms formed in the lungs of cystic fibrosis patients caused predominantly by *Pseudomonas aeroginosa* (Bjarnsholt *et al.*, 2009).

Initially, individual or clumps of planktonic cells come into contact with either the native tissue, the surface of the implanted device, or the host proteins that rapidly coat the device on implantation (Fig 1.4) (Mack, 1999). These adherent cells then proliferate to form a mature multi-layered biofilm of bacterial cells encased in a matrix composed of bacterial polysaccharide, host and bacterial proteins (Donlan and Costerton, 2002; Vuong and Otto, 2002). Clinically, biofilms are unusual as they are usually made up of a single species; although on occasion mixed species biofilms are associated with chronic wound infections (Gjodsbol *et al.*, 2006) where *S. aureus* can be found with *Pseudomonas aeruginosa* and other bacteria. *S. aureus* are an important human pathogen, and is responsible for a large proportion of infections in the hospital setting. Infection and disease caused by *S. aureus* is facilitated by biofilm formation, except in cases of bacteraemia. However, bacteraemia, an infection of the blood stream, can often occur as a secondary infection which is believed to arise from dissociation of cells from a biofilm infection elsewhere (Fux *et al.*, 2004). Single species *S. aureus* biofilm formation is the focus of the work in this study.

1.7 Factors currently implicated in *S. aureus* biofilm formation.

The factors described in the previous section all interact to make *S. aureus* a highly adaptable pathogen. Studies specifically concentrating on *S. aureus* biofilm formation have been plentiful over the past 10 years and biofilm production has been found to be a critical part of *S. aureus* virulence. Recently it appears from *in vitro* assays and animal studies that there are two major ways in which biofilm formation occurs, one is dependant on the *ica* operon and PNAG production, while the other is *ica* independent.

Biofilm formation has been observed in PNAG deficient strains, although the alternative factors involved have not been fully characterised. However they have been identified in some cases as being proteinaceous, as proteinase treatment removed the adherent film (Cucarella *et al.*, 2001; Fitzpatrick *et al.*, 2005; Toledo-Arana *et al.*, 2005; O'Gara, 2007; O'Neill *et al.*, 2007; Lauderdale *et al.*, 2009). The following section will first discuss PNAG dependant biofilm formation, and then secondly, PNAG independent biofilm formation will be briefly discussed.

PNAG

PNAG has been well studied and is the best understood factor involved in biofilm formation in both *S. epidermidis* and *S. aureus*; although its role in this has become less clear over recent years. In early studies, all *S. aureus* strains tested carried the *ica* operon including clinical isolates (Cramton *et al.*, 1999; Fowler *et al.*, 2001). In addition mutants of both *S. epidermidis* and *S. aureus* defective in the production of PNAG (PIA) were incapable of forming a biofilm under standard conditions (Heilmann *et al.*, 1996; Cramton *et al.*, 1999). However, other studies found that only some

isolates tested positive for *ica* genes by PCR (Arciola *et al.*, 2001), although it was suggested that this was probably due to the primers used, as they were designed against the sequence of *S. epidermidis ica* operon, and were not thought to be a true reflection of the *ica* status of the *S. aureus* strains (Rohde *et al.*, 2001). Furthermore PNAG has been shown to be expressed in *S. aureus in vivo* (McKenney *et al.*, 1999), and de-acetylation of the polymer by the IcaB protein was found to be essential for key virulence mechanisms of *S. epidermidis*, namely biofilm formation, colonization, and resistance to neutrophil phagocytosis and human antibacterial peptides. Furthermore, persistence of the *S. epidermidis icaB* mutant strain was significantly impaired in a murine model of device-related infection (Vuong *et al.*, 2004b). However, recent increasing reports of *ica* independent biofilm formation in *S. aureus* (Cucarella *et al.*, 2001; Fitzpatrick *et al.*, 2005; Toledo-Arana *et al.*, 2005; O'Neill *et al.*, 2007) might indicate that PNAG is a minor component of *S. aureus* biofilm formation and more important in *S. epidermidis* biofilm formation (Izano *et al.*, 2008).

S. aureus surface proteins also play a role in biofilm formation due in part, to their binding activity of host matrix proteins, as described earlier. However the only direct link with biofilm formation in *S. aureus, in vitro* has been the biofilm associated protein Bap, although this protein has so far only been identified in bovine strains (Latasa *et al.*, 2006). Bap positive strains were able to form a biofilm in the absence of *ica* (Cucarella *et al.*, 2004), suggesting that bovine isolates are also capable of *ica* independent biofilm formation. These findings suggest that biofilm formation in *S. aureus* is not due to the expression of a single factor, and that in different conditions, different factors may be important (Toledo-Arana *et al.*, 2005). It also suggests that the loss of some factors can

be compensated for by others, under certain conditions, implying that many factors may be required for biofilm production depending on the infection site (O'Neill *et al.*, 2007).

Teichoic acids in biofilm formation

Cell wall teichoic acids have also been implicated in biofilm formation. In a *S. aureus* strain with altered cell wall teichoic acid production, adhesion *in vitro* to glass and plastic was reduced (Gross *et al.*, 2001). This was found to be due to the overall increase in negative cell surface charge caused by the loss of D-alanine in the teichoic acid (Gross *et al.*, 2001). D-alanine cell wall teichoic acids have also been linked to *S. aureus* survival in the host (Peschel *et al.*, 1999).

eDNA in biofilm formation.

Extracellular DNA (eDNA) has recently been implicated in biofilm formation in many microbial species such as *Pseudomonas aeruginosa, Candida albicans, Bacillus cereus* and *S. aureus* (Whitchurch *et al.*, 2002; Rice *et al.*, 2007; Izano *et al.*, 2008; Paramonova *et al.*, 2009; Vilain *et al.*, 2009). In *S. aureus* secretion of eDNA is controlled in part by *cidA*, which is part of an operon mediating cell lysis (Rice *et al.*, 2007). *cid*A is regulated by the catabolite control protein A, CcpA. Carbon catabolite repression in bacteria allows the expression of factors involved in the utilisation of preferential carbon sources. For example in the presence of glucose, a preferential carbon source, components of the TCA cycle are repressed (Blencke *et al.*, 2003; Seidl *et al.*, 2006; Seidl *et al.*, 2008). In addition CcpA has been found to regulate virulence factors such as TSST1 and biofilm formation through its influence on *ica* transcription (Seidl *et al.*, 2008) and via its control of CidA production. Suggesting that, nutritional

signals influence the expression of virulence factors. The role of eDNA in biofilm formation is not fully understood; it is possible that it is a structural component resulting from programmed cell lysis, or that it serves a more complex purpose in facilitating gene diversity (Steinberger and Holden, 2005).

1.8 Regulation of virulence determinants

The production of virulence factors, as with any non-essential gene product, is not only costly to the cell, but may also be disadvantageous depending on the surrounding environment. Therefore, the expression of essential and non-essential factors is tightly co-ordinated by regulators that have both positive and negative effects on the expression of genes depending on temporal and environmental signals. Regulation of *S. aureus* gene expression is facilitated by a growing number of regulators. Many *S. aureus* regulators have been shown to interlink co-regulating the expression of virulence factors in a complex manner (Arvidson and Tegmark, 2001; Goerke *et al.*, 2001; Manna and Cheung, 2003; Li and Cheung, 2008; Lauderdale *et al.*, 2009). *S. aureus* has 16 two component regulator systems (based on N315 and MRSA252 sequence data) (Kuroda *et al.*, 2001), various DNA binding proteins, and other regulatory systems such as small RNA regulators. In this section the main focus will be the regulators which have been studied in more detail such as the sigma factors, Agr, SarA and Sae, which have been implicated in virulence. To date findings have suggested that Agr together with the SarA family of regulators and Sae are the three major regulatory systems involved in *S. aureus* virulence regulation (Lauderdale *et al.*, 2009). However in addition the ferric uptake regulator Fur which controls uptake of the essential nutrient iron, and has also

been implicated in virulence, is discussed as a separate regulator at the end of this section.

1.8.1 Sigma factors.

S. aureus has regulatory elements that control gene expression at all levels, e.g. transcription, translation and post-translation. Important regulators that control transcription are the sigma factors. Sigma factors are responsible for attaching RNA polymerase to specific promoter regions of target genes, initiating transcription (Wosten, 1998). Most bacteria produce several sigma factors which identify different promoter sequences facilitating variable function. In *S. aureus* sigma factor A (σ^A) is responsible for transcription of essential housekeeping genes, and an alternative sigma factor (σ^B), regulates the transcription of genes involved in the general stress response. In addition a third *S. aureus* sigma factor (σ^H) has been identified, SA0492 which is an orthologue of σ^H found in *B. subtilis* (Morikawa *et al.*, 2003). σ^H is responsible for the regulation of genes associated with genetic competence, which involves the uptake and usage of foreign DNA (Morikawa *et al.*, 2003). *S. aureus* is unusual in that only these three sigma factors have been identified. Other bacteria have many more sigma factors, for example *B. subtilis* has 17 sigma factors (Yoshimura *et al.*, 2004). It is possible that the wealth of other regulators which exist in *S. aureus* compensate for the lack of sigma factors.

1.8.2 Quorum sensing.

Sensing and responding to the surrounding environment is key to *S. aureus* adaptability, and is achieved, in part, by quorum sensing via the two component regulator (TCR)

system Agr (Projan and Novick, 1997). *S. aureus* has two quorum sensing systems, *luxS* and *agr*, but *luxS* has been shown to be non-functional in a quorum sensing capacity (Doherty *et al.*, 2006). Quorum sensing systems involve the production of low molecular weight molecules, which are secreted and detected by the bacteria, in a concentration dependant manner.

The Agr system comprises an operon of four genes, *agrBDCA* and a small non coding RNA, RNAIII, which also encodes δ-haemolysin *(hld)* (Fig 1.5). These are controlled by two divergent promoters P2 and P3. The promoter P2 produces RNAII, the transcript of *agrBDCA; agrB* encodes a transmembrane protein which is believed to be involved in the modification and export of the auto inducing peptide, AIP, produced by *agrD* (Novick and Muir, 1999). AgrA and AgrC are responsible for the classical response regulation associated with TCR's, AgrC being a histidine kinase sensor (Lina *et al.*, 1998), which when activated by the binding of API becomes phosphorylated. The N-terminus of AgrC resides in the cytoplasm and upon activation causes phosphorylation of AgrA, the response regulator. Activated AgrA binds the region between P2 and P3, increasing transcription from the two promoters. Activation of the P3 promoter produces transcripts for the δ-haemolysin and the sRNA molecule RNAIII. It is the production of RNAIII that leads to the majority of positive and negative regulatory effects of the *agr* system, although recently there has been a report of *agr* regulation independently of RNAIII, due to the direct binding of AgrA to promoter regions of target genes (Queck *et al.*, 2008).

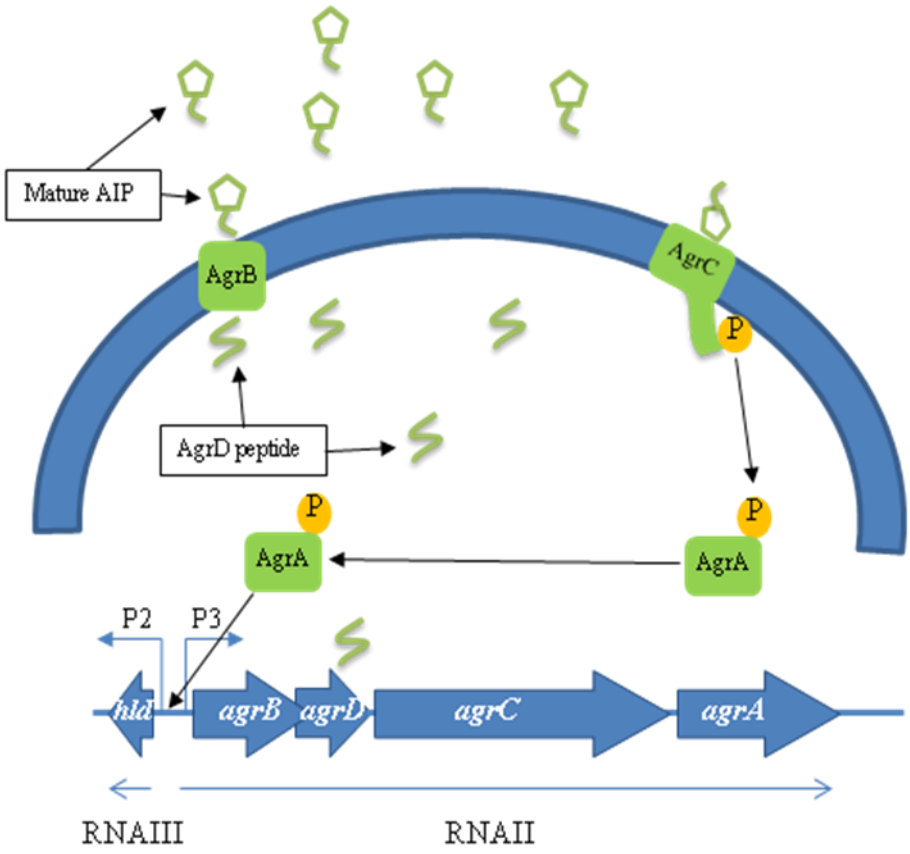


Figure 1.5. Schematic diagram of the *S. aureus agr* operon.
AgrA and AgrC are responsible for the classical response regulation associated with TCR's, AgrC being a histidine kinase sensor and AgrA is the response regulator. *agrB* encodes a transmembrane protein which modifies and aids the export of the auto inducing peptide, AIP, produced by *agrD* (Novick and Muir, 1999; Lina *et al.*, 1998). The accumulation of AIP causes phosphorylation of AgrC which then leads to the phosphorylation of AgrA. Activated AgrA binds the region between P2 and P3, increasing transcription of RNAII and RNAIII. Production of RNAIII leads to the majority of positive and negative regulatory effects of the *agr* system, although direct binding of AgrA to promoter regions of target genes has also been observed (Queck *et al.*, 2008).

Agr in *S. aureus* has been studied in great detail, and is thought to be a central regulator controlling virulence gene expression *in vitro* (Novick and Geisinger, 2008). *In vitro*, Agr expression allows the production of cell surface adhesins during the early stages of growth. When cell densities increase and the level of AIP reaches predetermined threshold, the production of surface proteins is repressed by the negative regulatory effects of RNAIII, and the production of toxins and other secreted proteins is up regulated (Janzon and Arvidson, 1990; Novick *et al.*, 1993). Regulation via RNAIII has been shown to be largely due to antisense base pairing, typically associated with regulation via small RNAs modulating the expression of many targets, sometimes via other regulators (Queck *et al.*, 2008). Small regulatory RNAs are described in more detail in section 1.10. Despite these findings, the role of Agr *in vivo* has been doubted. The questionable role of Agr *in vivo* was reviewed by Kong *et al.* in 2006 who concluded that quorum sensing was essential for biofilm related infection (Kong *et al.*, 2006). However a report by Traber *et al.* in 2008, recognised that whilst most clinical isolates were *agr* positive, some strains isolated from sites of infection were *agr* negative (Traber *et al.*, 2008). The study postulated that the *agr* negative isolates may have originated post infection, and continued to support the requirement for a functional Agr in early stages of infection (Traber *et al.*, 2008). In addition it has been hypothesised that active Agr is required for biofilm detachment and colonisation of new sites, although this has not been confirmed *in vivo* (Yarwood *et al.*, 2004; Boles and Horswill, 2008). Hopefully the further study of Agr and other *S. aureus* virulence regulators, with which Agr interacts, such as SarA and Sae (which are discussed in more detail in the following sections), will clarify the role of Agr in infection.

As well as its role in virulence regulation the *agr* system can also facilitate cross inhibition (Otto, 2001). This is a term usually applied to microbes which produce antimicrobial substances, favouring the growth and survival of one organism over another. However in this case, the term refers to the production of surface adhesins and excreted proteins, allowing then to compete for sites of colonisation where multiple species or strains might exist. Different AIPs are secreted by different strains, which are recognised by a specific type of AgrC sensor, and as such Agr systems can be split into four distinct groups based on the sequence of the AIP and sensor specificity (Ji *et al.*, 1997). The recognition of an AIP by an AgrC sensor of a different group leads to an altered response from the sensor molecule (AgrC) depending on the strain and the AIP (Ji *et al.*, 1997). These AIP groups can both inhibit systems belonging to other AIP groups, and activate *agr* systems of the same group (Ji *et al.*, 1997). The precise binding of the AIP to AgrC has not been fully elucidated for all groups, but specific hydrophilic and hydrophobic regions in the C-terminus, trans-membrane domain of AgrC have been identified as targets for group I and group IV AIP interactions respectively (Wright *et al.*, 2004). With this in mind studies have been carried out in an attempt to link *agr* types to specific diseases. These have produced some evidence to suggest an association between *agr* type and disease although the relationship does not appear to be straight forward. One study found that *agr* types seemed to be well distributed between sites of carriage and different infection sites (Jarraud *et al.*, 2002), whereas another suggested a link between type IV *agr* and skin infections (Garbacz *et al.*, 2009). Another study found that among asymptomatic nasal carriage isolates only *agr* types I-III were identified (Shopsin *et al.*, 2003). In addition, *agr* types have been associated with the expression of toxins, some of which are associated with particular

disease; for example *agr* type IV has been associated with exfoliative toxin production which causes scalded skin syndrome (Jarraud *et al.*, 2000; Jarraud *et al.*, 2002). In addition different Agr groups have been shown to produce different levels of toxin (Collins *et al.*, 2008). This may suggest that the regulation of toxin production by different Agr types could be related to disease. It is possible that a larger scale assessment of strains may give a clearer picture of the relationship between *agr* type and disease.

1.8.3 SarA family of regulators

SarA belongs to a family of 11 DNA binding proteins that regulate transcription, including the products of 9 *sar* genes, *sarA, sarR, sarS, sarT, sarU, sarV, sarX, sarY, sarZ,* and MgrA and Rot (Cheung *et al.*, 2008a). SarA is the most widely studied of the SarA family; far less is known about the other *S. aureus sarA* regulator homologues that act directly on specific targets or via *agr*. The Sar family have interlinking regulatory roles which are highly complex as illustrated in Fig 1.6. This complex network of pathways involving the Sar homologues will be addressed briefly, however SarA, as it has been more widely studied, will be covered in greater detail.

SarT represses α-haemolysin (*hla*), and RNAIII transcription, but is also repressed by SarA and Agr (Schmidt *et al.*, 2001). In addition SarT increases SarS transcription, resulting in increased levels of protein A and repression of *hla*. SarV regulates the production of genes involved in autolysis (cell division) and protease production (*scdA, splA, lrgB, atl* and *aur*) (Manna *et al.*, 2004). Initial investigations into the role of SarZ

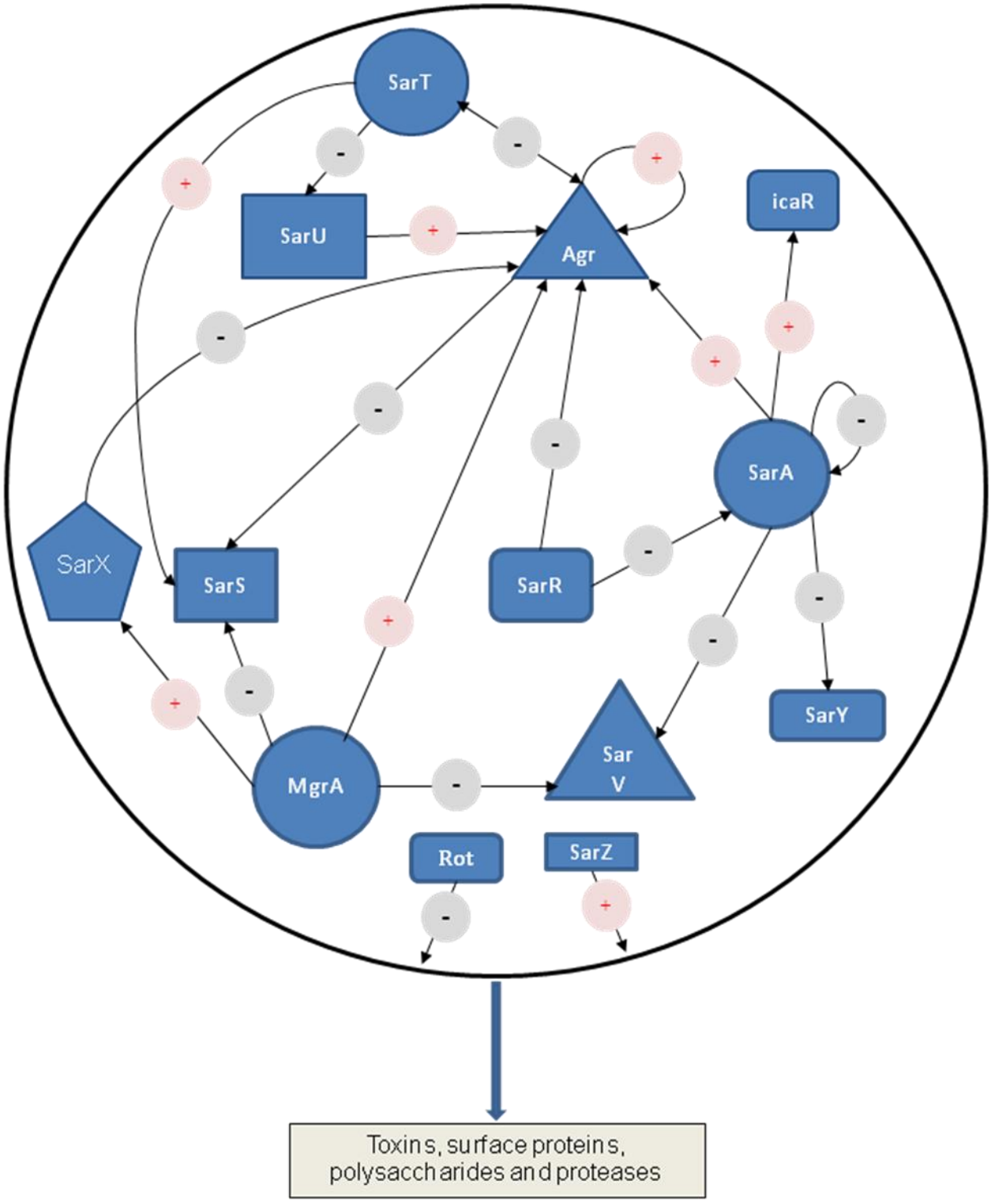


Figure 1.6. A schematic simplified model representing the highly complex regulatory interactions of the SarA family of regulators with Agr, and their combined regulatory affect on gene expression.

suggest that it, or its downstream genes may positively regulate haemolysin production (Cheung *et al.*, 2008b). In *S. epidermidis* SarZ has been found to influence the expression of lipases, haemolysins and proteases, and to influence resistance to a human antimicrobial peptide, but as yet its role in *S. aureus* is undetermined (Wang *et al.*, 2008). Rot, or repressor of toxins, negatively regulates α and ß-haemolysins (*hla* and *hlb*), lipase (*geh*), and serine proteases (*sspB* and *splA*) which are implicated in virulence (Shaw *et al.*, 2004). It also positively regulates the genes for cell surface proteins ClfB, Spa and SdrC (Said-Salim *et al.*, 2003). SdrC is implicated in calcium binding and is a putative adhesin required for infections of bone (Sabat *et al.*, 2006). More recently, MgrA (also called Rat or NorR), has been found to negatively regulate autolysin genes which control cell division and lysis, independently of SarA and Agr (Ingavale *et al.*, 2003); its effect on autolysis may be indirect and due to SarV which positively regulates several autolysins. The *mgrA* gene also impacts upon the expression of type 8 capsular polysaccharide (*cap8*) and *norA*, a gene encoding an efflux pump that confers resistance to the quinolone antibiotics, lipase, protease, as well as coagulase (Yoshida *et al.*, 1990; Truong-Bolduc *et al.*, 2003). Furthermore MgrA has been found to be capable of detecting deleterious reactive oxygen species, causing dissociation of the protein from DNA and therefore affecting gene regulation (Chen *et al.*, 2006).

1.8.4 SarA

SarA has three distinct promoters, P1, P2 and P3 (Fig 1.7) encoded within a region of approximately 800 bp, which generate three overlapping transcripts (Bayer *et al.*, 1996), and all three promoters are required for the production of functional SarA (Cheung and

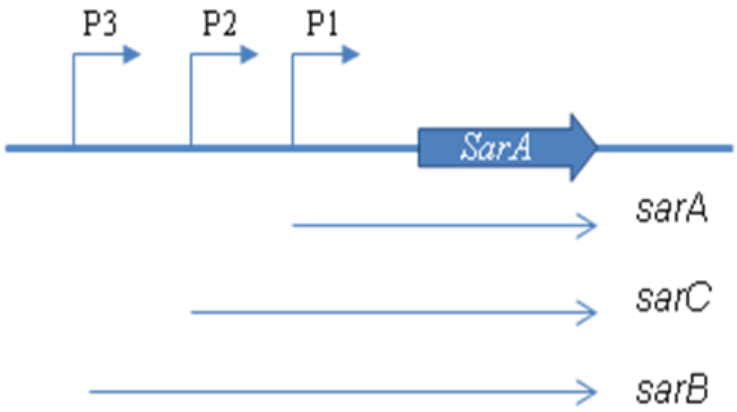


Figure 1.7. Schematic diagram of the arrangement of *S. aureus sarA*. Indicating the multiple transcripts and promoter sites. *sarA* is 0.5 kb, *sarC* is 0.8 kb and *sarB* is 1.2 kb in length. All transcripts arise from individual promoters but terminate at the same transcriptional terminator (Bayer *et al.* 1996).

Manna, 2005). s*arA* expression is auto regulated, with SarA binding to 2 sites within the promoter regions P1 and P3, repressing *sarA* transcripion (Cheung *et al.*, 2008a). SarA can act directly via binding promoter regions of target genes (Roberts *et al.*, 2006), indirectly via its regulation of *agr* (Cheung and Projan, 1994; Heinrichs *et al.*, 1996), or by stabilising mRNA possibly via direct binding to the mRNA (Roberts *et al.*, 2006). SarA regulates the production of Agr by binding to the same region between P2 and P3 of the *agr* operon as AgrA, positively regulating *agr* expression (Cheung and Projan, 1994; Koenig *et al.*, 2004). Binding sites for SarA have been identified in the promoter regions of *hla* and *spa*, and putative binding sites in the promoters of *fnbA* and *fnbB* have also been identified (Chien *et al.*, 1999). SarA also has negative regulatory effects on the expression of protein A and proteases such as aureolysin, cysteine protease and V8 protease (Manna and Cheung, 2006); (Cheung *et al.*, 2004). In addition SarA has also been shown to be essential for biofilm formation, via its effects on the *ica* operon. Deletion of *sarA* reduced *ica* transcription and PNAG production in four different strains. The *ica* operon has a 70 bp region between *icaR* and *icaA* which has a potential *sarA* binding site, suggesting that SarA might directly affect the expression of the operon. However, whether SarA binding affects *ica* directly or via its affects on *icaR* is not yet known (Valle *et al.*, 2003).

1.8.5 The *sae* Operon.

sae was first identified by Giraudo *et al.* in 1994 who found that a *S. aureus* strain containing a Tn*551* insertion mutation in what turned out to be the *sae* operon caused altered exo-protein production (Giraudo *et al.*, 1994) different to that witnessed in other regulatory mutants such as *agr* and *sarA*, which also regulate toxin production (Cheung

et al., 1992; Tegmark *et al.*, 1998). This region was therefore named *sae* for *S. aureus* exoprotein expression. There are four open reading frames (Fig 1.8), *saeP*, *saeQ*, *saeR* and *saeS*. SaeR and SaeS act as the classic two component signalling system (Novick and Jiang, 2003; Steinhuber *et al.*, 2003; Goerke *et al.*, 2005) in which SaeS is the receptor kinase and SaeR is the response regulator (Giraudo *et al.*, 1999). The system contains two distinct promoters P_A*sae* (P3) and P_C*sae* (P1) (Steinhuber *et al.*, 2003; Adhikari and Novick, 2008). A possible third promoter had been proposed, from sequence analysis and transcriptional mapping, P2, situated between P_C*sae* (P1) and P_A*sae* (P3) (Steinhuber *et al.*, 2003). However in a chromosomally integrated promoter-*lacZ* fusion assay, no β-galactosidase activity could be detected using different sized cloned fragments including P2 but excluding the other two promoters, suggesting that the P2 promoter was not active (Geiger *et al.*, 2008). P_A*sae* is located within *saeQ* coding sequence and is expressed *in vitro* during early growth, producing a transcript covering part of *saeQ* and the *saeR* and *saeS* genes, referred to as transcript A. P_C*sae* gives rise to three transcripts, transcript C, which covers the entire operon, transcript B, which covers *saeQRS*, and transcript D, which covers only *sae*P. Transcript B is likely to be derived from C as a result of transcript processing. Transcript D may also be derived from C or may be an original transcript (Adhikari and Novick, 2008). The products of *saeRS* auto regulate the operon (Novick and Jiang, 2003). During the later stages of growth *in vitro*, P_A*sae* is repressed by *saeRS* and P_C*sae* is activated (Geiger *et al.*, 2008). This switch is reported to be dependant on *agr* (Novick and Jiang, 2003). P_C*sae* is reported to respond to a variety of signals, including sub inhibitory concentrations of antibiotics, low pH, high salt, hydrogen peroxide and α-defensins, and to Agr, SarA, SaeP or SaeP targets (Novick and Jiang, 2003; Steinhuber

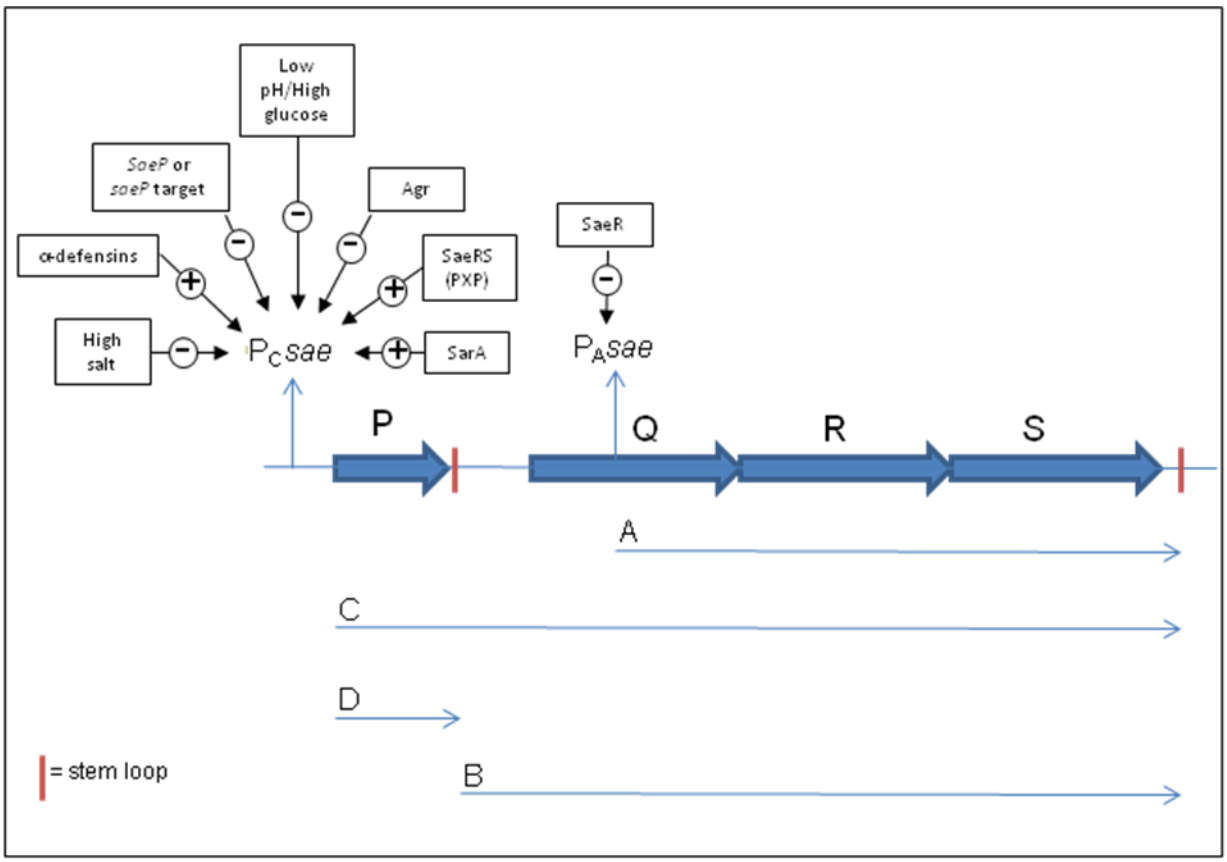


Figure 1.8. A schematic representation of the *sae* operon. Known regulation of the two promoters is indicated, and general regulatory affects on the operon are also shown. In addition the regulatory impact of Sae on the expression of virulence factors is indicated. SaeS is the receptor kinase and SaeR is the response regulator, which make up a classic two component signalling system (Giraudo *et al.,* 1999). The system contains two distinct promoters P_A*sae* (P3) and P_C*sae* (P1) (Adhikari *et al.*, 2008; Steinhuber *et al.*, 2003). P_A*sae* is located within *saeQ* and is expressed during early growth, producing a transcript covering part of *saeQ* and the *saeR* and *saeS* genes, referred to as transcript A. The products of *saeRS* auto regulate the operon, during later stages of growth $Psae_A$ is repressed by *saeRS* and $Psae_C$ is activated. This switch is reported to be dependant on *agr* (Novick *et al.*, 2003). $Psae_C$ produces transcript C, covering the entire operon, transcript B, which covers *saeQRS,* and transcript D, which covers only *sae*P. Transcript B is likely to be derived from C as a result of transcript processing. Transcript D may also be derived from C or may be an original transcript.

et al., 2003; Adhikari and Novick, 2008; Geiger *et al.*, 2008). In addition, transcription of the *sae* operon has been shown to be repressed by σ^B (Novick and Jiang, 2003). The roles of SaeP and SaeQ are not yet fully understood, but SaeQ is believed to be involved in resetting the response regulator after activation (Adhikari and Novick, 2008).

The effects of *sae* expression on gene regulation are global; for example Sae activates the production of β-haemolysin, DNase, protease, protein A and coagulase production at the transcriptional level (Giraudo *et al.*, 1994; Giraudo *et al.*, 1997). In addition *sae* has been shown to positively regulate the genes for the toxins γ-haemolysin, α–haemolysin and lukSF (PVL), and for the surface proteins, FnbB, FnbA, Eap and Emp, and to negatively regulate the genes for capsular polysaccharide 5 (CP5) (Giraudo *et al.*, 1999; Steinhuber *et al.*, 2003; Blickwede *et al.*, 2005; Goerke *et al.*, 2005; Harraghy *et al.*, 2005; Yamazaki *et al.*, 2006; Adhikari and Novick, 2008). Sae also regulates the genes for the immune modulators SCIN and CHIPS (Rahimpour *et al.*, 1999; Chavakis *et al.*, 2002; Rooijakkers *et al.*, 2006). Although Sae expression is affected by other regulators such as SarA, SigB and Agr, Sae does not appear to have any affect on the expression of *agr*, RNAIII or *sarA* (Giraudo *et al.*, 1997; Giraudo *et al.*, 2003; Novick and Jiang, 2003; Steinhuber *et al.*, 2003; Adhikari and Novick, 2008; Geiger *et al.*, 2008). Given the involvement of Sae with the regulation of such important virulence factors it is not surprising that Sae expression affects the pathogenicity of *S. aureus*. Sae has been shown to be essential for virulence in an ovine peritonitis model (Rampone *et al.*, 1996). It has also been suggested that *in vivo S. aureus* might activate different regulatory pathways, implicating *sae* as the dominant regulator (Goerke *et al.*, 2001). The gene for α-haemolysin is positively regulated by Agr and Sae and *in vitro*

transcripts for both *agr* and *sae* have been shown to be high. Despite this, strain Newman produces little α-haemolysin *in vitro* (Blickwede *et al.*, 2005; Geiger *et al.*, 2008). By contrast *in vivo* levels of RNAIII were decreased and *hla* was increased. This suggests that Sae regulated *hla* may play a role in virulence (Goerke *et al.*, 2001). This also suggests that the regulatory affects of Sae predominate over Agr *in vivo*, implying that there is a regulatory hierarchy in virulence regulation which may have implications on previous *in vitro* findings.

1.9 Overcoming the low iron status of the host.

In addition to the wealth of virulence factors described in the previous section, *S. aureus* has also evolved mechanisms that allow it to survive and flourish in the low iron environment of the host. Iron is an essential trace element to almost all organisms including the mammalian host and invading bacteria (Litwin and Calderwood, 1993). Iron is used in many cellular metabolic processes due to its high redox potential (Litwin and Calderwood, 1993). Iron is a highly abundant element on earth; it exists in the soluble reduced ferrous form (Fe^{2+}) in the absence of oxygen, but is insoluble as Fe^{3+} at neutral pH in the presence of oxygen (Andrews *et al.*, 2003). It is hypothesised that during evolution, iron was selected for use due to its abundance, but as photosynthetic organisms evolved the production of oxygen led to oxidation of iron to the ferric state (Fe^{3+}) making it biologically scarce (Andrews *et al.*, 2003). When ferrous iron is exposed to hydrogen peroxide (H_2O_2), a natural by product of cellular metabolism, the iron is oxidised to Fe^{3+} and the H_2O_2 is converted to a hydroxide ion and a hydroxide free radical in a process known as the Fenton reaction, as summarised in the reaction below (Halliwell, 1978).

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

This highly reactive free radical form of hydroxide can cause a wide range of cellular damage leading to cell death. Superoxide free radicals (O_2^-) generated from metabolic processes (Scott *et al.*, 1987) convert Fe^{3+} back to Fe^{2+} further fuelling the process of cellular damage (Benov, 2001). In addition, superoxides are capable of cellular damage in their own right (Benov, 2001). Therefore not only is iron essential, but it is also extremely toxic in oxygen containing environments and so is bound to high affinity iron storage proteins (Litwin and Calderwood, 1993) in both bacteria and the host (MacKenzie *et al.*, 2008). As a defence against free radical attack, cells have developed a process to neutralise superoxide. The enzyme superoxide dismutase (SOD) converts reactive O_2^- to non reactive O_2, and H_2O_2 (Scott *et al.*, 1987); H_2O_2 is then also deactivated by converting it to water by catalase (Clements *et al.*, 1999).

The binding of iron to protein not only protects the host from cellular damage but also limits the amount of free iron available to invading bacteria. In humans iron is bound to haem, transferrin, lactoferrin and ferritin, each having a specialised role. Haem proteins are multi functional in the mammalian host, one of their major roles is oxygen transport, which is achieved when haem is encorporated into the protein haemoglobin. Transferrin is found in the blood, cerebrospinal fluid and extracellular secretions (Jordan and Saunders, 2009). Lactoferrin is found in milk, tears, saliva, other bodily fluids (Brown and Holden, 2002), and on mucosal membranes (Naidu *et al.*, 1991). Ferritin is found in the liver, spleen, muscle, bone marrow and at low levels in the blood

(Jordan and Saunders, 2009), limiting the amount of free iron in host serum to approximately 10^{-18} M, far too low to sustain bacterial proliferation (Bullen *et al.*, 1978). During the onset of an infection, iron is further limited by the host (hypoferraemia) in order to prevent bacterial colonisation (Weinberg, 1978; Letendre and Holbein, 1983). The process of hypoferraemia is not fully understood, but it is proposed that lactoferrin released from phagocytocic cells during the inflammatory response may be responsible for reducing the available iron, as it has a higher affinity for iron than serum transferrin (Van Snick *et al.*, 1974). Despite these attempts to discourage bacterial infection many bacteria including *S. aureus* can grow and cause infection in the human host. This is achieved by the action of iron sequestering systems.

S. aureus has four main ways of acquiring iron from the surrounding environment (Fig 1.9). Like many bacteria *S. aureus* produces low molecular weight molecules with a high affinity for ferric iron, called siderophores. *S. aureus* produces four siderophores, staphyloferrins A and B, aureochelin and staphylobactin, which are taken into the cell via a number of specific transporters (Fig 1.9) (Konetschny-Rapp *et al.*, 1990; Drechsel *et al.*, 1993; Courcol *et al.*, 1997; Morrissey *et al.*, 2000; Sebulsky and Heinrichs, 2001; Dale *et al.*, 2004). *S. aureus* has also been shown to utilise hydroxamate siderophores produced by other bacteria, effectively hijacking the iron supply, which may be advantageous in colonisation of sites with mixed populations (Sebulsky *et al.*, 2000; Lindsay, 2008). Iron loaded siderophores are then transported into the cells via specific surface bound receptors. Once internalised, the iron is

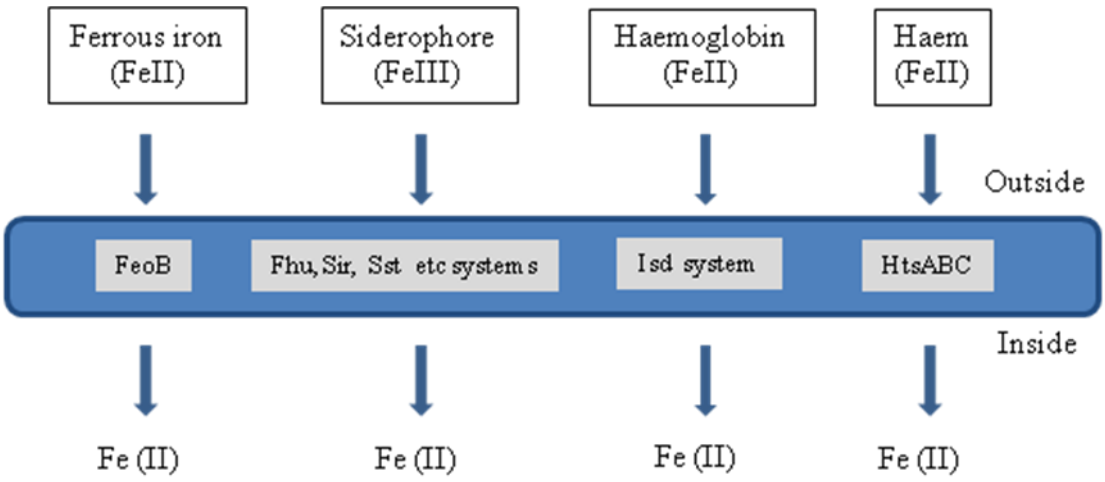


Figure 1.9. A summary of the many iron uptake systems in *S. aureus* illustrating the four main types of iron transporter systems. Free ferrous iron transport is likely facilitated by FeoB, although this has not been confirmed. Iron loaded siderophores are transported into the cell by a range of transporters, only some of which are illustrated. Haemaglobin and Heam are bound and transported into the cell by the Isd family of proteins and the HtsABC transporter respectively. All systems result in the release of ferrous iron into the cell.

liberated from the molecules and the iron is then either used directly, or stored in the iron storage proteins ferritin and Dps (Andrews *et al.*, 2003). *S. aureus* has ferritin and a Dps homologue MrgA, however MgrA has not been fully characterised and its role in iron storage has not been fully determined (Horsburgh *et al.*, 2001a; Morrissey *et al.*, 2004).

FeoB is a ferrous iron transporter, homologous to FeoB of *E. coli* which binds and imports free ferrous iron into the cell, and may play a similar role in *S. aureus*, although this has not been fully investigated (Lindsay, 2008). Another iron uptake mechanism is facilitated by the Isd (Frp) family of proteins (Mazmanian *et al.*, 2002; Morrissey *et al.*, 2002). The *isd* operon (iron regulated surface determinant), encodes haem-iron binding proteins (Mazmanian *et al.*, 2003). It is proposed that during an infection, *S. aureus* binds haemoglobin via IsdB and IsdH. Haem is then removed from haemoglobin via IsdA and transferred to the cell wall protein IsdC. Haem is then passed on to membrane translocation proteins IsdD, IsdE, and IsdF and transported into the cytoplasm (Mazmanian *et al.*, 2003). Isd acquired Haem can also be delivered into the cytoplasm by the HtsABC transporter system, however the release of iron from haem involves IsdG and IsdI which are thought to liberate iron by degrading haem (Skaar *et al.*, 2004; Skaar and Schneewind, 2004; Mason and Skaar, 2009).

In addition to haem and haemoglobin binding, Isd proteins also bind haptoglobin and the haemoglobin-haptoglobin complex that forms when haemoglobin is oxygen free prior to degradation (Dryla *et al.*, 2003). As well as their roles in iron uptake the Isd family of proteins have also recently been associated with virulence and immune

evasion, as IsdA and IsdB are required for abscess formation in a murine model of infection (Cheng *et al.*, 2009), and IsdH protected *S. aureus* cells from phagocytosis *in vitro* (Visai *et al.*, 2009).

The ferric uptake regulator Fur

Iron homeostasis is, not surprisingly, negatively regulated by iron. This avoids the accumulation of free iron, iron overload, which would cause damage to the bacterial cell (Braun, 1997; Xiong *et al.*, 2000), and is facilitated by the ferric uptake regulator, Fur (Xiong *et al.*, 2000). Fur is a DNA binding protein which negatively regulates the expression of genes in high iron (Fig 1.10). When iron is readily available, the Fur protein binds to ferrous iron causing a conformational change in the protein; this allows Fur to bind to the promoter region of target genes (Xiong *et al.*, 2000). These target genes have a specific Fur recognition site called a Fur or iron box, and binding of Fur prevents attachment of RNA polymerase, blocking transcription of the gene (Xiong *et al.*, 2000).

Organisms also utilise other metal ions for essential processes which also have similar toxic effects and are also regulated by Fur like proteins. *S. aureus* has three Fur homologues which control metal ion homeostasis, Fur, PerR and Zur. Zur is a zinc dependant repressor responsible for zinc homeostasis (Gaballa and Helmann, 1998; Lindsay and Foster, 2001). PerR is a transcriptional repressor associated with the regulation of iron storage proteins and with the production of proteins involved with the oxidative stress response (Horsburgh *et al.*, 2001a; Horsburgh *et al.*, 2001b). A third

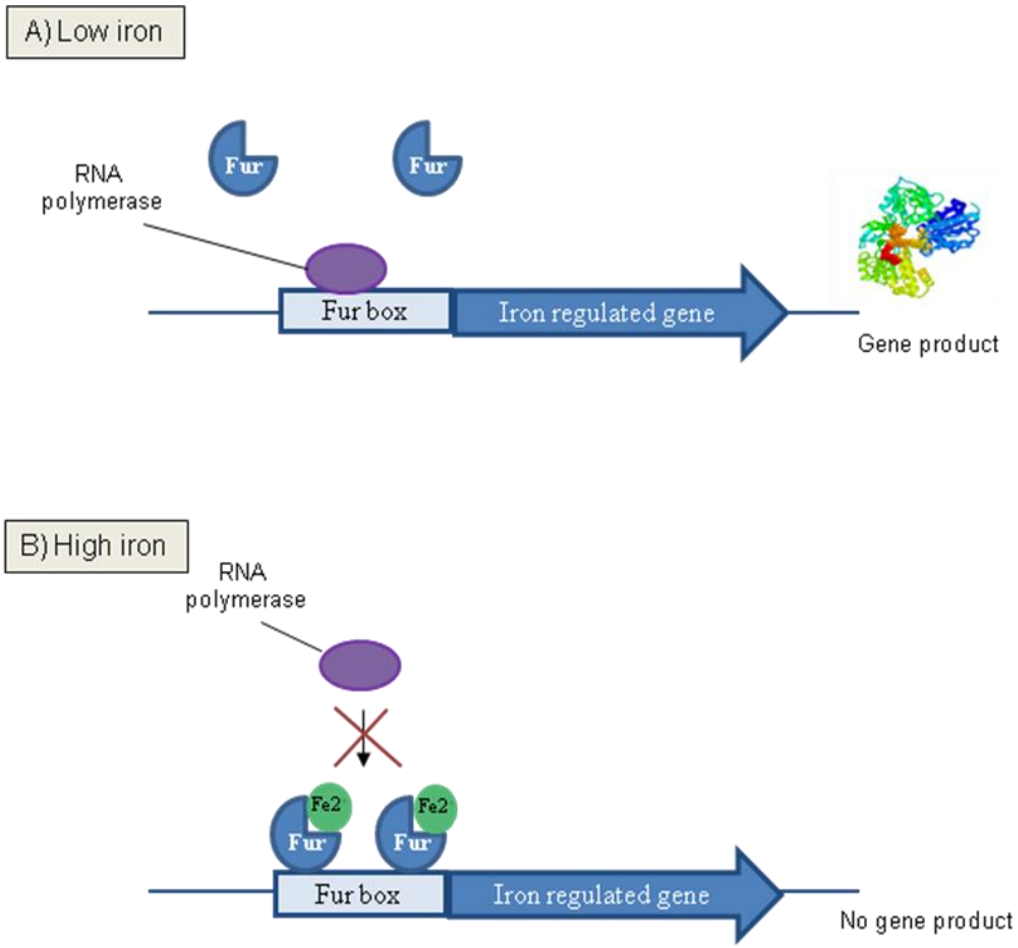


Figure 1.10 . A schematic representation of classical Fur repression.
A) In the absence of iron Fur remains unbound by its co-factor iron, preventing Fur from binding to the Fur box region of the promoter of the target gene. RNA polymerase can bind to the promoter and initiate transcription resulting in successful generation of the gene product. B) In high iron, Fur binds to ferrous iron causing a conformational change in the protein. This allows binding of the complex to the Fur box, preventing RNA polymerase from accessing the promoter region resulting in gene repression.

metal dependant regulator MntR, distinct from Fur, is associated with manganese homeostasis, and has also been shown to interact with Fur and PerR (Horsburgh *et al.*, 2002b). In *S. aureus*, in addition to controlling the expression of genes associated with iron uptake, Fur has been shown to regulate genes involved in virulence, iron storage and oxidative stress via its interactions with PerR, as well as general metabolism, and is therefore considered a global regulator (Horsburgh *et al.*, 2001b; Morrissey *et al.*, 2004; Friedman *et al.*, 2006).

Although Fur is often responsible for iron responsive gene regulation in microorganisms, Fur independent iron regulation has been observed in other bacteria. For example in *Bradyrhizobium japonicum*, a nitrogen fixing symbiont of the soy bean plant, the DNA binding protein Irr, a similar but distinct protein from Fur, is responsible for Fur independent iron regulation (Hamza *et al.*, 2000). In *Campylobacter jejuni*, iron regulation is both Fur dependant and Fur independent. Fur independent iron regulation was found to be due to PerR (van Vliet *et al.*, 1999). In *S. aureus*, as already mentioned, PerR is involved in iron storage and oxidative stress, but it does not appear to have a role in iron mediated gene regulation, therefore to date, Fur is the only regulator shown to mediate iron responsive gene regulation in *S. aureus.*

As previously mentioned Fur is a negative regulator of target gene expression in high iron. However, more recently there have been examples of non-conventional Fur regulation in other bacteria. For example, genes are now being identified which are negatively regulated by Fur in low iron, and positively Fur regulated in both high and

low iron (Gruer and Guest, 1994; Dubrac and Touati, 2000; Delany *et al.*, 2001; Grifantini *et al.*, 2003; Delany *et al.*, 2004; Ernst *et al.*, 2005; Mey *et al.*, 2005b).

Given that classical negative Fur regulation relies on iron as a co-factor, how positive Fur regulation is achieved in low iron in *S. aureus* is as yet not known. Negative Fur regulation in low iron has been observed in *Helicobacter pylori*. In this instance Fur binds to the Fur box sequence in the promoter region of the ferritin gene *pfr*, in low iron, directly repressing gene expression. In high iron the Fur/iron complex can no longer bind the Fur box, which alleviates repression, increasing gene expression (Delany *et al.*, 2001). This suggests that Fur can bind to the promoter region of some target genes without the conformational change in the protein induced by the binding of iron. Therefore it might be possible that Fur could also activate gene expression by direct binding in the absence of iron. There are also examples of positive Fur regulation in high iron and in some cases the mechanisms have not yet been identified. For example in *Neisseria meningitidis* Fur positively regulates transcription of the *Neisseria* surface protein A (NspA) in high iron, (Shaik *et al.*, 2007). Fur also positively regulates *Pasteurella multocida hbpA* which encodes a haemin binding protein that is regulated by iron in a Fur-independent manner (Garrido *et al.*, 2003). Positive Fur regulation in high iron has also been observed in *E. coli*, the mechanism for which has been identified. The expression of genes for the iron storage ferritin proteins, the gene for the oxidative stress factor SodB, and genes associated with the tricarbocylic acid cycle (TCA) cycle have all been shown to be positively Fur regulated, and dependant on iron as a co-factor (Niederhoffer *et al.*, 1990; Gruer and Guest, 1994; Dubrac and Touati, 2000; 2002; Masse and Gottesman, 2002). However these positive regulatory roles of

Fur have been subsequently found to be indirect, and due to the de-repression of the Fur regulated non-coding small RNA (sRNA) RhyB (Masse and Gottesman, 2002). The regulatory effects of sRNAs are described in more detail in the following section. In *E. coli* RhyB represses the expression of genes for iron containing and iron storage proteins (Masse and Gottesman, 2002). RhyB expression is itself classically Fur regulated in that it is repressed by Fur in high iron (Fig 1.11). Therefore in low iron, or in the absence of Fur, Fur repression of *rhyB* is alleviated leading to transcription of *rhyB.* This leads to repression of RhyB target genes such as ferritin. In high iron Fur represses *rhyB,* allowing transcription of RhyB repressed genes, giving the impression of positive Fur regulation (Masse and Gottesman, 2002).

1.10 Hfq and small RNAs

Many adaptive regulatory responses in bacteria have been attributed to small non coding RNA molecules such as small RNAs (sRNAs). As described above, the sRNA RhyB of *E. coli* plays an important role in the regulation of genes in low iron. sRNAs are produced by many organisms, and provide a vital transcriptional and posttranscriptional regulatory function, having both positive and negative regulatory roles by affecting the stability of mRNA, the activity of RNA polymerase and the functionality of proteins (Wassarman and Storz, 2000; Mehta *et al.*, 2008; Liu *et al.*, 2009). In other bacteria sRNAs have been implicated in iron responsive gene expression and positive Fur regulation, (Masse and Gottesman, 2002; Wilderman *et al.*, 2004; Davis *et al.*, 2005; Metruccio *et al.*, 2009), and, in addition, in *S. aureus* sRNAs have been implicated in virulence (Pichon and Felden, 2005). sRNAs are often reliant

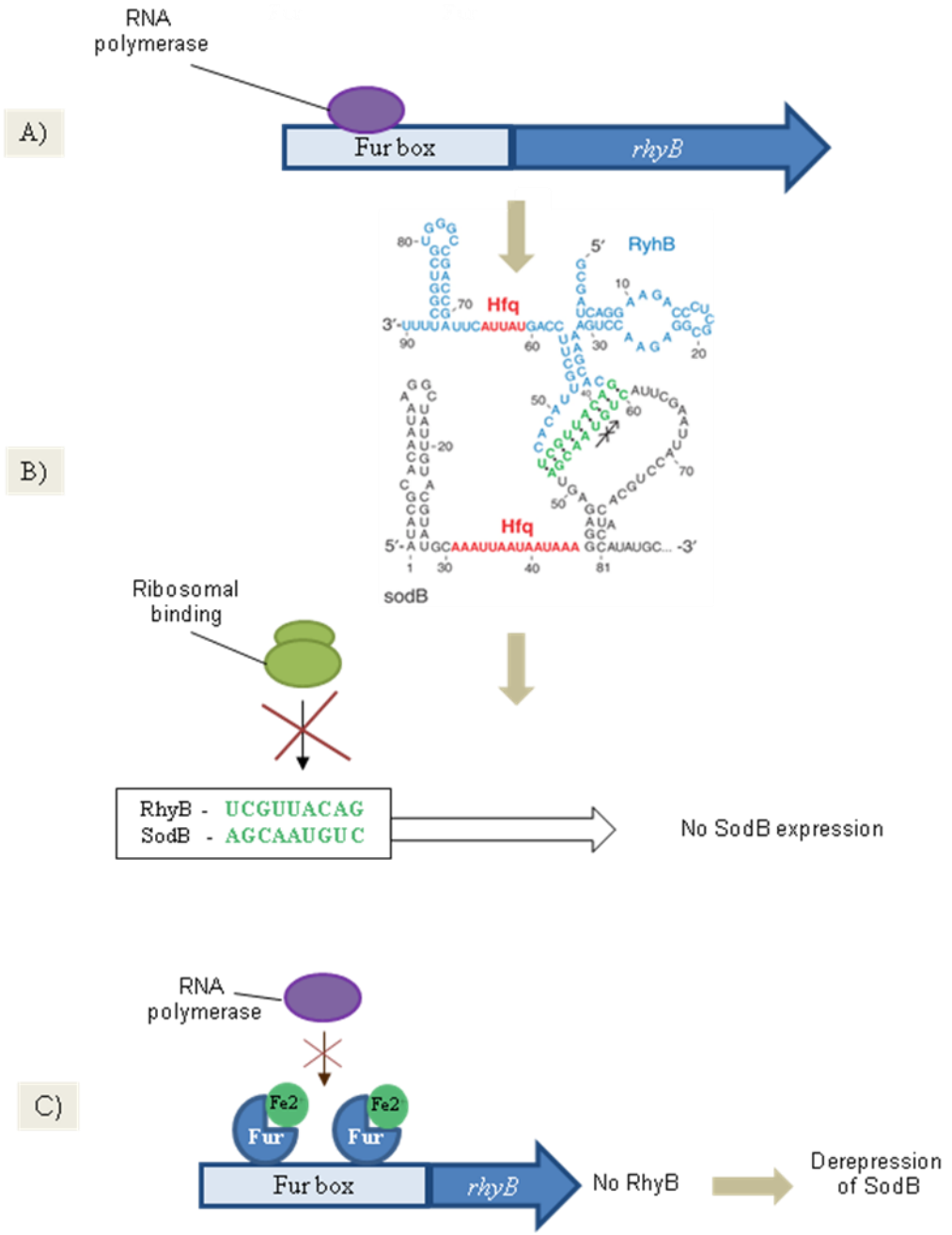


Figure 1.11. A schematic representation of Fur regulated RhyB expression repression. A) In low iron or the absence of Fur, RNA polymerase binds to the promoter region of *rhyB* initiating transcription, resulting in successful generation of the sRNA RhyB. B) RhyB and SodB mRNA are bound by the sRNA chaperone Hfq. This causes structural changes allowing the binding of RhyB to SodB mRNA, and preventing SodB translation. C) In high iron, Fur binds to iron causing a conformational change in the protein. This allows binding of the complex to the Fur box, preventing RNA polymerase from accessing the promoter region resulting in the repression of RhyB. The structural figure for RhyB and SpdB showing the Hfq binding site, and the RhyB/SodB binding sequence was taken from Geissmann *et al* 2004.

on the RNA binding protein Hfq for activity (Wassarman *et al.*, 2001; Geissmann and Touati, 2004). Hfq is a small protein that stabilises and mediates the interaction of sRNA, acting as a chaperone with target mRNAs. Hfq can also have an effect on protein expression without sRNAs by binding directly to mRNAs. This affects either mRNA ribosome binding or stability by tagging it for degradation (Tsui *et al.*, 1997; Vytvytska *et al.*, 2000; Le Derout *et al.*, 2003).

In *E. coli* Hfq is also implicated in Fur regulation via the regulation of RhyB, which regulates the expression of iron containing proteins such as SodB. In the absence of Hfq, the Fur regulated sRNA RhyB is very unstable (Masse *et al.*, 2003), and *in vivo* Hfq is required for RhyB activity (Geissmann and Touati, 2004). *E. coli* SodB expression is regulated by RhyB; Hfq binds to SodB and RhyB mRNA causing a structural change that facilitates the SodB/RhyB interaction (Fig 1.11) (Geissmann and Touati, 2004). Moreover, Hfq has been linked with Fur in *Neisseria meningitidis* via NrrF, a sRNA which binds Hfq and mediates Fur-dependent NrrF regulation of succinate dehydrogenase, an iron containing protein involved in electron transfer (Metruccio *et al.*, 2009). As a result Hfq has also been shown to be essential for virulence in many bacteria including *Salmonella typhimurium*, *Vibrio cholerae*, and *Shigella flexneri* (Ding *et al.*, 2004; Sharma and Payne, 2006; Sittka *et al.*, 2007).

However in *S. aureus*, the role of sRNAs (with the exception of RNAIII) and their dependency on Hfq have not been widely studied. In *S. aureus* only 12 sRNAs have been identified (Pichon and Felden, 2005), suggesting that their role in *S. aureus* regulation may be limited. This is unusual as many prokaryotes rely on sRNAs for

many regulatory events; *E. coli* for example has over 100 potential sRNA candidates. RNAIII is the only well documented *S. aureus* sRNA affecting the expression of many virulence genes via the quorum sensing *agr* operon (Novick, 2003). One study implied that RNAIII might be a possible target for Hfq interactions in *S. aureus* (Huntzinger *et al.*, 2005). However this has recently been disputed by Bohn *et al.* (2007), who failed to find a phenotype for an *hfq* mutant bringing into question the role of Hfq in *S. aureus*. It is possible that the multitude of other complex inter-linked regulatory pathways available to *S. aureus* makes sRNAs and Hfq less important. Alternatively the increased focus on sRNA regulation at present may mean that new sRNAs will be identified and their functions will become apparent.

1.11 Summary

In summary *S. aureus* is a highly adaptable organism. It not only adapts to its ever changing environment, causing disease at almost all sites in the mammalian host, but it can also adapt to resist our efforts to eradicate it. Resistance mechanisms that protect the bacteria from almost all aspects of our highly complex immune system, or from antimicrobials, ensure survival of the organism in compromised hosts which is to some extent understandable. However, recently it is becoming more common that *S. aureus* isolates can cause life threatening disease in otherwise healthy individuals. So far there is no clear answer as to why this is so. Recent advances in the study of bacterial infection have established the importance of biofilm formation in disease and *S. aureus* biofilm formation has been widely studied *in vitro*. Despite this there are still many unanswered questions regarding the expression of virulence factors and disease. It is

hoped that future studies will help to answer some of these questions so that infections can be prevented or treated by novel therapies.

1.12 The aims of this work.

With the increasing incidence of *S. aureus* infection hampered by antibiotic resistance it is vital that we have a better understanding of how *S. aureus* cause infections; what factors are required for infection to take place, and what controls the expression of these factors? Understanding this may lead to the discovery of novel preventative measures or new treatments being discovered. Many *in vitro* studies have revealed factors that facilitate *S. aureus* virulence, some of which have been confirmed in clinical studies. However, some *in vitro* studies which have suggested a role for virulence factors in disease have faced questions when applied to animal models or *in vivo* studies. This is likely to be due to the differential expression of factors in response to environmental conditions. It is now becoming more widely accepted in the staphylococcal research field that growth conditions can have a profound effect on the expression of proteins, polysaccharides and other factors which may be involved in virulence. Studies have shown that pathogenic bacteria use the low iron status of the host as a signal to up regulate genes associated with virulence, and whilst there have been many studies investigating factors involved in biofilm formation *in vitro*, there have been no studies investigating the direct effects of iron on biofilm formation or on the factors which have previously been implicated in biofilm formation. It is the aim of this work to further investigate the effect of environmental conditions such as low iron which would be encountered in a host infection on biofilm formation, and to establish what is responsible for biofilm formation under these conditions. Much of the work presented in the following chapters has been published or is in the process of submission (Johnson *et al.*, 2005; Johnson *et al.*, 2008).

Objectives.

1: Investigate the effects of iron on *S. aureus* biofilm formation.

2: Determine the role of PNAG, and identify novel factors involved in low iron biofilm production.

3: Investigate the regulatory mechanisms involved in biofilms formed in low iron.

Chapter 2

Materials and Methods

2.1 Growth & storage of bacterial strains

Frozen cultures of *S. aureus* were stored at -70˚C in Tryptic Soy Broth (TSB) containing 20% v/v glycerol in 1.5 ml aliquots. Cultures were recovered by scraping a small amount of the frozen stock onto a blood agar plate containing appropriate antibiotics where necessary and incubating overnight at 37^{o}C in 5% CO_2 in air. After incubation plates were examined for pure culture and used to inoculate liquid cultures. To help maintain reproducibility plate cultures were made from fresh frozen stocks for each assay replicate. *E. coli* strains were stored at -70˚C in Luria Bertani (LB) containing 20% v/v glycerol in 1.5 ml aliquots. Cultures were recovered by scraping a small amount of the frozen stock onto a LB agar plate containing appropriate antibiotics where necessary and incubating overnight at 37^{o}C in air. After incubation plates were examined for pure culture and used to inoculate liquid cultures. Plates were then stored at 4^{o}C until required for up to 1 month.

Growth of *E. coli strains*

E. coli TOPO10 was grown in LB medium at 37 ºC in air overnight. Liquid cultures were shaken at 200 rpm on an orbital shaker (New Brunswick Scientific). LB consists of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride. Solid media contained 1.5% (w/v) agar and all media components were purchased from Oxoid, except for sodium chloride which was obtained from Sigma. Solutions were autoclaved at 121^{o}C for 15 minutes to achieve sterility. Antibiotic solutions were added at appropriate concentrations where required (see table 2.2 below).

Growth of *S. aureus strains*

S. aureus strains were grown in liquid cultures in either BBL™ Trypticase™ Soy Broth (TSB), CRPMI, or LK media or on solid media blood agar (BA) or LKA as required with the addition of the appropriate antibiotics as required, concentrations of antibiotics are detailed in table 2.2.

2.2 Growth media

TSB

15 g of TBS powder consisting of Casein, 17.0 g/l, Papaic Digest of Soybean Meal, 3.0 g/l, sodium chloride, 5.0 g/l, dipotassium phosphate, 2.5 g/l and dextrose, 2.5 g/l, was added to 500 ml of deionised water and autoclaved at 121^{o}C for 15 minutes to

achieve sterility. The appropriate antibiotics were added as required at concentrations detailed in table 2.2. Cultures were incubated at 37^{o}C statically or shaking at 200 rpm in air or statically in 5 % CO_2 in air as appropriate. To change the pH of TSB to that of CRPMI, concentrated NaOH was added drop wise to TSB to a pH of 8.8 prior to sterilisation. After equilibration of the medium with CO_2 the pH was approximately 7.6, the same as CRPMI.

CRPMI

RPMI 1640 with sodium bicarbonate, without L-glutamine (R0833 see table 2.1), a low iron tissue culture media, was purchased from Sigma. To produce iron restrictive conditions for assays observing the effect of iron concentrations, RPMI 1640 was further depleted of iron by batch incubation with 6% (w/v) Chelex 100 (C7901, Sigma). Chelex was added to RPMI, incubated overnight with stirring at 4^{o}C, and then removed by filter-sterilisation using a 0.2 µm filter and a vacuum filtration system (Stericup, Millipore Ltd). 10% RPMI 1640 was then added back to the solution to provide trace elements required for growth. All cultures were incubated statically at 37^{o}C in 5 % CO_2 in air. Where indicated the medium was supplemented with varying concentrations of metal ions as described. Ions were dissolved in deionised water, filter sterilised with a 0.2 µM acrodisc (Millipore Ltd) and stored at room temperature for up to two weeks. Where increased glucose concentrations were required, 25% filter sterilised glucose was added to a final concentration of 1.25 %. Where increased salt concentrations were required, autoclaved 25% NaCl was added to a final concentration of 3% to the growth medium prior to inoculation.

Table 2.1 ingredients of RPMI 1640 R0883 (Sigma Ltd)

Component	**g/l**
$Ca(NO_3)_2 \cdot 4H_2O$	0.1
MgSO4 (anhydrous)	0.4884
KCl	0.4
NaCl	6.0
NaHPO4 (anhydrous)	0.8
Succinic Acid•$6H_2O$•Na	0.1
Succinic Acid (free acid)	0.075
L-Arginine	0.2
L-Asparagine (anhydrous)	0.05
L-Aspartic Acid	0.02
L-Cystine•2HCl	0.0652
L-Glutamic Acid	0.02
Glycine	0.01
L-Histidine	0.015
Hydroxy-L-proline	0.02
L-Isolucine	0.05
L-Leucine	0.05
L-Lycine•HCl	0.04
L-Methionine	0.015
L-Phenylalanine	0.015

Component	**g/l**
L-Proline	0.02
L-Serine	0.03
L-Threonine	0.02
L-Tryptophan	0.005
L-Tyrosine	0.02
L-Valine	0.02
D-Biotin	0.0002
Choline Bitarate	0.00544
Folic Acid	0.001
myo-Inositol	0.035
Niacinamide	0.001
p-Amino Benzoic Acid	0.001
D-Panthenic Acid•½Ca	0.00025
Pyridoxine•HCl	0.001
Riboflavin	0.0002
Thiamine•HCl	0.001
Vitamin B12	0.000005
D-Glucose	2.0
Glutathione (reduced)	0.001
Phenol Red•Na	0.00318

LK and LKA

LK broth was prepared in 500 ml volumes containing 1% tryptone, 0.5% yeast extract, 0.7% KCl in deionised water. In addition LK agar contained 1.5% agar. Solutions were autoclaved at 121^{o}C for 15 minutes to achieve sterility. All media components were obtained from Oxoid except for potassium chloride which was purchased from Sigma. LK agar was also supplemented with 0.05% sterile sodium citrate when cool. The appropriate antibiotics were added as required; concentrations of antibiotics are detailed in table 2.2 Broth cultures were then incubated in at 37^{o}C shaking at 200 rpm in air. Plate cultures were incubated statically at 37^{o}C in air.

Blood Agar

Blood agar base (CM0055) was purchased from Oxoid and prepared in 500 ml volumes as described by the manufacturer. 20 g of powder was added to 500 ml of deionised water and autoclaved at 121°C for 15 minutes to achieve sterility. The medium was cooled to 50^{o}C and supplemented with sterile 6% defibrinated horse blood (SR050C, Fisher Scientific) and with antibiotics where appropriate. Plates were stored until required at 4^{o}C for up to 2 weeks.

Table 2.2 Antibiotic concentrations.

Antibiotic	Preparation	Concentration used	
		E. coli	***S. aureus***
Ampicillin	100 mg/ml in sterile distilled water; filter sterilized and stored at 4˚C.	100μg/ml	
Chloramphenicol	10 mg/ml in 50% ethanol in water; stored at 4˚C.		10 μg/ml
Erythromycin	10 mg/ml in 100% ethanol; stored at 4˚C.		10 μg/ml
Kanamycin	30 mg/ml in 100% ethanol; stored at 4˚C.		30 μg/ml
Tetracycline	10 mg/ml in 100% ethanol; stored at 4^{o}C.		10 μg/ml

2.3 Growth curves

To increase reproducibility, growth assays were set up from fresh plates for each assay. Strains were taken from frozen stocks and plated onto blood plates containing the appropriate antibiotics where necessary, to single colonies. The plates were incubated at 37^{o}C in 5% CO_2 in air overnight. The following day the plates were checked for purity and used to inoculate 10 ml of the appropriate broth (TSB or CRPMI) and again incubated statically overnight (16-18 hours) at 37^{o}C in 5 % CO_2 in air. The next morning cultures were centrifuged briefly to pellet the cells. The supernatant was discarded and the cell pellet was re-suspended in the residual liquid. This heavy cell suspension was then added drop wise to fresh medium (the same type as for the overnight culture) to achieve an optical density of 0.1 $OD_{595\ nm}$ (checked by measuring

1 ml in a spectrophotometer). This culture was then added in 200 µl volumes to a flat bottomed 96 well microtitre plate (Nunc) in quadruplet. The plate was read in a Biorad 3550 plate reader at 595 nm to record the starting OD of the cultures. Plates were then incubated at 37°C in 5 % CO_2 in air. Plates were removed every hour from the incubator and read in the plate reader at 595 nm to record growth; plates were returned to the incubator as quickly as possible to minimise the effects of changes in temperature and atmosphere on growth. The quadruplet readings were then averaged. All growth assays were repeated at least three times and figures represent the average of at least three replicate assays; error bars represent the standard deviation of the mean.

2.4 Strains and plasmids used in this work

TABLE 2.3. Bacterial strains and plasmids used and constructed in this study.

Strain or Plasmid	Description	Reference or source
S. aureus		
RN4220	Restriction-negative strain, 8325 derivative	Laboratory stock
EMRSA-15 (PM25) †	English endemic isolate	Jodi Lindsay
EMRSA-16 (PM27) †	Scottish endemic isolate	Jodi Lindsay
Cowan I†	High protein A producer	Alan Cockayne
BB†	Bovine isolate	Alan Cockayne
8325-4†	Spontaneous mutation of *rsbU*⁻.	Laboratory stock
4532/7†	Clinical isolate	Queens Medical Centre, Nottingham
83†	Clinical isolate	Queens Medical Centre, Nottingham
84†	Clinical isolate	Queens Medical Centre, Nottingham
85†	Clinical isolate	Queens Medical Centre, Nottingham
86†	Clinical isolate	Queens Medical Centre, Nottingham

† strains tested in Chapter 3 for iron responsive biofilm formation (see page 131).

TABLE 2.3. Bacterial strains and plasmids used and constructed in this study cont.

Strain or Plasmid	Description	Reference or source
S. aureus		
90†	Clinical isolate	Queens Medical Centre, Nottingham
92†	Clinical isolate	Queens Medical Centre, Nottingham
B1003003†	Clinical isolate	Queens Medical Centre, Nottingham
B2202016†	Clinical isolate	Queens Medical Centre, Nottingham
B1203012†	Clinical isolate	Queens Medical Centre, Nottingham
B0903007†	Clinical isolate	Queens Medical Centre, Nottingham
D169/7†	Clinical isolate	Queens Medical Centre, Nottingham
MR1†	Clinical isolate	Queens Medical Centre, Nottingham
MR2†	Clinical isolate	Queens Medical Centre, Nottingham
Mn8†	High TSS toxin producer	(Kropec *et al.*, 2005)
Newman	Wild-type	(Duthie and Lorenz, 1952)
Newman *fur*	Newman *fur::tet*	This work
Newman *ica*	Newman Δ*ica::tet*	(Kropec *et al.*, 2005)
Newman *ica* repaired	Newman Δ*ica::tet* repaired	(Kropec *et al.*, 2005)
Newman *sae*	Newman *sae::Tn917*	(Goerke *et al.*, 2001)
ALC1342	RN6390 Δ*sarA::ermC*	(Cheung *et al.*, 2001)
Newman *sarA*	Newman Δ*sarA::ermC*	This work
RN6911	RN6390 Δ*agr::tetM*	(Novick *et al.*, 1993)
Newman *agr*	Newman Δ*agr::tetM*	This work
Newman *eap*	Newman Δ*eap::erm*	(Hussain *et al.*, 2002)
Newman *emp*	Newman Δ*emp::erm*	(Johnson *et al.*, 2008)
Newman *sigB*	Newman Δ*rsbUVWsigB* Em^r	(Giachino *et al.*, 2001)
E. coli		
TOP10	Cloning strain	Invitrogen
Plasmids		
pMK4	*E. coli/S. aureus* shuttle vector	(Sullivan *et al.*, 1984)
pEmp	PMK4 with *emp* gene, inc RBS	This work
pEap	PMK4 with *eap* gene, inc RBS	This work

† strains tested in Chapter 3 for iron responsive biofilm formation (see page 131).

2.5 Isolation, purification and manipulation of DNA

Plasmid extraction

Isolation of plasmid DNA from *E. coli* TOP10 and *S. aureus* RN4220 was achieved using the Qiaprep Spin Mini or Midi Prep kit (Qiagen), following the manufacturer's instructions, except 125 µg/ml lysostaphin was added to 250 µl of P1 buffer (Qiagen) for *S. aureus* cultures to lyse the cells.

Extraction of *S. aureus* genomic DNA

Genomic DNA was extracted using the CTAB method as described by Ausubel in 1994 with minor modifications (Ausubel, *et al* 1994) 5 ml of LB were inoculated with the appropriate strain of *S. aureus* with the appropriate antibiotics if required and incubated at 37^{o}C with shaking overnight. The next day 1.5 ml of the culture were decanted into an Eppendorf tube and centrifuged at 12,100 × g, the supernatant was discarded and the cell pellet was re-suspended in 250 µl of P1 buffer (Qiagen) containing 100 µg/ml lysostaphin. This was then incubated in a 37^{o}C water bath for 20 minutes or until the suspension became clear and viscous. After lysis 0.05 mg of proteinase K was added followed by 27 µl of 10 % SDS, mixed well by inversion and incubated at 37^{o}C for 20 minutes. 97 µl of 5M NaCl was then added and mixed well by inversion, after which 81 µl of CTAB (4.1% w/v NaCl, 10 % w/v cetyl trimethyl ammonium bromide) pre heated to 65 ^{o}C were added and mixed well by inversion. This was incubated for 20 minutes at 65^{o}C. An equal volume of 24:1 chloroform:isoamylalcohol was added and the mixture was centrifuged at 12,000 × g for 10 minutes. The upper layer was transferred to a fresh tube, avoiding any precipitate; if separation was poor the mixture

was mixed and spun for a second time for 10 minutes. An equal volume of propan-2-ol was added and mixed, and the mixture was centrifuged for a further 10 minutes; 100-300 µl of sterile deionised water was used to solubilise the resulting DNA pellet once all the alcohol had evaporated.

Restriction enzyme digestion of DNA

Restriction enzymes were purchased from New England Biolabs Ltd. and all digestions were carried out in 10× Multi-core buffer (Promega). Typically plasmid DNA and PCR products were digested for 1-3 hours with 10 units of restriction enzyme for every 5 µg of DNA.

Gel extraction

Fragments excised from agarose gels under UV visualisation were recovered and cleaned up using a Zymogen gel extraction kit (Cambridge Biosciences) as directed by the manufacturer. Recovered fragments were run on 1% agarose gels to determine the concentration, after which they were used in ligation or labelling reactions.

Ligation of DNA

The concentrations of restriction digested vector and insert DNA fragments were estimated by gel electrophoresis as explained below. A ratio of insert:vector of 3:1 was used in ligation reactions, typically using 50 ng of vector in a total volume of 20 µl

containing 400 units of T4 DNA Ligase (New England Biolabs Ltd.) and 1 × T4 DNA Ligase reaction buffer. Reactions were incubated overnight at 4ºC.

Agarose gel electrophoresis of DNA

DNA was separated and visualised using 1% agarose gels containing ethidium bromide. Agarose (Seakem LE agarose, Cambrex) was dissolved in 1 × TAE (9.68 g/l Tris base, 0.744 g/l EDTA adjusted to pH 7.7 with glacial acetic acid by heating in a microwave oven to boiling point. When the agarose solution had cooled to approximately 65^{o}C, 25 µg/ml of ethidium bromide was added, and the molten agarose was poured into an appropriate size gel casting tray containing a comb to create wells. When the gel had set the comb was removed and DNA samples were loaded into the wells mixed with 5 × loading buffer (1 × TAE, 1.5% glycerol and 0.5% orange G in water). Samples were subjected to electrophoresis in 1 × TAE buffer, typically at 100 volts for 15-20 minutes; gels were visualised using a UV transilluminator.

Estimation of DNA concentration using gel electrophoresis.

The concentration of DNA in samples was estimated by agarose gel electrophoresis. 1-5 µl of DNA solution was mixed with 5 × sample loading buffer and loaded onto a 1% agarose gel containing ethidium bromide. 5 µl of lambda *Hind*III molecular weight marker (Invitrogen) of known concentration was loaded into an adjacent well. The gel was subjected to electrophoresis at 100 V for approximately 15 minutes after which the

DNA was visualised under UV light. DNA concentration was estimated visually, by comparing the sample to the known concentration of the molecular weight markers.

2.6 DNA sequencing and PCR

Plasmid DNA was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The pre-mix terminator dye was diluted 1/8 with 5 × Sequencing Buffer (Applied Biosystems) before use. Sequencing reactions comprised 4 µl of diluted terminator pre-mix, 100 ng of template plasmid DNA and 2 pmoles of primer in a total volume of 10 µl of distilled deionised water.

Primers used for sequencing are shown in Table 2.4. Thermal cycler conditions were 29 cycles at 96 ºC for 10 seconds, 50 ºC for 10 seconds followed by 4 minutes at 60 ºC, carried out on a G-Storm GS1 thermal cycler. After completion, 2 µl of 2.2% (w/v) SDS and 8 µl of sterile distilled water were added to the reaction mixture, which was then heated to 98^{o}C for 5 minutes and cooled to room temperature. Unincorporated dye and excess primers were removed from the reaction using Performa® Gel Filtration Cartridges (Edge BioSystems). The purified reactions were analysed by the University of Leicester's Protein and Nucleic Acid Chemistry Laboratory using an Applied Biosystems 3730 sequencer.

Polymerase chain reactions (PCR).

PCR products that were to be used for cloning were generated with Bio-X-Act Long DNA Polymerase (Bioline) with PCR buffer Premix D (Cambio). All other PCR reactions were performed using *Taq* DNA Polymerase (ABgene). Typical reactions contained 0.5 units/µl of polymerase, 1× buffer and 1 pmol/µl of forward and reverse primers. Reactions contained 50-100 ng of template DNA in a total volume of 20-50 µl, made up with distilled deionised water.

Standard reaction conditions were performed on a G-Storm GS1 thermal cycler and involved 28 cycles of 95ºC for 30 seconds, 50-60ºC (depending on the annealing temperature of the primer pair, see table 2.4) for 30 seconds and 70 C for 1 minute. This was followed by a further incubation at 72^{o}C for 1 minute per 1 kb of PCR product.

Oligonucleotide primers for construction of complementing plasmids, confirmation of mutations in the genome of S. *aureus* and construction of probes for northern blotting are shown in Table 2.4. Primers were purchased from Invitrogen, Sigma or the University of Leicester's Protein and Nucleic Acid Laboratory.

Table 2.4. Oligonucleotide primers used in this study.

Primer	Primer sequence (5'-3')	Description	Annealing temp ^{o}C
JM Fur F	CTATCATTGCATTGCAAGAC	*fur* mutant confirmation	55
JM Fur R	CTGCGTCGCCTAATGGTAC		
ica F	GCCTGACATAAATGTGGATG	*ica* mutant confirmation	56
ica R	CATATCAGTATCCCAGTAGC		
EapF	CTCGGATCCTTCAGTTAATTCAAAAAATAG	*eap* complement construction	60
EapPF	CTCGGATCCAAAAGGAGAGATAATTTATGA	*eap* probe generation	52
EapR	CTCCTGCAGTTAAAATTTAATTTCAATGTCTACTTTTTTAATGTC	*eap* complement &probe generation	
EmpF	CTCGGATCCTGTTAAGACAACGTTTACT	*emp* complement construction	57
EmpPF	CTCGGATCCAAGGAGAAATAACAGATGAAAAG	*emp* probe generation	51
EmpR	CTCCTGCAGTTATACTCGTGGTGCTGGTAAGC	*emp* complement & probe generation	
SarR	GTTTGCTTCAGTGATTCG	*sarA* mutant confirmation	49
SarAR	TGGGATGTTTGGCCAGATTCTAATGGGC		
AgrF	CAGTAAGAACCCATTTCGCC	*agr* mutant confirmation	55
AgrR	GGCAATGAGTCTGTGAGATT		
saePF	GCAGCTGGTGCTGTTATCAC	*saeP* probe generation	55
saePR	CGAAGATGACGTGAGC	*saeP* probe generation	
saeRF	GGTGCAGATGACTATG	*saeR* probe generation	55
saeRR	GGTATACTCGATACGACG	*saeR* probe generation	
saeSF	GGTGTTATCAATTAGAAGTC	Sequence determination of *saeS*	
Sae flankR	GAGAAGGATACCCATAAAG	Sequence determination of *saeS*	
FurF	CATCTACACGACCACATTCC	*fur* probe generation	55
FurR	CGATTAAATCGCGTTAAGCAAC	*fur* probe generation	
xerDF	GTATCAATTCAGATACACGC	*xerD* probe generation	55
xerDR	CTCTGACATATCTACTACC	*xerD* probe generation	
HfqF	GGATCCCGACAAATGACATGGTCAAG	Sequencing	
HfqR	GAATTCGTCGTATCATATATTGACTCG	*hfq* mutant confirmation and sequencing	55
MiaF	ACGAGTTGAATGGGTTAACC	*hfq* mutant confirmation and sequencing	

Table 2.4. Oligonucleotide primers used in this study continued.

Primer	Primer sequence (5'-3')	Description	Annealing temp ^{o}C
isdAF	GGATGACTATATGCAACACC	*isdA* probe generation	57
isdAR	GTTCTTGAGCAGTTTGTG		
isdBF	CTGTTGAGTTCCATCTTTC	*isdB* probe generation	55
isdBR	CTACAACATGAACAAACGG		

2.7 Transformation of *E. coli* with plasmid DNA

Preparation of Electrocompetent *E. coli.*

E. coli TOP10 was grown overnight with shaking in LB. 1 ml of the overnight culture was used to inoculate a 500 ml sterile flask containing 100 ml of fresh LB pre-warmed to 37^{o}C. The culture was grown for approximately 3 hours or until it reached 0.6 $OD_{600\ nm}$ at which point the flask was plunged into a tub of ice and swirled to rapidly chill the culture to approximately 4^{o}C. The culture was then poured into sterile 50 ml tubes and centrifuged at 4^{o}C in a pre chilled rotor for 10 minutes at 3200 × g in an Eppendorf 5810R bench-top centrifuge to pellet the cells. The supernatant was discarded and the cell pellets were pooled and re-suspended in 25 ml of ice cold sterile deionised water. The tubes were spun again for 10 minutes at 4^{o}C to pellet the cells. The ice cold sterile water wash was repeated a further three times; after the final wash the cell pellet was re-suspended in approximately 200 µl of the final wash supernatant. The cells were then either transformed immediately or 20% (v/v) glycerol was added and the cells were stored in 50 µl aliquots at –80^{o}C. Cells were thawed on ice before use.

Preparation of Electrocompetent *S. aureus.*

The procedure for producing electrocompetent *S. aureus* cells is the same as for *E. coli* above except that *S. aureus* RN4220 was grown in TSB and washed in ice cold filter sterilised 0.5 M sucrose instead of water.

Electroporation of *S. aureus* and *E. coli* with plasmid DNA.

Before electporation salt was removed from plasmid DNA by dialysis. A 0.025 µm filter disc (Millipore; catalogue number VSWP 01300) was floated on the surface of sterile distilled water in a Petri dish and DNA was added to the filter disc. Samples were dialysed for at least 20 minutes. The DNA was then added to 50 µL of competent *E.coli* or *S.aureus* cells in a prechilled 2 mm electrocuvette (Geneflow Ltd, E6-0060) on ice. *E. coli* cells were transformed almost immediately whereas *S. aureus* cells and DNA were incubated together on ice for 25 minutes prior to electroporation. Electroporation conditions consisted of a pulse of 25µF, 2.5kV and 200Ω delivered using the BioRad Gene pulsar electroporator. Immediately after the pulse, 950 µl of sterile SOC was added to the cuvettes for both *E. coli* and *S. aureus* to aid recovery of the cells. The cells were then transferred to a sterile universal tube and incubated with shaking at 200 rpm at 37^{o}C for 2-4 hours. 100 µl aliquots of the cultures were spread onto LA plates containing the appropriate antibiotics and incubated overnight at 37^{o}C.

SOC Electroporation recovery medium

SOC consisted of 20 g tryptone, 5 g yeast extract, 0.5 g sodium chloride and 10 ml of 250 mM potassium chloride in 950 ml of distilled water, autoclaved at 121^{o}C for 15 minutes to achieve sterility. When cool, 20 ml of filter sterilised 1 M glucose were added with 5 ml of 2 M magnesium chloride; the volume was made up to 1000 ml with sterile distilled water and the medium was frozen in 20 ml aliquots until required. Aliquots were thawed and pre-warmed to 37^{o}C before use.

2.8 Construction of *eap*- and *emp*-complementing plasmids.

eap and *emp* fragments were PCR amplified with enzyme restriction sites compatible with the *E. coli/S aureus* shuttle vector pMK4. PCR products were cloned first into the pGEM T-Easy vector system (Promega), which improves the chances of successful cutting with the desired enzymes, compared to digesting fresh PCR products. pGEM T-Easy is a high copy number vector that, when transformed into *E. coli*, allows blue-white selection of colonies containing the desired insert. After successful restriction, fragments containing *eap* and *emp* were cloned into pMK4 and transformed into the appropriate *eap* or *emp* mutant strains.

The *eap* and *emp* genes were amplified by PCR from Newman wild-type DNA using Triple Master Mix (Eppendorf) with primer pairs EapF/EapR and EmpF/EmpR, respectively, which added a *Bam*HI restriction site to the 5′ end and a *Pst*I restriction site to the 3′ end of the PCR products. The reaction was subjected to electrophoresis on

a 1% agarose gel and the resulting DNA fragments representing *eap* and *emp* were recovered from the gel using a Zymogen gel recovery kit (Cambridge Biosciences) as directed by the manufacturer. The DNA polymerase in Triple Master Mix has exonuclease activity and therefore removes A-overhangs, the PCR products were therefore A-Tailed using Taq DNA polymerase (Applied Biosystems) by incubating with Taq at 70^{o}C for 30 minutes as directed by the vector manufacturer (Promega). This resulted in the addition of a single 3′-A overhang, allowing the PCR products to be ligated into the pGEM T-Easy vector system which contains a single 3´-T overhang, so improving the efficiency of the ligation reaction. The resulting ligation products were transformed into *E. coli* by mixing the reaction mixture with 100 µl of electro competent *E. coli* TOP 10 cells (Invitrogen) and electroporating as described above. Electoporated cells were allowed to recover in SOC medium for 4 hours, after which 100 µl of the mixture was plated onto LB containing 100 µg/ml Ampicillin, 20 mg/ml IPTG and 40 µg/ml X-Gal. Plates were incubated overnight at 37^{o}C, and the following day white colonies were screened by PCR, using the same primer pairs as for their construction. Plasmids containing *eap* and *emp* inserts were digested with *Bam*HI and *Pst*I; the resulting digest was separated by electrophoresis on a 1% agarose gel, and the *eap* and *emp* DNA fragments were recovered from the gel as previously described and ligated into similarly digested pMK4, creating plasmids pEap and pEmp respectively. These plasmids were then transformed into electrocompetent *E. coli* TOP10 cells, and spread onto plates containing 100 µg/ml ampicillin, and incubated overnight at 37^{o}C. Resulting colonies were screened for the correct insert by PCR as described before. Plasmids containing the relevant inserts were transformed into electrocompetent restriction deficient *S. aureus* RN4220. After recovery, 100 µl of cell suspension were

plated onto agar containing chloramphenicol at 10 μg/ml, and incubated overnight at 37°C. Resulting colonies were screened as before by PCR; plasmids containing the relevant complementing genes were then transduced into Newman *eap::erm* or Newman *emp::erm* as described below, using chloramphenicol at μg/ml for selection.

2.9 Phage transduction or *Staphylococcus aureus* cells

Phage Lysate Preparation

Donor cells containing the plasmid or mutation to be transduced were grown in 5 ml of TSB overnight with shaking at 200 rpm. This culture was then used to inoculate 25 ml of TSB^{++} to an OD_{600} of 0.05 (TSB^{++} is TSB supplemented with 10 mM $CaCl_2$ and 10 mM $MgSO_4$), and grown at 37°C with shaking to OD_{600} 0.2 . 10 ml of this culture was subcultured into 25 ml of fresh TSB^{++} and 1 ml of phage phi 11 of an appropriate titre was added. The culture was incubated at 37°C with shaking for 4 hours or until complete lysis was observed. The lysate was then filter sterilised using a 0.45 μm filter disc (Acradisc, Millipore Ltd) and stored at 4°C until required.

Determination of Phage Titres

For successful transduction the lysate should contain 10^7-10^{10} pfu (plaque forming units) per 100 ml. To determine the pfu an overnight culture of *S. aureus* 8325-4 was grown in 5 ml of TSB at 37°C with shaking. 100 μl of this was spread onto the surface of an LA plate and allowed to dry. 10 μl aliquots of tenfold serial dilutions of the phage lysate were spotted onto the seeded plate and allowed to dry before overnight incubation at 37°C. The following day dilution spots with clearly defined plaques were counted

and the number of pfu per ml was determined. If the number of pfu was below 10^7-10^{10} pfu/100 ml a second round of lysate production was carried out as described above with the lysate generated in the first round of lysate production, in order to increase the lytic phage count.

Phage Transduction

An overnight culture of recipient bacteria was grown at 37°C with shaking in 20 ml of LK broth. This was centrifuged at 3,200 × g for 10 minutes and the pellet was re-suspended in 1 ml LK broth. This was split into two 500 µl aliquots, one of which was used as a control to which no phage was added. Cells were treated as follows;

Test	**Control**
500 µl recipient cells	500 µl recipient cells
1 ml LK broth + 10 mM $CaCl_2$	1.5 ml LK broth + 10 mM $CaCl_2$
500 µl phage lysate	

The samples were incubated for 25 minutes at 37°C statically in a water bath followed by 15 minutes at 37°C with shaking. After incubation 1 ml of ice cold 0.02 M sodium citrate was added to the samples on ice, followed by centrifugation at 3,200 × g for 10 minutes. The pellets were re-suspended in 1 ml of ice cold 0.02 M sodium citrate and incubated on ice for 2 hours. 100 µl aliquots were spread on LK plates containing the appropriate antibiotics and incubated overnight at 37 °C.

2.10 Biofilm/Adhesion Assay

Cultures were inoculated into 96 well microtitre plates as described in section 2.3. To assess adhesion every hour one plate was inoculated for each time point required, and the OD_{595} was read as an inoculation control. After the required incubation time the plate was removed from the incubator, read at 595nm and then emptied into a trough of detergent by flicking the contents out. The wells were then washed with a constant flow of phosphate buffered saline (PBS) three times to remove non adherent cells and air dried at 60^{o}C for 30 minutes. When dry the plates were stained with 1% safranine at room temperature for 30 minutes, rinsed with tap water until the water ran clear and dried before reading at 490nm. Wells were also inoculated with medium only as a contamination control as a blank for adhesion. Assays were repeated at least three times and the average and standard deviations of the mean were calculated. To establish if variations in growth at 24 hours affected the outcome of the adhesion or biofilm assay the 490 nm value obtained for biofilm formation was divided by the 595 nm value obtained for growth of the corresponding strain, and then multiplied by an arbitrary value, in this case 0.1, to obtain a value for biofilm formation for a standard level of growth. In most cases variations in OD at 24 hours did not affect the outcome of the biofilm assay and the original data is presented, otherwise the adjusted data is presented. Where required, a students T-test was carried out and values were accepted as significant if $P = < 0.05$.

Addition of conditioning film

Microtitre wells were pre-coated with a conditioning film consisting of human extra-cellular matrix proteins by pre-incubating the 96 well plates with either 10 µg/ml human Fibrinogen (F4883, Sigma Ltd) or 100% human plasma (FFP). 200 µl of protein solution was added to each well and the plate was incubated overnight at 4^{o}C, following which the wells were emptied and blocked for 1 hour at 37^{o}C with 2% BSA (Bovine Serum Albumin, Sigma Ltd). After blocking the wells were emptied and 24 hour biofilm formation was assayed for strains Newman and 8325-4 with and without the addition of 50 µM Fe.

Preparation of fresh frozen plasma (FFP) from whole blood.

A fresh 50 ml sample of human blood was treated with heparin/sodium citrate to prevent clotting. Plasma was prepared by density gradient centrifugation. Blood was centrifuged at 13,000 × *g* for 10 minutes at 4^{o}C after which the top plasma layer was carefully removed so as not to disrupt the layer of white cells (buffy coat) between the plasma and the red cell layer. Aliquots of plasma were stored at –20^{o}C until required.

2.11 Protein Analysis

Staphylococcal Protein Fractionations

10-20 ml of 6 or 24 hour cultures were centrifuged at 3200 × g for 10 minutes to pellet the cells. The supernatant, which contained secreted proteins, was decanted, retained

and later concentrated (supernatant extract). The pellets were re-suspended in the residual supernatant (approx 200 µl) and transferred to a sterile pre-weighed Eppendorf tube. The cells were centrifuged at 11,300 × g for 5 minutes and all the supernatant was removed with a Gilson pipette and the Eppendorf tube weighed again to determined the pellet weight. The sample was then treated as follows depending on the protein fraction to be extracted. As boiling in sodium dodecyl sulphate (SDS) to remove surface proteins prevents proper lysis with lysostaphin, a double volume (20 ml) of cells was used; half was used for SDS protein extraction and the other half was used to obtain proteins from all the other fractions.

SDS extraction of non-covalently bound surface proteins

Cells were re-suspended in 100 µl of 2 % SDS per 10 mg of pellet, boiled for 3 minutes, immediately centrifuged for 1 minute at 11,300 × g, and the supernatant removed to a fresh tube. 20 µl of 2 × Laemmli sample loading buffer were added to 20 µl of SDS extract, boiled for 3 minutes, briefly centrifuged and then loaded onto a 10% separating SDS PAGE gel. 2 × Laemmli buffer consisted of 20% (v/v) glycerol, 4% (w/v) SDS, 200 mM Tris pH6.8, 2% (w/v) bromophenol blue and 200 mM dithiothreitol.

Cell Wall protein extraction

Cells were re-suspended in 250 µl of 30% (w/v) raffinose, 80 µg/ml lysostaphin and 1 mg/ml benzamidine per 10 mg of pellet, incubated at 37°C for 30 minutes and centrifuged 6,700 × g for 5 minutes. Cell wall extracts (supernatants) were removed to a fresh tube after centrifugation, while the remaining protoplast pellets were used to prepare membrane and cytoplasmic proteins as follows.

Membrane and cytoplasmic protein extraction

Protoplasts obtained from the removal of the cell wall (see above) were re-suspended in PBS depending on the previously determined cell pellet weight, based on 250 µl of PBS per 10 mg of cells. Protoplast suspensions were sonicated in a Biorupter sonicating waterbath (Diagenode) containing ice, for 30 seconds on and 30 seconds off for a total of 10 minutes. Cytoplasmic proteins were then removed to a clean tube after centrifugation at 6,700 × g for 10 minutes, and the remaining protoplast pellet was re-suspended in 1 × Laemmli sample buffer depending on the previously determined cell pellet weight based on 250 µl sample buffer per 10 mg of cells.

Protein gel electrophoresis

Proteins were separated on a SDS-polyacrylamide gel by electrophoresis (SDS-PAGE). 25 ml of 10% resolving gel was prepared per gel by mixing 12.3 ml of buffer A (9.1% Tris base, 0.2% SDS in water, pH 8.8), 8.3 ml of acrylamide mix (37.5:1 acrylamide:bisacrylamide), 864 µl of 1% ammonium persulphate (APS) and 3.4 ml of distilled water. 68 µl of N,N,N',N'-tetramethylethylenediamine (TEMED) was added directly before pouring the gel. The surface of the gel was then overlaid with isopropanol until polymerisation occurred, after which it was removed and a 5% stacking gel was poured on top of the resolving layer; a comb was inserted to create loading wells. The stacking layer was made up in 7 ml per gel and consisted of 1.2 ml of acrylamide mix, 3.5 ml of buffer B (3% Tris base and 0.2% SDS in deionised water, pH 6.8), 175 µl of 1% APS, 2.2 ml of deionised water and 14 µl of TEMED immediately before pouring. After polymerisation of the stacking layer, the wells were

washed out with deionised water and the protein samples were loaded after being boiled for 3 minutes with Laemmli buffer.

The gel was run in 1 × SDS-PAGE running buffer (0.025 M Tris, 0.192 M glyine, 0.19 % SDS in distilled water) at 45 mA per gel for approximately 90 minutes. Protein bands were visualised by overnight staining with Coomassie stain, followed by incubation in destain to remove non specific staining. Coomassie stain consisted of 0.25 % Coomassie Brilliant blue R250, Sigma, in destain (45% (v/v) methanol, 10% (v/v) glacial acetic acid, in water).

2.12 Transcriptional analysis

Extraction of total RNA from *S. aureus* (Schmitt *et al.*, 1990).

50 ml cultures were harvested after 6 hours of growth by centrifugation at 3,200 × g for 5 minutes in an Eppendorf benchtop microfuge, transferred to an RNase free 2 ml screw cap tube, re-suspended in 1 ml of RNA protect (Ambion) and stored at 4^{o}C overnight. The following day the cells were harvested by centrifugation at 11,300 × g and re-suspended in 400 µl of RNase free AE buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA). 20-40 µl of 10 mg/ml lysostaphin was added to the cell suspension and incubated for 20 minutes to lyse the cells. After lysis 80 µl of 10% (w/v) SDS were added and vortexed for 30 seconds. An equal amount (480 µl) of phenol equilibrated with AE buffer was added and vortexed for a further 30 seconds. Samples were incubated at 65ºC for 4 minutes before snap-freezing in a dry ice ethanol bath for 3 minutes. These hot and cold incubation steps were repeated a further three times with a

final incubation at 65ºC for 4 minutes. The samples were centrifuged at 11,300 × g for 5 minutes and the aqueous phase was transferred to a fresh tube to which 500 µl of phenol:chloroform:isoamylalcohol (25:24:1 ratio) were added. Samples were vortexed briefly and centrifuged at 11,300 × g for 10 minutes at room temperature. This step was repeated and the aqueous phase transferred to a clean tube until there was no further visible precipitate. The RNA was precipitated by adding 2 volumes of 100% ethanol and incubating overnight at -80ºC. The samples were centrifuged at 11,300 × g for 15 minutes at room temperature and washed with 500 µl of 80% ethanol. After centrifugation at 11,300 × g for 15 minutes at room temperature, the supernatant was removed and the pellets were air dried and re-suspended in 50 µl of RNase free water.

All RNase free solutions used were rendered RNase free by treatment with 0.1% diethylpyrocarbonate (DEPC) or were made with DEPC-treated water. After addition of DEPC solutions were mixed vigorously and left overnight in a fume hood; next day the DEPC was deactivated by autoclaving solutions at 121^{o}C for 15 minutes.

Determination of RNA concentration using a spectrophotometer.

1 µl of a solution of RNA was loaded onto the pedestal of a Nanodrop spectrophotometer (ND1000) which had been previously calibrated against the same DEPC treated water as the diluent. The sample was then subjected to analysis at various wavelengths to determine purity and concentration. Concentration was based on the calculation that 1 OD_{260} unit = 40 µg of RNA.

Denaturing formaldehyde agarose gel electrophoresis of RNA

Formaldehyde agarose gels for separation of RNA comprised 1.5% (w/v) agarose dissolved in 1 × MOPS (3[N-Morpholino]propanesulfonic acid) (0.2 M MOPS, 50 mM sodium acetate, 1 mM EDTA), 5% formaldehyde and 3 µl of 10 mg/ml ethidium bromide in RNase-free water. 10-30 µg RNA were mixed with 5 × RNA sample loading buffer, heated to 65^{o}C for 10 minutes and stored on ice for 10 minutes before loading onto the gel. 5 × RNA sample loading buffer contained 200 µl of saturated bromophenol blue solution, 80 µl of 500 mM EDTA pH 8.0, 720 µl of 37% formaldehyde, 2 ml of glycerol, 3.084 ml of formamide, and 10 ml of 10 × MOPS per 10 ml of RNase-free water. Electrophoresis was carried out in 1 × MOPS for 2-2½ hours at 90 volts, and visualised using a UV transilluminator.

Northern blotting

RNA was transferred to nylon membrane by Northern blotting immediately after denaturing agarose gel electrophoresis. After visualisation of RNA using a UV transilluminator the gel was transferred to the blotting apparatus. An inverted gel casting tray the same size as the gel was placed in a large tray containing 20 × SSC (SSC is 3.0 M NaCl and 0.3 M sodium citrate at pH 7.0). A piece of blotting paper (3M) was placed over the casting tray; this was as wide as the tray but long enough to touch the bottom of the tray containing the 20 × SSC, so acting as a wick. The gel was then placed on top of the 3M paper and a piece of positively charged nylon membrane (GRI Osmonics), cut to the same size as the gel, was pre-wet with 2 × SSC and placed over the gel. A second piece of 3M blotting paper the same size as the gel was also pre-

wet with 2 × SSC and placed over the top of the membrane. A stack of 5 sheets of QuickDraw extra thick pure cellulose blotting paper (Sigma Ltd P8046) was placed on top of the gel, membrane and 3M paper followed by a large glass plate. A weight was placed on top of the glass plate and transfer was performed overnight. When completed, the nylon filter was dried and the RNA fixed using 700,000 μJ cm^{-2} of energy in a UV crosslinker (Amersham Biosciences).

Probe synthesis and labelling.

Probes for detection or RNA transcripts were synthesised by PCR as previously described using the primer pairs as listed in table 2.4.

Radioactive labelling of probes

Radioactive α-^{32}P d-CTP was used to label probes for Northern blotting using random hexamers as primers (Feinberg and Vogelstein, 1983). 30 ng of DNA in a total volume of 16 μl were denatured by boiling for 5 minutes and chilling on ice for 5 minutes. The probes were labelled by adding 5 μl of OLB (ligolabelling buffer), 1 μl of BSA, 1 μl of Klenow fragment of DNA Pol I (New England Biolabs) and 2.5 μl of α-^{32}P d-CTP (specific activity 0.925 Mbq). OLB was made from a mixture of solutions A, B and C in the ratio 2:5:3. Solution A contained 1 ml of Solution O (1.25 M Tris-Cl, pH 8.0, 0.125 M magnesium chloride), 18 μl of β-mercaptoethanol and 5 μl each of dATP, dTTP and dGTP at an individual concentration of 100 μM. Solution B was 2 M HEPES (pH 6.6) and Solution C contained random hexamers (GE Healthcare) re-suspended in TE (pH 8.0) at a concentration of 90 OD_{600} units/ml. Probes were labelled for 1-2 hours

at 37ºC. Unincorporated nucleotides were removed using Illustra NICK™ columns (GE Healthcare) as directed by the manufacturer using elution buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.1 % SDS) to equilibrate the column and to recover the probe.

Hybridisation and stripping of radiolabelled probes

RNA crosslinked membranes were pre-hybridised with constant rotation for at least one hour at 65ºC in Church Gilbert's buffer (0.5 M Na_2HPO_4, 0.5 M NaH_2PO_4, 7% SDS, 1 mM EDTA). Radiolabelled probe was added and hybridised to the membrane overnight with constant rotation at 65ºC. The membrane was then washed at 65ºC with constant rotation with several changes of wash buffer (3 × SSC, 0.1% SDS) until no further radiation was detected in the wash removed from the membrane. Membranes were then wrapped in Saran wrap supported by a piece of damp 3M paper and placed into autoradiography cassettes containing intensifier screens with X-ray film. The X-ray films were exposed at –80ºC and then developed. Blots were stripped and re-probed with either a second target probe or with a *16S rRNA* DNA probe which was used as a loading control. Probes were stripped from membranes by adding boiling 0.1% w/v SDS to the membranes and incubating them at 65ºC with agitation with frequent changes of SDS until no further radiation was detected on the membrane. The membranes were then re-hybridised for at least one hour at 65ºC in Church Gilbert's buffer and probed as previously described.

2.13 PNAG extraction and detection.

Extraction of PNAG

PNAG was extracted from cell pellets as described by Cramton *et al*. (2001) with minor amendments. 10 ml of bacterial culture grown for 24 hours statically at 37^{o}C in CRPMI in 5 % CO_2 in air, with or without supplementation of iron, were centrifuged at 3,200 × g for 10 minutes to pellet the cells. The supernatant was removed by decanting and the cell pellet was re-suspended in the residual supernatant. The cells were then transferred to a pre-weighed microcentrifuge tube and centrifuged at 11,200 × g for 5 minutes to pellet the cells. As much as possible of the remaining supernatant was removed using a Gilson pipette, and the microcentrifuge tube was weighed again to determine the weight of the cell pellet. The cells were re-suspended in 0.5 M EDTA, pH 8, based on 50 µl of EDTA per 10 mg of cells. The suspension was vortexed briefly, boiled for 5 minutes and immediately centrifuged at 11,200 × g for 1 minute to pellet the cells. The supernatant containing the PNAG was removed to a fresh microcentrifuge tube, mixed with 4 mg of proteinase K and incubated at 37^{o}C for 30 minutes. The PNAG extract was stored at 4^{o}C until required for a maximum of 3 days.

Slot blotting of PNAG extracts

PNAG samples were diluted as required (10^{-1}/10^{-2}) in sterile distilled water in a volume of at least 50 µl. A piece of nitrocellulose membrane (Amersham Biosciences) and a piece of 3M blotting paper were cut to fit the size of the slot blot apparatus platform, soaked in 10 × SSC and placed over the wells of the blotter. The top of the blotting

apparatus was secured with the pins and screws as described by the manufacturer and suction was applied to the apparatus not exceeding 1 atmosphere of pressure. 50 µl of each sample were applied to the wells; 50 µl of 0.5 M EDTA were added to empty wells to act as a control. Once the sample had emptied from the wells 50 µl of 0.5 M EDTA were washed through the wells. Whilst still under pressure the apparatus was dismantled and the membrane was removed and left to air dry.

Detection of PNAG

The dry blot was soaked in blocking solution (3 % BSA, Sigma, in PBS) for 1 hour with agitation at room temperature, to prevent non-specific binding of the antibody. The membrane was rinsed twice with PBS containing 1% Tween® 20 (PBS + Tween) and then washed three times for 15 minutes each with 120 ml of PBS + Tween. Rabbit PNAG antibody, a kind gift from Kimberly Jefferson (Kropec *et al* 2005), was diluted with PBS + Tween at a ratio of 1:50,000 or 1:1000 and incubated with the membrane with agitation for 1 hour at room temperature. The blot was then rinsed twice with PBS + Tween.

Horse radish peroxidise (Sigma) was diluted 1:4000 in PBS + Tween (12.5 µl in 50ml) and incubated with the membrane for 1 hour at room temperature, followed by 2 rinses with PBS + Tween and three washes for 15 minutes each with 120 ml of PBS + Tween. The membrane was removed from the wash and allowed to drain; it was then placed on a piece of Saran wrap. Enhanced chemiluminescence (ECL) detection reagents

(Amersham: GE Healthcare) were mixed and added to the membrane as directed by the manufacturers' instructions; the Saran wrap was wrapped around the blot and air bubbles were excluded to ensure even contact between the membrane and the reagents. The membrane was then wrapped in a fresh piece of Saran wrap, exposed to X-ray film and then developed.

2.14 Densitometry.

Transcriptional differences and differences in the expression of PNAG were verified using a densitometry software package ImageJ version 1.41 (http://rsbweb.nih.gov/ij/). Values obtained for transcripts were normalised against those obtained with the *16S rRNA* DNA probe. Expression of PNAG was compared directly to that of the wild type.

Chapter 3

Biofilm formation: Establishing the assay.

3.1 Introduction

Biofilm formation is an essential process for many pathogenic bacteria, including *S. aureus,* as it facilitates colonisation. *S. aureus* is a human commensal and an opportunistic pathogen, with an arsenal of virulence factors at its disposal if it breaches our natural defences such as the skin and our immune system. Surgical procedures often lead to infection, a major cause of which is *S. aureus.* This leads to increased suffering of the patient, possibly death, and increases costs to the National Health Service (Surveillance of Healthcare Associated infections report: 2008). Treating *S. aureus* infections is not always straight forward. For example, colonisation of host or artificial surfaces as a thick multi-layered biofilm encased in protective polysaccharide makes treating the infection difficult (Williams *et al.*, 1997). Also, antibiotic resistance is a major concern in *S. aureus* infections as a growing number of strains are resistance to methicillin, one of the most widely trusted antibiotics used to treat Staphylococcal infections. The evolution of methicillin resistance was a major blow to health care practitioners, and led to the widespread use of vancomycin, another effective antibiotic in treating staphylococcal infections, including those which are resistant to methicillin.

Unfortunately this in turn led to vancomycin resistance, leaving a more limited number of last resort antibiotic treatments available for use. In addition to increased antibiotic resistance, reports of *S. aureus* infections occurring in otherwise healthy people are on the increase. This suggests that some community associated strains are more virulent, no longer requiring the host to be already compromised for an infection to take hold.

A major hurdle for bacteria to overcome in order to cause infection in the mammalian host is iron limitation. Iron is kept to a minimum in the host to protect host cells from damage and in an attempt to prevent infection. However, *S. aureus* has developed multiple iron scavenging mechanisms enabling it to survive in the host, and like other pathogenic bacteria, it uses low iron availability as a signal to up regulate genes associated with virulence. Recently it has become more apparent that *S. aureus* rapidly adapts and responds to its surrounding environment, and that different *in vitro* environments have a global impact on gene expression (Wilcox *et al.*, 1991; Morrissey *et al.*, 2002; Cirz *et al.*, 2007). The effects of iron on the virulence of other pathogenic bacteria, such as *E. coli*, have been studied in some detail. By contrast less is known about the affects of iron on *S. aureus* virulence, including biofilm formation. *S. aureus* biofilm formation has been widely studied but the regulatory mechanisms behind it are still not fully understood (reviewed by Gotz, 2002). The cell surface, being in direct contact with the host has been the main focus of many studies investigating biofilm formation, in which rich growth media have been routinely used. Iron depleted tissue culture medium RPMI 1640 (CRPMI) has been used in previous investigations to grow *S. aureus*, and has been shown to produce protein profiles more reflective of those

observed *in vivo* than was obtained from rich growth medium (Morrissey *et al.*, 2002). RMPI is a low iron tissue culture media that is further depleted of iron by the use of a chelating resin Chelex®100, and is then referred to as CRPMI (Chelex treated RPMI). It is the aim of this section of work to establish a method for analysing the effects of iron on *S. aureus* biofilm formation *in vitro*, and given its prior use for iron studies in *S. aureus*, CRPMI was chosen as a growth medium in this study. Much of the work presented in this chapter has been published (Johnson *et al.*, 2005; Baker *et al*., 2009).

3.2 96 well microtitre biofilm assay using rich growth medium (TSB).

Biofilm formation in bacteria can be assayed using a range of techniques which have different advantages and disadvantages. For example biofilms are commonly assayed using a flow cell, a chamber connected to inlet and outlet tubing into which seeded growth media can be introduced. The apparatus is then incubated and biofilms form on the inside of the chamber, which has a specially designed window through which biofilm formation can be viewed microscopically. In addition the chamber can be constructed out of various materials to observe the effects of different surfaces on biofilm formation. This method also allows the growth medium to be introduced at various pressures to recreate the flow which may be encountered inside a catheter. However the number of strains and conditions which can be tested at any one time is limited by the cost and space required for the flow cells and microscope equipment. The most favoured technique for assessing biofilm formation in a range of strains and growth conditions is the semi-quantative 96 well microtitre plate assay. The assay was first described by Christensen *et al.* in 1985 and has been widely used since with some

minor modifications. In this work six strains were used to test the assay (Table 2.3); these included two strains previously assessed for biofilm formation, 8325-4 and Newman (Lim *et al.*, 2004; Beenken *et al.*, 2003), laboratory strains Cowan I and BB, and two methicillin resistant isolates belonging to clonal types EMRSA-15 and EMRSA-16, MRSA PM25 and MRSA PM27 respectively. These two clonal types are responsible for the majority of MRSA infections in Scotland and in England respectively. *In vitro* biofilm assays are often conducted using the rich growth medium TSB (tryptic or trypticase soy broth), and published data exists for biofilm formation for strains 8325-4 and Newman in TSB. Therefore to determine if previous levels of biofilm formation for strains Newman and 8325-4 could be reproduced in this work the assay was first tested using TSB incubated at 37^{o}C in 5 % CO_2 in air.

Figure 3.1 shows that Newman forms a low level of biofilm in TSB and 8325-4 forms a high level of biofilm in TSB, similar to previous levels of biofilm formation reported for the two strains elsewhere (Lim *et al.*, 2004; Beenken *et al.*, 2003). This also highlighted that the ability to form a biofilm varied depending on the strain, as the other strains tested formed relatively poor biofilms compared to the laboratory strain 8325-4 in TSB (Fig 3.1 A). Although growth was similar for all stains tested (Fig 3.1 B), the values obtained for biofilm formation were adjusted to take into account these variations in cell numbers to determine if this affected the outcome of the biofilm assay (see section 2.10). Adjusting the data to take into accout growth differences had little effect on the overall pattern of biofilm formation between the strains (Fig 3.1 C), suggesting that the differences in biofilm formation were not related to variations in growth.

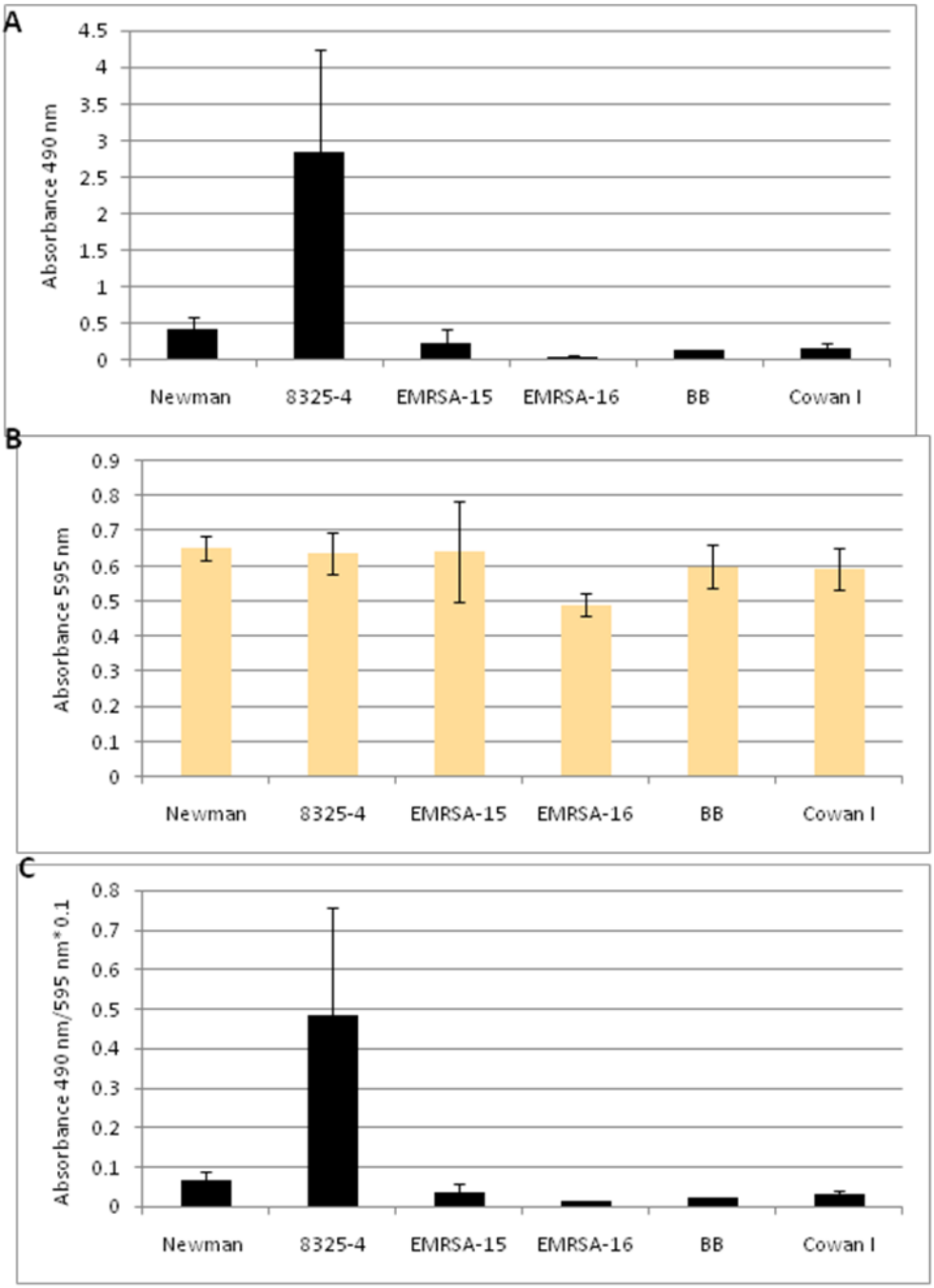


Figure 3.1. 24 hour biofilm formation of *S. aureus* strains in TSB growth medium. Overnight cell pellets were used to inoculate fresh TSB with the relevant strain to an OD 595 of 0.1. 200µL of culture was then inoculated into quadruplet wells of a 96 well microtitre plate. After 24 hours incubation in 5% CO_2 in air at 37°C, plates were read at 595 nm to measure growth and wells were emptied, dried and stained with safranin. Plates were then read at 490 nm to determine the level of biofilm formation. A) Raw 490 nm data. B) 24 hours growth at 595 nm C) 490 nm data adjusted for differences in growth. The OD 490 nm was divided by the OD 595 and multiplied by 0.1 to give a normalised value for biofilm formation relative to growth. Figures show the mean and standard deviation of at least three separate experiments.

3.3 96 well microtitre biofilm assay using low iron growth medium CRPMI.

As the assay in rich medium had proved successful in reproducing previously reported findings for Newman and 8325-4, the effect of low iron on biofilm formation of the six strains was observed using the growth medium CRPMI. The use of CRPMI had a significant effect on the biofilm capacity of the strains tested (compare Figs 3.1 A and 3.2 A). 8325-4 which produced a high level of biofilm in TSB, produced one of the lowest levels of biofilm in the iron restrictive growth medium CRPMI. Conversely Newman, produced only a moderate level of biofilm in TSB, yet produced the highest level of biofilm in CRPMI of all the strains in either growth medium. The other strains tested produced relatively poor levels of biofilm in both growth media. Growth at the end of the assay was also measured and there were variations in the end point optical density between the strains (Fig 3.2 B). Biofilm values at 490 nm were adjusted to take this variation into account as described previously (section 2.10). Here again, adjusting the data had very little effect on the overall pattern of biofilm formation (Fig 3.2 C).

As Newman and 8325-4 showed the most variation in biofilm formation in the two different growth media and because Newman produces the highest level of biofilm in the low iron medium, these two strains were selected for further observation. CRPMI is relatively low in nutrients compared to TSB which affects the growth and may impact on the outcome of the biofilm assay if the bacteria in the two different media are at different stages in the growth cycle. To investigate this, the proliferation of the two strains was compared in the two media at hourly intervals (Fig 3.3). There was, as expected, a difference in growth of strains in the different media. Approximately half

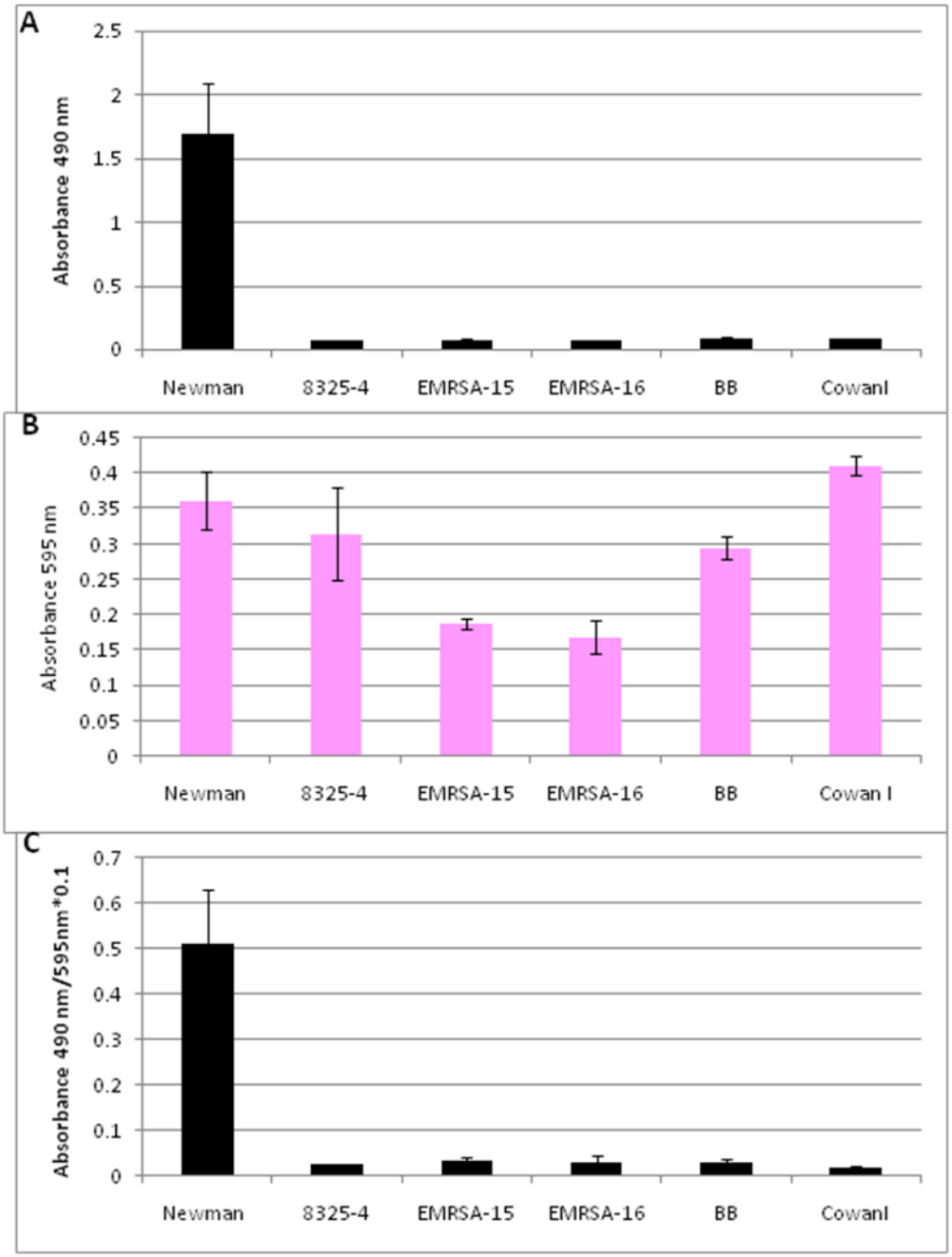


Figure 3.2. 24 hour biofilm formation of *S. aureus* strains in CRPMI growth medium. Overnight cell pellets were used to inoculate fresh CRPMI with the relevant strain to an OD 595 of 0.1. 200µL of culture was then inoculated into quadruplet wells of a 96 well microtitre plate. After 24 hours incubation in 5% CO_2 in air at 37°C, plates were read at 595 nm to measure growth and wells were emptied, dried and stained with safranin. Plates were then read at 490 nm to determine the level of biofilm formation. A) Raw 490 nm data. B) 24 hour growth at 595 nm. C) 490 nm data adjusted for differences in growth. The OD 490 nm was divided by the OD 595 and multiplied by 0.1 to give a normalised value for biofilm formation relative to growth. Figures show the mean and standard deviation of at least three separate experiments.

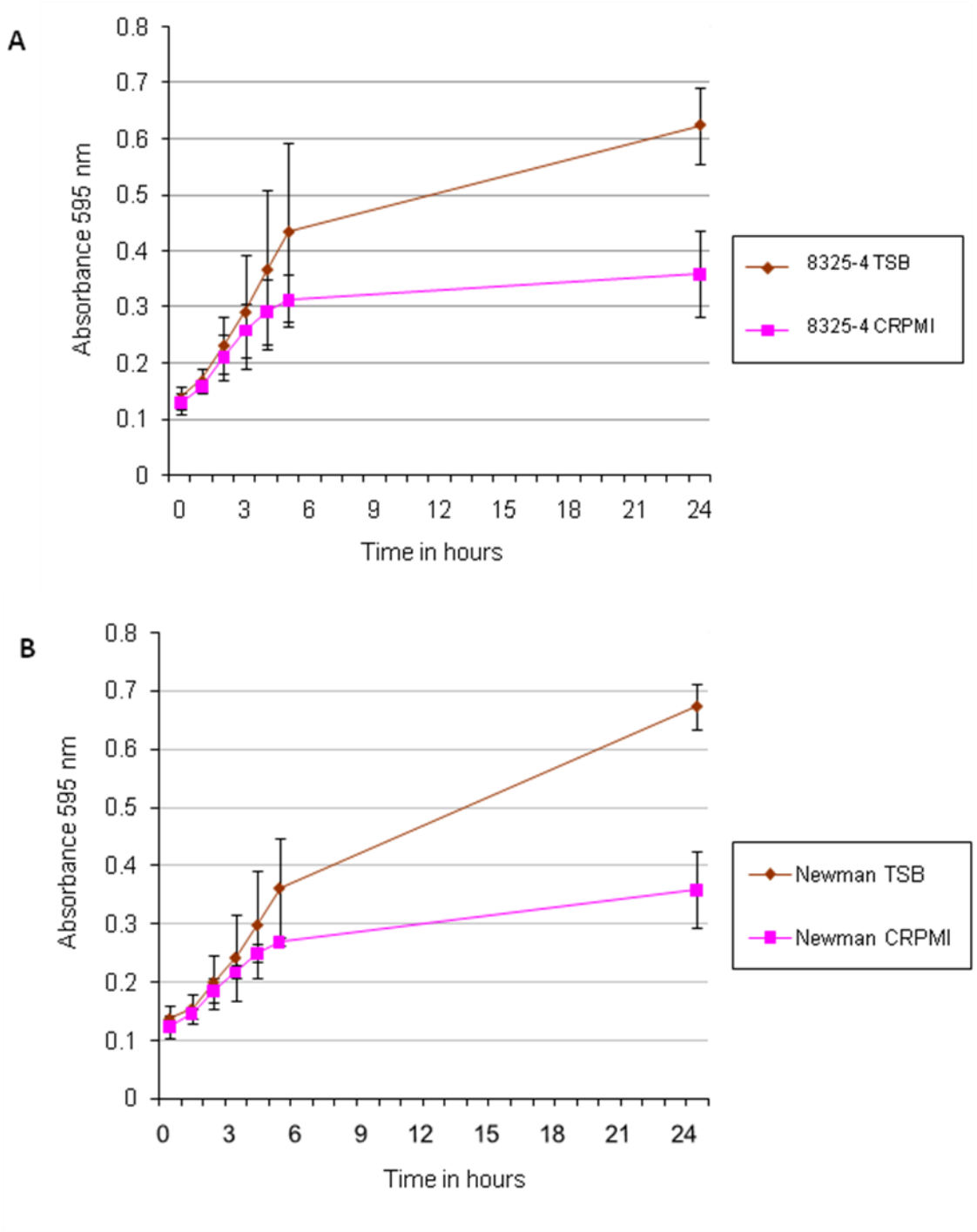


Figure 3.3. Comparison of growth of *S. aureus* strains Newman & 8325-4 in CRPMI & TSB growth media. Overnight cell pellets were used to inoculate fresh growth media with the relevant strain to an OD 595 of 0.1. 200µL of culture was then inoculated into quadruplet wells of a 96 well microtitre plate, incubated in 5% CO_2 in air at 37°C. At each time point the plate was removed and read in a plate reader at 595 nm to monitor growth. Figures show the mean and standard deviation of at least three separate experiments. A) Strain 8325-4. B) Strain Newman.

the optical density for each strain was observed in CRPMI compared to that in TSB at 24 hours, due to the lower nutritional content of the medium. *In vitro* bacterial cultures go through a series of recognised growth phases and it was observed that cultures grown in CRPMI reached, for example, log phase later than those grown in TSB. However at 24 hours, cultures in both growth media were in stationary phase suggesting that the contrasting biofilm profiles of the two strains in the different growth media were not due to variation in growth phase.

3.3.1 The effect of pH on biofilm development.

The fact that there is so much variation in biofilm formation between strains in the two different growth media is interesting. As TSB is not a defined growth medium it is difficult to ascertain what causes these differences. In a previous study it was found that pH influenced biofilm formation in *S. epidermidis* (Dunne and Burd, 1992). In addition to iron status, CRPMI and TSB vary slightly in pH. Fresh TSB equilibrated in 5 % CO_2 has a pH of 6.6, whereas CRPMI has a pH of 7.6. It could be that this difference causes the contrasting biofilm phenotypes for 8325-4 and Newman in the two media. To investigate this, the pH of TSB was adjusted to that of CRPMI to determine if this would induce biofilm production in strain Newman comparable to that observed in CRPMI. After 24 hours incubation there was an increase in biofilm formation for Newman in the pH adjusted TSB compared to the non-adjusted TSB, but, the level of biofilm was far from that observed in CRPMI (compare Fig 3.2A with 3.4). This suggests that pH alone is not responsible for the differences in biofilm production for the two strains in the contrasting growth media.

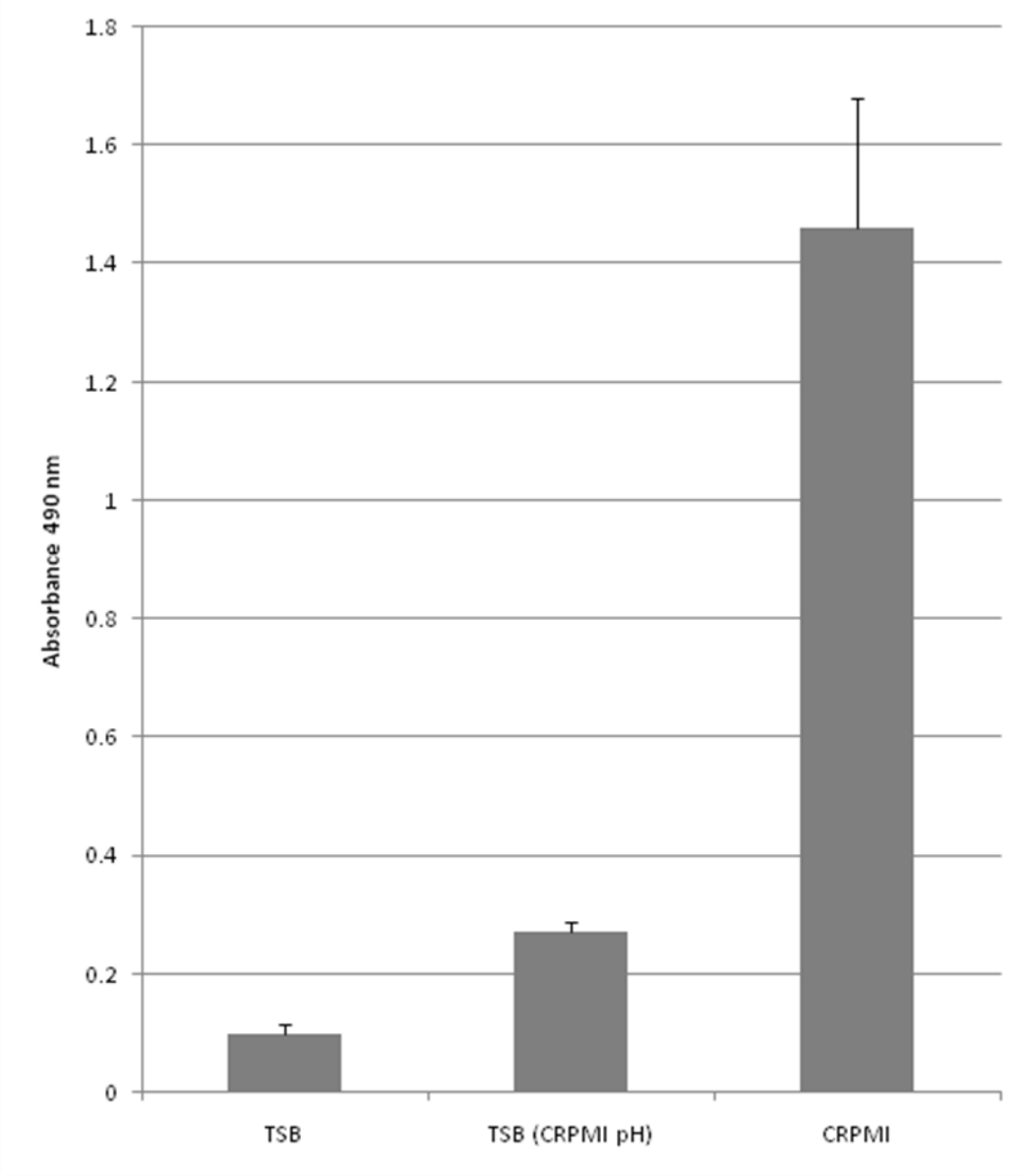


Figure 3.4. 24 hour biofilm formation of strain Newman in TSB (pH 6.6), in TSB with the pH adjusted to that of CRPMI (7.6), and in CRPMI. Overnight cell pellets were used to inoculate fresh TSB with the relevant strain to an OD 595 of 0.1. 200µL of culture was then inoculated into quadruplet wells of a 96 well microtitre plate. After 24 hours incubation in 5% CO_2 in air at 37°C, plates were emptied, dried and stained with safranin. Plates were then read at 490 nm to determine the level of biofilm formation. The figure represents the mean and standard deviation of at least three separate experiments.

3.4 *S. aureus* adhesion in low iron growth conditions.

Biofilm formation is a multi step process involving initial adhesion, mature multi-layered biofilm formation and dissemination. Strains that test negative in a biofilm assay may be deficient in either early attachment or mature biofilm formation, or they may detach before the end of the assay. In CRPMI at 24 hours, strain Newman forms a high level of biofilm whereas the biofilm formed by strain 8325-4 is very low. To establish if 8325-4 (a) was non-adhesive, (b) was capable of initial adhesion but unable to form a full biofilm, or (c) if the biofilm detached before the end of the assay, it was necessary to investigate earlier stages of biofilm formation.

Early adhesion is often measured using a separate assay from that used to observe biofilm formation, and this can be achieved in a number of ways. A Petri dish based adhesion assay involves a PBS suspension of cells pre-cultured overnight in TSB, which are then added to a Petri dish and incubated at 37^{o}C for 1-2 hours. After incubation non-adherent cells are washed off, and adherent cells are stained with safranin. Randomly selected microscopic fields of view are then counted and averaged. This method was tested with cells grown in CRPMI, and was found to be both time-consuming and lacking in reproducibility. Adhesion was variable both between assays and across the surface of individual dishes. This made counting subjective to the microscopic field of view chosen, and was therefore not reflective of the adhesive potential of the strain and reproducible results could not be obtained. In an attempt to rectify this, and to accommodate the comparison of multiple strains and conditions, the 96 well biofilm assay was adapted to observe early adhesion. A similar 96 well based

assay has been used to monitor adhesion in *Pseudomonas* spp (Cowan and Fletcher, 1987).

Initially overnight cultures grown in TSB and CRPMI were re-suspended in PBS. 200 µL aliquots of the cell suspensions were then added to the 96 well plates in quadruplet and the plates were incubated for 1 hour at 37^{o}C. After incubation the wells were washed and stained in the same way as was done in the biofilm assay, and adhesion was measured at 490 nm using a plate reader. Unfortunately this again was not reproducible, and no useful data was obtained. The assay was then repeated using different growth phases to determine if the cells are more adhesive during active growth so that more consistent data could be obtained. 20 mL TSB or CRPMI cultures were inoculated from an overnight cell pellet grown in the appropriate medium to an OD 595 of 0.1. The cultures were then incubated at 37^{o}C in 5% CO_2 and at hourly intervals 1 mL was removed. The cells were pelleted by centrifugation and the cell pellet was resuspended in 1 mL of sterile PBS. 200 µL of each suspension was inoculated into quadruplet wells of a microtitre plate and left to adhere for 1 hr at 37^{o}C in 5 % CO_2. After incubation the wells were washed and stained, and adhesion was measured at 490 nm using a plate reader as before. Data collected using this technique suggested that there were adhesive differences between stains and growth media, but the technique was not reproducible. Nevertheless, it was advantageous to utilise the 96 well plate format to measure adhesion, as it allowed multiple strains and different growth conditions to be assayed simultaneously. In an attempt to obtain reproducible results for early stages of biofilm formation the assay was altered again, this time to measure the adhesive

potential of actively growing cells in growth media. The assay was set up exactly as for the biofilm assay except multiple plates were inoculated at the start of the assay, depending on the number of time points required. Plates were then incubated as before, and at hourly intervals, growth and adhesion were measured as for biofilm formation. Although this did not allow the adhesive potential of cells to be measured at a single growth phase, i.e. it could not be determined if cells in exponential growth phase were more adherent than those in stationary phase, it did allow cumulative adhesion to be measured throughout biofilm formation, and standardised conditions for adhesion and biofilm formation.

Modifying the assay to include earlier time points proved successful. Figure 3.5 shows the adhesive profile of Newman and 8325-4 in CRPMI growth medium from 1-6 hours. Surprisingly 8325-4 had the same adhesive profile as Newman in the early stages of the assay. However, after 3 hours, 8325-4 adhesion was reduced compared to Newman, and at 6 hours, there was a significant difference in adhesion between Newman and 8325-4. Additional time points between 6 and 24 hours were also observed but have not been shown as there was no change in adhesion for strain 8325-4, while Newman adhesion steadily progressed to that observed at 24 hours (Fig 3.2A). This suggests that under iron-restrictive growth conditions 8325-4 is capable of initial adhesion, but lacks one or more components essential for the production of a mature biofilm.

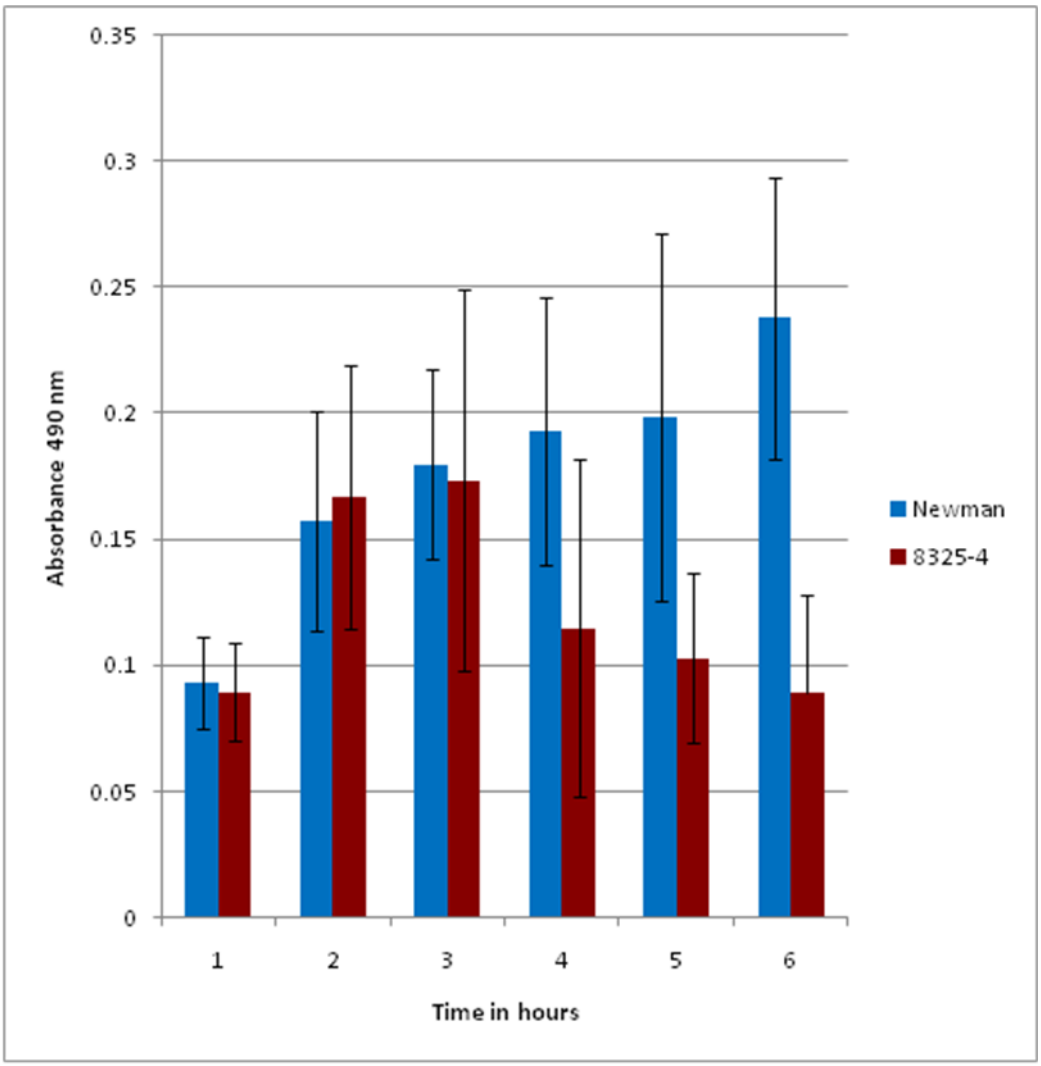


Figure 3.5. Early adhesion of strains Newman and 8325-4 growing in CRPMI. Multiple plates were inoculated with an OD of 0.1 of cells in CRPMI and incubated at 37°C in 5 % CO_2 in air. One plate was removed at each time point and wells were washed, dried and stained. Plates were then read at 490 nm to measure adhesion. The figure represents the mean and standard deviation of at least three separate experiments

3.5 Biofilm formation on surfaces coated with host proteins.

S. aureus biofilms form readily on implanted prosthetic devices and directly on host tissue, as observed in native valve infectious endocarditis (Lowy, 1998). As prostheses are inserted into the body they inevitably become coated with host proteins (McDevitt *et al.*, 1994). *S. aureus* has been shown to express surface proteins that recognise specific host proteins so aiding colonisation (Patti *et al.*, 1994; Chavakis *et al.*, 2005). Therefore in an attempt to encourage biofilm formation in strain 8325-4 under low iron conditions and to determine whether coating the 96 well plates with host proteins affected biofilm formation in strain Newman, human plasma and fibrinogen were chosen as candidates to be tested. Plasma was used to represent a non-specific mixture of host proteins, and fibrinogen to investigate a specific protein to protein interaction. Wells of 96 well plates were coated with the host proteins prior to inoculation (see section 2.10). No difference in 24 hour biofilm formation was observed with wells coated with fibrinogen in either Newman or 8325-4 (Fig 3.6); Newman biofilm production remained high and 8325-4 biofilm remained low. In addition coating the wells with plasma had no effect on 8325-4 biofilm formation. However, plasma coating severely inhibited Newman biofilm formation. As neither coating proved advantageous for biofilm formation under these conditions, coating the wells was not further pursued.

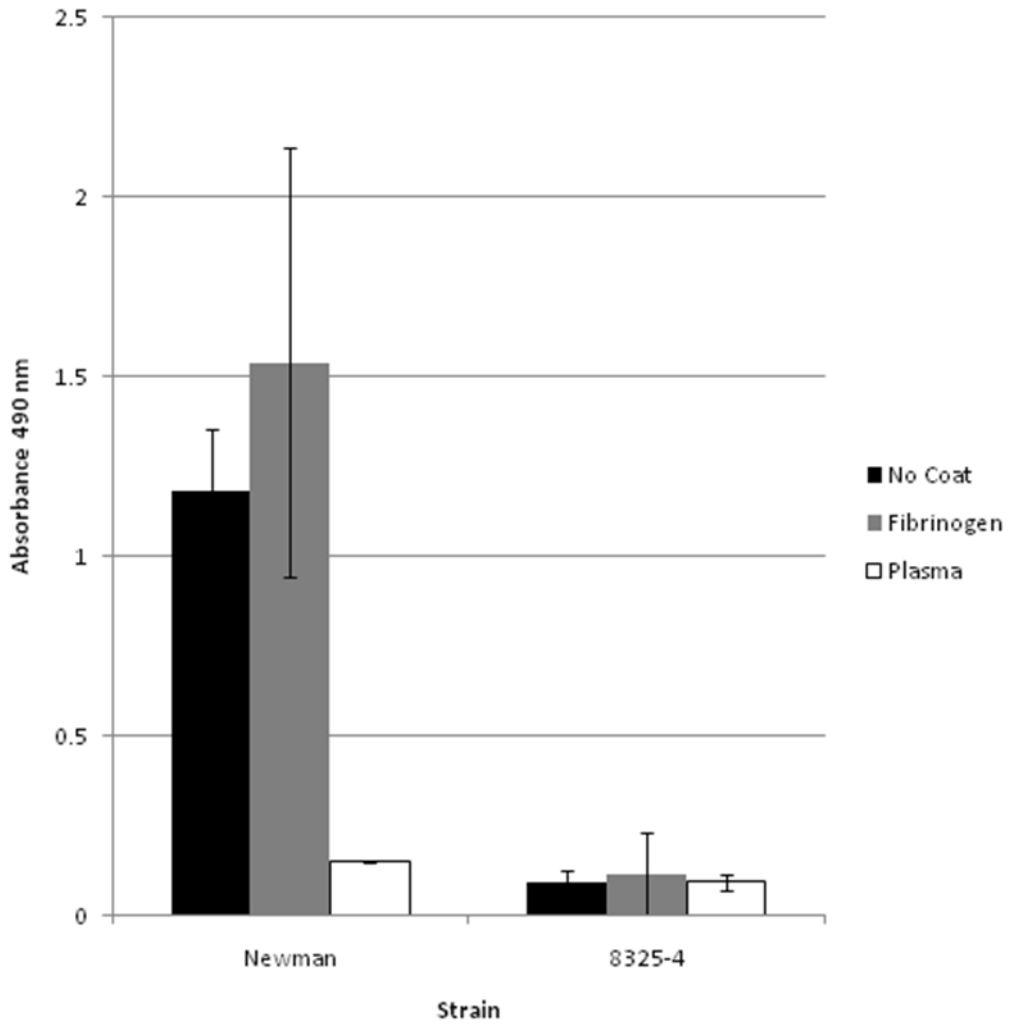


Figure 3.6. Newman and 8325-4 24 hour biofilm formation on wells coated with host proteins. Wells of the 96 well plate were coated with plasma or fibrinogen prior to inoculation. Plates were then inoculated and incubated for 24 hours in 5 % CO_2 in air at 37°C. Plates were then washed, dried, stained and read at 490 nm to determine biofilm formation. The figure represents the mean and standard deviation of at least three separate experiments.

3.6 The role of metal ions in the regulation of *S. aureus* adhesion and biofilm formation.

CRPMI is considered a low iron growth medium, and the work described in the previous section showed that biofilm produced by strain Newman was greatly increased in CRPMI compared to that formed in TSB. To determine if the increase in biofilm was due to the low iron status of the medium, increasing concentrations of iron were added to the cells prior to inoculation into the microtitre wells. Growth was also monitored to record any growth effects, as excess iron is toxic to cells.

3.6.1 The effect of iron on *S. aureus* Newman biofilm formation.

Fig 3.7A shows that biofilm formation is iron regulated and that the effects are dose dependant. Biofilm formation in strain Newman is progressively reduced with increasing concentrations of iron. As little as 5 µM iron reduces biofilm formation 6-fold compared to non-iron supplemented cultures. 50 µM iron, resulted in a value comparable to background (achieved by staining wells incubated with medium only), and iron concentrations in excess of 50 µM were therefore unable to further reduce biofilm formation.

Cultures containing iron grew slightly better than the non supplemented culture (figure 3.7 B). After 24 hours growth, the culture containing no additional iron appeared to have a significantly higher OD than all of the cultures with iron supplementation. However, this is not a true representation of the amount of cells present in the cultures,

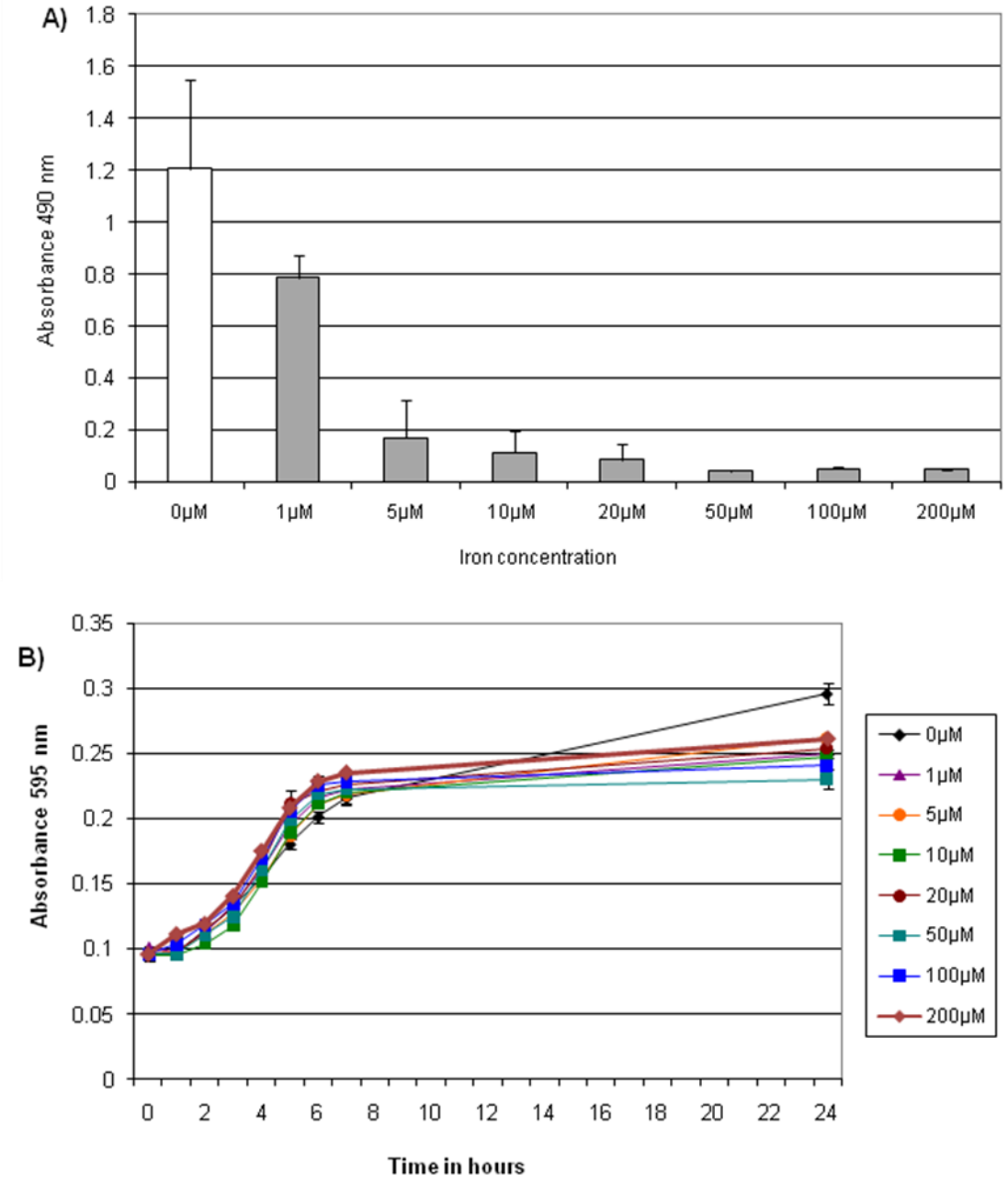


Figure 3.7. 24 hour biofilm formation and growth of *S. aureus* strain Newman in CRPMI in the presence of increasing concentrations of iron. Overnight cell pellets were used to inoculate fresh CRPMI with Newman cells to an OD 595 of 0.1. The cell suspension was split and increasing concentrations of iron were added prior to inoculation of the plate. 200μL of culture was inoculated into quadruplet wells of a 96 well microtitre plate. At hourly intervals the plate was read at 595 nm to monitor growth. After 24 hours incubation in 5% CO_2 in air at 37°C, plates emptied, dried and stained with safranin. Plates were then read at 490 nm to determine the level of biofilm formation. A) Biofilm formation. Figures represent the mean and standard deviation of at least three separate experiments. B) Growth (595 nm). Figures represent the mean and standard deviation of growth in a typical assay

as the addition of iron causes *S. aureus* Newman cells to clump together. This is visible by eye at high cell densities, and results in a reduced OD at 24 hours compared to cultures without iron. Cultures supplemented with more than 10 µM iron contained significantly more cells than non supplemented cultures. This was confirmed by the increased cell pellet weight obtained from cultures with the addition of 50 µM iron compared to those without iron, as observed during experiments comparing protein profiles (section 2.10, data not shown). Also, a previous study observed that cultures supplemented with iron grew better than cultures without additional iron (Horsburgh *et al.*, 2002a). Therefore it could be seen that the decrease in biofilm formation observed in the cultures supplemented with iron was not due to reduced cell numbers resulting from iron toxicity.

3.6.2 The effects of iron on adhesion of *S aureus* strains Newman and 8325-4

These results establish that iron greatly reduces the biofilm potential of strain Newman at 24 hours. To assess the effects of iron on the initial stages of biofilm development the assay was repeated using both Newman and 8325-4 with and without the addition of 50 µM iron. 8325-4 was included as it adheres to a similar level as Newman in the early stages of biofilm formation. 50 µM iron was chosen as it provided the maximum reduction and gave the most reproducible results of the concentrations tested. Multiple replica plates were inoculated at the start of the assay to provide the various time points required. The addition of iron to the growth medium inhibited adhesion at all of the time points assayed for both Newman and 8325-4 (Fig 3.8 A & B), including 24 hour biofilm formation (Fig 3.8 C). The addition of iron reduced adhesion to a level

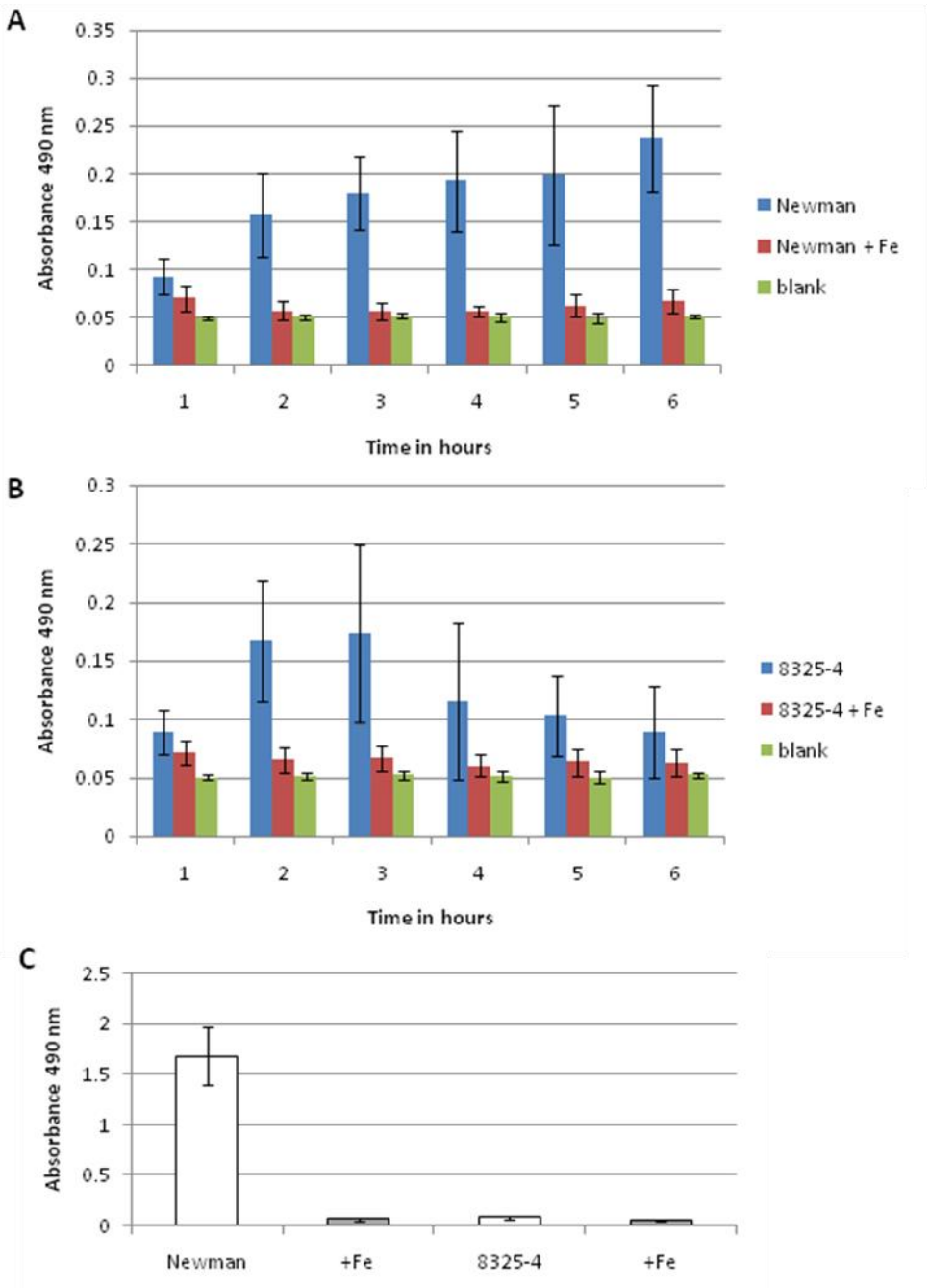


Figure 3.8. A comparison of the effect of iron on early adhesion (A & B) and 24 hour biofilm formation (C) in *S. aureus* strains Newman and 8325-4. Overnight cell pellets were used to inoculate fresh CRPMI with the appropriate strain to an OD 595 of 0.1. The cell suspension was split and iron was added to half the cell suspension prior to inoculation of the plate. 200µL of culture was inoculated into quadruplet wells of a 96 well microtitre plate. Plates were then incubated in 5% CO_2 in air at 37°C. At the required time point a plate was removed from the incubator, emptied, dried and stained with safranin. Plates were then read at 490 nm to determine the level of adhesion or biofilm formation. Results show the mean and standard deviation of at least three separate experiments.

comparable to background (stained wells incubated with medium only). This suggests that iron affects factors involved in early adhesion, preventing the bacteria from adhering to the wells. Growth was also measured for both strains with and without the addition of iron (Fig 3.9). This shows that, as observed with strain Newman, iron increases the growth of 8325-4. Interestingly in high iron, 8325-4 cells do not appear to form clumps at 24 hours in contrast to strain Newman, suggesting that the cell aggregations formed by strain Newman might be due to the expression of something absent or reduced in high iron in strain 8325-4.

3.6.3 The effect of other metal ions on biofilm formation.

It is well established that all organisms including bacteria rely on many transition metals in order to carry out a multitude of biological functions, and CRPMI is low in metal ions other than iron. To establish if biofilm formation and adhesion are specifically regulated by iron, a range of concentrations from 1 µM-1000 µM of different metal ions was added to aliquots of a suspension of Newman cells prior to inoculation of the microtitre plates. Growth was monitored by reading the plate at hourly intervals after inoculation of the plates.

Figure 3.10 shows the effects of increasing metal ion concentration on biofilm formation. The addition of manganese reduced biofilm formation at concentrations of 100 µM and above. However even the highest manganese concentration did not inhibit biofilm formation as much as far lower concentrations of iron (20 µM). Figure 3.11 A

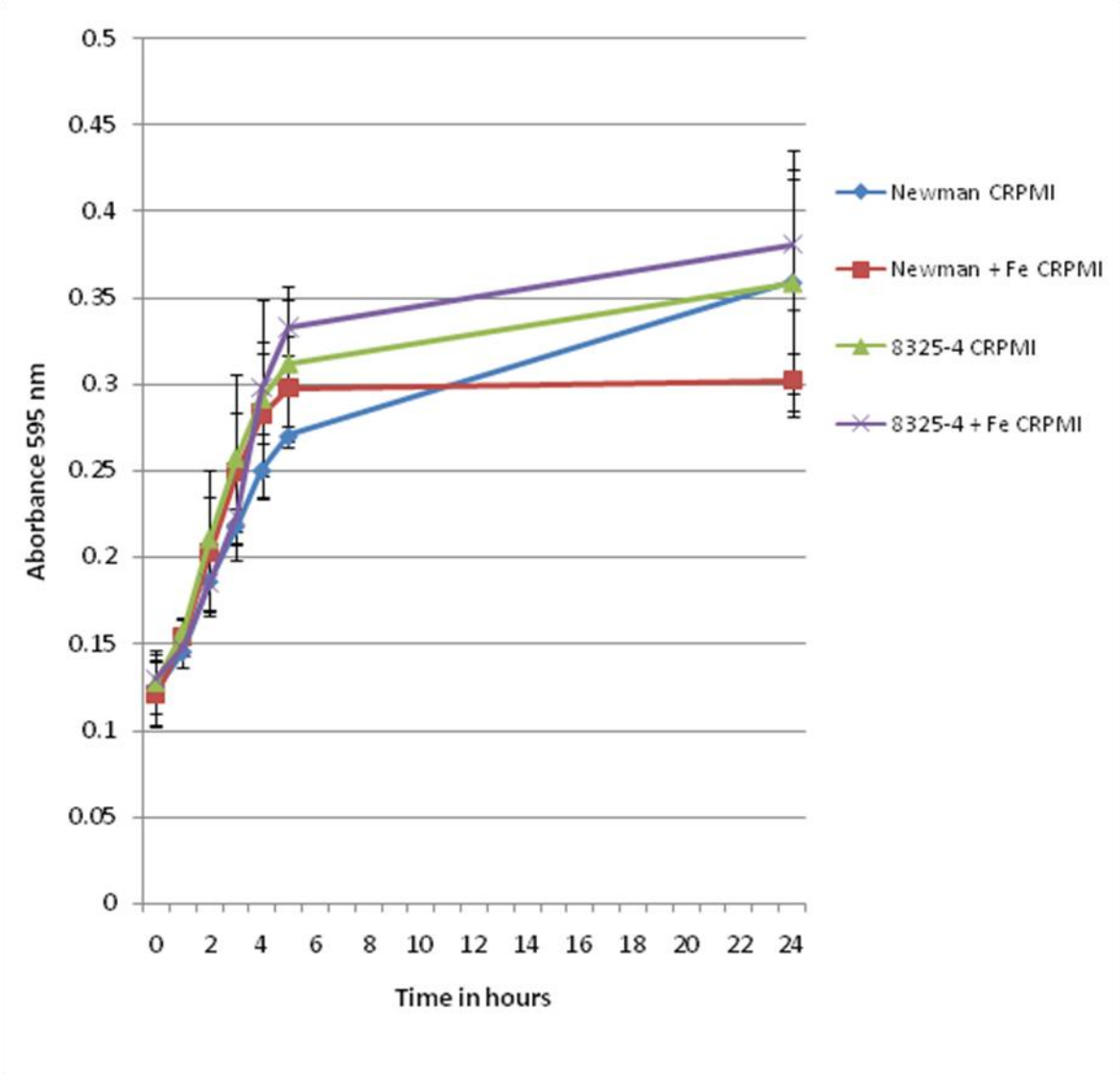


Figure 3.9. The effect of 50μM iron on the growth of *S. aureus* strains Newman and 8325-4. Overnight cell pellets were used to inoculate fresh CRPMI with the appropriate strain to an OD 595 of 0.1. The cell suspensions were split and iron was added to half of the culture for each strain prior to inoculation of the plate. 200μL of culture was inoculated into quadruplet wells of a 96 well microtitre plate, and plates were incubated at 37°C in 5 % CO_2 in air. At hourly intervals the plates were read at 595 nm to monitor growth. Results show the mean and standard deviation of at least three separate experiments.

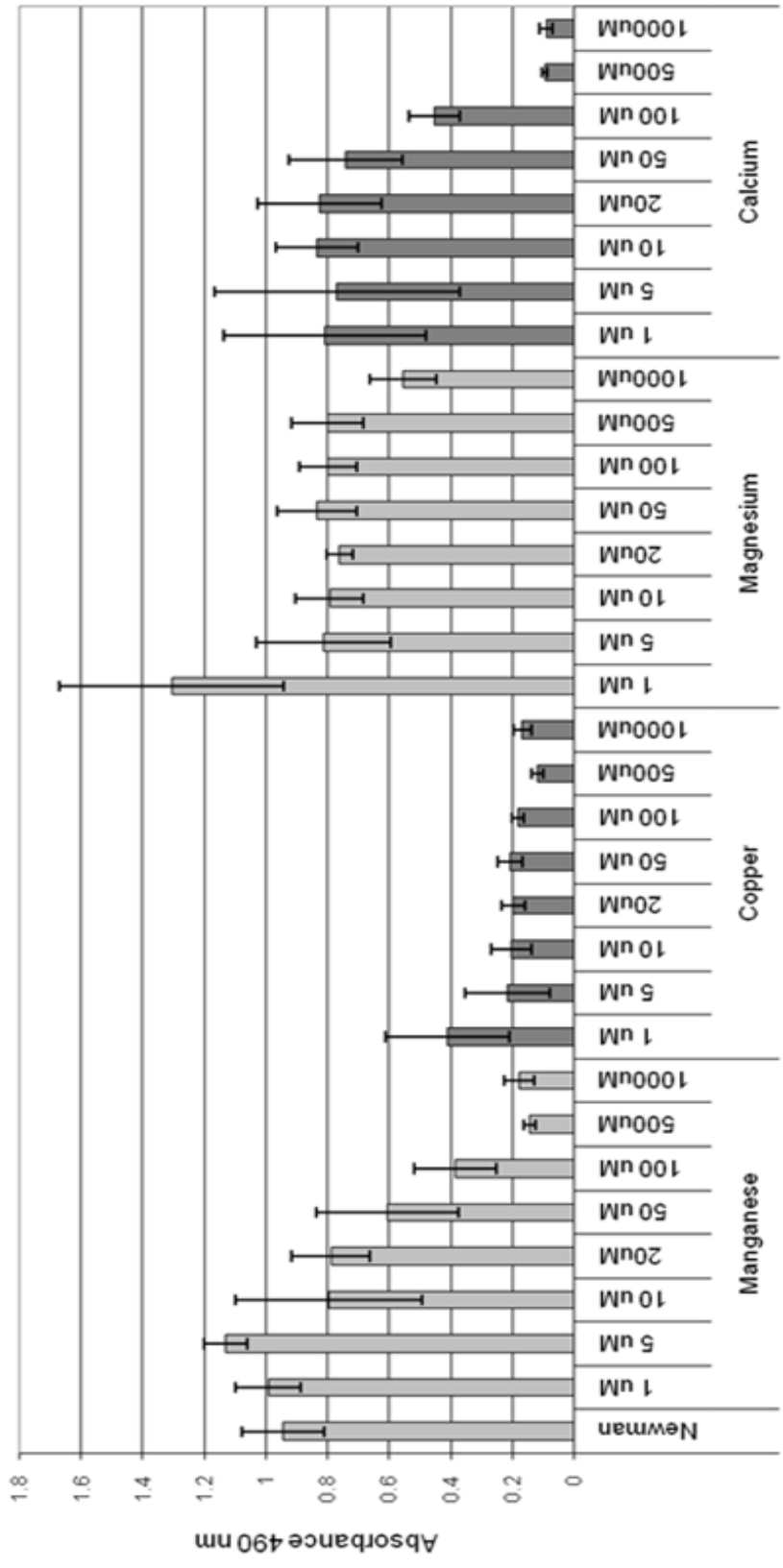


Figure 3.10. Biofilm formation of strain Newman with various concentrations of metal ions. A cell pellet from an overnight culture of strain Newman grown in CRPMI was used to create a suspension of cells at approximately 0.1 OD 595 nm in fresh CRPMI. The cell suspension was then split into aliquots and metal ions were added at various concentrations. 200 µL of each culture was added to quadruplet wells of a 96 well microtitre plate. The plate was then incubated for 24 hours at 37°C in 5% CO_2 in air. The graph represents the mean and standard deviation of at least three separate experiments.

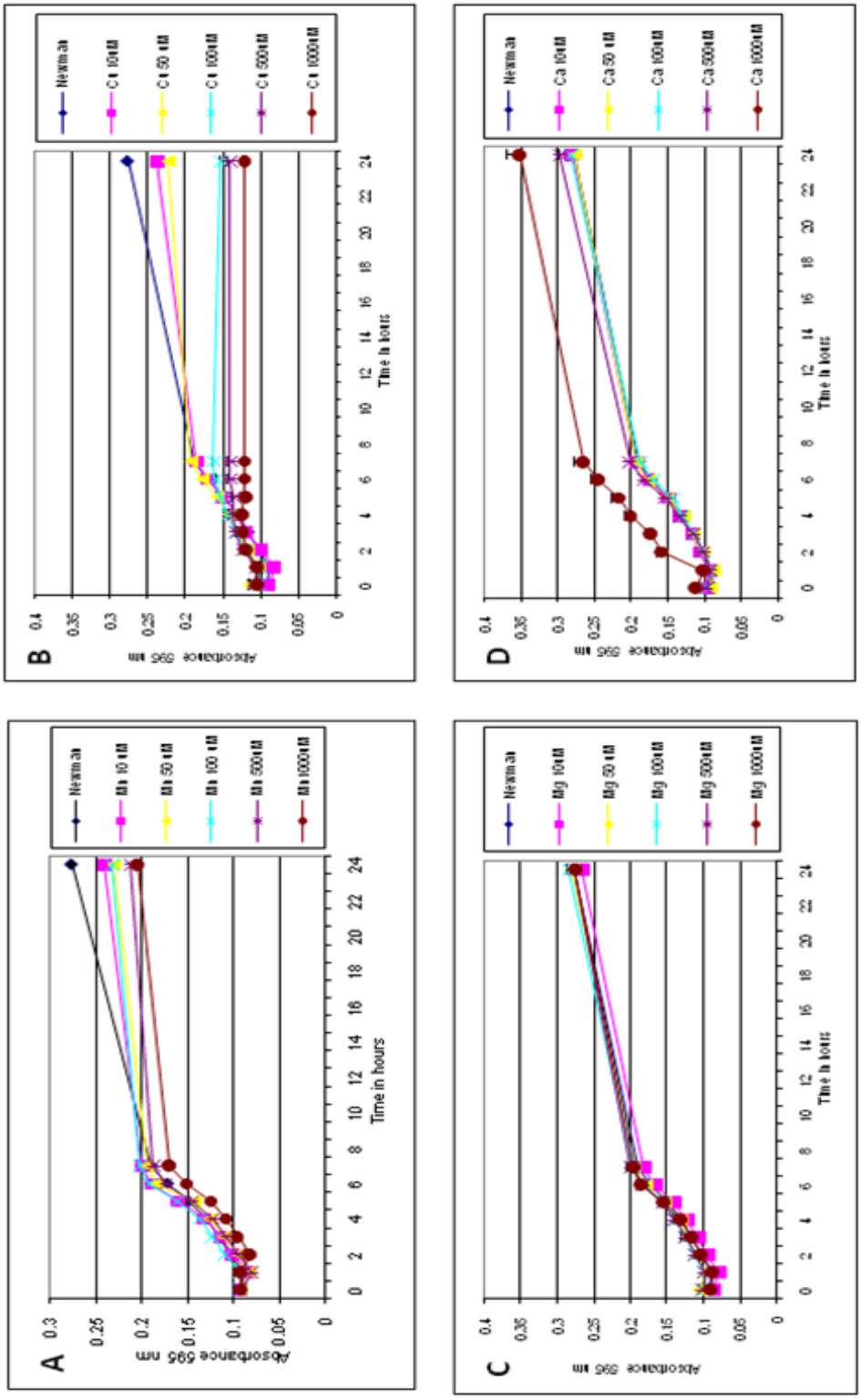


Figure 3.11. Graph showing growth of *S. aureus* strain Newman in CRPMI growth media with and without the addition of different concentrations of metal ions. Overnight cell pellets were used to inoculate fresh CRPMI with Newman cells to an OD 595 of 0.1. The cell suspension was split and increasing concentrations of ions were added to the aliquots prior to inoculation of the plate. 200µL of culture was inoculated into quadruplet wells of a 96 well microtitre plate which were incubated in 5% CO_2 in air at 37°C. At hourly intervals the plate was read at 595 nm to monitor growth. Figures are representative of typical growth, and have been adjusted for initial turbidity of the media caused by the addition of the ion where necessary. A) Manganese, B) Copper, C) Magnesium and D) Calcium.

shows growth of Newman in medium containing increasing manganese concentrations. To simplify the graphs, growth curves for 1, 5, and 20 µM concentrations have been omitted. Supplemented cultures grew slightly more slowly than un-supplemented cultures in a concentration dependant manner. This was particularly evident at 24 hours suggesting that manganese may be toxic at the concentrations used. Unlike iron, there was no visible clumping of the cells suggesting that the reduced OD values at 24 hours were due to fewer cells and not due to cell aggregation.

The addition of copper at all concentrations significantly reduced Newman biofilm formation (Fig 3.10). However, biofilm formation in cultures containing 500 µM and above were still higher than that observed with 20 µM iron. Cultures containing all concentrations of copper had reduced growth compared to the non supplemented culture Figure 3.11 B, and growth was reduced in a concentration dependant manner, and suggests that copper is toxic to *S. aureus* strain Newman. The reduction in biofilm formation observed with increasing copper concentration may be in part be due to the toxic effects of the metal ion at high concentrations, however at low concentrations of copper (10µM) biofilm formation was reduced significantly, despite only a small reduction in growth. This implies that other factors are involved in reduced biofilm formation in the presence of copper besides reduced growth.

Magnesium had no obvious effect on biofilm production until the concentration reached 1000 µM, with a 1.5 fold reduction ($P = 0.038$) (Fig 3.10). Addition of 20 µM

magnesium appears to result in significant reduction in biofilm formation ($P = 0.024$), but this may be anomalous since concentrations immediately above and below had no significant effect on biofilm formation ($P > 0.05$). The addition of magnesium at any of the concentrations used had no effect on growth (Fig 3.11 C), suggesting that the reduction in biofilm formation observed at 1000 µM was due to factors other than toxicity of the metal.

The addition of calcium significantly reduced biofilm formation only at concentrations of 100 µM and above (Fig 3.10). The addition of calcium at concentrations of 10 to 500 µM had no effect on growth, but 1000 µM calcium significantly increased growth (Fig 3.11 D). This suggests that very high concentrations of calcium are both beneficial and detrimental to *S. aureus* Newman, in that growth is increased but biofilm formation is compromised.

Adjusting the 24 hour biofilm data to accommodate the differences in growth at 24 hours in cultures with ion supplementation did not change the overall pattern of biofilm formation, although growth was clearly effected by copper, and high levels of manganese and calcium.

3.7 The effect of iron on biofilm formation in other *S. aureus* strains.

It has been shown in the previous section (3.6) that biofilm formation in strain Newman is negatively regulated by iron. To determine if this is a specific response by strain Newman or a general *S. aureus* response, biofilm formation was observed using a range of *S. aureus* strains. The experiment was performed as previously described with the presence and absence of 50 µM iron. Growth was monitored at 0 and 24 hours to determine any variations in growth. *S. aureus* strains assayed, listed in table 2.3(†), included laboratory strains and recent clinical isolates, and iron was seen to inhibit biofilm formation in all strains tested to varying levels. Figure 3.12 illustrates a sample of the variation in levels of biofilm and iron regulation observed. All of the strains tested produced a low to moderate level of biofilm formation in low iron which was reduced in all strains in high iron. Even the low level of biofilm formed by strain 4532/7 is still iron regulated ($P = 0.037$). It is noteworthy that none of the strains tested produced a level of biofilm comparable to strain Newman.

3.8 Discussion

The aim of the work described in this section was to establish an appropriate method for studying *S. aureus* biofilm formation and early adhesion, and to determine how biofilm formation was affected by iron. The 96 well microtitre plate assay was selected to observe biofilm formation, and adapting the assay to include early adhesion proved successful. Low iron is a major limiting factor for *S. aureus* survival in the host (Litwin and Calderwood, 1993), and there is a strong link between iron and virulence in other

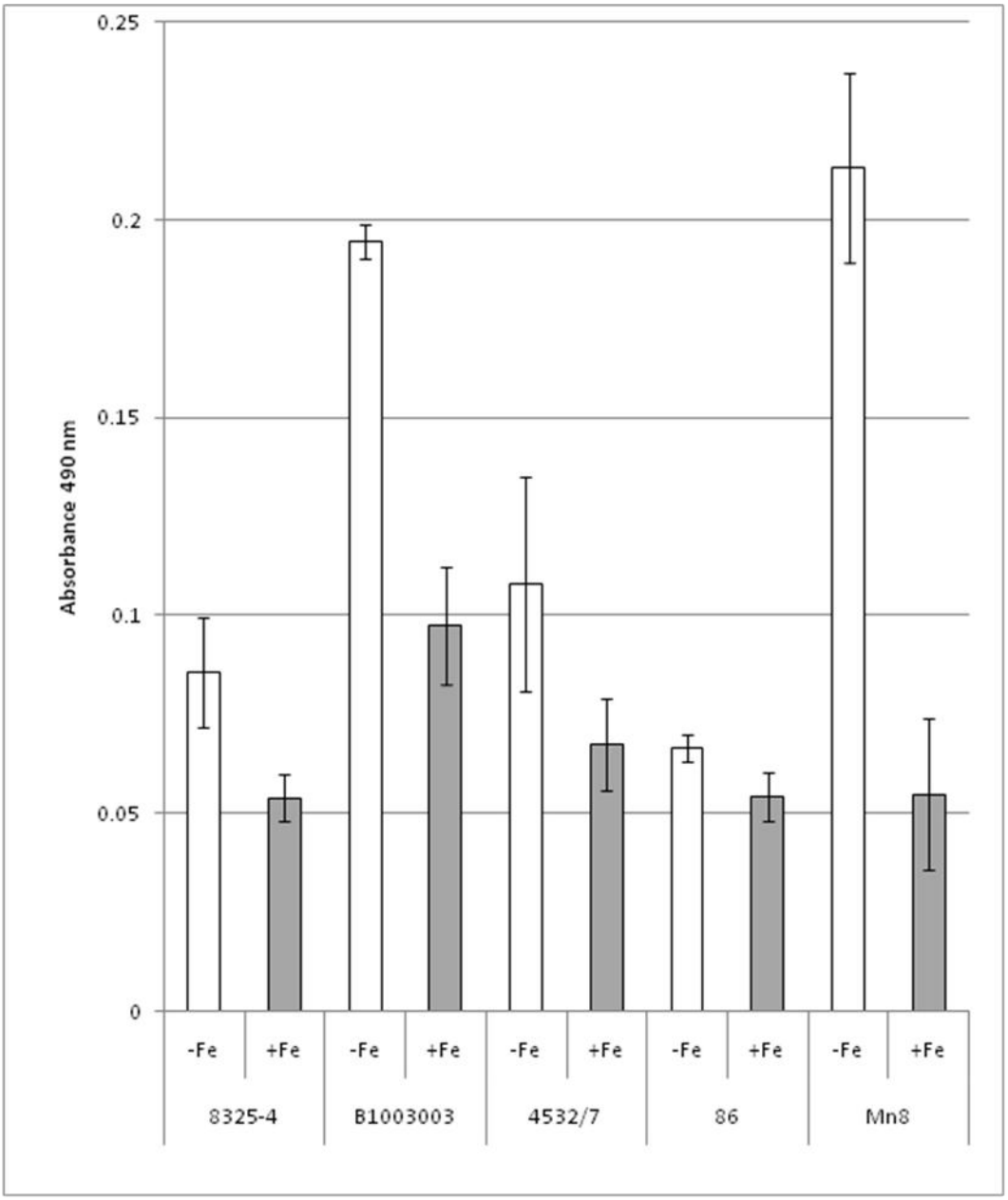


Figure 3.12. Biofilm formation of *S. aureus* strains in CRPMI growth medium in the absence and presence of 50 µM iron. Examples of the variation observed in biofilm formation and iron regulation amongst a range of *S. aureus* isolates. A cell pellet from an overnight culture of each strain grown in CRPMI was used to create a suspension of cells at approximately 0.1 OD 600 nm in fresh CRPMI. 50 µM iron was added to half of each suspension. 200 µL of each culture was added to quadruplet wells of a 96 well microtitre plate. The plate was then incubated for 24 hours at 37°C in 5% CO_2 in air. After 24 hours incubation the plates were washed, stained, dried and read at 490 nm to measure biofilm formation. Figures show the mean and standard deviation of at least three separate experiments. All strains assayed showed a significant reduction in biofilm formation in the presence of iron, including strain 4532/7 ($P = 0.037$) and 86 ($P = 0.033$). P values were determined using a students T test.

pathogens (Litwin and Calderwood, 1993). In this study *S. aureus* biofilm formation has been shown for the first time to be iron regulated in a dose dependant manner.

The 96 well assay is by far the most favoured of the methods available to study biofilm formation *in vitro*. Here it has been shown that the assay is reproducible using both rich and low nutrient growth media (Figures 3.1 and 3.2). Previously reported biofilm results were reproduced for strains Newman and 8325-4, confirming that Newman produces a low level of biofilm and 8325-4 produces a high level of biofilm in the rich growth medium TSB. This work also revealed two facts, important when studying biofilm formation in *S. aureus,* and probably other bacteria as well. Firstly, the ability of different strains to form a biofilm when grown under the same conditions is vastly different. This is a key observation suggesting that care should be taken when generalising observations if only one strain is tested. Secondly, changing the growth medium affects the biofilm formation of the strains tested considerably. For example, Newman and 8325-4 had almost opposite biofilm phenotypes in TSB and CRPMI. There are differences between the two media and aside from iron content, one other difference is the pH, with TSB being pH 6.6 and CRPMI being pH 7.6. pH has been implicated in influencing biofilm formation in *S. epidermidis* (Dunne and Burd, 1992). Adjusting the pH of TSB to that of CRPMI did slightly increase biofilm formation of strain Newman in TSB but did not account for the full increase in Newman biofilm production in the low iron medium CRPMI (Figure 3.4).

Early adhesive stages of biofilm formation were also investigated in CRPMI. Existing methods for measuring adhesion often use cells taken from overnight rich medium cultures suspended in PBS, so that the cells are not actively growing. For PBS suspensions of cells in CRPMI such methods proved inconsistent and time consuming and were not further pursued for the purpose of this study. This lead to the adaptation of the existing 96 well assay for measuring biofilm formation to include adhesion of actively growing cells. This not only gave reproducible results, but also allowed a comparison between the stages of early adhesion and biofilm formation to be made. This showed that in low iron CRPMI, both Newman and 8325-4 adhered to the surface at a similar level, and that at all time points assayed, adhesion was negatively regulated by iron in both Newman and 8325-4 (Figures 3.5 and 3.8). However, 8325-4 lacks the ability to produce one or more factors essential for the subsequent steps that facilitate biofilm production in low iron, and the level of adhesion decreased after 3 hours, suggesting that factors required for attachment were down regulated or not expressed. Strain Newman, by comparison, continued to proliferate and adhere, resulting in a high level of biofilm production at 24 hours.

The ability of *S. aureus* to bind host proteins has been shown to be an important factor in colonisation. However coating the plastic wells with fibrinogen in this study did not change the adhesive potential of the strains, Newman adhesion remained high and 8325-4 adhesion remained low (Figure 3.6). Newman has previously been reported to express a high level of fibrinogen binding proteins *in vitro* whereas in 8325-4 expression of fibrinogen binding proteins was reduced (McAleese *et al.*, 2001). This,

coupled with the already high level of biofilm produced on non coated wells by Newman may explain why coating the wells did not increase 8325-4 biofilm formation and remained unchanged in strain Newman. Coating wells with plasma did not change 8325-4 adhesion but significantly reduced biofilm formation of strain Newman. Human plasma contains proteases and peptidases (Ayache *et al.*, 2006); if adhesion to plastic in strain Newman is facilitated by surface proteins it may be that enzymes present in the plasma may have degraded these, so reducing adhesion. Alternatively the binding of plasma proteins under these conditions may block other factors responsible for binding *S. aureus* to the polystyrene surface, as is seen by the binding of SasG and Pls to host matrix proteins (Savolainen *et al.*, 2001; Roche *et al.*, 2003).

A major difference between the two growth media TSB and CRPMI is the iron content. To determine if iron had an influence on the outcome of the biofilm assay, iron was added to CRPMI. In all 21 *S. aureus* strains tested biofilm formation was reduced in high iron (Figure 3.12). The high level of biofilm formation witnessed in strain Newman in low iron CRPMI was negatively regulated by iron in a dose dependant manner, and was not due to inhibition of growth resulting from iron toxicity. The involvement of iron in biofilm formation in other pathogenic microorganisms has been reported and has been shown to have both negative and positive effects, depending on the species. Biofilm formation of *Candida albicans* (Baillie and Douglas, 1998), *Acinetobacter baumannii* (Tomaras *et al.*, 2003) and *Streptococcus mutants* (Berlutti *et al.*, 2004) is, as in *S. aureus* inhibited by high iron, whereas in *Vibrio cholerae* (Mey *et al.*, 2005a) and *E. coli* (Wu and Outten, 2009) biofilm formation is increased in high

iron. In *Pseudomonas* spp biofilm formation the role of iron may be more complex. Reports have shown that iron is required both for biofilm formation and for stabilisation of biofilm structures (Chen and Stewart, 2002; Singh *et al.*, 2002). However, high iron has also been shown to prevent biofilm formation, and to clear preformed *Pseudomonas* biofilms (Musk *et al.*, 2005). In the lungs of cystic fibrosis (CF) patients, where *Pseudomonas* is the predominating cause of infection (Moskowitz *et al.*, 2004), iron levels are high compared to iron levels in healthy people (Reid *et al.*, 2002). It is possible that *Pseudomonas* has adapted to the extremes of iron availability encountered in the body and in the CF lung, and utilises the iron in different sites to its advantage.

Results in this chapter show that other metal ions tested also had negative effects on biofilm formation (Figure 3.10), but much higher concentrations were required to cause inhibition compared to iron, with the exception of copper which was also toxic (Figure 3.11 B). Copper, like iron and other metal ions, is required for growth, but is also toxic and copper toxicity is thought to have similar mode of action to iron toxicity with regard to oxidative stress (Rodriguez-Montelongo *et al.*, 1993). Microbial copper toxicity is well known and has been exploited for many years as a way of limiting microbial growth, for example in fungicides (Michaud and Angela, 2003) and algaecides (Garcia-Villada *et al.*, 2004). Also, as a result of the recent increase in hospital 'super bugs', copper has been recommended for use as a coating for hospital door handles and other hospital fixtures to prevent cross contamination (Noyce *et al.*, 2006). A recent study in a Birmingham hospital demonstrated the efficacy of copper as an antimicrobial (www.uhb.nhs.uk/News/labels/Copper). However some pathogenic bacteria are

resistant to copper due to the acquisition of copper resistance genes sometimes carried on plasmids (Rouch *et al.*, 1985; Ciraj *et al.*, 1999; Hasman and Aarestrup, 2002), and some strains of *S. aureus* have been observed with increased copper tolerance and resistance (Ug and Ceylan, 2003; Noyce *et al.*, 2006).

In contrast to copper and manganese, high concentrations of calcium (1000 µM) were advantageous for growth, but also inhibited biofilm formation (Figures 3.10 and 3.11 A & D). Since these results have been obtained, another study has demonstrated that high concentrations (in excess of 6 mM) of calcium and manganese, but not magnesium, inhibited biofilm formation of a bovine strain of *S. aureus* grown in TSB (Arrizubieta *et al.*, 2004). The increased concentration required to inhibit biofilm formation compared to the concentrations used here may be due to the different strain and growth medium used in the two assays. The *S. aureus* surface protein Bap is associated with the formation of cellular aggregations and biofilm formation in bovine strains of *S. aureus*, but is not found in human isolates. In a recent study calcium was found to block Bap binding, preventing Bap dependant cell aggregation resulting in the loss of cellular clumps and biofilm formation (Arrizubieta *et al.*, 2004). In milk, where calcium is likely to be high, calcium may reduce biofilm formation and multi-cellular aggregation by inhibiting Bap production (Arrizubieta *et al.*, 2004). Despite this *S. aureus* is a major cause of mastitis in dairy cattle (Rainard, 2005). It is possible that inhibition of Bap in free living cells provides a survival advantage in the presence of high calcium (Arrizubieta *et al.*, 2004). Although human *S. aureus* isolates do not express Bap, it might be that another as yet unidentified surface protein may react to calcium in a

similar way and may be relevant in cases of human mastitis. Furthermore the reduction in biofilm formation witnessed in high calcium despite an increase in growth was interesting. Concentrations of calcium used were physiologically relavent with serum containing in excess of 2 mM calcium (Reichel *et al.*, 1992). This indicates that the role of caluium in biofilm control would benefit from further investigation.

In conclusion, these results suggest that biofilm formation is a multi-factorial process that is dependant on the iron status of the environment for all *S. aureus* strains tested. Although other metal ions inhibited biofilm formation in strain Newman, iron was chosen for further study, and will be the continued focus in subsequent chapters. It is proposed that the investigation of iron regulated biofilm formation will either reveal novel factors that can be targeted for biofilm prevention, or will confirm the involvement of already identified factors such as PNAG in biofilm formation, in the hope that they can be better understood. In addition, the observation of the effects of other metal ions on biofilm formation is also interesting as they may provide possible therapeutic targets for the future. Copper toxicity and resistance in *S. aureus* is the subject of research being currently carried out by a colleague.

Chapter 4

Iron regulation and biofilm formation in *S. aureus* strains.

4.1 The role of Fur and iron in the regulation of *S. aureus* adhesion and biofilm formation.

In the previous chapter biofilm formation in *S. aureus* was shown to be repressed by high iron. It is the aim of this chapter to further investigate how iron affects biofilm formation and what factors are involved. Although *S. aureus* sequesters iron from low iron environments to sustain growth and other biological functions, intracellular iron concentrations are tightly controlled within the bacterial cell to avoid toxicity. Processes involved with iron homeostasis are controlled by the ferric uptake regulator Fur (Xiong *et al.*, 2000). Fur also controls some factors responsible for *S. aureus* virulence (Horsburgh *et al.*, 2001b). To determine if iron regulated biofilm formation observed in strain Newman is Fur dependant, early adhesion and biofilm assays were performed with Newman wild-type and *fur* mutant strains, with and without the addition of iron. The *fur* mutation was transferred from strain 8325-4 (MJH010) to Newman via phage transduction as described in section 2.9. Acquisition of the mutation was confirmed by PCR using primers JM FurF and JM FurR (see table 2.4) which cover the flanking regions of *fur*. This resulted in a 1 kb PCR product for the wild type and a 2.5

kb PCR product in the mutant, due to the insertion of a tetracycline resistance cassette in the *fur* mutant. As a negative regulator of gene expression in high iron, if Fur were involved in iron-dependent repression of biofilm production then equal levels of biofilm in iron-restricted and iron-replete growth conditions would be expected. However, biofilm levels remained repressed by iron in the Newman *fur* mutant, suggesting that negative iron regulation of *S. aureus* Newman biofilm formation is not via Fur (Fig. 4.1 A). Nevertheless, Fur does appear to have a significant role in biofilm regulation in Newman. After 6 hours of growth, biofilm formation in the *fur* mutant was increased approximately 2.4-fold compared to the wild type (Fig 4.1 B). This suggests that Fur negatively regulates the early stages of biofilm formation in low iron. This is a novel and non-classical example of Fur repression in *S. aureus*, as repression is occurring in low iron. In contrast, after 24 hours, the *fur* mutant produced significantly less biofilm in low iron than the wild type strain. This suggests that Fur is an activator of the later stages of biofilm formation in low iron conditions, another novel example of non-classical Fur regulation in *S. aureus*.

It has previously been reported that the *S. aureus fur* mutant of strain 8325-4 has a growth defect, which was rescued by the addition of iron to the growth medium (Horsburgh *et al.*, 2001b). To determine if the observed decrease in biofilm formation in the Newman *fur* mutant was due to growth effects, growth was monitored for both

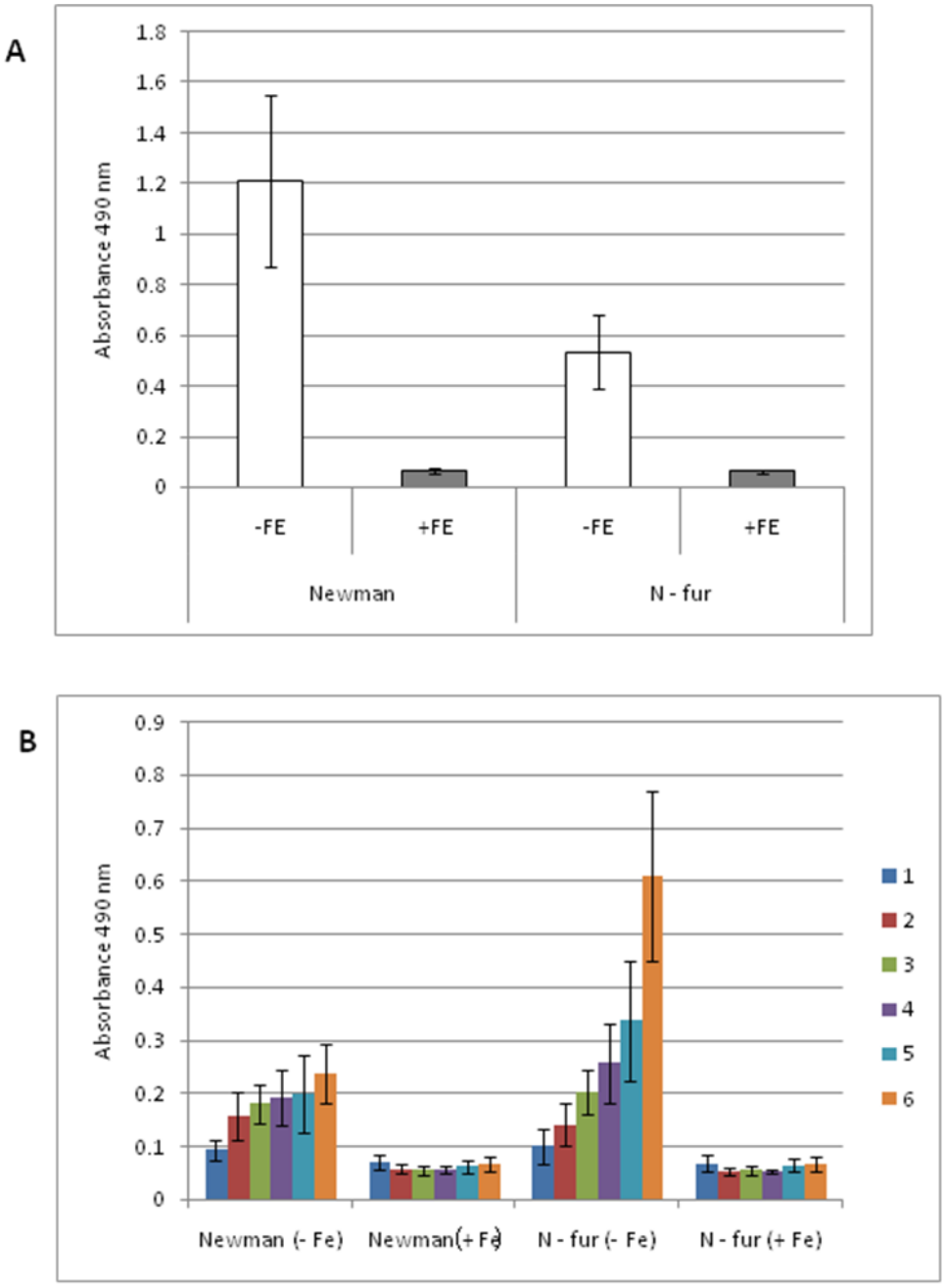


Figure 4.1. Graph showing early adhesion and 24 hour biofilm formation of *S. aureus* strains Newman wild type and *fur* mutant in CRPMI growth medium with and without the addition of 50 µM $FeSO_4$. A cell pellet from an overnight culture of each strain grown in CRPMI was used to create a suspension of cells at approximately 0.1 OD 595 nm in fresh CRPMI. Iron was added to half of each suspension. 200 µL of each culture was added to quadruplet wells of multiple 96 well microtitre plates. The plates were incubated for 24 hours at 37°C in 5% CO_2 in air. After the required time of incubation plates were washed, stained, dried and read at 490 nm to measure adhesion and biofilm formation. Figures represent the mean and standard deviation of at least 3 separate experiments. A) Biofilm formation 24 hours after inoculation. B) Early adhesion 1-6 hours after inoculation.

the wild type and *fur* mutant with and without the addition of iron. Figure 4.2 confirms that Newman *fur* also has a slight growth defect in early growth, which is rescued with the addition of iron. As observed in the previous chapter, Newman cells form clumps when grow in liquid culture in high iron, which results in a false OD value obtained at 24 hours. Increased cell numbers in high iron was confirmed by the increased cell pellet weight obtained from cultures with the addition of 50 µM iron compared to those without iron, as observed during experiments comparing protein profiles (section 2.10, data not shown). In addition pellet weights obtained from the *fur* mutant cultures were similar to those obtained from the wild type at 24 hours, and adjusting the biofilm data to take into account the reduction in cell numbers in the mutant does not account for the reduction in biofilm formation observed (not shown). This suggests that Fur has a dual role in *S. aureus* Newman biofilm formation, acting as a repressor in the early stages of biofilm formation, and as an activator in the production of mature biofilm. Much of the work in this chapter has been published (Johnson *et al.*, 2008).

4.2 The effect of osmotic stress on biofilm formation: the role of Sigma factor B.

Newman and 8325-4 have opposite biofilm profiles in TSB and CRPMI and, as yet, exactly what causes these different phenotypes is unclear. In low iron Newman produces a high level of biofilm which is negatively iron regulated and positively Fur regulated. Despite having a functional *fur* gene (Xiong *et al.*, 2000; Horsburgh *et al.*, 2001b) 8325-4 does not form a biofilm in low iron. Previous studies have

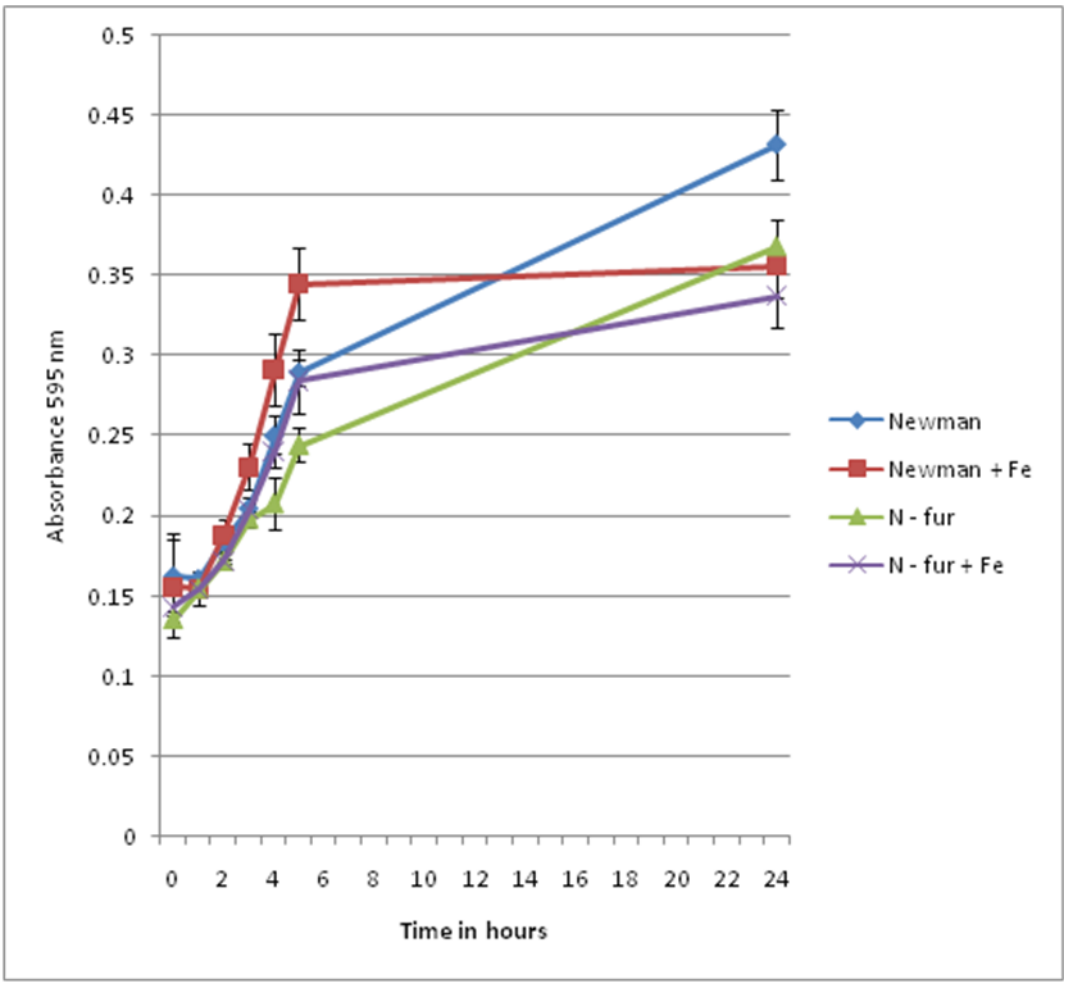


Figure 4.2. Growth of Newman and Newman – *fur* in CRPMI with and without the addition of 50 µM iron. A cell pellet from an overnight culture of each strain grown in CRPMI was used to create a suspension of cells at approximately 0.1 OD 600 nm in fresh CRPMI. Iron was added to half of each suspension. 200 µL of each culture was added to quadruplet wells of a 96 well microtitre plate. The plate was then incubated for 24 hours at 37°C in 5% CO_2 in air. At each time point the 96 well plate was read at 595 nm to record growth. The figure represents the mean and standard deviation of at least three separate experiments.

found that staphylococcal biofilm formation can be induced in TSB via stress responses caused by increased temperature (Rachid *et al.*, 2000) and osmotic stress, both of which might be encountered during an infection (Mack *et al.*, 1992; Conlon *et al.*, 2002; Lim *et al.*, 2004), or by high glucose concentrations (Christensen *et al.*, 1982; Mack *et al.*, 1992), which may be encountered during continuous ambulatory peritoneal dialysis. High concentrations of glucose are often used in dialysis fluid used to perform artificial cleansing of the blood via peritoneal dialysis. The high levels of glucose create a concentration gradient which facilitates dialysis (Mak *et al.*, 1997). During the dialysis process the fluid may become contaminated with *S. aureus,* causing peritonitis (Piraino, 1998; Szeto *et al.*, 2007).

Previous studies have shown that *S. aureus* biofilms induced by osmotic stress involve the alternative transcription factor sigma B (σ^B) (Rachid *et al.*, 2000). σ^B is responsible for the regulation of a general stress response in *S. aureus,* and is also believed to be important for survival and for expression of some virulence factors (Jonsson *et al.*, 2004). 8325-4 has been found to contain a deletion in *rsbU*, a positive regulator of σ^B (Kullik *et al.*, 1998). It is possible that 8325-4 is not able to form biofilms in low iron medium because of a deficiency in expression of σ^B. To determine if the *rsbU* mutation in 8325-4 had an effect on biofilm formation in low iron growth conditions, SH1000, a repaired *rsbU*$^+$ version of 8325-4 (Horsburgh *et al.*, 2002a) was assayed for biofilm production alongside an isogenic σ^B deficient mutant of strain Newman. In low iron medium, SH1000 produced the same level of biofilm as the σ^B deficient 8325-4 (Fig 4.3). In addition, the σ^B mutant of strain Newman had no significant effect on biofilm

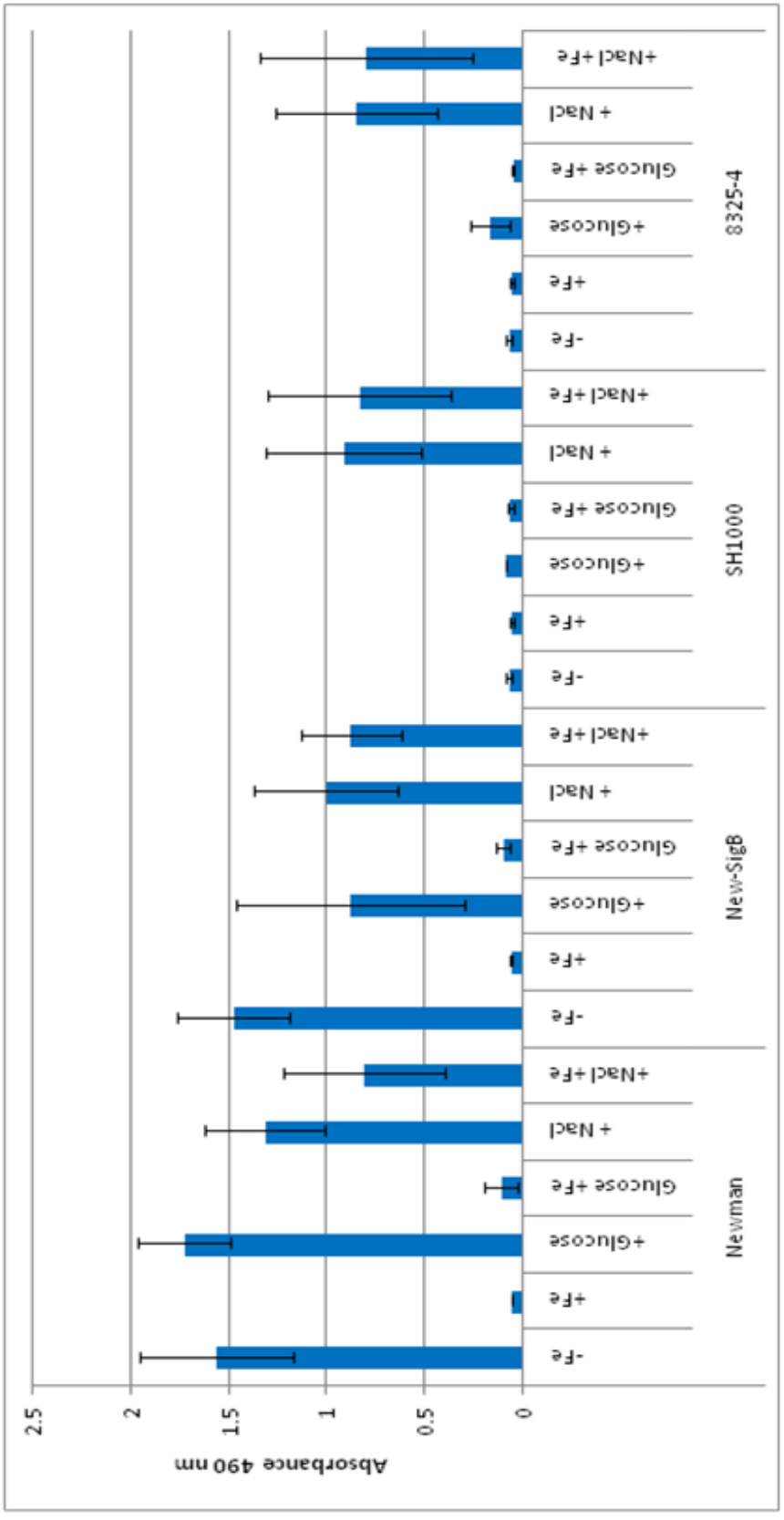


Figure 4.3. 24 hour biofilm formation of *S. aureus* strain Newman wild type and *sigB* mutant & 8325-4 *sigB* deficient strain and a repaired *sigB*+ strain SH10000 with and without the addition of salt and glucose, in low and high iron. A cell pellet from an overnight culture grown in CRPMI was used to create a suspension of cells at approximately 0.1 OD 600 nm in fresh CRPMI for each strain. The cell suspensions were then split into aliquots and glucose, salt and iron were added or omitted where appropriate. 200 µL of each cell suspension was added to quadruplet wells of a 96 well microtitre plate. The plate was then incubated for 24 hours at 37°C in 5 % CO_2 in air. The plate was then emptied, washed, stained and dried, and adherent cells were measured by reading the plate at 490 nm. The graph represents the mean and standard deviation of at least three separate experiments.

formation. This suggests that the different biofilm phenotypes observed between Newman and 8325-4 in low iron was not related to the σ^B defect present in 8325-4, and that in low iron σ^B does not regulate biofilm formation.

To further investigate the role of σ^B, and to establish if biofilm formation could be induced in strain 8325-4 in low iron by osmotic stress, the biofilm assay was repeated with growth medium supplemented with additional salt or glucose. Unsupplemented CRPMI contains 0.6 % salt and 0.2 % glucose. Overnight cultures were diluted to approximately 0.1 OD 600 nm as previously described, except that before inoculation final concentrations of either 3 % salt or 1.25 % glucose were added to the medium. The effect of iron on biofilm formation under conditions of osmotic stress was also observed. Biofilm formation could not be induced in either 8325-4 or SH1000 by the addition of extra glucose under low or high iron conditions (Fig 4.3). In wild type strain Newman and in Newman σ^B the addition of glucose did not significantly affect biofilm formation. Also biofilms formed by Newman in the presence of high glucose were repressed by iron, suggesting that the same factors are required for *S. aureus* Newman biofilm formation in both high and low glucose, and that under both conditions, biofilm formation is independent of σ^B, and dependant on the iron status of the media.

Low iron biofilm formation of Newman and Newman σ^B was not significantly affected by high salt. However in strains 8325-4 and SH1000, biofilm formation was increased 10 fold by the addition of salt. Moreover, iron regulation of biofilm formation was lost

in the presence of high salt in both strains. This suggests that factors involved in salt induced biofilm formation are different to those required for biofilm formation in low salt conditions, and are independent of σ^B.

4.3 PNAG production by *S. aureus* strains Newman and 8325-4.

A major staphylococcal cell surface polysaccharide, PNAG, has been heavily implicated in biofilm formation and virulence. It is possible that the level of PNAG expression is variable between Newman and 8325-4, and may explain the different biofilm potential of the two strains. In addition, since high salt concentrations induced biofilm formation in strain 8325-4, it is possible that *S. aureus* PNAG synthesis is also up regulated in high salt. PNAG produced by *S. epidermidis* and has previously been shown to be induced in response to high salt resulting in increased biofilm formation (Knobloch *et al.*, 2001), and increased salt was shown previously to increase transcription of *icaA* in *S. aureus* (Fitzpatrick *et al*., 2005).

To test this idea, levels of PNAG expression were measured in Newman and 8325-4. PNAG was extracted from the cell surface after 24 hours growth in high and low iron, and in growth medium containing high and low concentrations of salt. PNAG was also extracted from Newman and 8325-4 *icaR* mutants grown in low iron as a positive control for the assay. The *ica* operon which produces PNAG is negatively regulated by *icaR*, therefore deleting *icaR* results in the over production of PNAG. PNAG extracts were diluted and immobilised on nitrocellulose membrane using a slot blot apparatus, and detected using PNAG specific polyclonal antiserum (Maira-Litran *et al.*, 2002) and

ECL detection reagents (section 2.13). Surprisingly, the overall levels of PNAG expression were similar in 8325-4 and Newman in both high and low iron and in high salt (Fig 4.4). This suggests that the level of surface associated PNAG is not responsible for the difference in biofilm formation between strains 8325-4 and Newman in low iron, low salt conditions and that surface associated PNAG does not appear to be iron regulated. Also the induction of 8325-4 biofilm formation in low iron, high salt conditions is not due to increased PNAG expression, suggesting that *ica* expression, at least in *S. aureus* strains Newman and 8352-4, is not induced in high salt.

4.3.1 The role of *ica* in biofilm formation

Although the previous section has shown that the expression of surface associated PNAG was not different between the strong biofilm forming strain Newman and the poor biofilm producing strain 8325-4, it does not rule out a role for *ica* or PNAG in biofilm formation. To investigate this further, a Newman *ica* mutant, and a derivative that had been repaired by homologous recombination with a plasmid construct containing wild type *ica* gene (obtained from Kropec *et al.* (2005)), were assayed for their ability to form a biofilm, and compared to the wild type. Also to investigate how the over production of PNAG affected biofilm formation in low iron, *icaR* mutants of 8325-4 and Newman were also assayed.

Interestingly given the fact that PNAG levels were similar in Newman and 8325-4, there was a 10 fold reduction in biofilm formation in the Newman *ica* mutant compared to the wild type and the Newman *ica+* repaired strain (Fig 4.5 A). In addition, the *icaR*

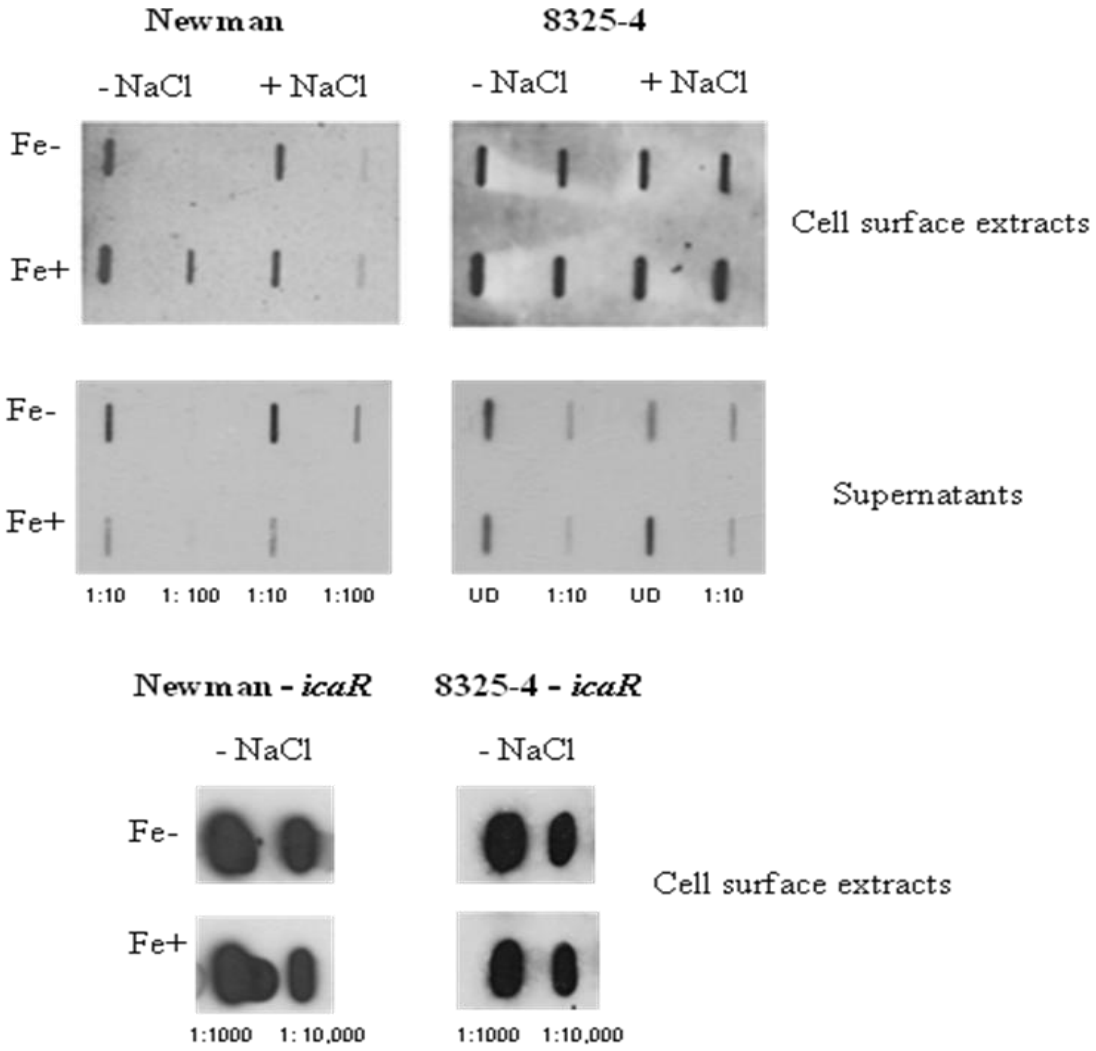
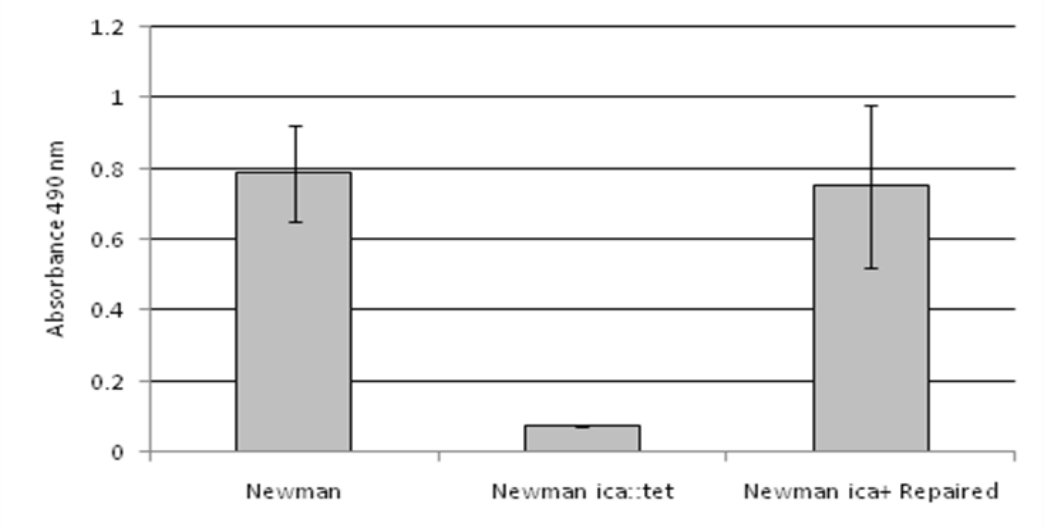


Figure 4.4. PNAG extracts from Newman and 8325-4 wild type and *icaR* mutants grown for 24 hours in CRPMI, low and high iron, low and high salt. PNAG was extracted from cultures grown for 24 hours in low or high iron and in low or high salt. Cells were centrifuged and the supernatant was retained. The cell pellets were weighed and surface extracts were prepared in a volume of EDTA based on the pellet weight. The cell surface extracts were then diluted 1 in 10 and 1 in 100 for wild type extracts, and 1 in 1000 and 1 in 10,000 for the *icaR* extracts. Supernatants were used undiluted (UD) and diluted 1 in 10. Samples were immobilised on nitrocellulose membrane and PNAG was detected with a PNAG specific antibody. Figure shown is representative of at least 3 repeat experiments.



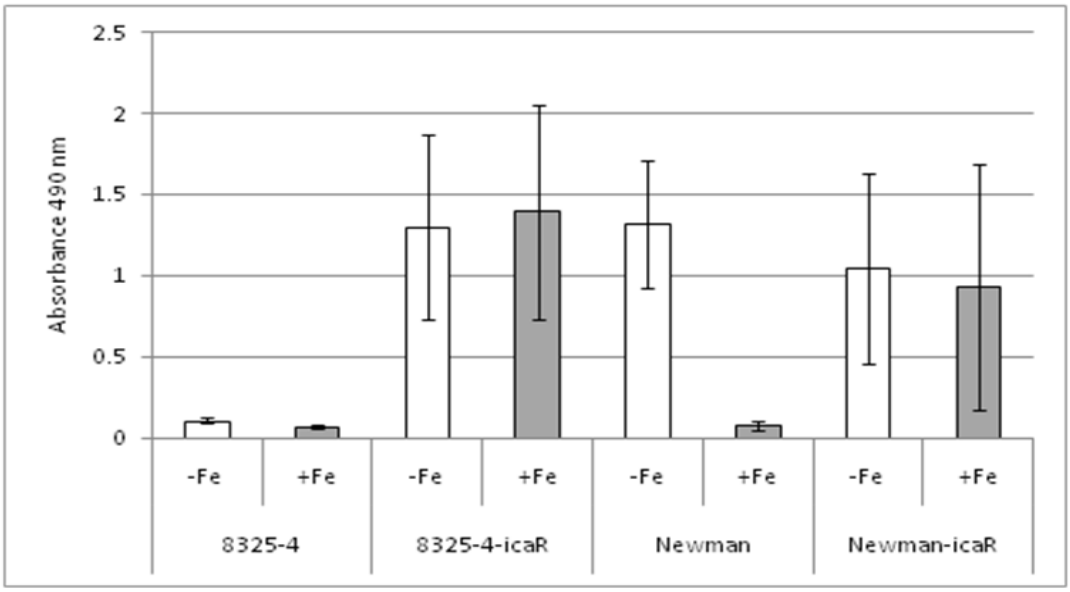


Figure 4.5. The effects of PNAG expression on 24 hour biofilm formation in Newman & 8325-4. A cell pellet from an overnight culture of each strain grown in CRPMI was used to create a suspension of cells at approximately 0.1 OD 600 nm in fresh CRPMI. Iron was added to half of each suspension where required. 200 µL of each culture was added to quadruplet wells of a 96 well microtitre plate. The plate was then incubated for 24 hours at 37°C in 5% CO_2 in air. After 24 hours incubation the plates were washed, stained, dried and read at 490 nm to measure biofilm formation. Figures show the mean and standard deviation of at least three separate experiments. A) Biofilm formation in Newman wild type, *ica* mutant and the *ica* repaired strain, in low iron. B) Biofilm formation of Newman and 8325-4 wild type and *icaR* mutant strains in high and low iron.

mutation in 8325-4 resulted in a 10 fold increase in biofilm production, to a level comparable to Newman wild type biofilm production (Fig 4.5 B). This suggests that despite the detection of similar levels of PNAG in both strong and weak biofilm forming strains, *ica* and PNAG do have a role in low iron biofilm formation. The *icaR* mutation of Newman did not show increased biofilm formation, but iron regulation of biofilm formation was lost in the *icaR* mutants of both Newman and 8325-4.

To further investigate the effect of the *ica* mutation on strain Newman, full protein profiles including (a) supernatants, (b) SDS extracts containing proteins not covalently attached to the cell wall, (c) cell wall extracts, (d) membrane preparations and (e) cytoplasmic extracts were prepared from the wild type and *ica* mutant strains after 24 hours growth in CRPMI. Figure 4.6 A-E shows the difference in expression of surface associated protein between the wild type and *ica* mutant in low iron. The most striking observation is that in the supernatant extract there is a protein of approximately 70 kDa produced in abundance in the wild type but missing in the *ica* mutant. This protein is also reduced or absent in all the other extracts obtained from the *ica* mutant. In addition, an approximately 40 kDa protein in the wild type supernatant extract was reduced in the *ica* mutant and was also reduced or absent in all other fractions.

These two proteins were excised from the SDS PAGE gel and identified by MALDI-TOF mass spectrometry analysis, performed by Andrew Bottrill and Shairbanu Ibrahim (PNACL, Proteomics Facility, Leicester University), as Eap (70 kDa) and Emp (38.5 kDa). Eap is well documented for its role in adhesion and virulence, as discussed in the Introduction (section 1.6.3). Emp has been studied in less detail, but has also been

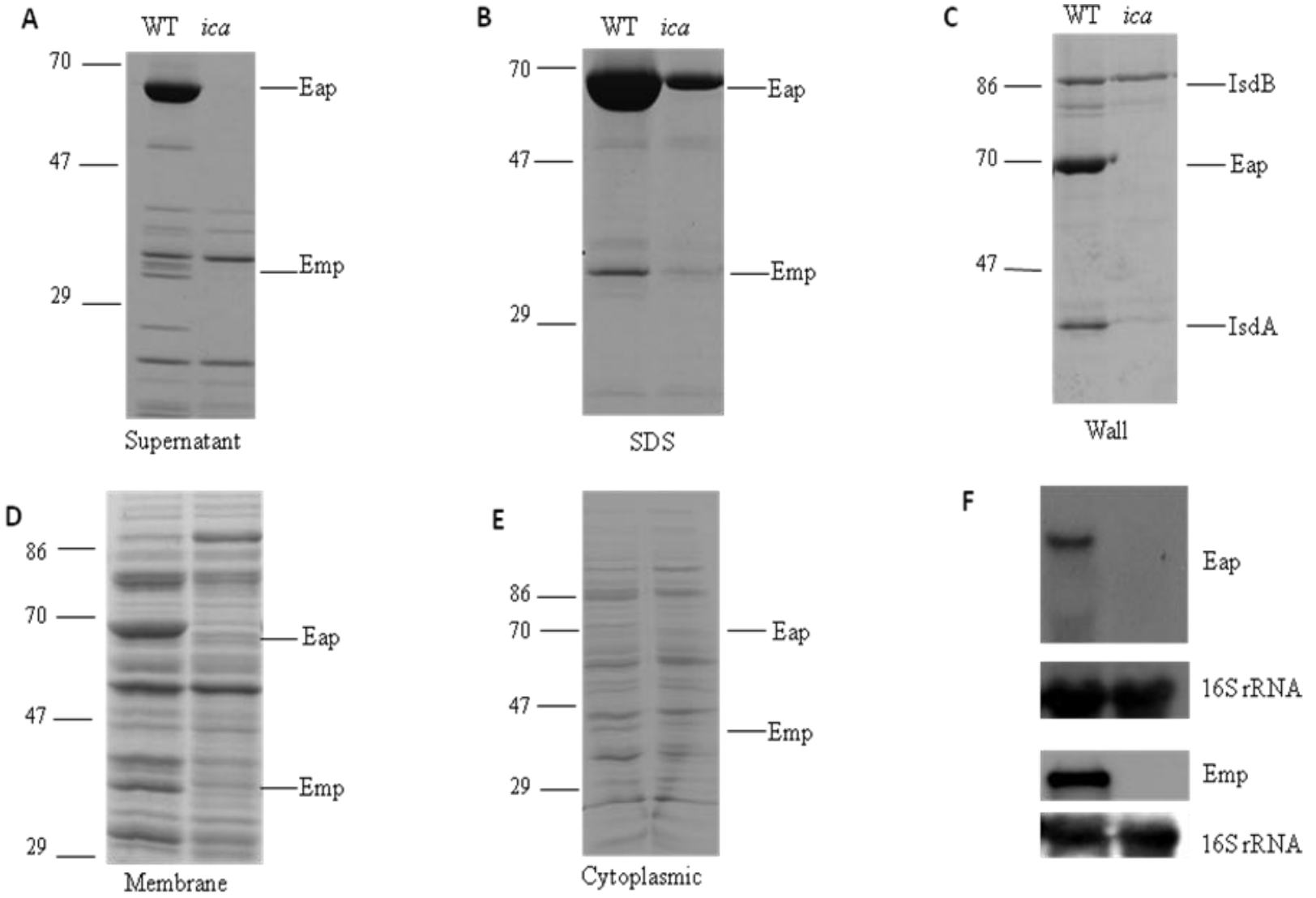


Figure 4.6. Expression of Eap and Emp in Newman wild type and *ica* mutant. A-E) Protein extracts from cells grown for 24 hour in CRPMI. F) Transcriptional analysis of *eap* and *emp* in low iron growth medium. 10µg of total RNA was used for each sample. Northern blots were hybridised with *eap* and *emp* specific probes; after detection blots were re-hybridised with 16S rRNA DNA probes as a loading control.

identified as a secreted protein which binds to various host proteins, implying that Emp has a role in virulence (Hussain *et al.*, 2001b).

Amongst other variations in protein expression in the wild type and the *ica* mutant one other distinct difference was the reduced expression of the iron regulated cell wall protein IsdA (Fig 4.6 C). However, IsdB, which is also an iron-regulated protein, was not affected by the *ica* mutation. This is an interesting observation given the association with IsdA\B proteins and virulence (Cheng *et al.*, 2009).

4.4 The role of Eap and Emp in biofilm formation.

The reduced cell surface expression of Eap and Emp in the *ica* mutant was unexpected. One possible explanation for this is that the proteins may be correctly expressed in the *ica* mutant but fail to correctly localise on the cell surface, and may have become trapped within the cell, however other protein fractions did not contain increased levels of Eap and Emp to account for this. This suggests that the reduced expression in the *ica* mutant might be due to decreased expression, although if the proteins had failed to localise, it is also possible that they might have been degraded. To investigate if the loss of surface expression of Eap and Emp in the *ica* mutant was due to transcriptional regulation, total RNA was extracted from both Newman wild type and Newman *ica* cultures grown for 6 hours in low iron. Northern blot analysis was carried out using specific *eap* and *emp* DNA probes generated using the primer pairs EapPF and EapR, and EmpPf and EmpR respectively (Table 2.4). In the *ica* mutant there was no detectable transcription of either *eap* or *emp* (Fig 4.6 F), even though there was still

some evidence of the two proteins visible in the surface protein extracts, especially in the SDS protein fraction. This suggests that some transcription must be occurring that is rapidly degraded before detection, and that *ica* is required for full expression of both *eap* and *emp*.

Loss of biofilm formation coupled with reduced expression of Eap and Emp in the absence of *ica* suggests that these two surface proteins may be involved in low iron biofilm formation in strain Newman. To further investigate this, mutant strains of Newman deficient in the production of Eap and Emp were screened for their ability to form a biofilm. The loss of expression of the two proteins was confirmed by preparing SDS extracts from the two mutant strains (Fig 4.7 A). Interestingly the absence of either protein significantly reduced biofilm production (Fig 4.7 B). The loss of Eap resulted in a 2-fold reduction in biofilm formation ($P = 0.007$), whereas loss of Emp resulted in a 10-fold reduction in biofilm. To verify that the effects observed were due to the loss of the proteins and not due to polar effects caused by the mutations, plasmids were constructed containing wild type copies of the respective genes under the control of their own promoters. The entire gene and putative upstream promoter regions were amplified by PCR and cloned into the *E. coli/S. aureus* shuttle vector pMK4, which was then transduced into the appropriate background (Newman *–eap* or Newman *–emp*). The plasmids containing the correct inserts were confirmed by PCR amplification of the inserted gene using plasmid construction primers, and by sequencing using the same primers. Unfortunately, this only partially restored both protein expression (Fig 4.7 A),

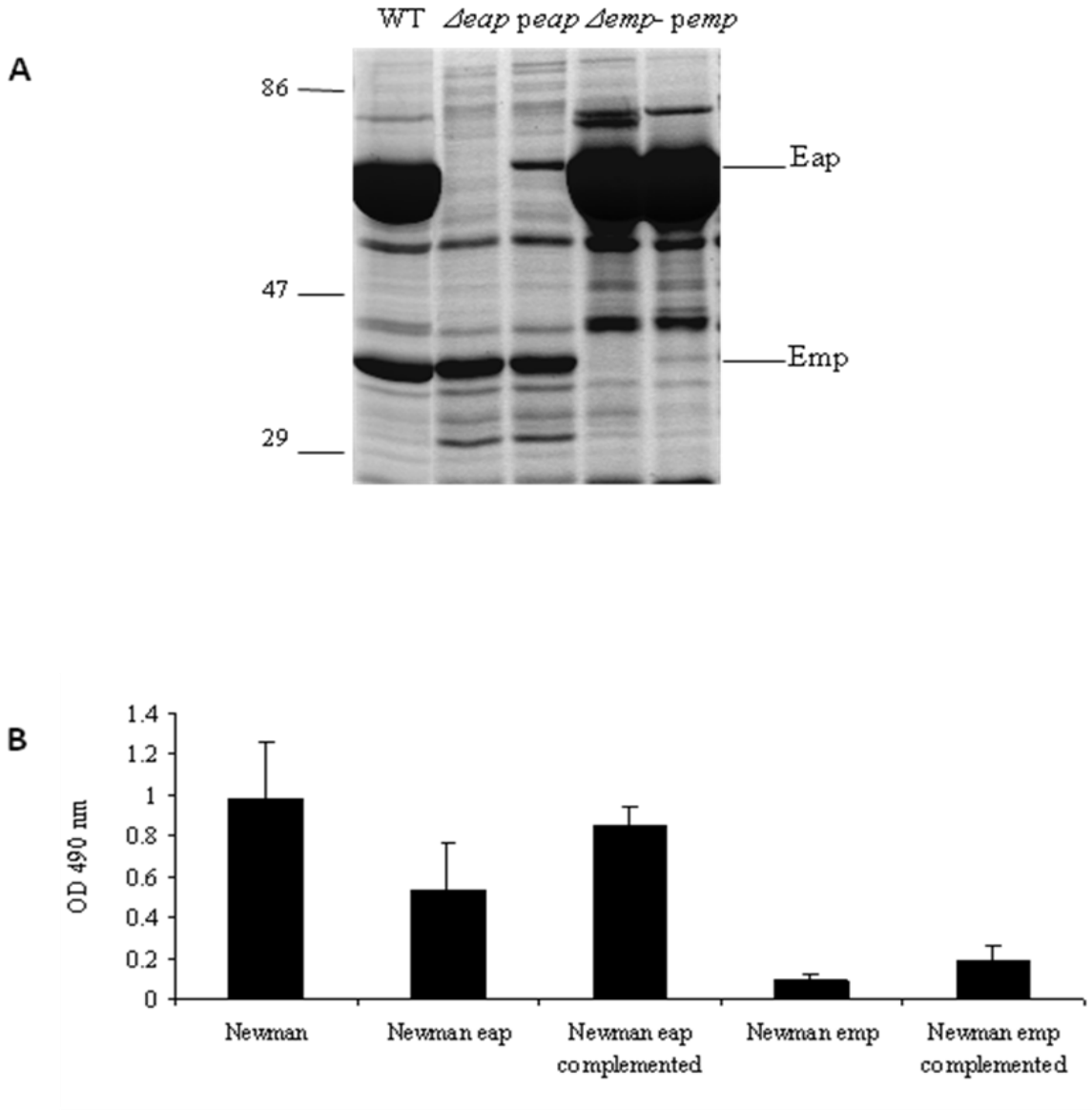


Figure 4.7. Biofilm formation and surface protein extracts from Newman wild type, *eap* and *emp* mutants and mutant strains containing complementing plasmids. A) SDS protein extracts showing the expression of Eap and Emp in the wild type, *eap* and *emp* mutants, and the partial complementation of Eap and Emp expression by plasmids containing wild type copies of either *eap* or *emp* under the control of their own promoters. B) 24 hour biofilm formation in low iron of Newman, and of *eap* and *emp* mutants with or without the corresponding complementing plasmids.

and biofilm formation (Fig 4.7 B). Nonetheless, these results confirm the importance of Eap and Emp in biofilm formation in strain Newman.

4.4.1 The expression of Eap and Emp in TSB and in high and low iron.

As both Eap and Emp have been shown to affect biofilm formation in strain Newman, the expression of the two proteins was investigated in strain 8325-4, which does not produce a biofilm in low iron. Also as 8325-4 and Newman have almost opposite biofilm phenotypes in rich growth medium, the expression of the two proteins was also examined in TSB. The two proteins were most abundant in the SDS protein extracts of strain Newman, and so SDS extracts were prepared from 8325-4 grown in low iron CRPMI and in TSB, and these were compared with those obtained from strain Newman, grown under the same conditions. Surprisingly, despite 8325-4 carrying genes encoding Eap and Emp, (determined by PCR), only a very low level of expression of either protein was visible in the extracts obtained from 8325-4 in low iron (Fig 4.8). In addition, Eap was reduced in the extract from Newman grown in TSB and levels of the two proteins in 8325-4 were very low, far lower than the expression levels observed in Newman. Despite this, 8325-4 produces a high level of biofilm in TSB, suggesting that factors other than Eap and Emp are responsible for biofilm formation in the nutrient rich growth medium TSB.

It has been demonstrated here that low-iron-induced biofilm formation is repressed in high iron, and both Eap and Emp are required for biofilm formation in strain Newman. Therefore, to determine the effect of iron on Emp and Eap expression, SDS surface

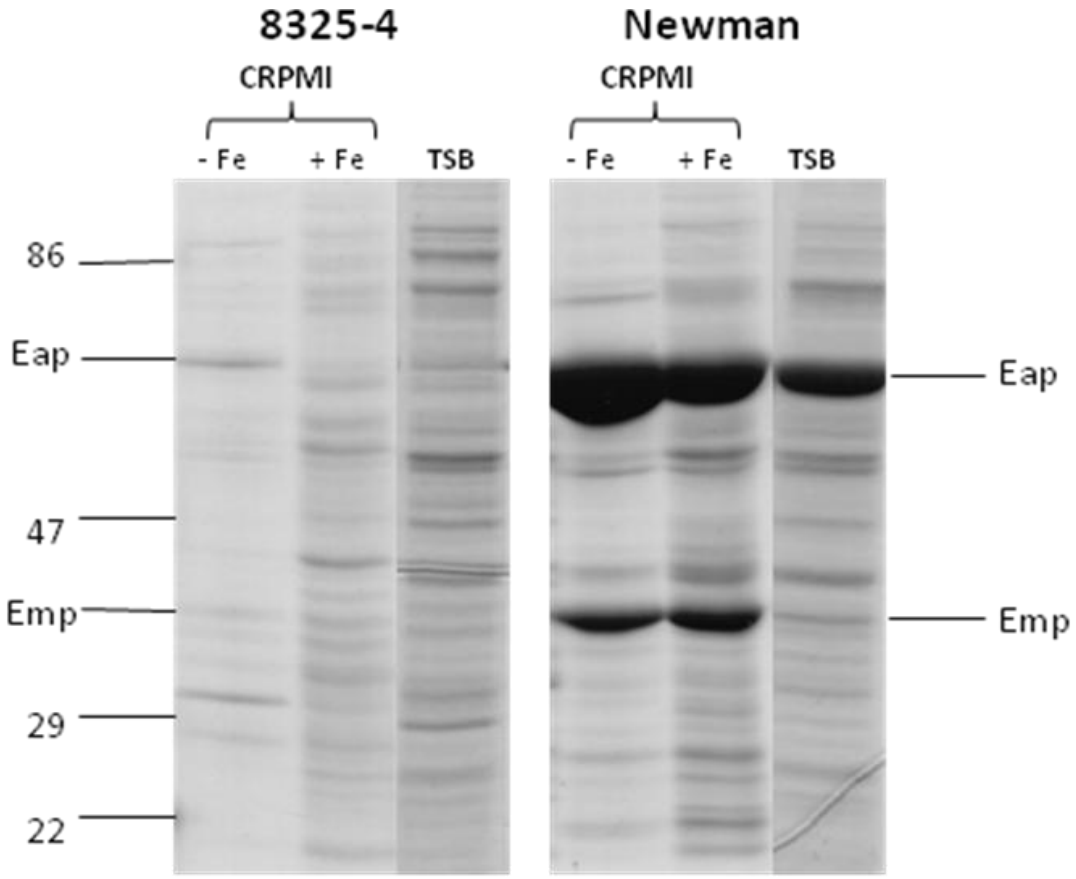


Figure 4.8. SDS surface protein extracts from Newman and 8325-4 after 24 hours growth in TSB, and in CRPMI with and without the addition of 50 µM iron.

extracts were prepared from Newman grown in high and low iron and the levels of Eap and Emp were observed, which revealed that both Eap and Emp were reduced in high iron (Fig 4.9 A).

4.4.2 The role of Fur in the regulation of Eap and Emp.

As described in the previous chapter iron regulated genes are usually also regulated by the iron dependant repressor Fur, and biofilm formation has also been seen in this work to be affected by Fur. To determine if *eap* and *emp* are iron regulated via Fur, the expression of the two proteins was investigated in a Newman *fur* mutant in high and low iron. This revealed that in a *fur* mutant, expression of Eap and Emp is reduced, but still repressed by high iron (Fig 4.9 B and D). The surface protein extracts also showed that expression of both proteins, like biofilm formation, remained iron regulated in the absence of *fur*. This suggests that Eap and Emp are dependant on *fur* for full expression, and that iron regulation of Eap and Emp is via an unknown regulator.

To further investigate the role of Fur in the regulation of Eap and Emp expression, transcriptional analysis was carried out using RNA extracted from Newman wild type and its isogenic *fur* mutant. Northern blot analysis using *eap* and *emp* specific probes confirmed that the iron and *fur* regulation of Eap and Emp witnessed in the protein extracts is as a result of transcriptional regulation (Figure 4.9 F). Transcripts of *eap* and *emp* were significantly reduced after growth in high iron, and were absent in the *fur* mutant. Again, as in the *ica* mutant, evidence of Eap and Emp proteins in the protein extracts obtained from the *fur* mutant suggests that there must be some transcription of both genes (Figure 4.9 A to E), but the transcript is degraded before detection. This

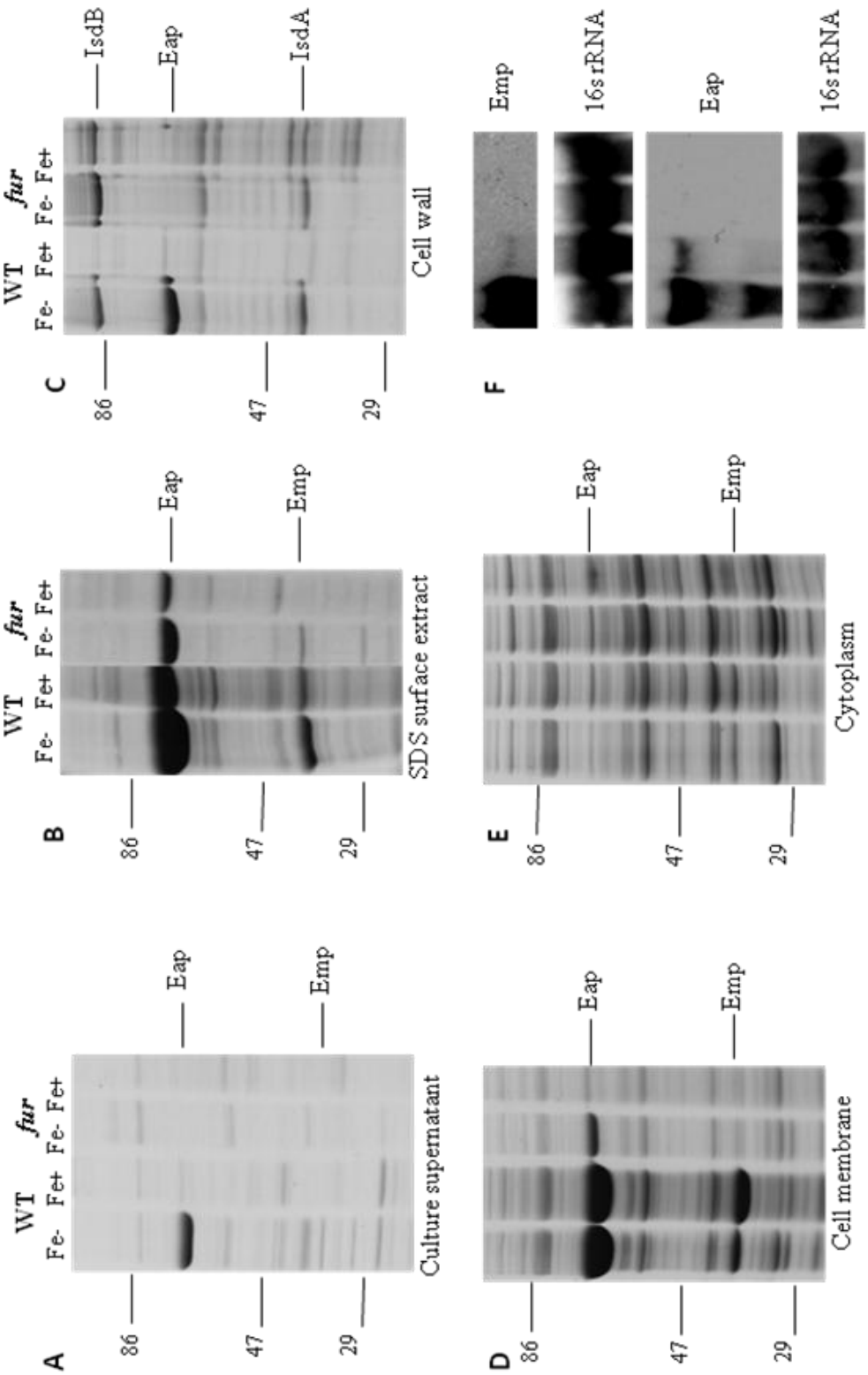


Figure 4.9. Expression of Eap and Emp in low and high iron in Newman wild type and the isogenic *fur* mutant. A-E) 24 hour protein extracts. F) Transcriptional analysis of *eap* and *emp* in low iron growth medium. 10 µg of total RNA was used for each sample and blots were hybridised with *eap* and *emp* specific probes; after detection blots were re-hybridised with 16S rRNA DNA probes as a loading control.

non-classical Fur regulation is likely to explain the non-classical Fur regulation of biofilm formation witnessed in the previous chapter. Fur would normally act as a transcriptional repressor in response to a high-iron environment. It would therefore be expected that Fur regulated genes would be constitutively expressed in a *fur* mutant irrespective of the iron status of the medium. However, here positive *fur* regulation of biofilm formation and Eap and Emp expression in a low iron environment has been demonstrated.

4.5 Discussion

It was the aim of this section to determine how iron affected biofilm formation and what factors were involved in low iron induced biofilm formation. In this chapter it has been seen that the iron regulated proteins Eap and Emp were required for biofilm and that *ica* is required for their expression. In addition it was seen that in high salt or in TSB factors other than Eap and Emp were responsible for biofilm production. It has also been shown that Fur has dual novel regulatory functions in *S. aureus* Newman biofilm formation, positively regulating Eap and Emp expression, and negatively regulating early adhesion. Also iron regulation of Eap and Emp was seen to be Fur independent, suggesting that *S. aureus* has a novel unidentified iron repressor.

PNAG is a well studied factor implicated in staphylococcal biofilm formation. It has previously been observed by others that Newman does not form a biofilm in TSB *in vitro*, nor does it produce *ica* transcripts or PNAG, yet it is capable of biofilm

formation, *ica* transcription and PNAG production *in vivo* (Fluckiger *et al.*, 2005). Low levels of PNAG were extracted from the cell surface of both Newman and 8325-4 in low and high iron, suggesting that PNAG does not have a great impact on biofilm formation in low iron, neither is it iron regulated. Moreover biofilm formation was induced in 8325-4 in high salt, and yet levels of PNAG remained the same (Figure 4.4). It has previously been demonstrated that *icaA* expression in clinical isolates, was increased in rich medium containing high salt, although this did not to biofilm formation. This suggests that increased PNAG was insufficient to induce biofilm formation under the conditions used (Fitzpatrick *et al*., 2005). The levels of biofilm formed by Newman and 8325-4 could not be explained by the levels of PNAG expressed, hyper production of PNAG in the *icaR* mutants resulted in a high level of biofilm production in strain 8325-4 which was not iron regulated. This suggests that high levels of PNAG can compensate for the absence of other factors required for biofilm formation, such as Eap and Emp. The level of PNAG expression in Newman and 8325-4 *icaR* mutants was similar, and not surprisingly, very high. By comparison, the level of expression in the wild type strains was very low, suggesting that PNAG expression is usually strongly repressed at 24 hours and implies that the role of PNAG in the later stages of biofilm development is limited. Nevertheless, the absence of the *ica* operon, which produces PNAG, was found to be essential for biofilm formation (Figure 4.5 A). This indicates that whilst *ica* is vital for biofilm formation, a factor or factors other than PNAG must also be involved. This may in part explain the variations in *ica* dependant and *ica* independent biofilm formation observed in the literature

(Cramton *et al.*, 1999; Arciola *et al.*, 2001; Cucarella *et al.*, 2001; O'Neill *et al.*, 2007; Fowler *et al.*, 2001; Fitzpatrick *et al.*, 2005; Toledo-Arana *et al.*, 2005).

Protein extracts obtained from Newman wild type and *ica* mutant strains revealed that the expression of the abundant surface proteins Eap and Emp was reduced in the *ica* mutant (Figure 4.6). Further characterisation of these two proteins revealed that they were required for full biofilm formation, as mutants in the genes for the individual proteins exhibited reduced biofilm production. In addition it has been shown here that under low iron growth conditions 8325-4 produces very little Eap or Emp, whereas in strain Newman these proteins are highly abundant on the cell surface (Figure 4.8). Levels of these two proteins were also reduced in rich growth media TSB. This suggests not only that factors other than Eap and Emp are required for biofilm formation in rich growth conditions, but also that the inability of strain 8325-4 to form a biofilm in low iron may well be due to its lack of induced Eap and Emp expression. Plasmids containing wild type copies of *eap* and *emp* partially restored both protein expression and biofilm production (Figure 4.7). Incomplete complementation was later discovered to be due to the inclusion of insufficient sequence upstream of the promoter regions, as functional plasmids for both Eap and Emp have since been constructed by Dr Alan Cockayne, University of Nottingham, which fully restore protein expression and biofilm formation.

Why the loss of the surface polysaccharide PNAG should affect the transcription of surface proteins is not known. PNAG is hydrophobic and it is possible that the loss of this polysaccharide on the cell surface causes localised environmental changes. Global regulators which detect and respond to fluctuations in the localised environment may

repress the expression of surface proteins. In *E. coli*, proteins such as the iron and Fur regulated protein FepA are regulated in response to changing external signals and this type of regulation is facilitated by small RNAs (Guillier and Gottesman, 2006). Also, capsular polysaccharide has been shown to protect bacteria against desiccation (Roberson and Firestone, 1992; Roberts *et al.*, 1996), and may be linked to survival in highly osmotic conditions (Gibson *et al.*, 2006). It is possible that in *S. aureus* localised changes in surface charge, hydrophobicity or internal concentrations of chemicals resulting from increased diffusion in the *ica* mutant may stimulate a regulatory response that results in the repression of *eap* and *emp*. It was also shown that mutation of *ica* affected the surface expression of IsdA but not IsdB. The difference may not be surprising, as the *isdA* and *isdB* genes are believed to be individually transcribed from separate promoters (Morrissey *et al.*, 2002; Mazmanian *et al.*, 2003) and are differentially regulated by other regulators. For example, σ^B has been reported to regulate *isdA* expression but not *isdB* expression (Bischoff *et al.*, 2004).

In chapter 3 it was seen that *S. aureus* strain Newman produces a high level of biofilm in CRPMI growth medium, but little in TSB, whereas the opposite was the case for strain 8325-4. Previous studies have shown that biofilm formation in rich media can be induced by osmotic stress caused by high concentrations of salt or glucose (Rachid *et al.*, 2000; Lim *et al.*, 2004), which is controlled in part by σ^B (Rachid *et al.*, 2000); in addition 8325-4 is a σ^B mutant (Kullik *et al.*, 1998). The presence of high glucose did not induce biofilm formation in 8325-4 or in the σ^B repaired strain SH1000 in low iron. Furthermore, biofilm formation in strain Newman was unaffected by high glucose and remained iron regulated (Figure 4.3). This indicates that biofilm formation in high or

low glucose in strain Newman is independent of σ^B. In contrast to glucose, biofilm formation in both 8325-4 and SH1000 was induced in high salt, although biofilm production in Newman wild type and Newman σ^B was unaffected. This indicates that σ^B does not regulate salt induced biofilm formation in either Newman or SH1000 in low iron, contrary to what was observed in different strains in rich growth medium TSB (Rachid *et al.*, 2000). The transcriptional regulator Rbf has been shown to positively regulate salt and glucose induced biofilm formation of 8325-4, a σ^B mutant, in rich growth media (Lim *et al.*, 2004), and it would be interesting to investigate the role of Rbf in low iron biofilm formation. Unlike in high glucose, biofilms formed in high salt were not significantly reduced by the addition of 50 µM iron. This suggests that biofilms formed in high salt require different factors to those required for biofilm formation in low salt, low iron conditions, or that an additional factor or factors are produced in high salt that block iron from inhibiting biofilm formation. Staphylococci have a higher than average tolerance to salt (Chapman, 1945), but so far it is unclear why this is so. As we sweat, salt concentrations build up on the skin; it could therefore be speculated that tolerance to high osmotic environments would be advantageous to a commensal of the human skin. High osmotic tolerance might also be advantageous in aiding *S. aureus* survival during continuous ambulatory peritoneal dialysis related infection, where high osmolarity is key to the artificial dialysis process

This work has shown that biofilm formation in *S. aureus* is iron regulated and dependant on the iron regulated proteins Eap and Emp. In *S. aureus* iron regulation is facilitated by Fur. However in this study, iron regulation has been shown to be independent of Fur, as in the *fur* mutant biofilm formation continued to be iron

regulated, due in part to Fur-independent iron regulation of Eap and Emp (Figures 4.1 A and 4.9). This suggests that there is a novel mode of iron dependant regulation occurring in *S. aureus.* In *Campylobacter jejuni*, Fur independent iron regulation is due to PerR (van Vliet *et al.*, 1999). *S. aureus* also has PerR, but it is a manganese dependant repressor of genes involved in the oxidative stress response, and there is no evidence in the literature to suggest that *S. aureus* PerR can negatively regulate genes in response to increased iron concentration in the absence of Fur. Indeed, the genes encompassed by the *perR* regulon show increased expression in response to high iron in *S. aureus* (Horsburgh *et al.*, 2001b). Nevertheless it would be interesting to investigate the effect of a *fur/per* double mutation on Fur independent iron regulation in *S. aureus.* *Bradyrhizobium japonicum,* a nitrogen fixing symbiont of the soy bean plant, expresses Irr, a Fur independent iron responsive regulator that performs a similar role to Fur, controlling iron dependant gene expression and is a PerR homologue (Hamza *et al.*, 2000).

Although Fur is not responsible for the iron regulation observed, it does have an important dual role in biofilm formation. Typically Fur acts as a negative regulator, and in high iron the Fur/iron complex binds to the promoters of target genes, blocking their transcription. Without iron, the Fur protein cannot bind and the genes are expressed. Here, two novel regulatory functions for Fur in *S. aureus* have been demonstrated. In early adhesion Fur negatively regulates biofilm formation in low iron, and in the later stages of biofilm development Fur acts as a positive regulator, also in low iron (Figure 4.1 B). There are examples of positive Fur regulation in other bacteria. In *Vibrio vulnificus* Fur positively regulates gene expression in low iron (Lee *et al.*, 2007), and

Fur has been shown to directly repress gene expression in low-iron conditions in *Helicobacter pylori* (Bereswill *et al.*, 2000; Lee *et al.*, 2007). It is possible therefore that in *S. aureus* the iron-free form of Fur can bind to DNA either as an activator or as a repressor. Other studies have been performed linking regulation of biofilm formation to other *S. aureus* global regulators such as Agr and SarA, but this is the first time that Fur has been implicated in biofilm formation, a major virulence determinant and survival mechanism of the staphylococcal species. The role of Fur in the regulation of Eap and Emp, suggests that Fur might be involved in a complicated network controlling the expression of *S. aureus* virulence determinants which requires further investigation.

Chapter 5

The regulation of Eap and Emp expression.

5.1 Regulation of Eap and Emp in low iron by SaeRS, SarA and Agr.

In the previous chapter iron and Fur were seen to play a role in the regulation of the genes encoding the biofilm dependant proteins Eap and Emp which have been shown by others to be regulated by Agr, SarA and Sae at the transcriptional level in nutrient rich growth media (Harraghy *et al.*, 2005). Eap and Emp are also regulated at the transcriptional level by Fur and by iron independently of Fur. It is the aim of the work in this chapter to further investigate the relationship between these regulators and their influence on Eap and Emp expression. Some of the work in this chapter has been published (Johnson *et al.*, 2008) and the remainder is in the process of being submitted for publication. Agr and SarA have also been implicated in biofilm formation, although there have been some discrepancies as to the role of Agr in some strains (Yarwood *et al.*, 2004; Boles and Horswill, 2008; Beenken *et al.*, 2003; Manago *et al.*, 2006). Sae is required for virulence in various animal models (Rampone *et al.*, 1996; Goerke *et al.*, 2001; Goerke *et al.*, 2005; Xiong *et al.*, 2006), however the role of Sae in biofilm formation has not been previously observed. In an attempt to further investigate the regulatory pathway involved in low iron induced biofilm formation, biofilm assays and

cell surface protein extractions were performed using wild type Newman and its isogenic mutants Newman *saeRS::Tn917*, Newman Δ*sarA::erm* and Newman Δ*agr::tet*. The *agr* and *sarA* mutants were constructed via transduction of the mutations from RN6911 and ALC1342 respectively, as described in section 2.9. Confirmation of the corresponding mutations was confirmed by PCR amplification of the genetic regions covering the insertions, using the primer pairs SarAF and SarAR, and AgrF and AgrR respectively (table 2.4).

SDS protein extracts prepared from the *agr* and *sae* mutant strains revealed that Eap was also almost completely eliminated on the cell surface, whereas Emp was undetectable (Fig. 5.1 B). SDS surface extracts obtained from the *sarA* mutant revealed a reduction of Emp on the cell surface, whilst Eap appeared to be unchanged. To further investigate the regulatory affects of Agr, SarA and Sae on *eap* and *emp*, proteins were extracted from the cell wall, cell membrane, cytoplasm and supernatants (Fig 5.1 A & C-E). This demonstrated that there was a slight increase in Eap expression in the membrane and cytoplasmic extracts obtained from the *sarA* mutant, despite a reduction in Eap detected in the supernatant and cell wall extracts, whilst Emp was reduced or absent in all fractions. This suggests that the localisation of Eap may be altered in the *sarA* mutant, possibly via the loss of a SarA dependant cell wall protein with which Eap may co-localise. In contrast levels of Eap and Emp were reduced or absent in the agr and sae mutant protein extracts, suggesting that the change in expression of Eap and Emp in these mutant strains was a result of regulatory effects and not due to defective localisation. This is in agreement with these regulators being known transcriptional

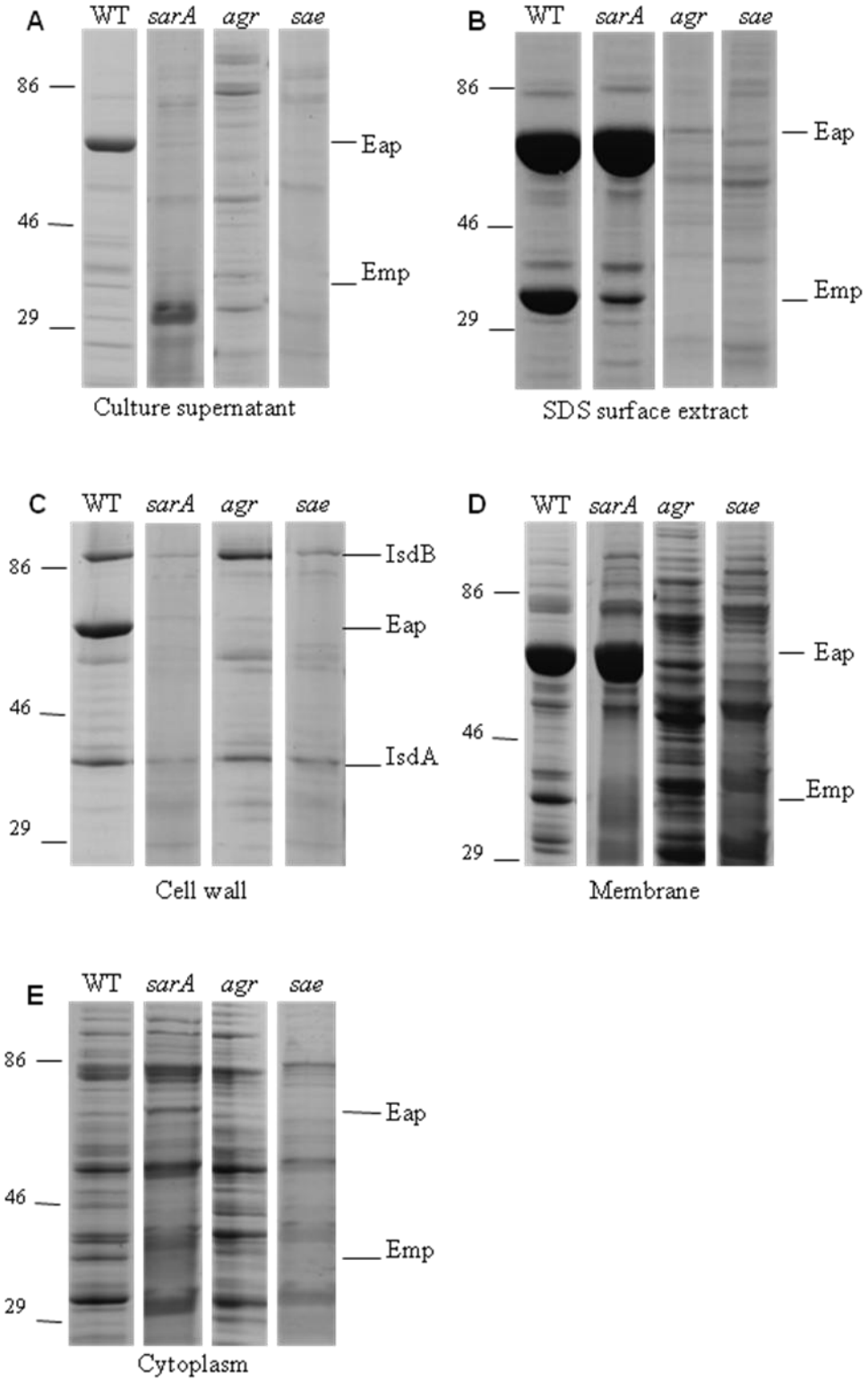


Figure 5.1. Comparison of Eap and Emp expression in Newman wild type and isogenic *sarA*, *agr* and *sae* mutants. After 24 hours incubation, CRPMI cultures were centrifuged and the supernatants were retained and concentrated. Cell pellets were then subjected to the relevant extraction technique as described in section 2.11.

regulators of *eap* and *emp* expression, except that SarA was previously found to activate both *eap* and *emp* in rich medium (Harraghy *et al.*, 2005).

5.2 Regulation of low iron induced biofilm formation by SaeRS, SarA and Agr.

In the previous chapter it was demonstrated that Eap and Emp are required for full biofilm production; it was therefore expected that the loss or reduction of expression of these proteins in the regulator mutants *sarA*, *agr* and *sae* would have a negative affect on biofilm production. Biofilm formation in Newman Δ*sarA::erm* was reduced 3.4-fold compared to the wild-type strain (Fig. 5.2), which is consistent with the reduced Emp expression previously witnessed in the *sarA* mutant (Fig 5.1 A, B, D & E). As expected biofilm formation in Newman *sae* and *agr* mutants was almost completely eliminated, with 8 and 10-fold reduction in biofilm respectively compared to the wild-type (Fig. 5.2), corresponding to almost complete loss in surface expression of both Eap and Emp. These findings suggest that Sae, Agr and SarA all play important roles in the complex regulatory pathway that leads to low iron induced biofilm formation and that these effects are probably via Eap and Emp.

5.3 The regulation of *sae* expression by high iron and by SarA, Agr and Fur in low iron.

In this and previous chapters it has been shown that the surface proteins Eap and Emp, required for biofilm formation in strain Newman, are regulated by iron, Fur, SarA, Agr and Sae. It has also been shown here that in low iron and in the *fur* mutant regulation of these proteins is at the transcriptional level, also elsewhere Agr, SarA and Sae have

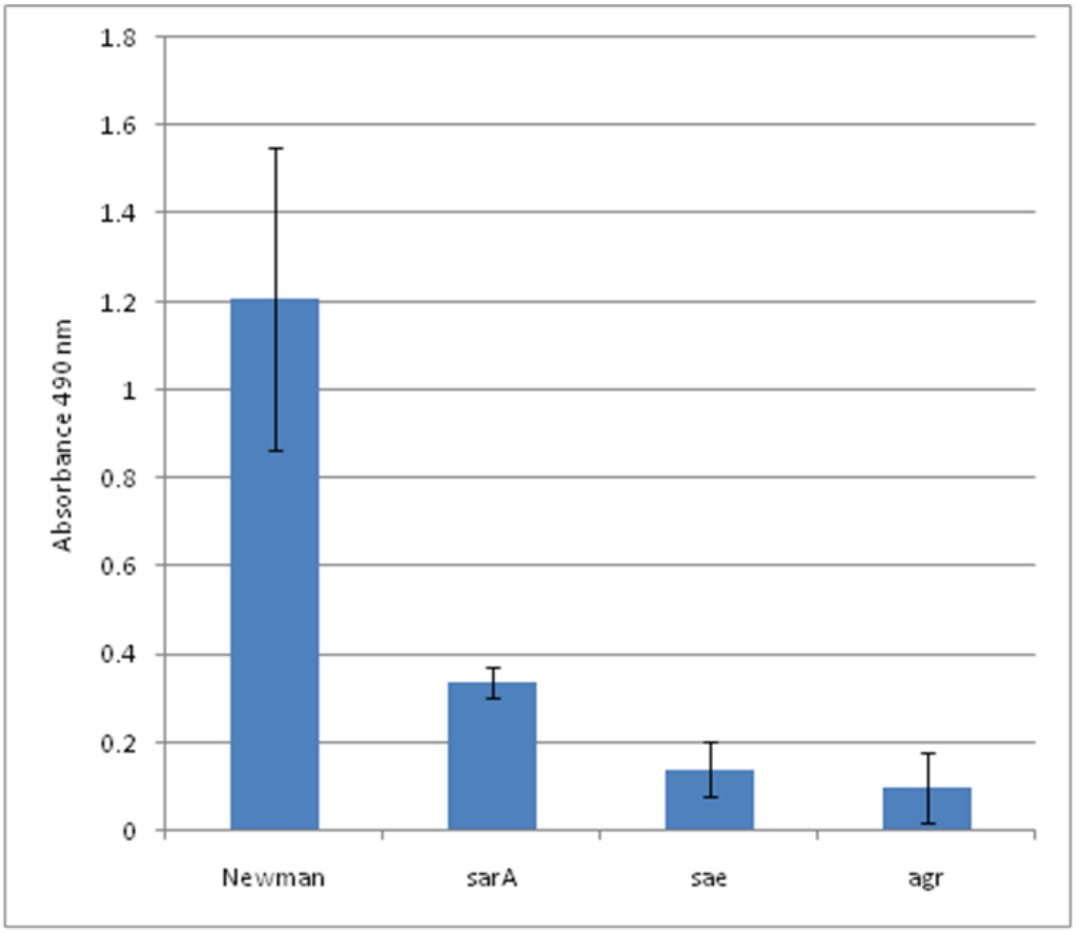


Figure 5.2. The effect of *sarA*, *sae* and *agr* regulators on biofilm formation. A cell pellet from an overnight culture of each strain grown in CRPMI was used to create a suspension of cells at approximately 0.1 OD 600 nm in fresh CRPMI. 200 µL of each culture was added to quadruplet wells of a 96 well microtitre plate. The plate was then incubated for 24 hours at 37°C in 5% CO_2 in air. After 24 hours incubation the plate was washed, stained, dried and read at 490 nm to measure biofilm formation. Figures show the mean and standard deviation of at least three separate experiments.

been shown to be transcriptional regulators of *eap* and *emp* (Harraghy *et al.*, 2005), although how these regulators interact is not yet known. Sae and Fur have a strong association with virulence, but their expression in response to low iron has not been investigated. In an attempt to further explore the regulatory network involving Sae, Fur, Agr and SarA in response to iron, the expression of Sae and Fur was observed in low and high iron in the wild type and *fur* mutant, and in low iron in the *sarA* and *agr* mutants.

To investigate *sae* transcription total RNA was extracted from cultures grown for 6 hours in low or high iron as required from Newman wild type, Newman *fur*, Newman *agr* and Newman *sarA*, and from Newman *sae* as a negative control. Northern blot analysis was then carried out using two *sae* specific probes (see table 2.4), one designated saeP which detects transcripts D and C (Fig 5.3 A), and another designated saeR which detects transcripts A, B and C (Adhikari and Novick, 2008). These four transcripts are expressed from two promoters, P_A*sae* and P_C*sae*. Transcriptional analysis showed that all four transcripts produced by the *sae* operon were absent in the *sae* mutant (Fig 5.3 B), confirming findings of others (Steinhuber *et al.*, 2003). All four transcripts were reduced in high iron, and densitometry performed on the northern blot revealed that transcripts C, B and A were reduced approximately 3-fold in high iron, whereas transcript D was reduced 1.4-fold (Table 5.1 A). This suggests that the transcripts arising from P_C*sae* (C, B and D) are differently posttranscriptionally regulated in response to iron.

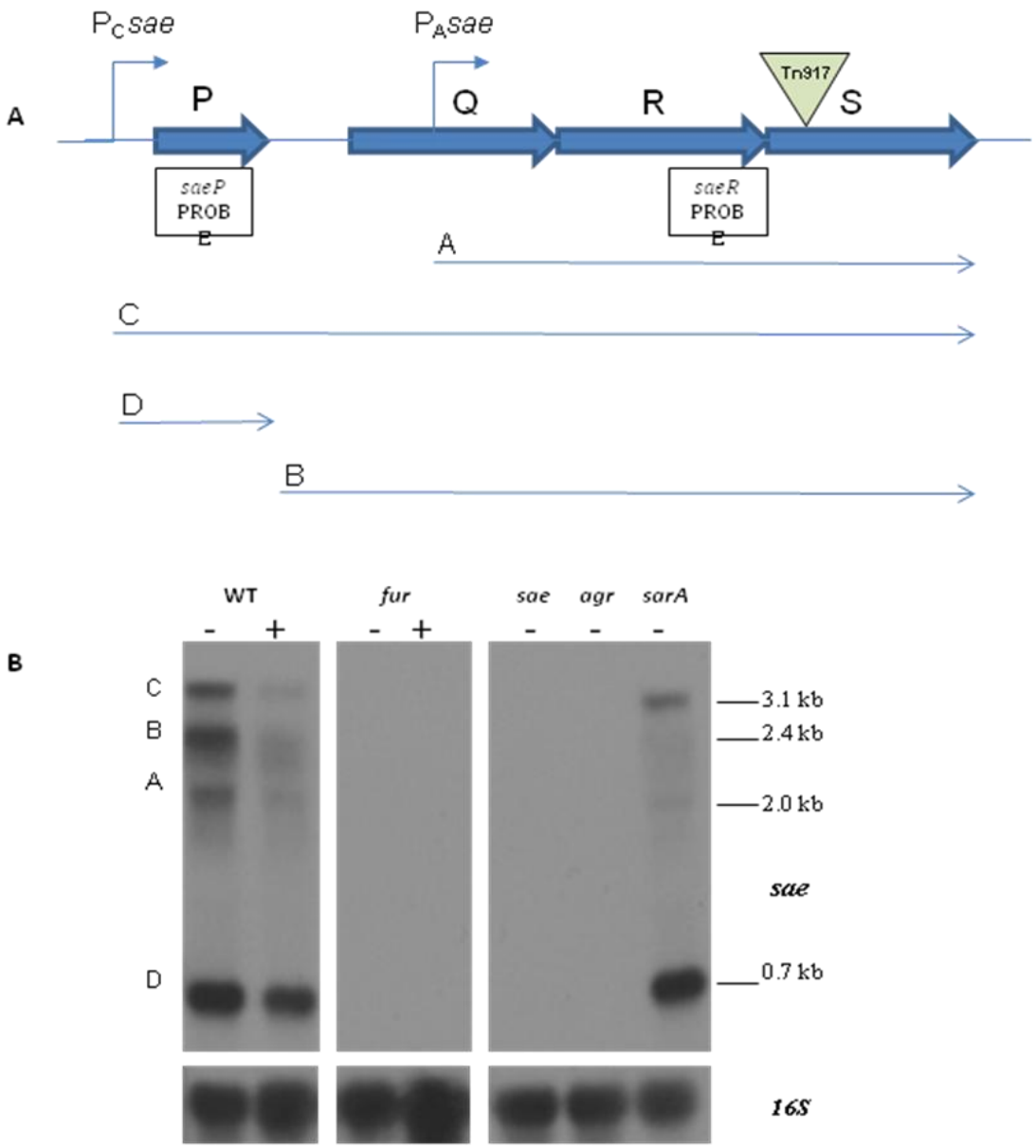


Figure 5.3. Analysis of *sae* transcription. A) A schematic representation of the *sae* operon showing the two promoter regions and the resulting four transcripts. Annealing sites for the two probes are shown and the insertion site for the *Tn917* transposon in the *sae* mutant is indicated. B) Northern blot analysis of *S. aureus* Newman wild type and its isogenic *fur, sae, agr* and *sarA* mutants in high (+) or low (-) iron. Total RNA (10µg) was prepared from 6 hour cultures grown in CRPMI. Blots were hybridised with saeP and saeRS DNA probes specific for *sae*. The blots were then stripped and rehybridised with a *16S* rRNA DNA probe as a loading control. Figure shown is representative of at least three repeat experiments, and patterns of expression were reproducible between repeats.

A

sae Transcripts					
Strain		C	B	A	D
WT	- Fe	0.62	0.87	0.62	1.11
	+ Fe	0.12	0.27	0.21	0.78
fur	- Fe	0.0	0.0	0.0	0.03
	+ Fe	0.0	0.0	0.0	0.0
sae	- Fe	0.0	0.0	0.0	0.0
agr	- Fe	0.0	0.0	0.0	0.0
sarA	- Fe	0.48	0.17	0.16	1.30

B

sae Transcripts					
Strain		C	B	A	D
WT	- NaCl	0.66	0.84	0.40	1.13
	+ NaCl	0.11	0.28	0.17	0.51

Table 5.1. Densitometry analysis performed on Northern blot data. Expression levels of the genes are shown as a proportion of the expression level of the 16S control. A: *sae* expression in the wild type high and low iron, and in low iron in *fur*, *agr*, and *sarA* regulator mutants (Fig. 5.3B). B: *sae* expression in high salt (Fig 5.4A).

In addition *sae* transcripts were absent in the *agr* mutant and reduced in the *sarA* mutant in low iron. This shows that in strain Newman Agr and SarA are required for activation of *sae* transcription in low iron. This supports previous findings in rich medium which suggest that P_A*sae* is activated by SarA (Novick and Jiang, 2003; Geiger *et al.*, 2008; Steinhuber *et al.*, 2003; Adhikari and Novick, 2008), but disagrees with *sae* being unresponsive to Agr signals in strain Newman in rich medium (Geiger *et al.*, 2008). Interestingly, *sarA* affected the four *sae* transcripts differently. Transcript C was reduced 1.2-fold, transcript B 5-fold, transcript A 3.9-fold and transcript D 1.2-fold, again suggesting that different posttranscriptional modification of *sae* transcripts may occur. Unexpectedly, *sae* transcription was completely absent in the *fur* mutant. This suggests that Fur is essential for the activation of *sae* transcription. Also a greater reduction in *sae* transcription was observed in the *agr* and *fur* mutants than in the *sarA* mutant, particularly for transcript D, which suggests that *sarA* may act in fine tuning the regulation of *sae* or that transcript D is regulated by additional factors.

5.4 Sequence analysis of the Newman *sae* operon.

It has previously been observed that *sae* transcription is independent of *sarA* and *agr* in some strains (Novick and Jiang, 2003; Giraudo *et al.*, 1997; Geiger *et al.*, 2008), and that high salt represses *sae* transcription (Novick and Jiang, 2003). In strain Newman it has been reported that an amino acid substitution in SaeS renders the sensor kinase insensitive to signals from Agr and high salt conditions (Adhikari and Novick, 2008).

This leads to *sae* transcription independent of *agr,* and prevents repression of *sae* transcription by osmotic stress (Adhikari and Novick, 2008). Findings shown here demonstrate that under low iron growth conditions, *sae* expression in *S. aureus* strain Newman is dependant on SarA as well as Agr, contrary to findings of other groups. This led to the evaluation of the isolate of Newman used in this study. It is possible that over time this isolate of Newman had lost the amino acid substitution in SaeS, resulting in the *agr* responsive phenotype. To investigate this, the sequence of *saeS* in this isolate of Newman was determined by Joanna Purves by amplifying part of the *saeR* gene and the entire *saeS* region using primers saeRF and saeFlankR (see table 2.4). The purified PCR product was then subjected to sequence analysis using the two primers used to generate the PCR product and an additional primer saeSF, using the method described in section 2.6. This revealed that the amino acid substitution was still present in this isolate, and that the region of the operon sequenced was the same as that published in the Newman genome project, GenBank accession number NC_009641 (Baba *et al.*, 2008).

5.5 Factors involved in the induction of biofilm formation by osmotic stress in low iron growth conditions.

As this study has shown that Newman is responsive to Agr in low iron despite having the amino acid substitution in SaeS, the response of *sae* in strain Newman to osmotic stress was also examined in low iron. RNA was extracted from 6 hour cultures of Newman wild type in CRPMI with and without the addition of salt, and *sae* transcripts were detected as described above. Under conditions causing osmotic stress, *sae*

transcription was reduced (Fig 5.4 A). The negative regulatory effect of high salt on *sae* transcription was different for each transcript, with transcripts C, B, A and D being reduced by 5.2, 3, 2.4 and 2.2-fold respectively (Table 5.1 B), demonstrating that under low iron growth conditions, Newman *sae* is not only responsive to Agr but also to osmotic stress, regardless of the amino acid substitution in SaeS. As *sae* transcription has been shown here to be repressed in high salt, it would be expected that expression of the *sae* regulated surface proteins Eap and Emp would also be reduced. To investigate this, cell surface proteins were extracted from strain Newman wild type grown in high and low salt conditions. Figure 5.4 B shows that, as expected, Eap and Emp surface expression was reduced in high salt conditions, probably due to the negative regulatory effects of high salt on *sae* expression. The fact that Eap and Emp are essential for biofilm formation in low iron and low salt conditions, but are repressed by growth in high salt, suggests that biofilm formation under osmotic stress is independent of *eap, emp* and possibly *sae,* as transcription of *sae* is also reduced under osmotic stress. In addition, Agr, SarA and Fur have been shown to affect *sae* transcription and *eap* and *emp* expression in low iron. To further investigate this, biofilm formation in individual mutants of these regulators and in individual mutant strains of *eap, emp* was observed in high salt. Figure 5.4 C shows that biofilm formation can be induced by high salt in the absence of *eap* and *emp*, but only partial induction was possible in the *sae* mutant. This demonstrates that biofilm formation in low iron, high salt conditions is independent of *eap,* and *emp,* but requires *sae* for full induction, even though *sae* transcription is reduced in high salt conditions. Figure 5.4 C also shows that biofilm formation can be induced in the regulator mutants of *sarA* and *fur*. However full induction was not achieved in the *agr* mutant, suggesting that although Agr, SarA, Fur and Sae all interact,

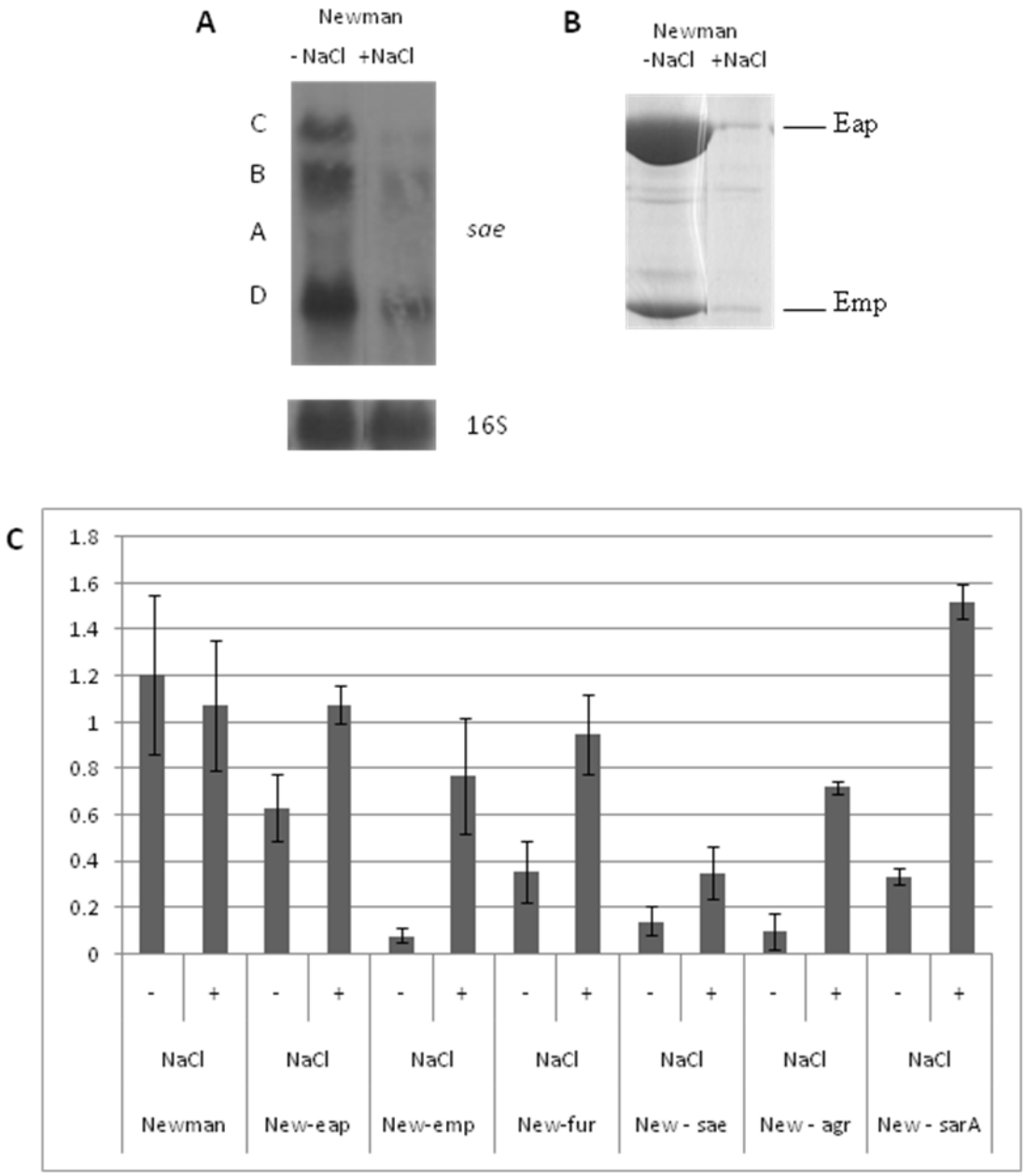


Figure 5.4. The effect of osmotic stress on *sae* transcription, Eap and Emp surface expression and biofilm formation in Newman wild type and its isogenic *eap, emp, fur sae, agr* and *sarA* mutants in low iron. (A) Northern blot analysis of *sae* transcription in Newman wild type in high (+) and low (-) salt. Total RNA was extracted after 6 hours growth, and 10 µg of total RNA was loaded for each sample. Transcripts were detected using saeP and saeRS probes. After detection blots were then stripped and rehybridised with *16S rRNA* DNA probes as a loading control. (B) SDS cell surface protein extracts, from Newman wild type in high and low salt after 24 hours growth. Extracts were prepared as described in section 2.11 (C) 24 hour biofilm assays in high and low salt. The assays and proteins extracts were performed as described in section 2.10. The figure represents the mean and standard deviation of at least three separate experiments.

the production of biofilm formation under conditions of osmotic stress is dependant only on Agr and Sae out of the four regulators assayed.

5.6 The regulatory effect of high and low iron, Agr and Sae on *fur* transcription.

To further investigate how Fur interacts with *sae*, *agr* and *sarA* to coordinate their regulatory response, the effect of *sae*, *agr* and *sarA* on the transcription of *fur* was examined. To achieve this, total RNA was extracted from Newman wild type, Newman Δ*fur*, Newman Δ*agr*, Newman Δ*sarA* and Newman Δ*sae* grown for 6 hours in CRPMI. The expression of *fur* in low and high iron was also compared by the omission or addition of 50 µM iron. Northern blot analysis was then carried out using a *fur* specific probe generated using the primers FurF and FurR (Fig 5.5 A). Transcriptional analysis revealed two transcripts of approximately 1.3 kb and 0.5 kb detected with the *fur* specific probe present in the wild-type strain (Fig 5.5 B). The detection of these two transcripts suggested that *fur* might be co-transcribed with the downstream gene *xerD*, which encodes a site specific recombinase, despite *xerD* having its own proposed promoter region (Xiong *et al.*, 2000). Northern blot analysis using a *xerD* specific probe generated by primers xerDF and xerDR (Table 2.4), confirmed that *fur* and *xerD* are co-transcribed. The *xerD* gene is 885 bp, *fur* is 449 bp, and so it was estimated that a combined *fur/xerD* transcript, including the intergenic region, would be approximately 1.3 kb. This was confirmed, as in the wild type there was a single transcript of approximately 1.3 kb detected with the *xerD* specific probe (Fig 5.5 C). In the *fur* mutant there was single larger transcript detected by the *xerD* probe, of approximately

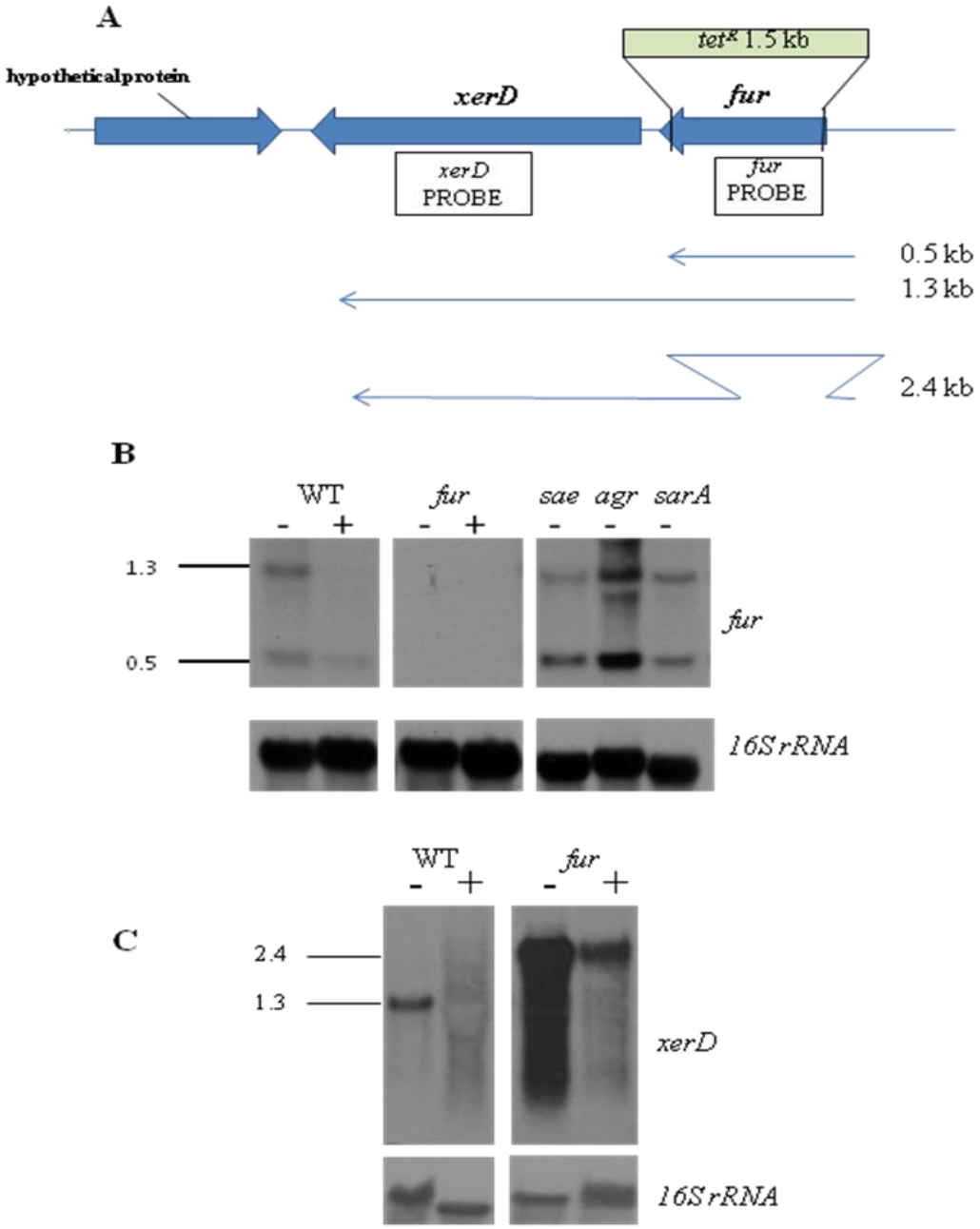


Figure 5.5. Transcription analysis of the *fur* gene A) Schematic diagram of the arrangement of Newman *fur* and *xerD* genes showing the regions amplified by PCR for probe generation. B) Northern blot analysis of *fur* expression in *S. aureus* Newman wild type and its isogenic *fur, sae agr* and *sarA* mutants in high or low iron as indicated with a *fur* specific probe. C) Northern blot analysis of *xerD* expression in *S. aureus* Newman wild type and its isogenic *fur* mutant in high or low iron as indicated using a *xerD* specific probe. Total RNA (10µg) was prepared from 6 hour cultures grown in CRPMI. Blots were hybridised with *fur* (B) or *xerD* (C) specific DNA probes as indicated. The blots were then stripped and rehybridised with a *16S rRNA* DNA probe as a control. Figure shown is representative of at least three repeat experiments, and patterns of expression were reproducible between repeats.

2.4 kb corresponding to the few remaining bases of *fur*, the 1.5 kb tetracycline cassette used in the construction of the *fur* mutant (Horsburgh *et al.*, 2001b) and *xerD*. As *fur* and *xerD* are co-transcribed, the increase in *xerD* transcription in Newman *fur* suggests that *fur* might be auto-regulated, which in turn represses *xerD*. Furthermore the *xerD* transcript is still iron regulated in the *fur* mutant, suggesting that *xerD* and possibly *fur* are iron regulated independently of Fur.

In addition to repression by iron, the 0.5 kb *fur* transcript is also regulated by Agr, Sae and possibly SarA. Densitometry performed on the Northern blot showed that the 0.5 kb *fur* transcript was increased 4-fold in the *agr* mutant (Table 5.2), 2-fold in the *sae* mutant and 1.3-fold in the *sarA* mutant, indicating that Agr and to a lesser extent Sae and SarA repress *fur* expression, and suggests that there is a regulatory hierarchy involved in this complex regulatory network.

5.7 The role of Hfq in the regulation of *S. aureus* Newman surface proteins Eap, Emp, IsdA and IsdB.

In the previous sections it has been seen that the expression of Eap and Emp is positively regulated in the *fur* mutant, which is a novel function of Fur in *S. aureus* in low iron. In other bacteria such as *E. coli* positive Fur regulation has been shown to involve the regulatory protein Hfq (Vecerek *et al.*, 2003; McNealy *et al.*, 2005). To investigate if Hfq is involved in the positive Fur regulation of *eap* and *emp* in strain Newman, the transcription and expression of these surface proteins/genes were observed in a Newman *hfq::Kan* and a Newman *hfq/fur* double mutant (Dr Julie

		***fur/xerD* Transcripts**		
Strain	**Condition**	*fur* T1 **1.3 kb**	*fur* T2 **0.5 kb**	*xer*D **1.3 kb**
WT	**- Fe**	0.30	0.2	0.67
	+ Fe	0.06	0.1	0.0
fur	**- Fe**	0.1	0.0	1.60
	+ Fe	0.0	0.02	1.46
agr	**- Fe**	0.66	0.81	-
sae	**- Fe**	0.22	0.42	-
sarA	**- Fe**	0.25	0.27	-

Table 5.2. Densitometry analysis of Northern blot data.
Expression levels of the genes in Fig. 5.5 B & C are shown as a proportion of the expression level of the 16S rRNA control.

Morrissey, Department of Genetics, Leicester University). On obtaining the mutants the *hfq* mutation was verified by PCR amplification of the region encompassing *hfq* using the primers MiaF and HfqR (table 2.4). The PCR product was sequenced using primers MiaF, HfqF and HfqR as described in section 2.6. The *hfq* mutation was then transferred to the Newman *fur* mutant by phage transduction as described in section 2.9, and verified by PCR.

Surface protein extracts from the wild type and mutant strains were prepared from 24 hour cultures grown in CRPMI plus and minus iron. Fur positively regulates the surface expression of Eap and Emp as, in the *fur* mutant, expression of both proteins was reduced as shown previously (Fig 4.10 B, Fig 5.6 A). In the *hfq* mutant the level of surface expression of Eap and Emp was comparable to the wild type (Fig 5.6 A); surprisingly, however, in the *hfq/fur* double mutant, the surface expression of Eap and Emp was completely lost. To further investigate this, Northern blot analysis was carried out on total RNA extracted from cultures grown for 6 hours in CRPMI with and without the addition of iron. Blots were hybridised with DNA probes specific for *eap* and *emp* as described previously. Figure 5.6 B shows that transcription of *eap* and *emp,* as seen previously in chapter 4, is positively regulated by Fur and negatively regulated by iron.

In addition Hfq also positively regulates the transcription of both *eap* and *emp,* although this was not reflected in the surface proteins extracted after 24 hours growth (Fig 5.6 A). The discrepancy between the levels of transcript and protein expression of Eap and Emp in the *hfq* mutant suggests that *eap* and *emp* transcripts must be translated and then rapidly degraded. This is similar to what was seen in the *fur* single mutant, although in

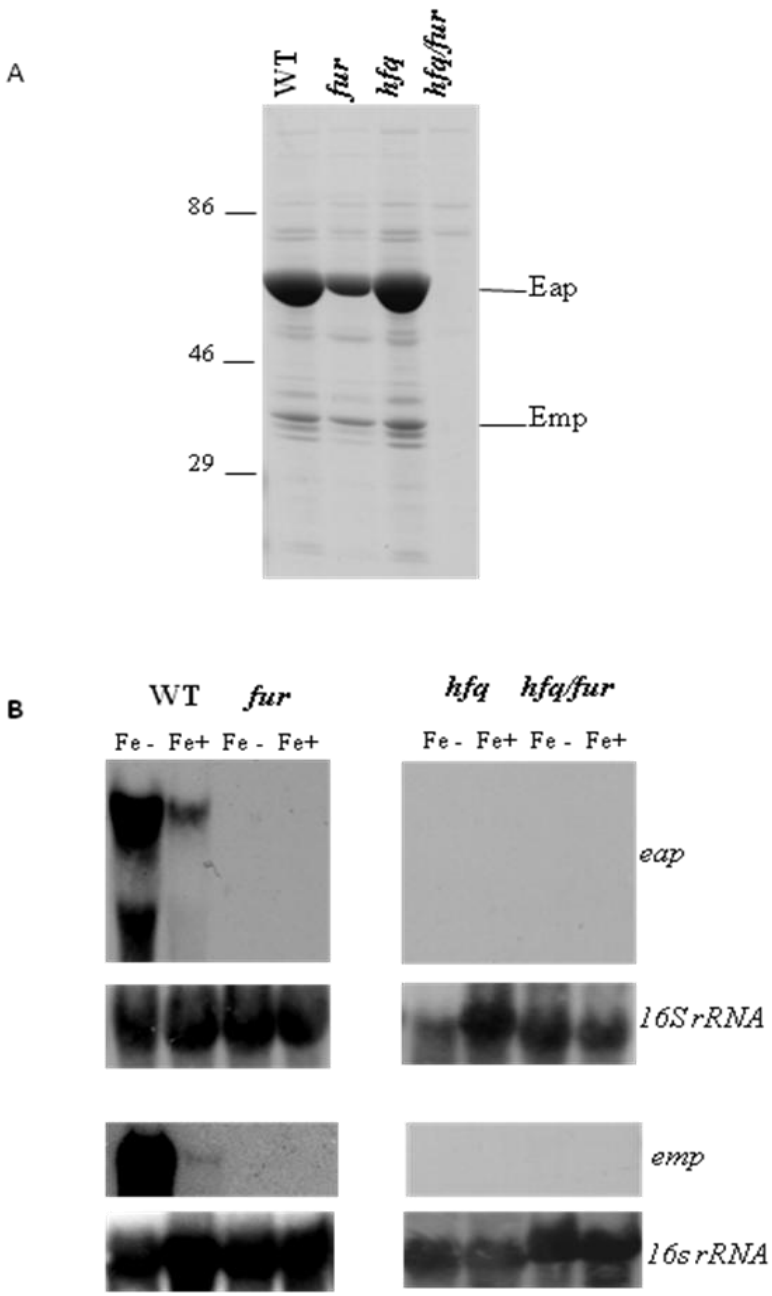


Figure 5.6. Expression of Eap and Emp in Newman wild type and its isogenic mutants *fur*, *hfq* and the *hfq/fur* double mutant. A) SDS surface protein extracts. A cell pellet from an overnight culture of each strain grown in CRPMI was used to create a suspension of cells at approximately 0.1 OD 600 nm in fresh CRPMI. Cultures were then incubated for 24 hours at 37°C in 5% CO_2 in air. After 24 hours surface proteins were extracted. B) Total RNA (10µg) was prepared from 6 hour cultures grown in CRPMI in high or low iron as indicated. Transcripts were detected with *emp* and *eap* specific probes as described earlier, after which blots were stripped and rehybridised with a *16S rRNA* DNA probe as a loading control.

the *fur* mutant there must also be reduced *eap* and *emp* transcription to explain the reduced protein expression compared to the wild type or *hfq* mutant. Both *eap* and *emp* transcripts were also absent in the *hfq/fur* double mutant, but since the transcripts for neither gene was detected in the *fur* or *hfq* single mutants, loss of transcription in the *fur/hfq* double mutant was not surprising. However it was surprising that in the double mutant loss of detectable transcripts for *eap* and *emp* also resulted in loss of expression of the proteins on the cell surface, suggesting that Fur and Hfq interact in the regulation of Eap and Emp.

As *eap* and *emp* are non classically Fur regulated, the role of *hfq* in the transcriptional regulation of the classically Fur regulated genes *isdA* and *isdB* was further investigated (Mazmanian *et al.*, 2002; Morrissey *et al.*, 2002). Total RNA was extracted from the *hfq* and *fur* single and double mutants after 6 hours growth in high and low iron. *isdA* and *isdB* specific probes were generated from PCR products using the primers isdAF and isdAR and isdBF and isdBR respectively (table 2.4). The *isd* gene cluster consists of eight open reading frames and three promoters (Fig 5.7 A). The *isdA* and *isdB* genes are transcribed divergently from the rest of the operon, each having their own putative promoter with a Fur recognition box (Morrissey *et al.*, 2002). It was therefore surprising to find from Northern blot analysis that both *isdA* and *isdB* had multiple transcripts (Fig 5.7 B). In the wild type, the *isdB* specific probe detected three transcripts T1, T2 and T3, of which T2 and T3 were the most prominent, and all three transcripts were absent in high iron (Fig 5.7B & Table 5.3). The *isdA* specific probe detected four transcripts T1, T2, T3 and T4 in the wild type, which like *isdB* transcripts,

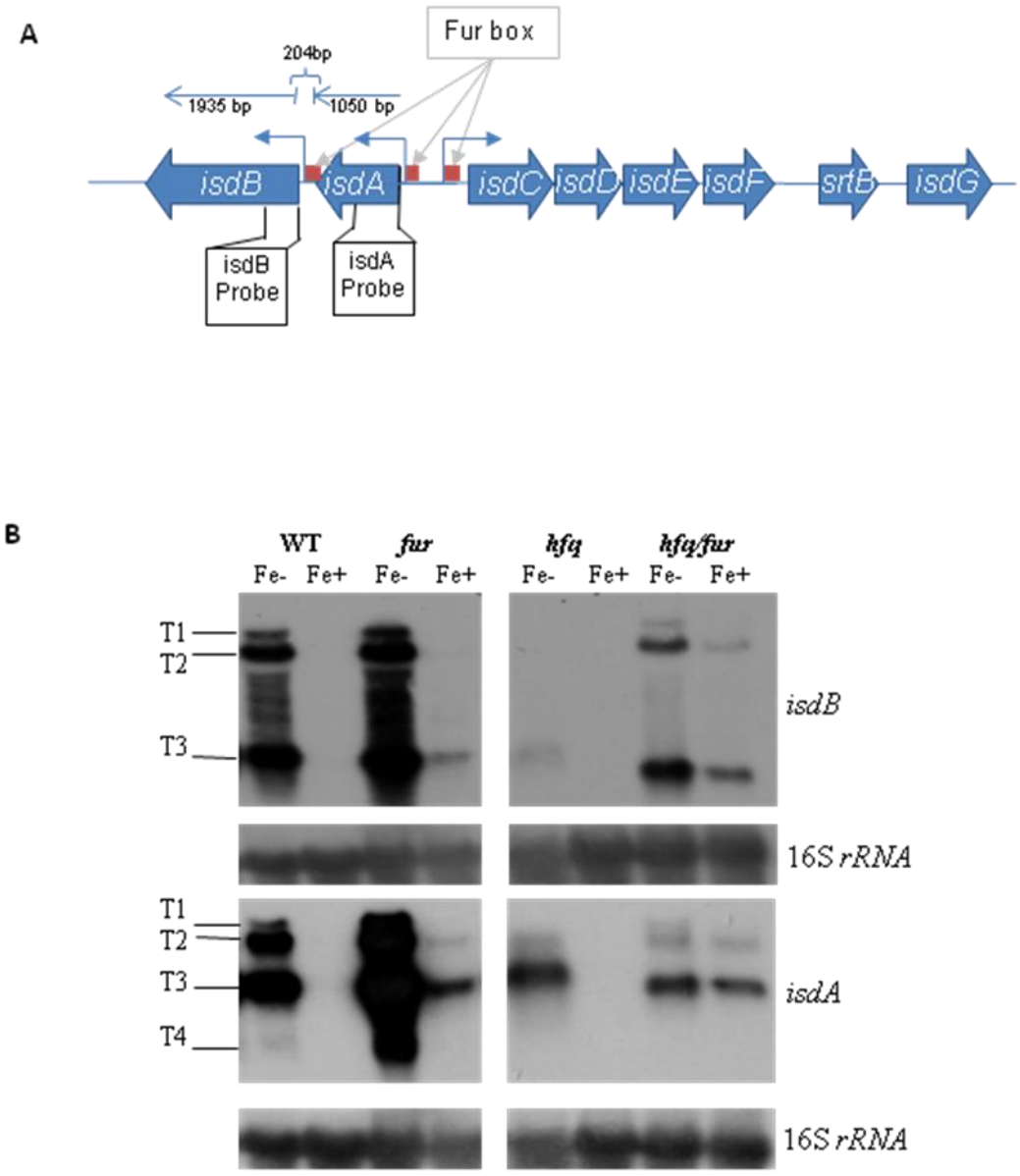


Figure 5.7. *isdA* and *isdB* expression in Newman wild type and its isogenic *fur, hfq and hfq/fur* mutants in high or low iron. A) Schematic diagram of the arrangement of the *S. aureus isd* locus showing the regions where the probes anneal, and the putative Fur regulated promoter regions for *isdA* and *isdB*. B) Northern blot analysis. Total RNA (10µg) was prepared from 6 hour cultures grown in CRPMI in high or low iron as indicated. Transcripts were detected with *isdA or isdB* specific DNA probes as indicated, after which blots were stripped and rehybridised with a *16S rRNA* DNA probe as a loading control. The figure shown is representative of at least three repeat experiments and the expression patterns shown were reproducible.

		***isdB* Transcripts**		
Strain	**Condition**	*isdB* T1	*isdB* T2	*isdB* T3
WT	**- Fe**	0.95	2.6	2.76
	+ Fe	0.0	0.0	0.0
fur	**- Fe**	1.77	3.3	3.56
	+ Fe	0.0	0.0	0.61
hfq	**- Fe**	0.0	0.0	0.52
	+ Fe	0.0	0.0	0.0
hfq/fur	**- Fe**	0.46	1.59	2.38
	+ Fe	0.0	0.39	1.09

		***isdA* Transcripts**			
Strain	**Condition**	*isdA* T1	*isdA* T2	*isdA* T3	isdA T4
WT	**- Fe**	0.77	1.47	1.8	0.19
	+ Fe	0.0	0. 0	0.0	0.0
fur	**- Fe**	1.45	1.87	1.99	1.46
	+ Fe	0.0	0.23	1.37	0.0
hfq	**- Fe**	0.0	0.54	1.28	0.0
	+ Fe	0.0	0.0	0.0	0.0
hfq/fur	**- Fe**	0.22	0.32	0.78	0.0
	+ Fe	0.0	0.23	0.58	0.0

Table 5.3 Densitometry analysis performed on Northern blot data. Expression levels of the genes in Fig. 5.7C are shown as a proportion of the expression level of the 16S control.

were all absent in high iron. In the *fur* mutant transcripts for both genes were derepressed, showing that *isdA* and *isdB* are negatively regulated by iron at the transcriptional level (Fig 5.7 B). However in the *fur* mutant transcripts for both *isdA* and *isdB* were expressed at a lower level in high iron than in low iron suggesting that additional Fur independent regulation of *isdA/B* may be occurring at the transcriptional level, in high and low iron. Alternatively, in the previous chapter (Fig 4.10 C), cell wall extracts obtained from the *fur* mutant shows that IsdA and IsdB are translated in high iron at a similar level to low iron, this could suggest that transcripts are destabilised in high iron, or stabilised in low iron, in the absence of Fur.

In the *hfq* single mutant only very low levels of T3 were evident for *isdB,* and only low levels of T2 and T3 were present for *isdA,* far lower than transcripts present in the *fur* mutant or wild type for either gene. This suggests that Hfq is required for full expression of both *isdA* and *isdB.* In high iron, all transcripts were absent for both genes in the *hfq* mutant, suggesting that Hfq is not required for iron regulation of *isdA/B.* In the *hfq/fur* double mutant transcripts for *isdA* and *isdB* are derepressed in high iron as seen in the *fur* single mutant. However in comparison to the *fur* single mutant levels of *isdA/B* in low iron are much lower in the double mutant. Thus Fur is shown to repress *isdA/B* whilst Hfq appears to be required for the full induction of *isdA* and *isdB* expression in low iron.

5.8 Discussion

It was the aim of the work in this chapter to further investigate the relationship between the regulators Fur, Sae, Agr and SarA, and their influence on Eap and Emp expression. This work has shown that the iron and Fur regulated surface proteins Eap, Emp, IsdA and IsdB are also regulated by Hfq, a novel function for the regulator in *S. aureus*. In the previous chapter the surface proteins Eap and Emp were reported to be essential for biofilm formation in low iron. A previous study has shown that *eap* and *emp* are regulated by Agr, SarA and Sae (Harraghy *et al.*, 2005), but their regulation in low iron was not determined. Further investigation of proteins extracted from these regulator mutants revealed that Agr and Sae activate *eap* and *emp* expression whereas SarA activated *emp* expression but slightly repressed that of *eap*. This is contrary to previous observations that SarA activated both proteins (Harraghy *et al.*, 2005), and could be due to the different growth media used. Iron has been shown to override some signals (Bollinger *et al.*, 2001), and so it is possible that in low iron SarA has a different role to that observed in iron replete conditions. In addition protease production is up-regulated in *sarA* mutants (Chan and Foster, 1998), which could explain why, although Eap levels were increased in the membrane, cytoplasm and possibly in the cell surface extracts of the *sarA* mutant, Eap in the supernatant of the *sarA* mutant was lost.

In previous studies *sae* has been shown to be regulated by Agr and SarA (Giraudo et al., 2003; Novick and Jiang, 2003). However in strain Newman, *sae* was said to be insensitive to Agr and high osmolarity caused by high concentrations of salt, due to an amino acid substitution in the sensor kinase SaeS. The sequence of *saeS* in the isolate

of Newman used in this work has been verified as containing the same mutation, yet it is still responsive to both Agr and high salt. This could also be due to different growth media used in this study, and the possible overriding effects of iron. However, it would be interesting to sequence the rest of the operon, including the promoter regions, to determine if there are variations elsewhere that might account for the discrepancy in Agr and salt response, although differences in promoter sequence may be unlikely as Eap and Emp are expressed at high levels in this Newman isolate, as previously reported for Newman containing the SaeS amino acid substitution elsewhere (Adhikari and Novick, 2008). In addition, the regulatory affects of SarA on *sae* expression witnessed in this study were interesting. Previously SarA has been shown to repress transcripts A, B and C (Novick and Jiang, 2003; Steinhuber *et al.*, 2003), which is confirmed here in low iron. However, the level of the smallest transcript, transcript D, is the same as that observed in the wild type. All other effectors, including iron, salt, Fur, and Agr, had an effect on all transcripts. Other studies have attempted to assign roles to the multiple *sae* transcripts and have found that transcript A, which covers *saeRS,* is required for *sae* expression from either promoter. However, a role for transcript D, encompassing *saeP,* has not yet been fully determined, although a mutant in *saeP* was shown to exhibit increased expression of coagulase (Adhikari and Novick, 2008), suggesting that that transcript D might indeed have a regulatory role.

The repression of *sae* in high salt corresponds to a reduction in the expression of cell surface Eap and Emp, although in high salt Newman forms a level of biofilm equal to that in low iron, low salt conditions. This supports the proposal that factors other than

Eap and Emp are required for biofilm formation in high salt. This was confirmed by observing biofilm formation in high salt in mutants of *eap* and *emp*. Biofilm was also produced by the *sarA* mutant, moderate biofilm formation was achieved in the *agr* mutant and only a low level of biofilm was formed in the *sae* mutant in high salt. This suggests that factors involved in biofilm formation in high salt are independent of SarA but partially dependant on Agr, and dependant on Sae despite *sae* being repressed in high salt.

This work has also shown that the *fur* gene is iron regulated, co-transcribed with *xerD*, possibly auto-regulated and regulated by the global regulators Sae, Agr and SarA. In addition, this work has shown that *sae*, a major staphylococcal regulator, requires Fur for expression. Also the previously determined regulation of *sae* by Agr and SarA in rich growth medium has been confirmed in low iron.

In this chapter, it has been observed that Fur in *S. aureus* strain Newman acts as a positive transcriptional regulator of *eap* and *emp* in low iron, but the mechanisms behind this are not yet known. In *E. coli*, positive Fur regulation is achieved via the Hfq dependant sRNA RhyB, in which the absence of Fur apparently positively regulates the expression of SodB. However, the regulation is indirect and via the derepression of *rhyB*, which is a repressor of *sodB* (Masse and Gottesman, 2002). If positive Fur regulation of *eap* and *emp* was occurring in a similar way to the *E. coli* Hfq/RbyB example it would be expected that wild type levels of Eap and Emp expression would

observed in the *hfq/fur* mutant. However it appears that this is not the case in *S. aureus*, as in the *hfq/fur* mutant there is no transcription of *eap* or *emp*. Therefore Hfq does play a role, but it is possibly positive in *S. aureus*. The fact that Eap and Emp proteins are seen on the cell surface suggests that Hfq is not acting at the level of transcription of these two genes, but maybe responsible for mRNA stability. It is possible that after 6 hours, when RNA was extracted, transcription of *eap* and *emp* might increase in the *hfq* mutant leading to the level of protein observed in the surface protein extracts at 24 hours.

In the *fur* mutant there was no detectable *eap* or *emp* mRNA, yet reduced levels of both Eap and Emp proteins were present in the cell wall extracts. The discrepancy between the high level of Eap and Emp proteins observed and the level of transcripts detected in the *fur* mutant may also be explained by the difference in time points between RNA extraction and protein extraction. Alternatively, as Fur is required for full expression of Eap and Emp, transcription may occur in the absence of Fur which is degraded prior to detection, accounting for the level of protein observed.

In the *hfq/fur* double mutant no Eap or Emp protein was detected in the cell surface extracts, and no detectable transcripts were present for either gene. Therefore it is possible that the basal level of transcription in the absence of Fur, coupled with destabilisation of transcripts in the absence of Hfq, may prevent translation so that surface expression of the proteins is lost. It is also possible that Hfq acts to stabilise

mRNA of *eap* and *emp* via sRNA, as is the case for other examples of Hfq regulation (Le Derout *et al.*, 2003), or that Hfq independent sRNAs are responsible for positive Fur regulation of *eap* and *emp*. Only a few sRNA molecules have been so far identified in *S. aureus;* it would be interesting to see if any of these are involved in the Hfq regulation witnessed here.

In addition to Eap and Emp regulation, Hfq also has a role in the transcriptional regulation of the classically Fur regulated cell wall proteins IsdA and IsdB, which have been implicated in iron uptake and immune evasion (Cheng *et al.*, 2009). Interestingly, the *isdA* gene is directly upstream of *isdB,* and both genes are transcribed in the same direction with individual putative Fur responsive promoter regions (Morrissey *et al.*, 2002). However both *isdA* and *isdB* specific probes revealed multiple transcripts. Whether these include down stream regions in addition to the target gene, or whether they are a result of posttranscriptional modification is not yet known. Transcriptional data also shows that Fur and Hfq have an antagonistic relationship with regard to the regulation of *isdA/B.* Hfq has a positive effect on the level of *isdA* and *isdB* transcription, as in the *hfq* mutant the transcripts were reduced. Whereas in the *fur* mutant the expression of both *isd* genes was dramatically increased, confirming that Fur negatively regulates transcription of these two genes.

In all conditions the transcripts for both *isdA* and *isdB* genes remained iron regulated, although the iron regulation of transcripts is less obvious in the *fur/hfq* double mutant.

This suggests that Hfq or Hfq dependant sRNAs might play some role in posttranscriptional iron regulation in the absence of Fur, at least for *isdA* and *isdB*.

By contrast, the level of iron regulation of both *isdA* and *isdB* transcripts in the *fur* mutant are obvious in the northern blot, but iron regulation at the protein level was less obvious in the cell wall extracts, as observed in the previous chapter (Fig 4.9 C). This implies that the transcripts for both genes in high iron must be somehow stabilised in the *fur* mutant as, although the transcripts are reduced in high iron in the *fur* mutant, relatively high levels of protein are observed in high iron in the *fur* mutant. This suggests that additional factors may be involved in the posttranscriptional or posttranslational regulation of IsdA and IsdB. Also in the *fur* mutant, in low iron, mRNA levels of both *isdA* and *isdB* were increased, but this resulted in only a small increase in cell wall IsdB (as seen in the previous chapter), and no increase in IsdA protein, despite the detection of similar levels of transcript for each gene. This is again suggestive of post-transcriptional or post-translational modification, and indicates that translation of IsdA might either be blocked or the protein might be degraded more readily than IsdB in the *fur* mutant.

This data has shown that Hfq interacts with Fur to regulate the expression of *isdA, isdB, eap* and *emp*, and that iron regulation of *eap*, *emp*, *isdA* and *isdB* is, to some extent, Fur independent. These data also show that *eap* and *emp* are differently regulated to *isdA* and *isdB*, which suggests that other factors may be involved in the regulation of iron

responsive genes. These factors may act to fine tune the expression of genes in response to the iron status of the environment, and may occur at different levels, from transcription and translation to post-translation. It is possible that some of these regulatory effects may be due to Hfq dependent sRNAs, as iron regulation was less obvious for *isdA/B* in the *hfq/fur* double mutant, and that different sRNAs may be responsible for differential regulation depending on the iron status of the surroundings. Although only a few sRNA have been identified in *S. aureus* they could be involved in the regulatory circuit in *S. aureus*. In *E. coli* the sRNA DsrA regulates the expression of two regulatory proteins RpoS and HNS (Masse and Gottesman, 2002) and it is possible that sRNA and Hfq have a role in the regulatory network observed in this work.

Chapter 6

Final discussion and future work.

The work carried out in this study has revealed that *S. aureus* biofilm formation is iron regulated, an important discovery in the understanding of biofilm production in this pathogenic organism. Different strains of *S. aureus* were found to form biofilms to varying levels, which changed in response to the growth medium used. The impact of growth medium on phenotypic observations in *S. aureus* is becoming more widely accepted, and this work highlights the need for the awareness of this. This work has revealed two novel roles for Fur in *S. aureus* strain Newman. Fur was shown to positively regulate the expression of *eap* and *emp* leading to induction of 24 hour biofilm formation in low iron, and negatively regulate early adhesion in low iron. This is unusual as Fur is usually regarded as an iron dependant repressor. In addition, the regulators Fur, Sae, Agr and SarA have been implicated in the regulation of biofilm formation and *eap* and *emp* expression. Furthermore *fur* and *sae* are iron regulated and regulated by each other and by Agr and SarA by an as yet undetermined mechanism. It has also been demonstrated that Hfq has an important role in the expression of key virulence factors *eap, emp, isdA* and *isdB*, the first reported phenotype for Hfq in *S. aureus*, and it has been observed that repression of *eap* and *emp*, and therefore biofilm formation, occurs independently of Fur in high iron, suggesting that *S. aureus*

strain Newman is capable of Fur independent iron regulation via an as yet unknown mechanism. The growing *S. aureus* regulatory network is summarised in figure 6.1.

In this work, biofilm assays have demonstrated that strain 8325-4 produced a high level of biofilm in the rich growth medium TSB, but failed to produce a mature biofilm in the low iron growth medium CRPMI, despite early adhesion patterns being the same as in strain Newman. Newman by comparison produced a moderate level of biofilm in TSB, and a high level in low iron CRPMI. This suggested that strain 8325-4 lacked one or more factors responsible for full biofilm formation in low iron conditions. Iron regulated biofilm formation in strain Newman was found to be dependant on the expression of surface proteins Eap and Emp which were highly expressed on the surface of strain Newman in low iron. In 8325-4 the surface expression of Eap was low compared to Newman, and Emp could not be detected. The lack of these two proteins on the surface of 8325-4 probably explains why 8325-4 was unable to form a biofilm in low iron. In addition Eap and Emp expression was repressed in the rich growth medium TSB in both Newman and 8325-4. Despite this 8325-4 forms a high level of biofilm in TSB, showing that biofilm formation occurs in response to different environmental cues and are dependant on the expression of different factors.

The production of Eap and Emp has been implicated in virulence *in vivo* (Chavakis *et al.*, 2002; Scriba *et al.*, 2008; Garzoni and Kelley, 2009; Voyich *et al.*, 2005; Cheng *et al.*, 2009). The two proteins are secreted and reattached to the cell surface; however the mechanism for their attachment has not been fully characterised. Eap has been shown to bind *in vitro* to a surface expressed neutral phosphatase and to

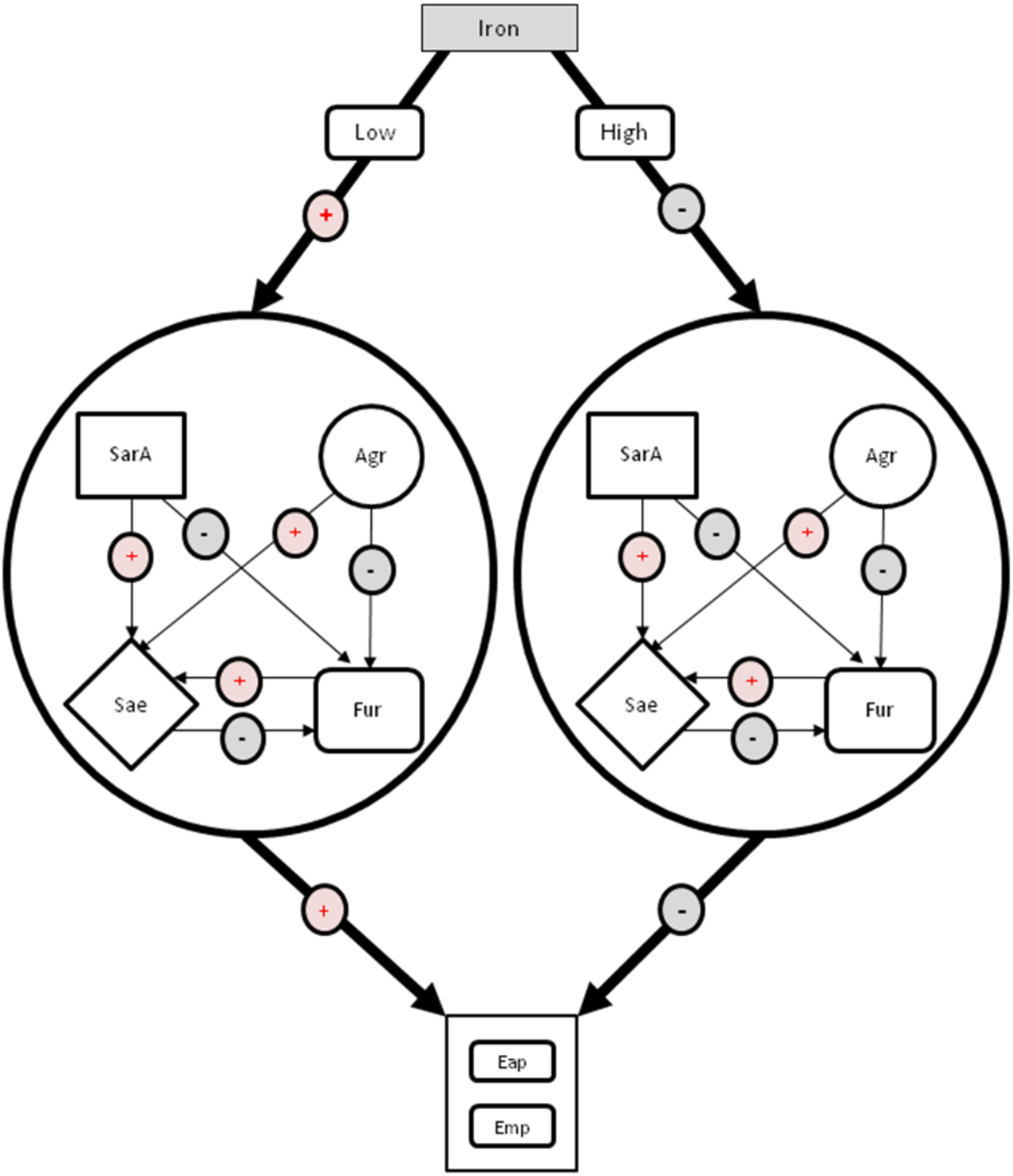


Figure 6.1. A Schematic model of Eap and Emp expression coordinated by Sae, Fur, Agr and SarA in response to iron as demonstrated in this work. Sae, Fur, Agr and SarA positively regulate Emp expression, Sae, Fur and Agr also positively regulate Eap expression. In turn the regulators Sae and Fur are negatively regulated by high iron, reducing the expression of Eap and Emp. In addition to the regulation shown above, Hfq also has a role in the expression of *eap, emp* and *isdA/B*.

also bind to itself (Flock and Flock, 2001), whereas Emp binding has not yet been investigated. Determining how Eap and Emp localise to the cell surface would be very interesting, if their re-attachment to the cell surface could be blocked this may prove advantageous in the treatment of *S. aureus* infection, given the established role of Eap and the predicted role for Emp in *S. aureus* virulence. In protein extracts it was observed that not all of the secreted protein Eap was removed from the cell surface in the SDS extracts, a substantial amount was retained in the cell wall at 24 hours. It is possible that secreted Eap rebinds to the surface via the cell wall associated Eap. To investigate if Eap and Emp bind to specific targets on the cell surface under low iron conditions, Eap and Emp proteins could be tagged and immobilised on an affinity column, through which different protein fractions could be passed. The bound protein could then be separated from the tagged Eap or Emp protein and sequenced.

Early studies into *S. aureus* biofilm formation showed that PNAG was essential for biofilm formation and that the *ica* operon encoding the polysaccharide was found in most strains tested. More recently the role of PNAG and *ica* in biofilm formation has become more controversial with biofilm negative, *ica* positive strains being identified as well as biofilm formation occurring in the absence of *ica*. The *ica* operon consists of four genes, *icaADBC*, under the control of the divergently transcribed repressor, *icaR*. Despite the limited role of wild type levels of PNAG in low iron biofilm formation, in this study the surface expression of Eap and Emp was found to be dependant on an intact *ica* operon.

Why the expression of a surface associated polysaccharide should affect the expression of surface proteins such as Eap and Emp is not known. The loss of the charged surface polysaccharide may result in localised environmental changes. As shown in the case of high osmolarity, environmental signals can have implications for the transcription of *sae* which in turn reduces the expression of Eap and Emp (Novick and Jiang, 2003). The *ica* deficient strain used in this study contained an insertion deletion that replaced the entire *icaADBC* operon, including the *icaR* repressor gene. Therefore to further determine the role of *ica* in low iron biofilm formation it might be useful to assess biofilm formation using different *ica* mutants; for example, it would be interesting to observe the effects of over expressing *icaR* on *eap* and *emp* expression. Increasing IcaR would prevent or greatly reduce *icaADBC* expression and may allow the effects of reduced polysaccharide to be observed on *eap* and *emp* without disrupting the operon. Alternatively an *icaB* mutant might also be useful. IcaB has been previously shown to be responsible for surface attachment and deacetylation of polysaccharide residues (Vuong *et al.,* 2004; Cerca *et al.*, 2007). Therefore an *icaB* mutant would still express PNAG, but it would be secreted into the culture medium. This might give a better understanding of the effects of loss of surface PNAG expression on *eap* and *emp* expression. It might also be interesting to observe the expression of other genes associated with osmotic or environmental stress in the *ica* mutant, such as the osmotically regulated gene *ahpC*, which encodes an alkyl hydroperoxide reductase (Armstrong-Buisseret *et al.,* 1995). If the expression of *ahpC* is also altered in the *ica* mutant it may indicate that the loss of surface expression of PNAG causes osmotic changes within the cell, altering *eap* and *emp* expression. Osmotic changes in cells can be observed microscopically as changes in osmolarity of the surroundings can cause

cells to increase or decrease in volume. Microscopic evaluation of wild type and *ica* mutant cells could confirm whether loss of the polysaccharide was causing such effects to occur. In addition, potassium ions, which are involved in turgor changes induced by changes in osmolarity, can also be measured using flame photometry (Meury *et al.*, 1985).

Biofilm formation in strain 8325-4 was induced in high salt, despite the lack of Eap and Emp surface expression. In addition, Eap and Emp were shown to be reduced in high salt in strain Newman, probably via reduced *sae* expression in high salt. Biofilm formation also remained high in strain Newman even though Eap and Emp were reduced in high salt in strain Newman. Furthermore, repression of biofilm formation in high iron was lost in both strains in high salt. As PNAG expression was not increased in high salt, this suggests that multiple factors can induce biofilm formation under different conditions. It is possible that a proteinaceous factor is responsible for the increase in biofilm formation observed in high salt, as increased salt concentrations have been shown elsewhere to induce the expression of a protein involved in biofilm formation (Lim *et al.*, 2004). To further investigate this, preformed biofilms could be treated with proteinase; if proteins were involved the biofilms should dissociate.

In the 8325-4 *icaR* mutant, over expressing PNAG also resulted in biofilm induction in low iron. In the Newman *icaR* mutant biofilm formation remained high, and, as in high salt, iron regulation was lost. It would be useful to determine if the increase in PNAG expression alone was responsible for the increase in biofilm formation in the *icaR* mutants, or whether it was due to another factor. It is important to remember that

expression of certain factors decreases biofilm formation, for example, SasG and Pls (Roche *et al.*, 2003; Savolainen *et al.*, 2001), which block the interactions of some surface adhesins with their target. It is therefore possible that the increase in biofilm formation observed under conditions of high PNAG expression might be due to the decrease in expression of a factor or factors which block biofilm formation. To further investigate the role of polysaccharides in low iron, low salt biofilm formation, preformed biofilms could be treated with metaperiodate, which degrades polysaccharides thus detaching preformed biofilms that are dependant on polysaccharides for attachment (Kogan *et al.*, 2006). In addition preformed biofilms could be treated with proteinase to determine the role of proteinaceous factors in high PNAG induced biofilm formation as described above.

Recent advances in the study of biofilm production have shown that extracellular DNA (eDNA) is a structural component of biofilms formed by many micro-organisms (Whitchurch *et al.*, 2002; Izano *et al.*, 2008; Vilain *et al.*, 2009), including *S. aureus* (Rice *et al.*, 2007). In *S. aureus* eDNA has been shown to be released via cell lysis. The two component regulator LytSR, regulates the *lrgAB* operon which operates in conjunction with the *cidABC* operon to control peptidoglycan cleavage, which regulates cell growth, division and lysis releasing eDNA (Groicher *et al.*, 2000). In a biofilm assay, both *lytS* and *lrgAB* mutants exhibited increased *in vitro* biofilm formation, which was proposed to be via increased eDNA release (Sharma-Kuinkel *et al.*, 2009; Mann *et al.*, 2009).

Pseudomonas has three interlinking quorum sensing (QS) systems that control the production of eDNA (Allesen-Holm *et al.*, 2006); one of these systems, Pqs, is iron regulated (Bollinger *et al.*, 2001). Therefore, it is possible that eDNA may have a role in iron regulated biofilm formation in *S. aureus* which may involve the quorum sensing system Agr. Simple biofilm assays involving the treatment of preformed biofilms with DNAse would indicate the involvement of eDNA in *S. aureus* low iron biofilm formation. Furthermore, the level of eDNA produced in wild type and *agr* mutant strains could be examined using DNA specific fluorescent dyes which may then be observed microscopically. However the specific regulatory interactions of Agr with eDNA production, if relevant, will require more involved investigation as the genes involved in *S. aureus* eDNA production have not yet been fully characterised (Rice *et al.*, 2007).

In addition to being iron regulated, *eap* and *emp* are also regulated by Sae, Fur, Agr, Hfq and *emp* is also regulated by SarA. Sae regulation has already been demonstrated in rich media and has been confirmed in this work in low iron. Recently the role of Sae in virulence has been confirmed in various animal models and here *sae* has been shown to be repressed in high iron and to be dependant on Fur for expression. The interaction of these two major virulence regulators is important when characterising virulence determinants *in vitro*, this interaction may be missed as the effects of Fur are not routinely examined in rich growth medium. In previous studies Sae has been shown to be expressed at different levels in different strains, this suggests that there may be additional regulators involved in this increasingly complex network which have yet to be identified. It may be that different regulatory patterns influence the expression of

certain genes in response to particular conditions, and unravelling the complex regulatory cascade may explain the huge amount of strain variation in the expression, rather than possession, of some virulence genes and may well be key to developing treatments and in the prevention of *S. aureus* infection.

The observation that *eap* and *emp* are positively regulated by Fur is unusual as Fur normally binds DNA when complexed with iron, negatively regulating gene expression. However in this work Fur positively regulates the expression of *eap* and *emp* in low iron. It is possible that the *S. aureus* iron-free form of Fur can bind DNA either as an activator or as a repressor, as Fur has been shown to directly repress gene expression in low-iron conditions in *Helicobacter pylori* (Delany *et al.*, 2001). Interestingly although the transcription of *sae, eap* and *emp* are all regulated by Fur in this work, there is no evidence of Fur boxes in the promoter regions of these genes. In *Neisseria meningitidis,* Fur binds to operators upstream of the nitric oxide reductase (*norB*) gene promoter, inducing transcription (Delany *et al.*, 2004). Therefore, in *S. aureus,* Fur may bind to regions outside the predicted promoter regions of target genes. Mobility shift assays would be useful for determining specific interactions using promoter sequences and purified Fur protein. Alternatively examples of positive Fur regulation in other organisms have been observed and to be both direct and indirect. Indirect mechanisms have been identified as requiring Hfq dependant sRNAs. The Fur/iron complex binds to the sRNA promoter sequence preventing its expression. In low iron or in the absence of Fur, repression is released and the sRNA is produced, allowing the sRNA/Hfq complex to exert its negative regulatory effects on target genes giving the false impression of positive Fur regulation. However, this does not appear to be the case in this study, as

eap and *emp* expression was reduced in the *hfq* mutant, suggesting an important role for Hfq in the expression of *eap* and *emp*. In addition Hfq has been shown to be required for the expression of *isdA* and *isdB*, and although only a few sRNAs have so far been identified in *S. aureus* it would be interesting to determine in more detail how they and Hfq are involved in *S. aureus* regulation. As the findings of this work suggest that the role of Hfq in *eap, emp, isdA* and *isdB* expression is post transcriptional it would be useful to create transcriptional and translational fusion reporter constructs containing the promoter regions of selected genes fused to a reporter, for example *gfp*. Changes in activity detected from the transcriptional reporter would suggest that the Hfq regulation observed might be indirect via another regulator. However, changes in reporter activity detected from the translational fusion would suggest that the regulatory effects of Hfq were posttranslational, via mRNA stability, or via translational interference, and would therefore require further characterisation. The role of Hfq in *S. aureus* gene regulation is currently the focus of the work of another PhD student in our research group, Emma Tarrant. It is also possible that reduced *eap* and *emp* expression in the *fur* mutant is due to the positive regulatory effects of Fur on *sae* and mobility shift assays using *sae* promoter sequences and purified Fur protein will also help to establish if the interaction between Fur and *sae* is direct.

The work carried out in this study has revealed that, in conjunction with Sae, Agr and SarA, Fur is an important global regulator in *S. aureus*, forming a complicated network controlling the expression of virulence genes. It has also shown that Fur is not the only regulator associated with iron responsive regulation of genes. Fur is a regulator common to many bacterial species and controls the expression of iron responsive genes.

However, it has been seen here that in the absence of Fur there is clear iron regulation of staphylococcal genes including *eap* and *emp*. In the absence of Fur, expression of the genes is de-repressed in high iron. However, repression of *eap* and *emp* in high iron still occurs at the transcriptional level in the absence of Fur. In *Campylobacter jejuni* PerR is responsible for Fur independent iron regulation (van Vliet *et al.*, 1998). A *perR* mutant already exists in *S. aureus* strain 8325-4 which needs to be transduced into strain Newman and into Newman *fur* to investigate how iron regulation of the genes encoding the surface proteins Eap and Emp are affected at the transcriptional level in the absence of PerR and Fur. In addition a DtxR homologue MntR should also be investigated given that it has been implicated as an iron responsive repressor of iron uptake genes in *S. epidermidis* (Hill *et al.*, 1998). Alternatively, should it turn out that PerR and MntR are not responsible for Fur independent iron regulation in *S. aureus,* a transposon library could be created in a Newman *fur* mutant background. However as iron regulation is most apparent at the protein or transcriptional level a suitable screening assay would need to be determined to allow a large throughput of colonies to be assessed for mutants with a defective response to high iron, such as a reporter based transposon library, where a known iron regulated gene such as *eap* could be fused to a reporter such as *gfp,* and changes in expression under high and low iron could be monitored.

Sae expression in this work has been shown to be iron regulated. Newman has previously been described as expressing high levels of Sae *in vitro* (compared to strain 8325-4) (Adhikari and Novick, 2008; Geiger *et al.*, 2008), which is responsible for the increased surface expression of Eap and Emp in strain Newman. Increased Sae expression in strain Newman is reported to be due to an amino acid substitution in SaeS

which results in a loss of sensitivity of the two component regulator to various stimuli (Novick and Jiang, 2003; Geiger *et al.*, 2008). Despite this other strains, including recent clinical isolates, have been shown to express similar *in vitro* levels of Sae as Newman (Steinhuber *et al.*, 2003). Moreover the isolate used in this work contained the amino acid substitution, and responded to various signals such as Agr that Newman had been previously reported as being insensitive to. Although the sequence of SaeS has not been investigated in the clinical isolates, this suggests that the level of expression of Sae witnessed in strain Newman is not as unusual as previously thought and it may be that Sae expression is increased *in vivo* in virulent strains increasing the expression of Eap and Emp. It would be interesting to determine if the over expression of Sae in 8325-4 would compensate for the expression of Eap and Emp, and induce biofilm formation, or whether the expression of other regulators is also different between the two strains. Alternatively the expression of another regulator may also vary between Newman and 8325-4, causing the reduced surface expression of Eap and Emp in 8325-4. Unpublished data obtained in our laboratory indicates that the level of Fur expression varies between different strains *in vitro,* and there is less Fur expression in 8325-4 than in Newman. It is therefore possible that reduced Fur expression might also reduce the expression of Sae dependant factors, and might explain the difference in expression of *eap* and *emp* between the two strains. Virulence of strain Newman has been demonstrated *in vivo* in animal models (Rampone *et al.*, 1996; Goerke *et al.*, 2001; Goerke *et al.*, 2005; Xiong *et al.*, 2006). However 8325-4 was found to be avirulent in a murine foreign body infection model (Luong *et al.*, 2009); it would be worthwhile to determine if this was related to the reduced surface expression of Eap and Emp in 8325-4 in low iron witnessed here, by observing the expression of *eap* and *emp* in 8325-4 and

other strains *in vivo*. A previous study observed the *in vivo* expression of *hla* using a GFP reporter plasmid in a rabbit model of infective endocarditis (Xiong *et al.*, 2006). The same technique could be used to asses the expression of *eap, emp* and other genes such as *sae, fur, agr* and *sarA in vivo.*

The regulator Agr is one of the best studied *S. aureus* regulators, but its role in virulence has been debated in the literature. Here it is shown that in strain Newman, Agr increases the expression of *sae* and represses the transcription of *fur*, two major virulence regulators. The fact that *agr* gene products are iron regulated and that *sae* and *fur* are regulated by Agr further supports a role for Agr in virulence. The fact that Fur and Agr are linked in the *S. aureus* regulatory circuitry may not be surprising. In *Pseudomonas aeruginosa* quorum sensing (QS) has been linked with the iron status of the environment. In conditions of iron scarcity, genes controlled by iron were regulated in favour of the nutritional requirements of the cell, and QS was overridden (Bollinger *et al.*, 2001). This study has found that in low iron Agr positively regulates *fur* in *S. aureus* strain Newman. It would be interesting to investigate the effects of Agr on *fur* in high iron, and to observe the effects of Fur and iron on *agr* expression. Nutrient limitation and QS have also been linked in other bacteria such as in *Bacillus subtilis* where QS and nutritional regulatory circuits have been seen to regulate each other (Lazazzera, 2000). Also in *Ps. aeruginosa,* QS is most active in the stationary phase of growth, where cell densities are high and nutrients are low (Brint and Ohman, 1995). In *S. aureus* the role of the Agr QS system *in vivo* has been debated and it is thought that Agr probably has a role in early colonisation (Traber *et al.*, 2008) and later in the dispersal of biofilms (Yarwood *et al.*, 2004; Boles and Horswill, 2008). If this is the

case, it is likely that the effects of Agr on *fur* may well change with the nutritional status and cell density of the biofilm. It would therefore also be worthwhile to investigate the expression of *agr* in response to iron availability and cell density, which may be achieved in a reporter based assay.

Environmental signals have an effect on gene expression and high salt (4%) has been shown to repress RNAIII transcription in *S. epidermidis* (Stevens *et al.*, 2009). However salt did not appear to affect RNAIII expression in *S. aureus* strain 8325-4 at the concentrations used (1.2%) (Regassa and Betley, 1993), and it would be interesting to observe if higher concentrations of salt affect RNAIII expression in *S. aureus*. As salt represses *sae* at the transcriptional level, and Agr is required for *sae* expression, the affect of salt on *sae* may be indirect and via Agr. Therefore, it would be interesting to investigate the transcriptional affects of high salt on *sae* transcription in an *agr* mutant. Observations have been made by others that strain Newman *sae* is unresponsive to both salt and Agr in nutrient rich media (Novick and Jiang, 2003). If the QS system is overridden in high nutrient media the regulatory effects of Agr on *sae* might be misinterpreted. Therefore it would also be of use to determine if the Agr regulation of *sae* in low iron is via direct binding of AgrA or RNAIII to the promoter regions of *sae*, or whether the regulation is indirect via another regulator. This could be achieved by sequence analysis and by mobility shift assays using purified AgrA protein or by DNA protection assays.

The positive regulatory effects of SarA on Emp expression have been demonstrated in rich media and confirmed here in low iron; however previously SarA was shown to also

activate *eap* expression, whereas here Eap expression was increased in the *sarA* mutant. To further understand the interactions of SarA with *eap* and *emp* it would be useful to confirm the regulatory affects of SarA and Agr on Eap and Emp at the transcriptional level using northern blot analysis, and to examine how the regulators interact with their targets. It would be of interest to establish if direct binding of SarA, AgrA or Fur occurred in *eap, emp, sae* and *fur* where applicable. The availability of the Newman genome sequence makes searching for possible predicted Fur, Agr and SarA binding sites a possibility. Mobility shift assays can also be performed with purified regulator protein as is often used to determine the interactions of Fur with target sequences. In addition DNAse protection assays can be used to asses if promoter regions are bound by protein.

Since the completion of this work micro-array data has confirmed some of the findings of this study relating to gene expression in relation to iron and Fur, using a different low iron growth medium (unpublished data, personal communication, Jayaswal Radheshyam). Micro-array data was collected from low and high iron growth conditions, using *S. aureus* strain SH1000 and its isogenic *fur* mutant. This showed that in the *fur* mutant, transcription of *fur* and *xerD* was increased, further suggesting that Fur is auto-regulated, and that *fur* and *xerD* are iron regulated independently of Fur. Also the microarray data suggests that like *sae* and *fur, agr* is also negatively regulated by iron; in addition *saeS, saeR* and *agr* also appear to be positively regulated by Fur, as in the *fur* mutant *agrA* expression was significantly less than that obtained in the wild type. Decreased expression of other identified Sae regulated genes was also observed in the *fur* mutant, such as the surface associated protein *mapW*, an *eap* homologue, and

haemolysins *hla* and *hlgC*. This further supports the work in this study, increasing the evidence suggesting a pivotal role for *fur* in *S. aureus* virulence regulon.

This work has begun to illustrate how complex the regulatory network in *S. aureus* is and highlights the central role of Fur and Sae. The interaction of these and the regulators SarA and Agr requires further investigation. In *Pseudomonas* nutritional requirements, in particular iron availability, are prioritised, overriding the quorum sensing regulatory circuit (Bollinger *et al.*, 2001). Also Sae has been reported to override other *S. aureus* regulators *in vivo* (Goerke *et al.*, 2005). For example, in a guinea pig model of device related infection, the regulatory effects of σ^B on target genes were overridden by Sae resulting in reduced σ^B activity *in vivo* compared to that *in vitro* (Goerke *et al.*, 2005), suggesting that *S. aureus* regulatory systems have a hierarchy which can vary depending on different environmental cues. The discovery that *S. aureus* biofilm formation is iron regulated *in vitro*, in addition to the finding that factors associated with virulence *in vivo* are also iron regulated may be important in the therapeutic treatment of *S. aureus* infections. Although iron itself may not be suitable as a therapeutic agent due to its toxic effects on mammalian cells and due to that fact that higher than physiological concentrations facilitate microbial growth, it is possible that iron regulated genes could be targeted. Small interfering (siRNAs) are short double stranded RNA molecules of approximately 21-22 nucleotides in length that interfere with, or silence mRNA transcripts, quenching the gene output (Yanagihara *et al.*, 2006). Regulation by siRNAs is specific and relies on antisense binding of the siRNA with the target mRNA. The double stranded siRNA binds to a protein complex, which uses one strand as a template to locate target mRNAs with homologous sequence to the siRNA

by an unknown mechanism, targeting it for degradation. Although most bacteria possess RNAse III, a potent enzyme which degrades short sequences of RNA (Blaszczyk *et al.*, 2001), this type of interference has been proposed as a therapeutic agent for cancer, viral infections and neurological disorders (Ryther *et al.*, 2005). It has also been suggested as a possible therapeutic agent for the treatment of MRSA via the inhibition of coagulase production (Yanagihara *et al.*, 2006). It is possible that the identification of other virulence genes associated with disease such as *eap, emp* or important regulators such as Sae or Fur may be used as a target in a similar way.

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