INVESTIGATION OF A NOVEL IRON-UPTAKE SYSTEM AND OTHER GENOMIC FEATURES IN *mecC STAPHYLOCOCCUS AUREUS*

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DECLARATION

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is a significant pathogen that causes a wide variety of disease in humans and animals. Methicillin resistant *S. aureus* (MRSA) isolates carrying *mecC,* the gene that confers resistance to the antibiotic, have been isolated from humans but also from diverse animal species covering livestock, domestic and wild animals throughout Europe. Many of the known MRSA *mecC* isolates have been whole-genome sequenced by our group to gain insight into the evolution and epidemiology of these emerging lineages.

For microbes and humans alike, iron is an essential cofactor in many biochemical reactions and *S. aureus* requires iron for colonisation and subsequent pathogenesis. The success of *S. aureus* is partly attributed to its ability to exploit the host iron pool. It does this through multiple iron uptake mechanisms, including at least two high-affinity iron scavenging siderophores (staphyloferrins A and B) and an iron-regulated surface determinant (Isd) pathway for haem-iron acquisition.

Here I describe the identification of a novel locus encoding a siderophore-like non-ribosomal peptide synthetase (NRPS), directly downstream of the *SCCmec* insertion site in *mecC S. aureus* isolates. A homologous region was identified in *Streptococcus equi* 4047 (*S. equi)* which encodes a NRPS termed 'equibactin' that is involved in iron acquisition. I have therefore named the NRPS product 'staphylobactin' in MRSA, and the aim of this study was to determine the function of the staphylobactin biosynthesis cluster: is this region involved in iron acquisition and how might it be regulated?

Analysis of the prevalence of isolates containing the staphylobactin locus showed it to be present in a large number of *mecC* strains in our collection but also identified homologues in other staphylococcus isolates. The region is highly conserved in all *S. aureus* isolates belonging to clonal complex (CC) 130 (broad host range lineage), suggesting that staphylobactin might impact on *S. aureus*'s ability to infect a broad range of host species.

The staphylobactin gene cluster contains 14 coding sequences, *stbB-F, F1, G-M* and *O*. Bioinformatic analysis results in predictions of domain and gene functions associated with iron acquisition. I hypothesized that staphylobactin might have been acquired to

compensate for the lack of another siderophore, such as staphyloferrin B, but the staphyloferrin B biosynthesis cluster and transport is present in nearly all *S. aureus* strains, ruling out this model. Unlike the equibactin locus, however, the staphylobactin locus lacks a homolog for the iron-dependent regulator *eqbA*. Instead, expression of this locus appears to be regulated by MntR, a DtxR-like regulator. The staplylobactin gene cluster is flanked by direct repeats which suggest staphylobactin could have been gained by horizontal gene transfer.

In order to study the role of the staphylobactin gene cluster, deletion mutants of MntR, the staphylobactin locus and staphyloferrins A and B, were generated using the pIMAY two step gene deletion procedure in the previously un-manipulated *mecC S. aureus* CC130 strains – a challenging protocol that required significant optimization given the difficulties with manipulating this bacterium. Analysis of the MntR mutant suggests that the staphylobactin operon is regulated by MntR, acting as a positive regulator, in an iron-dependent manner. By RT-PCR, I found that expression of the staphylobactin NRPS genes is increased when cultures are grown in the absence of iron, suggesting an involvement with iron acquisition. Genomic inactivation of the staphyloferrins resulted in a mutant severely incapacitated for growth in serum and transferrin as the sole iron source, and addition of iron reversed this phenotype. However, deletion of staphylobactin alone or in addition to the staphyloferrins, lacked an iron-dependent growth defect, and numerous assays failed to identify a clear role for staphylobactin in iron metabolism. Therefore, further experiments are needed to elucidate the function of this siderophore like NRPS.

Analysis of the same sequenced CC130 *mecC* isolates from our strain collection in which the staphylobactin locus was found, led to the identification of a novel Von Willebrand (*vwb)* gene. In order to investigate possible reasons for these isolates to infect a wide range of host species, wild-type and *vwb* deletion mutant strains, along with the novel *vwb* expressed in *lactococcus*, were tested using a coagulation assay and were able to clot plasma from a broad range of host species. Thus the specificity of vWbp proteins can be used to infer the host specificity and evolutionary history of the *S. aureus* strains that harbour them.

Although I was unable to generate definitive evidence revealing the biological role for the staphylobactin locus this study has generated valuable tools for further studies and thoroughly tested a number of hypotheses concerning its role in cation metabolism.

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1 INTRODUCTION

1.1 *Staphylococcus aureus*

1.1.1 *Staphylococcus aureus* infections

Staphylococcus aureus (*S. aureus*) is a Gram-positive, non-motile coccal bacterial pathogen. A common human commensal, it is often found as a part of the human skin microbiota and on mucous membranes. It colonises the nares of nearly a third of the world's population (1-4). Most *S. aureus* infections cause skin and soft tissue infections, including furunculosis (5), impetigo (6), cellulitis (7), mastitis (8) and flesh eating disease (9, 10). It is the most common cause of hospital infections affecting the treatment of patients, especially in individuals suffering from chronic illness, traumatic injury, burns or immunosuppression (11, 12). Breach of skin and mucosal membranes can cause bacteraemia, toxic shock (13), pneumonia (14), deep abscesses and septic shock (15) and sepsis, which can lead to osteomyelitis (16), septic arthritis (17), and endocarditis (18, 19). *S. aureus* is also a common cause of infections associated with indwelling devices such as joint prostheses, cardiovascular devices and artificial heart valves. Invasive *S. aureus* infections are a source of considerable morbidity and mortality (20). *S. aureus* colonises and sometimes causes infections in a wide variety of animals, including household pets, farm animals and wild animal species, and can be transmitted among diverse species. However, in general isolates obtained from different species differ at the genome level, suggesting the existence of specific host genotypes (21). Since *S. aureus* possesses a highly conserved core genome, host specific adaptions are likely to be carried by mobile genetic elements (MGEs). This implies that the organism may switch host specificity the acquisition (or loss) of one or more such elements. In dairy farms, *S. aureus* is among the most common causes of clinical and subclinical mastitis, which is a disease of considerable economic importance (22).

1.1.2 Antibiotic resistance

When Penicillin was introduced in 1941, over 94% of *S. aureus* isolates were susceptible to it (23). However, penicillin resistant strains were soon reported and an alternative therapy was required (23). Although initially prevalent only in hospitals, penicillin resistance is now present in over 90% of community isolates.

1.1.2.1 Penicillin resistance

Initial *S. aureus* resistance to penicillin was mediated by a plasmid-encoded penicillinase (β-lactamase) that cleaves the β-lactam ring of penicillin, rendering the antibiotic ineffective. This led to the search for β-lactamase-resistant agents, which resulted in the synthesis of the semisynthetic antistaphylococcal penicillins, beginning with methicillin and subsequently with methicillin derivatives such as oxacillin, cloxacillin, dicloxacillin, flucloxacillin and nafcillin.

1.1.2.2 Methicillin resistance

Methicillin, a penicillinase stable β-lactam, was first used clinically in 1959. However, only 2 years later, Jevons *et al*. first described methicillin-resistant *S. aureus* (MRSA) (24). Resistance to methicillin and other β-lactam antibiotics is conferred by the *mecA* gene (25). *mecA* encodes an alternative penicillin-binding protein, PBP2a or PBP2', that differs from other penicillin-binding proteins by an active site having a decreased affinity for binding β-lactams such as penicillins, cephalosporins, and carbapenems (26-28). As such, PBP2a can continue to catalyse the transpeptidation reaction required for peptidoglycan cross-linking, enabling cell wall synthesis in the presence of penicillins (29). Therefore, acquisition of *mecA* allows for resistance to all β-lactam antibiotics, and obviates their clinical value for treating MRSA infections (30).

1.1.3 Staphylococcal Cassette Chromosome (SCC*mec*)

The *mecA* gene is carried on a mobile genetic element called the staphylococcal cassette chromosome (SCC) that is a member of a broader SCC family of elements. SCC*mec* also contains the genes *ccrA* and *ccrB*, which encode the recombinases that mediate the site-specific integration and excision of the SCC*mec* element from the *S. aureus* chromosome (31, 32). There are 11 variants of SCC*mec* (types I to XI) currently defined, ranging in size from 21-67 kb and distinguished by variation in *mec* and *ccr* gene complexes (33). Chromosomal insertion is at the same position in all strains, the integration site sequence (ISS) within an open reading frame (ORF) designated *orfX* in the *S. aureus* chromosome (34). Different SCC*mec* types confer different phenotypic characteristics, such as antimicrobial resistance rates (35). Different SCC*mec* types are also associated

with strains causing different epidemiological patterns of infections. Typically, hospital acquired MRSA (HA-MRSA) carry SCC*mec* types I-III, which are large elements including multiple resistance determinants. Community-associated MRSA (CA-MRSA) is associated with SCC*mec* types IV and V, which are smaller and carry fewer resistance elements than those seen in hospital-associated MRSA strains. The smaller size of SCC*mec* type IV has been suggested to promote its mobility; in support of this proposal, SCC*mec*IV has been inserted into multiple lineages of *S. aureus* whereas SCC*mec*II and SCC*mec*III have only been found in three and two lineages, respectively (36).

1.1.4 Detection and molecular typing of MRSA

Most isolates of MRSA in the UK that have been cultured from blood, infected wound sites and body fluids, are identified by antimicrobial susceptibility testing, by measuring zones of growth inhibition around antibiotic-impregnated discs on agar plates, or through measurement of minimum inhibitory concentrations (MIC). Detection of the *mecA* gene by PCR (37, 38) or of PBP2a in a slide agglutination assay (39) can be used to confirm MRSA when phenotypic results are borderline. These two assays are regarded as the gold standard for the detection of MRSA. In a clinical setting, the use of automated antibiotic susceptibility testing, such as Vitek (40), are becoming more commonly used than MIC.

In *S. aureus*, commonly used molecular typing methods include pulsed-field gel electrophoresis (PFGE) (41), *spa* typing (42) and multilocus sequence typing (MLST) (43). For MRSA, Staphylococcal Cassette Chromosome *mec* (SCC*mec*) analysis is also widely being carried out. MLST is a highly discriminatory method of characterising isolates on the basis of the nucleotide sequence of internal fragments of seven housekeeping genes (44). The sequences of each gene fragment are assigned as distinct alleles, and the sequence type (ST) or allelic profile of each isolate is defined by the alleles at each of the seven housekeeping loci. Isolates with the same allelic profile for five or six out of seven genes are assigned as members of the same clonal complex (CC) (45). MLST can be used to study the evolution and population biology of *S. aureus*.

1.1.5 Discovery of a novel *mecA* homologue *mecC*

In 2011, Garcia-Alvarez *et al.* discovered MRSA with a novel *mecA* homologue in human and bovine populations in the United Kingdom and Denmark (46). The original strain (LGA251) was isolated from bulk milk in England and was phenotypically resistant to β-lactams but tested negative by PCR for the *mecA* gene and by PBP2a slide agglutination tests. This isolate was genome sequenced to find the genetic basis of the methicillin resistance. A divergent *mecA* homologue: *mecC* (previously known as *mecA*LGA251 (47)) was discovered in a novel SCC*mec* element, designated SCC*mec* type XI. Published at the same time, work in the Republic of Ireland independently described *mecC* in human MRSA strains isolated in 2010 (48). *mecC* exhibits 69% nucleotide identity with the conventional *mecA* gene. The PBP2a protein encoded by *mecC* has distinct biochemical properties in comparison to mecA-PBP2a, showing a higher relative affinity for oxacillin compared to cefoxitin and being unstable at 37 °C (49). Since the initial discovery of this *mecC*, it has been reported in several European countries (50) including Denmark (51-53), France (54), Spain (55-57), Sweden (58), Austria (59), Finland (60), The Netherlands, Ireland, Germany (61-63), Belgium (64), Slovenia (65), Switzerland (66), Norway and the United Kingdom (67, 68), showing a wide geographical spread of these isolates. MRSA harbouring *mecC* has been reported from humans but also from diverse animal species including livestock, domestic, and wild animals (69). Host species include dogs, cats (70, 71), rat, rabbit, seal (72), wood mice (57), cattle (73-77), hedgehogs (78), small ruminants (53, 79), guinea pig and chaffinch (72, 80, 81) hares, otters, fox, deer (82, 83) and mara (84). *mecC* isolates have been found belonging to clonal complexes (CCs) 49, 130, 425, 599 and 1943, with the majority belonging to CC130. This is interesting as different *S. aureus* lineages normally show selective affinity towards specific animal hosts. However, the broad host spectrum, including a list of wild species supports the idea that *mecC* MRSA is a generalist lineage. The initial discovery and subsequent studies have demonstrated that *mecC* MRSA is frequently found in dairy cattle, suggesting that cows might provide a reservoir of infection (67). Evidence of transmission of *mecC* MRSA from livestock to humans has been provided by whole genome sequencing (WGS) in two cases in Denmark, showing that *mecC*-MRSA has the potential for zoonotic transmission (51, 85, 86). Gomez *et al*. (82) found *mecC*-MRSA in red deer in Southern Spain,

suggesting that red deer could be acting as *mecC*-MRSA hosts and could share these genes with other animals when they coexist in the same habitat. In Sweden, Unnerstad *et al.* (58) reported *mecC*-MRSA in domestic animals suggesting them as potential reservoirs with associated risks to humans. When wildlife and livestock share the same habitat, interspecies transmission can occur, either directly or indirectly. A mara in Copenhagen zoo was found to be carrying *mecC*-MRSA; it was suggested that the possible transmission route is likely to have involved either zoo personnel or wildlife such as birds and small rodents, both of which have been shown to carry *mecC*-MRSA (57, 72). In humans, *mecC*-MRSA prevalence is uncommon. It has been mostly found in skin and soft tissue infections (73), but has been responsible for fatal bacteraemia (55) and osteomyelitis (87). In Denmark, the number of *mecC*-positive MRSA cases in humans (including colonisation and infection) increased from 9 in 2009 to 24 in 2012. However, none of the 24 human cases registered reported any contact with livestock (81), supporting previous evidence that this lineage is able to transmit between humans (55). In Germany, only two *mecC*-MRSA isolates were found among 3207 MRSA isolates and there was no change in prevalence between 2004-2011 (61, 62). In the UK, 0.45% of human MRSA isolates were found to carry *mecC* in 2011 (88). Meta-analysis carried out in 2016 showed that the overall pooled prevalence of *mecC* was 0.009% (89). The estimated prevalence in the human subgroup was 0.004%, as compared to 0.10% in the animal subgroup. Studies have also reported isolation of *mecC*-MRSA from river water and urban waste water (56, 90). These findings highlight the potential role of environmental dissemination of *mecC*-MRSA in surface water but the origin, epidemiology and impact of *mecC* is still unclear. Recently, a *Staphylococcus xylosus* isolate was found to harbour a new allotype of the *mecC* gene, *mecC1*. The *mecC1* gene has 93.5% nucleotide identity to the *mecC* gene in *S. aureus* LGA251. WGS revealed that *mecC1* forms part of a class E *mec* complex (*mecI-mecR1-mecC1-blaZ*) located at the *orfX* locus as part of a likely SCC*mec* remnant, which also contains a number of other genes present on the type XI SCC*mec* (91). Also recently, *mecC* genes have been found in other coagulase-negative *Staphylococcus* species (92) including *Staphylococcus stepanovicii* (83), *Staphylococcus sciuri* (93) and *Staphylococcus saprophyticus* (94, 95) isolates

suggesting that coagulase-negative staphylococci (CoNS) may have provided the original source of *mecC* in *S. aureus*.

1.1.6 Host cell interactions of *S. aureus*

Following infection with *S. aureus*, a large inflammatory response occurs. The host's innate immune response is the recruitment and activation of neutrophils through induction of cytokines, chemokines, and chemokine receptors (96, 97). These neutrophils can phagocytose opsonised bacteria and trigger oxidative killing and other antimicrobial mechanisms such as NETs (neutrophil extracellular traps) (98, 99). To counter neutrophil attack, *S. aureus* produces factors to subvert the immune system, via the secretion of staphylococcal superantigen-like 5 and 10 (SSL5 and SL10), extracellular adherence protein (Eap) (100) and the chemotaxis inhibitory protein of *S. aureus* (CHIPS) (101-103). *S. aureus* secretes nuclease to degrade NETs, allowing it to escape entrapment, but also to cause death of immune cells (104).

Neutrophil phagocytosis and bacterial clearance are enabled when microbes are opsonised by components of the complement system or immunoglobulins (Igs) (105). Compliment can quickly recognise and opsonise bacteria directly or kill bacteria by the formation of membrane attack complexes (106). *S. aureus* reduces or delays opsonisation by targeting complement activation via staphylococcal complement inhibitor (SCIN) and extracellular complement binding protein (105). *S. aureus* secretes aureolysin which recruits complement inhibitory factors and clumping factor A (ClfA) (107). To prevent opsonisation by Ig, *S. aureus* evolved Ig-binding factors, such as staphylococcal protein A (SpA) and staphylococcal binder of Ig (108).

S. aureus produces cytolytic toxins, including Panton-Valentine leukocidin (PVL) (109), α-haemolysin (110) and phenol-soluble modulins (PSMs) (111). These are pore-forming toxins that can lyse host cells (103), converting local host tissue into nutrients required for bacterial growth (112) or destroying immune cells. *S. aureus* uses α-haemolysin to lyse erythrocytes and release haemoproteins that are a source of iron. This allows staphylococcal iron acquisition systems, such as siderophores and haem transports, to overcome the host attempts to starve it of this essential nutrients.

Once phagocytosed by neutrophils, monocytes and macrophages, *S. aureus* are able to survive and proliferate before lysing the cell and escaping back into surrounding tissue (113). To survive within neutrophils, *S. aureus* produces factors such as staphyloxanthin (114) and superoxide dismutase that inhibit reactive oxygen species (ROS)-mediated killing.

1.1.6.1 Aggregation and coagulation mechanisms

Following entry into the blood stream of infected hosts, *S. aureus* disseminates into tissues and seeds abscesses. Staphylococci replicate as a bacterial community at the centre of these abscesses, protected by an inner pseudocapsule and an extended outer dense microcolony-associated meshwork (MAM) containing fibrin. The bacteria produce a number of factors that target coagulation as an immune evasive strategy. They do this by secreting two proteins that promote coagulation, coagulase (Coa) and von Willebrand factor-binding protein (vWbp) (115). Both Coa and vWbp interact with prothrombin, causing the conversion of fibrinogen to fibrin promoting the clotting of plasma (116, 117) (Figure 1.1). Both Coa and vWbp are required for the formation of abscesses and bacterial persistence in host tissues (118). Coa is involved in formation of pseudocapsule whereas MAM formation depends on vWbp. This clotting may help protect invading *S. aureus* from the host immune defenses, such as opsonisation or phagocytosis (119).

Figure 1.1 *S. aureus* produces coagulase (Coa) and von Willebrand factor-binding protein (vWbp) to clot blood.

vWbp binds to prothrombin causing its activation and leading to the conversion of fibrinogen to fibrin, causing the clotting of blood, which probably help protect the invading *S. aureus* from phagocytosis. Image adapted from Crosby *et al.* Adv Appl Microbiol. 2016 (120).

Coagulase is secreted by nearly all *S. aureus* strains and coagulates blood and plasma from various animals (121). *S. aureus* also produces clumping factor A (ClfA), which binds fibrinogen and contributes to platelet aggregation via a fibrinogen or complement dependent mechanism (122). In addition, vWbp binds von Willebrand factor (vWf) (123), a blood glycoprotein that is upregulated on host platelets and endothelial cells during haemostasis to promote blood coagulation (124). The ability of vWbp to bind this glycoprotein may have a role in localising prothrombin activation and therefore clot formation in the vicinity of the bacterium. The interaction between vWbp and vWf is highly specific and is mediated by a region of 26 amino acid residues in the C-terminal part of vWbp (123). When establishing endovascular infections, *S. aureus* adheres to the vessel wall under shear forces of flowing blood by vWbp-mediated binding to vWf (125, 126). The secreted vWbp interacts with vWf and ClfA (a Sortase A-dependent staphylococcal surface protein) to form a complex that anchors *S. aureus* to vascular endothelium under shear stress (127).

In order to disseminate to organ tissue and seed abscess lesions, *S. aureus* needs to leave the bloodstream. *S. aureus* induces its uptake by non-phagocytic cells, such as epithelial and endothelial cells (128) by the production of adhesins that enable the attachment to the host cell surface. The major adhesins are fibronectin-binding proteins (Fnbps), collagen-binding protein, and fibrinogen binding proteins (ClfA and ClfB) (129). These factors belong to a major class of *S. aureus* adhesins, termed MSCRAMMs (microbial surface component recognising adhesive matrix molecules), which are covalently anchored to cell peptidoglycans and recognise the most prominent components of the extracellular matrix or blood plasma, including fibrinogen, fibronectin, and collagens (12).

1.1.6.2 Regulation of virulence factors

The ability of *S. aureus* to cause a variety of infections and its dynamic adaption to extracellular and intracellular host niches is dependent on the timely and coordinated expression of virulence factors by the pathogen. *S. aureus* coordinates expression of virulence genes mainly by two regulatory mechanisms, dependent on genes encoded within the *agr* (accessory gene regulator) locus and on the *sarA* (staphylococcal accessory regulator) gene (130-132). Additional mechanisms also fine tune expression of virulence factors. The two component regulator SaeRS is responsive to stimuli associated with phagocytic immune cells, such as hydrogen peroxide (133), leading to expression of secreted toxins and capsule (134).

Iron limitation is also a key sensory trigger for the expression of staphylococcal virulence factors. Ferric uptake regulator (Fur) bound Fe^{2+} indirectly contributes to coordinated repression of secreted hemolysins and cytotoxins, and under sustained conditions of iron limitation upregulation of these factors occurs. On the other hand, Fur-Fe^{2+} contributes to positive regulation of immunomodulatory proteins, including superantigens, protein A, complement inhibitory protein, and chemotaxis inhibitor (135). A *fur* inactivated mutant was shown to be less virulent in a murine pneumonia model of infection (135), suggesting that in the absence of this key regulatory protein, virulence is compromised due to improved clearance by immune cells despite the enhanced cytotoxicity of the mutant.

1.2 Iron

Iron is an essential nutrient for all microbial pathogens, with the exception of *Borrelia burgdorferi* and *Treponema pallidum*. In *S. aureus*, metal ions, such as iron, participate in diverse biochemical processes such as DNA synthesis, regulation of virulence factor expression, and defence against oxidative stress.

The utility of iron in biological processes is due to its ability to participate in single electron transfers to interconvert between ferrous (Fe^{3+}) and ferric (Fe^{2+}) redox states. However, through this activity, excess or 'free' iron is capable of generating highly toxic reactive oxygen species (ROS) that can compromise cellular integrity through damage to lipids, proteins, and nucleic acids. Therefore, a delicate balance exists in *S. aureus* between the acquisition of iron for essential cellular processes and the sequestration of iron to avoid toxicity. The ability to acquire iron is required in *S. aureus* for colonization and disease pathogenesis.

1.2.1 Nutritional Immunity

As part of their innate immune response to bacterial infection, vertebrates have evolved sophisticated systems that sequester excess iron intracellularly to restrict the amount of free iron available for invading pathogens. This process is referred to as nutritional immunity (136) and extends to other transition metals, including zinc and manganese (137).

1.2.1.1 Transferrin and Lactoferrin

Extracellular iron is rapidly sequestered by high affinity iron binding proteins such as transferrin in serum which binds free iron (136). In addition to binding free iron, transferrin functions as an iron transport protein, moving iron to peripheral tissues through receptor-mediated endocytosis of transferrin receptor 1 (TfR1) upon complexation with holo-transferrin (138). Iron levels are also limited in lymph and mucosal secretions through lactoferrin, which quickly binds all free iron. Additionally, lactoferrin is a major antimicrobial constituent of lysosomes within phagocytes, ensuring that engulfed pathogens have limited access to intracellular iron.

1.2.1.2 Haem and Haem carrier proteins

The majority of iron within vertebrates is complexed into the tetrapyrrole ring of haem, and is bound within haemoproteins such as haemoglobin, which contains around two-thirds of the total body reservoir (69). To further prevent pathogen access to iron, haemoglobin is sequestered intracellularly within erythrocytes. More than 90% of iron within the human body is located intracellularly making it inaccessible to extracellular pathogens unless mechanisms are employed to liberate these rich sources of nutrient iron (139). *S. aureus* and many other bacterial pathogens lyse erythrocytes to release haemoglobin; however the host mitigates this through the action of the high-affinity haemoglobin binding protein haptoglobin (88). Haptoglobin is abundant in serum and this is increased during inflammation (140). The haemoglobin-haptoglobin interaction is one of the strongest noncovalent interactions reported in serum, ensuring that the complex does not dissociate until the proteins and haem are recycled in the liver (141). Lysed erythrocytes also release free haem which is then bound by haemopexin with high affinity and taken to the liver where it is endocytosed by hepatocytes and cleared from the serum (142).

1.2.1.3 Ferritin

Ferritin is an important component of iron homeostasis in the body, specifically in regards to storage of surplus iron in a soluble and nontoxic form inside cells (143, 144). In animals, ferritin is most abundant in liver hepatocytes, which act as the body's primary iron reserve (145). Release of ferritin into serum may aid in iron delivery from the liver to other tissues (146, 147). More importantly, elevated serum ferritin serves to reduce iron overload caused by hemochromatosis or some cancers (148).

1.2.2 Iron homeostasis

Iron homeostasis in the host is strictly regulated to make sure that iron is available for essential biochemical reactions while preventing iron-associated toxicity and bacterial growth. Iron toxicity results from the accumulation of excess iron that surpasses the binding capacity of transferrin causing non-bound iron to catalyse the production of highly reactive oxygen species (ROS). This leads to damage of the liver, heart and

endocrine organs as well as potentially providing invading pathogens with a growth-essential nutrient. The importance of iron homeostasis in health and disease is made clear when it is disrupted. For example, disruption of iron homeostasis can lead to increased severity of *S. aureus* infections (149). Iron overload, as manifested in haemochromatosis (caused by a mutation within the *HFE* gene leading to excessive iron in the liver and tissues) (150) or β-thalassaemia (caused by a mutation in the β globin gene that affects haemoglobin synthesis (151)), results in tissue damage and organ dysfunction such as liver failure and cardiovascular disease, causing increased risk of bacterial and viral infections (152, 153). Liver transplantations can also result in iron overload (139). The mortality rate in liver transplant patients that suffer from *S. aureus* bacteraemia is over 80% (154). Iron deficiency, encountered in patients with chronic inflammation or inadequate dietary intake, results in ineffective erythropoiesis (155).

In order to overcome nutritional immunity, *S. aureus* has evolved specialised systems for the acquisition, processing and detoxification of iron and iron containing host molecules and proteins.

1.3 Bacterial strategies for acquisition of host iron

1.3.1 *S. aureus* response to iron starvation

Iron is a cofactor of numerous proteins involved in central metabolism and respiration. *S. aureus* responds to iron limitation by reduced expression of non-essential iron-containing pathways, including the tricarboxylic acid (TCA) cycle, while also upregulating glycolytic and fermentative metabolism (156).

1.3.2 The ferric uptake regulator response to iron limitation

S. aureus responds to the iron-restricted environment of the host by dramatically altering its genetic expression of iron uptake mechanisms and virulence factors that play roles in liberating host iron stores or circumventing host immune strategies. This change is mediated, in large part, by the iron-dependent Fur (156). Fur is an iron-dependent transcriptional regulator that is conserved among Gram-positive and Gram-negative bacteria (157). The genes involved in both siderophore-mediated iron scavenging and

the haem-iron acquisition systems are under the control of this transcriptional repressor. In the presence of iron, Fur binds a consensus DNA sequence known as the Fur box, upstream of Fur regulated genes. This blocks RNA polymerase, leading to transcriptional repression.

In iron-deplete conditions, Fe^{2+} dissociates from Fur-Fe complexes, Fur is released from Fur boxes, and transcription of Fur regulated genes can proceed (158). In this way, *S. aureus* can initiate a transcriptional program based on the amount of iron available in the environment (159). Fur also regulates the expression of virulence factors involved in attachment to host cells, biofilm formation, and manipulation of host wound healing (160-162). It additionally controls the expression of secreted cytolytic and immunomodulatory toxins such as haemolysins that increase erythrocyte lysis, and regulates hydrolases (for example cytolysins and proteases) that modulate the host immune response such as chemotaxis inhibitory protein (CHIP). This plays an important role in decreasing the host immune response and promoting bacterial survival (163). Inactivation of *fur* and the peroxide stress regulator (*perR)*, a homologue of *fur*, leads to a reduction in virulence in a mouse model of *S. aureus* infection (164) thus emphasising the importance of Fur in *S. aureus* pathogenesis.

1.3.3 Alternate examples of regulatory mechanisms contributing to iron homeostasis

Some organisms contain multiple *fur* homologues, including *Bacillus subtilis* that encodes three *fur* homologues (*fur*, *perR* and *zur*) (165) and a homologue of the *Corynebacterium diphtheriae* iron-regulator *dtxR* (*mntR*). *B. subtilis* Fur binds Fe^{2+} and regulates the expression of iron-uptake genes (166). PerR regulates the peroxide regulon and binds to Mn^{2+} or Fe^{2+} (166), while Zur down-regulates two Zn^{2+} uptake pathways in the presence of Zn^{2+} (167). MntR binds Mn^{2+} and regulates Mn^{2+} transport (168). The MntABC system competes with calprotectin for manganese. The transcriptional repressors MntR and Zur allow *S. aureus* to respond to manganese-deplete and zinc-deplete environments, and regulate the expression of high affinity metal transporters. Like iron, free Zn^{2+} is found in low concentrations *in vivo*. *S. aureus* has ORFs corresponding to *fur*, *perR*, *zur* and *dtxR* which are predicted to be involved in

the regulation of these iron responses, and in the regulation responses to other metal ions (169-171).

1.3.4 Direct uptake of Fe^{3+} from transferrin

Some bacteria secure transferrin and lactoferrin iron through direct binding and manipulation of these serum proteins using cell surface receptors.

1.3.5 Indirect uptake of Fe^{3+} from glycoproteins: siderophores

Siderophores are small, high affinity, ferric iron-scavenging molecules produced and secreted by numerous bacteria, fungi, and plants in response to iron deprivation. The affinity of siderophores for ferric iron affords them the capacity to scavenge residual free iron from the environment, as well as steal it from host glycoproteins, such as transferrin and lactoferrin. Broadly, siderophores are classified based on functional groups involved in iron coordination, and include catecholate, hydroxamate and α-hydroxycarboxylate types, as well as 'mixed type' siderophores when more than one type of coordinating moiety is employed.

1.3.6 Indirect uptake of Fe^{3+} from transferrin: catecholamine stress hormones

The low affinity of transferrin and lactoferrin for Fe^{2+} means their function may be compromised by interaction with agents that reduce Fe^{3+}. Studies have shown that the catecholamine stress hormones, including adrenaline (epinephrine), noradrenaline (norepinephrine), dopamine, and L-3,4-dihydroxyphenylalanine (L-DOPA), are capable of forming direct complexes with holotransferrin and hololactoferrin, and reductively liberating iron as Fe^{2+} (172, 173). Catecholamine stress hormones may also act as biological chelators that compete with transferrin.

1.3.7 Uptake of Fe^{2+}

Bacteria also have mechanisms to transport the low levels of ferrous (Fe^{2+}) iron that may be available in their environments. Transport of Fe^{2+} is an iron uptake strategy for many bacteria, which involves the Feo (ferrous iron) transport system. First characterised in *E.*

coli (174, 175), this comprises of two or three proteins: FeoB (175, 176), FeoA and FeoC
(177). FeoB-mediated iron uptake from the gastrointestinal tract contributes to
colonization by and pathogenesis of *Campylobacter jejuni* (178) and *Helicobacter pylori*
(179). FeoB also contributes to intracellular growth of *Legionella pneumophila* (180).

1.4 Siderophores

1.4.1 Biosynthesis of siderophores

Siderophores are assembled from amino acids, organic acids, and other small organic
metabolites, and their biosynthesis usually occurs through one of two modes of
assembly, characterised by the requirement, or lack thereof, for large multifunctional
enzyme assembly lines known as nonribosomal peptide synthetases (NRPS) (181).

1.4.1.1 Nonribosomal peptide synthetase assembly

The majority of siderophores classes are synthesised by NRPS-based siderophore
synthesis. This involves the activation and incorporation of non-proteinogenic amino
acids into an elongating chain, in the absence of an RNA template (182). Independently
of a ribosome, NRPS siderophores are condensed and modified stepwise on a
multimodular enzyme, the NRP synthetase, for which the roles of functional domains
can be summarized as follows (183):

1) Activation of an amino acid or growing intermediate aminoacyl-AMP by adenylation
(A) domains;

2) Transfer to peptidyl carrier protein (PCP) domains;

3) Condensation of PCP-bound amino acids by condensation domains;

4) Amino acid modifications, for example by epimerization domains;

5) Transesterification (TE) of the peptide chain from the terminal PCP onto the TE
domain, with subsequent liberation of the mature metabolite through hydrolysis or
macrocyclization.

The modular arrangement of the NRPS synthetase essentially creates a protein assembly
line, allowing metabolite assembly and modification to occur in a progressive, stepwise

fashion. The coordinated biosynthesis of NRPSs (involved with siderophore synthesis) is activated by promoters which are sensitive to iron depletion. The NRPS genes for a certain peptide are usually organised in one operon.

In addition to the synthesis of numerous macrocyclic, or 'aryl capped', peptide siderophores (206), including enterobactin (184), yersiniabactin (185), and vibriobactin (186), NRPS enzymes also produce antimicrobial peptides (187) and peptide-based antibiotics such as penicillin (188), daptomycin (189), and vancomycin (190).

1.4.1.2 Nonribosomal peptide synthetase independent assembly

In contrast, NRPS-independent siderophore (NIS) occurs through condensation of alternating amino alcohols, alcohols, dicarboxylic acids, and diamines through synthetase enzymes that catalyse amide and ester bond formation between these siderophore subunits (191). The majority of carboxylate and α-hydroxycarboxylate type siderophores are synthesised using NIS-type pathways, including alcaligin (192), rhizobactin (193) and vibrioferrin (194). Notably, petrobactin, which is produced by pathogenic *Bacillus* spp., is synthesized via a NIS/NRPS hybrid system (195, 196).

S. aureus is known to produce two α-hydroxycarboxylate-type siderophores; staphyloferrin A (197), and staphyloferrin B (198). They scavenge soluble ferric iron or compete for extracellular iron with host iron-binding proteins such as transferrin and lactoferrin. Both siderophores are synthesised by the nonribosomal peptide synthetase independent pathway (NIS). Biosynthesis and import of staphyloferrin A and staphyloferrin B are regulated by Fur in response to environmental iron concentrations (199, 200). An additional putative *S. aureus* siderophore, aureochelin, has been found to be produced in iron-restricted medium, but has not been fully characterised (201). Studies of culture collections have so far demonstrated that staphylococcal isolates do not produce two other classes of siderophore molecules, namely hydroxamates and catecholates (201-203). Bioinformatic analysis uncovered a NRPS pathway (gene *aus*) encoded within all sequenced genomes. The NRPS synthetase has been characterised and does not produce an iron-binding metabolite or antibiotics, but functions as regulators of virulence factor expression and are necessary for productive infections (204, 205).

In *S. aureus*, inactivation of staphyloferrin B biosynthesis but not staphyloferrin A biosynthesis leads to impaired growth in iron-restricted media. Double mutants of both staphyloferrins are severely growth attenuated in iron-deplete media and are resuscitated to wild-type levels only if the growth media is replete with iron (199) suggesting that staphyloferrin B plays the more prominent role in staphylococcal growth during iron limitation, at least under the conditions tested. However, both staphyloferrin A and B can remove iron from human holo-transferrin to support *S. aureus* growth in iron-deplete conditions (206).

1.4.2 *S. aureus* siderophores: Staphyloferrin A

Staphyloferrin A has been identified in supernatants from both *S. aureus* and CoNS (197, 207, 208). It is a 479 Da molecule, encoded by the *sfaABCD* operon (209-211), that is composed of a molecule of D-ornithine linked with two molecules of citrate via amide bonds (212). The staphyloferrin A encoding locus lacks a dedicated citrate synthase and citrate for staphyloferrin A production is derived from the TCA cycle citrate synthase, CitZ (213). Following the binding of iron by staphyloferrin A, transport across the bacterial cell membrane is mediated by the ABC transporter HtsABC in a process powered by a separate ATPase, FhuC (Figure 1.2A) (214). The staphyloferrin A operon encodes two NIS synthetases: SfaB and SfaD and is chromosomally adjacent to the *HtsABC* operon. The name of the *htsABC* operon is derived from its initial characterisation as a haem transport system (215). Crystallographic studies of HtsA demonstrate that it binds staphyloferrin A (216). Thus HtsABC is involved in the transport of multiple iron sources into the *S. aureus* cell.

1.4.3 *S. aureus* siderophores: Staphyloferrin B

Staphyloferrin B was first purified from supernatant extracts of *Stapylococcus hyicus* (CoNS) (198). It is a 448 Da molecule composed of a L-2,3-diaminopropionic acid (Dap), 1,2-diaminoethane (Dae), and α-ketoglutaric acid (217). Staphyloferrin B production is encoded by the *sbnABCDEFGHI* operon (202, 218, 219). Only the NIS synthetases SbnC, SbnE, SbnF, and the decarboxylase SbnH are required for *in vitro* synthesis of staphyloferrin B (220). Import of staphyloferrin B into the bacterial cell is mediated by the staphylococcal iron-regulated transporter SirABC system (Figure 1.2B). The *sirABC*

operon is located next to the *sbn* operon on the *S. aureus* chromosome. The upstream DNA sequence encodes a consensus Fur box, and the *sirABC* operon is upregulated under conditions of iron limitation (221). SirBC is predicted to be a membrane permease that is involved in the transport of staphyloferrin B into the cytoplasm. SirA is the lipoprotein receptor component of the staphyloferrin B import system (222). *sbn* genes and SB production have also been described for two proteobacteria: the plant pathogen *Ralstonia solanacearum* (223), and the heavy metal tolerant saprotroph *Cupriavidus metallidurans* (224).

1.4.4 Transport of siderophores across the membrane

In Gram-negative bacteria, siderophore import to the periplasm is mediated by outer membrane receptors and the Ton system. In Gram-positive bacteria, transport of molecules across the cytoplasmic membrane requires energy, therefore ABC transporters are normally associated with an ATPase. However, neither the *sirABC* or *htsABC* operon encodes a putative ATPase (214). The ferric hydroxymate uptake system, Fhu, is comprised of two permease units (FhuBG), an ATPase (FhuC), and two substrate-binding proteins, FhuD1 and FhuD2 (214, 225-228). The ATPase, FhuC, is necessary for the transport of staphyloferrin A and staphyloferrin B (214).

Figure 1.2. A model of the *S. aureus* iron acquisition pathways.

S. aureus produces two α-hydroxycarboxylate-type siderophores. Staphyloferrin A and Staphyloferrin B are taken up by HtsABC (A) and SirABC (B) respectively. Xenosiderophores produced by other bacteria are taken up by the FhuCBG and SstABCD systems (C). The energy required for siderophore transport is provided by the FhuC or the SstC ATPase. Haem acquisition is mediated by the Isd system (D). IsdB binds haemoglobin and IsdH binds haemoglobin-haptoglobin. Haem is passed through the near iron transporter (NEAT) domains of IsdB, IsdC, IsdA and IsdH. Haem transport across the membrane occurs through IsdDEF. Degredation of haem leads to the release of iron. Figure adapted from Hammer & Skaar, Annu Rev, Microbiol 2011 (229).

Investigation of a novel iron-uptake system and other genomic features in *mecC Staphylococcus aureus*

Siderophore-mediated iron acquisition

Staphyloferrin A +Fe **A** HtsA HtsBC FhuC

Staphyloferrin B +Fe **B** SirA SirBC FhuC

Xeno-siderophore transport

Hydroxamates +Fe **C** FhuD FhuBG FhuC

Catechols +Fe SstD SstAB SstC

Haem-mediated iron acquisition

Haemoglobin **Haem** **Haptoglobin**

IsdB **D** IsdA IsdH

IsdC

Cell Wall

IsdDEF

Cell Membrane

Cytoplasm

IsdG IsdI

Claire Raisen - January 2019
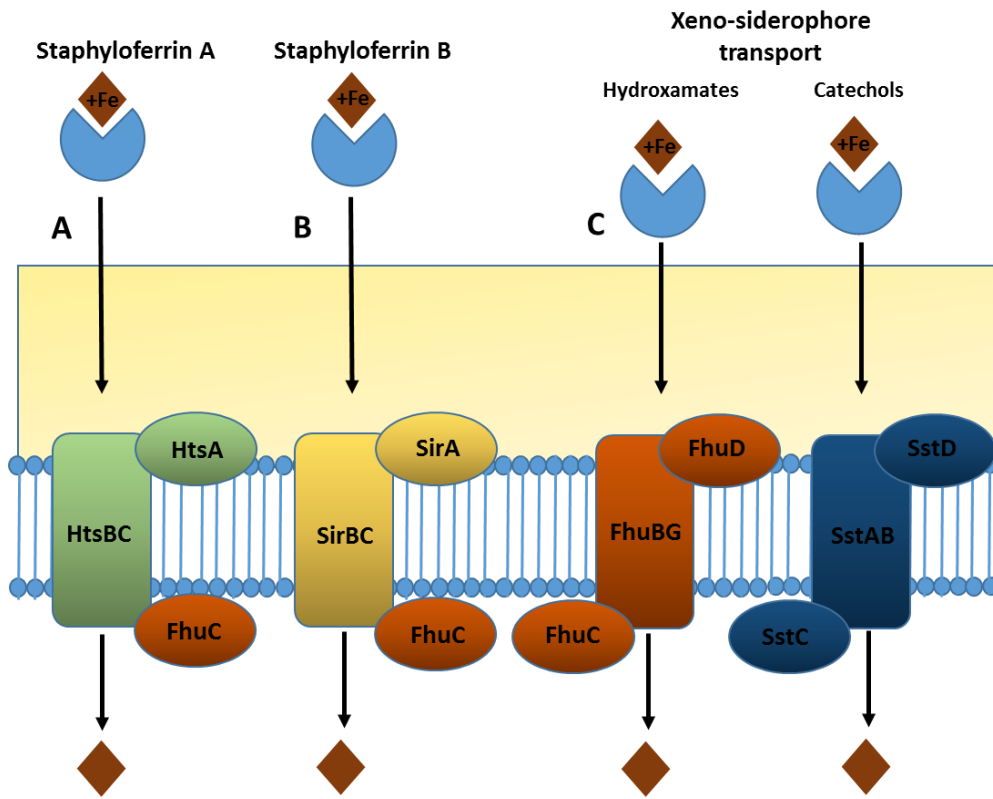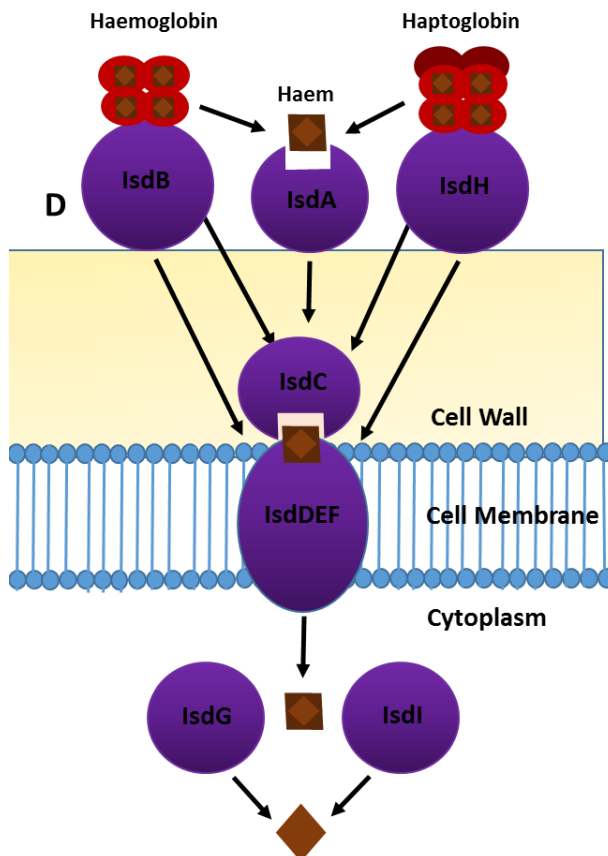
1.4.5 Xenosiderophores

In addition to endogenous siderophore production and utilisation, some bacteria are able to import siderophores produced by other bacteria as a source of iron, these are known as 'xenosiderophores' (230) (Figure 1.2C). Acquisition of xenosiderophores often occurs through uptake pathways with broad specificity for iron-loaded hydroxamate, catechol or α-hydroxycarboxylate-type siderophores, rather than any one specific siderophore. Bacteria capable of acquiring exogenous siderophores may have a competitive advantage within heterogeneous bacterial populations, where they are able to exploit multiple iron-chelating molecules as an iron source without the energetic demands involved in their synthesis and secretion (231-233). *S. aureus* imports xenosiderophores through the binding activity of FhuD1 and FhuD2 receptor lipoproteins and the FhuBG permease (230) (Figure 1.2C). Unlike SirA and HtsA, FhuD1 and FhuD2 undergo only modest conformational change upon siderophore binding (226, 234) which probably facilitates the binding of a broad spectrum of xenosiderophores. *S. aureus* is unable to synthesise endogenous-hydroxamate siderophores and FhuD2 facilitates the uptake of a wide variety of hydroxamate-type siderophores, including ferrichrome, desferrioxamine B, aerobactin and coprogen (214, 225, 227, 228, 235), whereas FhuD1 appears to accommodate a restricted subset of these siderophores (e.g. ferrichrome and desferrioxamine B). *S. aureus* is also known to use catecholate siderophores, such as enterobactin, for growth under iron restriction (225, 228). Bioinformatic analysis of the *S. aureus* genome uncovered the *sstABCD* operon, which includes genes for a dimeric permease (SstAB), an ATPase (SstC), and a lipoprotein sharing sequence identity with bacterial transport systems for iron ligands (SstD) such as *Bacillus subtilis* petrobactin receptor YclQ (236) (Figure 1.2C). This operon was shown to be iron regulated *in vitro* and *in vivo* (237) and is a broad specificity transporter of catecholate iron-coordination ligands. Catecholamine-mediated acquisition of serum iron is secondary to staphyloferrin-mediated iron uptake *in vitro* and can only be observed in the absence of siderophore production using *sfa/sbn* mutants. Genomic inactivation of *sstABCD* enhanced the attenuation phenotype of siderophore biosynthesis and transporter mutants in the mouse systemic model of infection,

suggesting the ability of *S. aureus* to acquire iron from host serum is the sum of multiple mechanisms and not exclusively limited to staphyloferrin-mediated uptake (238).

1.4.6 Contributions to virulence

A number of studies have demonstrated the impact of siderophore-mediated iron uptake on virulence, even among organisms capable of extracting host haem iron (Table 1.1).

Bacteria may also produce siderophores for survival outside a host niche, and rely on other iron uptake strategies *in vivo*. For example, production of a catecholate siderophore is important for survival of the veterinary pathogen *Rhodococcus equi* as an environmental saprotroph, while having no significant impact on virulence in a murine infection model (239).

Table 1.1: Siderophores contributing to the virulence of bacteria in animal models of colonisation or infection.

Species	Siderophore	Animal model	Ref
Acinetobacter baumannii	Acinetobactin	Mouse, Wax moth larvae, Human epithelial cells	(240)
Actinobacillus pleuropneumoniae	Tbp	Pig	(241)
Bacillus anthracis	Petrobactin	Mouse	(195)
Bordetella bronchiseptica	Alcaligin	Pig	(242)
Bordetella pertussis	Alcaligin	Mouse	(243)
Burkholderia cenocepacia	Ornibactin	Rat	(244)
Campylobacter jejuni	Enterobactin	Chick	(245)
Dickeya dadantii	Chrysobactin	African violet	(246)
Escherichia coli	Aerobactin	Mouse	(247)
	Salmochelin	Mouse	(248)
Klebsiella pneumoniae	Aerobactin,	Mouse	(249)
	Yersiniabactin	Mouse	(250)
Legionella pneumophila	Legiobactin	Mouse	(251)
Mycobacterium tuberculosis	Mycobactin	Mouse	(252)
Neisseria gonorrhoeae	Gonobactin	Chicken embryo	(253)
	Tbp	Human	(254)
Pantoea stewartii	Aerobactin	Sweet corn	(255)
Pseudomonas aeruginosa	Pyoverdin	Mouse	(256)
	Pyoverdin/Pyochelin		(257)
Salmonella typhimurium	Enterobactin	Mouse	(258)
	Salmochelin		(259)
Salmonella enterica	Salmochelin	Mouse	(260)
Staphylococcus aureus	Staphyloferrin B	Mouse	(202)
	Staphyloferrin A		(238, 261)
Vibrio cholera	Vibriobactin	Mouse	(262)
Vibrio vulnificus	Vulnibactin	Mouse	(263)
Yersinia pestis	Yersiniabactin	Mouse	(264, 265)

1.4.7 Siderocalin and stealth siderophores

Part of the human host response to siderophores is to tightly sequester iron in proteins such as transferrin; however, the mammalian host also produces siderocalin, a molecule that binds bacterial siderophores, rendering them inaccessible to bacteria as iron-scavenging agents [220]. Siderocalin is secreted by macrophages and neutrophils during the inflammatory response to bacterial antigens such as lipopolysaccharide, leading to rapid rises in serum levels (266). It is known to sequester a variety of bacterial siderophores, primarily those with catecholic or phenolic moieties and has been shown to bind enterobactin (267), bacillibactin, carboxymycobactin, and parabactin (268, 269). Siderocalin-deficient mice are more susceptible to sepsis and pneumonia caused by *E. coli* strains that rely on enterobactin for siderophore-mediated iron uptake (266, 270). Siderocalin is also capable of binding catecholamine stress hormones (271), further enhancing bacteriostasis through iron sequestration. Bacteria have evolved ways of avoiding this innate immune response, either by producing alternate, unrecognisable siderophores, or by incorporating structural modifications or substitutions within pre-existing siderophore molecular templates. For example, bacteria including *S. aureus* (staphyloferrin A and staphyloferrin B), *C. diphtheria* and *B. anthracis* (petrobactin and bacillibactin) produce one or more siderophores that cannot be bound by siderocalin (267, 268, 272). These siderophores have been termed 'stealth siderophores'. Mycobacteria usually colonize intracellular compartments of macrophages, which protects their siderophores and allows them to exploit iron-transferrin uptake inside phagosomes (273). Some bacteria that produce siderophores that can be bound by siderocalin occupy niches such as intestinal sites where they avoid siderocalin in plasma.

1.5 Haem

1.5.1 Haem uptake

In addition to siderophore-mediated iron acquisition, *S. aureus* has the ability to acquire iron through the extraction of haem from host erythrocytes and serum haemoproteins by haem acquisition systems. Haem is the primary reservoir of iron in vertebrates. *S. aureus* preferentially imports and utilises haem-iron when grown in the presence of

both haem and haemoglobin (215). *S. aureus* lyses erythrocytes through the secretion of haemolysins, such as α-hemolysin, to release haemoglobin. The iron-regulated surface determinant (Isd) system mediates the binding of haemoglobin and the transport of haem across the cell wall and plasma membrane (Figure 1.2D). The Isd system consists of a membrane transporter (IsdEF), cell wall anchored haem-binding proteins (IsdA and IsdC), haem/haptoglobin receptors (IsdB and IsdH), two haem oxygenases (IsdG and IsdI), and sortase B (SrtB), a transpeptidase that anchors specific substrate proteins to the cell wall (274). A consensus Fur box is located upstream of each of these operons, and these are therefore iron regulated (275). IsdA, IsdB and IsdH are covalently anchored to the cell wall by sortase A (SrtA) while IsdC is anchored by SrtB (274, 276). The current model for import of haem across the membrane and into the cytoplasm is: haem extracted from haemoproteins by IsdB and IsdH at the cell surface is relayed through the cell wall by IsdA and IsdC. IsdC then channels haem to the IsdDEF ABC transporter system (277, 278), which transports haem through the membrane, powered by an undefined ATPase (Figure 1.2D). Once internalised, haem is degraded by one of two haem oxygenases, IsdG or IsdI, releasing free iron from the tetrapyrrole ring for use by *S. aureus* (279). Haem degradation catalysed by the haem oxygenases results in the production of a chromophore unique to the IsdG family of haem oxygenases called staphylobilin (280). The binding of haemoproteins, extraction of haem and subsequent shuttling of haem through the cell wall by Isd proteins is facilitated by near iron transporter (NEAT) domains. IsdB and IsdH possess two and three NEAT domains, whereas IsdA and IsdC each possess one (281). These NEAT domains bind haemoglobin and haemoglobin-haptoglobin (282-284).

1.5.2 Haem uptake by haemophores

Haemophores are secreted proteins that extract haem from solution or from haem carrier proteins and deliver it to the bacterial cell. Haemophore production by Gram-positive bacteria has been documented for *Bacillus anthracis* and *Listeria monocytogenes*, which secretes soluble proteins with haem-binding motifs homologous to those found in its Isd system (285, 286).

1.5.3 Haem toxicity

Haem is used as a cofactor in many biological systems due to the redox potential of the iron atom. However, this redox-active molecule that can be toxic in high levels. *S. aureus* prevents haem toxicity through the activation of an ABC-type transporter called haem-regulated transporter (*hrt*) composed of a permease (HrtB) and an ATPase (HrtA) (Figure 1.3). The ATPase activity of HrtA is influenced by various physiochemical conditions including ATP concentration, temperature, pH, and metal cofactors (287). It is thought that HrtAB acts as an efflux pump that expels excess toxic haem. The upregulation of HrtA in response to haem is dependent on a two-component regulatory system, HssSR, composed of a intracellular haem accumulation sensor (HssS) and a transcriptional regulator (HssR) that function to activate expression of the *hrt* operon. Haem exposure results in the autophosphorylation of HssS which is followed by transphosphorylation of HssR. Phosphorylated HssR binds to a direct repeat sequence within the *hrtAB* promoter in order to induce transcription of *hrtAB* (Figure 1.3) (288).

Figure 1.3. A model for sensing and alleviating Haem toxicity.

HssR senses exposure to haem resulting in the expression on *hrtAB.* HrtA and HrtB transport haem across the cell membrane to alleviate haem mediated toxicity. Adapted from Hammer & Skaar, Annu Rev, Microbiol 2011 (229).
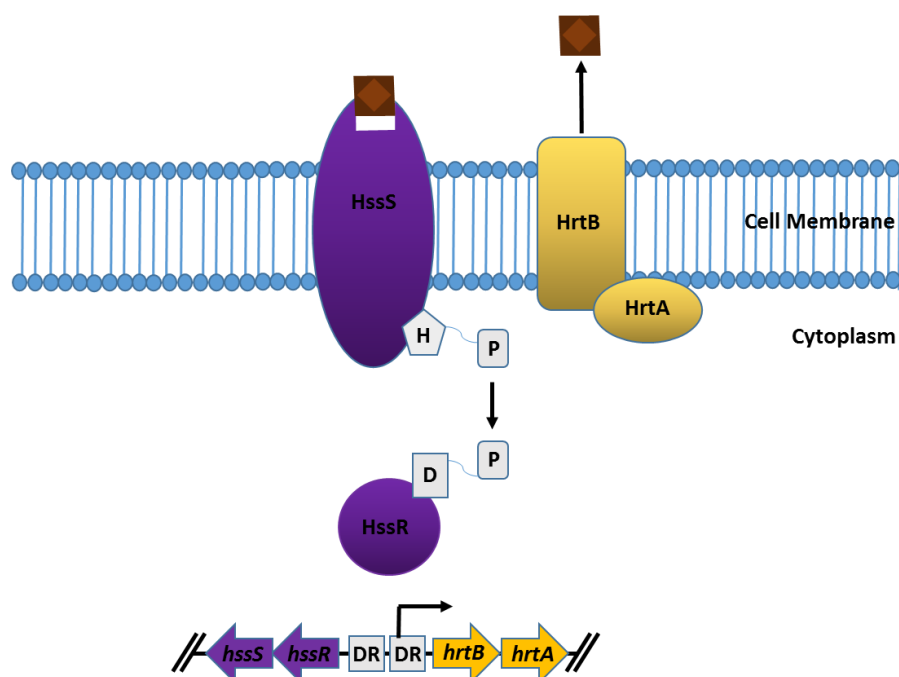
1.6 Transition metals

Metal-ion homeostasis is important for bacterial life. The transition metals manganese and zinc, along with iron, play key roles in the metabolism of bacteria. While copper serves as a cofactor in some proteins, bacteria also possess transporters to avoid copper toxicity. In addition to restricting iron availability, the host also sequesters other transition metals. The host protein calprotectin inhibits the growth of *S. aureus* by sequestering manganese and zinc. Calprotectin is a major protein constituent of the neutrophil cytoplasm.

1.6.1 Manganese

Bacteria must acquire and regulate their intracellular concentration of manganese. It is necessary for a variety of processes including carbohydrate and amino acid metabolism and defence against oxidative stress (289). *S. aureus* encodes *mntR*, a manganese-modulated transcriptional regulator, along with *mntABC* and *mntH*, manganese transporters. The transcriptional regulator MntR affects expression of both *mntABC* and *mntH*. In manganese-replete conditions, transcription of *mntABC* is repressed by MntR. In contrast, *mntH* transcription is reduced in the absence of MntR, suggesting that MntR may be a bifunctional regulator of *S. aureus*, as previously described for *B. subtilis* (168). In addition to MntR, *mntABC* is negatively regulated by PerR, a manganese and iron-dependent Fur homologue that controls responses to oxidative stress (171, 290).

1.6.2 Copper

Copper is an important cofactor for a number of bacterial enzymes, but is toxic if concentrations are not carefully regulated. The mechanism by which copper homeostasis is achieved in *S. aureus* has not been fully elucidated. CopA has been shown to be involved in copper efflux (291) and its regulation has been shown to be regulated by the copper-sensitive operon repressor (CsoR) (292).

1.6.3 Zinc

Zinc is an essential element that participates in diverse processes such as regulation of virulence factors, metabolism, and inactivation of antibiotics. Zinc concentrations must

be carefully regulated and homeostasis is achieved through coordinated regulation of import and export. *S. aureus* possesses two zinc importers, AdcABC and CntABCDF, which are induced in response to zinc limitation (293). The *crzC* gene encodes zinc resistance and is genetically located within the type V SCC*mec* elements. The *crzC* gene is widespread among CC398 LA-MRSA isolates and might have contributed to the emergence of CC398 MRSA (294).

A promising strategy to combat bacterial infections is to inhibit the procurement of the nutrients necessary for growth. The decreasing efficacy of available antibiotics underscores the need to increase our understanding of the fundamental processes that promote *S. aureus* pathogenesis, as these processes represent targets for novel therapeutics.

1.7 Thesis overview

1.7.1 Identification of a novel ICE encoding a siderophore-like NRPS in *S. aureus*

Comparison of the *S. aureus mecC* strain LGA251, the first *mecC* isolate discovered by García-Álvarez *et al.* (67), and newly sequenced *mecC S. aureus* genomes from our strain collection using the Artemis Comparison Tool (295) led to the identification of a 23 kb region in all CC130 strains that was absent from the LGA251 genome (Data not published). Analysis of the locus showed that it contained coding regions with similarity and conserved gene order to coding sequences (CDs) of a non-ribosomal peptide synthetase (NRPS) siderophore system called equibactin from *Streptococcus equi* 4047 (296).

1.7.2 *Streptococcus equi* and equibactin

Streptococcus equi (*S. equi*) is a host-restricted pathogen that causes a highly contagious equine disease called strangles. The disease is characterised by abscessation of the lymph nodes in the head and neck, causing swelling and restriction of the airway, leading to significant economic and welfare cost (297, 298). *S. equi* establishes persistent infections within the guttural pouches of apparently healthy horses that can result in

direct transmission to naïve horses or via indirect transmission due to contamination of human attendants, objects or the environment. *S. equi* is thought to have evolved recently from an ancestral strain of *Streptococcus zooepidemicus* (299), which is an opportunistic pathogen causing a wide variety of diseases in horses and other animals. Comparison of the genomes of *S. equi* strain 4047 with those of *S. zooepidemicus* strain H70 identified gene loss due to nonsense mutations and deletions, and gene gain through the acquisition of mobile genetic elements (MGEs), including four prophages and two novel integrative conjugative element (ICE). One of the ICEs, ICE*Se*2, encodes a NRPS (non-ribosomal peptide synthetase) that produces a putative siderophore, named equibactin. Equibactin has been shown to enhance the ability of *S. equi* to acquire iron *in vitro* (296) (Figure 1.4) and is the first example of a streptococcal siderophore (300). The encoded NRPS system of *S. equi* shows homology to other systems found in *Clostridium kluyveri* and *Yersinia* spp. that produce an unnamed siderophore (301) and the ferric iron-binding siderophore yersiniabactin (302). Siderophore biosynthesis plays an important role in the virulence of many important pathogens (303), including Yersiniabactin by *Yersinia pestis*, the causative agent of bubonic plague (304). The equibactin locus is absent from all *S. zooepidemicus* strains examined to date, and the acquisition of this locus is thought to represent the main speciation event in the evolution of *S. equi*, as a host-restricted pathogen of horses, from an ancestral *S. zooepidemicus* strain (305). The acquisition of virulence genes carried on ICE*Se2* in *S. equi* may have contributed significantly to the survival fitness and influence niche adaption due to more efficient acquisition of iron that could enhance survival within the lymph nodes. This is critical to the establishment of long term carriage and vital to the success of this bacterium (305). Deletion of the equibactin locus occurs solely in isolates from persistently infected horses and stops these isolates from being able to cause severe disease and therefore transmitted to other horses. The *S. equi* genome encodes other metal acquisition systems, including a haem-binding system (306), a putative Mn^{2+} and Fe^{3+} metal transport system and a putative Fe^{3+} ferrichrome transport system.

Figure 1.4. The ICE*Se2* locus in *S. equi* produces a siderophore, named equibactin, which improves iron acquisition.

The *S. equi* NRPS system carried on the ICE*Se2* locus produces a secreted siderophore, named equibactin, which enhances the ability of *S. equi* to acquire iron *in vitro* (296)*. The genes *eqbH*, *eqbI* and *eqbJ* encode a putative ABC transporter, which transport iron bound equibactin into the *S. equi* cell. Figure adapted from Waller *et al.* Journal of Medical Microbiology 2011 (307).
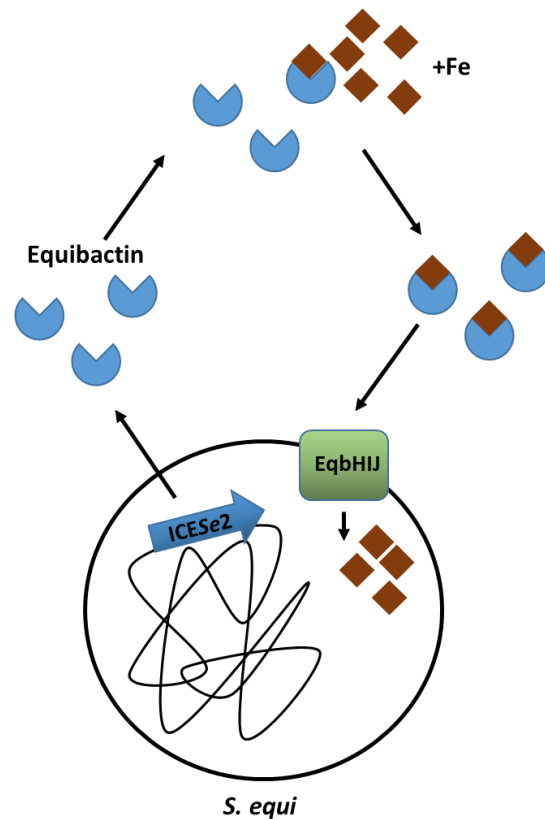
1.7.3 Thesis objectives and summary

This thesis focusses on the *S. aureus* homologue of the equibactin cluster identified through sequencing of the *mecC S. aureus* genomes in our strain collection.

This project will address the research questions:

Chapter 1: Introduction

What is the role of the homologous equibactin biosynthesis cluster found in *mecC S. aureus* CC130 isolates?

Is this region involved in iron acquisition and how might it be regulated?

Does this region contribute to growth of *S. aureus* and to virulence?

What is the advantage for CC130 isolates that contain this region and how has it contributed to its ability to infect a broad range of host species across Europe?

Chapter 2, I describe computational analysis of the *mecC* strains in our collection, looking at the host and geographical range of these isolates specifically from CC130. I also looks at the prevalence of this 'equibactin' locus in *S. aureus* and other bacterial strains. I discuss predicted functions of the gene cluster by sequence analysis, as well as how this region might be regulated and substrate specificity of the binding pockets of the A domains in the NRPS.

In Chapter 3, I describe the process of genetic manipulation of *mecC S. aureus*. In order to study the function of genes, genetic manipulation is required and this is the first time it has been carried out in *mecC* strains. This chapter includes discussion of how I overcame barriers encountered when creating deletion mutants in extremely difficult bacteria to manipulate, to create the optimal protocol.

In Chapter 4, characterisation of the 'equibactin' locus was carried out. *S. aureus* is already known to produce two siderophores, staphyloferrin A and staphyloferrin B. Mutagenesis was performed to eliminate the iron acquisition loci from the *S. aureus* chromosome to dissect their individual and combined contributions toward growth in iron-restricted media, and to assess their roles in virulence. I determined whether *mecC S. aureus* produce siderophores and which siderophores they produce. I also analysed whether the 'equibactin' genes are expressed under iron-limited conditions and how this NRPS might be regulated and transported across the bacterial membrane.

In Chapter 5, I describe a von Willebrand factor-binding protein (vWbp) that is produced by *mecC S. aureus* with the ability to clot plasma from a broad range of host species.

Finally, Chapter 6 summarises the main findings and conclusions drawn from this project, including future investigative foci.

2 SEQUENCE ANALYSIS OF STAPHYLOBACTIN

2.1 Introduction

2.1.1 Identification and prevalence of a novel ICE encoding a siderophore-like NRPS in *S. aureus*

The following text in this introduction section describes unpublished work that was carried out by researchers, Dr Ewan Harrison and Dr Gavin Paterson, in our group prior to my PhD studies. Comparison of the *mecC S. aureus* LGA251 and newly sequenced *mecC S. aureus* genomes from our strain collection using the Artemis Comparison Tool (ACT) (295) led to the identification of a 23 kb region in all CC130 strains that was absent from the LGA251 genome (CC425) (Figure 2.1). The region was found downstream of the SCC*mec* (Type XI) insertion site and is highly conserved in all CC130 isolates in our collection. The CC130 strains have been isolated from diverse host species including bovine, human, cat and deer, and from a broad geographical area. Basic Local Alignment Search Tool (BLAST) analysis of all the sequenced isolates in our collection (~1200) identified one other *mecC S. aureus* isolate with the same region, strain 8572, with approximately 130 single nucleotide polymorphisms (SNPs) over 23 kb. Strain 8572 belongs to CC350 and was isolated in the UK from a horse tracheal wash.

Figure 2.1. Comparison of strain LGA251 (CC425) and strain C03 363 (CC130) genomes using ACT and showing the 23 kb region (orange) present in all CC130 genomes. The SCC*mec* Type XI element highlighted in green.
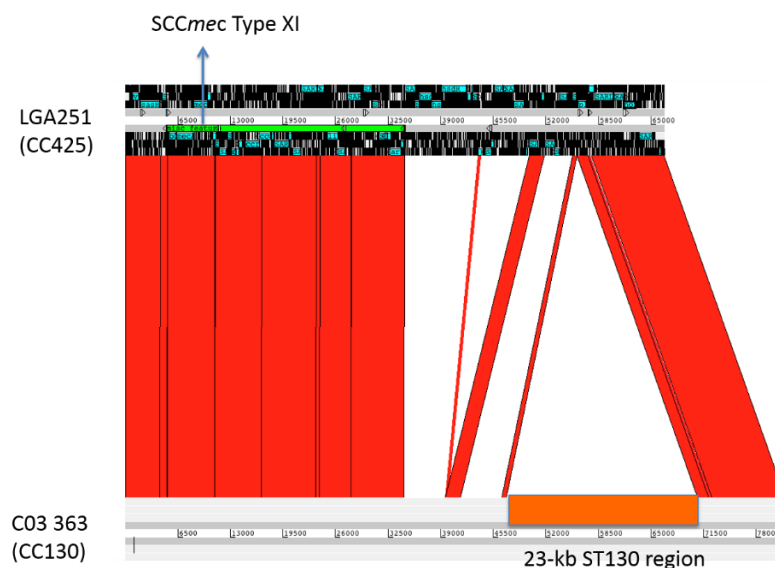
TBLASTX and FASTA (308) analysis against UniProt determined that the 23 kb locus contained coding regions with similarity to (43% aa identity) and gene order conserved with the *Streptococcus equi* 4047 (*S. equi*) equibactin locus. Equibactin is a non-ribosomal peptide synthetase (NRPS) siderophore system contained within a integrative conjugative element (ICE) ICE*Se*2 in the *S. equi* genome (296). We therefore named this locus in *S. aureus*: staphylobactin (stb). Note that the name staphylobactin had previously been used for staphyloferrin B before its identity was confirmed by mass spectrometry (202).

A closer homologues region (54% aa identity) was also found to be present in the genome of *Staphylococcus lugdunensis* (*S. lugdunensis*) strain N920143 but this region is currently uncharacterised (Figure 2.2).

Figure 2.2. TBLASTX comparison of the 'equibactin' region in *Streptococcus equi*, *S. aureus* and *S. lugdunensis* (Displayed using ACT).

Red and blue lines between the elements represent protein similarity. The genes involved in equibactin biosynthesis are underlined in dark blue and those encoding the ABC transporters associated with the equibactin locus are underlined in light blue.
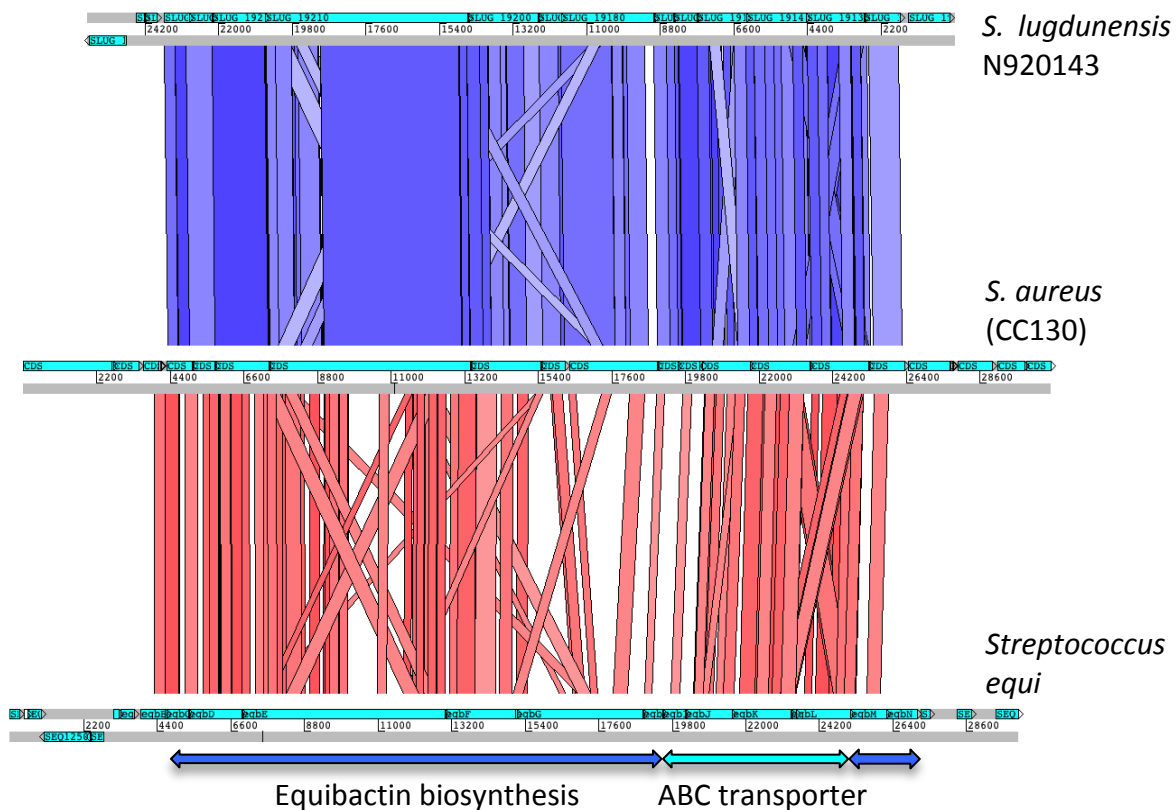
The *stb* gene cluster contains 14 coding sequences *stbB-F, F1, G-M, O* (Figure 2.3). Comparison of the *stb* gene cluster and the *eqb* gene cluster shows that the staphylobactin locus is lacking homologues of *eqbA*, which encodes a DtxR-like repressor, and *eqbN*, an α/β hydrolase (Table 2.1). However, the *stb* gene cluster contains two additional genes that are not present in the *eqb* cluster, *stbF1* and *stbO*. BLASTp and FASTA analysis of these genes predict the function of *stbF1* as a glyoxalase-like domain protein, with its closest homologue found in *S. lugdunensis* VCU148 (62% identity). *stbO* is predicted to be a putative salicylate synthase and its closest homologue was found in *Staphylococcus hyicus* (71% identity).

Table 2.1. Comparison of the protein sequences contained within the staphylobactin cluster with the equibactin proteins.

Their percentage amino acid identity and predicted functions using BLASTp. The organism showing identity and the length of amino acids for that protein.

Protein	Amino acid % identity	Function	Organism	Length (a.a)
StbB	54%	Putative non-ribosomal peptide synthesis thioesterase	*Streptococcus equi*	255
StbC	35%	Putative 4'-phosphopantetheinyl transferase superfamily protein	*Streptococcus equi*	227
StbD	54%	Putative salicylate-AMP-ligase	*Streptococcus equi*	535
StbE	43%	Equibactin nonribosomal peptide synthase protein	*Streptococcus equi*	2005
StbF	38%	Thiazoline reductase	*Streptococcus equi*	704
StbF1	62%	Glyoxalase-like domain protein	*Staphylococcus lugdunensis*	230
StbG	44%	Equibactin nonribosomal peptide synthase protein	*Streptococcus equi*	874
StbH	28%	ABC transporter permease	*Streptococcus equi*	192
StbI	25%	ABC transporter permease	*Streptococcus equi*	225
StbJ	39%	ABC transporter ATP-binding protein	*Streptococcus equi*	495
StbK	55%	ABC transporter ATP-binding protein	*Streptococcus equi*	584
StbL	56%	ABC transporter ATP-binding protein	*Streptococcus equi*	577
StbM	32%	Putative oxidoreductase	*Streptococcus equi*	350
StbO	71%	Putative salicylate synthase	*Staphylococcus hyicus*	445

The staphylobactin region is flanked by direct repeats (Figure 2.3). The left and right repeats show almost complete identity over 56bp, with just a single base pair difference. The direct repeats also have a 50 bp identity with a single base pair difference with the LGA251 chromosome (Figure 2.3). LGA251 is a *mecC* positive *S. aureus* strain that does not contain the staphylobactin locus. These direct repeats flanking staphylobactin are not identical to the region flanking equibactin.

Figure 2.3. The staphylobactin locus containing genes *stbB-stbO* bordered by direct repeats, DR-L and DR-R.

Below shows the direct repeat left and right sequence homology and the direct repeat left matching the sequence from the strain LGA251.

2.2 Bioinformatic analysis

Illumina library preparation and Hi-Seq sequencing were carried out by the core sequencing and informatics team at the Wellcome Trust Sanger Institute. Maximum likelihood phylogenetic trees were constructed from a core genome alignment produced by Roary (309) using Randomised 'Axelerated' Maximum Likelihood MLST (RAxML) (310), and displayed and annotated using Interactive tree of life (iTOL) (311). BLAST and FASTA was used to find regions of similarity between biological sequences (312), and along with the UniProt Knowledgebase (UniProtKB) (313), was used to predict functions of proteins. Amino acid sequence alignment was carried out using SeaView applying the Clustal method (314). Identification of adenylation domains with their known substrates was carried out with: Non-Ribosomal Peptide Synthase Substrate Predictor (NRPSsp) (315), Latent Semantic Indexing (LSI) based A-domain function

predictor (316), PKS/NRPS Analysis (317). NRPSpredictor2 (318, 319), Genes encoding NRPS and modular PKS assembly lines, predictive sequence analysis tools (320-326) and Pfam (327). Comparison of genomes were displayed using ACT (295). Sequences were downloaded from the UniProt website. Staphylobactin gene sequences were extracted using Artemis (328) Genome browser and annotation. The sequences of A domains were retrieved from publicly accessible databases (NCBI, Swiss-Prot etc).

2.3 Results

2.3.1 Identification of staphylobactin locus in our strain collection

Over the course of this project and during the initial identification of this region, many genomes have been sequenced by our group. These genomes are predominantly *S. aureus* but include other staphylococcal species. In order to investigate the prevalence in our current strain collection, I repeated this search for the genes in the staphylobactin locus, using 90% sequence similarity as the cut-off, in all our *S. aureus* isolates (2434 genomes).

In our entire strain collection there are a total of 226 sequence types, the largest number of strains belonging to ST130 (407 isolates), followed by ST22 (241 isolates), ST133 (232 isolates), ST425 (200 isolates) and ST398 (122 isolates).

The staphylobactin locus was found to be present in 582 genomes, of which 565 isolates belonged to clonal complex (CC) 130, 409 belonging to ST130 and 156 belonging to 40 other different sequence types, including ST521 and ST1944. These isolates are all the CC130 that have been sequenced by my group and contained the whole gene cluster and were highly conserved within ST.

The *S. aureus* CC130 isolates are interesting as they are from a diverse host species including: bovine, human, cat and deer, and from a broad geographical area: mainly the UK and Denmark, but also Germany, Norway and Spain. There are a number of isolates, inside and outside of the CC130 clade, containing the staphylobactin locus that do not contain the *mecC* gene. The *mecC* gene in the CC130 isolates was acquired once, and

has been lost in a small number of isolates, mainly from wild animals such as rat, squirrel and seal (E. M. Harrison, unpublished data).

2.3.1.1 Staphylobactin in other sequence types

The staphylobactin locus was found in other sequence types as follows:

- ST177: the *mecC* positive *S. aureus* ST177 isolate (8572, 9061_8#79) came from a horse tracheal wash in the UK in 2005. This strain was the single locus variant of ST350 originally identified in the initial discovery of this locus in *S. aureus*.

- ST2639: isolate ST2639 (DICM09/00289-9 HAS 12162_2#48) came from a nasal swab from an Iberian ibex, in 2009, Spain.

- ST136: the ST136 isolate (178_B09 12448_6#83) came from a goat nasal swab in the Netherlands.

- ST479: there are 14 isolates belonging to ST479 that contain the entire locus, these are all the ST479 isolates in our collection, they are all bovine isolates from the Netherlands provided by Ruth Zadoks.

- ST425: there are 200 isolates belonging to ST425 in our collection, and a single ST425 isolate contains the entire staphylobactin locus (H091100226 7092_7#55). This was isolated from a human skin swab, in the UK during 2009.

All the strains that contain the staphylobactin locus have it in its entirety.

2.3.1.2 Identification of other genomic features

Isolates were also investigated for the presence of the *eqbA* homologue, which encodes a DtxR-like repressor, but none of the strains contained this gene. The staphylobactin locus was found in either strains containing *mecC* or related MSSA strains, but no strains carrying the *mecA* gene. The presence of the staphyloferrin B biosynthesis cluster and transport was also searched for and found to be present in nearly all *S. aureus* strains except three ST that did not contain the *sbnA-I* genes and one ST that did not contain either *sbnA-I* and *sirABC*; however, none of these four STs contained the staphylobactin locus. This suggests that the presence of staphylobactin has not been acquired to compensate for the lack of the staphyloferrin siderophore. The direct repeats either side of the staphylobactin locus were searched for in all the strains and was found to be

present in all the CC130 isolates, along with the single ST425. ST479 was found to have one of the repeat regions missing. A maximum likelihood phylogenetic tree based on concatenated MLST allele sequences was constructed using RAxML illustrating the presence of this gene cluster within our sequenced *S. aureus* collection to show the population structure context of the five staphylobactin lineages (Figure 2.4)

Figure 2.4: Maximum likelihood phylogenetic tree representing all the sequence types of *S. aureus* in our strain collection.

An alignment of the core genome for all the sequence types of *S. aureus* in our strain collection was produced using Roary and MAFFT (309) using default settings. RaxML (310) was used to generate a maximum likelihood tree which was annotated using iTOL (311). The presence of methicillin resistance encoding genes *mecA* and *mecC*, staphyloferrin B biosynthesis and transport genes *sbnA-I*, *sirABC*, the equibactin DtxR-like repressor, *eqbA* and the individual genes of the staphylobactin locus *stbB-O* were searched for using 90% sequence identity in the whole of our strain collection. Sequence types in bold highlight the presence of the staphylobactin locus. Empty boxes show the absence of that gene in the genome. To the right of each sequence type, I give the number of strains containing the staphylobactin locus followed by the total number of strains belonging to that sequence type.

Although our strain collection predominantly comprises *S. aureus* isolates, we have sequenced a large number of other staphylococci, mainly coagulase negative isolates. These isolates (~200) were searched for the presence of all the staphylobactin genes, equibactin genes, and *lugZ* (predicted to encode an NADH dehydrogenase) using a 50% similarity threshold. This revealed 35 isolates of interest. The presence of the *mecA* and *mecC* genes was also determined, along with the staphyloferrin B biosynthesis and

transport cluster, *sbnAI* and *sirABC*. These strains were speciated using Kraken (329), however this proved unsuccessful for a large number of isolates, therefore 16s ribosomal RNA gene sequence analysis (16s) was carried out. All the strains searched contain *lugZ*, 70 strains contain *mecA*, and one *S. xylosus* isolate contains *mecC*. The remaining strains in this collection do not contain either gene and are therefore assumed to be methicillin susceptible. One *S. schweitzeri* isolate contains *sbnA-I* encoding staphyloferrin B biosynthesis and 40 strains contain the staphyloferrin B transporter, *sirABC*, including *S. hyicus*, *S. schleiferi* and *S. agnetis*. Four *S. lugdunensis* contain the entire homologous staphylobactin region, along with one *S. hyicus* strain that contains all staphylobactin genes (*stbB-stbL*) except *stbM*. There are many isolates with just one or two of the genes present in the following species: A *S. agnetis* and a *S. auricularis* strain contains *stbH-L*, predicted to encode an ABC transporter. Strains that contain individual staphylobactin genes include, six strains containing the *stbH* gene with 77% similarity from *S. haemolyticus*, *stbI* and *stbJ* (*S. xylosus*) and *stbL* (*S. pettenkoferi*) homologues. Isolates that contained two homologues include five strains containing *stbH* and *stbJ* with 52-56% similarity from *S. saprophyticus*, *stbK* and *stbL* (*S. argentus* and *S. shweitzerei*) and, *stbI* and *stbJ* (*S. cohnii* and *S. gallinarum*) homologues. The strains with two or more of the staphylobactin genes present are co-located in the genome. The non *S. aureus* isolates in our collection come from broad host species, including sheep, goat and cows, and from a large geographical area in Europe, including Turkey, Belgium and France.

A maximum likelihood phylogenetic tree of the staphylococcal strains in our collection containing genes of the staphylobactin locus was constructed from a core genome alignment produced by Roary using RAxML and is shown in Figure 2.5.

Figure 2.5 Phylogenetic analysis of the staphylobactin biosynthetic cluster in non-*S. aureus* staphylococcal species in our strain collection.

An alignment of the core genome for all the sequence types of *S. aureus* in our strain collection was produced using Roary and MAFFT (309) using default settings. RaxML (310) was used to generate a maximum likelihood tree which was annotated using iTOL (311). Searches were performed for the presence of methicillin resistance encoding genes *mecA* and *mecC,* all genes in the equibactin, staphylobactin and lugdunbactin (see section 2.3.2) loci plus the staphyloferrin B biosynthesis and transport genes *sbnA-I* and *sirABC* and the *lugZ* gene with 50% sequence identity. The tree is based on core genome and lists the sequence ID of that isolate or staphylococcal species if known (strains were speciated using Kraken (329) and 16s analysis). Sequence types in bold highlight the presence of the staphylobactin or lugdunbactin genes. *eqbA* and *stbO* was not found to be present in any of the genomes searched. Empty boxes show the absence of that gene in the genome.
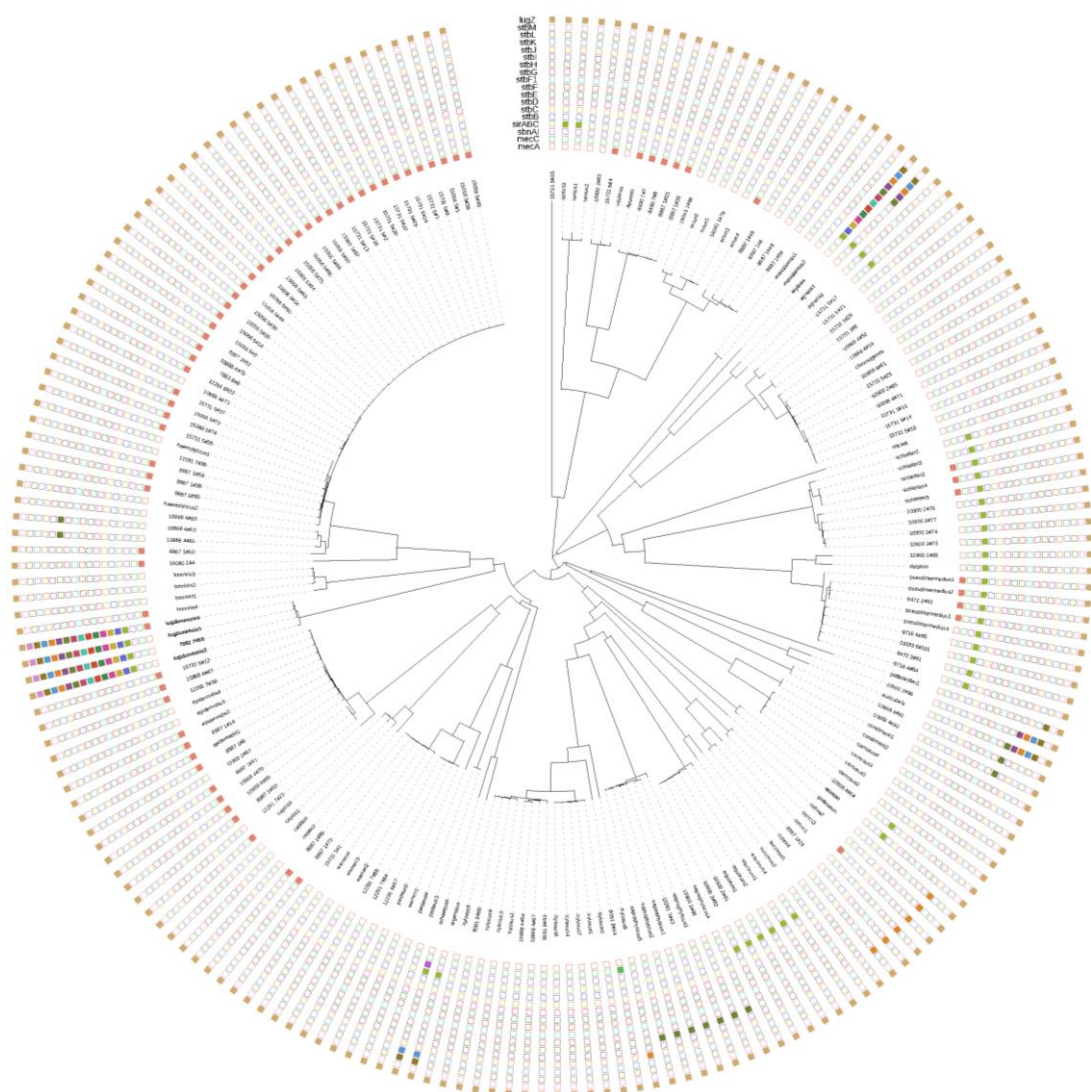
2.3.2 Further analysis of the homologous staphylobactin region in *S. lugdunensis*

Our strain collection contains one sequenced *S. lugdunensis* isolate, H042360302, which was isolated from a human skin swab in England, in 2004. Two isolates that have been sequenced are available in the public domain; N920143, a human breast isolate (330) (NCBI reference sequence NC_017353.1) and HKU-09 (331) (NCBI Reference Sequence: NC_013893.1). These strains are probably the most well studied sequenced *S. lugdunensis* strains (332-334). Other *S. lugdunensis* genomes currently available in the public domain are; VCU150, VCU148, M23590 and ACS-027-V-Sch2.

BLASTn of the whole staphylobactin region from *S. aureus* strain 71277 (CC130, *mecC*) against the *S. lugdunensis* genome N920143 showed that it had 71% sequence identity, whereas BLASTn of the equibactin region from *S. equi* had 72% sequence identity. All of the *S. lugdunensis* genomes available including the one from our strain collection were searched for the presence of all the equibactin genes, including *eqbA*, the staphylobactin genes along with the staphyloferrin B biosynthesis and transport genes (*sbnA-I* and *sirABC*). These *S. lugdunensis* strains contained the whole staphylobactin gene cluster, in the same order, except for the presence of *stbO*, and had sequence identity ranging from 37% for *stbM* to 76% for *stbL* (Table 2.2). The homologous staphylobactin locus in *S. lugdunensis* was termed lugdunbactin and the genes labelled *lugB-lugZ*.

Table 2.2. Comparison of the protein sequences contained within the lugdunbactin cluster with the staphylobactin proteins.

Their percentage amino acid identity and predicted functions using BLASTp. The organism showing identity and the length of amino acids for that protein.

Protein	Amino acid % identity	Function	Organism	Length (a.a)
LugB	62%	Thioesterase	*S. aureus*	250
LugC	41%	4'-phosphopantetheinyl transferase	*S. aureus*	232
LugD	63%	2,3-dihydroxybenzoate-AMP ligase	*S. aureus*	527
LugE	54%	non-ribosomal peptide synthetase	*S. aureus*	2007
LugF	52%	Polyketide synthase	*S. aureus*	702
LugF1	57%	Hypothetical protein	*S. aureus*	230
LugG	53%	Polyketide synthase	*S. aureus*	920
LugH	75%	Membrane protein	*S. aureus*	192
LugI	68%	Energy-coupling factor transporter transmembrane protein EcfT	*S. aureus*	243
LugJ	58%	ABC transporter	*S. aureus*	486
LugK	74%	ABC transporter ATP-binding protein	*S. aureus*	583
LugL	76%	ABC transporter ATP-binding protein	*S. aureus*	577
LugM	37%	Saccharopine dehydrogenase	*S. aureus*	345
LugZ	89%	NADH dehydrogenase	*S. haemolyticus/ S. aureus*	402

Comparison of *lugB* shows that it is well conserved both at the nucleotide and protein level, 62% aa identity with StbB and 57% aa identity with EqbB, and is predicted to encode a thioesterase. *lugC* is predicted to encode a 4'phosphopantetheinyl transferase. BLASTn with *stbC* and *eqbC* show this gene is less well conserved, 67% nucelotide identity and only 39% coverage with *stbC* and BLASTp 41% aa identity, 99% coverage. *lugC* was found to be highly divergent from the staphylobactin and equibactin homologues, and so was not originally identified when searching the genome, however, I was able to identify the genes by looking for the ORF in this region and searching with a 50% identity limit. Using this search method, *lugE-L* were identified. *lugD* is predicted to encode a 527 amino acid 2,3-dihydroxybenzoate-AMP ligase; BLASTn with *stbD* shows 71% nucleotide identity and BLASTp with StbD 63% aa identity. The largest gene in the locus, *lugE* (6021bp) has 66% identity at a nucleotide level with *stbE*, and 54% amino acid identity (43% with EqbE) and is predicted to encode a non-ribosomal peptide synthetase. *lugF* (68%), *lugF1* (73%) and *lugG* (69%) have varying nucleotide identity and are predicted to encode polyketide synthase proteins. Like *stbH-L*, *lugH-L* are predicted to encode an ABC transporter with similarity ranging from 67-76%. *lugM* is not highly conserved with either *stbM* or *eqbM*, sharing only 37% amino acid identity. The next ORF after *lugM* is a completely different gene to *stbO*, which encodes a 402 amino acid protein predicted to be a NADH dehydrogenase, which I termed *lugZ*. This gene is not exactly in the same location in the genome as *stbO*, but was included in the searches with other staphylococcal species and was found to be a highly conserved gene that is present in all staphylococcal species investigated. Despite the low percentage identity between some genes in this cluster, they are all predicted to produce a protein with the same function as the homologous gene in staphylobactin and equibactin (Table 2.1 and Table 2.2) Searching this region I could not find direct repeats identified in *S. aureus*, either side of this locus in either *S. lugdunensis* or *S. equi*. *sirABC*, but not *sbnA-I* were identified in *S. lugdunensis*, as previously reported (334) and shared 77% sequence identity.

2.3.3 Functions of the staphylobactin gene cluster as predicted by sequence analysis.

BLASTp was used to find sequence similarity and predict functions of the staphylobactin gene cluster by comparing the amino acid sequence of the individual genes in the staphylobactin cluster to sequence databases (Table 2.3). Amino acid sequences for each staphylobactin gene was taken from the genome of *mecC* CC130 strain 71277 isolated from human blood in Denmark, and were used for the predicted function sequence analysis.

Table 2.3. BLASTp and FASTA analysis of the amino acid sequence of the individual genes of the staphylobactin cluster

S. aureus	Amino acid % identity	Function	Organism
StbB	100	Iron acquisition yersiniabactin synthesis enzyme (thioesterase)	*Staphylococcus aureus* O11
	68	Thioesterase	*Staphylococcus hyicus*
	63	Thioesterase	*Staphylococcus lugdunensis*
	55	Thioesterase	*Anaerococcus lactolyticus*
	54	Thioesterase	*Peptoniphilus harei*
	54	Putative non-ribosomal peptide synthesis thioesterase type II	*Streptococcus equi*
StbC	100	4'-phosphopantetheinyl transferase	*Staphylococcus aureus* O11/O46
	50	Hypothetical protein	*Staphylococcus hyicus*
	41	4'-phosphopantetheinyl transferase	*Staphylococcus lugdunensis*
	38	4'-phosphopantetheinyl transferase	*Streptococcus anginosus*
	41	Hypothetical protein	*Clostridium difficile*

StbD	100	2,3-dihydroxybenzoate-AMP ligase	*Staphylococcus aureus* O11/O46
	70	2,3-dihydroxybenzoate-AMP ligase	*Staphylococcus hyicus*
	64	2,3-dihydroxybenzoate-AMP ligase	*Staphylococcus lugdunensis*
	58	2,3-dihydroxybenzoate-AMP ligase	*Clostridiodes difficile*
	57	2,3-dihydroxybenzoate-AMP ligase	*Anaerococcus tetradius*
StbE	99	Non-ribosomal peptide synthetase	*Staphylococcus aureus* O11/O46
	61	Non-ribosomal peptide synthetase	*Staphylococcus hyicus*
	54	Non-ribosomal peptide synthetase	*Staphylococcus lugdunensis*
	45	Non-ribosomal peptide synthetase	*Anaerococcus lactolyticus*
	43	Non-ribosomal peptide synthetase	*Streptococcus equi*
	42	Non-ribosomal peptide synthetase	*Peptoniphilus* spp.
	40	Non-ribosomal peptide synthetase	*Clostridium kluyveri*
	34	Non-ribosomal peptide synthetase	*Staphylococcus epidermidis*
StbF	100	Polyketide synthase	*Staphylococcus aureus* O11/O46
	62	Polyketide synthase	*Staphylococcus hyicus*
	52	Polyketide synthase	*Staphylococcus lugdunensis*
	38	Thiazoline reductase	*Streptococcus equi*
	36	Equibactin biosynthetic protein	*Anaerococcus lactolyticus*
StbF1	100	Hypothetical protein	*Staphylococcus aureus* O11/O46
	63	Hypothetical protein	*Staphylococcus hyicus*
	57	Hypothetical protein	*Staphylococcus lugdunensis*
	33	Hypothetical protein	*Lysinibacillus macroides*
	27	Hypothetical protein	*Streptomyces* sp.
StbG	100	Polyketide synthase	*Staphylococcus aureus* O11/O46
	61	Polyketide synthase	*Staphylococcus hyicus*
	53	Polyketide synthase	*Staphylococcus lugdunensis*
	44	Equibactin nonribosomal peptide synthase protein	*Streptococcus equi*

	32	Condensation domain-containing protein	*Anaerococcus prevotii*
	37	Pyochelin synthetase	*Clostridium butyricum*
StbH	100	Hypothetical protein	*Staphylococcus aureus* O11/O46
	82	Hypothetical protein	*Staphylococcus sciuri*
	80	Hypothetical protein	*Staphylococcus haemolyticus*
	78	Membrane protein	*Staphylococcus hyicus*
	78	Membrane protein	*Staphylococcus agnetis*
	68	Membrane protein	*Staphylococcus auricularis*
	76	Uncharacterised protein	*Staphylococcus lugdunensis*
	55	Hypothetical protein	*Clostridium* spp.
	55	Hypothetical protein	*Enterococcus faecalis*
	40	Membrane protein	*Lachnospiraceae bacterium*
StbI	99	Protein of energizing module of ECF transporter	*Staphylococcus aureus* O11/O46
	79	ECF transporter	*Staphylococcus sciuri*
	74	Putative cobalt uptake protein	*Staphylococcus agnetis*
	75	Protein of energizing module of ECF transporter	*Staphylococcus hyicus*
	68	Protein of energizing module of ECF transporter	*Staphylococcus lugdunensis*
	44	Cobalt transporter	*Clostridium* spp.
	44	Membrane protein	*Streptococcus constellatus*
StbJ	100	ABC transporter	*Staphylococcus aureus* O11/O46
	69	ABC transporter	*Staphylococcus sciuri*
	67	ABC transporter	*Staphylococcus agnetis*
	67	ABC transporter	*Staphylococcus hyicus*
	58	ABC transporter	*Staphylococcus lugdunensis*
	49	ABC transporter, ATP-binding protein	*Finegoldia magna*
	48	ABC transporter	*Clostridium* sp.

StbK	99	Multidrug ABC transporter permease	*Staphylococcus aureus* O11/O46
	80	Multidrug ABC transporter permease	*Staphylococcus hyicus*
	80	Multidrug ABC transporter permease	Staphylococcus agnetis
	74	Multidrug ABC transporter permease	*Staphylococcus lugdunensis*
	67	Multidrug ABC transporter permease	*Staphylococcus pettenkoferi*
StbL	100	Multidrug ABC transporter ATP-binding protein	*Staphylococcus aureus* O11/O46
	82	Multidrug ABC transporter ATP-binding protein	*Staphylococcus sciuri*
	81	Multidrug ABC transporter ATP-binding protein	*Staphylococcus hyicus*
	76	Multidrug ABC transporter ATP-binding protein	*Staphylococcus lugdunensis*
	67	Multidrug ABC transporter ATP-binding protein	*Staphylococcus pettenkoferi*
StbM	100	Hypothetical protein	*Staphylococcus aureus* O11/O46
	49	Hypothetical protein	*Staphylococcus hyicus*
	37	Saccharopine dehydrogenase	*Staphylococcus lugdunensis*
	32	Oxidoreductase	*Streptococcus equi*
	31	Oxidoreductase	*Anaerococcus lactolyticus*
	24	Saccharopine dehydrogenase	*Brevibacillus laterosporus*
StbO	100	Salicylate synthase	*Staphylococcus aureus* O11/O46
	71	Salicylate synthase	*Staphylococcus hyicus*
	44	Salicylate synthase	*Desulfitobacterium hafniese*
	44	Salicylate synthase	*Clostridium termitidis*
	43	Salicylate synthase	*Clostridium saccharobutylicum*

Claire Raisen - January 2019

The *stbB* gene is predicted to encode a type II thioesterase similar to the thioesterase, YbtT, from the yersiniabactin synthesis cluster. StbB shared 68% and 63% amino acid identity with other staphylococcal species (*hyicus* and *lugdunensis*) and 55% aa identity with a predicted thioesterase in *Anaerococcus lactolyticus*. StbC is predicted to function as a 4'-phosphopantetheinyl transferase, the same as EqbC in *S. equi*. Similar to EqbC, StbC is lacking sequence homology to the 4'-phosphopantetheinyl transferase, YbtD, of the yersiniabactin locus (24% identity, 38% coverage). StbC shows 50% and 41% aa identity with *S. hyicus* and *S. lugdunensis*, and 38% aa identity with *Streptococcus anginosus.* StbD shares 70% aa identity with *S. hyicus* and 64% aa identity with *S. lugdunensis* and is predicted to encode a 2,3-dihydroxybenzoate (DHB) AMP ligase. StbD shares sequence identity (39% identity, 94% coverage) with the characterised YbtE salicylate-AMP ligase from *Y. pestis* (335) and 38% sequence identity and 95% coverage with DHB AMP ligase, DhbE from *B. subtilis* (336). StbE, encoded by the largest gene in the staphylobactin cluster (6018bp), is predicted to encode a non-ribosomal peptide synthetase (NRPS) with 61% and 54% aa sequence identity with *S. hyicus* and *S. lugdunensis*, and 45% with *Anaerococcus lactolyticus*. StbF and StbG show 62% and 61% aa identity with polyketide synthase. *stbF1* shows identity with other staphylococcal species, as well as *Lysinibacillus macroides* and *Streptomyces* spp. The sequence encodes a small protein made of 230 aa but currently has no predicted function using BLASTp. StbH, StbI, StbJ, StbK and StbL are predicted to be involved in transport. All five proteins show high sequence identity with *S. aureus* O11/O46, S. *hyicus* and S. *lugdunensis* species. StbH is predicted to encode a membrane protein, the same predicted function as EqbH, with sequence identity to *Clostridium beijerinckii*. StbI is predicted to encode a protein of energizing module of ECF transporter or a cobalt uptake/transporter protein. StbJ shows 69% aa identity with ABC transporters. StbK shared 80% aa identity with a multidrug ABC transporter permease from five different staphylococcal species; *S. aureus, S. hyicus, S. agnetis, S. lugdunensis and S. pettenkoferi*. StbL shared aa identity with the same staphylococcal species with 100 - 67% aa identity and is predicted to encode multidrug ABC transporter ATP-binding protein. StbM is predicted to encode an oxidoreductase, sharing 32% and 31% aa identity with EqbM of the equibactin gene cluster from *Streptococcus equi* and an oxidoreductase in *Anaerococcus lactolyticus,*

respectively. The staphylobactin gene cluster is missing EqbN, predicted to encode an α/β hydrolase, but encodes an extra gene, *stbO*. StbO is predicted to encode a salicylate synthase.

2.3.3.1 Identification of the staphylobactin locus in publicly available bacterial genome databases

S. aureus from many different lineages along with other bacterial genomes have been sequenced and are available in the public domain. BLASTp analysis of the whole staphylobactin region was carried out and found to be highly conserved in the *S. aureus* strains O11 and O46 (337). These two ovine strains induce severe (O11) and mild (O46) mastitis (338). Sequence analysis of the genomes showed that the two strains are closely related and cluster with isolates from the bovine CC130 lineage (339). The amino acid sequence of the staphylobactin locus shared the highest percentage sequence identity with coagulase-negative staphylococci (CoNS), *S. hyicus (*61% aa identity*)*, *S. lugdunensis* (54% aa identity) and *S. sciuri*. The staphylobactin locus shared 45% aa identity with *Anaerococcus lactolyticus* and 43% aa identity with the equibactin region of *S. equi.* BLASTn of the whole staphylobactin region from strain 71277 was carried out. *S. hyicus* strain ATCC 11249, had the highest sequence identity of 77% but only 46% coverage. All the other top hits were *S. aureus* strains with 78% identity but only 14% coverage. After taking a closer look at this region, two genes adjacent on the chromosome outside the staphylobactin locus had showed sequence homology with *stbL* and *stbK*.

2.3.4 Analysis of protein sequences of StbE and StbG, which are predicted to be a non-ribosomal peptide synthetase

BLASTp analysis of the staphylobactin locus predicts that StbE and StbG encode a NRPS. The number of NRPS modules and their domain organisation within the enzymes determine the structure of the final peptide product. Using analyses of the NRPS genes, these conserved features within the NRPS machinery allow prediction of the type, and sometimes the structure, of the synthesized polypeptide. It is therefore possible to investigate the biosynthetic potential of a given bacterium by analysis of the architecture of its NRPS gene clusters. A basic module of nonribosomal peptides and polyketides (PK) contains an initiation module consisting of adenylation (A) responsible

for amino acid activation, and peptidyl carrier protein (PCP) domains for thioesterification of the activated amino acid. This is followed by an elongation module consisting of a condensation (C) domain, which performs transpeptidation between the upstream and downstream peptidyl and amino acyl thioesters to elongate the growing peptide chain. Finally there is a chain-terminating thioesterase (TE) domain that is responsible for detachment of the mature polypeptide (181, 340, 341).

While carrying out BLASTp analysis on StbE, putative conserved domains were identified and classified as a NRPS, condensation and phosphopantetheine (PP)-binding domains. Using PKS/NRPS and Pfam analysis, StbE is predicted to be a protein of 2005 amino acids, containing one adenylation domain within a total of seven domains. The eight residues lining the A-domain binding pocket are DLYNFSLI. Further analysis using NRPSsp and LSI based A-domain function indicated that the StbE A-domain starts at position 572 and ends at position 971 in the protein, and its predicted substrate is cysteine. Sequences yielding significant alignments for the A-domain prediction included myxothiazol synthetase, epothilone synthetase, bacitracin synthetase, pyochelin synthetase (a siderophore produced by *P. aeruginosa*) and syringomycin synthetase. The seven domains are predicted to be phosphopantheine (PP), Cyclization (Cy), A, unknown, PP, Cy, PP (Figure 2.6). There may be one extra domain between the A and T domains at position 902 and 1381.

Figure 2.6. Organisation of modules and domains of StbE.
StbE is comprised of three phosphopantetheine (PP) attachment sites (Green), two condensation (C) domains (Red) and a AMP-binding enzyme (Blue).
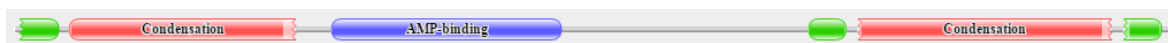
In the equibactin locus, EqbE is predicted to comprise of four carrier domains, three cyclization domains, an epimerisation domain, a single adenylation domain, a methyltransferase domain and a type I thioesterase domain.

StbG is 922 amino acids in length and is also predicted to have a Cy domain and a thioesterase domain (Figure 2.7). Using the NRPSpredictor2, the StbG A-domain is located between 702-847, with its binding pocket signature being DLYNFSLIWK and predicted substrate also being cysteine. This analysis shows similar domain order and structure to that found in equibactin NRPS.

Figure 2.7. Organisation of modules and domains in StbG.

StbG protein is comprised of a condensation domain (Green) and a thioesterase domain (Red).

2.3.5 Prediction of substrate specificity and conservation of the substrate binding pockets of A-domains in StbD and StbE

At the core of each module is an adenylation domain that recognises and activates the substrate by reaction with ATP. Critical residues in all known NRPS A-domains have been identified that align with eight binding-pocket residues in the gramicidin S synthetase (GrsA) A-domain, from *Bacillus brevis*, and define sets of highly conserved recognition templates. Prediction of the substrate specificity of A-domains of unknown function can therefore be predicted by analysing a set of 8–10 critical residues in the binding-pocket of the A domain (321, 342). In the equibactin and yersiniabactin biosynthetic clusters, the single A-domain in HMWP2/EqbE is probably responsible for the aminoacylation of all three different PCP domains (two in HMWP2/EqbE and one in HMWP1/EqbG) (296, 343). The sequences of 20 A-domains were retrieved from publicly accessible databases and aligned using the program SeaView (314) (Figure 2.8) and an update of the table from the Heather *et al.* (296) paper describing equibactin, including the staphylobactin proteins, StbD and StbE, for comparison (Table 2.4). Based on the structural data of DhbE and GrsA, residues conferring substrate specificity were identified (321, 336).

StbD

StbD shares 39% aa identity to the YbtE salicylate-AMP ligase (335), 38% sequence identity with 2,3-dihydroxybenzoate (DHB) AMP ligase, DhbE from *B. subtilis* (336), and 51% aa identity with the uncharacterised CKL_1504 of *C. kluyveri* (301). 38% aa identity with the pyochelin biosynthesis protein, PchD of *P. aeruginosa*, 36% aa identity with the enterobactin synthase component E, EntE from *E. coli* and 63% aa identity with LugD of *S. lugdunensis*. Based on the alignment of staphylobactin D (StbD) against these other A-domain containing proteins, StbD is predicted to activate salicylate as it shares the conserved cysteine (C) at residue 239, present in other NRPS systems that utilises salicylate. The conserved alanine (A) is also present at position 278 in StbD, which is changed to glycine in EqbD. At positions 322,330 and seen in EqbD, both residues are isoleucine (I) (Figure 2.8A and Table 2.4). These residues are conserved in StbD.

StbE

By comparison to the GrsA APhe prototype, StbE is predicted to mediate cysteine recognition, as it shares the critical residues lining the substrate-binding pocket of the A-domain with other A-domains that utilise cysteine, including the yersiniabactin synthetase; Irp2, bacitracin synthetase BacA, anguibactin synthetase AngR and pyochelin synthetases, PchE and PchF (321, 342, 343). Unlike EqbE, StbE has the conserved asparagine (N) at position 278, which is changed to aspartate in EqbE and LugE. The universally conserved Asn278 is presumably involved in substrate selection through interaction with the cysteine thiol. Other than a single change to phenylalanine from either leucine or methionine at position 299, StbE is conserved in all other residues in respect to other critical residues lining the A-domain binding pocket (Figure 2.8B and Table 2.4).

Figure 2.8: Alignment of staphylobactin homologues: (A) StbD in comparison to EqbD, DhbE, PchD, YbtE, EntE and LugD. (B) StbE in comparison to EqbE, AngR, PchE, BlmIV and LugE.

The predicted amino acid sequences of the StbD and StbE A-domains were aligned, using clustal method in SeaView, to A-domains or aryl-AMP ligase homologues with > 30% sequence identity to StbD or StbE (321). The key amino acids residues lining the substrate binding pockets of A-domains conferring substrate specificity are indicated with arrows and correspond to the same numbered residues in Table 2.4 (321, 336). StbD and StbE of *S. aureus*, EqbD and EqbE of *S. equi*, LugD and LugE of *S. lugdunensis*, DhbE of *B. subtilis,* PchD and PchE of *P. aeruginosa,* YbtE of *Yersinia* spp., BlmIV from *Streptomyces verticillus* and EntE from *E. coli*.

A
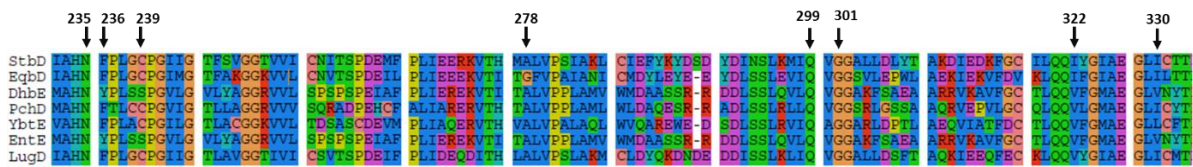
B

Table 2.4: Prediction of StbD and StbE A-domain substrate specificity

a. Protein name: Irp2 (HMWP2), yersiniabactin NRPS, *Y. pestis* (343); BacA, bacitracin NRPS, *Bacillus licheniformis* (344); AngR, anguibactin NRPS, *Vibrio anguillarum* (345); PchE and PchF, pyochelin NRPSs, *Pseudomonas aeruginosa* (346); CtaC, cystothiazole A NRPS, *Cystobacter fuscus* (347); MtaC, myxothiazol NRPS, *Stigmatella aurantiaca* (348); BlmIV, bleomycin NRPS, *Streptomyces verticillus* (349); DhbE, bacillibactin DHB-AMP ligase, *Bacillus subtilis* (336); EntE, enterobactin synthetase, *Escherichia coli* (350); MxcE, myxochelin DHB-AMP ligase, *S. aurantiaca* (351); VibE, vibriobactin DHB-AMP ligase, *Vibrio cholerae* (352); AngE, anguibactin DHB-AMP ligase, *V. anguillarum* (353); YbtE, yersiniabactin salicyl-AMP ligase, *Y. pestis* (343); PchD, pyochelin salicyl-AMP ligase, *P. aeruginosa* (346); SnbA, pristinamycin I 3-hydroxy-picolinic acid-AMP ligase, *Streptomyces pristinaespiralis* (354). LugE and LugD, *Staphylococcus lugdunensis* (331). Substrate: Activated amino acid by adenylation domain.

b. Amino acids at positions 239, 330 discriminate salicylate (Sal) from 2,3 –dihydroxy benzoic acid (DHB) (336). *S. equi* residues defined in bold differ from the consensus code of characterised substrate-activating proteins.

Accession number for the protein sequence from UniProtKB. EqbE predicted substrate is cysteine, however there is a change from asparagine to aspartate at residue 278. The critical residues lining the A-domain of StbE and StbD are highlighted in yellow.

Investigation of a novel iron-uptake system and other genomic features in *mecC* *Staphylococcus aureus*

| A-domain[a] | Substrate | Residue (according to A_{Phe} numbering) | | | | | | | | Accession number |
		235	236	239[b]	278	299	301	322	330[b]	
Irp2	Cys	D	L	Y	N	M	S	M	I	Q9Z399
BacA	Cys	D	L	Y	N	L	S	L	I	O68006
AngR	Cys	D	L	Y	N	M	S	M	I	P19828
PchE	Cys	D	L	F	N	L	S	L	I	Q9RFM8
PchF	Cys	D	L	Y	N	L	S	L	I	Q9RFM7
CtaC	Cys	D	L	Y	N	M	S	L	V	Q5MD35
MtaC	Cys	D	L	Y	N	M	S	L	I	Q9RFK9
BlmIV	Cys	D	L	Y	N	L	S	L	I	Q9FB18
LugE	Cys	D	L	Y	D	L	S	M	I	RS09450
EqbE	Cys?	D	L	Y	D	M	S	M	I	C0M6N2
StbE	Cys	D	L	Y	N	F *	S	L	I	
DhbE	DHB	N	Y	S	A	Q	G	V	V	P40871
EntE	DHB	N	Y	S	A	Q	G	V	V	P10378
MxcE	DHB	N	F	S	A	Q	G	V	V	Q9F638
VibE	DHB	N	F	S	A	Q	G	V	V	O07899
AngE	DHB	N	F	S	A	Q	G	V	V	Q5DK17
YbtE	Sal	N	F	C	A	Q	G	V	L	Q56950
PchD	Sal	N	F	C	A	Q	G	V	I	Q9RFM9
LugD	Sal	N	F	C	A	Q	G	V	I	EFU84524
EqbD	Sal?	N	F	C	G	Q	G	I	I	C0M6N3
StbD	Sal	N	F	C	A	Q	G	I	I	
SnbA	3-Hydroxy-picolinic acid	N	F	C	S	Q	G	V	L	P95819

* Phenylalanine present at position 299 in cyclosporin synthetase (CssA) in (321)

2.3.6 Proposed mechanism of staphylobactin biosynthesis

Greg Challis and Lijiang Song from the University of Warwick attempted to identify the metabolic product of the equibactin biosynthetic gene cluster in *S. equi*, but without success (296). Although the biological data indicated that the genetic locus predicted to produce equibactin appears to be involved in iron uptake, they were not able to show evidence of the action of a specific iron-chelating siderophore molecule. They attributed this to the fact that a key catalytic serine residue within the thioesterase domain of EqbG is mutated to alanine in *S. equi*, strongly suggesting the NRPS assemble line is impaired or even non-functional. So I addressed the question of whether the TE domain in the MRSA ortholog of EqbG carries the same serine to alanine mutation.

The biosynthesis of NRPs shares characteristics with the polyketide and fatty acid biosynthesis. Biosynthesis of a NRPs starts by the activation of the first amino acid with ATP by the A-domain and loaded onto the serine-attached 4'-phospho-pantethine (4'PP) sidechain of the PCP-domain, catalysed by the PCP-domain. The elongation is followed by the C domain catalysing the amide bond formation between the thioester group of the growing peptide chain from the previous module with the amino group of the current module. The extended peptide is then attached to the current PCP-domain. This cycle is repeated for each elongation module. Finally termination occurs by the release of the polypeptide chain from the PCP-domain through the activity of a terminal thioesterase (type I) (355), forming cyclic amides or cyclic esters. The final peptide is often modified by glycosylation, acylation, etc. The enzymes responsible are usually associated with the synthetase complex and their genes are organised in an operon or gene cluster.

In equibactin, the C-terminal domain of EqbG has similarity with type I thioesterases. However, sequence alignment of this domain to thioesterases in Pfam showed a serine to alanine mutation in the critical serine residue (GXSXG motif) of the predicted serine, histidine, aspartate catalytic triad essential for function (302, 356). Sequence alignment of the StbG, EqbG and LugG amino acid sequences was performed to see if StbG contained the same serine to alanine mutation as in EqbG which showed that StbG

possessed the critical serine residue (Figure 2.9). This suggests that the NRPS assembly line or staphylobactin is not impaired and should be functional.

Figure 2.9. Amino acid sequence alignment of StbG, EqbG and LugG.

The GXSXG motif circled with red circle. Black arrow points to serine (green) to alanine (blue) mutation in EqbG of the serine, histidine, aspartate catalytic triad. The predicted amino acid sequences of StbG, EqbG and LugG were aligned using clustal method in SeaView.

2.3.7 Other iron acquisition genes

S. aureus possesses several key iron acquisition loci that are well-characterised. It expresses an effective haem and haemoglobin acquisition system (Isd system), produces two carboxylate-type siderophores, staphyloferrin A and staphyloferrin B, and expresses transporters for many other siderophores that it itself does not synthesize (229, 357). The staphyloferrin A locus is conserved across all sequenced staphylococcal genomes, as is the *htsABC* operon encoding its ABC transport. The staphyloferrin B locus (*sirABC* and *sbnA-I*) was shown to be conserved in all the *S. aureus* isolates in our sequenced strain collection, regardless of the isolate having the staphylobactin locus (Figure 2.4). This suggests that the staphylobactin locus is not present to compensate for lack of other siderophores expressed by *S. aureus*, but may compliment their activity or be more beneficial in different niches or during the infection process. In contrast to *S. aureus*, *S. lugdunensis* makes neither staphyloferrin A nor staphyloferrin B, due to the absence of the SB gene cluster, and a large deletion in the SA biosynthetic gene cluster. This was confirmed when the staphyloferrin B biosynthesis and transport was included in the search of the CoNS in our strain collection. *S. lugdunensis* and *S. hyicus* which are the only two species to contain the staphylobactin locus in its entirety, were found to contain the homologous staphyloferrin B transport locus (*sirABC*) but not its biosynthetic

gene cluster (Figure 2.5). Other CoNS that did not contain the staphylobactin locus but did contain a homologous SB transport locus included *S. lentus*, *S. agnetis* and *S. schleiferi*. The only isolate shown to contain both the homologous *sirABC* and *sbnA-I* was *S. schweitzeri*. While carrying out sequence analysis of strains containing the staphylobactin locus, other key iron acquisition loci were found to be present, including the Isd and Fhu locus.

2.3.8 Genome mining

To identify secondary metabolite gene clusters, genome mining was carried out. Additional biosynthetic gene clusters found in the *S. aureus* CC130 genomes may contribute to its success and its ability to adapt to multiple species. Microorganisms produce a wealth of structurally diverse specialised metabolites with a remarkable range of biological activities. Previously, genome-mining approaches have been used to predict NRPs that are highly conserved within *S. aureus* (204). A widely used computational tool for biosynthesis gene cluster (BGC) identification is antiSMASH (antibiotics and secondary metabolite analysis shell) (358-362), allowing direct analysis of protein sequences and detection of additional classes of specialised metabolite BGCs (360). Scanning sequenced genomes using antiSMASH from our strain collection (C03 365, LGA251, 71277 and KO), S. *lugdunensis* strains (N920143 and HKU-09), *S. aureus* strains in the public domain (MRSA 252, Newman and USA300) and *S. equi 4047* led to identification of BGCs. All the *S. aureus* genomes searched, staphyloferrin A and staphyloferrin B were identified, and as expected not present in *S. lugdunensis or S. equi.*

2.3.8.1 Bacteriocins and microcins

Bacteriocins, which are antimicrobial peptides or proteins produced by bacteria that can enhance environmental adaption, were identified in all the *S. aureus* isolates from our collection (C03 365, LGA251, 71277 and KO), along with two in MRSA 252 and five in *S. equi*. Using BAGEL3 (BActeriocin GEnome mining tooL), a bacteriocin search software, analysis of the 71277 genome identified two putative bacteriocins or modified peptides. Terpene was identified in every staphylococci genome searched, along with lantipeptide

in LGA251, Newman and USA300. A single microcin was identified in *S. aureus* strains 71277 and KO, five were identified in *S. lugdunensis* strain N920143. Microcins are very small bacteriocins, for example microcins produced by commensal *E. coli* strains help outcompete pathogenic strains (363).

2.3.8.2 NRPS

A NRPS was identified in isolates C03 365, 71277, KO, MRSA252, Newman, USA300 and two in N920143. On closer inspection of the NRPS identified in the genome of 71277, the NRPS was identified as the Aureusimine BGC. These are not antibiotics, but function as regulators of virulence factor expression (204). antiSMASH analysis also led to the identification of an as yet undescribed NRPS gene cluster. This cluster contains 14 genes transcribed in the same direction in *S. aureus* isolates C03 365, 71277 and KO and orthologs are present in *S. lugdunensis* and *S. equi*, but is absent in other *S. aureus* isolates, MRSA252, USA300, Newman and LGA251. After taking a closer look, this was found to be the staphylobactin gene cluster. Gene neighbourhood alignments of this NRPS cluster were gathered using the Integrated Microbial Genomes-Joint Genome Institute (IMG-JGI) database (364) and the Gene Ortholog Neighbourhood Finder program (365). This identified this NRPS gene cluster in *S. aureus* strains O11 and O46 and *S. lugdunensis* strains UCIM6116, VCU139, VCU150, N920143, ACS-027-V-Sch2, VCU148, M23590 and HKU-09.

2.3.9 Regulation of the staphylobactin operon

Upstream of the *eqb* operon is a metal ion-dependent repressor, EqbA. Heather *et al.* (296) described the equibactin locus reporting that the *eqbA* gene showed 42% and 40% amino acid sequence identity with two putative repressors in *S. agalactiae* and *Clostridium kluyveri* (301, 366). It was also shown to be homologous to MntR, a manganese-dependent repressor, in *Bacillus subtilis*, and DtxR, an iron-dependent repressor in *Corynebacterium diphtheriae* (168, 367). An updated BLASTp of EqbA shows:

- 52% identity to a transcriptional regulator belonging to the MarR family in *Anaerococcus prevotti*.

- 48% identity to the iron-dependent repressor in *Streptococcus anginosus*.

- 48% identity to a transcriptional regulator in *Clostridium novyi*.

- 50% identity to an iron-dependent repressor in *Tissierellia bacterium*.

This provides further supporting evidence that EqbA acts as an iron-dependent repressor of the equibactin operon.

Sequence alignment of the equibactin locus and the staphylobactin locus shows that *S. aureus* is missing the homologous *eqbA* gene. To identify a potential regulator of the staphylobactin locus, tBLASTn of the EqbA sequence against a *S. aureus* strain C03 365 (CC130) was carried out and identified the protein with the highest identity (21/71 (30%) is MntR (SARLGA251_05680); a DtxR-like protein. MntR regulates the expression of the *mntABC* and *mntH* genes, which encode putative manganese transporters in *S. aureus* (290). Analysis of the region immediately upstream of *stbB* in strain C03 365 identified a 57 bp sequence 27 bp upstream of the *stbB* start codon that shares 29/57 (50.8%) nucleotide identities with the MntR box upstream of *mntABC* in *S. aureus*. MntR represses expression of the *mntABC* operon by binding to the MntR box upstream of *mntABC,* which contains a somewhat conserved DtxR box (Figure 2.10A) (368). The 49 bp inverted repeat region upstream of *stbB* overlaps the 36bp imperfect palindrome that is -73 bp to -38 bp upstream of *eqbB* (Figure 2.10B). It was previously shown using an electrophoretic mobility shift assay that the DNA palindrome is essential for EqbA binding to the *eqbB* promoter region (296).

Figure 2.10. Putative DtxR-like regulator of staphylobactin

(A) Nucleotide alignment of the six DtxR binding sites of staphylococcal species. Unique SNPs are highlighted in green. SNPs present in more than one other alignment are highlighted in blue. The 19bp DtxR consensus sequence is indicated below the alignment (368).

(B) An alignement of *S. epidermidis* SirR box (top) (369) with the *S. aureus* 5'-*stbB* sequence (bottom). The 49 bp conserved imperfect repeat and the putative ribosomal binding start and ATG translation start codon of *stbB* are shown. Conserved bases between the two alignements are highlighted in bold.

A

S. epidermidis	TATTATAAAATAAATTAGGTTAACCTAAACTTTTTATTA-GGAGGTTTTAATATTTG
S. aureus	TATTATACAATTAATTAGGTTAGCCTAAACTTTTAATTA-GGAGGTATAAACGTTTG
S. warneri	TATTATAAAATAAATTAGGTTAACCTAAACTTTTAATTA-GGAGGTATTAATATTTG
S. lugdunensis	TATCATGAAATAAATTAGGTTAACCTAAACTTTTTATTA-GGGGGTGTAAACATTT
S. saprophyticus	TATTATAAATTAAATTAGGTTAGCCTAAAAATATTATTAAGGAGGTTTAAAATGTTA
S. aureus 5'*stbB*	AATATTATCAAAAAATATTTAATCATACACCTATTGATA-ACCGTTATCAATAGGTG
DtxR consensus	**TTAGGTTAGCCTAACCTAA**

DtxR box

B

5'-T**ATT**AT**AAAAT**A**AAA**TTAGG**TT**AACC**TA**AA**C**TTTTT**ATT**A**GGA**G**GT**TTT**AATA**TTT**G** -3'

5'-GACACA**AT**ATT**A**TC**AA**AA**AA**ATA**TTT**AATCATACACCT**ATT**GAT**A**ACC**GTT**ATC**AATA**GG**TG**TATGATTAAAAA**TGTAAG**TTTTTATAA**ATG**-3'

49 bp imperfect repeat   RBS   →

Met

2.4 Discussion

2.4.1 Prevalence

Whole genome sequencing of our strain collection to investigate evolution and epidemiology of a novel zoonotic strain of MRSA was performed. From sequence analysis we noted most isolates from a specific MRSA lineage, CC130, have a region homologous to the equibactin synthesis loci in *S. equi*. This 23 kb region consisting of 14 genes, all in the same orientation, organisation and similar to that of the equibactin locus, was termed staphylobactin. The genes of this cluster show 54% aa identity and 64% nucleotide identity (43% coverage) with the equibactin locus and exhibit the potential to produce a siderophore. Three of the predicted polypeptides, StbE, StbF and StbG, share sequence similarity with predicted NRP and polyketide synthetases in other bacteria. The region encodes components of an ABC transporter, which has been proposed to transport the iron bound siderophore into the cell. The predicted StbH, StbI and StbJ proteins share similarity with membrane transporter proteins. The staphylobactin locus resides on the genome 3.9 kb downstream of the *mecC* gene and the sequences flanking the staphylobactin cluster were homologous with the *S. aureus* LGA251 genome, suggesting that the cluster was inserted into a conserved genomic region. These flanking regions were found in other *S. aureus* isolates, suggesting that gene sharing might have occurred via homologous recombination. Mobile genetic elements and plasmids can transfer important genetic elements, with the potential to facilitate adaption not only within one species but also between two closely related species, which has possibly happened with *S. aureus* and the other CNS containing the staphylobactin locus (370).

2.4.1.1 Identification of staphylobactin locus in our strain collection

There are a broad number of sequence types in our strain collection. However it should be noted that there is a sampling bias towards CC130 and CC425 *mecC* isolates associated with bovine hosts from a broad geographical area across Europe. When presenting the results for the prevalence of the staphylobactin locus in our strain collection, the number of strains belonging to each sequence type is given. For example,

all 408 strains in our collection belonging to ST130 contained the *stb* locus, but only one out of 200 strains belonging to ST425 contained this region. The phylogenetic tree gives an overall population structure of our strain collection, showing the prevalence of the *stb* locus. The staphylobactin locus is present in all *S. aureus* CC130 isolates from a diverse host species including bovine, human, cat and deer. With many of the strains isolated from mastitis and milk that are particularly low iron environments and nearly all isolates containing the staphylobactin locus coming from animals, this suggests an important role for staphylobactin in adaption of *S. aureus* to animal hosts. The region was also found sporadically in other isolates. In ST425, there are 200 strains in our collection with only one isolate containing the staphylobactin region, suggesting this was recently acquired. In all the *S. aureus* containing *stb*, there appears to be a direct repeat either end of the locus, allowing for the region to be gained or lost. *S. lugdunensis* was shown to contain the closest homologue to the staphylobactin region. In *S. lugdunensis*, there are no direct repeats found either side of the locus, suggesting that the lugdunbactin region is probably not mobile. In evolutionary terms, this gene complex probably started out in a non-*S. aureus* strain. Through random interactions with phages or plasmids, eventually this region got put into an ICE (self-transmissible mobile genetic element with predicted or demonstrated integrative and conjugative properties (371, 372)), which allowed it to transfer to other species. This is normally a key factor in the emergence of new pathogenic strains. The staphylobactin locus probably came from an ancestral strain such as *S. sciuri* or *S. xylosus,* where homologous staphylobactin genes have been identified (Figure 2.5).

The staphylobactin locus may contribute to the biological success of these strains, either by conferring a selective advantage through adaption of the strains to their specific environments. Or has some advantage in some ecological niches or it would be unlikely to be conserved, and where it has moved it has moved in its entirety. However, after searching all the available bacterial genomes in our sequence collection and in the public domain, staphylobactin was not identified in a large number of bacterial genomes. Other than equibactin found it *S. equi*, it is not a common gene cluster and is probably not highly valuable to the general success of the bacteria, as we would see it in more species. Therefore it seems likely to be of advantage for a particular ecological niche. There is

not much in the way of variation within the region, and it is highly conserved within sequence types. This is often the case through acquisition of antibiotic resistance mechanisms or the production of new fitness factors, such as the *mecA* and *mecC* genes that exist on mobile genetic elements (MGE), allowing them to jump in and out independently of the core genome. The *stb* locus was found in MSSA and *mecC* strains in our strain collection and BLAST identified the region in *mecA* strains O11 and O46, suggestion the *stb* locus was gained independently of the *mecA*/*mecC* genes.

2.4.2 Function

BLASTp analysis of the proteins produced by the staphylobactin cluster to predict their functions suggested that StbB encodes a thioesterase. Thioesterases are enzymes that optimise NRPS-dependent peptide siderophore synthesis (373), such as PchC thioesterase of the siderophore, pyochelin, in *Pseudomonas aeruginosa* (356). Though, the thioesterase*, YbtT,* has been found not to be necessary for both product release and full *in vitro* activity of the yersiniabactin assembly machinery (185).

StbC is predicted to function as a 4'-phosphopantetheinyl transferase. These proteins transfer the 4'-phosphopantetheine (4'-PP) moiety from coenzyme A (CoA) to the invariant serine in an acyl carrier protein (ArCP) and has been found to be essential for siderophore biosynthesis in *Aspergillus nidulans* (374). In *S. equi*, EqbC is predicted to activate the ArCP domain of EqbE and the peptide carrier domains (PCP) of EqbE and EqbG (296). StbC is highly likely to carry out the same function in Stb.

StbD is predicted to encode a DHB-AMP ligase. This enzyme participates in biosynthesis of siderophores by activating 2,3-dihydroxy-benzoic acid and has been shown to be involved in the biosynthesis of the siderophores; bacillibactin and enterobactin (375, 376).

StbE is predicted to encode a NRPS, similar to that of the iron acquisition yersiniabactin synthesis enzyme, Irp2. As previously mentioned in the introduction, NRPs are synthesised by one or more specialised NRPS enzymes via a ribosome- and mRNA-independent mechanism. The NRPs produced have a broad range of biological activates, including antibiotics and siderophores. The NRPS genes for a certain peptide are usually

organised in one operon, and this seems to be the case with the genes of the staphylobactin locus.

StbF and StbG shows aa identity with polyketide synthases (PKSs), including the yersiniabactin synthetase, thiazolinyl reductase component, Irp3, and the iron acquisition yersiniabactin synthesis enzyme (Irp2). PKSs are multi-enzyme complexes that are responsible for the biosynthesis of polyketides and are very similar to NRPS. Many metabolites are combinations of NRPs and polyketides, normally when the PK module follows the NRP module. For example, yersiniabactin synthesis occurs by a mixed NRPS/PKS mechanism. The Stb locus shows similarity to the yersinabactin locus, suggesting Stb could be synthesised via a similar mechanism.

StbF1 encodes a small protein that currently has no predicted function, and is possibly a gene remnant that does not have a function within the locus.

StbH-L encode components of an ATP-binding cassette transporter (ABC transporter). ATP-binding cassette transporters (ABC transporters) are members of a transport system superfamily present in a range of phyla from prokaryotes to humans (377). ABC transporters often consist of multiple subunits, one or two of which are transmembrane proteins and membrane-associated ATPases and can actively transport chemically diverse substrates across the lipid bilayers of cellular membranes. The ABC uptake system of staphylobactin is predicted to be involved in iron uptake by an iron-siderophore complex. While searching for the staphylobactin genes in our strain collection, as well as publicly available genomes. All *S. aureus* genomes searched contained genes homologous to *stbK* and *stbL* (76% and 75% identity) next to each other on the genome, not in association with the staphylobactin complex and are also predicted to encode components of an ABC transporter. This possibly occurred through recombination from a common ancestor and possibly carries out a different transport function.

StbM is predicted to encode an oxidoreductase. An oxidoreductase is an enzyme that catalyses the transfer of electrons from one molecule to another and usually uses NADP or NAD^+ as cofactors.

There is no *eqbN* homologue in the staphylobactin locus. EqbN was predicted to encode a putative α/β hydrolase, suggested to provide a potential mechanism for hydrolytic chain release similar to coelichelin (296). The *eqbN* gene was required for optimal production of the *eqb* NRPS product(s) by *E. coli* and induction of increased streptonigrin sensitivity in cross-feeding assays. However, absence of *eqbN* did not abolish production of the NRPS product(s) in this system suggesting that *E. coli* may produce an endogenous hydrolase that can complement the activity of EqbN. *S. aureus* may also produce an endogenous hydrolase, therefore abolishes the need of the *stbN* gene.

StbO is predicted to encode a salicylate synthase. All proteins encoded by the staphylobactin gene cluster, except StbF1, were putatively linked to siderophore production based on further analyses.

BLASTp analysis of the *stb* locus found *S. aureus* ovine strains O11 and O46 were highly conserved. These two strains induce severe and mild mastitis. Comparison of O11 and O46 revealed 103 truncated genes present in one strain or the other, corresponding to point mutations or indels causing frameshifts or leading to a premature stop codons. Among these truncated genes, 3% were involved in iron metabolism, *isdH*, *feoA* and *hrtB* (337). The *stb* locus was identified in a number of CoNS; *S. hyicus, S. lugdunensis and S. sciuri.* Compared to *S. aureus*, less is known about these strains when searching literature, especially about their iron acquisition systems. *S. hyicus* was originally isolated from skin infections in pigs. It is a known animal pathogen that can cause skin disease in cattle, horses and pigs. Both staphyloferrin A and staphyloferrin B siderophores have been isolated and characterised in *S. hyicus* (197, 203, 378). *S. sciuri* is also animal associated and commonly present on skin and mucosal surfaces of a wide range of pets, livestock and wild animals. *S. lugdunensis* seems to be the most widely studied of the CoNS in terms of iron acquisition systems. *S. lugdunensis* is commonly found as a normal constituent of the skin microbiota and able to cause a wide variety of infections, mainly human skin and soft tissue infections (SSTIs) but also infections of the blood and indwelling medical devices (379). Two genomes of *S. lugdunensis* strains, N920143 and HKU09-01 (330, 331) were released and allowed the identification of molecular determinants, including an Isd system for haem acquisition comparable with

S. aureus (333). Strain N920143 contains one of these Isd locus, however strain HKU09-01 contains a duplicated Isd locus (332). The Isd system of *S. lugdunensis* effectively facilitates the use of both haem and heamoglobin as iron sources *in vitro*, and may represent the preferred iron sources of this pathogen, as it is unable to synthesize endogenous siderophores (334). The staphylobactin locus shared 45% and 43% aa identity with *Anaerococcus lactolyticus* and the equibactin region of *S. equi.*

2.4.3 Substrate predictions

The staphylobactin NRPS has two A-domains that have conserved NRPS codes for cysteine and salicylate for all staphylococci containing the NRPS. In the equibactin locus, EqbD is predicted to activate salicylate since it shares the conserved cysteine at position 239, which is then transferred to the phosphopantotheine thiol of the ArCP domain of EqbE (Table 2.4). According to bioinformatics based predictions, StbD is predicted to activate salicylate, and also has the conserved alanine at position 278 (according to GrsA Aphe numbering), that is changed to glycine in EqbD. This differs from other NRPS systems that utilise salicylate. At positions 322 and 330 both residues are isoleucine, the same as EqbD. Evidence for the addition of salicylate is required for the production of equibactin due to salicylate being required for optimum production of equibactin in conditioned medium when expressed in *E. coli*. The single A-domain of EqbE is predicted to activate cysteine which is then transferred to the phosphopantotheine thiol of each of the three PCP domains of the NRPS (two in EqbE and one in EqbG). Unlike EqbE, StbE has the conserved asparagine at position 278, which is changed to asparate in EqbE. Other than a single change to phenylalanine from either leucine or methionine at position 299, StbE is conserved in all other residues in respect to other critical residues lining the A-domain binding pocket. Yersiniabactin is released from the last PCP domain by a C-terminal thioesterase domain. However, the C-terminal thioesterase domain of EqbG has the critical serine residue (S1092) of the predicted serine, histidine, aspartate catalytic triad essential for function (GXSXG motif) mutated to alanine. StbG does not contain this mutation and maintains the serine residue. This analysis adds weight to the notion that the staphylobactin cluster is likely to be functional.

2.4.4 Regulation

MntR, a DtxR-like regulator in *S. aureus* shares the greatest identity to EqbA. The *S. aureus* MntR protein controls manganese homeostasis by the regulation of the *mntABC* and *mntH* operons. Expression of *mntABC* is repressed in the presence of Fe^{2+} and Mn^{2+} ions by MntR (380), but functions as a positive regulator of *mntH*. MntR also regulates genes involved in manganese homeostasis in *B. subtilis* (168) and *E. coli* (381). The control of iron and manganese uptake in bacteria is mediated by members of the Fur and DtxR families (382-386). In *Treponema denticola*, the DtxR-like transcriptional regulator (TroR) is a manganese and iron-dependent transcriptional repressor (387). Therefore MntR in *S. aureus* is likely to play an important role in regulating both manganese and iron homeostasis in these isolates containing the staphylobactin region. DtxR controls expression by binding to a 19-bp operator sequences. DtxR-type metal ion-dependent repressors, present in many bacterial pathogens, not only regulate genes for iron acquisition but also regulate expression of virulence genes. For example the diphtheria toxin gene in *Corynebacterium diphtheriae* (388). The sequence 27 bp upstream of *stbB* contains a 57 bp sequence that is highly similar to consensus DtxR box sequences (389), suggesting that staphylobactin transcription is iron-regulated via the activity of the DtxR repressor protein. While carrying out antiSMASH analysis of the CC130 genomes, a region encoding an Arc family transcriptional regulator adjacent to the staphylobactin region was highlighted, representing a possible alternative promoter similar to that in *Yersinia pestis* that induces yersiniabactin synthesis or uptake of siderophore similar to that in *B. subtilis*. In *Yersinia pestis*, as well as Fur repression of the yersiniabactin operons, transcription is regulated by YbtA, a member of the AraC family of transcriptional regulators. YbtA in the presence of yersiniabactin induces yersiniabactin operons involved in yersiniabactin biosynthesis, iron-yersiniabactin uptake and salicylate synthesis, and the Fe-yersiniabactin receptor. In *B. subtilis,* Fur represses bacillibactin synthesis in iron-replete conditions. Btr, a member of the AraC regulators encodes an activator that binds to a conserved direct repeat promoter region and activates transcription of the siderophore uptake system (390). It is encoded adjacent to the uptake system. The 49bp imperfect repeat that is upstream of *stbB* could possibly be used to identify the regulator of the staphylobactin operon.

3 GENETIC MANIPULATION OF *MECC S. AUREUS*

Claire Raisen - January 2019

3.1 Introduction

Molecular genetic manipulation is required to study the function of genes in *S. aureus.* The gene of interest can be inactivated and mutants tested for function in phenotypic assays. To do this, directed mutagenesis by allelic replacement is used to alter the genome. Compared to laboratory strains of *E. coli*, and many other pathogens, *S. aureus* can be difficult to genetically manipulate because of its intrinsic restriction modification systems, low transduction frequency and low efficiency of recombination. Functional genomic analysis of *S. aureus* has been limited by an inability to genetically manipulate the majority of clinical isolates. Therefore, many methods and molecular tools have been developed by various researchers for genetic manipulation.

3.1.1 Restriction Modification Systems

The majority of *S. aureus* isolates have restriction modification (RM) systems consisting of enzymes that act like an immune system by attacking 'foreign' DNA entering the cell. This makes *S. aureus* difficult to genetically manipulate as it is necessary to be able to transform *S. aureus* with plasmids that have been constructed in *E. coli* strains, hindering exchange of DNA (391).

Restriction modification systems recognise the methylation status of DNA at their specific target sequence. Fully methylated DNA is recognised to be part of the host genome and hemimethylated DNA is recognised as newly replicated host DNA. The methylation status of the genomic DNA is maintained by the methyltransferase (MTase). Incoming unmethylated DNA, such as that of plasmid or bacteriophages, is recognised to be foreign and cleaved by the restriction endonuclease (REase).

Due to the strong restriction barrier present, this has limited mutagenesis to a small number of transformable laboratory strains such as Newman and 8325-4 that are amenable to genetic manipulation. The majority of *mecA* MRSA isolates mainly cluster into five clonal complexes (CC5, CC8, CC22, CC30 and CC45) of which until recently, only CC5 and CC8 had been successfully manipulated genetically.

3.1.1.1 Types of restriction modification systems

Restriction modification systems are currently classified into four different types (Type I-IV) based on their subunit composition, cofactor requirements, target recognition, and DNA cleavage mechanism.

Of these four known types only three are found in *S. aureus* and type I and IV are the most common.

3.1.1.1.1 Type I

Type I RM systems is encoded by three genes, *hsdR* (restriction), *hsdS* (specificity) and *hsdM* (modification) (392). The products of these genes form complexes. The specificity subunit (S) is required for recognition of a specific DNA sequence, the MTase subunit (M) catalyses DNA methylation and the REase subunit (R) is essential for DNA cleavage (392).

3.1.1.1.2 Type II

The majority of Type II RM systems consist of separate REase and MTase proteins that recognise the same DNA sequence. In *S. aureus*, the REase cleaves both DNA strands of the unmodified palindromic GATC sequence just 5' to the guanine nucleotides. The MTase protects DNA from cleavage by methylating the recognition site at the C5 position of cytosine (393).

3.1.1.1.3 Type IV

Type IV systems are modification-dependent REases that cleave DNA substrates only when their recognition sites have been modified. The REases recognise modified DNA with low sequence specificity. The Type IV system in *S. aureus* is SauUSI (394). It comprises of SauUSI endonuclease, which specifically recognise cytosine methylated DNA. SauUSI, which is highly conserved in *S. aureus*, has been found to be a major barrier for DNA uptake in *S. aureus*.

3.1.1.2 Overcoming the restriction barrier

To overcome this barrier, *E. coli* and *S. aureus* strains have been developed as cloning intermediates. Passage of DNA through these strains modifies the foreign DNA to resemble the hosts before being transferred into the recipient *S. aureus* strain.

3.1.1.2.1 Bypassing type I RM using RN4220 *S. aureus*

S. aureus strain RN4220 is deficient in type I restriction HsdR but retains the modification function.

It was obtained by extensive chemical mutagenesis of strain 8325-4 (395). RN4220 has been used extensively as an initial recipient before transferring DNA to other strains. However, many strains accept DNA isolated from RN4220 at an extremely low frequency and is therefore only useful for a small subset of closely related strains.

3.1.1.2.2 Bypassing type IV RM using DC10B *E. coli*

Monk *et al*. (396) created a DNA cytosine methyltransferase (*dcm*) deletion mutant in the high efficiency *E. coli* cloning strain DH10B (called DC10B). The absence of cytosine methylation allows plasmid DNA to bypass the type IV restriction system, but not type I. Plasmids isolated from DC10B can be directly transformed into clinical isolates of *S. aureus* without the need for using RN4220 as the intermediate host. DNA isolated from DC10B was introduced into 15 strains from different clonal complexes that were chosen to represent a diverse selection of *S. aureus* lineages and cover laboratory and clinically relevant clonal complexes.

3.1.1.2.3 Bypassing type I RM from CC8 and CC30 using SA30B or SA08B *E. coli*

E. coli strain DC10B was modified to express either CC8 or CC30 type I RM genes *HsdMS* to create strains SA08B and SA30B (397). These strains mimic the clonal complex specific methylation profile, improving the transformation rate further of *S. aureus* isolates of clinical importance. CC8 strains include USA300, Newman and 8325-4 and CC30 isolates include strains MRSA252 and Cowan.

3.1.2 Electroporation of *S. aureus*

Electroporation is the method of choice for introducing plasmid DNA into *S. aureus* cells, as this process is approximately ten times more effective than chemical transformation.

3.1.2.1 Preparation of electrocompetent *S. aureus*

The most widely used protocol involves the growth of cells into early logarithmic phase followed by washing with a hypertonic buffer (e.g. 500 mM sucrose) to remove salts and stabilise the cells (398). The cells are concentrated to 1-3 x 10^{10} CFU/ml and purified plasmid DNA added. A defined electric pulse is discharged through the cells to facilitate the uptake of DNA. The cells are then grown in broth for a short period of time to allow recovery and for the growth to begin prior to plating on media containing an antibiotic that selects for the plasmid containing transformants. In 2012, Monk *et al.* (396) modified an electroporation protocol published by Löfblom (399) that gave a 50-fold improvement in the transformation frequency over the sucrose wash method, showing that improving the efficiency of electroporation allowed the type IV and type I RM systems to be bypassed. The protocol was modified as follows: Preparing electrocompetent cells by washing in water then 10% glycerol. Treatment of cells prior to electroporation. Recovery of transformed cells after electroporation.

3.1.3 Plasmids

The majority of methods for inactivating a chromosomal gene exploit the cells' homologous recombination machinery. A wide variety of naturally occurring and engineered plasmids have been developed for genetic manipulation of the *S. aureus* genome. These plasmids have different characteristics including different copy numbers and temperature sensitivities. Clinically isolated *S. aureus* are often resistant to several antibiotics, therefore multiple gene deletion in a strain may be extremely difficult due to the limited number of available antibiotic markers. In order to overcome this problem, drug-resistant markerless gene deletion vectors have been developed. These include *E. coli/S. aureus* shuttle vectors such as pMAD (400), pKOR1 (401), pIMAY (396), that can be used to facilitate the construction of mutations by allelic exchange in the *S. aureus* genome. These plasmids normally encode for replication in both *E. coli* and *S.*

aureus and a multiple cloning site to facilitate insertion of DNA sequences. For selection within the host strain, these plasmids carry genes conferring antibiotic resistance markers and are temperature sensitive. The plasmids used in this study are described below.

3.1.3.1 pMAD

Arnaud *et al.* (400) created the allelic replacement vector, pMAD. A temperature sensitive plasmid that was made by joining pE194ts to pBR322 with the addition of a *bgaB* gene encoding a constitutively expressed thermostable β-galactosidase (Figure 3.1). This enables the use of a blue-white colony assay on plates containing X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) to identify plasmid free bacteria. Plasmid free colonies appear white, while colonies that retain the plasmid appear blue.

Figure 3.1. Physical map of the pMAD plasmid

Figure from (400). pMAD plasmid consisting of a multiple cloning site, with unique restriction sites indicated bottom left. The *bgaB* gene encoding a thermostable β-galactosidase. Antibiotic resistance genes, *ermC* and *bla*, for the selection of the plasmid.
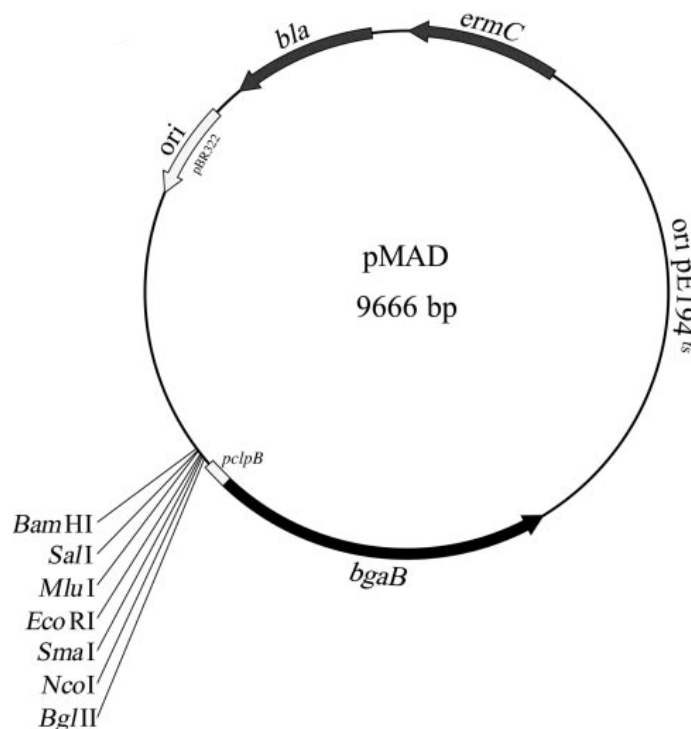
3.1.3.2 pIMAY

pIMAY was developed by Monk *et al.* (396). It has a highly expressed chloramphenicol resistance (*cat*) gene which allows selection as a single copy when integrated into the chromosome. It has the pVWO1ts replicon, which is highly temperature sensitive in Staphylococci. The replicon is functional at the permissive temperature (30 °C), but the plasmid cannot replicate at the restrictive temperature of 37 °C, allowing plasmid integrants to be selected. The plasmid also carries the tetracycline-inducible *secY* antisense counterselection determinant (Figure 3.2).

Figure 3.2. Genetic map of pIMAY

Figure from (396). Temperature sensitive plasmid pIMAY consists of the highly expressed *cat* gene, the temperature sensitive replicon repBCD and the tetracycline-inducible antisense *secY* region.
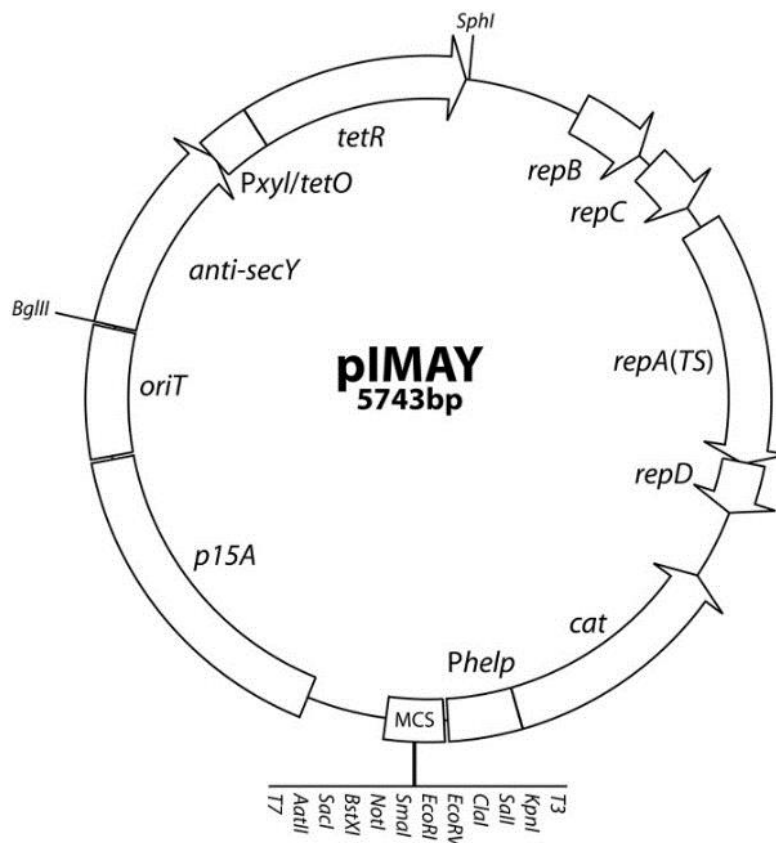
3.1.3.3 Steps for genetic manipulation

1. The procedure for creating a mutation is an integration and excision process represented in Figure 3.3.

2. Plasmids carrying the mutational cassette are introduced into *S. aureus* by electroporation, and the bacteria are grown at the permissive temperature for replication, selecting for the phenotype conferred by the marker that resides on the plasmid.

3. Integration by a single crossover (SCO) event at either the upstream or downstream region for homology is selected by growing at a non-permissive temperature for replication.

4. Plasmids that have integrated into the chromosome are selected for antibiotic resistance encoded by the plasmid.

5. Bacteria are then grown at the permissive replication temperature without antibiotic selection. This allows for excision of the plasmid by a SCO event and loss of plasmid.

6. This results in either a wild-type or mutant strain which can be verified by PCR.

Figure 3.3. Allelic exchange in *S. aureus*

Figure adapted from (391). The two-step approach (integration/excision) is shown above for the creation of directed mutations. A deletion construct consisting of fragments flanking the gene of interest is assembled in the multiple cloning site of a plasmid and then transformed into the target strain at a temperature permissive for replication. A temperature shift, to one non-permissive for plasmid replication (in the presence of selection for antibiotic resistance encoded by the plasmid), stimulates integration through either the upstream (AB) or downstream (CD) region of cloned homologous DNA. Decreasing the temperature and removing antibiotic selection stimulates replication which leads to vector excision. If the plasmid integration event occured through the AB side, plasmid excision through the AB side recreates the wild-type locus, while CD excision creates a mutated gene (shown in figure).

The different allelic exchange plasmids developed contain additional features which aid in discrimination of colonies lacking the plasmid post excision. (a) pMAD: constitutive *bgaB* for the detection of white colonies on agar containing X-Gal, (b) pIMAY: ATc inducible secY antisense to repress growth of plasmid-containing strains.
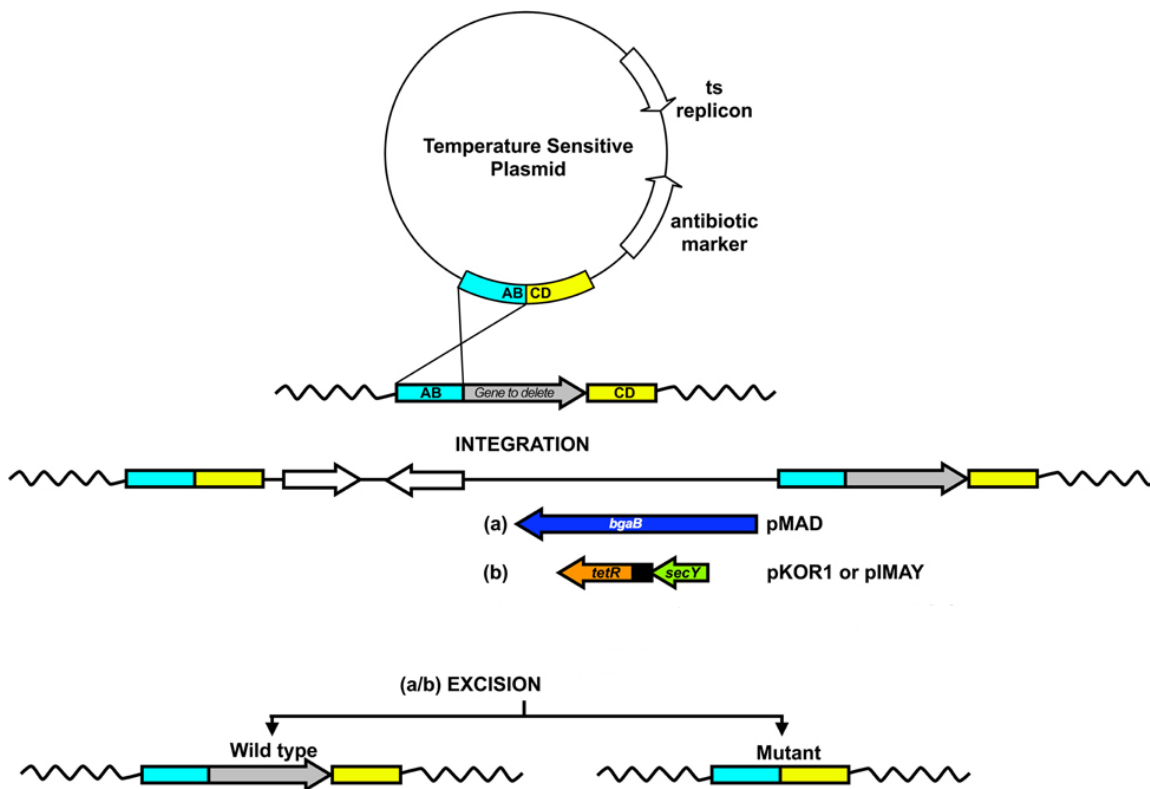
3.1.4 Objectives

The aim of this work was to create gene deletion mutants in the *mecC* CC130 *S. aureus* strains that had previously not been genetically manipulated, by developing and optimising a method using the current tools and techniques available. Knocking out the genes of the staphylobactin region, along with other genes involved in iron acquisition, will allow for testing of the CC130 strains in phenotypic assays in order to determine the function of the staphylobactin locus.

Claire Raisen - January 2019

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids and growth media

Bacterial strains and plasmids used are described in Table 3.1. DNA was extracted using Epicenter MasterPure Gram Positive DNA Purification Kit according to the manufacturers protocol and diluted to 50 µg/ml for PCR amplification. The identification of *S. aureus* strains were confirmed by plating onto DNase agar methyl, Brilliance MRSA2 and Brilliance Staph 24 Agar plates (Oxoid, UK) and tested using Staphylase Test for agglutination (Oxoid, UK). Oligonucleotides were purchased from Sigma-Aldrich. Primers were designed using PrimaClade (primaclade.org) to include the desired restriction sites and additional bases to facilitate direct cloning or splicing by overlap extension (SOE) PCR (Table 3.1). For routine culture, *E. coli* was grown in Luria Broth (LB) or on LB agar (Oxoid, UK) at 37 °C, shaking at 200 rpm. *S. aureus* was grown on Tryptone Soya Agar (TSA) or Brain Heart Infusion Agar (BHI) or in Tryptone Soya Broth (TSB) (Oxoid, UK) shaking at 200 rpm, incubated at 28 °C or 37 °C accordingly.

Bacterial stocks were stored in glycerol at -80 °C. Antibiotics were purchased from Sigma-Aldrich and used in the following concentrations as appropriate: ampicillin (100 µg/ml) or chloramphenicol (10 µg/ml) for *E. coli*; erythromycin (5 µg/ml) or chloramphenicol (10 µg/ml) for *S. aureus*. For colorimetric blue-white screening of bacteria, 40 µl of X-Gal (5-bromo-4-chloro-3-indolyl--D-galactopyranoside; Melford) (200 mg/ml in dimethyl sulfoxide (DMSO)) was spread onto agar plates. All solutions and media were made with water purified through a Milli-Q water purification system (Millipore).

Table 3.1. Strains, plasmids and oligonucelotides used in this study.

Strain/Plasmid	Description	Source or Reference
E. coli strains		
DC10B	Δ*dcm* in the DH10B background; Dam methylation only	(396)
TOP10	Host for DNA cloning	Invitrogen
C2984	Turbo Competent *E. coli* cells	New England Biolabs
SA08B	DC10B expressing *hsdMS* genes from CC8	(397)
SA30B	DC10B expressing *hsdMS* genes from CC30	(397)
S. aureus strains		
LGA251	ST425, *mecC* reference strain	(67)
Enr. 7594/1975	ST130, *mecC* clinical isolate	This study
MS-18.31	ST130, *mecC* clinical isolate	This study
8572	ST130, *mecC* clinical isolate	This study
08-02004	ST130, *mecC* clinical isolate	This study
2383/03	ST130, *mecC* clinical isolate	This study
C03 365	ST130, *mecC* clinical isolate	This study
71277	ST130, *mecC* clinical isolate	(67)
71277Δ*sfa*	71277 with *sfa* deletion	This study
71277Δ*sbn*	71277 with *sbn* deletion	This study
71277Δ*sfa*Δ*sbn*	71277 with *sfa* and *sbn* deletion	This study
71277Δ*stbE*	71277 with *stbE* deletion	This study
71277Δ*stbHIJ*	71277 with *stbHIJ* deletion	This study
71277Δ*stbE-O*	71277 with *stbE-O* deletion	This study
71277Δ*stbB-O*	71277 with *stbB-O* deletion	This study
71277Δ*stbE*Δ*stbHIJ*	71277 with *stbE* and *stbHIJ* deletion	This study
71277Δ*stbE*Δ*stbHIJ*Δ*sfa*Δ*sbn*	71277 with *stbE*, *stbHIJ*, *sfa* and *sbn* deletion	This study
71277Δ*stbE-J*	71277 with *stbE-J* gene deletions	This study

71277ΔstbE-JΔsfaΔsbn	71277 with stbE-J, sfa and sbn gene deletions	This study
71277ΔstbB-OΔsfaΔsbn	71277 with stbB-O, sfa and sbn gene deletions	This study
RN4220	Restriction-defective derivative of 8325-4 ST 8	(395)
Plasmids		
pMAD	Shuttle vector for generating gene inactivation mutants	(400)
pCR2 TOPO	Cloning vector	Invitrogen
pCR4 TOPO	Cloning vector	Invitrogen
pLI50	E. coli - S. aureus shuttle vector; Ap^R/Cm^R	(402)
pLI50stbE	pLI50 with stbE gene from 71277	This study
pLI50sfa	pLI50 with sfa gene from 71277	This study
pLI50sbn	pLI50 with sbn gene from 71277	This study
pRMC2	E. coli - S. aureus shuttle vector, Amp^R, Cm^R	(403)
pXB01	E. coli - S. aureus shuttle vector pRMC2 with bla deletion; Cm^R	(404)
pXB01stbHIJ	pXB01 with stbHIJ genes from 71277	This study
pIMAY	pIMC5 with tetracycline inducible secY antisense from pKOR1; Cm^R	(396)
pIMAYΔstbE	Modified pIMAY for deleting stbE gene	This study
pIMAYΔstbHIJ	Modified pIMAY for deleting stbHIJ genes	This study
pIMAYΔstbE-J	Modified pIMAY for deleting genes stbE-J	This study
pIMAYΔstbE-O	Modified pIMAY for deleting genes stbE-O	This study
pIMAYΔstbB-O	Modified pIMAY for deleting genes stbB-O	This study
pIMAYΔsfa	Modified pIMAY for deleting gene sfa	This study
pIMAYΔsbn	Modified pIMAY for deleting gene sbn	This study
Primers		
ΔstbE_A	ATAT<u>GGTACC</u>TTTATGAGACGCAAGGAAAGC	KpnI
ΔstbE_B	GCTAAACTCCCCTTTCCAATTCAATAAG	
ΔstbE_C	<u>GAAAGGGGAGTTTAGC</u>AACATGAAGAAGTGCATAGTTTGTGG	
ΔstbE_D	ATAT<u>GAGCTC</u>AACCTGGTATGGTCCTTCTTCTC	SacI

Δ*stbE*_outF	CGGCCAAAGATATTGAAGACA	
Δ*stbE*_outR	TTCTTCGGAAATAACACTTGGC	
Δ*stbHIJ*_A	ATAT<u>GGTACC</u>TATAGGATATTGTTCCGGGGG	KpnI
Δ*stbHIJ*_B	ATTACTAACTCCTATTCATTAC	
Δ*stbHIJ*_C	AATAGGAGTTAGTAATATTGTGTTGAAGAAAGGAGCC	
Δ*stbHIJ*_D	ATAT<u>GAGCTC</u>CGATGCTACTTGGTGCTCAT	SacI
Δ*stbHIJ*_outF	GCGGAGCTTAAAAAGGATGATT	
Δ*stbHIJ*_outR	GCTGAAACCAAATACATGCCTAC	
Δ*stbEJ*_C	GAAAGGGGAGTTTAGCATTGTGTTGAAGAAAGGAGCC	
Δ*stbEO*_C	GAAAGGGGAGTTTAGCGGTTAATTTATTTAGGTGAAGC	
Δ*stbEO*_D	ATAT<u>GAGCTC</u>GCCGTGATGAACTGAAATCAT	SacI
Δ*stbEO*_outR	GCTAAACTCCCCTTTCCAATTCAATAAG	
Δ*sfa*_A	ATAT<u>GGTACC</u>GCAATACCAACTGGTTTAACGTC	KpnI
Δ*sfa*_B	TATATTAATCAATAAGTCTAAG	
Δ*sfa*_C	TAGACTTATTGATTAATATAAAATTATAGAATTTTATTAATC	
Δ*sfa*_D	ATAT<u>GAGCTC</u>TTGGGTATATGAAAGACCGTAAGC	SacI
Δ*sfa*_outF	GCAACAACTGCTGGAAGCAC	
Δ*sfa*_outR	GTTTCCTAGCGTTTCTATTAATCGC	
Δ*sbn*_A	ATAT<u>GGTACC</u>GCGACAATTAACTCCGGTTTT	KpnI
Δ*sbn*_B	AAAGCGCTTCCTCCTCAAATTTAAA	
Δ*sbn*_C	GAGGAGGAAGCGCTTTGTTTTACTGTGATGTTGAGGG	
Δ*sbn*_D	ATAT<u>GAGCTC</u>TTTTCCTCATGCTCATTCCC	SacI
Δ*sbn*_outF	CCAGCATATCCACCAGCAT	
Δ*sbn*_outR	GCAGCAATAATCGCATATACA	
IM151 (pIMAY MCS F)	TACATGTCAAGAATAAACTGCCAAAGC	(396)
IM152 (pIMAY MCS R)	AATACCTGTGACGGAAGATCACTTCG	(396)
pMAD stbE_A	AGTC<u>GGATCC</u>CAGGTGTTGAATTTCAAAGAGAGATAAGAGCG	BamHI
pMAD stbE_B	GTTCTAAATGACACCTTCTTCGTTTGC	
pMAD stbE_C	AGGTGTCATTTAGAACCTATGTATAGAGTAGGTCATTTGCAATATAAAGTG	

pMAD stbE_D	AGTCCCATGGACGTCATATGATTAGTTAATCTAAATAATTCGCC	NcoI
pMAD stbHIJ_A	AGTCGGATCCGAGAATATTGAAAACGCAAGTTATGTTGCG	BamHI
pMAD stbHIJ_B	GAAATTTGATAAAAATTCAAACGAGACCCGCCTTTTTGAAATG	
pMAD stbHIJ_B1	GAAATTTGATAAAAATAATTCAGTTGGCGAATTCAAACGAGACCCG	
pMAD stbHIJ_C	ATTTTTATCAAATTTCAATGATAAAGGAGGATACC	
pMAD stbHIJ_D	AGTCCCATGGCACCAATTAACGCTAACAATGAGACCTCCC	NcoI
T7F	TAATACGACTCACTATAGGG	Sequencing of SOE insert
M13R	CAGGAAACAGCTATGACC	Sequencing of SOE insert

3.2.2 PCR Amplification of homologous regions for cloning

PCR was performed upstream and downstream of the target gene to be deleted. PCR reactions were performed in 50 µl. Where required PCR reactions were optimised using a Biometra T Gradient PCR machine. When using pMAD for gene deletion, Velocity High Fidelity DNA Polymerase (Bioline) was used on genomic DNA from strain C03 365. When using pIMAY for gene deletion, PCR was performed using KOD Hotstart DNA polymerase (Novagen) or Phusion DNA polymerase (Finnzymes) on genomic DNA from strain 71277.

3.2.3 DNA visualisation and purification

Agarose gels between 1%-2% w/v were used depending on the size of the product (UltraPure Agarose, Invitrogen) and 1 x TBE buffer (Crystal 10xTBE Buffer, Bioline) was used for gel electrophoresis. DNA was stained using SYBR Safe DNA gel stain (Invitrogen) within the agarose gel and visualised with a UVP UV Transilluminator or a BioRad Gel Doc camera system. For size analysis, either Hyperladder IV (1 kb) or Hyperladder I (10 kb) was used (Bioline). DNA samples were mixed with 6x Blue/Orange loading dye (Promega). Electrophoresis was performed at 100-120 V for 45 mins-1hr depending on

the degree of resolution required. DNA bands of interest were excised from the gel with a scalpel and purified using a QIAquick Gel Extraction Kit (Qiagen) and Qiagen MinElute PCR Purification Kit (Qiagen) according to the manufacturer instructions or using the Gel/PCR DNA Purification Kit (YORBIO).

3.2.4 Splicing by overlap extension (SOE) PCR

Fusion of PCR products was either carried out using Velocity DNA Polymerase (Bioline) or Accuprime Pfx (Invitrogen) followed by the addition of 1μl BIOTAQ DNA Polymerase (Bioline) and 1μl dNTPs (Bioline) for 10 min at 72˚C for the addition of an 'A' overhang for integration into TA cloning vector (pCR2 or pCR4). For direct cloning into pIMAY, KOD Hot Start DNA Polymerase (Merck, UK) was used for SOE-PCR of products. For performing SOE-PCR, the Master Mix consisted of 5 μl KOD buffer (5X), 5μl dNTPs, 3 μl $MgSO_4$, 0.7 μl KOD enzyme and 26.4 μl Milli-Q water. Template DNA (5μl, 2.5 AB, 2.5μl CD PCR product) was added and SOE-PCR was run for 10 cycles under the following conditions: 94 °C for 5 min; 10 cycles of 94°C for 30 s, 60°C for 1.5 min and 72 °C for 2.5 min. An extra step of 10 °C for 5 min was added in the program for adding 2.5 μl primer A and 2.5 μl primer D in the reaction tube. SOE-PCR then was continued under the following conditions: 95 °C for 2 min; then 35 cycles of 95 °C for 20 s, 59 °C for 10 s and 70 °C for 50 s; and one final extension step of 70 °C for 5 min.

3.2.5 DNA Restriction digests

Restriction enzymes from New England Biolabs were used and reactions were performed according to the manufacturer's instructions in 50 μl volumes. DNA was digested at 37 ˚C for 2 hours followed by heat inactivation for 10 min at 65˚C. Digests were separated by gel electrophoresis. The bands containing the insert and linearized plasmid were extracted and purified.

3.2.6 Plasmid isolation and purification

Plasmid DNA was isolated from *E. coli* using the QIAprep Spin Miniprep Kit (Qiagen), following the manufacturer's guidelines. To isolate plasmids from *S. aureus*, cells were incubated for 30 min at 37˚C in P1 buffer containing 50 mg/ml lysostaphin prior to addition of lysis buffer P2 before proceeding with extraction using QIAprep Spin

Miniprep Kit (Qiagen) as per the manufacturer's guidelines. *S. aureus* plasmid DNA was also isolated by QIAprep Spin Midiprep Kit, HiSpeed Plasmid Midi Kit (Qiagen) or NucleoBond PC500 Kit using the AX500 Columns (Macherey-Nagel) following low-copy plasmid purification protocol. Plasmid DNA was concentrated by ethanol precipitation (with Pellet Paint, Millipore) or by a rotating evaporator. DNA quantity and purity were determined by optical density measurements using a NanoDrop spectrophotometer (Thermo Scientific). The plasmid DNA was eluted in sterile water to reduce the conductivity and minimise the risk of arcing caused by salt contaminants in the electroporation step.

3.2.7 Dephosphorylation and purification of vector

pMAD was dephosphorylated to increase the efficiency of ligation with an insert. pMAD was treated with alkaline phosphatase (Roche) using 1 unit per µg of vector DNA. This was carried out immediately after digestion, by incubation with alkaline phosphatase at 37°C for 1 hour followed by inactivation at 70°C for 10 min. Purification of the dephosphorylated cut pMAD vector was carried out using a standard Phenol/Chloroform extraction protocol, followed by concentration of the sample by ethanol precipitation.

3.2.8 DNA Sequencing

DNA sequencing of the insert was carried out by Source Bioscience. Sequence data was analysed using FinchTV and BLAST. Primers used for sequencing are T7F and M13R for pCR4 TOPO plasmid and IM151/IM152 for sequencing of the insert in pIMAY.

3.2.9 Ligation reaction

Ligation reactions were performed using T4 DNA Ligase (Thermo Scientific) according to the manufacturers instructions, incubated at 16°C overnight and heat inactivated at 65˚C for 10 min.

3.2.10 Colony PCR of positive clones

A colony PCR with MCS primers was used to confirm the presence of replicating plasmid.

For screening of plasmids in *E. coli* cells, MyTaq DNA Polymerase (Bioline) was used by mixing a very small amount of cells with the PCR master mix and cycling at the following conditions. 95 °C 5 min, 35 cycles (95 °C- 15 s, 58°C – 10s, 72°C 30s), 72°C for 10 min. For *S. aureus* cells, a pipette tip was touched on a single colony and a very small amount was mixed with 3 µl of water in a PCR tube. 47 µl of Phire Hot Start II DNA polymerase (Thermo Fisher) master mix was added to the PCR tube and PCR was carried out with the following cycling conditions. 95 °C 10 min, 98 °C 30 s, then 35 cycles (98°C- 10s, 59°C – 10s, 72°C 20s), 72°C for 5 min.

3.2.11 Preparation of *E. coli* chemical competent cells

E. coli cells were made heat-shock competent by growing a single colony in LB overnight at 37 °C, shaking at 200 rpm. The overnight culture was inoculated 1:2000 into LB and grown at 37 °C, 200 rpm until they reached an OD_{600} of 0.4. Cells were pelleted at 3,000 x g, 4 °C for 10 min. The supernatant was removed and the bacterial pellet was washed with 100 mM ice cold $MgCl_2$ and centrifuged (3,000 x g, 4 °C, 10 min). The pellet was washed in 100 mM ice cold $CaCl_2$, incubated on ice for 10 min and centrifuged as above. Cells were resuspended in 4 ml of ice cold 100 mM $CaCl_2$ in 15% glycerol (w/v) and frozen at -80 °C in 50 µl aliquots.

3.2.12 Heat shock transformation of *E.coli*

50 µl of chemically competent *E. coli* were defrosted on ice, 10 µl of plasmid DNA was added and incubated on ice for 30 mins. The cells were placed in a heat block at 42 °C for 45 seconds, and then immediately returned to ice for 2 mins. 1 ml of SOC or LB was added and cells were incubated at 37 °C for 1 hour, 200 rpm. The cells were plated on selective plates and grown overnight.

3.2.13 Preparation of electrocompetent *S. aureus* and electroporation (Oskouian and Stewart)

This was done according to the method adapted from Oskouian and Stewart (398). *S. aureus* was grown in TSB for 16 hrs at 37 °C shaking at 200 rpm. This was diluted 1:100 into 100 ml LB, incubated at 37 °C, 200 rpm until OD_{450} reached 0.2. Culture was stood

on ice for 10 min, followed by a 10 min centrifugation at 3000 xg, 4 °C to pellet bacteria. The bacterial pellet was washed twice in 10 ml ice cold 500 mM sucrose. The pellet was resuspended in sucrose and snap frozen. For electroporation, 5 µl of plasmid DNA was added to 200 µl electrocompetent *S. aureus* cells in a 0.2 cm cuvette and pulsed at 2.5 kV, 25 µF and 400 Ω with a Bio-Rad MicroPulser (Bio-Rad Lab) followed by the addition of 1 ml TSB. This was incubated at 37 °C for 1 hour and plated on X-Gal selective plates and incubated overnight at 37 °C.

3.2.14 Preparation of electrocompetent *S. aureus* and electroporation (McNamara)

This was done according to the method by McNamara (405). A single colony was grown overnight in TSB. 4 ml of overnight culture was added into 200 ml TSB and grown to an OD_{660} of 0.4 at 37 °C, 200 rpm. The culture was centrifuged at 5,000 xg for 20 min at 4 °C and the pellet was resuspend in 200 ml ice-cold 0.5 molar (M) sucrose. Centrifugation was repeated. The pellet was resuspended in 100 ml 0.5 M sucrose and stood on ice for 30 min. Cells were centrifuged as above. 20 ml ice cold 0.5 M sucrose was added and centrifugation was repeated as above. The cells were resuspend in 300 µl ice cold 0.5 M sucrose. Cells were frozen at -80 °C in 80 µl aliquots or used immediately. For electroporation, 1 µg of plasmid DNA was added to 80 µl of electrocompetent cells in a 0.2 cm cuvette and electroporated at 100 Ω resistance, 25 mF capacitance, and a charge voltage of 2.5 kV. Immediately after electroporation, 1 ml of SMMP broth was added and transferred to a 1.5 ml Eppendorf. This was incubated at the appropriate temperature with shaking followed by plating on selective media.

3.2.15 Preparation of electrocompetent *S. aureus* and electroporation (Löfblom)

Adapted by Monk et al from the Löfblom protocol (399). 5 ml of overnight culture of *S. aureus* cells were diluted to an optical density of 0.5 OD_{600} in 45 ml of prewarmed TSB and incubated at 37 °C, 200 rpm for 30 min. The culture was placed on ice for 10 min, and then harvested by centrifugation (3,900 x g, 10 min at 4 °C). Supernatant was discarded and cells were washed twice with 50 ml of ice cold sterile water. The cells

were then washed with 5 ml followed by 1 ml of sterile ice cold 10% (w/v) glycerol. Finally the pellet was resuspended in 250 µl of 10% glycerol, 50 µl aliquots of cells were immediately transferred to -80 °C. Electrocompetent cells were thawed on ice for 5 min and incubated at room temperature for 5 min. Cells were centrifuged at 5,000 x g for 1 min, the supernatant discarded and the cells resuspended in 50 µl of 10% glycerol/500 mM sucrose. Added to the cells was 5 µl of purified plasmid (up to 5 µg) and mixed by gentle flicking. Cells were transferred to a 0.1 cm electroporation cuvette (Biorad) and electrotransformed using 21 kV/cm, 100 Ω and 25µF, with a time constant around 2.0-2.4 ms. Immediately 1ml TSB with 500 mM sucrose was added and transferred to an Eppendorf tube, incubated at 28 °C for 2 hrs and 200 µl plated out on TSA with 10 µg/ml chloramphenicol and incubated at 28 °C for 48hr. Putative positive colonies are streaked out and grown overnight at 28 °C on TSA + Cm10.

3.2.16 Generating gene deletions using pMAD

Transformants from the electroporation of *S. aureus* with modified pMAD were selected by plating on X-Gal TSA plates containing erythromycin (5 µg/ml) at 30 °C for 48 hours. Single blue colonies which contain the modified pMAD plasmid were restreaked onto X-Gal TSA with erythromycin (5 µg/ml) and incubated at 30 °C. The plasmid was recombined into the bacterial chromosome by inoculating a blue colony into 5 ml TSB without antibiotic and incubating the culture for 2 hours at the permissive temperature (30 °C) with shaking, followed by 6 hours at the restrictive temperature (42 °C). Serial dilutions of this culture were plated on X-Gal TSA plates with erythromycin (5 µg/ml) and incubated at 42 °C overnight. To resolve the plasmid out of the chromosome, and generate possible deletion mutants, single colonies were inoculated into 5 ml TSB grown at 30°C for 6 hours and serial dilutions were plated on to X-Gal TSA plates at 37 °C overnight. White colonies were screened by streaking onto X-Gal TSA plates at 37 °C and tested for erythromycin sensitivity. Colonies that are white on X-Gal and erythromycin sensitive were likely to be deletion mutants (confirming loss of plasmid) and were confirmed by colony PCR and sequencing.

3.2.17 Generating gene deletions using pIMAY

A single colony from the 28 °C plate containing modified pIMAY, was vortexed in 200 µl of TSB and then diluted 10^{-3} and all dilutions plated on TSA with chloramphenicol (10 µg/ml) and incubated at 37 °C. Colonies from the overnight plate were restreaked onto TSA with chloramphenicol (10 µg/ml) at 37 °C. Confirmation of plasmid integration was confirmed by colony PCR using primers external to the MCS of the plasmid, with a negative result showing plasmid integration into the chromosome. Clones that gave a negative result were screened for the site of integration, using primers OUT FW/D or OUT RV/A. A single colony from either side of integration was inoculated into 5 ml TSB and grown at 28 °C, 200 rpm for 2 hours. The broth was diluted 1:10 to 10^{-6} and 50 µl aliquots were plated onto BHI containing 1 µg/ml anhydrotetracycline (ATc) (Sigma) and incubated at 28 °C for 48 hours. Colonies were restreaked onto BHI with ATc (1 µg/ml) and incubated at 37 °C overnight to obtain single colonies. Single colonies were then restreaked onto BHI with ATc (1 µg/ml) and TSA with chloramphenicol (10 µg/ml) plates, incubated at 37 °C overnight. Colonies sensitive tochloramphenicol were screened by colony PCR using the OUT primers for confirmation of the deletion. DNA sequencing across the deletion site was also used to verify chromosomal deletions.

3.3 Results

To determine the function of the staphylobactin cluster and to establish if it encodes putative siderophore synthetase involved in iron acquisition, staphylobactin gene deletion mutants were constructed in the *S. aureus* CC130 chromosome. Originally, seven strains containing the staphylobactin locus were chosen to target for gene deletion, six CC130 strains and the single CC350 strain. These strains were selected because they cover a broad range of host species, sites of isolation and country of origin (Table 3.2).

Table 3.2. *S. aureus* **strains containing the staphylobactin locus to target for gene deletion**

Strain	Country	Origin	Isolated from	Date	CC
08-02004	Germany	Roe Deer	Nasal Swab	25/08/2008	130
71277	Denmark	Human	Blood	12/05/2010	130
2383/03	Denmark	Human	Unknown	03/09/2003	130
MPS–18.31	England	Bovine	Milk	2012	130
Enr.7594/1975	Denmark	Human	Blood	1975	130
C03 365	England	Bovine	Milk	07/2006	130
8572	England	Horse	Tracheal Wash	16/11/2005	350

These seven *mecC* wild-type isolates were streaked from glycerol stocks onto blood agar plates and genomic DNA was extracted. To confirm that the strains were *S. aureus*, single colonies were streaked onto identification plates and tested for agglutination. The first genes to be targeted for deletion in the staphylobactin region were the *stbE* gene (6018 bp) which is predicted to encode a non-ribosomal peptide synthetase (NRPS) and *stbH, stbI and stbJ* genes (2763 bp), which are predicted to encode an ABC transporter associated with iron import. The homologous equibactin genes were shown to be required for iron import (296).

3.3.1 Generating a deletion mutant cassette

To obtain gene deletion mutants, firstly an insert was made carrying the upstream and downstream sequence of the target gene to be deleted. The size of the flanking regions was approximately 600 bp long (400 bp being the minimum length of PCR fragment for recombination to occur, longer fragments may increase recombination frequency) and contained the additional bases for SOE PCR and restriction sites for cloning (Figure 3.4).

Figure 3.4. Schematic of the homologous regions upstream and downstream of the gene to be deleted, including restriction sites and overlapping base pairs for SOE PCR of the primers.
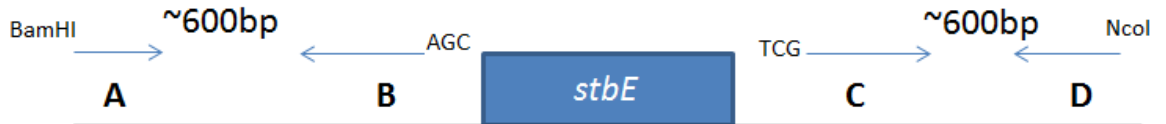
The genomic DNA was amplified using a high fidelity polymerase to avoid generating mutations in the homologous sequence that would affect the genes surrounding the target gene. Primer annealing temperature was optimised using a PCR gradient machine (Figure 3.5). The PCR was run on an agarose gel and bands of the correct size were extracted.

Figure 3.5. Optimisation of primer annealing temperature for PCR of upstream and downstream fragments of target gene for primers AB and CD.

Primers for the pMAD vector were used targeting *stbE* and *stbHIJ* genes for deletion.
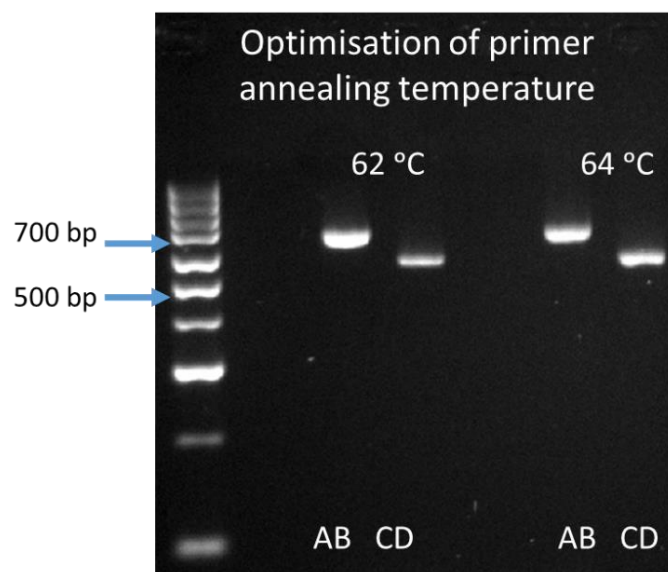
The two fragments were then joined by SOE PCR, mediated by an overlap between primer C and primer B. The expected product size was 1.2 kb, however using Velocity High Fidelity Polymerase did not produce any product, despite optimisation using a gradient PCR machine. When the SOE PCR was run on an agarose gel, only bands around 600 bp appeared. The SOE PCR was also run with standard polymerase, this produced bands of the correct size. These PCR fragments were ligated into the pCR2.1 TOPO cloning vector and the inserts sequenced. However, when the sequencing data was analysed, all inserts contained at least one or more base pair mutations, so could not be used for future work. Five different high fidelity taq polymerase were tested, Platinum Taq, Pfx50, Accuprime, PCR Platinum Supermix (ThermoFisher) and Velocity along with 5 PCR cycles prior to the addition of primers to allow annealing of PCR fragments (Figure 3.6). This protocol worked very well for SOE PCR using the Accuprime Taq for both targets. Platinum, Pfx50 and Platinum SuperMix did not produce any bands of the correct size and Velocity only produced one band for the *stbE* cassette.

Figure 3.6. Optimisation of SOE PCR with High Fidelity Taq Polymerase

SOE PCR run with five different High Fidelity Taq polymerase according to manufacturers protocols. For each polymerase, SOE PCR was run for both *stbE* (left) and *stbHIJ* (right). Bands that are approximately 1.5 kb in size show successful SOE PCR, whereas bands around 600 bp are PCR fragments AB and CD.
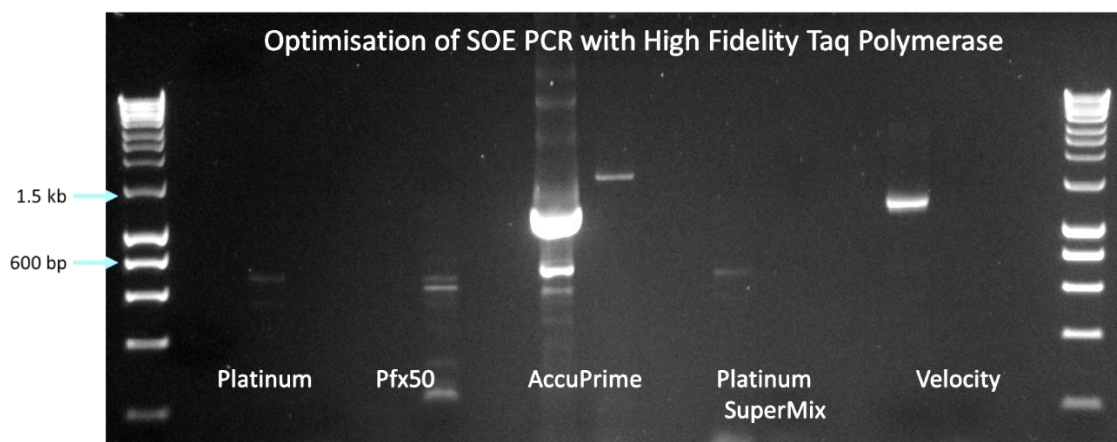
Therefore AccuPrime High Fidelity Polymerase was chosen for future work, along with the modified PCR protocol. The addition of standard polymerase at the end of the PCR cycle along with dNTPs to the PCR reaction allowed for the addition of 'A' overhangs to the PCR fragment which enabled cloning into the pCR vector. The SOE PCR was ligated to the pCR4 TOPO vector and then transformed by heat shock into TOP10 *E. coli* cells. The cells containing the plasmid plus insert were selected using plates containing ampicillin. Originally the vector pCR2.1 TOPO was used, however this required both ampicillin and X-Gal plates to select for cells containing the plasmid. pCR4 TOPO contains a lethal gene that only allows growth of positive recombinants, therefore blue/white screening is not required making it quicker and easier to select for colonies containing the plasmid plus insert. To confirm the plasmid was carrying the correct insert, plasmid was extracted from four colonies for each construct, *stbEΔ* and *stbHIJΔ*. They were digested with *EcoRI* which cuts either side of the insert and with *BamHI/NcoI* which cuts the restriction sites in the primers (Figure 3.7). The plasmids with the correct restriction bands were also sent for sequencing using primers T7F and M13F.

Figure 3.7. Restriction digest of vector pCR4 TOPO containing the insert fragment using *NcoI/BamHI* and *EcoRI*.

EcoRI cuts 7-10bp upstream and downstream of the PCR insert producing 2 bands. The insert 1300bp and plasmid 4000bp in size. *BamHI* and *NcoI* cut either side of the insert and one other place in the vector producing 3 bands, 1300bp, 1500bp and 2500bp in size.
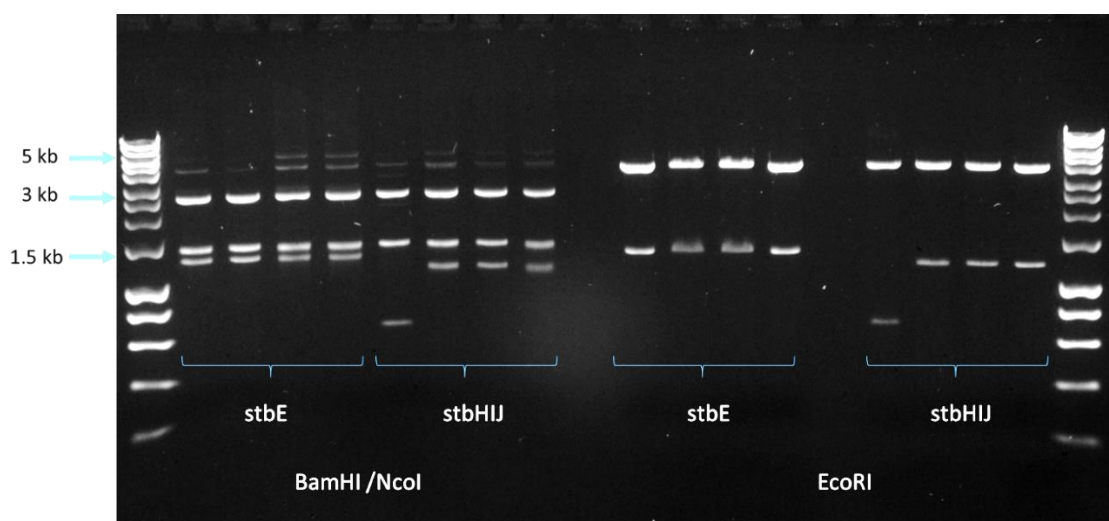
The plasmid available in the lab at the start of this project to carry out allelic replacement was the pMAD system developed by Arnaud *et al.* (400). pMAD can be used to generate gene inactivation mutants, with or without an associated antibiotic resistance gene, that will not disrupt downstream genes. The absence of a selection marker means I could make multiple mutations and potentially delete whole regions in a stepwise deletion. This avoids the issues with creation of multidrug resistant MRSA and potential artefacts caused by resistance markers.

The full length 1.3 kb fragment containing perfect copies of the target region in the pCR4 vector was restricted with NcoI/BamHI, along with the pMAD vector, and run on an agarose gel. The correct restriction bands were extracted and purified. The fragments were ligated to the cut pMAD vector and transformed into C2984 *E. coli* cells and plated onto LB agar containing ampicillin for selection of cells containing the plasmid. The ligation was unsuccessful, as colonies contained empty plasmid. Therefore, pMAD was treated with alkaline phosphatase to prevent self-ligation and then cleaned up with a phenol/chloroform extraction followed by concentration using ethanol precipitation. The phenol purification step was used as it increases the likelihood that the purified DNA will be amenable to further manipulation. This step proved to be successful in creating modified pMAD vector containing the correct insert. Ligation reactions were typically transformed into *E. coli* first. This step takes advantage of the high transformation/electroporation frequency associated with *E. coli* but not *S. aureus*. The plasmid was then grown in *E. coli* under selection, purified, and used to electroporate into *S. aureus*. Four colonies from each ligation were extracted with the Qiagen Mini Kit, plasmid was digested with NcoI and BamHI, and the amount of plasmid extracted checked using a NanoDrop. The yield of modified pMAD extracted was very low (less than 30 ng/µl), and a higher yield was needed for cloning to be successful. This may have been due to pMAD being produced at a low copy number, even in *E. coli*. The extraction was repeated with the Qiagen Midi and Qiagen HiSpeed Midi kit. These still produced a low yield so the extracts were concentrated using a rotating evaporator.

3.3.2 Passage of plasmid through restriction deficient strains

The plasmid was passaged through *S. aureus* RN4220 (which had been made electrocompetent using the Oskouian & Stewart protocol) to modify the methylation status of the DNA, therefore bypassing the type I RM system before transferring to wild-type CC130 strains. pMAD containing the insert was electroporated into RN4220 and selected for on TSA plates containing erythromycin, at 30 °C for 48 hours. Plasmid was extracted from RN4220 using the Qiagen mini kit, however this produced a very low yield (less than 12 ng/μl) with 5 μg/ml needed for transformation. Extraction was repeated with the Qiagen Midiprep Kit and the HiSpeed Plasmid Midi Kit, to try and increase the amount of plasmid, though still very low concentrations were extracted. Therefore plasmid was concentrated in a rotating evaporator prior to electroporation. The NucleoBond kit was also tested with the protocol for low copy number plasmids. This produced a higher yield and plasmid was concentrated using ethanol precipitation. Presence of the correct modified pMAD containing the deletion insert from the extraction was confirmed either by restriction digest with *EcoRI* (Figure 3.8) or PCR using primers that amplify the SOE insert.

Figure 3.8. Restriction digest with *EcoRI* of modified pMADΔ*stbE* extracted from restriction deficient *S. aureus* RN4220.
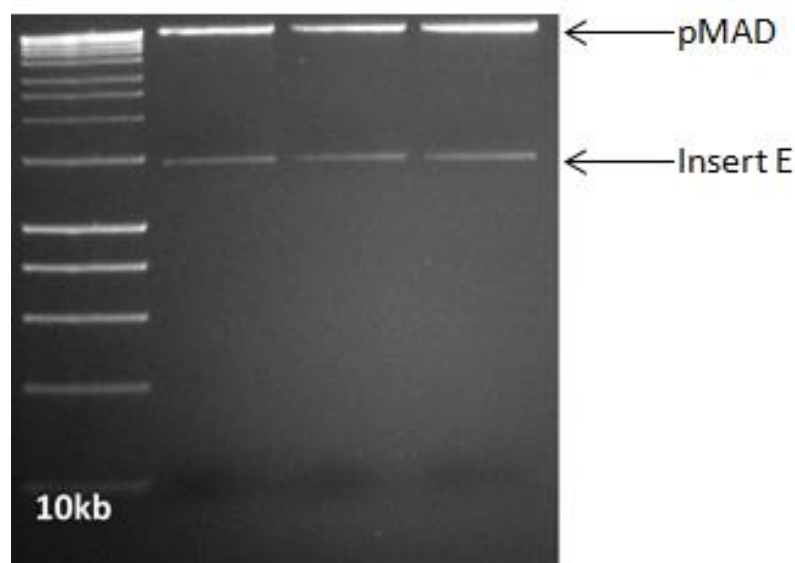
The plasmid pMADΔ*stbHIJ* was also passaged through DC10B restriction deficient *E. coli* cells (therefore bypassing the type IV RM system), producing a high enough yield when extracted with the Qiagen Mini Kit without the need to be concentrated. This was due to the high efficiency cloning background (DH10B) of DC10B, allowing for good quality plasmid preps.

3.3.3 Generating deletion mutants using pMAD

The modified recombinant pMAD vector was used in a two-step strategy for allele replacement in *S. aureus* CC130 strains. Both pMADΔ*stbHIJ* (extracted from DC10B) and pMADΔ*stbE* (extracted from RN4220) were electroporated into the seven strains containing the staphylobactin region (made electrocompetent using the Oskouian & Stewart protocol) and selected for on X-Gal plates containing erythromycin at 30 °C, a temperature permissive for replication. Positive colonies containing the modified pMAD vector appeared blue for strains 8572, 2383/03 and 7594/1975 when targeting for *stbHIJ* deletion, however the transformation frequency was very low with only one blue colony per transformation. No colonies were obtained from cells transformed with pMADΔ*stbE*. Discrimination of blue white colonies on X-Gal plates was difficult. This was possibly due to pMAD having a low copy number and not being strongly expressed, or the white *S. aureus* CC130 cells do not contain the plasmid and are intrinsically resistant to erythromycin. This low frequency of transformation may be due to not enough plasmid so a higher concentration may increase successful transformation, or that it is not possible to transform pMAD into CC130 cells, due to their specific restriction modifications or other barriers. Blue colonies containing the plasmid were integrated into the chromosome by homologous recombination between a target gene and homologous sequences carried on a plasmid by a single or consecutive double crossover. Cells were grown at the permissive temperature then at the non-permissive temperature, followed by plating on X-Gal plates and incubation at the non-permissive temperature. Light blue colonies were due to the integration of the vector by a single crossover event, or white colonies represent candidate clones resulting from a double crossover event and loss of the vector. However, no colonies were visible on the agar plates after 2 hours at 30 °C followed by 6 hours at 42 °C.

3.3.4 Optimisation of transformation frequency

To address the issues that I had encountered in making deletion mutants, a number of areas were researched to improve protocols and try to overcome these problems.

As already mentioned, *S. aureus* have strong restriction barriers, making them difficult to genetically manipulate, so the low transformation frequency may be due to inability to bypass this. pMADΔ*stbHIJ* was isolated from strain DC10B which modifies DNA to bypass the type IV RM system and pMADΔ*stbE* was isolated from strain RN4220, which modifies DNA so that it can bypass type I RM system. Whereas strains SA30B/SA08B can bypass type I restriction systems but specifically target strains from CC8 and CC30. The type IV restriction system has been identified as the major barrier to transformation with foreign DNA. When the strains belonging to CC130 are mapped on a phylogenetic tree compared to strains from CC8 (Newman, USA300 and 8325-4) and CC30 (MRSA252) which have been successfully genetically manipulated, it can be seen that these strains are widely separated phylogenetically and likely to have very different genetic backgrounds (Figure 3.9).

Figure 3.9. Phylogenetic tree of the major MRSA lineages and other animal associated lineages.

S. aureus isolates beloning to CC8 and CC30 that have previously been genetically manipulated are on divergent branches of the *S. aureus* phylogenetic tree from CC130 suggesting very different genetic backgrounds. Clonal complex 8, 30 and 130 are highlighted with blue arrows. Phylogenetic tree created using RaxML (310).

The type I RM system comprises three genes, *hsdM*, *hsdR* and *hsdS* which encode a host specificity determinant (hsd). The recognition sequence for *hsd* methylation is clonal complex specific, preventing transfer between lineages of *S. aureus* and from *E. coli*. These genes were found to be present in the CC130 strains, allowing comparison to their homologues in strains 8325-4, Newman, USA300 (CC8) for which RN4220 has previously been used for genetic manipulation, and MRSA252 (CC30) - using BLAST to assess their amino acid identity (Table 3.3). The *hsdM* gene is 1557 bp in size for all strains observed,

with a 96% amino acid sequence identity when the CC130 *hsdM* gene compared to the other four strains. The *hsdR* gene is also the same size in all strains (2790 bp), with a 99% sequence identity when compared to the four strains. The *hsdS* gene had the biggest difference between strains from the different clonal complexes. Strains from CC8 have an *hsdS* gene consisting of 1,199 bp, CC30 is 1,155 bp and CC130 is 1,221 bp. When compared to other strains, the CC130 *hsdS* gene only had a very small homologous region of 176 bp of 96%, which matched the central region of the gene sequence. When a BLAST search of the *hsdS* gene from CC130 strain 71277 was carried out, very few strains showed sequence homology with the whole region, with the closest strain scoring 98% sequence identity (1198/1221 bp). This large difference in sequence homology compared to the CC8 strains may account for the inability for plasmid passaged through RN4220 to be modified.

Table 3.3. Sequence identity of the type I restriction modification genes from CC130 strains compared to CC8 and CC30 strains. Nucleotide BLAST.

Nucleotide identity, % identity in brackets.

Strain	*hsdM*	*hsdR*	*hsdS*
MRSA252 (CC30)	1491/1557 (96%)	2764/2790 (99%)	161/180 (89%) 68/71 (96%)
USA300 (CC8)	1489/1557 (96%)	2762/2790 (99%)	166/176 (94%) 156/171 (91%)
8325.4 (CC8)	1492/1557 (96%)	2762/2790 (99%)	166/176 (94%) 156/171 (91%)
Newman (CC8)	1489/1557 (96%)	2762/2790 (99%)	166/176 (94%) 156/171 (91%)

To improve the transformation frequency, certain parts of the protocol were tested.

3.3.5 Ability of wild-type CC130 strains to accept plasmid

mecC CC130 strains had never been genetically manipulated before, therefore experiments were carried out to assess which of the wild-type strains listed in Table 3.4 were able to accept empty plasmid.

pRMC2 (a non-temperature sensitive shuttle plasmid) was passaged through *E. coli* strain DC10B. 5 µg/ml of isolated pRMC2 plasmid was electroporated into the CC130 strains and plated on TSA plates containing chloramphenicol for selection. Out of the seven CC130 strains, five were found to be to acquire plasmid, producing between 1 to 6 colonies per transformation. Strain Newman and RN4220 were also included as a comparison. Newman produced 9 colonies per transformation and RN4220 produced over 200 colonies per transformation, 32 when diluted 1 in 10 (Table 3.4). This highlighted strains 2383/03, 71277, 18.31 and 08-02004 as targets for genetic manipulation.

Table 3.4. Ability of wild-type CC130 strains to accept plasmid isolated from DC10B

Strain	Number of colonies (pRMC2)	Number of colonies (-ve)
Newman	9	0
RN4220	200+	0
7594/1975	0	0
2383/03	6	0
71277	4	0
8572	0	0
C03 365	1	0
18.31	4	0
08-02004	5	0

3.3.6 Plasmid isolation from restriction modification strains

The CC130 strains with higher transformation frequencies were used to test which "restriction modification" strains used for the isolation of plasmid DNA were accepted. pRMC2 plasmid isolated from *E. coli* strains: DC10B, SA08B or SA30B, were electroporated into wild-type CC130 strains, along with the routinely manipulated strain Newman as a positive control. Water was added to the *S. aureus* cells to act as a negative control, and the cells were plated for selection of plasmid. Of the restriction strains tested, no colonies were produced from strain SA08B along with the negative control. Strain 71277 and Newman had a small number of colonies when plasmid was isolated from SA30B, but all strains grew a bacterial lawn when pRMC2 had been isolated from DC10B. Therefore, DC10B was the restriction deficient strain for isolation of plasmid for the targeting of CC130 cells.

3.3.7 Preparation of electrocompetent *S. aureus* and electroporation using McNamara protocol

A different protocol (McNamara Protocol) for making electrocompetent cells was tried along with heat shock of electrocompetent cells at 56 °C for 2 min prior to electroporation to temporarily inactivate the restriction modification systems and allow for better uptake of plasmid. These made no detectable difference to the transformation frequencies, so were not included in future protocols.

3.3.8 Intrinsic antibiotic resistance of *S. aureus* CC130 strains

The background resistance on CC130 strains may have been affecting the screening of colonies containing the plasmid if they have intrinsic resistance to the same antibiotics as the plasmid for gene deletion. To test their susceptibility to certain antibiotics used in the generation of gene deletion, these strains were streaked onto plates containing kanamycin, erythromycin, chloramphenicol and plates containing no antibiotic. No colonies grew on the erythromycin and kanamycin plates and so were all considered susceptible, however there was a small amount of growth on plates containing chloramphenicol for strains RN4220, LGA251, 08-02004, 2383/03 and C03 365.

Therefore only strains 71277 and 18.31 were used to target for gene deletion with plasmid passaged through strain DC10B.

3.3.9 Increasing frequency of cointergrate resolution

The modified pMAD vector had been successfully introduced to wild-type CC130 strains at very low frequency for strains 2383/03, 7494/1975 and 08-02004 for pMADΔ*stbHIJ*. However, once the plasmid had been introduced into the target strain for deletion and generation of deletion mutants by incubation at 30 °C for 2 hours followed by 6 hours at 42 °C had taken place, no colonies were produced. Kato and Sugai (406) reported that sequential non-selective passage at a reduced temp (2 x 25 °C) improves frequency of cointegrate resolution. This was tested with these strains containing pMAD by subculturing twice at 37 °C to try to increase the chances of integration into the chromosome. This was unsuccessful as no colonies were detected. It was also not possible to check for cointegration of the plasmid into the chromosome by PCR as both crossover steps were carried out together in broth at 42 °C.

3.3.10 Testing temperature sensitivity to replicate

pMAD was tested for its sensitivity to replicate at 42 °C. In order for homologous recombination to occur, pMAD needs to not be replicating. This was tested along with the temperature sensitive plasmid pIMAY, which can also be used to target gene deletion. *E. coli* cells containing the empty plasmids were streaked onto LB containing appropriate levels of antibiotic for selection and incubated overnight at their permissive (30 °C for pIMAY and 37 °C for pMAD) and non-permissive temperature for replication (37 °C for pIMAY and 43 °C for pMAD). Both plates with *E. coli* cells containing the plasmid grew at the temperature permissive for replication. However, when pMAD was grown at 43 °C which should be non-permissive for replication, colonies were present on the plate suggesting that pMAD was not sufficiently temperature sensitive for successful recombination. Therefore pIMAY was used to generate gene deletion mutants, as no colonies appeared on the plate at the non-permissive temperature.

3.3.11 Construction of *S. aureus* mutants using pIMAY

The pIMAY plasmid is temperature sensitive at 37 °C and also has counter selection. PCR can be easily applied to demonstrate that extrachromosomal plasmid is no longer present with primers external to the multiple cloning site (MCS) on pIMAY (IM151 and IM152), and determine whether plasmid integration has occurred via the upstream or downstream region of homology cloned into the plasmid. Sequences upstream and downstream were amplified using primers A/B and C/D that contain the new restriction sites (KpnI/SacI) for direct cloning into pIMAY on genomic DNA from strain 71277. The PCR was optimised by testing the different high fidelity taq polymerase used previously, along with KOD (Merck), Phusion and Q5 (NEB). A small amount of PCR product was run on an agarose gel to check the presence of bands around 600 bp long, and the remaining PCR product was purified using YORBIO Gel/PCR Purification Kit for better recovery of product, compared to running the whole PCR sample in an agarose gel followed by band extraction and purification. These fragments were used as a template for SOE PCR with primers AD using the KOD polymerase SOE PCR method, resulting in fragment AD. The PCR product of about 1.2 kb was purified from agarose gel using YORBIO Gel/PCR Purification Kit. pIMAY and AD fragments were both digested with SacI and KpnI followed by ligation. The resulting plasmid was designated pIMAYΔ*stbHIJ* or pIMAYΔ*stbE*. The plasmid was directly transformed into chemically competent DC10B *E. coli* cells (bypassing the need for extra cloning intermediates) and selected for on plates containing chloramphenicol. Colonies were screened by PCR for the presence of the insert using primers IM151/IM152 with the MyTaq colony PCR protocol (Figure 3.10). Positive clones were extracted using the Qiagen Mini Prep Kit and analysed by restriction digest and sequencing of the insert.

Figure 3.10. Colony PCR of *E. coli* DC10B for the presence of pIMAY deletion cassette.

Eight colonies were screened for pIMAY deletion cassette from each transformation using MyTaq PCR and MCS primers. Empty plasmid produced bands at approx. 200 bp and successful constructs containing the deletion cassette produced bands of approx. 1.5 kb.
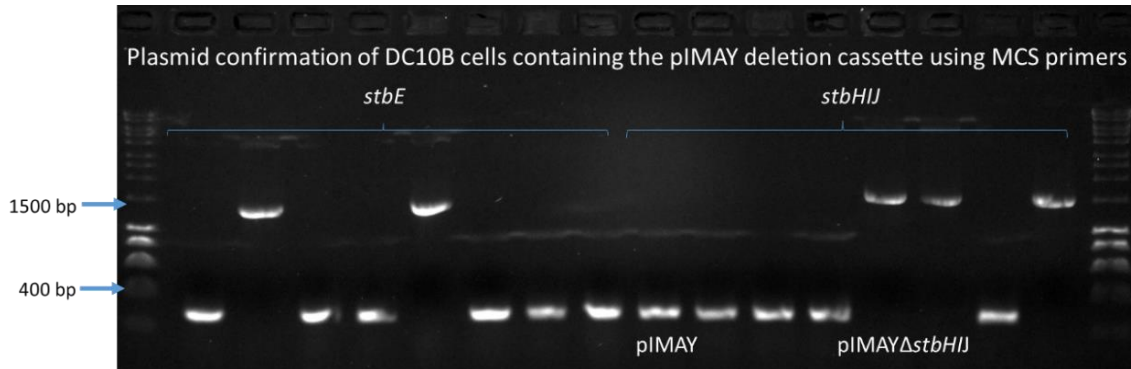
Confirmed plasmid pIMAYΔ*stbE* or pIMAYΔ*stbHIJ* was electroporated into the target *S. aureus* strain (71277 and 18.31) and plated onto TSA with chloramphenicol (Cm) 10 µg/ml, at 28 °C for 48 hours. Colonies were produced for strain 71277 and 18.31. Colony PCR was used to check for the presence of the plasmid containing the insert by MyTaq colony PCR with MSC primers. Colony PCR was found to produce clearer results if the plate was placed at 4 °C for a few hours prior to carrying out the PCR. To integrate pIMAY into the chromosome, a single colony from the transformation plate was homogenized in TSB and spread onto TSA+Cm10, and incubated overnight at 37 °C to stimulate single crossover integration. Large well isolated colonies were picked from these plates and restreaked onto TSA+Cm10 at 37 °C overnight to make sure the colonies were well isolated and then MyTaq colony PCR was used to determine the absence of extrachromosomal DNA. PCR was also used to check whether plasmid integration happened upstream or downstream of the gene. Colonies negative for replication plasmid and containing the integration upstream and also downstream were picked and grown in TSB at 28 °C, on a roller (200 rpm) for a few hours to stimulate replication. Cultures were then diluted and spread onto BHI with 1 µg/ml ATc, incubated at 28 °C for 48 hours. Expression of the *secY* antisense RNA inhibits the growth of cells maintaining the plasmid. Colonies were restreaked onto BHI with 1 µg/ml ATc at incubated at 37 °C. The colonies incubated at 28 °C for 48 hours were mixed, containing both wild-type and

mutants, and therefore produced a false result. Restreaking these colonies improved success rate as this produced well isolated single colonies that could be easily identified for the mutation on selective plates (BHI+ATc1 and TSA+Cm10) (Figure 3.11).

Figure 3.11. Screening of potential mutant colonies using chloramphenicol sensitivity
Single colonies were streaked onto BHI+ATc1 (left) and TSA+Cm10 (right). Colonies sensitive to chloramphenicol indicate the loss of the pIMAY plasmid.
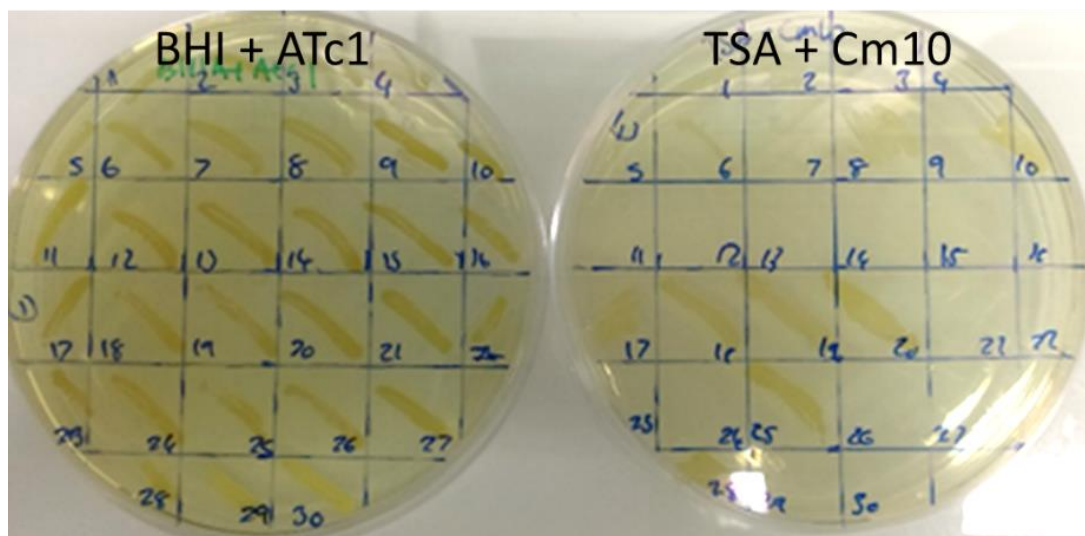
Colonies sensitive to chloramphenicol, indicating the loss of the pIMAY plasmid, were screened by colony PCR using primers Δ*stbE*_outF/R (Figure 3.12) or Δ*stbHIJ*_outF/R (Figure 3.13) depending on the expected mutation using the Phire colony PCR protocol. Colony PCR was also run in parallel using the MyTaq protocol, however most of the time no PCR product was produced, especially for the wild-type genes. This may be due to Phire Taq being able to handle 'dirty' template better and also produce larger PCR fragments when using this protocol. Therefore MyTaq was used for colony PCR for confirmation for the presence of extrachromosomal DNA and Phire was used for the confirmation of deletion mutants. Deletion mutants gave a PCR product around 1.5 kb, while wild-type colonies gave a larger fragment. Chromosomal deletions were confirmed through sequencing of PCR amplicons generated from across the deleted regions. The genes *stbHIJ* were easier to delete in terms of the number of deletion

mutants obtained when screened by colony PCR and chloramphenicol sensitivity. This may be due to the *stbE* gene being twice the size of the region being deleted for the *stbHIJ* genes.

Figure 3.12. Colony PCR of strain 71277 screening for the deletion of *stbE* using Phire Taq.

Colony PCR using primers Δ*stbE*_outF/R for confirmation of gene deletion run on agarose gel. Bands at 6 kb represent wild-type strains, bands of approx. 1.5 kb show strains containing the deletion.
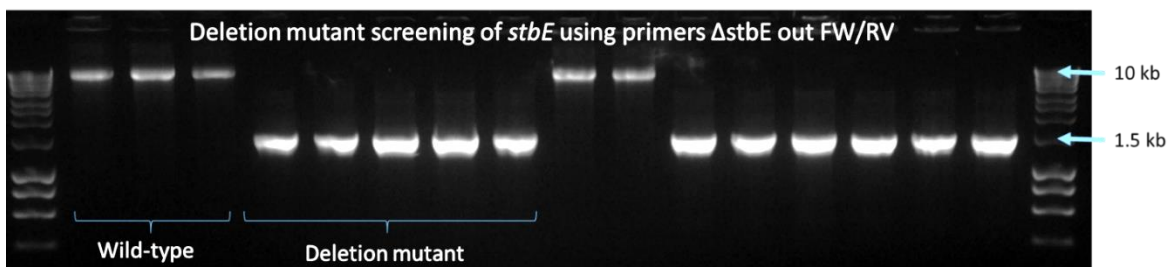
Figure 3.13. Colony PCR of strain 71277 screening for the deletion of stbHIJ using Phire Taq.

Colony PCR using primers Δ*stbHIJ*_outF/R for confirmation of gene deletion run on agarose gel. Bands at 3 kb represent wild-type strains, bands of approx. 1.5 kb show strains containing the deletion.
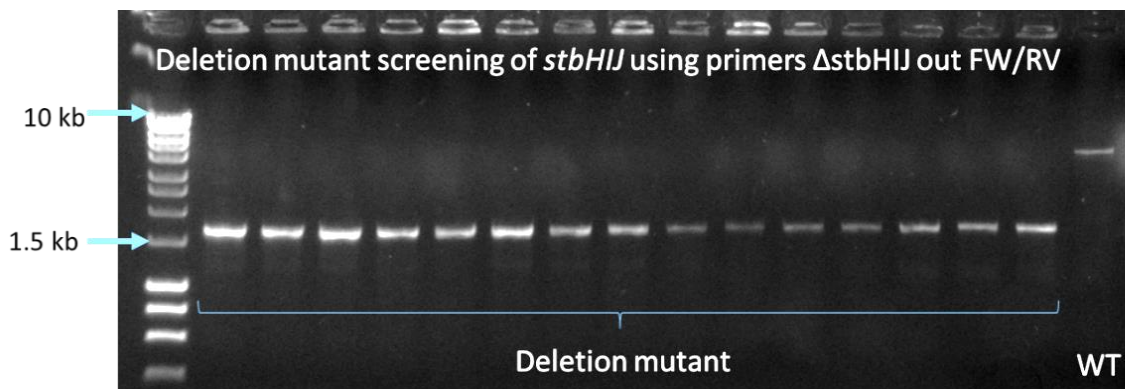
Double deletion mutants were generated by deleting genes *stbE* (6018 bp) and *stbHIJ* (2763 bp) using plasmids pIMAYΔ*stbE* and pIMAYΔ*stbHIJ* followed by deleting genes stb*F, F1, G* (5577 bp) using plasmid pIMAYΔ*stbE-J*. Subsequently genes *stbE-O* (5880 bp) were deleted using plasmid pIMAYΔ*stbE-O* and then the whole staphylobaction region (genes *stbBCDEFF1GHIJKLMO* consisting of 23,298 bp), by plasmid pIMAYΔ*stbB-O* (Figure 3.14). The SOE PCR using KOD protocol, modified pIMAY passaged through DC10B, electroporation using the updated Löfblom protocol and colony PCR using Phire Taq were all used as described above to create the resulting deletion mutants listed in Table 3.1. These deletions were carried out in the *mecC* strain 71277 as this strain was the most amenable to genetic manipulation of the CC130 strains targeted, resulting in the whole staphylobactin region to be deleted in a stepwise manner without antibiotic resistance markers that can be tested in a number of phenotypic assays to determine the role of this region.

Figure 3.14. Schematic representation of the deletion of the staphylobactin region in a stepwise manner.

Deletion of *stbE* and *stbHIJ* was followed by deletion of *stbF,F1, G* using plasmid pIMAYΔ*stbE-J*. This was followed by deletion of *stbK, L, M, O* using plasmid pIMAYΔ*stbE-O*.
 Finally the whole staphylobactin region was deleted by using plasmid pIMAYΔ*stbB-O*.
Genes of the staphylobactin locus to be deleted in blue.
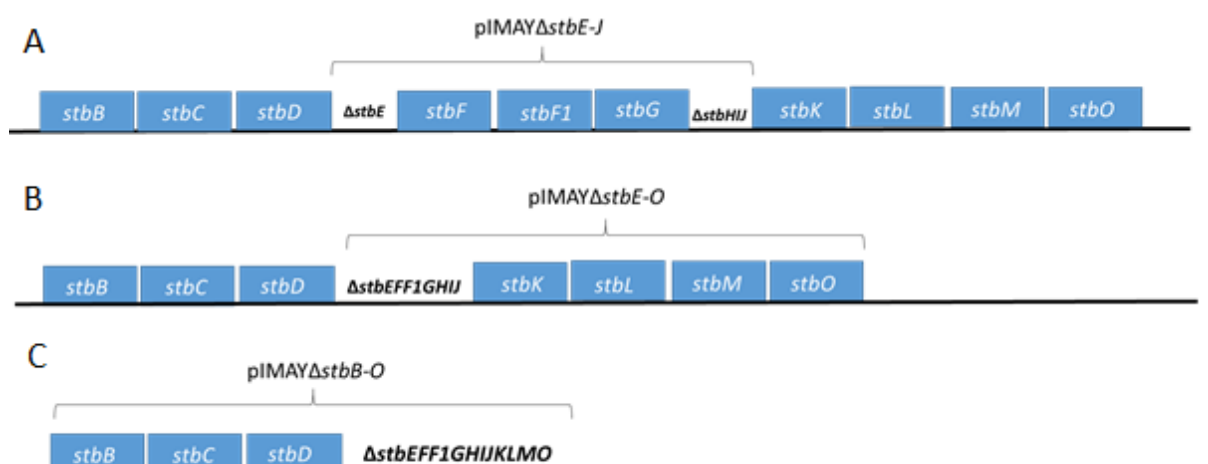
Once the staphylobactin mutants were generated using this optimised protocol, it was quicker and easier to create deletion mutants in other genes of interest using the exact same protocol. Staphylobactin is predicted to encode a NRPS that is involved in iron acquisition. *S. aureus* is known to produce the siderophores, staphyloferrin A (encoded by the genes *sfaABCsfaD*) and staphyloferrin B (*sbnABCDEFGHI*). These genes were targeted in the wild-type and staphylobactin mutant strain, resulting in a CC130 *S. aureus* strain 71277Δ*sfa*Δ*sbn* and 71277Δ*stbB-O*Δ*sfa*Δ*sbn* devoid in siderophores known to be involved in iron acquisition.

3.4 Discussion

The construction of gene deletion mutants is one of the important techniques for assessing the gene function in bacteria. Genetic manipulation is frequently impeded by the lack of efficient transformation protocols, native genetic elements interfering with available vector systems, or other limiting factors including cell envelope composition or DNA methylation patterns that mean only a small number of strains have been amenable to genetic manipulation. During my project these have been one of the biggest obstacles to overcome, taking considerable time and work in order to create deletion mutants in the *mecC* CC130 strains for phenotypic studies.

During this study I have developed a methodology using a variety of techniques and tools to create gene deletions in *S. aureus* CC130 chromosomes. To achieve this I have assessed and optimised many protocols in order to facilitate genetic manipulation of strains that had not previously been genetically altered, enabling the study of 'wild' or clinical isolates rather than just the existing small subset of laboratory strains amenable to genetic manipulation. This will speed up the process of mutant construction and the understanding of *S. aureus mecC* strains in future studies.

3.4.1 Bypassing the restriction barrier

Restriction modification systems of most *S. aureus* strains act as an efficient barrier against foreign DNA.

3.4.1.1 RN4220

For transformation of foreign DNA into *S. aureus*, RN4220 (which has an impaired ability to restrict foreign DNA, therefore allowing *E. coli*-derived plasmid DNA to be properly methylated) has been extensively used as an initial recipient before transferring DNA to other *S. aureus* strains. However, many strains accept DNA isolated from RN4220 at an extremely low frequency, if at all. Passage of the pMAD vector through RN4220 and then electroporation into wild-type CC130 strains occurred at an extremely low frequency or not at all. When looking at the genome sequences of the CC130 strains and comparing the similarity of the *hsdS*, *hsdR* and *hsdM* genes against strains from CC8 and CC30 that have previously been manipulated using RN4220. The *hsdS* gene from CC130 was dramatically different from those from CC8 and CC30 strains. In *S. aureus*, the *hsdM* and *hsdR* genes appear to be conserved (407), while the *hsdS* genes share much less homology among various clonal complexes. However, *hsdS* is conserved within each clonal complex, suggesting a specificity of the RM system within the same clonal complex (407). This may explain the inability to transform plasmid isolated from RN4220 into CC130 strains. Also, plasmid isolation from RN4220 produced a very poor yield and quality compared to that isolated from *E. coli.* RN4220 is not an ideal cloning intermediate. Firstly, maintenance of plasmid in *S. aureus* is not ideal. Secondly the ability to bypass using it will reduce the time involved for plasmid transfer, due to additional time for growth at 30 °C because of the temperature sensitive plasmid. This reduces the cost due to lesser need for extensive extraction and purification of plasmid.

3.4.1.2 DC10B

The type IV RM factor SauUSI has been found to be a major barrier for DNA uptake in *S. aureus* and *E. coli* DC10B was constructed to overcome this. When pRMC2 was isolated from DC10B and introduced into CC130 strains it proved to be very successful and was used as a cloning intermediate for bypassing the restriction modification systems for the generation of gene deletions in these strains. High concentrations of good quality plasmid DNA can be quickly and efficiently isolated from DC10B using the Qiagen Miniprep Kit, overcoming poor transformation efficiency and direct transformation can be performed with plasmid isolated from DC10B. This reduces the need to pass through

RN4220 as the intermediate host and other intermediate steps through TOP10 and C2984 *E. coli* cells.

The transformation rate was not improved using DNA isolated from SA30B (type I RM genes *hsdMS* cloned into DC10B) and plasmid isolated from SA08B was unable to be transformed into CC130 at all.

Comparison of *S. aureus* genome sequences has found that both type I and/or type IV systems are highly conserved. However, some strains encode additiional type I or II RM systems, which can impair transformation. Understanding the biology of RM in various clinical isolates is important for overcoming the RM barrier for DNA uptake and developing better genetic engineering tools and techniques.

If CC130 strain were found to be refractory to genetic manipualtion using the RM strains described above, then a number of approaches could have been tried to overcome this.

In 2015, Monk *et al.* (397) described the construction and characterisation of the IMXXB series of *E. coli* strains that mimic the type I adenine methylation profiles of *S. aureus* clonal complexes 1, 8, 30, and ST93. The IMXXB strains enable direct, high-efficiency transformation and genetic manipulation of major *S. aureus* lineages. If CC130 strains were found to be completely refractory to transformation with plasmid DNA isolated from RN4220, DC10B, SA08B and SA30B plasmid then Pacbio Single-Molecule Real-Time (SMRT) Sequencing could have been used to detect the adenine methylation pattern of the CC130 strains (408). *E. coli* strains such as DH10B could then have been modified to express the adenine methylation profile from CC130 strains as detailed for CC1 and CC30 (397). In 2012, Morikawa *et al* (409) reported that expression of the *sigH* gene renders *S. aureus* cells competent for transformation by plasmid or chromosomal DNA. This could be exploited for genetic manipulation of *S. aureus* instead of the need to be transformed by electroporation protocols.

3.4.2 Plasmids for genetic manipulation: pMAD vs pIMAY

3.4.2.1 pMAD

The plasmid available in the lab at the start of my project was the pMAD system (400) consisting of a temperature sensitive plasmid and a blue/white screening for mutant

identification. Plasmid constructs were made targeting the deletion of the *stbE* and *stbHIJ* genes in the CC130 strains and were successfully electroporated into three strains, producing blue colonies. However, shifting the temperature from 30 °C to 42 °C to allow a single crossover event to take place, failed to produce cointegrants. Subculturing twice at 25 °C of the single crossover integrants, in an attempt to improve the frequency of cointegration resolution, was unsuccessful in promoting allelic exchange in *S. aureus* CC130. When the pMAD plasmid was tested for temperature sensitivity at 42 °C (the non-permissive temperature for replication), colonies still grew. This may be the reason for the inability to generate deletion mutants as the plasmid has to not be replicating for homologous recombination to take place.

Screening of colonies didn't work because pMAD does not contain a counterselectable marker for mutant identification so plasmid excision cannot be selected and relies on colour screening of colonies when plated on X-Gal plates. Blue colonies indicate that the plasmid is still in its episomal state, light blue represents colonies with chromosomal integration (single crossover) and white suggest successful replacement and loss of the plasmid (double crossover). I found colour of colonies difficult to distinguish making it harder to identify the state of the plasmid inside the cell.

3.4.2.2 pIMAY

pIMAY was selected as the alternative choice for making gene deletions as it has a counter-selective device for selecting successful mutants and is temperature sensitive for replication at 37 °C. This was screened for its ability to replicate at 37 °C. No colonies appeared on the plate at the non-permissive temperature. Having a lower selection temperature compared to that of pMAD means *S. aureus* is less prone to second site mutations when incubated at temperatures non-physiological to the cell. Additionally a high level of chloramphenicol resistance is obtained by expression of the *cat* gene from a strong promoter. This reduces the pressure for a selection of variants with increased chloramphenicol resistance, which, combined with the low copy number of the plasmid, lessens the chance of tandem duplication occurring during chromosomal integration and aids in selection of correct single-copy integrands. pIMAY is considerably smaller than pMAD, which has advantages for transformation and stability. pIMAY was

successfully used to make deletion mutants in CC130 strains without associated antibiotic resistance. This facilitated the deletion of multiple genes in a single strain, including the whole staphylobactin region in a stepwise fashion. Screening of mutants was easy when streaked on selective plates and confirmation by colony PCR. The ability to check for replicating plasmid and sites of integration at the first crossover event using colony PCR allowed improved mutant generation. Monk *et al.* (396) found that resuspension of a colony and direct plating rather than broth grown at the nonpermissive temperature for plasmid replication improves the isolation of both single crossovers and this was incorporated into the protocol for generating deletions.

3.4.3 Optimisation of transformation frequencies

In order to reach transformation frequency levels that were required to make gene deletion mutants in CC130 strains, the various parameters in the transformation process were investigated and evaluated. This included the preparation of electrocompetent cells, the electroporation procedure and the recovery of cells after transformation. The modified protocol published by Löfblom (399) that was adapted by Monk *et al.* (396) resulted in significantly higher electrocompetence of the *S. aureus* cells compared to the sucrose wash method descried by Oskouian (398) and an updated version by McNamara. Another advantage of this protocol is the reduced time required for the production of competent cells, taking 2 hours instead of 4 hours.

3.4.4 Summary

In summary, I facilitated genetic manipulation of *mecC* strains of *S. aureus*. This was done by using the temperature sensitive plasmid pIMAY, combined with an improved transformation protocol, and the ability to bypass the restriction barrier by using plasmid DNA isolated from the DC10B strain of *E. coli*.

The timescale from start of cloning through to mutant confirmation, can be completed in under two weeks. This methodology should be useful for other researchers going forwards and could be used for other *S. aureus* strains that have not been genetically manipulated. After this long process, I was finally in a position to analyse the function of the staphylobactin locus.

4 PHENOTYPING THE STAPHYLOBACTIN LOCUS

4.1 Introduction

Prior to this study, *S. aureus* was known to produce two characterised α-hydroxycarboxylate-type siderophores, termed staphyloferrin A and staphyloferrin B, which are induced under conditions of iron limitation (410). In this study, mutagenesis was performed to eliminate *sfa*, *sbn* and *stb* loci from the staphylococcal chromosome in an attempt to investigate the entire complement of known *S. aureus* siderophore biosynthesis systems, as well as to dissect their individual and combined contribution to growth of *S. aureus* in iron-restricted environments, and to assess their roles in virulence.

4.1.1 Regulation and expression of siderophores in an iron-dependent manner

Sequence analysis of the staphylobactin region showed that it was missing the *eqbA* homologue, a DtxR-like regulator. BLAST of EqbA against the *S. aureus* CC130 genome identified MntR, an iron-dependent repressor, as a possible regulator. The intergenic region immediately upstream of the *stbB* operon contains a 57 bp sequence that has a 50% nucleotide identity with the MntR box sequence in *S. aureus*, suggesting that the staphylobactin transcripts are regulated by the activity of the MntR protein. In *S. aureus*, MntR has been shown, by gel shift assay, to bind to the *mntABC* operator in the presence of Fe^{2+} or Mn^{2+} and transcription of the *mntABC* operon is repressed in the presence of Fe^{2+} or Mn^{2+} (380). PerR acts with MntR to control the expression of *mntABC* and manganese uptake. The expression of PerR regulated genes, including *fur* (ferric uptake regulator), was diminished in Δ*mntR* strains when grown in excess Mn^{2+}. Therefore, the control of Mn^{2+} associated members of the PerR regulon and the Fur protein are modulated by MntR through its control of Mn^{2+} uptake.

In *S. equi*, transcriptional regulation of the NRPS gene (*eqbE*) by the putative DtxR-like repressor (EqbA) was assessed through the comparison of *eqbE* transcript levels in the Δ*eqbA* and parent strain when grown in Todd-Hewitt broth (THB)(296). Deletion of *eqbA* resulted in a 13 fold increase in *eqbE* transcript levels relative to the wild-type strain. Binding of EqbA to the *eqbB* promoter was increased in the presence of additional Fe^{2+} or Zn^{2+} and to a lesser degree by Mn^{2+}, but not by Fe^{3+} or Cu^{2+}. Deletion of *eqbA* resulted

in a small colony phenotype in *S. equi*, possibly due to iron toxicity resulting from overproduction of the product(s) of the equibactin cluster and increased iron uptake. Supplementing of Todd-Hewitt Agar (THA) with an iron chelator restored near normal colony size in the Δ*eqbA* strain. The increased production of equibactin in the Δ*eqbA* mutant was further supported by ^{55}FeCl$_3$ incorporation assays, which showed an almost twofold increase in intracellular iron in the repressor deletion strain, compared with wild-type or the Δ*eqbAE* mutant. Δ*eqbAE* crossfed with filter sterilised stationary phase culture supernatant from the Δ*eqbA* strain but not the Δ*eqbAE* strain, showed a similar increase in ^{55}FeCl$_3$ accumulation (296).

In *S. aureus*, all the iron uptake systems studied to date have been shown to be regulated by Fur. Staphyloferrin A and B are expressed under conditions of iron restriction in a Fur/Fe dependent fashion. Real-time PCR analysis confirmed that transcripts are upregulated in an iron deprivation dependent fashion, i.e. they are repressed by iron saturation of the culture medium, but continue to be readily expressed regardless of iron availability in a *fur* inactivated mutant (210, 411).

To optimise their ability to acquire environmental iron, some bacteria have evolved systems to prioritise the production and transport of specific siderophores. As a result, many siderophore systems are regulated at two different levels. Firstly iron-dependent repression mediated by Fur and substrate induction mediated by an activator protein whose activity is controlled by the cognate siderophore (412-415). The second class involves the "iron subfamily" of AraC-type transcription activators. In these systems, an AraC-type regulator mediates the siderophore dependent induction of the uptake system and, in some cases, of the corresponding biosynthetic operon. Siderophore-mediated regulation by AraC-type transcription factors has been documented in *Yersinia pestis* (YbtA), *P. aeruginosa* (PchR), and *Bordetella* spp. (AlcR and BfeR) (416-419). All four promoter regions of the yersiniabactin region (*psn*, *irp2*, *yntA* and *ybtP*) possess a Fur binding site and are negatively regulated by this repressor in the presence of iron. In the presence of yersiniabactin, YbtA (a member of the AraC family of transcriptional regulators) activates expression from the *psn*, *irp2* and *ybtP* (transport and biosynthetic genes) promoters but represses expression of its own promoter (420).

4.1.2 Growth in minimal media

Many siderophores are competitive with transferrin and lactoferrin for iron binding, and several studies have demonstrated that microbial siderophore production contributes to the growth of pathogens in the iron-restricted milieu of serum (Table 4.1). Chromosomal inactivation of staphyloferrin biosynthesis loci *sfa* and *sbn* was reported to severely restrict growth of *S. aureus* under conditions of severe iron chelation (410). However, deletion of individual loci had mild to unnoticeable effects on growth of *S. aureus* in mammalian serum (210).

Table 4.1. Siderophores contributing to growth of bacteria in the presence of transferrin or in serum

Species	Siderophore	Ref.
Aeromonas spp.	Amonabactin	(421)
Bordetella bronchiseptica	Alcaligin	(422)
Burkholderia cepacia	Salicylic acid	(423)
Escherichia coli	Aerobactin, enterobactin	(424)
Mycobacterium spp.	Exochelins	(425)
Pseudomonas aeruginosa	Pyoverdin, pyochelin	(426)
Pseudomonas pseudomallei	Malleobactin	(427)
Salmonella enterica	Salmochelin, enterobactin	(260)
Staphylococcus aureus	Staphyloferrins A and B	(210, 238)
Vibrio parahaemolyticus	Vibrioferrin	(428)
Vibrio vulnificus	Vulnibactin	(263)
Yersinia pestis	Yersiniabactin	(264)

Growth of *S. equi* in minimal media containing serum, transferrin or lactoferrin as an iron source and equibactin's contribution to growth have not been reported (296).

When *S. equi* was grown *in vitro*, deletion of *eqbE* had no effect on the growth rate on nutrient rich THA. This may be due to import of cations through the activity of alternative cation transport systems. The *S. equi* genome encodes an HtsABC haem-binding system

(306), a putative MtsABC Mn^{2+} and Fe^{3+} metal transport system and a putative FtsABCD Fe^{3+} ferrichrome transport system that share sequence identity to cation transport systems of *S. pyogenes* (429, 430). Deletion of the *eqbA* promoter produced a small colony phenotype and the double deletion strain Δ*eqbAE*, had a large colony phenotype when grown on THA. This proved that the slow growth rate in the Δ*eqbA* strain was due to the function of the *eqbE* gene product. Complementation of the Δ*eqbA* strain with a plasmid that contained a second copy of *eqbA* under the control of the *eqbA* promoter produced a large colony phenotype when grown on THA. Transformation of the Δ*eqbAE* strain with a plasmid that contained a second copy of *eqbE* under the control of the *eqbB* promoter produced a small colony phenotype (296).

4.1.3 Transport

The antibiotic streptonigrin interacts with intracellular iron to form reactive oxygen species that leads to DNA damage and eventual cell death (431, 432). Bacteria that contain intracellular iron are therefore sensitive to its effects, while iron transport and siderophore mutants are more resistant due to decreased iron content. Numerous bacterial iron transport mutants have been isolated and characterised using streptonigrin, including the identification of the aerobactin transport system (433), transferrin-specific iron uptake in *Neisseria meningitidis* (434) and *Haemophilus influenza* (435). This assay was used in *S. equi* and demonstrates that equibactin is secreted by *S. equi* and that the *eqbH*, *eqbI* and *eqbJ* genes are required for its associated iron import (296). Quantification of ^{55}Fe accumulation by different *S. equi* strains showed reduced levels of ^{55}Fe in the Δ*eqbHIJA* strain, suggesting that the *eqbH*, *eqbI* and/or *eqbJ* gene products are essential for the majority of eqb product-dependent iron accumulation.

The *S. aureus* genome encodes at least six known and predicted ATP-binding cassette (ABC) transporters for iron or haem acquisition. Haem acquisition is mediated by the Isd system (436), while iron siderophore uptake systems (Sir, Hts and Fhu) have been characterised (218, 238, 437, 438). Streptonigrin sensitivity has been used in *S. aureus* to demonstrate that *sirABC* is involved in transport of staphyloferrin B, with *sirA* or *sirB* deletion mutants exhibiting increased resistance to iron-starved *S. aureus* to

streptonigrin (218, 228). From computer analysis of the staphylobactin region, *stbHIJKL* is predicted to encode an ABC transporter and therefore it was hypothesised that *stbHIJ* is responsible for transport of staphylobactin.

4.1.4 Virulence

As already shown in Table 1.1 (Chapter 1), numerous studies have demonstrated the impact of siderophores on virulence in both animals and plants. In *S. aureus*, the *sbn* gene cluster that encodes staphyloferrin B biosynthesis and efflux have been implicated in virulence in both the mouse bacteraemia and the rat infective endocarditis models (202, 439). In a murine sepsis model, single-locus deletions for either staphyloferrin biosynthesis (*sfa*, *sbn*) or uptake (*sirA*, *hts*) did not produce statistically significant reductions of bacterial counts in any organ, while deletion of catechol iron uptake (*sst)* alone or deletion of the sfa-sbn or hts-sir combination did yield significant reductions in heart colonisation. Combined inactivation of sst with staphyloferrin biosynthesis resulted in a noticeable decrease in heart and liver colonisation, but combining sst with the staphyloferrin uptake mutant resulted in an even larger drop in bacterial counts recovered from the heart, liver and kidneys (238).

Wild-type *S. equi* and *eqbE* deletion mutant strains were tested for virulence in ponies. The Δ*eqbE* strains significantly reduced the amount of pyrexia and pathology at post mortem examination. This suggests that deletion of the equibactin locus renders *S. equi* significantly less able to cause acute disease in the natural host and may enhance survival within, and abscessation of, lymph nodes (305). It was therefore hypothesised that staphylobactin plays a role in virulence. In this study the wax moth virulence model was used to study the impact of staphylobactin on virulence. This model has been shown to provide an insight into the pathogenesis of a wide range of microbial infections including mammalian fungal and bacterial pathogens including *S. aureus* (440) and *Campylobacter jejuni* (441), and has previously been used in our lab to study antibiotic treatment of *mecC* MRSA strains (404). Specifically, bacterial iron acquisition systems have been studied for their effect on virulence in the wax moth model (240, 442, 443).

4.2 Materials and Methods

4.2.1 Bacterial growth conditions

Bacterial strains and plasmids used are summarised in Table 4.2. For routine culture, all bacteria were cultured at 37 °C, shaking at 200 rpm unless otherwise indicated. *E. coli* was grown in LB broth or on L-agar (Oxoid, UK). *S. aureus* was grown in TSB (Oxoid, UK) or as specified below in: Tris-minimal succinate broth (TMS) (61); TMS chelated with 2,2'-dipyridyl (Sigma-Aldrich) or the non-metabolisable iron chelator ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid (EDDHA) (LGC Standards); a 40:60 mixture of TMS and heat inactivated horse serum (Sigma-Aldrich) (HoS-TMS); TMS treated for 24 h at 4 °C with 10% w/v Chelex-100 resin (Bio-Rad) prior to addition of post-autoclaving nutrients (C-TMS). For iron repletion of restricted growth media. $FeCl_3$ was added to a final concentration of 50 to 100 μM. For selection of plasmids and recombinant alleles, antibiotics were added to media at the following concentrations; ampicillin (100 μg/ml), chloramphenicol (10 μg/ml). All media was made with water purified through a Milli-Q water purification system (Millipore). Plastic wear was used for growth of cultures to avoid residual iron contamination.

Table 4.2. Bacterial strains and plasmids used in this study

Strain/Plasmid	Description	Source or Reference
S. lugdunensis N920143	*Staphylococcus lugdunensis* isolated from a breast cancer abscess	(330)
S. aureus **strains**		
Newman	Wild-type clinical osteomyelitis isolate	(444)
8325-4	Prophage-cured laboratory strain	(445)
8325-4Δ*sfa*Δ*sbn*	8325-4Δ*sbn*::Tet Δ*sfa*::Km; Tet^R Km^R	(210)
LGA251	ST425, *mecC* reference strain	(67)
LGA254	ST425, *mecC* clinical isolate	(67)
H093880936	ST425, *mecC* clinical isolate	This study
m-MR-6AA	ST133, *mecC* clinical isolate	This study
PI 41/95	ST425, *mecC* clinical isolate	This study
C05 232	ST151, *mecC* clinical isolate	This study
Enr. 7594/1975	ST130, *mecC* clinical isolate	This study
MS-18.31	ST130, *mecC* clinical isolate	This study
8572	ST130, *mecC* clinical isolate	This study
08-02004	ST130, *mecC* clinical isolate	This study
2383/03	ST130, *mecC* clinical isolate	This study
C03 365	ST130, *mecC* clinical isolate	This study
KO	ST130, *mecC* clinical isolate	This study
71277	ST130, *mecC* clinical isolate	(67)
71277Δ*sfa*Δ*sbn*	71277 with *sfa* and *sbn* deletion	This study
71277Δ*stbE*Δ*stbHIJ*	71277 with *stbE* and *stbHIJ* deletion	This study
71277Δ*stbE*Δ*stbHIJ*Δ*sbn*	71277 with *stbE*, *stbHIJ* and *sbn* deletion	This study
71277Δ*mntR*	71277 with *mntR* deletion	This study
71277Δ*sfa*	71277 with *sfa* deletion	This study
71277Δ*sbn*	71277 with *sbn* deletion	This study
71277Δ*stbE*	71277 with *stbE* deletion	This study
71277Δ*stbHIJ*	71277 with *stbHIJ* deletion	This study

71277Δ*stb*	71277 with *stbB-O* deletion	This study
71277Δ*stbE-J*	71277 with *stbE-J* gene deletions	This study
71277ΔstbE-JΔsfaΔsbn	71277 with *stbE-J*, *sfa* and *sbn* gene deletions	This study
71277ΔstbΔsfaΔsbn	71277 with *stbB-O, sfa* and *sbn* gene deletions	This study
RN4220	Restriction-defective derivative of 8325-4 ST 8	(395)
LS-1	Spontaneous murine arthritis isolate	(446)
LS-1Δ*HtsA*	LS-1 derivative, Δ*htsA*	(447)
Plasmids		
pLI50	*E. coli - S. aureus* shuttle vector; Ap^R/Cm^R	(402)
pLI50*stbE*	pLI50 with *stbE* gene from 71277	This study
pLI50*sfa*	pLI50 with *sfa* gene from 71277	This study
pLI50*sbn*	pLI50 with *sbn* gene from 71277	This study
pRMC2	*E. coli – S. aureus* shuttle vector. Amp^R, Cm^R	(403)
pXB01	*E. coli - S. aureus* shuttle vector pRMC2 with *bla* deletion; Cm^R	(404)
pXB01stbHIJ	pXB01 with *stbHIJ* genes from 71277	This study

4.2.2 Real-time PCR

S. aureus cells were grown to stationary phase overnight in TMS with 100 µM 2,2-dipyridyl. Iron-starved cells were washed twice in saline buffer and diluted 1:100 into HoS-TMS broth. Cultures were grown at 37 °C to mid-logarithmic phase (OD$_{600}$ ~ 1.0) then partitioned into two aliquots of equal volume. One aliquot from the pair was spiked with 100 µM FeCl$_3$. Growth was allowed to resume for 1 h and then pelleted by centrifugation with the addition of RNA Protect Bacteria Reagent (Qiagen). Total RNA was extracted from bacteria using RNeasy kit (Qiagen) according to the manufacturers' instructions. RNA extraction was preceded by mechanical cell disruption through two 20 second treatments in 500 µl RLT buffer in a 2 ml Precellys ceramic tube (Peqlab). An additional DNAse treatment was carried out using Turbo DNA-free kit (Ambion) before being reverse transcribed with ProtoScript II (NEB) as directed, with primers listed in Table 4.3 using universal primers for *gryB* as a control. Quantitative real-time PCR (qPCR) was performed with a Rotor-Gene Q instrument (Qiagen) using SensiFAST SYBR No-ROX

Kit (Bioline). For the qPCR, template cDNA was diluted five-fold with nuclease free water. Quantification relative to the housekeeping gene *gryB* was performed. For each representative RNA extraction, one-step reactions were performed in triplicate. Reverse transcriptase-negative controls were included to confirm the absence of contaminating DNA from RNA samples.

Table 4.3. Oligonucleotides used in this study

Primer	Sequence
stbE-RT-PCR-F	AACAGGTTCTCAGGTTAATTCTGC
stbE-RT-PCR-R	GCATGTCAGTCACAACATCGT
stbG-RT-PCR-F	GAGGGGTGTGAAGCTACTCG
stbG-RT-PCR-R	AATCACTTCGTTTTCCACGC
sfaD-RT-PCR-F	CCTCTAATGCAATGCCATATTTA
sfaD-RT-PCR-R	ACAATGAATCACCTATCGTGACA
sfaA-RT-PCR-F	AGTCTATCATGCGCCAACAAC
sfaA-RT-PCR-R	AACCTGTCGCCATAATCAATAA
Newman*htsA*-RT-PCR-F	TTTAAATCCAGAGCGTATGATCA
71277*htsA*-RT-PCR-F	GCTGACTTAAATCCTGAACGC
htsA-RT-PCR-R	CAGAAGAAATTAAGCCACGAGAT
gyrB-RT-PCR-F	ATAATTATGGTGCTGGGCAAAT
gyrB-RT-PCR-R	AACCAGCTAATGCTTCATCGATA
StbE_KpnI FW	ATATGGTACCGGAAAGGGGAGTTTAGCATGGG
StbE_SacI RV	ATATGAGCTCGCACTTCTTCATGTTCTAAATG
StbHIJ_KpnI FW	ATATGGTACCGAATAGGAGTTAGTAATATG
StbHIJ_SacI RV	ATATGAGCTCGGCTCCTTTCTTCAACACAAT
pLI50MCS_FW	GCGCACATTTCCCCGAAAAG
pLI50MCS_RV	TCCAATACAAAACCACATAC
pRMC2MCS-FW	TCCCCTCGAGTTCATGAAAA
pRMC2MCS-RV	GTTGTAAAACGACGGCCAGT

4.2.3 Bacterial growth curves

For each strain, a single isolated colony grown on TSA plates was inoculated into 5 ml C-TMS broth and incubated at 37 °C, 200 rpm shaking until it reached stationary phase. From this culture, cells were washed twice in sterile phosphate buffered saline (PBS), normalised to an OD_{600} of 0.1 and diluted 1:100 into C-TMS, 60:40 HoS-C-TMS, 20:80 HoS-C-TMS broth or C-TMS broth containing human transferrin (Sigma-Aldrich). Culture medium was used as described (iron deplete) or supplemented with 50 µM or 100 µM $FeCl_3$ (iron-replete) or with $NiCl_2$, $CoCl_2$, $MnSO_4$, $MnCl_2$, $ZnCl_2$ or $FeSO_4$ added to C-TMS at concentrations of 0.001 µM to 1000 µM. Cultures were grown under constant high amplitude shaking in a Bioscreen C machine at 37 °C, with absorbance measured at 600 nm every 30 minutes for 48 hours. Each strain was tested in triplicate. For growth in milk, sterilised liquid milk was purchased at a retail store and added to C-TMS.

4.2.4 Streptonigrin sensitivity

Streptonigrin sensitivity was performed using microbroth dilution for minimum inhibitory concentrations (MIC) according to BSAC protocols (http://bsac.org.uk/wp-content/uploads/2012/02/Chapter-2-Determination-of-MICs 2006updated.pdf). Test strains were grown on TSA overnight at 37 °C. At least four morphologically similar colonies were touched with a sterile loop and inoculated into 5 ml Iso-Sensitest broth (Oxoid, UK). Inoculated broth was incubated at 37 °C with 200 rpm shaking until the visible turbidity was that of a 0.5 McFarland standard. This culture was diluted 1:100 in Müller-Hinton broth (MHB) (Oxoid, UK), for inoculation. A flat-bottomed 96 well cell culture plate (Greiner Bio-One, CELLSTAR®) was loaded with appropriate antibiotic dilutions. For each test isolate, 75 µl of each antibiotic dilution were added to a row of wells and 75 µl diluted test isolate culture was added into the wells. The 96 well plate was then placed in a plastic bag to minimise evaporation. Each isolate was tested in triplicate for streptonigrin minimum inhibitory concentration (MIC) by incubation at 35 °C for 20 hours before readings were recorded. Antimicrobial susceptibility was interpreted by comparing growth from a well containing no antibiotic. The antibiotic ranges were prepared in MHB a step higher than the final concentrations 64 – 8 ng/ml for streptonigrin.

For bacterial growth curves in the presence of streptonigrin, cultures were grown overnight in TSB or TMS to late logarithmic phase under aerated conditions at 37 °C. Cells were diluted 1:100 into fresh test broth of either TSA, C-TMS, 60:40 HoS-TMS, C-TMS plus 250 µg/ml transferrin, C-TMS plus 50 µM $FeCl_3$ containing increasing concentrations of streptonigrin (1 – 20 ng/ml) and grown under constant high amplitude shaking in a Bioscreen C machine at 37 °C, with absorbance measured at 600 nm every 30 minutes for 48 hours. Each strain was tested in triplicate.

4.2.5 Bioactivity test

The antimicrobial activity of staphylobactin containing isolates was determined using a variety of bacterial isolates for the capacity to inhibit growth. Overnight cultures of the test strains were grown in TSA and diluted 1:1000 into 0.9% NaCl and streaked onto either Basic Medium (BM) agar (448), supplemented with 200 µM 2,2'-Bipyridine for iron limiting conditions. 10 µl of *S. aureus* CC130, 71277Δ*stb* or *S. lugdunensis* culture grown in C-TMS overnight was spotted onto sterile paper discs. The plates were incubated for 24 hours at 37 °C and observed for a zone of inhibition around the paper disc to confirm antimicrobial activity.

4.2.6 Chrome-azurol S assay for siderophore activity

Quantification of siderophore output from *S. aureus* strains was performed by testing the iron-binding activity of culture supernatants, using a chrome-azurol S (CAS) shuttle solution (449). *S. aureus* cells were grown to stationary phase in C-TMS and washed twice in saline buffer. These cultures were diluted 1:100 into C-TMS containing either 50 µM $FeCl_3$ or C-TMS only (iron deplete). Cultures were grown at 37 °C, shaking at 200 rpm. Readings were taken at 24 and 48 hours by adding equal volumes of culture supernatants to CAS shuttle solution and incubated in darkness at room temperature with gentle shaking for 15 min before the absorbance was taken at 630 nm. With an empty cuvette serving as the blank, siderophore units were calculated as follows:

Siderophore units = $\dfrac{100 \text{ x dilution factor x (A630 of control solution} - \text{A630 of sample)}}{\text{A630 of control solution}}$

For the determination of siderophore activity of raw culture supernatants, siderophore units were normalized to a culture OD_{600} of 1.0.

4.2.7 Production of siderophore from culture supernatants and high resolution liquid chromatography mass spectrometry (LC-MS) analysis

S. aureus cells grown to stationary phase in C-TMS and washed twice in saline buffer. These cells were diluted 1:100 into TMS or C-TMS and grown at 37 °C, 200 rpm to post exponential phase (approximately 40 h) in plastic Falcon tubes. Cells were removed by centrifugation at 5000 rpm, 4 °C and culture supernatants (50ml) were frozen using dry ice. Comparative metabolic profiling of the wild-type 71277, 71277Δ*stbE* strains were performed using LC-MS with Dionex 3000RS UHPLC coupled with Bruker MaXis Impact Q-TOF mass spectrometer by staff of the laboratory of Greg Challis (Warwick University) as follows. An Agilent Zorbax Eclipse plus column (C18, 100x2.1mm, 1.8μm) was used. Mobile phases are consisted of A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). After 5 minutes of isocratic run at 5% B, a gradient of 5% B to 100% B in 15 minutes was employed with flow rate at 0.2ml/min, then the column was washed with 100% B for 5 minutes before equilibrate back to initial condition in 3 minutes. UV detector was set at 210 nm. Mass spectrometer was operated in electrospray positive mode with a scan range 50-3,000 m/z. Source conditions are: end plate offset at -500V; capillary at -4500V; nebulizer gas (N_2) flow at 1.4bar; dry gas (N_2) flow at 8L/min; dry Temperature at 180 °C. Ion transfer conditions as: ion funnel 1 RF at 200Vpp; ion funnel 2 RF at 200vpp, hexapole RF at 200Vpp; quadruple ion energy at 5ev, quadrupole low mass set at 55 m/z; collision energy at 5.0ev; collision RF ramping from 800 to 1500 Vpp; transfer time set at from 100 to 155 μs; pre-Pulse storage time set at 5 μs. Calibration was performed with sodium formate (1 mM) through a loop injection of 20 μl of standard solution at beginning of each run. Sample injection volume was 2 μl.

4.2.8 Wax moth larvae (*Galleria mellonella*) infection assay

The wax moth larvae assay was based on a method previously described by Desbois *et al*. (450). *Galleria mellonella* larvae were purchased in bulk from Livefood UK. Larvae were stored at 4°C upon arrival and kept at 37°C during the course of the assay. Wild-

type and deletion mutant *S. aureus* strains were selected to evaluate the contribution of staphyloferrins and staphylobactin to virulence. Single bacterial colonies were picked to inoculate 5 ml of TSB, and cultures were grown overnight (~16 hours) at 37°C and 200 rpm shaking. Cultures were then diluted 1:100 into 5 ml of fresh TSB and grown for a further 4 hours at 37°C and 200 rpm shaking. Cultures were then centrifuged at 2,500 g for 10 minutes, the supernatant was removed, and pellets resuspended in sterile PBS to an OD_{595nm} of 0.2, giving approximately 1.3×10^6 CFU/ml (range: $1.0 – 1.4 \times 10^6$ CFU/ml). For each strain, groups of *G. mellonella* (n=10 in each group) were injected with 10 µl aliquots of resuspended culture or PBS behind the rear thoracic segments using a Tridak Stepper Pipette Dispenser (Dymax, UK). Once infected, larvae were incubated at 37°C for 5 days, and survival rate scored every few hours. Larvae were considered dead when they did not respond to touch to the head. The experiment was performed three times. All the strains were tested in three independent experiments and the combined results of all three experiment are presented in the results.

4.2.9 Construction of *S. aureus* gene deletion mutants

Oligonucleotide primer sequences are listed in Table 3.1. All deletion mutants in *mecC*-MRSA strains were generated by allelic exchange with the temperature-sensitive vector pIMAY, as described in Chapter 3.

4.2.10 Complementation of mutant strains.

For complement expression of *stbE* and *stbHIJ*, these genes were cloned into expression plasmid pLI50 or pXB01, a derivative of the tetracycline-inducible expression vector pRMC2 with the *bla* gene deleted (403, 404). Both genes were amplified from the strain 71277 genome DNA with primer pairs StbE_KpnI FW/StbE_SacI RV or StbHIJ_KpnI FW/StbHIJ_SacI RV (Table 4.3) using high fidelity Taq polymerase. PCR products were digested with KpnI and SacI and ligated with the pXB01 or pLI50 vector cleaved with the same enzymes, generating plasmids pXB01-*stbHIJ* and pLI50-*stbE*. The plasmids were transformed into *E. coli* DC10B, and plasmid DNA was then extracted and electroporated into mutant strains for complementation with expression induced with 200 ng/ml anhydrotetracycline (Sigma-Aldrich, United Kingdom). Confirmation of mutant strains

containing the plasmid complement was performed by colony PCR using MyTaq polymerase with primers pLI50MCS-FW/RV or pRMC2MCS-FW/RV Table 4.3.

4.3 Results

4.3.1 Regulation of the staphylobactin locus by MntR

To determine if the regulation of the staphylobactin (stb) non-ribosomal peptide synthesis (NRPS) is achieved through the action of transcriptional repression by MntR in an iron-dependent manner, deletion mutants of *mntR* were constructed using the pIMAY plasmid as previously described in *S. aureus* CC130 strain 71277. Wild-type strain 71277 and 71277Δ*mntR* were grown in minimal media with or without iron. RNA was extracted and qPCR was carried out for the expression of the predicted NRPS, *stbE* and the predicted polyketide synthase (PKS), *stbG* under these conditions (Figure 4.1A).

Figure 4.1. Expression of *stbE* and *stbG* genes in wild-type and *mntR* deletion mutant

(A) Regulation of *stbE* and *stbG* by MntR in *S. aureus* strain 71277 (CC130). Real-time PCR of wild-type and a *mntR* inactivated mutant demonstrates upregulation in the absence of iron, or in the absence of the MntR protein when iron is absent. Error bars represent standard deviation from the mean. (n = 3). Strains were grown in HoS-TMS medium. The '+ Fe' cultures were spiked with 100 µM $FeCl_3$ for one hour at mid-log phase prior to cell harvesting. Quantification relative to housekeeping gene *gryB* was performed. Statistical differences between mutants and wild-type parent strain were calculated using the Student's unpaired *t*-test, *stbE* P = 0.1483, *stbG* P = 0.5475. P value: significant difference is P<0.05. Results presented in linear scale. (B) Gene expression fold change for *stbE* and *stbG* calculated using double delta Ct analysis. Transcription levels of *stbE* (left) and *stbG* (right) with and without iron supplementation in wild-type and the *mntR* mutant. Results presented in linear scale.

A.
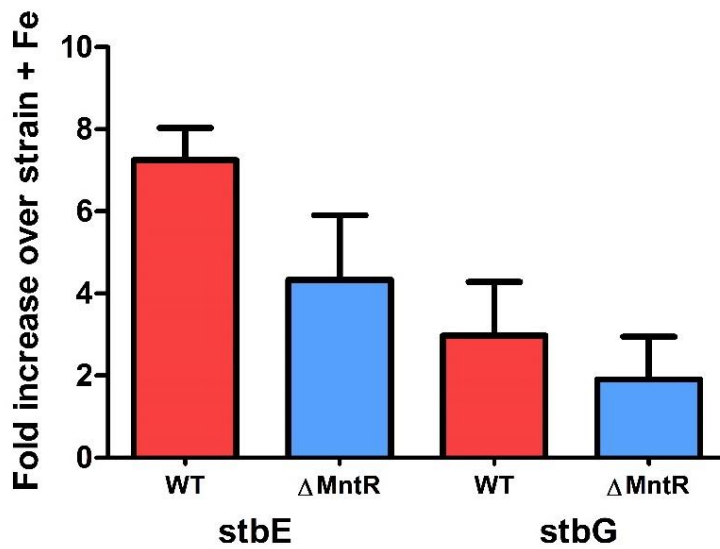
B.
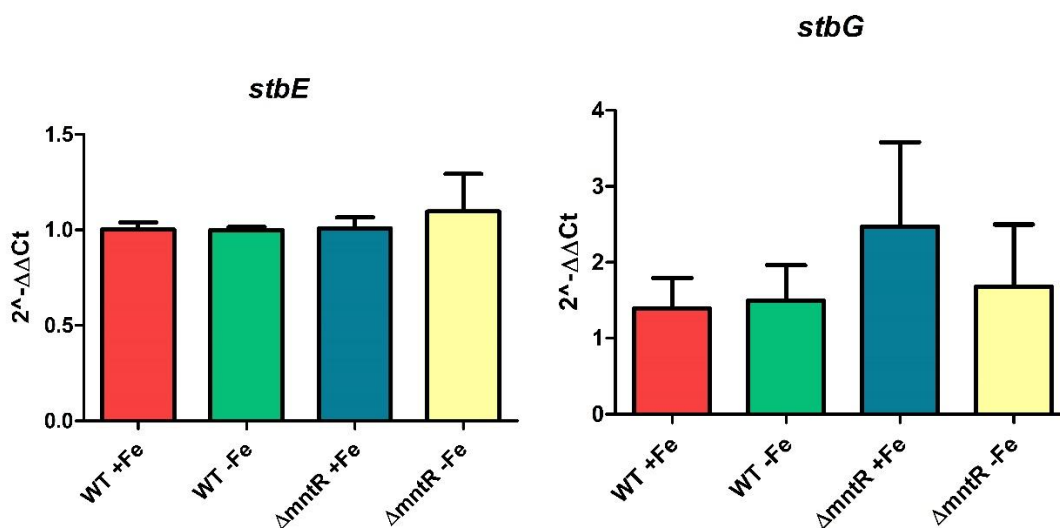
RT-qPCR revealed a reduction in the *stbE* and *stbG* transcripts in 71277Δ*mntR*, from 7 fold to 4 fold, and 3 fold to 2 fold compared with 71277 when grown in minimal media without iron compared to iron supplemented media (Figure 4.1). This suggests that MntR is a positive regulator of the staphylobactin operon in an iron-dependent manner. Also, no small colony phenotype was observed in the 71277Δ*mntR* which was observed in the *eqbA* deletion mutants. I cannot conclude anything for certain because there was no statistically significant difference between the wild-type and mutant strain

expression of *stbE* and *stbG* using the Students' unpaired *t*-test. However, the results show a trend so further investigative work should be carried out.

4.3.2 Expression of staphylobactin NRPS in an iron-dependent manner

Previous work demonstrated that the *sfa* and *sbn* operon is expressed under conditions of iron restriction in a Fur/Fe-dependent fashion (202). It was of interest to determine if *stb* NRPS is expressed under conditions of iron restriction. Expression of the NRPS genes *stbE* and *stbG* were assessed in the wild-type strain 71277 when grown in iron deplete media compared to iron-replete media and results are shown as fold increase of expression (Figure 4.1). This was done using real-time PCR analysis to confirm if they are upregulated and expression of *sfaD*, *sfaA* and *htsA* were also assessed in the presence and absence of iron in the 71277 CC130 strain, to provide positive controls (Figure 4.2).

Figure 4.2. Expression of the *hts* and *sfa* genes in an iron-dependent manner

Regulation of *htsA, sfaA* and *sfaD* by iron in strain 71277. Real-time PCR of wild-type demonstrates upregulation in the absence of iron. Error bars represent standard deviation from the mean (n = 3). Strains grown in HoS-TMS medium. + Fe denotes cultures spiked with 100 µM $FeCl_3$ for one hour at mid-log phase prior to cell harvesting. Quantification relative to housekeeping gene *gryB* was performed. Results presented in logarithmic scale.
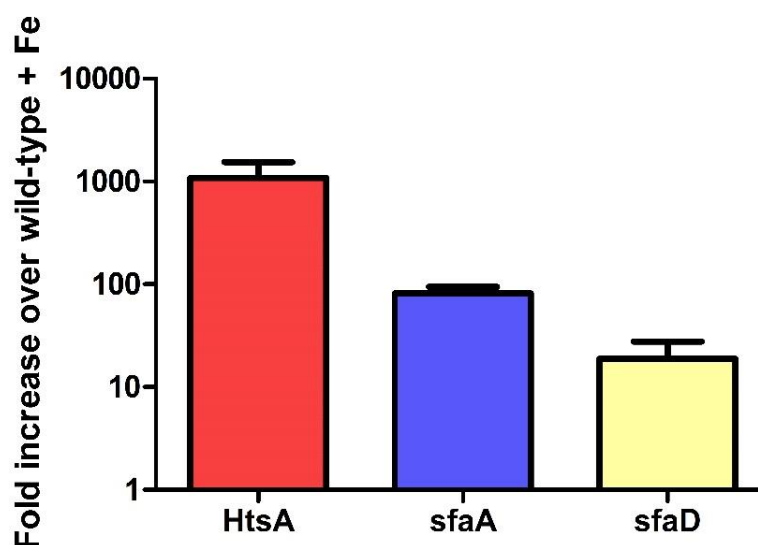
Both sets of transcripts are upregulated in an iron-deprivation-dependent fashion, i.e. they are repressed by iron saturation of the culture medium. The staphylobactin genes, *stbE* and *stbG*, had a 7 and 3 fold increase but less than the staphyloferrin A genes (Figure 4.2). The staphyloferrin biosynthesis genes *sfaA* and *sfaD* had an 80 fold and 20 fold increase and the staphyloferrin A transport gene *HtsA* had a 1000 fold increase over the same strain grown in iron-replete conditions (Figure 4.2).

RT-PCR experiments were also carried out with deletion mutants 71277Δ*sfa*Δ*sbn*, 71277Δ*stbE-J* and wild-type 71277 in iron-replete and deplete minimal media to see if deletion of either staphyloferrins (*sfa*, *sbn*) or staphylobactin (*stb*), compromising iron uptake and exacerbating iron starvation, would consequently enhance expression of the unchanged iron acquisition genes, including those involved in staphylobactin production (218). Expression of *sfaA*, *sfaD* and *HtsA* in the 71277Δ*stbE-J* strain, *stbE* and *stbG* in the 71277Δ*sfa*Δ*sbn* strain were observed and compared to expression in wild-type 71277. Mutation of either locus had no obvious effect on the expression of the alternative locus in an iron-dependent manner compared to wild-type expression (results not shown).

4.3.3 Growth of *S. aureus* in serum

Previous studies have demonstrated that, in comparison to wild-type *S. aureus*, mutants lacking the ability to produce the two staphyloferrin siderophores (Sfa and Sbn) grow poorly in iron-restricted media containing transferrin or horse serum (as a source of transferrin) (210, 238). Given that *S. aureus* CC130 genomic information suggests that staphylobactin may produce a siderophore involved in iron acquisition, it was hypothesised that deletion of the locus would lead to a drop in siderophore production, and consequently this would result in a strain deficient in its ability to grow in iron-chelated culture media (e.g. serum). To address this hypothesis, strains 71277Δ*stb*, 71277Δ*sfa*Δ*sbn* and 71277Δ*sfa*Δ*sbn*Δ*stb* were created and the growth of *S. aureus* 71277 compared to that of its staphyloferrin and/or staphylobactin deficient mutants in iron-restricted media was observed.

In comparison to the wild-type CC130 strain, staphyloferrin mutants (71277Δ*sfa*Δ*sbn* and 71277Δ*sfa*Δ*sbn*Δ*stb*) grew poorly in medium comprised of 40% low-iron Chelex-

treated TMS broth (C-TMS) with 60% complement-deactivated horse serum (HoS) (Figure 4.3A). Even after extended incubation periods (48 hrs), the staphyloferrin mutants never reached a final biomass equivalent to that of the wild-type, only reaching an OD_{600nm} of approx. 0.4. However, wild-type strain 71227 and 71277Δ*stb* grew at an approximately equivalent cell density and showed no growth impairment. This implies that the growth deficiency of 71277Δ*sfa*Δ*sbn* and 71277Δ*sfa*Δ*sbn*Δ*stb* was due to a deficiency in the ability to scavenge trace amounts of iron by the staphyloferrins and that staphylobactin did not contribute to iron-restricted growth in these conditions. Supplementation of the growth media with 50 µM (Figure 4.3B) or 100 µM of $FeCl_3$ (data not shown) promoted rapid growth of all strains at the same rate and biomass.

Figure 4.3. Growth of *S. aureus* CC130 71277 in serum.

(A) *S. aureus* strain 71277 and staphylobactin (Δ*stb*), and/or staphyloferrin A (Δ*sfa*) and B (Δ*sbn*) mutants were cultured in 60:40 HoS-TMS medium. Lack of staphyloferrin production severely restricted growth, however lack of staphylobactin did not. (B) All mutant kinetics were ameliorated to wild-type with the addition of 50 µM FeCl₃.

(A)

(B)
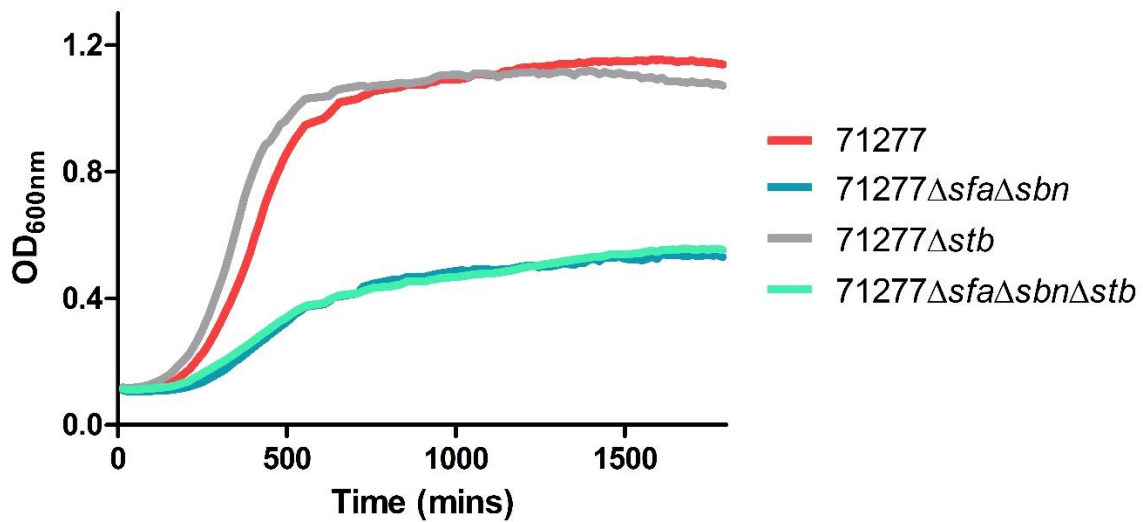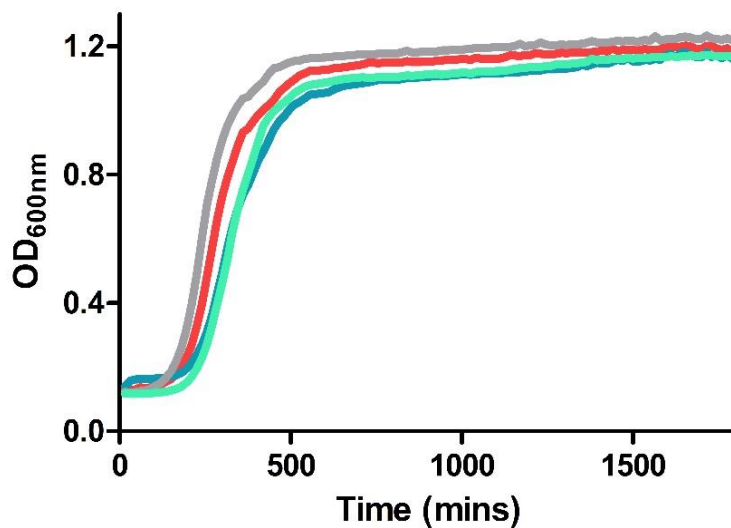
This growth curve was repeated using the full complement of staphyloferrin and staphylobactin deletion mutants, including the strains 71277Δ*sfa*, 71277Δ*sbn*, 71277Δ*sfa*Δ*stbE*Δ*stbHIJ* and 71277Δ*sbn*Δ*stbE-J*. Previously it was demonstrated that deletion of only the staphyloferrin A locus had no effect on growth, and deletion of staphyloferrin B only revealed reduced growth in serum, but not to the same extent as the severe growth defect observed with a strain lacking both staphyloferrin operons. When grown in minimal media containing serum as an iron source, strains 71277Δ*sfa*, 71277Δ*sfa*Δ*stbE*Δ*stbHIJ* and 71277Δ*stb* grew at the same rate and similar cell density at the wild-type 71277 strain, reaching an OD_{600nm} of approx. 1 after 500 minutes. This demonstrated that deletion of staphyloferrin A and/or staphylobactin did not affect growth of bacteria in serum. Deletion of staphyloferrin B (71277Δ*sbn*) alone or in combination with staphylobactin (71277Δ*sbn*Δ*stbE-J*) did affect the growth rate which was slower compared to the wild-type strain, reaching an OD_{600nm} of approx. 0.8 after 1000 minutes. This suggested that staphyloferrin B is required for full growth in serum.

To ensure these results were not specific to *mecC* CC130 strain 71277 carrying the additional staphylobactin locus, growth experiments were repeated using *mecA* CC8 control strain 8325-4 and its staphyloferrin mutant. Similar growth dynamics were observed (Figure 4.4A). Equivalent cell density for the staphyloferrin mutants was achieved for both strains, 71277 and 8325-4, although 8325-4 wild-type strain grew at a slower rate and did not reach the same cell density as wild-type strain 71277 that contains the staphylobactin region. Growth could be corrected to wild-type levels if the medium was saturated with free iron (Figure 4.4B), suggesting that strains containing the staphylobactin locus may have enhanced growth over strains lacking this locus in iron-replete conditions.

Figure 4.4. Growth of *S. aureus* CC8 8325-4 in serum.

(A) *S. aureus* strain 8325-4 and staphyloferrin A and B mutants were cultured in HoS-TMS medium. Lack of staphyloferrin production severely restricted growth. (B) All mutant kinetics were ameliorated to wild-type with the addition of 50 µM $FeCl_3$.

(A)
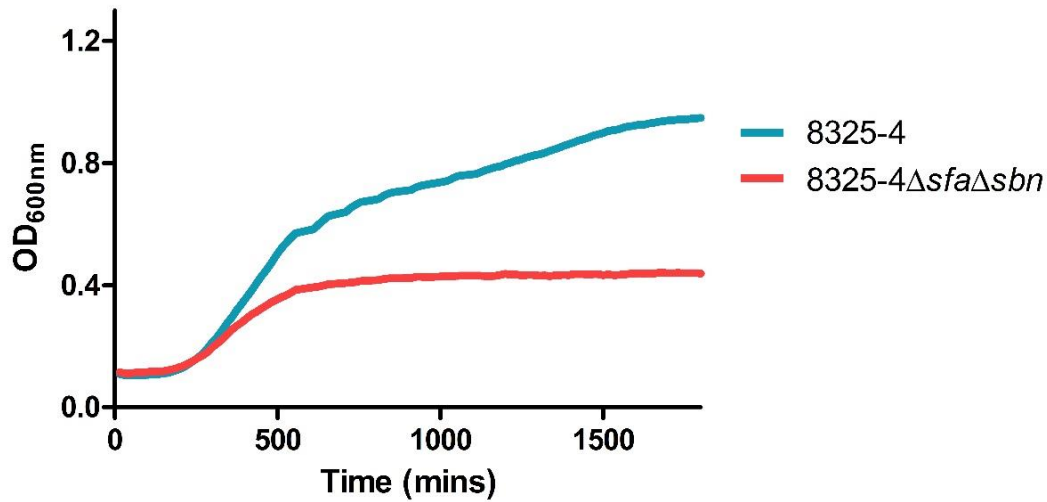
(B)
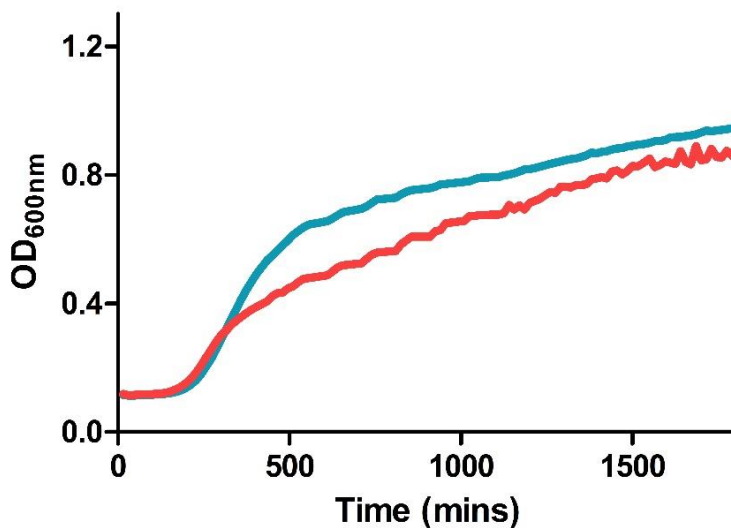
The same results were achieved when repeated in medium comprised of 80% C-TMS: 20% HoS. The wild-type and 71277Δ*stb* strain did not reach the same cell density or growth rate as the same strains grown in the serum rich minimal media. This is probably due to the lack of nutrients available in this low serum minimal media. Strains lacking staphyloferrins, with or without staphylobactin, showed severely attenuated growth. This defect could be corrected to wild-type levels if the medium was saturated with free iron. Strain 8325-4 and 8325-4Δ*sfa*Δ*sbn* produced nearly exactly the same results when grown in high or low serum minimal media, growing at the same rate and cell density (results not shown). Again this suggests that the presence of the staphylobactin region enhances growth in iron-replete conditions over those lacking this region, or this CC130 strain contains other mechanisms to allow for enhanced growth compared to the CC8 strain tested.

Due to the growth dynamics observed between strains 71277 (CC130) and 8325-4 (CC8) in minimal serum media supplemented with iron, it was of interest to see if there was a difference in growth for strains from different clonal complexes, containing *mecC* (with or without the staphylobactin locus), *mecA* or CoNS (containing the homologous staphylobactin region, but not the staphyloferrin loci) from a range of host species. *mecA* strains; 8325-4 (CC8), Newman (CC8), RN4220 (CC8), BCVA92 (CC398) and LS-1 (CC unknown) were grown in 60:40 HoS-TMS medium with or without iron (Figure 4.5), along with *mecC* CC130 strains; 71277 and 2383/03 isolated from human, C03 365 and KO isolated from bovine and 08-02004 isolated from deer. *mecC* strains from other clonal complexes; LGA254 and LGA251 from bovine, H093880936 from human and PI 41/95 from rabbit (all CC425), C05 232 from bovine (CC151) and m-MR-6AA from bovine (CC133) were also tested. Growth of *mecA* strains varied largely in both the growth rate and final optical density, from strains of the same clonal complex, ranging from OD_{600nm} 0.8-1.2 at 2000 minutes, even when media was supplemented with iron. The experiment was repeated and very similar results for all strains were seen when grown in either high or low serum minimal media, with or without iron supplementation (results not shown). This suggested that staphyloferrin biosynthesis and uptake, or other iron acquisition systems vary in their ability to utilise transferrin as an iron source for growth in these conditions and strains tested.

Figure 4.5. Growth of *S. aureus mecA* strains in serum.

(A) *S. aureus mecA* strains: 8325-4, Newman, RN4220, BCVA92 and LS-1 grown in 60% HoS-40% TMS medium. (B) Addition of 50 µM $FeCl_3$ to the 60% HoS-40% TMS medium. All strains encode for the staphyloferrins but not staphylobactin.

(A)

(B)
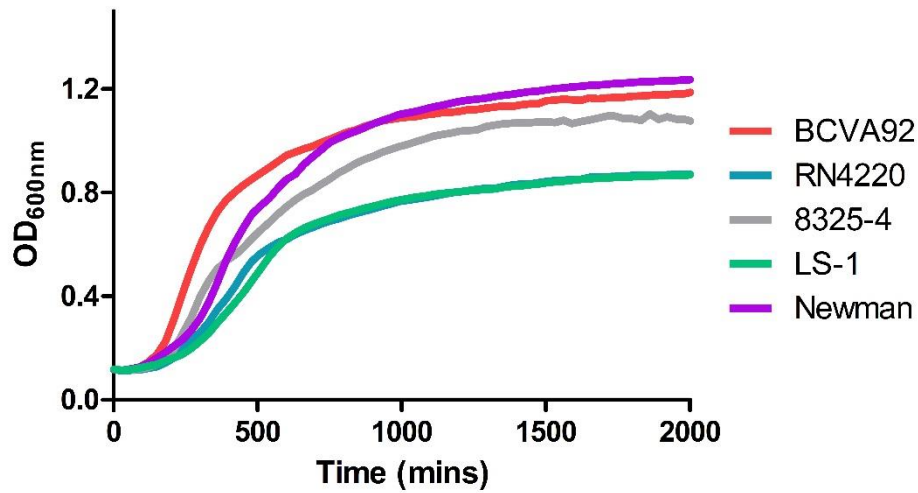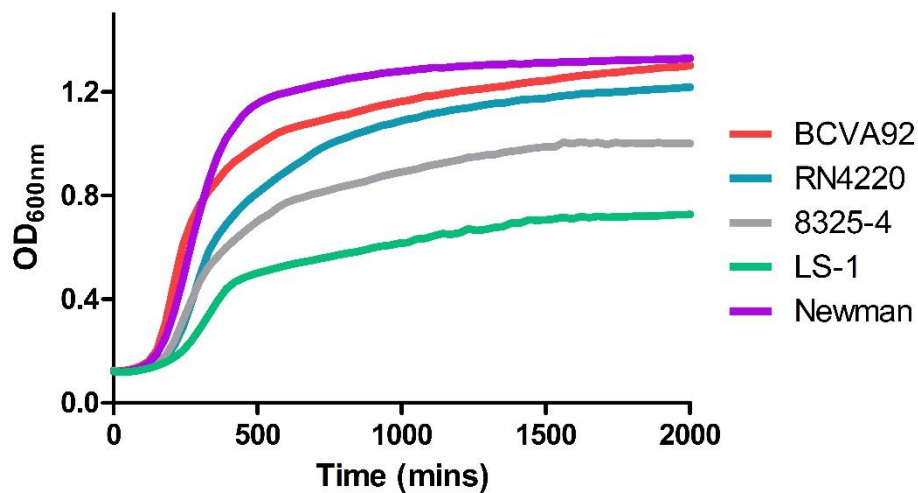
I tested strains from CC130, from a range of host species, containing both staphyloferrins and the staphylobactin loci, to see if growth in serum differed with the host or was enhanced by the presence of the staphylobactin locus. Less difference was seen in growth rates (OD_{600nm} at 2000 minutes = 1.1-1.2), especially strains 71277, 08-02004 and KO which grew at the same rate and optical density in both conditions. Strain 2383/03 grew the fastest and to the highest OD, reaching a very high OD (OD_{600nm} = 1.5) when supplemented with iron (Figure 4.6). The same growth trends were seen when repeated and grown in low serum media (results not shown). These results suggest that the *mecC* strains containing the staphylobactin and staphyloferrin loci have the ability to utilise transferrin in serum more efficiently to enhance growth compared to the *mecA* strains. *mecC* strains' growth is not affected by the host from which the strain was isolated.

Figure 4.6. Growth of *S. aureus* CC130 containing the staphyloferrins and staphylobactin locus in serum.

(A) *S. aureus* CC130 strain 71277, 2383/03, KO and 08-02004 containing the staphylobactin region were cultured in 60% HoS-40% TMS medium. Letters in brackets indicate the host that the strain was isolated from; H= Human, D= Deer, B= Bovine (B) Addition of 50 µM FeCl$_3$ to the 60% HoS-40% TMS medium.

(A)
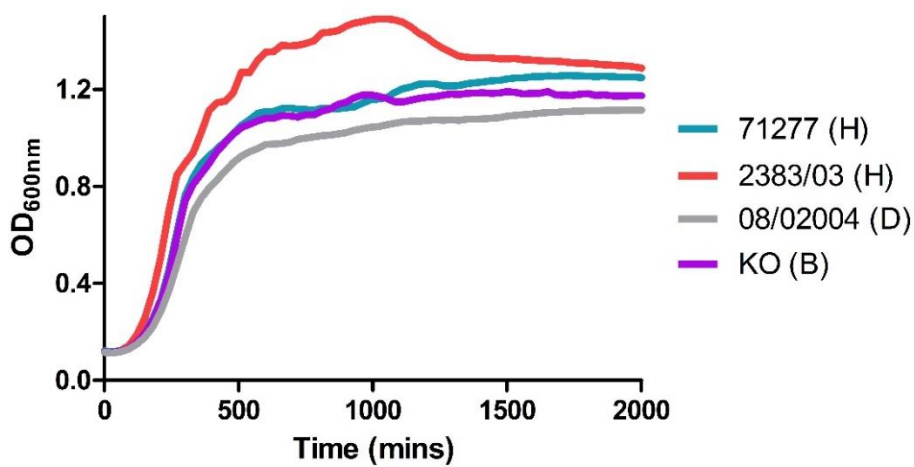
(B)
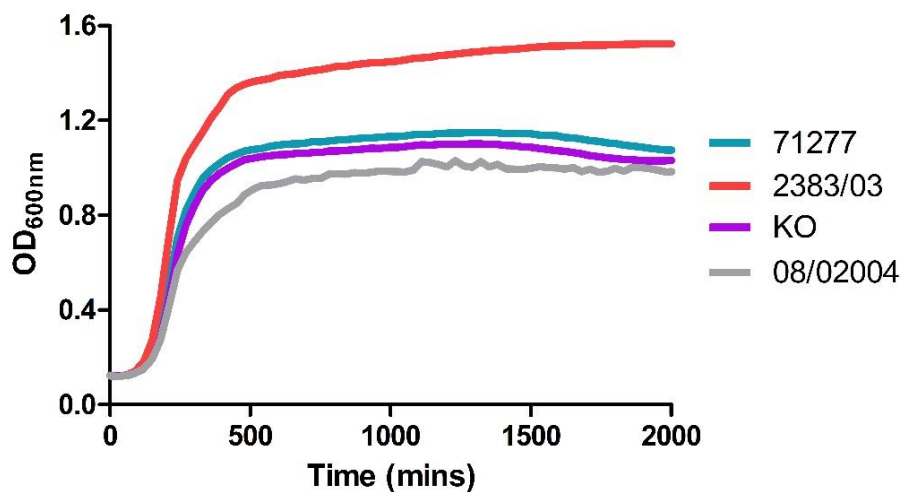
mecC strains lacking the staphylobactin locus, from various clonal complexes and host species were also grown in serum containing media. Similar to the *mecA* strains, the *mecC* strains lacking the staphylobactin locus did not reach such a high optical density reading at 2000 minutes: OD600$_{nm}$ between 0.8 and 1, compared to 1.1-1.2 for the *mecC* strains containing the staphylobactin locus. Strain m-MR-6AA grew poorly, with a delayed log growth phase, along with LGA251, which only reached a stationary phase of OD600$_{nm}$ 0.57 (Figure 4.7). Results show that both *mecC* and *mecA* strains lacking the staphylobactin region take longer to reach the stationary phase of growth, 750 minutes or more without the *stb* locus and around 500 minutes with the *stb* locus. These strains do not reach the same biomass density as strains containing the *stb* locus despite being inoculated at the same optical density at the start of the growth curve.

Figure 4.7. Growth of *S. aureus mecC* strains lacking the staphylobactin locus in serum. (A) *S. aureus* strain LGA251, LGA254, H093880936, m-MR-6AA, PI 41/95 and C05 232 lacking the staphylobactin region were cultured in 60% HoS-40% TMS medium. Letters in brackets indicate the host that the strain was isolated from; H= Human, R= Rabbit, B= Bovine (B) Addition of 50 μM FeCl$_3$ to the 60% HoS-40% TMS medium.

(A)
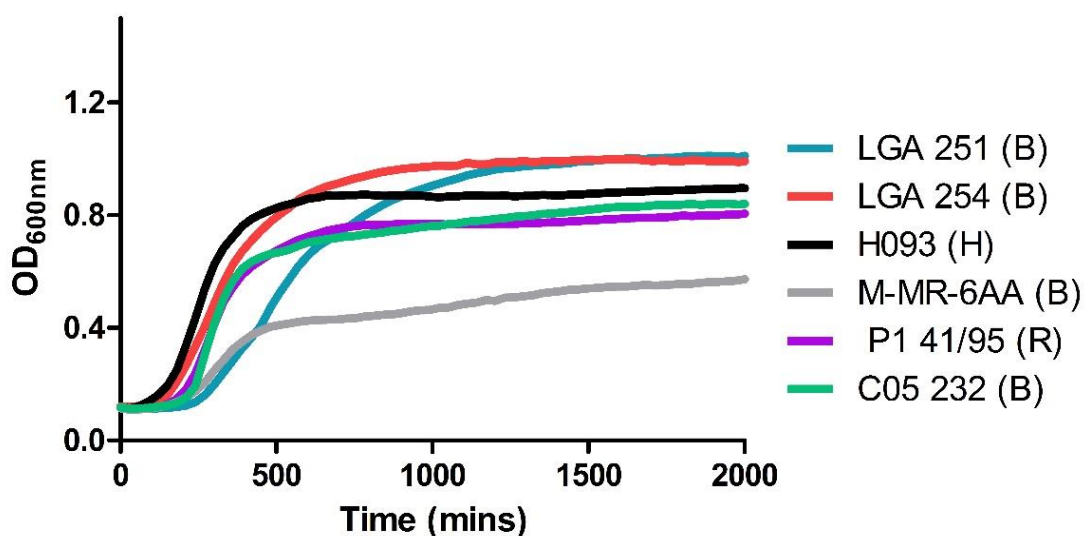
(B)
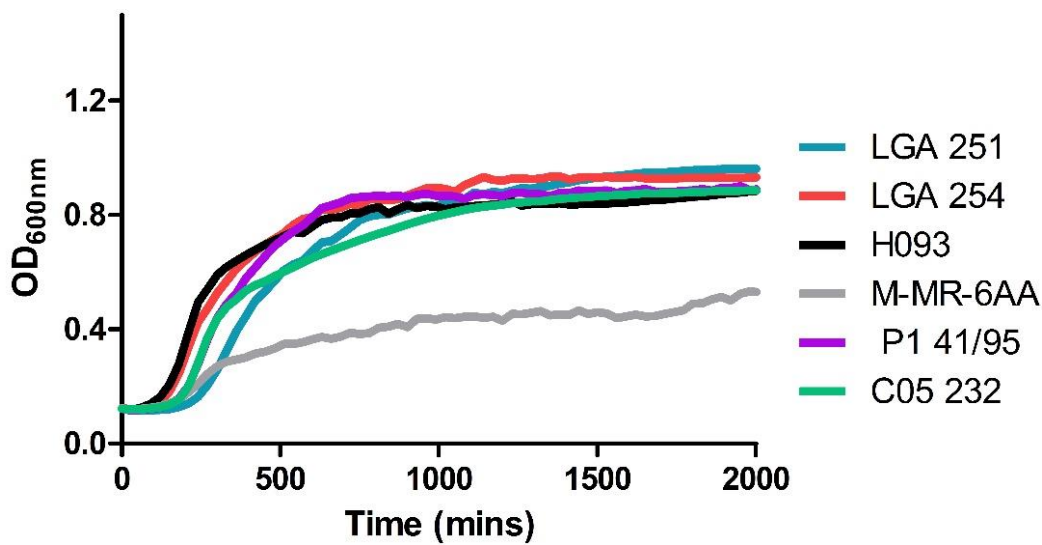
4.3.4 Growth of *S. aureus* with human transferrin

Bacteria with transport systems for certain siderophores can survive in the bloodstream using transferrin as a source of iron. Previously it has been shown that a deletion mutant of *sfa* and *sbn* had severely reduced growth in serum or where human transferrin was the sole source of iron; and that growth in the presence of transferrin could be rescued by the addition of saturating concentrations of iron (210). Numerous microbial siderophores contribute to the extraction of iron from the serum sequestration protein transferrin (Table 1.1). To understand if staphylobactin might play a similar role in *S. aureus* CC130 strains, deletion mutants were grown in C-TMS with the addition of varying concentrations of human transferrin (5 μg to 5 mg) (Figure 4.8). The transferrin was partially iron saturated, acting as a source of iron for strains with suitable extraction strategies, and as a sink for strains unable to extract iron from transferrin. Strains tested for their ability to grow in minimal media supplemented with transferrin were wild-type CC130 strain 71277, single deletion mutants *sfa*, *sbn* or *stb*, double deletion mutants *sfa/sbn*, *sbn/stbE-J*, or triple deletion mutants *sfa/sbn/stb* or *sfa/stbE/stbHIJ*.

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Figure 4.8. Growth of *S. aureus* CC130 71277 in human transferrin.

(A) *S. aureus* strain 71277 and staphylobactin (*stb*), and/or staphyloferrin A (*sfa*) and B (*sbn*) mutants were cultured in C-TMS medium supplemented with 50 μg/ml of human transferrin (partially iron saturated). No production of the staphyloferrins resulted in delayed growth kinetics, ending in a culture density nearly equivalent to wild-type. Strains lacking production of just staphylobactin were sufficient for wild-type growth. (B) All mutant kinetics were amended to wild-type with the addition of 50 μM $FeCl_3$. Each line represents mean OD for 3 samples per strain.

(A)
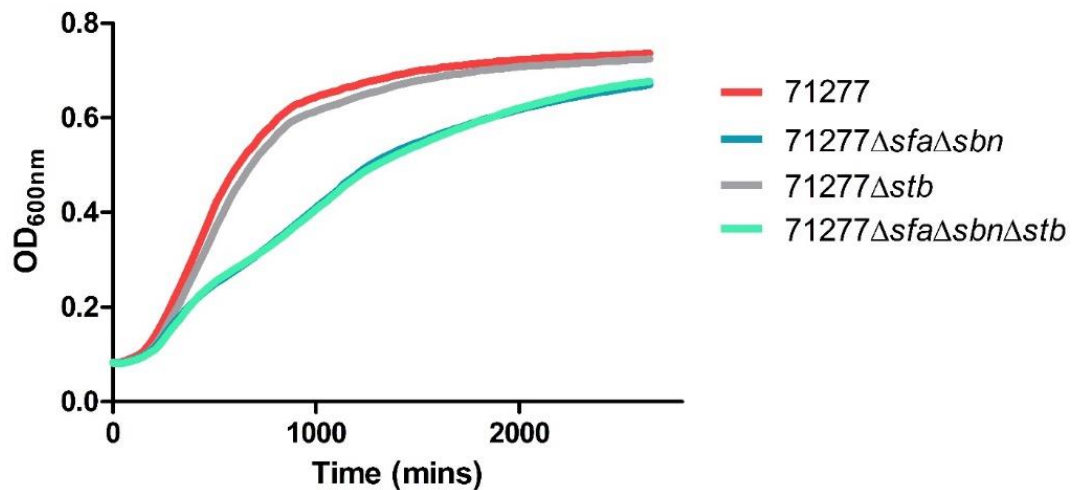
(B)
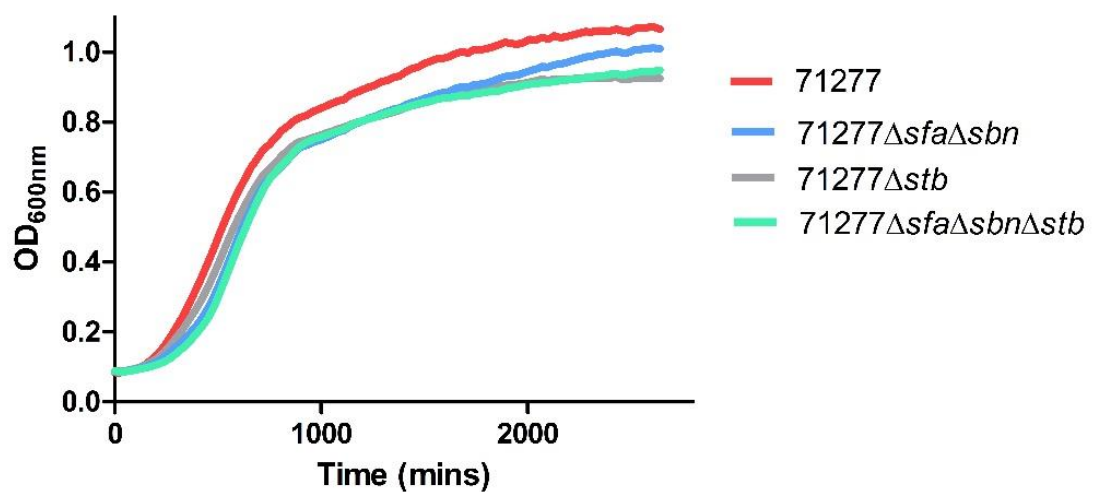
Deletion of staphyloferrin A and B slightly delays the onset of exponential growth (Figure 4.9A). It has already been demonstrated that iron extraction from transferrin is a redundant process mediated by both SA and SB. There was no difference between growth of the wild-type strain and strains lacking single deletions of the *sfa*, *sbn* or *stb* locus. When the concentration of transferrin was reduced to 5 µg/ml, no growth difference was observed between strains. When the concentrations were 5 mg/ml or 500 µg/ml, the growth for strains *sfa*/*sbn* and *sfa*/*sbn*/*stb* was reduced overall but otherwise no differences were seen (results not shown).

In this study, the strains 71277Δ*sfa*Δ*sbn* and 71277Δ*sfa*Δ*sbn*Δ*stb* demonstrated markedly impaired growth compared to its wild-type parent and 71277Δ*stb* within the first 44 h of incubation, but subsequently recovered to reach an approximately equivalent 48h cell density. Supplementation of the medium with free iron oblated this defect.

Growth of the same *mecA*, *mecC* CC130 and *mecC* strains lacking the staphylobactin region were carried out in C-TMS supplemented with 250 µg/ml transferrin showed similar growth kinetics when grown in serum as the iron source. *mecA* strains and *mecC* strains had a delayed onset of exponential growth and did not reach the same OD_{600nm} as the *mecC* CC130 strains. Also, there was greater variability in growth rates and final OD with strains lacking the staphylobactin locus (results not shown).

4.3.5 Growth of *S. aureus* in the presence of metal ions

Siderophores can chelate metals other than iron including copper, zinc and manganese. To test if staphylobactin might be involved in the uptake of metals other than iron, the staphyloferrin and staphylobactin mutants were tested in Chelex-treated minimal media, without serum and supplemented with various ions.

4.3.5.1 Ferrous Iron

Iron has two oxidation states: ferrous (Fe^{2+}) or ferric (Fe^{3+}). Ferrous iron predominates in low oxygen, low pH environments. Bacteria can utilise the small amounts of soluble ferrous (Fe^{2+}) iron and may be directly imported by way of high affinity ferrous iron transporters (174).

Wild-type strain 71277 and its MntR, staphylobactin and staphyloferrin deletion mutants were grown in minimal media supplemented with 0.001 to 1000 µM FeSO$_4$ as a ferrous iron source. No difference was seen in growth of strains at 0.001 to 1 µM and 1000 µM. At concentrations 10 and 100 µM only strains 71277$\Delta sfa\Delta sbn$ and 71277$\Delta sfa\Delta sbn\Delta stb$ were able to grow to the same OD as these strains grown in Chelex treated minimal media (Figure 4.9).

Figure 4.9. Growth of *S. aureus* strains in minimal media in the presence of ferrous iron
S. aureus strain 71277 and mntR, staphylobactin, and/or staphyloferrin A and B mutants were cultured in C-TMS medium supplemented with 100 µM FeSO$_4$. Strains lacking the production of staphyloferrins were able to grow in minimal media supplemented with ferrous iron. Each line represents mean OD for 3 samples per strain.
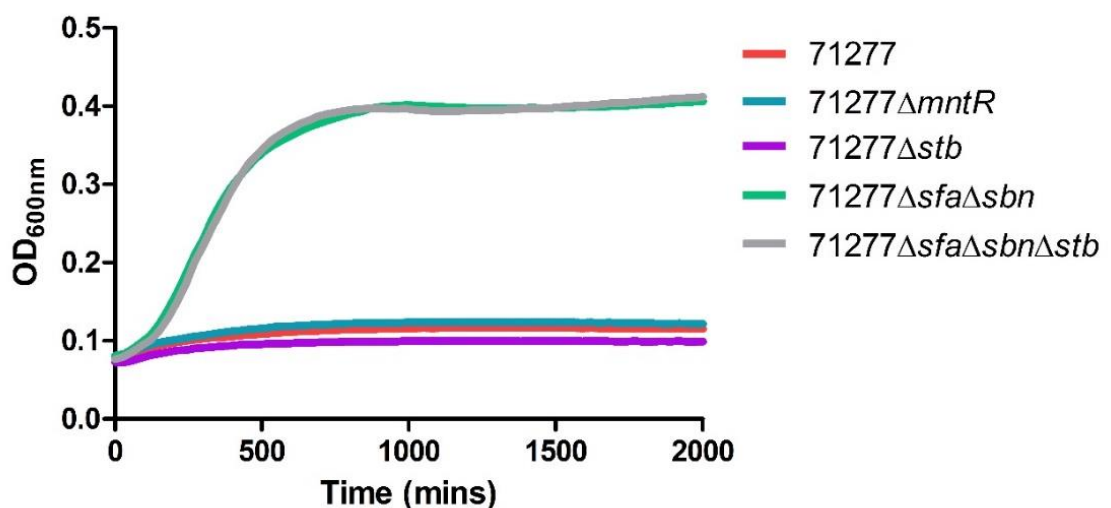
4.3.5.2 Manganese

Manganese plays a central role in cellular detoxification of reactive oxygen species (ROS). Therefore, manganese acquisition is considered to be important for bacterial pathogenesis by counteracting the oxidative burst of phagocytic cells during host infection.

Strain 71277, 71277Δ*mntR* and 71277Δ*stb* were tested for their ability to grow in C-TMS supplemented with 0.001 to 1000 μM of manganese. When grown in concentrations ranging from 0.001-0.1 μM, the wild-type strain grew slightly quicker and to a higher OD than the deletion mutants. From 1-1000 μM, the wild-type 71277 grew well in this media. 71277Δ*stb* grew at a slower rate but reached roughly the same OD as the wild-type strain (Figure 4.10)

Figure 4.10. Growth of *S. aureus* strains in minimal media in the presence of manganese

S. aureus strains 71277, 71277Δ*mntR* and 71277Δ*stb* were cultured in C-TMS medium supplemented with 100 μM $MnSO_4$. Deletion of the manganese transcriptional regulator resulted in the ability to grow in culture medium containing 1 μM $MnSO_4$ or more. Each line represents mean OD for 3 samples per strain.
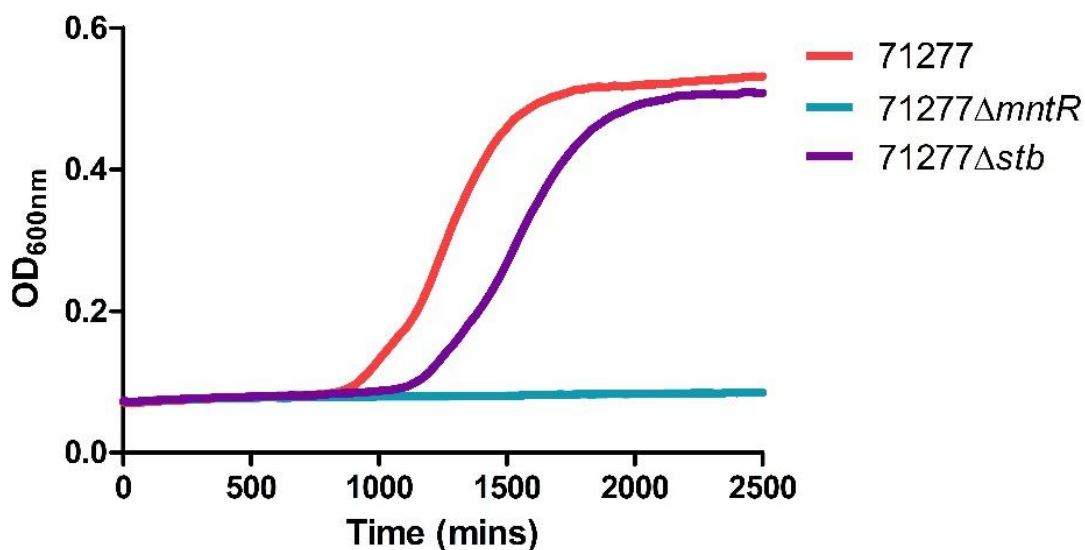
71277 displayed no obvious growth defects under any of the conditions tested. 71277Δ*stb* displayed delayed growth kinetics, but reached the eventual culture density nearly equivalent to wild-type after 2000 minutes. By adding a range of manganese, it was determined that 71277Δ*mntR* had a higher growth yield at low concentrations of Mn^{2+} and exhibited a reduced growth yield at raised Mn^{2+} levels. This result may be due to manganese toxicity at higher concentrations.

4.3.5.3 Zinc, Copper, Nickel and Cobalt

S. aureus isolate 71277, 71277Δ*mntR* and 71277Δ*stb* were grown in C-TMS with metal ions zinc, copper, nickel and cobalt at concentrations 0.001-1000 µM and their growth monitored.

The staphylobactin homologous siderophore, yersiniabactin, was shown to play a role in zinc acquisition in *Y. pestis* (265). Yersiniabactin is required for growth under zinc deficient conditions in a strain lacking the zinc ABC transporters ZnuABC, suggesting staphylobactin could play a similar role in *S. aureus*. No difference in growth between the three strains was observed at any of the concentrations, suggesting that staphylobactin does not play a role in zinc, copper, nickel or cobalt acquisition, or any growth defect is masked by another zinc transporter.

4.3.5.4 Milk

A natural niche of *S. aureus* is the mammalian skin and mucous membranes. Many of the strains containing the staphylobactin region were isolated from cows with infected mammary glands. Milk contains the iron binding protein lactoferrin, which is distinct from transferrin in that it releases iron at a much lower pH (pH 3.0 vs 5.5), therefore rendering it a useful chelator in various host niches (451). To study the impact of the staphylobactin locus on growth in milk, strains 71277, 71277Δ*stb*, 71277Δ*sfa*Δ*sbn*Δ*stb* were monitored for growth in media comprised of 0.5% milk to 100% milk with C-TMS, with or without the addition of iron. When grown in the Bioscreen machine, in all strains grown in 20% milk or more, there was no difference between wild-type and mutant strains, in either iron-deplete/replete conditions. This was due to the media being too opaque to take accurate readings. From 0.5% to 10% milk, media containing iron grew to a higher optical density than strains grown in deplete media, however, no difference in growth was seen between wild-type and mutant strains in either conditions. In an attempt to see if there was a difference in growth in 100% milk, aliquots were plated over a 24 hour time course to look at difference in CFU. No difference was seen between strains. This may be due to low resolution in results due to the sample time being less frequent than the Bioscreen machine and the results based on CFU counts instead of optical density readings taken from samples grown in triplicate.

4.3.6 Transport and intracellular iron content

Sensitivity to the antibiotic streptonigrin was used as a measure to compare intracellular iron content of the wild-type and the mutant strains that are lacking in siderophore production. Bacterial strains wild-type 71277, along with deletion mutants 71277Δ*sfa*Δ*sbn*, 71277Δ*stb*, 71277Δ*sfa*Δ*sbn*Δ*stb,* and control strains 8325-4 and 8325-4Δ*sfa*Δ*sbn* were tested using the microbroth dilution for minimum inhibitory concentrations (MIC) assay according to BSAC. Plates contained streptonigrin (20 ng/ml-0.625 ng/m) in Mueller-Hinton (MH) broth and erythromycin as a control (0.5 mg/ml-5 ng/ml). Each strain was tested in duplicate and the assay was repeated three times with no change in results (Table 4.4).

Table 4.4. MIC of *S. aureus* strains and their siderophore mutant strains to streptonigrin and erthyromycin.

Wild-type strains 71277 and 8325-4, and their siderophore mutant strains were tested to determine their MIC to the antibiotics streptonigrin and erythromycin.

Bacterial Strain	Streptonigrin MIC (ng/ml)	Erythromycin MIC (µg/ml)
71277	20	0.25
71277Δ*sfa*Δ*sbn*	20	0.25
71277Δ*stb*	20	0.25
71277Δ*sfa*Δ*sbn*Δ*stb*	20	0.25
8325-4	20	0.125
8325-4Δ*sfa*Δ*sbn*	20	0.125

There was no obvious difference in MIC of streptonigrin between wild-type and siderophore mutant strains, other than a lower MIC in strains 8325-4 (CC8) and 71277 (CC130) for erythromycin.

To investigate further, these strains were grown in iron-limited minimal media (C-TMS) and nutrient rich TSA media with varying concentrations of streptonigrin (1-20 ng/ml)

and their optical density measured every 30 minutes by a BioScreen analyser. In iron-limited media, none of the test strains grew at a concentration of 20 ng/ml, confirming the results for the BSAC MIC assay. When the concentration was reduced to 10 ng/ml, all strains except 8315-4Δ*sfa*Δ*sbn* (not producing staphyloferrin A and B, but lacking the staphylobactin region) were sensitive to this concentration and were not able to grow over the 30 hour time period (Figure 4.11A). The concentration was reduced to 5 ng/ml, allowing for the growth of all strains except wild-type CC130 strain 71277. All deletion mutants grew to a higher OD compared to their wild-type parent strain. The 71277 deletion mutant strains grew to approximately the same optical density over the 30 hours, similar to that of the wild-type strain 8325-4. Again, strain 8315-4Δ*sfa*Δ*sbn* was shown to be the most resistant to the effects of streptonigrin at a concentration of 5 ng/ml in minimal media (Figure 4.11B).

Figure 4.11. Growth of *S. aureus* strains in minimal media in the presence of streptonigrin.

S. aureus strains 71277 *mecC* and 8325-4 *mecA* and their siderophore deletion mutants grown in minimal media in the presence of streptonigrin. **(A)** OD_{600nm} readings over 30 hours growth in the presence of 10 ng/ml streptonigrin. **(B)** OD_{600nm} readings over 30 hours growth in the presence of 5 ng/ml streptonigrin. Each strain grown in triplicate, readings taken every 30 mins and each line represents average reading for each time point.

(A)
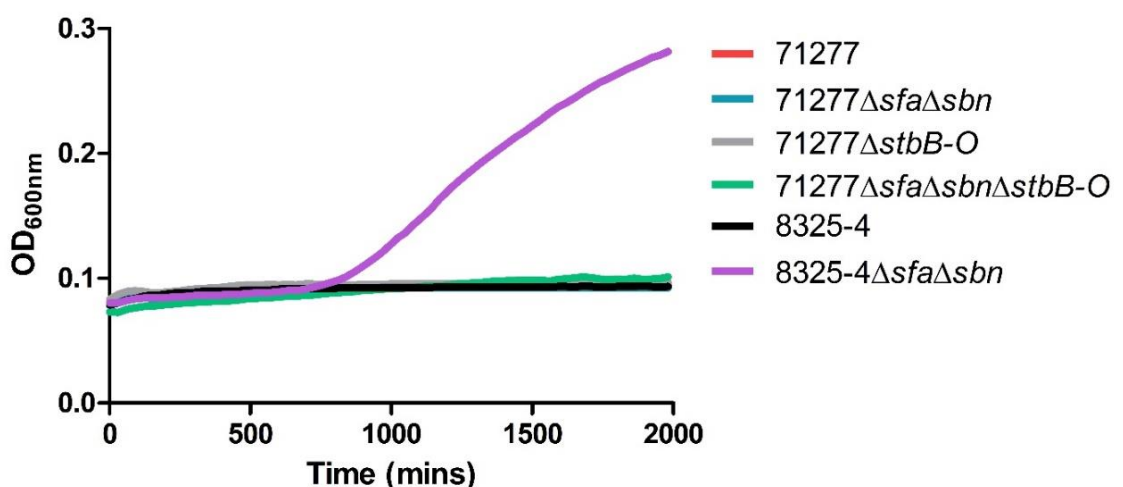
(B)
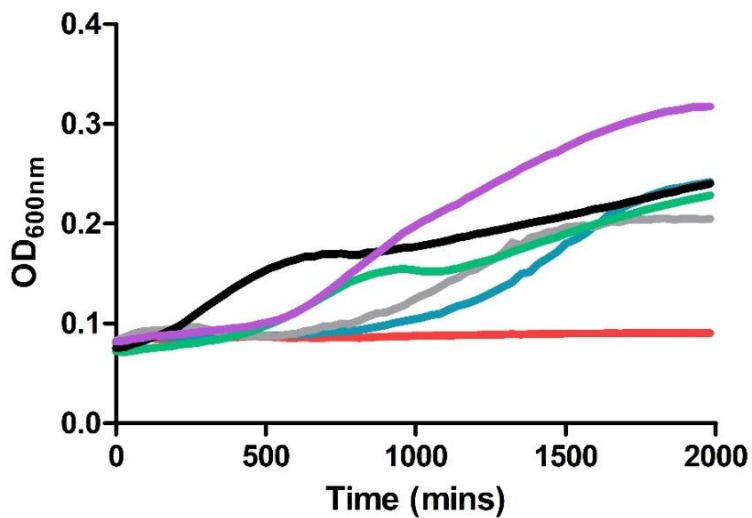
Very little difference was seen in growth between strains grown with 1 ng/ml of streptonigrin, and all strains had the same impaired growth when grown in minimal media alone (OD$_{600}$ 0.36-0.42). This OD is very low, but what would be expected for strains grown in minimal media without the supplementation of iron. Growth was repeated with strains grown in iron-replete TSB media. Strain 8325-4 was the only strain to grow well when the concentration was 20 ng/ml. When the concentration was reduced to 5 or 10 ng/ml, the deletion mutant strains grew to the same optical density as their wild-type parent strains. This shows no clear difference in growth due to the effects of streptonigrin. This suggests the same levels of intracellular iron concentrations in all strains (Figure 4.12). All strains grew well in 1ng/ml and TSB alone.

Figure 4.12. Growth of *S. aureus* strains in TSB in the presence of streptonigrin

S. aureus strains 71277 and 8325-4 and their siderophore mutants grown in TSB in the presence of streptonigrin. OD$_{600nm}$ readings over 25 hours growth in the presence of 5 ng/ml streptonigrin. Each strain grown in triplicate, readings taken every 30 mins and each line represents average reading for each time point.
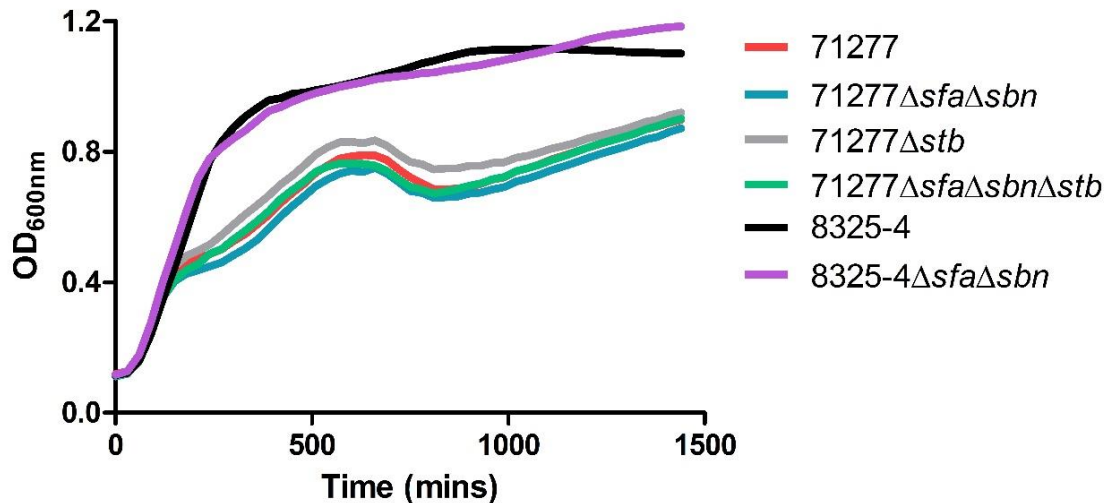
It was expected that if staphylobactin mediated iron accumulation, the mutant would be more resistant to streptonigrin relative to the parent strain, due to a reduction in intracellular iron concentrations. All strains tested, however, were equally resistant to streptonigrin, as demonstrated in Figure 4.12 except when grown in C-TMS at 5 ng/ml (Figure 4.11B). The parent wild-type strain 71277 was more sensitive than the deletion mutants, but there was no difference in resistance between the single, double and triple deletion mutants of staphyloferrins and/or staphylobactin. All CC130 strains were equally resistant to streptonigrin when these experiments were repeated in iron-replete media (TSB). In control experiments, 8325-4 was sensitive to low concentrations of streptonigrin, while the mutant 8325-4Δ*sfa*Δ*sbn*, deficient in the siderophores, staphyloferrin A and B, was predictably more resistant to the antibiotic when grown in minimal media, as shown in Figure 4.11A. Surprisingly, when this was repeated in iron-replete media, the wild-type strain was more resistant than the deletion mutant.

To characterise the role of *stbHIJ* in staphylobactin transport, a Δ*stbHIJ* mutant was constructed. If *stbHIJ* is involved in iron transport then mutants lacking these genes will be less sensitive to streptonigrin. Growth of strain 71227 and its staphylobactin transport mutant 71277Δ*stbHIJ*, along with the *S. aureus* isolate LS-1 and its staphyloferrin A transport mutant, LS-1Δ*htsA,* were observed in a variety of media containing 10 ng/ml of streptonigrin to see if the iron source or growth conditions

affected their susceptibility. When grown in minimal media (TMS) supplemented with either 50 µM FeCl$_3$ or 250 µg/ml transferrin as an iron source, wild-type isolates grew quicker and to a higher OD than their mutant counterparts. The strains lacking staphylobactin grew better than the strains containing the staphylobactin locus. The same growth for both wild-type and their transport mutant strains were seen when grown in 60:40 TMS or TSA. Again, strains that do not contain the staphylobactin locus grew quicker and to a higher final OD. Growth in just TMS media was so poor that no difference could be seen. These results were not as would be predicted if *stbHIJ* or *HtsA* are involved in transport of siderophores.

4.3.7 NRPS antibiotic

Whilst trying to investigate the possible role of staphylobactin, a study by Zipperer *et al*. (448) found that *S. lugdunensis* strains produce a NRPS, termed lugdunin, an antibiotic that prohibits colonisation by *S. aureus*. Lugdunin was only produced under iron limiting conditions and on solid agar surfaces, not in liquid culture. It was of interest to see if staphylobactin plays a similar role as a NRPS bioactive compound. Wild-type *S. aureus* CC130 strains and its Δ*stb* mutants were tested against a panel of other *S. aureus* and bacterial isolates in a bioactivity assay. *S. aureus* strain Newman and RN4220 were streaked onto basic agar and the *S. aureus* CC130 strains containing; the staphylobactin locus, 7594/1975, 2383/03, C03 365, 71277 and its deletion mutant, 71277Δ*stb,* were spotted onto paper discs to screen for their antimicrobial activity. All strains, including 71277Δ*stb* displayed a zone of inhibition around the paper disc, showing antimicrobial activity towards *S. aureus* strain Newman and RN4220 with or without the addition of the iron chelator to the basic agar. *S. lugdunensis* strain N920143 did not show any antimicrobial activity. The *S. aureus* CC130 strains were also tested against other bacteria that were tested with lugdunin producing *S. lugdunensis*, *E. coli* DC10B, *Enterococcus faecalis* h-Tr119 and *Pseudomonas aeruginosa* PA01, but no antimicrobial activity was observed.

The *S. aureus* CC130 strains seem to have the ability to kill other *S. aureus*, which may contribute to their success, but this doesn't seem to be the role of the staphylobactin locus, as no loss of activity is seen in the Δ*stb* strain versus the wild-type. Iron-replete or

deplete media had no effect of the zone of inhibition. This activity could be attributed to a biosynthetic gene cluster identified during antiSMASH analysis (360).

4.3.8 Chrome-azurol S assay for siderophore activity

To access the production and function of staphylobactin as a siderophore, supernatants of iron starved wild-type and deletion mutant CC130 strains were tested for iron-binding compounds using the chrome azurol S (CAS) shuttle solution. Wild-type strain 71277 along with deletion mutants of staphyloferrin A/B and staphylobactin were grown in iron-replete/deplete and culture supernatant siderophore activity was sampled at two time points (24 and 48 hours) (Figure 4.13). The CAS assay can detect total siderophores in chemically defined media irrespective of the chemical nature of the siderophores. The assay is based on the colour change of CAS-iron complex from blue to orange after chelation of the bound iron by siderophore. Strains 8325-4 and 8325-4Δ*sfa*Δ*sbn* were also used as controls as these have previously been tested using the CAS assay.

The *sfa/sbn* mutant in strain 71277 showed a significant reduction in siderophore output. If staphylobactin is produced in iron deplete media and functions as a siderophore then I would expect a reduction in iron-binding compounds of the supernatant from the Δ*sfa*Δ*sbn*Δ*stb* strain. The wild-type and staphylobactin deletion mutant produced around 150 units at 24 hours. The deletion of staphyloferrin A and B reduced the siderophore units down to 50, with the addition of the staphylobactin knockout this further reduced the siderophore units down to 30. An almost identical result was produced at 48 hours. While the *sfa/sbn* mutant significantly impaired siderophore output at both sampling times, it nevertheless secreted quantifiable amounts of (an) alternate iron chelator(s) (Figure 4.13. Top graph, green bar), while its Δ*stb* counterpart performed at a level comparable to the wild-type parent. Culture supernatant siderophore activity was repressed for all strains by the addition of 50 μM FeCl$_3$ to the minimal media, confirming that the media was iron-replete, allowing for the production of siderophores.

Figure 4.13. *S. aureus* produce siderophores.

Wild-type and mutants secreted iron-chelating metabolite(s) into the supernatant, detected using the CAS assay at 24 hours. Error bars represent standard error from the mean (n = 3). Statistical differences between mutants and the wild-type parental strain were calculated using the Student's unpaired *t*-test, 71277 vs 71277Δ*sfa*Δ*sbn* P = 0.0001, 71277 vs 71277Δ*sfa*Δ*sbn*Δ*stb* P = 0.0002, 71277 vs 71277Δ*stb* P = 0.1753, 71277Δ*sfa*Δ*sbn* vs 71277Δ*sfa*Δ*sbn*Δ*stb* P = 0.021, 8325-4 vs 8325-4Δ*sfa*Δ*sbn* P = 0.0101. P < 0.05.
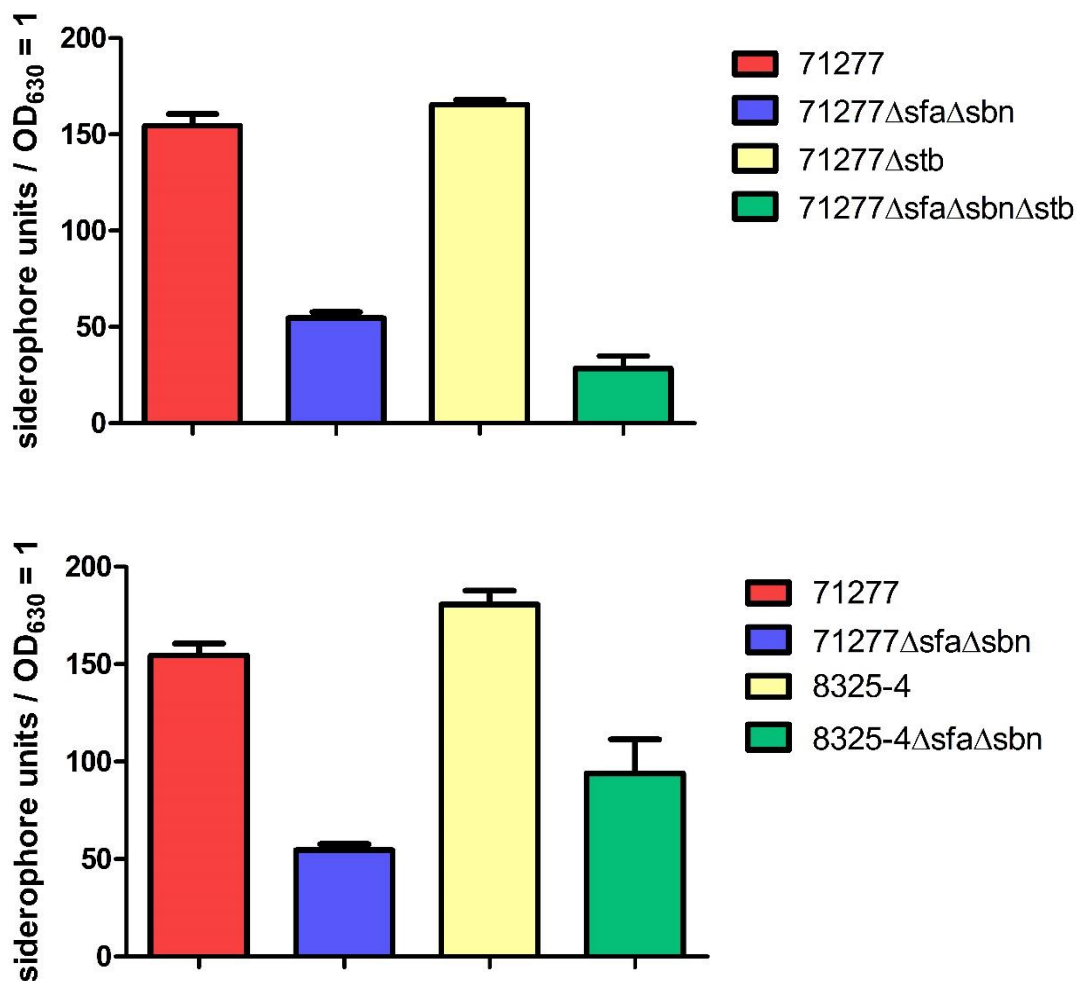
4.3.9 Liquid chromatography mass spectrometry analysis of culture supernatants

Comparative metabolic profiling of the 71277, 71277Δ*stbE* and 71277Δ*sfa*Δ*sbn* mutant strains and 18.31 and 18.31Δ*stbE* strains culture supernatants was performed using LC-MS and were examined for differences in mass ion composition, especially for those of known structures of staphylococcal siderophores. However, despite siderophore activity

being detected in the CAS assay using the same medium, repeated LC-MS analysis of culture supernatants from minimal media (TMS) failed to identify staphylobactin and staphyloferrins. No difference in profiling was observed between wild-type and mutant strains in TMS media, suggesting that the culture was not iron deficient enough to elicit good levels of siderophore production. TMS media was treated with Chelex resin to render the cultures even more iron deficient. This would allow for at least the detection of the staphyloferrins that are known to be produced under these conditions. LC-MS analysis from culture supernatants from C-TMS failed to identify staphylobactin and staphyloferrin B. Though, there is a possibility that staphyloferrin A may have been detected from spent culture from wild-type strain 71277 in C-TMS medium, but was not identified in TMS medium. A peak eluted from the 71277 C-TMS preparation, while no peak for that mass, above background levels of detection, was observed for the Δ*sfa* preparation (Figure 4.14).

Figure 4.14. Identification of staphyloferrin A production by *S. aureus* strain 71277

Top: Spent culture supernatant from the iron starved 71277 strains was subjected to LC-MS analysis. **Bottom:** Similar analysis to 71277 spent culture supernatant from the iron starved Δ*sfa* mutant. The selected ion chromatogram for the two samples is shown.
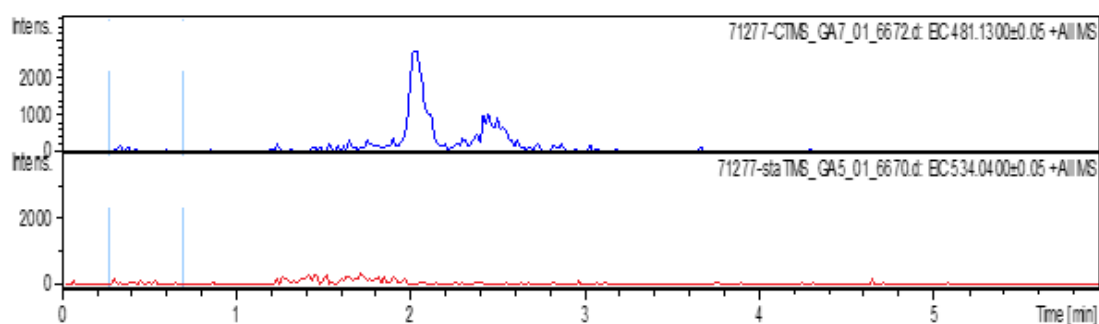
The level of staphyloferrin A identified is slightly lower in the 71277Δ*stbE* mutant than the wild-type 71277 strain. Even though not strictly quantitative, there is a possibility you would expect more to be produced by the mutant strain. The UV absorbance spectrum shows λmax 285 nm rather than the expected 320 nm (Figure 4.15).

Figure 4.15. Comparison of staphyloferrin A production by 71277 and 71277Δ*stbE* in C-TMS

Left: Spent culture supernatant from iron-starved 71277 (top) and the Δ*stbE* mutant (bottom) subjected to LC-MS analysis. The selected ion chromatogram for the two samples is shown, with the spectrum of that peak displayed on the right.
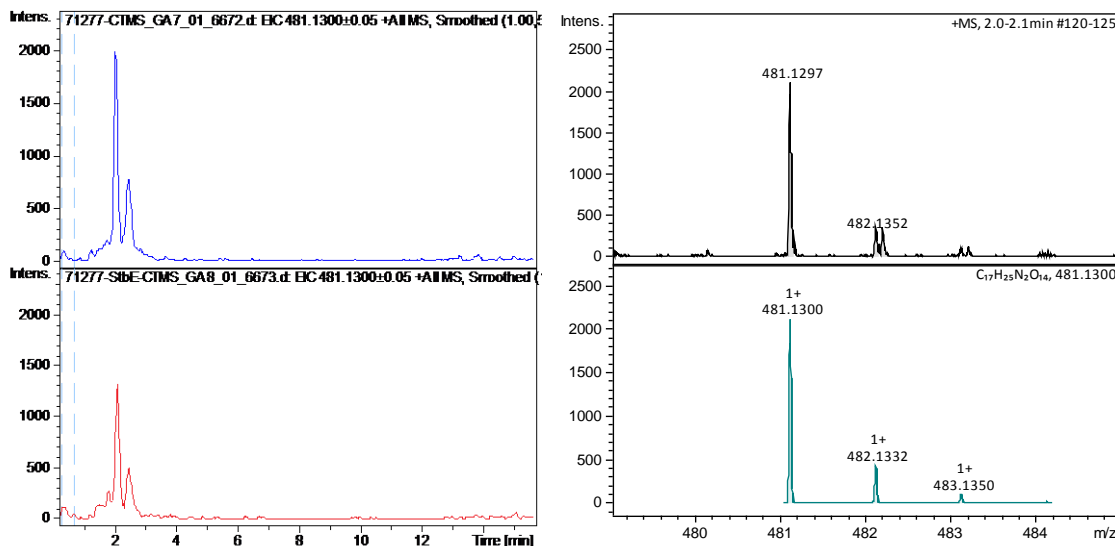
4.3.10 The contribution of staphylobactin to virulence *in vivo*

To investigate whether the staphylobactin locus and the staphyloferrins had an effect on virulence *in vivo*, the wax moth larvae infection model was used. *S. aureus mecC* CC130 strain 71277 along with deletion mutants for the siderophores, staphyloferrin A and B mutants were assayed by injection into the haemocoel of *Galleria mellonella*. Three separate experiments were carried out and the combined results are presented in Figure 4.16. Each test strain and a PBS control was injected into 10 larvae and the number of living larvae was recorded for seven days. For all strains, larvae started to die around 20 hours post infection. Wild-type strain 71277 dropped to 40% survival by 30 hours, then plateaued at 10% survival from 72 to 130 hours. Strain 71277Δ*stb* had a slightly higher survival rate (60%) at 30 hours compared to the wild-type strain. However, at 72 hours the survival had dropped to 7% where it remained until 130 hours post infection, showing very similar final survival rates to the wild-type strain. Strain 71277Δ*sfa*Δ*sbn* had the highest survival rate of all the strains tested at 30 hours at 70%,

dropping to 30% by 50 hours and the plateauing at 17% by 78 hours. Finally 71277Δ*sfa*Δ*sbn*Δ*stb* had a very similar survival rate to the wild-type strain 71277 until 50 hours, reaching 17% survival at 72 hours. Strain absent of the staphyloferrins had a slight better survival rate (17% survival) compared to the wild-type strain (10% survival). All three experiments showed broadly identical results.

Figure 4.16. *Galleria mellonella* **larvae infected with** *mecC* **MRSA strains 71277, 71277Δ*stb*, 71277Δ*sfa*Δ*sbn* and 71277Δ*sfa*Δ*sbn*Δ*stb***

Ten larvae in each group were experimentally infected and control larvae were injected with PBS alone. Figure shows the combined results of 3 separate experiments. CFU/ml 5×10^7 - 2×10^8
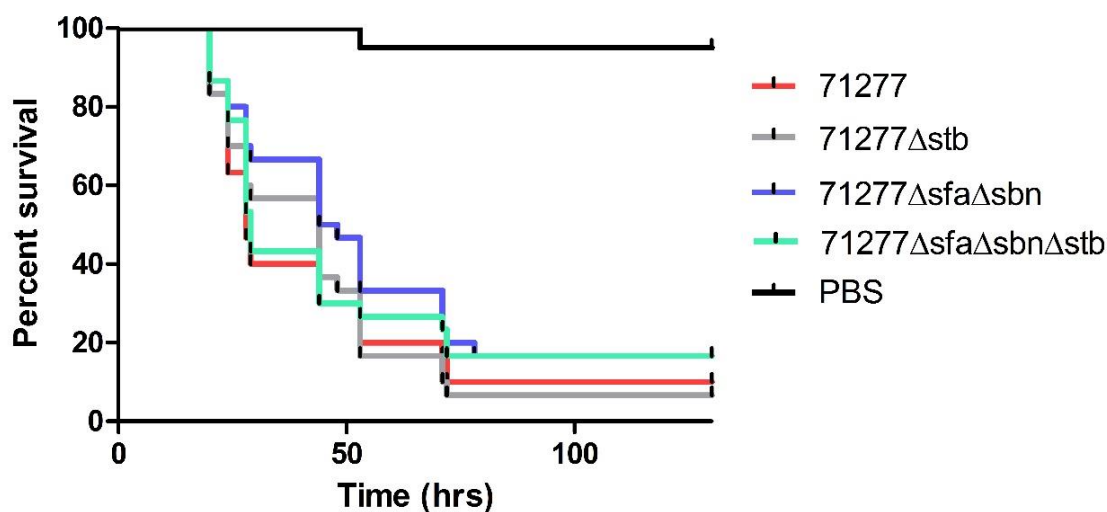
The wild-type parent strains 71277 (*mecC*) and 82325-4 (*mecA*) were compared to their staphyloferrin deletion mutants, 71277Δ*sfa*Δ*sbn* and 8325-4Δ*sfa*Δ*sbn* to see if the result was the same for deletion mutants from different strain backgrounds. Strain 8325-4 had very little effect on survival, maintaining around 90% survival over the seven days post infection. Strain 8325-4Δ*sfa*Δ*sbn* dropped to around 60% survival after 7 days post infection, approximately 30% lower than its wild-type strain. No drop in survival rate was seen in these strains until around day 3 post infection. For deletion mutant strains,

both were significantly more virulent than the wild-type strain (Figure 4.17). Both *mecC* strains were more virulent than the *mecA* equivalents, but showed very similar trends with the wild-type strain being less virulent than the staphyloferrin A and B deletion mutant.

Figure 4.17. *Galleria mellonella* infected with MRSA strains and their staphyloferrin A and B deletion mutants.

Ten larvae in each group were experimentally infected and PBS alone. Figure shows the combined results of 3 separate experiments.
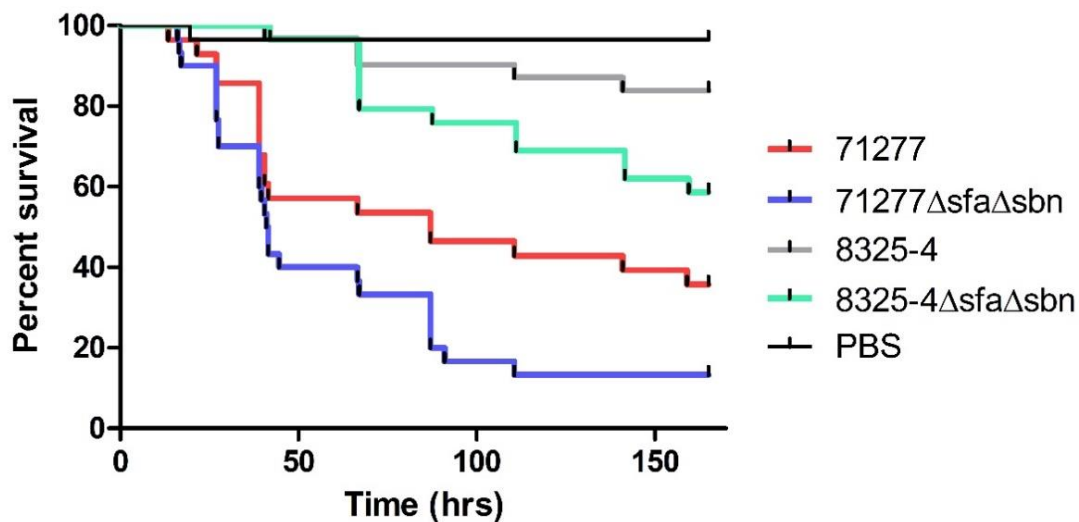
Due to the difference in virulence observed between the wild-type 71277 and 8325-4 strains, *mecC* CC130 strains (71277, KO and MS-18.31) were compared to strain Newman (*mecA*) from CC8 to see if virulence varied between strains from the same clonal complex and a different clonal complex, and try to understand why *mecC* strains are so virulent. Strain KO and 71277 killed over 60% of the larvae in 48 hours, MS-18.31 took a longer time but killed the same amount of larvae as KO and 71277 after 7 days. Strain Newman was far less virulent than CC130 strains, only killing 20% after 7 days of the experiment (Figure 4.18).

Figure 4.18. *Galleria mellonella* **infected with** *mecC*-**MRSA and** *mecA*-**MRSA strains.**

Ten larvae in each group were experimentally infected and PBS alone. Figure shows the combined results of 3 separate experiments. CFU/ml 4.2×10^8 to 6.4×10^8
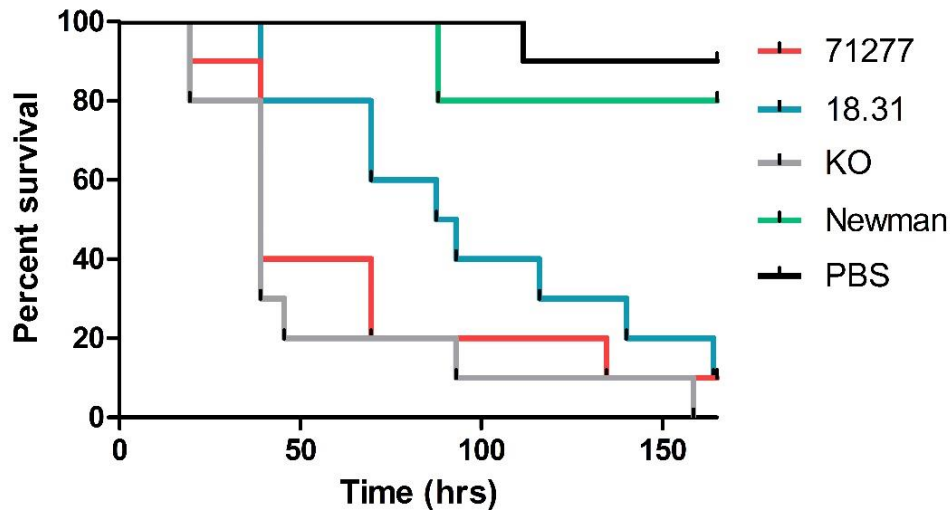
4.4 Discussion

4.4.1 Regulation

I expected MntR to act as a transcriptional repressor on the *stb* locus in an iron-dependent manner. This is because equibactin and both the staphyloferrins have been shown to be under the control of iron-dependent negative repressors, EqbA and Fur, and deletion of these regulators results in upregulation of their siderophore biosynthesis genes (219). RT-qPCR revealed a reduction in the *stbE* and *stbG* transcripts in *mntR* deletion mutants compared to its wild-type parent strain, suggesting that MntR is a positive regulator of the staphylobactin operon in an iron-dependent manner. However, MntR is already known to act as a negative and positive regulator of the expression of *mntABC* and *mntH* genes, which encode manganese transporters and that expression of *mntR* is constitutive in *S. aureus* (290).

It would be interesting to see how deletion of *mntR* would affect other iron acquisition gene transcription due to its interaction with PerR, which in turn regulates *fur*. As well as looking at expression of *mntABC* and *mntH* with the addition of Mn^{2+} or Fe^{2+} to the minimal media. This will act as a control, as *mntABC* expression is repressed in the presence of these ions, and will confirm the results that MntR acts as a positive regulator of the staphylobactin region. This could be done using transcriptomics techniques such as DNA microarrays or RNA-Seq.

Although not statistically significant the results are consistent and show a trend. I consider this trend unlikely to be the result of random variation, but further analysis would be required to gain a definitive result.

When deletion mutants of *mntR* in *S. aureus* were grown on nutrient rich TSB agar, no obvious growth defect was observed, unlike *S. equi* which produced very small colonies when grown on Todd-Hewitt Agar. This may be due to the fact that: *S. aureus* has been shown to have many iron acquisition systems so can possibly compensate for iron toxicity; or because MntR is possibly a positive regulator of *stb*, then deletion of *mntR* would not result in overproduction of *stb*.

4.4.2 Expression of the *stb* genes

RT-qPCR results showed both *stbE* and *stbG* expression was increased slightly in iron-deplete conditions compared to replete by 7 fold to 3 fold. This suggests that staphylobactin is expressed in an iron-dependent manner. These results are as expected. The genes are upregulated and the controls confirm that the correct genes are upregulated.

In previous studies, when wild-type strain Newman was grown in serum minimal media with or without the addition of 100 µM iron, it was shown that *sfaA* expression increased approx 3 fold, *sfaD* approx 2 fold and *htsA* approx 20 fold when grown in iron-deplete media compared to replete conditions. When this experiment was carried out in strain 71277, *HtsA* increased 1000 fold, *sfaA* 80 fold and *sfaD* 20 fold. These findings are in agreement with previous studies that used *S. aureus* Newman, suggesting that staphyloferrin A expression is upregulated in iron deplete conditions, even in strains containing the staphylobactin region. It would be of interest to look at the expression of

the staphyloferrin B operon in these conditions and examine the expression of the staphyloferrin A and staphylobactin operon. This may give insight into the dominant siderophore produced in these conditions and further confirm the results already obtained. RT-qPCR of *eqbE* in *S. equi* was carried out in THB, not a minimal media, it was not found if the regulation of equibactin was repressed in an iron-dependent manner via this method. However, when electrophoretic shift assays with EqbA were carried out, binding of EqbA to the *eqbB* promoter was increased on the presence of iron. The small colony phenotype of the *eqbA* mutant, due to the expression of the *eqb* genes leading to increased iron uptake, was reversed when media was supplemented with an iron chelator. These results demonstrate that expression of the *eqb* genes was regulated by EqbA in an iron-dependent manner (296).

4.4.3 Growth in minimal media

Growth of strain 71277 and its *sfa*, *sbn* and *stb* mutants were recorded over a 48h period to identify deficiencies in the growth rate that correlated with the loss of *sfa*, *sbn* or *stb* function. I showed that deletion of the staphylobactin locus did not independently demonstrate any detectable iron-restricted growth in either high or low serum, unless introduced into a strain bearing the *sbn* deletion. This indicates that staphylobactin has no significant role in Fe^{3+} uptake. The *stb*, *sfa*, *sbn* triple mutant was predicted to be crippled in its ability to obtain Fe^{3+} and the results show that this mutant does not grow well in iron-limited media, however, its growth is just as attenuated as the *sfa* and *sbn* mutant, suggesting that staphylobactin does not contribute to iron acquisition in serum.

A single deletion of *sbn* resulted in delayed growth kinetics and deletion of the *sfa* locus did not affect growth in iron deplete media, confirming results previously reported (210, 218, 238). This suggests that staphyloferrins are the predominant siderophores produced by *S. aureus*. Staphylobactin could be a secondary mechanism for acquiring iron as the staphyloferrin-possessing strains are able to grow well in low iron conditions, and loss of either staphyloferrin A and staphylobactin are compensated for by the action of staphyloferrin B. In iron-replete growth media, the growth of mutant strains was unaltered in comparison to that of 71277. This shows that it's an iron-dependent mechanism that's causing the growth defect in iron-deplete media.

Investigation of a novel iron-uptake system and other genomic features in *mecC* *Staphylococcus aureus*

The *S. aureus* strain 8325-4 (*mecA*), a background that has been used to study the staphyloferrins previously, and strain 71277 (*mecC*) harbouring deletions in both *sfa* and *sbn* loci, grew very poorly in iron-deplete media containing serum or transferrin. The *mecA* strain lacking the *stb* locus grew at a slower rate and to a lower cell density than the *mecC* strain. This could suggest that staphylobactin plays a part in enhanced growth under these conditions. Maybe the strains containing the staphylobactin region are able to upregulate and efficiently utilise the staphyloferrins better than those strains lacking *stb* in these conditions.

Comparison between *mecA* strains from different clonal complexes and host species varied largely in both growth rate and final optical density, suggesting that iron acquisition systems vary in their ability to utilise transferrin as an iron source. *mecC* CC130 strains from a variety of host species had the ability to utilise transferrin in serum more efficiently than *mecA* strains, but the ability to grow was not affected by the host that the strain was isolated from.

mecC strains lacking the staphylobactin locus displayed similar growth to *mecA* strains, which grow slower with a lower final optical density, suggesting staphylobactin plays a part in enhanced growth. No conclusions can be drawn from the difference in growth rates and final optical density of strains isolated from different host species due to small sample size from each species.

While carrying out growth curves, iron in its ferric state was added to the serum minimal media, allowing the mutant strains to recover to wild-type levels. The redox state of iron may have an effect on the results obtained and should be taken into consideration when carrying out future work. Addition of ferrous iron at varying concentrations to minimal media showed no difference in growth between strains at low (0.001), high (1 µM) and saturated (1000 µM). Only Δ*sfa*Δ*sbn* mutants were able to grow at 10 and 100 µM concentrations. This may be due to the continued import of iron in the strains containing staphyloferrin biosynthesis, leading to toxic levels of intracellular iron. These results suggest staphylobactin is not involved in ferrous iron uptake.

With the addition of manganese, 71277Δ*stb* grew at a slower rate than wild-type but eventually reached the same OD. It was previously demonstrated that deletion of *mntH*

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in *S. aureus* does not lead to an appreciable growth defect in metal depleted media, suggesting that MntABC may be the dominant manganese transporter in *S. aureus* under *in vitro* conditions (290). Staphylobactin may play a role in manganese acquisition, but only a slightly reduced growth defect was observed and any involvement in manganese acquisition may be masked by the more dominant manganese transporter. By adding a range of manganese, it was determined that 71277Δ*mntR* had a higher growth yield at low concentrations of Mn^{2+} and exhibited a reduced growth yield at raised Mn^{2+} levels. This is not surprising as MntR is known to be a manganese-modulated transcriptional regulator (290). At high concentrations of Mn^{2+}, 71277Δ*mntR* had very limited growth, which may be due to manganese being constantly imported into the cell due to the manganese transporter not being repressed by MntR.

Adding zinc, copper, nickel and cobalt ions to the minimal media had no effect on growth suggesting staphylobactin is not involved in acquisition of these ions. Low iron, lactoferrin containing milk displayed no difference in growth between strains, suggesting staphylobactin is not responsible for iron acquisition in these conditions.

4.4.4 Transport- Sensitivity to streptonigrin

Staphylobactin mutants were tested for sensitivity to streptonigrin to try and determine the contribution of staphylobactin locus to intracellular iron accumulation. When strains were tested for streptonigrin MIC in minimal media (iron deplete) and Mueller-Hinton broth (iron-replete) there was no obvious difference between strains. If staphylobactin was involved in iron acquisition you would expect the wild-type strain to have a lower MIC than the deletion mutants when grown in iron-restricted TMS, this was previously observed for Δ*sirA* and Δ*sirB* (involved in staphyloferrin B transport), but not in iron-rich media. This may be due to no difference between strains of intracellular iron concentrations.

When these strains were monitored for growth in minimal media and TSA at a range of streptonigrin concentrations. No growth of any strain was observed at 20 ng/ml. At 10 ng/ml, all strains except 8315-4Δ*sfa*Δ*sbn* were sensitive to the antibiotic, suggesting this strain does not accumulation iron making it reasonably resistant, compared to the wild-

type strain, which is what would be expected, however, there is no difference to the iron that is accumulated between strain 71277 and its siderophore deletion mutants, suggesting there could be another mechanism involved in iron accumulation. At 5 ng/ml, wild-type strain 71277 was the only strain unable to grow in iron-limited media. This result may be due to the minimal media having a very small iron source, leading to lower intracellular iron pool concentrations, and therefore being more resistant to the toxic effects of streptonigrin. Where there was a difference in growth between strains in minimal media in the presence of streptonigrin, it was insignificant. The OD_{600} was between 0.1-0.3 compared to growth without streptonigrin (OD_{600} 0.36-0.42). These differences are too small to be statistically significant and draw any conclusions. This slight reduction in growth compared to the minimal media control could be due to the toxic effects of streptonigrin on the cells as they have intracellular iron pools. When these strains were tested in the high iron media, TSB, allowing strains to accumulate higher intracellular iron pool concentrations, and be more sensitive to the damaging effects of streptonigrin than bacteria grown in low-iron media, highlighting the difference between wild-type and mutants.

There was no difference between parent strains and their deletion mutants of staphyloferrins and/or staphylobactin, suggesting that staphylobactin does not contribute to iron acquisition by the organism, or any contribution is too small to measure by this assay. However, there was a difference between lineages, with 71277 being slightly more susceptible than the 8325-4 background, which might suggest that there are other genes involved in iron accumulation or that the 8325-4 background could be metabolically less active, and just not accumulating iron. This result is the opposite of what I would expect because this wild-type strain has all its functioning siderophore transport systems allowing for transport of iron into the cell and therefore should have a higher intracellular concentration of iron making it more sensitive to streptonigrin.

Annotation of the genes in the staphylobactin locus predicted that *stbHIJ* genes encode proteins with similarity to components of a putative iron uptake ABC transporter. As there was no difference between strains in the conditions tested suggests that staphylobactin or its predicted ABC transporter does not contribute to intracellular iron

accumulation. It has been shown that Fhu encodes proteins that are involved in hydroxamate siderophore iron transport in *S. aureus*. However, the Fhu mutant was equally sensitive to streptonigrin as the wild-type strain, suggesting that the fhu mutant was not reduced for intracellular iron concentrations in the absence of exogenous siderophore (452). This may be the case for staphylobactin mutants and why I see no difference between wild-type and stbHIJ or staphylobactin mutants. *S. aureus* encodes a multitude of ABC transporters involved in iron uptake, deletion of one may have little or no effect on intracellular iron concentrations due to compensation by other ABC transporters and this is why no major difference were seen in MIC or growth in media containing streptonigrin between wild-type and staphylobactin mutants. If StbHIJ is not functional, there is a possibility that the other known or an unknown siderophore transporters are responsible for the import of staphylobactin, for example Fhu which is known to import siderophores produced by other bacterial species. This assay could be repeated with strains that have had all the siderophore transports deleted, including Sir, Hts, Fhu and the predicted staphylobactin transporter *stbHIJ*. This may allow for better phenotyping of the staphylobactin region for iron import. The interpretation of the results from this assay are complicated by the number of different factors involved, including growth in minimal or iron-rich media, varying concentrations of streptonigrin and different combinations of siderophore biosynthesis and transport deletion mutants.

4.4.5 Siderophore production-CAS assay

The CAS assay can be used as a quantitative method for siderophore production. The compounds identified through this method belong to both hydroximate and catechol types and are identified by a colour change of the CAS medium from blue to orange. The CAS assay has become widely used since it is comprehensive, exceptionally responsive, and more convenient than other microbiological assays which, although sensitive, may be rigidly specific.

No statistically significant difference in iron-chelating activity could be detected in the Δ*stb* mutant compared to the wild-type. In fact there was a slight increase in siderophore production. In previous work with single staphyloferrin mutants, endpoint supernatant siderophore activity of the Δ*sbn* mutant was equivalent to that of its parent

strain, as measured using the CAS assay, implying that staphyloferrin A might account for this recovery. This could be a possible reason why I did not see a difference between the wild-type and Δ*stb* mutant. The deletion of *sfa* and *sbn* locus from the 71277 and control 8325-4 strain showed significantly reduced production of iron-chelating activity. These are the results I would expect to see and showed that the assay was working. There was only a small reduction detected in the siderophore output of the Δ*sfa*Δ*sbn*Δ*stb* mutant compared to Δ*sfa*Δ*sbn*, however, this difference was statistically significant, providing some evidence that there is less siderophore produced and staphylobactin may have iron-chelating activity. The slight difference suggests that the concentration of staphylobactin in culture supernatant is low. It may not be produced in the conditions tested or the product of the *stb* cluster may have a low affinity for iron.

No siderophore activity was seen in the equibactin mutant Δ*eqbAHIJ* when using the CAS assay (296), but deletion of *eqbA* would suggest an overproduction of *eqbE* according to the qPCR results. Therefore it would be more likely to detect equibactin in the culture supernatant. There is no information on whether the *S. equi* was grown in iron deplete media, allowing for the production of siderophore. The culture supernatants of *S. lugdunensis* grown in C-TMS (containing the staphylobactin homologue), tested negative in the CAS assay, indicating a lack of secreted iron binding molecules (334).

A paper published by Ford *et al*. (453) used the SideroTech Kit (EmergenBio) to detect siderophore activity from culture supernatants. The kit works in the same way as the CAS assay but allows for the concentration of siderophore produced to be estimated from a reference curve produced by desferoxamine siderophore of varying concentrations. The CAS assay only detects siderophore concentrations above 2 μM, which could be well above the amount of staphylobactin produced. A high sensitivity modification of the CAS assay was developed by Andrews and Duckworth (454) for the detection of siderophore in natural waters that can be used to detect and quantify siderophore activity in the nM concentration range. The SideroTech kit or the high sensitivity assay could be used to try and detect if there were significant differences in siderophore activity in the mutant strains. Siderophores have variable affinity to iron, for example aerobactin and pyochelin have lower and others (e.g enterobactin) have

higher affinities to iron. If the staphylobactin cluster has a low affinity or acquires other metals there will be no detected result using these assays. Pyochelin, one of the two major siderophores produced and secreted by *Pseudomonas aeruginosa*, has been shown to bind and uptake other essential metals (455, 456) and this may also represent an alternative function for the *stb* NRPS product(s).

4.4.6 Mass spectrometry

Using LC-MS there is almost no difference mass ion composition of culture supernatants between wild-type and mutants with or without iron in the medium. This is despite siderophore activity using the CAS assay and also growth in minimal media being reduced for strains lacking the staphyloferrins.

The same was observed for equibactin, when comparative metabolic profiling of the Δ*eqbA*, Δ*eqbAE* and Δ*eqbHIJA* strains using LS-MS failed to identify equibactin. This was unexpected due to the effects of the streptonigrin sensitivity on strain Δ*eqbAE* in cross-feeding assays. Examination of biologically active conditioned LB and minimal media from *E. coli* containing the reconstituted equibactin NRPS by LC-MS failed to identify equibactin. This suggests that the concentration of equibactin in culture supernatant is low or the product of the *eqb* cluster may have a low affinity for iron and a structure different from that proposed (296).

Previous work carried out by Beasley *et al*. (210) examined low iron (C-TMS) culture supernatant concentrates of the *S. aureus* strain 8325-4, 8325-4Δ*sfa*Δ*sbn,* 8325-4Δ*sfa* and 8325-4Δ*sbn* for differences in mass ion composition using LC-ESI-MS identified staphyloferrin A and staphyloferrin B synthesised metabolite. A peak at m/z 447.14 corresponded to the published structure of staphyloferrin B and a peak at 479.110 m/z represented staphyloferrin A.

My results and previous work suggest that the media is iron deplete enough to elicit the production of staphyloferrins. Maybe the time from when the culture supernatant is removed, even though immediately frozen on dry ice, allows the siderophores to degrade before they are processed for LC-MS. In terms of staphylobactin production,

perhaps the concentration of staphylobactin is low or not produced in these conditions, therefore I have been unable to identify the metabolic product of staphylobactin.

4.4.7 Virulence

Finally, I sought to determine if the staphylobactin locus had an effect on *S. aureus* virulence *in vivo* using the wax moth larvae infection assay. Mammalian models of infection are costly and may raise ethical issues, therefore insects as infection models provide a valuable alternative. I chose to use larvae of the wax moth *Galleria mellonella* to study virulence as they are convenient and easy to handle, ethically acceptable, relatively inexpensive and a permissive host to study a variety of human and veterinary infectious diseases. The wax moth immune system shares a high degree of structural and functional similarity with the vertebrate innate immune system, and results have shown a strong correlation in virulence between mammalian models and wax moth (457). If the staphylobactin and staphyloferrin siderophores contributed to virulence then strains lacking either or both of these loci were expected to be less virulent than the wild-type strain. However, in the first set of experiments, strains lacking the ability to synthesise *sfa*, *sbn* and/or *stb* demonstrated no statistically significant difference in virulence compared to the wild-type isolate.

Previously, staphyloferrin B mutants have shown attenuated virulence in a murine kidney abscess model of infection (202). However, inactivating siderophore biosynthesis has minimal or no impact on virulence in a mouse sepsis or skin abscess model of infection (238). Although, inactivating siderophore transport has a significant impact on virulence on a mouse sepsis model. This suggests that the model of infection used will have a big impact on the results obtained. All the *S. aureus* isolates containing the staphylobactin locus were isolated from mammals, so wax moths are not their natural host. A large proportion of these isolates came from mastitic cows. It would be of interest to carry out any future work on virulence in a mouse mastitis model of infection (458).

Comparison of wild-type *mecC* strains and wild-type *mecA* strains, found that *mecC* isolates were significantly more virulent than the *mecA* strains tested, suggesting that these strains may contain additional virulence factors or the *mecA* background is

intrinsically less virulent. This was only tested in a small number of different isolates, so more work would need to be carried out with a broader number of isolates to draw any conclusions.

When *S. equi* and its *eqbE* deletion mutants were tested for virulence in its natural host, deletion of the equibactin locus renders *S. equi* significantly less able to cause acute disease (305). The staphylobactin locus may be important during different stages of infection. The yersiniabactin system has been shown to be essential in the early lymphatic stages of bubonic plague, irrelevant in the septicaemic stage, and critical in pneumonic plague. Two siderophores produced by *B. cereus*, petrobactin and bacillibactin, were shown to play different roles in virulence in the insect model. Bacillibactin is an important adaption factor, allowing *B. cereus* to disseminate into the low iron environment encountered in the insect haemocoel. *S. aureus* which produce staphylobactin may be adapted to acquire iron and cause disease in a certain niche, which the staphyloferrins are unable to. In *S. aureus*, haem transport mutants were studied in the murine pneumonia and a systemic infection models. A difference in bacterial load was only observed in the systemic infection model, suggesting that haem iron is not required for infection in the pneumonia model in the first 24 hours, but required by *S. aureus* to establish and maintain infection in the longer systemic infection model (459). Interestingly, when I carried out the second experiment (Figure 4.17), comparison of isolates 71277 and 8325-4 lacking staphyloferrin production were found to be more virulent than their wild-type counterparts. By contrast siderophore mutants of other bacteria tested in the wax moth model displayed reduced virulence (240, 442, 443). These results are difficult to interpret as I would expect the opposite result, suggesting the wax moth larvae model may not be particularly effective here.

To date, I have not been able to demonstrate a role for stb *in vivo* in preliminary experiments using wax moth larvae. However, depending on the host infected, the type of tissues, and the available iron source, variation in the virulence phenotype of strains containing stb will be affected. Further studies in mammalian models are needed to elucidate this possibility. If staphylobactin is found to affect virulence then it could be used as a therapeutic target.

4.4.8 Conclusions

To conclude, experiments showed weak but not convincing evidence that the staphylobactin gene cluster is involved in iron acquisition. If it is involved in iron acquisition then it must be interacting with other mechanisms which may explain the complexity and inconsistency of the results. At this point, it is not possible either to confirm or dismiss the hypothesis that the staphylobactin gene cluster is involved in iron acquisition.

Claire Raisen - January 2019

5 CHARACTERISATION OF VON WILLEBRAND FACTOR-BINDING PROTEIN IN BOVINE AND HUMAN ISOLATES OF *S. AUREUS*

5.1 Introduction

Coagulase is secreted by nearly all *S. aureus* strains and coagulates blood and plasma from various animals (121). *S. aureus* also produces clumping factor A (ClfA), which binds fibrinogen and contributes to platelet aggregation via a fibrinogen or complement dependent mechanism (122). In addition, vWbp binds von Willebrand factor (vWf) (123), a blood glycoprotein that is upregulated on host platelets and endothelial cells during haemostasis to promote blood coagulation (124). The ability of vWbp to bind this glycoprotein may have a role in localising prothrombin activation and therefore clot formation in the vicinity of the bacterium. The interaction between vWbp and vWf is highly specific and is mediated by a region of 26 amino acid residues in the C-terminal part of vWbp (123). When establishing endovascular infections, *S. aureus* adheres to the vessel wall under shear forces of flowing blood by vWbp-mediated binding to vWf (125, 126). The secreted vWbp interacts with vWf and ClfA (a Sortase A-dependent staphylococcal surface protein) to form a complex that anchors *S. aureus* to vascular endothelium under shear stress (127).

5.1.1 The contribution of vWbp and Coa to virulence

Virulence studies with strains lacking the *coa* gene have been performed. Early work reported contributions of coagulase to the pathogenesis of staphylococcal infections (121, 460). In a mouse model, a role for coagulase in the development of pneumonia was suggested (461). Absence of coagulase activity improved outcome in models of skin infection, sepsis, catheter infection, and endocarditis (462). However, in murine models of subcutaneous and intramammary infection, no decrease in virulence was detected (463). Also, *coa* deletion mutants in *S. aureus* strain 8325-4 were still virulent in endocarditis, skin abscess and mastitis mouse models (462, 463). A paper published by Cheng *et al.* (118) suggested that Coa and vWbp are important for the pathogenesis of *S. aureus* infections, when measured as staphylococcal survival in the bloodstream, lethal bacteraemia or the ability of *S. aureus* to form abscesses and persist in mouse models.

5.1.2 Species specificity

S. aureus can cause disease in a wide range of species, and certain *S. aureus* lineages (as defined by MLST) appear to be associated with a particular host species, providing evidence that *S. aureus* strains may adapt to their hosts by genetically determined mechanisms. The von Willebrand factor binding gene (*vwb*) has been linked with a role in host species specificity of *S. aureus* because distinct alleles of the gene appear to have varying abilities to coagulate different animal plasmas (464, 465). This specificity correlates with the host from which the strains were isolated, for example isolates from ruminants or horses can be differentiated from closely related human strains (466). Thus, the specificity of vWbp proteins can be used to infer the host specificity and evolutionary history of the *S. aureus* strains that harbour them.

Many *S. aureus* strains have a highly conserved chromosomal *vwb* gene as well as a *coa* gene (123), which is not found in other *Staphylococcus* such as *S. epidermidis*. In addition to the chromosomally encoded *vwb* found in most *S. aureus* strains, many strains also possess a second *vwb* gene encoded on a mobile pathogenicity island (SaPI). The chromosomally encoded vWbp does not show species-specific coagulation activity (464). The SaPI-encoded *vwb* gene is widely distributed among strains infecting ruminant or equine hosts (467, 468).

Most of the variation in vWbp from different strains is found in the N-terminal end, while the C-terminal end is more conserved and includes the vWf binding domain. vWbp shares 30% amino acid identity to the prothrombin binding domains of coagulase (123). The species-specific coagulation by vWbp has been shown to depend on its interaction with prothrombin (469) (Figure 5.1). Prothrombin is a conserved protein but species differences still exist (for example human and mouse prothrombin show 81% identity). The SaPI-encoded vWbps possess a unique N-terminal region specifically for the activation of ruminant and equine prothrombin (464). Equine SaPI vWbp can clot ruminant and equine plasma, however ruminant SaPI vWbp can only coagulate ruminant plasma. These SaPI-encoded vWbps may represent an important host adaption mechanism for *S. aureus* pathogenicity.

Figure 5.1 Alignment of vWbp and coagulase from *S. aureus*

Part of the vWbp of *S. aureus* shows homology to coagulase. Amino acids (aa), von Willebrand factor-binding region (vW), fibrinogen-binding repeats (Fg) and prothrombin binding domain (prothrombin). Image modified from Bjerketorp *et al*. FEMS Microbiol Lett 2014 (469).

5.1.3 Host species adaption of *mecC* strains

The *mecC* MRSA strains that have been sequenced by our group belong to a number of MLST sequence types including CC130, CC705, and CC425 lineages (470). These sequence types are able to cause infections across a broad range of host species. Analysis of the sequences in our strain collection led to the identification of strains within CC130 which have a chromosomal *vwb* allele that is divergent from chromosomal *vwb* in other *S. aureus* lineages. In this divergent *vwb* gene, a number of strains contain a non-synonymous mutation, exchanging adenine to thymine at position 398, which causes the generation of a premature stop-codon into the 5' end of the gene. Strains with this mutation are found across the phylogeny, suggesting there is a selective advantage to loss of the *vwb* gene (86). As these isolates are commonly found in ruminants, repeated loss suggests that coagulation activity might be selected against, suggesting that the chromosomally encoded vWbp in CC130 has activity against ruminant plasma, unlike chromosomally encoded vWbp from other *S. aureus* lineages.

Therefore I aimed investigate this, and specifically aimed to establish whether the chromosomal *vwb* alleles encoded by *mecC* positive strains have the ability to coagulate a broad range of animal plasmas, including human and/or bovine plasma and if the alleles encoded by these strains can contribute to host specific coagulation activity. This will provide evidence as to whether these strains are adapted to humans or cows, thus giving insight into strains' likely origins and direction of transmission (humans to cows or cows to humans). In addition, the study also aimed to investigate the effects of the premature stop codon on the ability of strains to coagulate different animal plasmas. To do this, a range of *mecC* strains were tested for their ability to clot plasma from different species using a coagulation assay (Table 5.1). The following text in this introduction section describes unpublished work that was carried out by researchers, Dr Ewan Harrison, Alexandra Riddell and Martin Schulze in our group prior to my PhD studies.

Table 5.1. *S. aureus* isolates used in previous work performed by our group

List of strains, the host species they were isolated from, geographical location, and sample site,

year isolated with MLST and clonal complex. Strains with (*) contain the premature stop codon.

Strain	Host	Location	Sample	Year	MLST	CC
LGA251	Bovine	Somerset, England	Bulk Milk	2007	425	425
LGA254	Bovine	Somerset, England	Bulk Milk	2007	425	425
M4A	Bovine	UK	Milk	2012	425	425
H093880936	Human	Southeast England	Ear swab	2009	425	425
H102840444	Human	Southwest England	Nasal swab	2010	425	425
MRSA P1 41/95	Cuniculi	Belgium	N/A	1995	425	425
C03 365*	Bovine	Sutton Bonington, England	Milk	2006-07	1245	130
C03 362*	Bovine	Sutton Bonington, England	Milk	2006-07	1245	130
C04 831*	Bovine	Truro, England	Milk	2006-07	1245	130
10.1799.W*	Human	Tayside, Scotland	Nasal swab	2010	1245	130
73415	Human	Herning, Denmark	Wound	2010	1245	130
KO	Bovine	Slagelse, Denmark	Nasal swab	2011	130	130
H061860351	Human	Northwest England	Sputum	2006	130	130
70355*	Human	Hillerød, Denmark	Nasal swab	2010	130	130
51049	Human	SLA, Denmark	Wound	2006	130	130
RN4220	Human	N/A	N/A	1983	8	8
8_4	Human	UK	Nasal swab		398	398
MRSA 1410	Murine	Belgium	N/A	2008-09	2273	49
41764	Human	SLA, Denmark	Urine	2004	1943	1943/ 1946
76626	Human	Slagelse, Denmark	Screen	2011	130	130
25044	Canine	Bavaria, Germany	Abscess	2010	599	599

5.1.4 Optimisation of coagulation assay performance

Coagulation assays have previously been used to assess to ability of bacteria to

coagulate plasma (464, 465, 471, 472). Firstly the coagulation assay described by Viana

et al. (464) was tested for assay performance (description of clotting grading can be

found in Table 5.2), including the number of colony forming units (CFU) added and effect on clotting time. The time taken for a given strain to coagulate bovine plasma was found to vary between repeats of the assay, from 4 hours to 24 hours. It was noted that strain RN4220 did not form clots within the 24 hour period, regardless of the CFU added. This is because some *S. aureus* isolates, RN4220 for example, have been found to contain an inactivating mutation in its chromosomal *vwb* gene and can only coagulate cuniculine plasma and not caprine, ovine or bovine plasma (464). However, when strain 8_4 was added to plasma there were clear differences in the rate of clot formation depending on the numbers of the bacteria used. It was able to form a grade 4 clot (fully clotted) at 2 hours when $1 \times 10^8 - 2 \times 10^8$ bacteria were added. If more than 4×10^8 or less than 5×10^7 were added to plasma, this reduced the formation of clots to grade 3 or 2, and no clotting was seen when greater than 1×10^9 or less than 1×10^7 added. Though after 24 hours all strains formed grade 4 clots, showing how the number of bacteria affects the kinetics of clot formation but does not alter the results after 24 hours. The protocol described by Viana *et al.* was updated to reduce variation in CFU added, by incubating the culture at 37 °C, shaking at 200 rpm for 14 hours. The clotting assay was carried out as described in the materials and methods section.

5.1.5 Screening of *mecC* strains

Once the coagulation assay had been optimised, screening of *mecC* strains were undertaken in order to determine whether the *vwb* alleles in the *mecC* positive strains accounted for differential coagulation activity. A selection of isolates from various animal origins (Table 5.1) were tested for their ability to coagulate a panel of mammalian plasmas. Comparison of the *mecC* strains sequenced show that their MLSTs separate into four clonal complexes and representative isolates from these clonal complexes were used. Analysis of the chromosomal *vwb* gene identified that the CC130 *vwb* is divergent (75% aa identity with strain Newman chromosomal *vwb* and 69% aa identity to the Equine SaPI vWbp (464)) from the *vwb* in other sequence types. Furthermore, a number of strains were identified that have the premature stop codon mutation (adenine to thymine at position 398) within their *vwb* gene. Representative isolates from

these four clonal complexes along with CC130 strains with or without the stop codon in the *vwb* gene were screened. RN4220, which contains an inactivating mutation in its *vwb* gene but has functional Coa activity (464) was used to help distinguish between those animal plasmas which are predominantly coagulated by Coa and those that are not. Results are shown in Table 5.2. *S. aureus* strains tested for their ability to coagulate plasma of different mammalian species.

Table 5.2. *S. aureus* strains tested for their ability to coagulate plasma of different mammalian species

Degree of clot formation after 24 hours incubation at 37 °C. Clot formation was graded on a scale of 0 - 4: 0 = no coagulation, comparable appearance to negative control tubes; 1 = minimal coagulation; 2 = less than 50% of the plasma has coagulated; 3 = more than 50% of the plasma has coagulated; 4 = complete coagulation with no displacement of clot on tube inversion, comparable in appearance to strain 8_4 (positive control). RN4220 is the *vwb* negative control. Strains with (*) contain the premature stop codon. Strain 8_4 with ($^+$) contains the SaPI associated *vwb* gene.

Strain	MLST	Bovine	Cuniculine	Equine	Human	Ovine	Porcine
LGA251	425	0	0	0	0	0	0
H102840444	425	0	4	3	1	0	3
RN4220	8	0	4	3	4	0	3
8_4$^+$	398	4	4	3	4	4	3
KO	130	4	4	3	4	3	3
76626	130	4	4	3	4	2	3
H061860351	130	4	4	3	4	3	3
70355*	130	1	4	3	4	2	3
MRSA 1410	49	0	4	3	3	2	3
10.1799.W*	1245	1	4	3	4	1	3
41764	1943	0	4	3	3	1	3
25044	599	0	4	2	2	3	3

Chapter 5: Characterisation of von Willebrand factor-binding protein in bovine and human isolates of S. aureus

The *mecC* positive strains that were tested against different animal plasmas were found to cause coagulation to a varying extent. All MRSA except LGA251, coagulated cuniculine, equine and porcine plasma within the 24 hour incubation period to some extent. RN4220 showed the ability to coagulate all of plasma types, except bovine and ovine, demonstrating the role for Coa in clot formation. Coa is known to be a weak coagulator of ruminant plasma (473) and so the grade 4 clots formed by strains 8_4, KO, 76626 and H061860351 suggest that vWbp is likely to have been largely responsible for the coagulation observed. Strain 8_4 clotted bovine plasma after 2 hours incubation. It was the most efficient at coagulation, and the only strain that contains the SaPI associated *vwb* gene. Strains from CC130 demonstrate the ability to clot a broad range of hosts´ plasma including ruminant plasma, despite lacking ruminate SaPI *vwb*. With the exception of strain 8_4, all the other strains which coagulated bovine plasma belonged to clonal complex 130. Of the CC130 strains tested, none of the strains giving grade 4 coagulation at 24 h contained the premature stop codon in *vwb,* while the two CC130 strains (70355 and 10.1799.W) which only inefficiently clotted the plasma possessed the premature stop codon (Table 5.2).

After the initial screening of these strains, I carried out a more detailed investigation into the clotting ability of this novel *vwb* gene and the effect of the premature stop codon, in the work detailed below.

5.2 Materials and Methods

Table 5.3. List of strains, plasmids and oligonucleotides used in this study

Strain/Plasmid	Description	Reference
S. aureus **strains**		
KO	ST130, *mecC* clinical isolate, Denmark, nasal swab, bovine	This study
KOΔ*vwb*	KO with *vwb* gene deletion	This study
KOΔ*vwb* + pXBO1-*vwb*	KOΔ*vwb* complemented with pXB01-*vwb*	This study
KOΔ*vwb* + pXBO1	KOΔ*vwb* with empty plasmid pXB01	This study
BCVA92	ST 398, *mecA* clinical isolate, human, nasal swab, UK	(68)
LGA254	ST 425, *mecC* clinical isolate, 2007, bovine, bulk milk, Somerset, UK	(67)
76626	ST130, *mecC* clinical isolate, Denmark, human	This study
70355	ST130, *mecC* clinical isolate, Denmark, human, containing the premature stop codon	(67)
51049	ST130, *mecC* clinical isolate, Denmark, human	This study
73415	ST130, *mecC* clinical isolate, Denmark, human	This study
C03 362	ST1245, *mecC* isolate, England, bovine, containing the premature stop codon	This study
C03 365	ST1245, *mecC* isolate, England, bovine, containing the premature stop codon	This study
RN4220	Restriction-defective derivative of NCTC8325-4	(395)
Newman	*S. aureus* reference strain	
LGA251	ST425, *mecC* reference strain	(67)
PI 41/95	ST425, *mecC* isolate, Belgium, rabbit	This study
H093880936	ST425, *mecC* isolate, England, human	This study
M4A	ST425, *mecC* isolate, England, bovine	This study
71277	ST130, *mecC* clinical isolate, Denmark, human, containing the premature stop codon	This study
L. lactis **strains**		
L. lactis cremoris 1363	*Lactococcus lactis* cremoris strain 1363	(474)
L. lactis + pORI123	*L. lactis* containing pORI23 plasmid	(475)
L. lactis + pORI123-*vwb*-KO	*L. lactis* containing pORI23 plasmid *vwb* gene from KO	This study

L. lactis + pORI123-*vwb*-71277	*L. lactis* containing pORI23 plasmid *vwb* gene from 71277 containing the premature stop codon	This study
***E. coli* Strains**		
DC10B	Δ*dcm* in the DH10B background; Dam methylation only	(396)
Plasmids		
pXB01	*E. coli - S. aureus* shuttle vector pRMC2 with *bla* deletion; Cm^R	(404)
pXBO1 *vwb*	pXB01 with *vwb* gene from strain KO	This study
pIMAY	pIMC5 with tetracycline inducible *secY* antisense from pKOR1; Cm^R	(391)
pIMAYΔ*vwb*	Modified pIMAY for deleting *vwb* gene	This study
pORI23-*vwb*-71277	Modified pORI23 with *vwb* gene from strain 71277	This study
Primers		
vWBF A	ATAT<u>GGTACC</u>GCATGTGGTAGTGTTGGGTTA	KpnI
vWBF B	AACATGTTGAATCAATATCCAGG	
vWBF C	GGATATTGATTCAACATGTTCTTCCATATGTAATTTGAAGTAAT	
vWBF D	ATAT<u>GAGCTC</u>GCATTGATGTCACTAATCTC	SacI
vWBF OUT FW	GCTATGCACTTAATTACTGGTGTGA	
vWBF OUT RV	GGATTTCTAGTGTATGAGTCGTTGAG	
vWBF COMP FW	ATAT<u>GGTACC</u>GGATATTGATTCAACATGTTTTGTAAAGC	KpnI
vWBF COMP RV	ATAT<u>GAGCTC</u>CTTCAAATTACATATGGAAGGTCG	SacI
IM151	TACATGTCAAGAATAAACTGCCAAAGC	(391)
IM152	AATACCTGTGACGGAAGATCACTTCG	(391)

5.2.1 Preparation of bacterial cultures

Bacterial strains, plasmids and primers are listed in Table 5.3 For routine culture, *Escherichia coli* (*E. coli*) was grown in Lysogeny broth (LB) or on L-agar (Oxoid, UK) at 37 °C. *S. aureus* from glycerol stocks were streaked onto Columbia blood agar plates (Oxoid, UK) or Tryptone Soy Agar (TSA) and grown overnight at 37ºC. *L. lactis* was grown at 30 °C in M17 medium (Oxoid) supplemented with 0.5% glucose (GM17). For the coagulation assay, bacteria were prepared as follows: single colonies of *S. aureus* were

used to inoculate 5 ml of Tryptic Soy Broth (TSB, Oxoid) and grown for 15 hours at 37 °C with 200 rpm. *L. lactis* colonies were inoculated into GM17 broth and grown at 30 °C, 200 rpm overnight. The overnight cultures were centrifuged at 3000 rpm for 5 minutes and the supernatants discarded. The pellets were resuspended in 4 ml sterile phosphate buffered saline (PBS, Oxoid).

Whenever appropriate, antibiotics were added to the media as follows: erythromycin at 5 µg/ml for *L. lactis* and at 300 µg/ml for *E. coli* containing pORI23. *S. aureus* and *E. coli* were supplemented with 10 µg/ml chloramphenicol (Cm10) as appropriate.

5.2.2 Generating *vwb* deletion mutant

Oligonucleotides primer sequences are listed in Table 5.3. *vwb* deletion mutants in KO *mecC*-MRSA strain were generated by allelic exchange with the temperature-sensitive vector pIMAY, as previously described. Sequences upstream and downstream of *vwb* gene to be deleted were amplified with primers A/B or C/D using Phusion High Fidelity DNA Polymerase (ThermoFisher). PCR products AB and CD were used as templates to obtain deletion construct AD with primers A/D in a splicing overlap extension (SOE) PCR. For performing SOE-PCR, KOD Hot Start DNA Polymerase (Merck, UK) was used. Separate master mix A and B were prepared. Master mix A consisted of 5 µl KOD buffer (5X), 5 µl dNTPs, 3 µl $MgSO_4$, 0.7 µl KOD enzyme and 26.3 µl Milli-Q water. Master mix B consisted of 2.5 µl primer A and 2.5 µl primer D. Template DNA was prepared by mixing 2.5 µl AB, 2.5 µl CD PCR product. Template DNA (5 µl) was added into master mix A and SOE-PCR was run for 10 cycles under the following conditions: 94°C for 5 min; 10 cycles of 94°C for 30s, 60°C for 1.5 min and 72°C for 2.5 min. An extra step of 10°C for 5 min was added in the program when adding master mix B in the reaction tube. SOE-PCR then was continued under the following conditions: 95°C for 2 min; then 35 cycles of 95°C for 20s, 59°C for 10s and 70°C for 50s; and one final extension step of 70°C for 5 min. The SOE-PCR product of about 1.6 kb was isolated from agarose gel using YORBIO Gel/PCR DNA Purification Kit (YORBIO, UK). Product AD was digested with restriction enzymes KpnI and SacI (New England BioLabs) and ligated using T4 DNA Ligase (Life Technologies, UK) to pIMAY digested with the same enzymes. The resulting plasmids were designated pIMAYΔ*vwb*. The plasmid was transformed into *E. coli* DC10B (a *dcm* deletion mutant of

DH10B, allowing the plasmid to be directly transferred into *S. aureus* strains) and plasmid DNA extracted using QIAprep Spin Miniprep Kit (Qiagen) from DC10B was then electroporated into *S. aureus* CC130 strain KO to create knockout mutants. Figure 5.4 shows the *vwb* gene deletion in KO as an example.

5.2.3 Complementation of deletion mutant

For complement expression of *vwb*, the gene was cloned into expression plasmid pXB01, a derivative of the tetracycline-inducible expression vector pRMC2 with a deleted *bla* gene (404). The *vwb* gene was amplified from KO genome DNA with primers *vwb* COMP FW / *vwb* COMP RV. PCR products were digested with KpnI and SacI and ligated with the pXB01 plasmid cut with the same enzymes, generating plasmid pXB01*vwb.* The sequence was verified and the plasmid was transformed into *E. coli* DC10B. The plasmid DNA was then extracted and electroporated into mutant strains for complementation with expression, induced with 200 ng/ml anhydrotetracycline (Sigma-Aldrich, UK).

5.2.4 Preparation of electrocompetent *Lactococci*

100 ml of SGM17 media was inoculated with 1.5 ml of overnight *L. lactis* culture grown in SGM17 and incubated at 30 °C and 200 rpm. The culture was incubated until the OD_{595nm} reached ~0.5 and centrifuged for 10 min, 5000 rpm at 4°C. The supernatant was removed and the pellets resuspended in 50 ml of ice cold 0.5M sucrose containing 10% glycerol, centrifuged again under the same conditions and the cell pellets resuspended in 25 ml of ice cold sucrose containing 10% glycerol, followed by another centrifugation and the cells resuspended in 1 ml of 0.5M sucrose containing 10% glycerol, stored at -80 °C in 40 µl aliquots.

5.2.5 Electroporation of plasmid pORI23-vwb-71277 into *L. lactis*

The plasmid pORI23 containing the *vwb* gene cloned from strain 71277 created during previous work on this study was extracted from *E. coli* cells using QIAprep Spin Miniprep Kit (Qiagen) and 5 µl was added to 40 µl of electrocompetent *L. lactis* cells in an electroporation cuvette and electroporated under the conditions of 25 µF, 200 Ω and

2.0 kV. 950 µl of SGM17MC medium were then added immediately and the cuvettes put back on ice for 5 min. The mixture was incubated at 30 °C for 2 h slightly rotating. 100 µl of the culture and 10^{-1}, 10^{-2}, 10^{-3} dilutions were plated on streptococcus regeneration medium (SRM) with 5 µg/ml erythromycin and incubated at 30 °C for 48 h. The presence of the plasmid was confirmed by colony PCR with primers for the MCS for pORI23.

5.2.6 Coagulation Assay

Adapted from (464). Cuniculine, bovine, human, equine, and ovine plasma were used for the coagulation assays. Blood from these animals was collected in ethylenediaminetetraacetic acid (EDTA) and centrifugation (3500 rpm at 4 $^{\circ}$C for 10 minutes) was used to separate out the plasma from the other blood constituents. Plasma was either used fresh on the same day as collected or was stored at -20 and defrosted to room temperature when required. The coagulation assay was carried out by mixing 300 µl of plasma with 50 µl (1×10^8) of PBS-washed *S. aureus* from an overnight culture in a sealed glass test tube. The contents were mixed by gentle agitation and incubated at 37 °C and the levels of coagulation was observed in comparison to controls by tilting the tubes at hourly intervals for four hours and then at 24 hours of incubation. For the negative control, 50 µl of PBS was added to the plasma. Positive controls were dependent on the plasma being used. For ruminant plasma strain BCVA92 (ST398) was used. Every strain was tested in triplicate and each assay was repeated at least twice. A positive result was obtained if a visible clot was formed in the contents of the tube at any one of these time points. They were scored as follows: minimal clotting apparent (1), less than 50% of the suspension has clotted (2) more than 50% of the liquid in the tube has clotted (3), and complete clot with no liquid on inversion (4).

5.3 Results

5.3.1 Identification of stop codons in *vwb* gene in CC130 isolates

Genomic analysis of CC130 isolates from humans and animals identified that the *vwb* genes in distinct clades, including a large clade from cattle, had an inactivating mutation in *vwb*, which should have no coagulation activity in cattle (Figure 5.2). This led to two hypotheses. Firstly, it could be that because the *vwb* gene was not required in cattle its loss was a random mutation. However, as this had happened on multiple occasions this seemed unlikely. Therefore the second hypothesis was that it actually functioned by promoting clotting in cattle and was being selected against. Thus the multiple inactivations were evolutionarily convergent due to a selective pressure.

Figure 5.2 Phylogenetic tree showing the human and bovine CC130 isolates

Maximum likelihood, midpoint rooted phylogeny based on core genome SNPs. Blue arrows and brackets indicate the strains containing the stop-codon within their chromosomal *vwb* gene. Colour of branches shows source of isolate: invasive disease (red), skin infection (yellow), human nasal carriage (orange), milk (blue), dog nasal swab (purple). Strain LGA251 included in the tree as a reference.

5.3.2 CC130 strains and the effect of the stop codon on coagulation of ruminant plasma

A previous study on SaPI-encoded *vwb* has demonstrated that the ruminant coagulating activity conferred by *vwb* is not species specific (e.g. there is no difference between bovine or ovine plasma) (465). To confirm this, coagulation assays using bovine and ovine plasma were set up with a selection of CC130 strains along with the relevant controls (Table 5.4). CC130 strains containing the divergent *vwb* gene were able to clot bovine and ovine plasma after 24 hours. Comparison of five CC130 strains with the premature stop codon and five without, confirmed that the presence of the stop codon dramatically reduces the ability to coagulate bovine plasma (Table 5.4).

Table 5.4. *S. aureus* CC130 strains ability to coagulate ruminant plasma

To each tube of 300 μl of plasma, between 7 x10^8 – 9 x 10^9 CFU of *S. aureus* were added. Degree of clot formation at 24 hours incubation at 37 °C of *S. aureus* CC130 strains either with or without the presence of the *vwb* stop codon. Clot formation was graded on a scale of 0 - 4: 0= no coagulation, comparable appearance to negative control tubes; 1 = minimal coagulation; 2 = less than 50% of the plasma has coagulated; 3 = more than 50% of the plasma has coagulated; 4 = complete coagulation with no displacement of clot on tube inversion, comparable in appearance to strain BCVA92 (positive control for ruminant plasma). Strain RN4220 and PBS (negative control). Clotting assays were carried out in triplicate for each strain. N/A, this test was not done.

Claire Raisen - January 2019

Strain	MLST	Stop codon	Bovine	Ovine
KO	130	No	4	3
MRSA 1410	130	No	4	3
H061860351	130	No	4	3
51049	130	No	4	3
73415	1245	No	3	3
C03 362	1245	No	1	3
C03 365	1245	No	1	3
C04 831	1245	Yes	0	N/A
10.1799.W	1245	Yes	1	1
70355	130	Yes	0	2
RN4220 (-)	N/A	No	0	0
BCVA92 (+)	398	No	4	4

5.3.3 Evidence of host range of CC130 *vwb* in comparison to other lineages.

5.3.3.1 Plasmid and deletion mutant construction

In order to confirm the ability of the *vwb* gene to clot a variety of mammalian plasma in *mecC* CC130 strains, a *vwb* gene deletion mutant was constructed in strain KO that was isolated by nasal swab from a bovine host in Denmark. Strain KO has been shown to fully clot bovine plasma after 24 hours (Table 5.2) and does not contain the premature stop codon in the *vwb* gene that has been shown to reduce the ability to clot bovine plasma (Table 5.3). Figure 5.3 shows the construction of the *vwb* gene deletion in KO.

Figure 5.3 Generating *vwb* gene deletion in *S. aureus* strain KO

(A) PCR amplification of upstream fragment AB and downstream fragment CD of the *vwb* gene from KO genomic DNA using Phusion High Fidelity DNA Polymerase. Fragment AB and CD then were joined together using primer A/D in a Splice Overlap Extension (SOE) PCR, resulting in fragment AD. Plasmid pIMAY and fragment AD were both digested with SacI / KpnI and ligated together using T4 DNA ligase (Life Technologies, UK). Resulting construct, pIMAYΔ*vwb* was transformed into *E. coli* DC10B. pIMAYΔ*vwb* extracted from DC10B was electroporated into KO at 28 °C.

(B, C) Single crossover integration was stimulated by growth at 37 °C on TSA (Cm10). Then loss of replicating plasmid was confirmed by colony PCR with MCS primers (IM151/152). Colonies negative for replicating plasmid were then screened for the side of integration with primers Δ*vwb*_outF/D (AB side integration) and Δ*vwb*A/outR (CD side). A clone from either AB or CD integration event was grown at 28 °C in broth without antibiotic selection to stimulate rolling circle replication and then plated on TSA with 1 µg/ml ATc. Expression of the *secY* antisense RNA (*a-secY*) inhibits growth of cells maintaining the plasmid. If the plasmid integration event occured through the AB side, plasmid excision through the AB side recreates the wild-type locus, while CD excision yields a mutated gene. Figure 5.3B was sourced from (396).

(D) Colony PCR screening of deletion mutants using primers Δ*vwb*_outF/R, deletion mutants gave a PCR produce about 1.5 kb, while wild-type colonies gave a larger fragment.
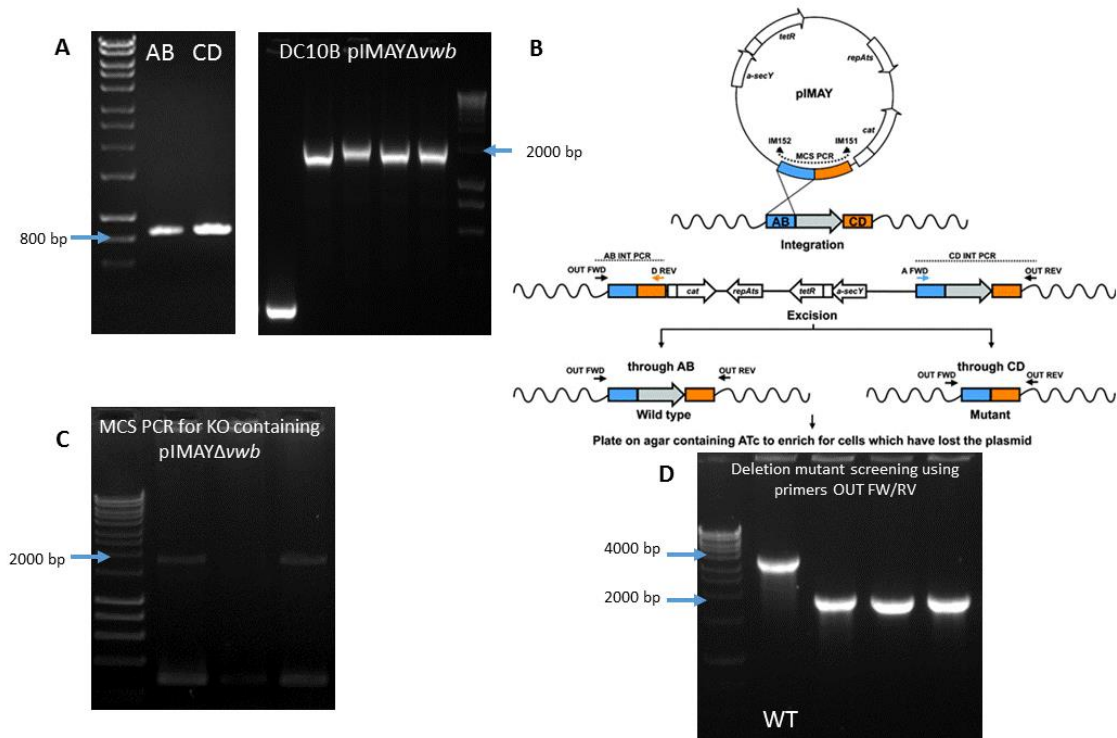
To confirm if any change in coagulation ability is due to the loss of the *vwb* gene, a deletion complement was made using the plasmid pXB01 that expressed the KO *vwb* gene which had been deleted from the chromosome. The strains that were generated, along with *mecA* strain BCVA 92 (ST 398) which has shown to be very efficient at clotting bovine plasma, LGA251, LGA254 and 76626 were tested with the coagulation assay using bovine plasma. Results are shown in Table 5.5.

Table 5.5. Role of *vwb* in coagulation of bovine plasma in CC130 *S. aureus.*

Strain BCVA92, positive control for ruminant plasma. PBS (negative control).

Strain	Bovine clotting after 24 hours
KO	3
KOΔ*vwb*	0
KOΔ*vwb*+pXB01	1
KOΔ*vwb*+pXB01*vwb*	3
BCVA92	4
LGA254	4
LGA251	0
76626	3
PBS	0

As expected strain LGA254 and BCVA 92 were fully able to clot bovine plasma after 24 hours, whereas strain LGA251 and the PBS control showed no clotting ability. The wild-type strain KO and 76626, both *mecC* CC130 strains, were able to clot to grade 3 (more than 50% of the liquid in the tube has clotted), which were similar to previous results, but not to the full extent seen at 24 hours incubation. The deletion strain showed no clotting ability, and the strain containing the empty pXB01 plasmid showed grade 1 clotting (very minimal clotting). The clotting ability was restored on complementation of the *vwb* mutant with the vwb containing plasmid pXB01. This data indicates that the vWbp is responsible for *S. aureus* strain KO ability to clot bovine plasma, and is not due to the activity of Coa. The clotting assay was not carried out with ovine plasma due to lack of available ovine plasma at the time.

5.3.4 Expression of *vwb* CC130 in *L. lactis*

To understand the ability of vWbp from CC130 strains to coagulate a broad range of animal plasma, and to eliminate the possibility that the native *coa* gene or other proteins expressed by *S. aureus* contributed to the results, the *vwb* genes were cloned into the *E. coli* pORI23 plasmid and expressed in *Lactococcus lactis* subsp. *cremoris* 1363. *L. lactis*

was used as it has a well understood genetic background, and lacks other factors that could affect the clotting activity of vWbp, while being able to secrete *S. aureus* proteins. Also the pORI23 *E. coli – L. lactis* shuttle and expression plasmid has been used in other projects with ClfA (475). This experiment allows a way to study individual staphylococcal pathogenic factors, complementing the classical knockout mutagenesis strategy. The *L. lactis* strains containing the pORI23 plasmid which constituently expresses the *vwb* gene from CC130 strain KO were tested for their clotting ability. The strains were tested for the coagulation of bovine plasma at 1, 2, 3, 4 and 24 hours incubation at 30 °C (Table 5.6). No clotting was observed for any strains between 1-4 hours incubation (results not shown). *L. lactis* expressing the *vwb* gene from CC130 can fully clot bovine plasma by 24 hours, whereas *L. lactis*, *L. lactis* containing the empty pORI23 plasmid and the PBC control were unable to clot bovine plasma suggesting that the vWbp produced by the chromosomally divergent *vwb* gene is solely responsible for the clotting of bovine plasma. In order to provide further evidence of this divergent vWbp's ability to clot plasma from a broad host range and the effect of the stop codon, a strain of *L. lactis* was created containing the pORI23 plasmid expressing the *vwb* gene from strain 71277, which is known to contain the premature stop codon.

Table 5.6. Coagulation of bovine plasma by *L. lactis* expressing the CC130 *vwb* gene

L. lactis expressing vWbp clots bovine plasma. Strains were tested in triplicate and clotting was graded after incubation at 30 °C for 24 hours. *L. Lactis 1363*, *L. Lactis* +pORI23, *L. Lactis* +pORI23-*vwb*-71277 (contains stop codon) and PBS are the negative controls.

Strains	Bovine clotting after 24 hr
L. Lactis 1363	0
L. Lactis +pORI23	0
L. Lactis +pORI23-*vwb*-KO	4
L. Lactis +pORI23-*vwb*-71277	0
PBS	0

Finally the KO strain and its *vwb* deletion mutants, along with the *L. lactis* strains containing the *vwb* gene, and control strains were tested against a broad panel of mammalian plasma to assess the full extent of the *vwb* gene of CC130 strains to coagulate plasma (Table 5.7).

Table 5.7. Role of *vwb* in coagulation of plasma from a broad range of host species.

Each strain tested in duplicate for each plasma. Results represent the outcome of three separate experiments. Between $3.35 \times 10^7 – 2.75 \times 10^8$ CFU of *S. aureus* was added to 300 μl of plasma. Strain BCVA92 was the positive control for ruminant plasma. Strain RN4220, *L. Lactis 1363,* PBS and PBS + Atc were negative controls. Strains with (*) contain the premature stop codon in the *vwb* gene.

Strain	Bovine	Human	Ovine	Porcine	Cuniculine	Equine	Canine	Feline
KO	3	4	4	3	4	4	2	4
KOΔ*vwb*	1	4	4	3	4	4	2	4
KOΔ*vwb*+ pXB01*vwb*	2	4	4	4	4	4	3	4
KOΔ*vwb*+pXB01	1	4	3	3	4	4	2	3
L. Lactis 1363	0	0	0	0	0	0	0	0
L. Lactis +pORI23	0	0	0	0	0	0	0	0
L.Lactis+pORI23-*vwb*-KO	4	4	4	4	4	4	4	4
L. Lactis+pORI23 -*vwb*-71277*	0	0	0	0	0	0	0	0
RN4220	0	0	0	2	3	0	0	4
Newman	0	1	3	3	4	4	0	1
BCVA92	4	1	4	3	4	4	3	4
LGA251	0	2	1	0	0	0	0	0
71277*	0	4	4	3	4	4	1	3
PBS+ Atc	0	0	0	0	0	0	0	0
PBS	0	0	0	0	0	0	0	0

For each assay tested, none of the negative control tubes (either PBS+Atc or PBS in plasma) showed signs of coagulation after 24 h incubation. RN4220, which has been found to have an inactivating mutation in its chromosomal *vwb* gene, coagulated only cuniculine, porcine and feline plasma. Therefore, this clotting phenotype can be attributed to its Coa activity. CC130 strain KO showed the ability to clot plasma from all species tested. Human, ovine, cuniculine, equine and feline plasma all fully clotted. Bovine and porcine plasma clotted to grade 3 (more than 50%) and canine to grade 2 (less than 50%). However, deletion of the *vwb* gene only reduced the clotting of bovine plasma to minimal clotting, and the other species tested clotted to the same extent as the wild-type. Complementation of the gene deletion by pXB01 expressing the *vwb* gene increased its ability to clot bovine and canine plasma compared to the deletion mutant. The deletion control strain containing the empty vector displayed the same clotting phenotype as the *vwb* deletion mutant. CC130 strain 71277, which contains the premature stop codon in its *vwb* gene, was unable to clot bovine plasma. It demonstrated the same clotting ability as strain KO for the other plasma tested, except for canine and feline, which has slightly reduced clotting ability.

This suggested that the stop codon in this divergent *vwb* gene only affects the strains' ability to coagulate bovine plasma. When strain LGA251 (ST425) was previously tested with a broad panel of animal plasma species, it was unable to form any clots. After it was retested, along with addition of canine and feline plasma, it displayed minimal clotting for human and ovine plasma, but no visible clots for the other plasmas. *L. lactis* strains containing either no vector (*L. Lactis 1363*), empty vector (*L. Lactis +pORI23*) or vector containing the *vwb* gene with the premature stop codon (*L. Lactis +pORI23-vwb-71277*) did not clot plasma from any species tested. These results confirmed that *L. lactis* containing the pORI23-*vwb*-KO plasmid expressed a vwb protein able to fully clot plasma from all species tested.

These results suggest that the divergent *vwb* is able to clot plasma from a wide variety of species, and the introduction of the stop codon renders the gene incapable of clotting any plasma, shown by the clotting phenotype of *L. lactis* expressing the *vwb* gene from strain 71277.

5.3.5 Screening of ST 425 strains

The two ST 425 strains tested against a variety of mammalian plasma showed different coagulation abilities (Table 5.2). To determine if either of these strains showed a coagulation phenotype representative of ST 425, additional strains were tested for their ability to clot bovine, equine, ovine, porcine, cuniculine and human plasma (Table 5.8). These strains are interesting as they were isolated from a broad range of host species but also possess the ability to clot plasma of different species to varying degrees.

Table 5.8. ST425 *S. aureus* strain's tested for their ability to coagulate a broad range of host plasma.

Between 1.5×10^8-3.5×10^8 CFU of *S. aureus* was added to each test tube. PBS was used as a negative control.

Strain	Host	MLST	Bovine	Cuniculine	Equine	Human	Ovine	Porcine
LGA251	Bovine	425	0	0	0	0	0	0
LGA254	Bovine	425	4	4	3	3	4	3
PI 41/95	Rabbit	425	0	4	3	2	0	3
H093880936	Human	425	0	4	3	2	0	3
H102840444	Human	425	0	4	3	1	0	3
M4A	Bovine	425	0	4	3	2	0	3

Strain LGA251 was unable to coagulate any of the plasma tested. This may be due to a nonsense mutation at codon 240 in the *coa* gene. Strain LGA254 was found to have a distinct coagulation phenotype as it was able to rapidly coagulate all the plasma tested (Table 5.8) suggesting that this strain is a good producer of the *S. aureus* coagulases. Study of the LGA254 genome identified a second novel SaPI *vwb* allele. This may explain why LGA254 can clot plasma from diverse species, including equine.

The other ST 425 strains are unable to clot ruminant plasma but clotted equine, porcine and cuniculine to the same extent as strain LGA254. They were also able to clot human plasma to a grade 2 clot (less than 50%).

5.4 Discussion

When Bjerketorp *et al* (469) expressed the *vwb* gene from strain Newman and 8325-4, and tested their ability to coagulate plasma from many different animal species they found it did this with varying efficiency. The vWbp from human-adapted strains was a very efficient coagulator of human and porcine plasma, but a poor coagulator of goat, sheep, equine and bovine plasma (466). All *S. aureus* produce coagulase, which does not effectively induce clotting of ruminant plasma (473). This suggests the species-specific determinant is responsible for the difference in coagulation efficiency. Ruminant and equine isolates containing the additional SaPI encoded *vwb* gene that encodes a vWbp that can specifically clot ruminant and equine plasma (464), suggesting that the SaPI-encoded vWbps may represent an important host adaption mechanism for *S. aureus* pathogenicity, and therefore that acquisition of vWbp-encoding SaPIs may be determinative for animal specificity.

5.4.1 Identification of stop codons in *vwb* gene in CC130 isolates

Whole genome sequencing of the *mecC* isolates in our collection from a broad range of host species revealed a unique *vwb* gene compared to other *vwb* genes characterised to date. *S. aureus* normally contains a highly conserved chromosomal *vwb* gene in addition to their *coa* gene (123). Basic screening showed that *mecC* CC130 strains isolated from human and bovine hosts, possessing the divergent *vwb* were able to coagulate bovine, cuniculine, equine, human, ovine and porcine plasma, unlike other *mecA* strains studied that have been shown to clot species specific plasma. These CC130 strains could coagulate ruminant plasma, despite lacking the SaPI encoded *vwb*. While clear differences were observed in the ability of the strains to coagulate bovine plasma, there was little variation in the ability of the strains to coagulate porcine, equine and cuniculine plasma, despite the fact that they have a divergent *vwb*. This is probably due to this divergent vWbp having the ability to interact with a broad range of prothrombin from different species. Prothrombin is not well conserved between different animal species (464) and vWbp that interacts optimally with one animal prothrombin may be less able to interact with prothrombin from another species. A number of CC130 strains,

including bovine isolates, were found to have a premature stop codon mutation within their *vwb* gene, causing a loss of their ability to clot bovine plasma, but had little effect on their ability to clot ovine plasma.

5.4.2 CC130 strains and the effect of the stop codon on coagulation of ruminant plasma

These results provide preliminary evidence supporting a role for chromosomal vWbp in host species coagulation activity. The reason for this selection against the production of vWbp is not obvious and might be a sign of a host specialisation where the vWbp is not needed. One theory is that the ability to clot blood is largely redundant as a fitness factor in mammary gland infections. Therefore, the presence of this particular *vwb* allele within the CC130 lineage may not only reflect host adaptation but may also be an example of an adaptation for a particular ecological niche i.e. the bovine mammary gland.

5.4.3 Knock out and complementation in *S. aureus*

The presence of the divergent vWbp in the CC130 isolates was found to correlate with an ability to coagulate bovine plasma and its deletion from a bovine isolate was found to abolish the bovine plasma coagulation phenotype. Re-complementation of *vwb* into the deleted strain was found to restore the ability to coagulate bovine plasma, further supporting the conclusions that the vWbp is responsible for the species-specific coagulation activity. The deletion did not alter this strain's ability to coagulate all the other species plasma, especially ovine and equine plasma which is normally associated with the SaPI encoded *vwb*, which this strain is lacking. But given that these strains can clot the other plasma maybe the *coa* gene is divergent as well.

5.4.4 *L. lactis* confirms the divergent vWbp to coagulate a broad range of animal plasma and the effect of the stop codons.

When testing these strains, it is possible that coagulation arising from Coa activity could mask any variation in coagulation capacity due to vWbp. It has been observed that purified Coa is more efficient at coagulating rabbit plasma than vWbp, but it is unknown whether both proteins are secreted by the bacterium to the same extent. The results of

this assay could not determine whether the difference in clotting was due to vWbp or Coa, as their activities could not be separated. *coa* expression could also be responsible for the difference in coagulation, especially in strains containing the stop codon in the *vwb* gene. This would compensate for vWbp not being produced, allowing these strains to coagulate ovine plasma.

The ability to test *S. aureus* mutants of the *vwb* gene was limited as other factors might overcome and compensate for the function of the missing or mutated factor. To circumvent this problem, and to allow Coa activity to be distinguished from vWbp activity, I expressed the divergent *S. aureus vwb* gene, with and without the premature stop codon in a less virulent organism. These strains were tested for this specific determinant to clot plasma from a broad range of host species. I hypothesised that expression of *vwb* in *Lactococci* would result in their ability to clot plasma from a broad range of host species, even though staphylococcal knockout mutants are not defective in clotting. *L. lactis* is devoid of virulence factors and has a well characterised genetic background. This bacteria has already been used to express single staphylococcal surface proteins (127, 475). Genes are expressed when useful for the bacteria and downregulated when unnecessary. Therefore, expressing these factors constitutively in a surrogate bacterium might help discriminate between their intrinsic contribution when taken alone and the importance of their regulation in the staphylococcal context. The promoters of the pORI23 plasmid expressed the *vwb* gene constitutively, leading to the over expression of vWbp. *L. lactis* alone is unable to cause coagulation of any species plasma, therefore the expression of the CC130 *vwb* from pORI23 in *L. lactis* is responsible for the complete clotting of all plasma tested.

5.4.5 Conclusions

Mobile genetic elements play a central role in the adaption of *S. aureus* strains to different host species (467, 476). The chromosomal *vwb* and *coa* confers species specific coagulation activity. The SaPI vWbp confers the specific activation of ruminant and equine prothrombin. It has been suggested that the equine SaPI *vwb* gene evolved from an ancestral bovine or ovine SaPI *vwb* gene, maybe by recombination, to enhance its

adaption to an equine host (464). However, it has been shown that porcine isolates had only a weak clotting activity with porcine plasma (464). But in the case of *mecC* CC130 strains, the vWbp seems responsible for clotting in a broad range of host species plasma. The divergent *vwb* chromosomal genes may have evolved from the human adapted strains by SNPs to allow this strain to infect different hosts. Overall, these divergent *vwb* genes encode host-specific functional activity likely to be important to *S. aureus* CC130 host adaption.

5.4.6 Future work

Further work that could be carried out on this project would be to transfer the *vwb* genes, chromosomal and SaPI, from strains covering a variety of backgrounds, into *L. lactis* and test their coagulation activity for proof that the clotting of plasma in difference animal species is due to the CC130 *vwb* gene only and no other factors, such as coagulase.

A possible strain to carry this out with is LGA251 (ST425), a bovine associated strain that has no clotting ability of the plasma tested, except very minimal clotting of human and ovine plasma, despite being cattle associated. It has been suggested that this may be due to a nonsense mutation at codon 240 in the *coa* gene, so any clotting activity would be due to its chromosomal *vwb* gene. Both the *coa* gene and the *vwb* gene could be cloned into *L. lactis* to assess their coagulation activity. Strain LGA254 (ST425) was found to have a distinct coagulation phenotype as it was able to rapidly coagulate all the plasma tested. This strain contains both the chromosomal and SaPI *vwb* genes. Both *vwb* genes could be cloned into *L. lactis* and their individual coagulation activity could be assessed. Other strains of interest would be Newman (human isolate), m-MR-6AA (ST133) which is ruminant-associated and C05 232 (ST151) which is bovine-associated. In this study, strain BCVA92 was the only isolate of human origin to effectively coagulate bovine plasma. It belongs to ST398, a sequence type that has a particularly diverse host range (477, 478). This may explain why this strain efficiently coagulated all the plasmas tested. Work already carried out in our group has cloned *vwb* from strains 70355 (CC130, human isolate), 41764 (human isolate), 10.4137.F (human isolate, ST22). These have already been cloned into pORI23 and can be transferred to *L. lactis* for expression.

Strain RN4220 has an inactivating mutation in its chromosomal *vwb* gene and has been shown to only coagulate cuniculine plasma and not ruminant through the activity of Coa. It would be of interest to create Δ*coa*, and Δvwb/coa in strain KO to see the contribution of each coagulase to clotting ability of different animal plasmas, and to eliminate the possibility that the native *coa* gene contributed to this result. In this study, CC130 isolates containing the divergent *vwb* gene isolated from humans and cows were tested for their ability to coagulate a broad range of animal plasma. Since these strains are not restricted to just human or bovine infections it would be interesting to study isolates from different species and investigate if the coagulation specificity is shifted towards the corresponding prothrombin.

6 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Claire Raisen - January 2019

6.1 Summary

MRSA continues to be a significant cause of infections both in humans and animals. Since 2011, *mecC*-carrying strains have been isolated from a broad range of host species, including humans, livestock, companion animals, and wildlife in more than 10 western European countries, presenting new challenges in MRSA diagnosis and treatment (59). The *mecC* isolates are capable of causing a wide variety of infections, including severe and fatal disease. *mecC* strains sequenced by our group have been shown to contain additional genetic features, such as the staphylobactin locus and the novel *vwb* gene, which may increase *S. aureus* ability to cause disease in a wide range of host species and niches.

As detailed in Chapter 1, *S. aureus* possesses a multitude of mechanisms by which it can obtain iron from an iron-restricted host. It exploits multiple host iron sources by expressing a haem acquisition system (Isd), produces two α-hydroxycarboxylate-type siderophores, staphyloferrin A and staphyloferrin B which extract iron from host glycoproteins (197, 198, 203, 378, 479-481), and it expresses transporters for many other siderophores that it does not synthesise. The success of *S. aureus* as a pathogen is partly attributable to its numerous and often functionally redundant mechanisms for iron acquisition within the host (261).

In Chapter 2 of this study, I have described the identification of the staphylobactin region in the genomes of *mecC* CC130 isolates, a non-host specific lineage, and investigated its possible role using sequence analysis. The novel locus encoding a siderophore-like non-ribosomal peptide synthetase (NRPS) was found directly downstream of the *SCCmec* insertion site and is flanked by direct repeats, which suggest that staphylobactin could have been acquired by horizontal gene transfer. A homologous region was identified in *Streptococcus equi*, which encodes a NRPS termed 'equibactin' which is involved in iron acquisition (296). The region has the same gene order and predicted functions as the equibactin locus, with *stbE* and *stbG* genes predicted to encode NRPSs, suggesting a possible role in iron acquisition. Of the 2,343 genomes that have been sequenced in our collection, 24% of these strains contained the staphylobactin region (97% belonging to CC130), covering a broad geographical and host range. The organisation of the *stb*

biosynthetic gene cluster is highly conserved in all CC130 *S. aureus* isolates. All these genomes contain the staphyloferrin B biosynthesis and transport genes, therefore staphylobactin was not acquired to compensate for lack of siderophore. When searching the genomes of other Staphylococcus spp., mainly CoNS, the staphylobactin locus was found in its entirety in all *S. lugdunensis* genomes searched and one *S. hyicus* isolate. Unlike the equibactin locus, the staphylobactin locus lacks a homolog for the iron-dependent regulator EqbA. Instead, expression of this locus appears to be regulated by MntR, a DtxR-like regulator. A DtxR box upstream of *stbB* was identified. The substrate specificity of the two staphylobactin NRPS A-domains have conserved NRPS codes for cysteine and salicylate, suggesting that the staphylobactin cluster is likely to be functional.

In Chapter 3, I discuss how I overcame barriers encountered to intrinsic restriction modification systems, low transduction frequency and low efficiency of recombination, when creating deletion mutants in bacteria which were extremely difficult to manipulate. I achieved this through developing and optimising a protocol, including an improved electroporation method, using the current tools and techniques available. This allowed the genetic manipulation of previously unmanipulated *mecC* strains, in order to study the function of genes. These staphylobactin and staphyloferrin mutants were used extensively throughout this thesis and will be indispensable for identifying the role of this gene cluster, assessing their role in pathogenicity and investigating how they can infect a broad range of host species. The protocols I developed have been used by Ba *et al*. (404) to study *mecC* strains.

In Chapter 4, a number of assays were performed to access the possible function of the staphylobactin gene cluster in iron acquisition and their impact on virulence. Regulation and expression of siderophores in an iron-dependent manner were assessed using RT-qPCR. MntR is possibly a positive regulator of the staphylobactin operon in an iron-dependent manner. There was a slight increase in expression of staphylobactin NRPS in an iron deprivation-dependent fashion. Mutagenesis was performed to eliminate the siderophore biosynthetic loci from the *S. aureus* genome to dissect their individual and combined contributions toward growth in iron-restricted media, containing serum, transferrin, milk and metal ions. Deletion of the *stb* locus did not demonstrate any

detectable growth impairment in any of the conditions tested, unless introduced into a strain bearing the *sbn* deletion. Staphylobactin mutants were tested for sensitivity to streptonigrin to determine the contribution of the staphylobactin locus to intracellular iron accumulation. There was no difference detected between wild-type, staphylobactin biosynthetic and transport mutants in the conditions tested, suggesting that staphylobactin or its predicted ABC transporter does not contribute to intracellular iron accumulation. I evaluated whether staphylobactin could function as a NRPS antibiotic. The *S. aureus* CC130 strains seem to have the ability to kill other *S. aureus* isolates, which may contribute to their success. This doesn't seem to be the role of the staphylobactin locus, as no loss of activity is seen in the Δ*stb* strain versus the wild-type. I determined whether *mecC S. aureus* produce siderophores using the CAS assay, and which siderophores they produce using LC-MS analysis of culture supernatants. There was only a small reduction detected in the siderophore output of the Δ*sfa*Δ*sbn*Δ*stb* mutant compared to Δ*sfa*Δ*sbn*, however, this difference was statistically significant, providing some evidence that there is less siderophore produced and that staphylobactin may have iron-chelating activity. The slight difference suggests that if the concentration of staphylobactin in culture supernatant is low, it may not be produced in the conditions tested or the product of the *stb* cluster may have a low affinity for iron. Despite the changes in siderophore activity detected using the CAS assay and growth in minimal media being reduced for strains lacking the staphyloferrins, almost no difference was detected from culture supernatants between wild-type and mutants with or without iron in the medium using LC-MS. Staphylobactin and the staphyloferrins do not affect virulence in the wax larvae infection model. Although I was unable to generate definitive evidence revealing the biological role for the staphylobactin locus this study has generated valuable tools for further studies and thoroughly tested a number of hypotheses concerning its role in cation metabolism.

In addition to the staphylobactin region, genomic sequence comparison revealed a novel *vwb* gene in the genomes of these isolates, which may explain how they have adapted to infect a broad range of host species. Strains from CC130 demonstrate the ability to clot a broad range of hosts´ plasma including ruminant plasma, despite lacking the ruminant and equine specific SaPI *vwb*. Identification of a stop codon in the *vwb*

gene of CC130 isolates from human and bovine hosts suggests convergent evolution. The presence of this stop codon affected coagulation of bovine plasma, but had little effect on ovine plasma. Knockout and complementation of the *vwb* gene in *S. aureus* CC130 strains showed that the loss of *vwb* affected the ability to clot plasma from a broad host range. *L. lactis* expressing the divergent *vwb* gene was used to confirm this in the absence of any other *S. aureus* proteins. *mecC* strains from ST425 were also tested for their ability to clot plasma. Strain LGA251 was unable to clot any plasma at all, due to an inactive *coa* gene. Other strains from ST425 were able to clot certain plasmas to varying degrees. However, strain LGA254 was efficient at clotting all plasma tested, including ruminant. This strain was found to contain both the chromosomal and SaPI *vwb* genes.

6.2 Why more than one siderophore?

The adaptive significance of *S. aureus* CC130 strains producing three siderophores, two of which are the α-hydroxycarboxylate-type staphyloferrins with redundant functions, is not clear. Many bacteria secrete multiple siderophores, such as uropathogenic strains of *E. coli* and Mycobacteria produce three siderophores (482), which may differ both in chemical properties and affinity for ferric iron. From my results using the CAS assay and growth in serum and transferrin, I know that both staphyloferrins are produced and functional in the strains containing the staphylobactin locus. So why would the *mecC* CC130 strains require more than one siderophore, in spite of the perceived costs of maintaining and expressing additional genes, gene products and metabolites?

6.2.1 Siderocalin

The production of more than one siderophore of different classes is an adaptive mechanism among pathogens and represents one mechanism by which the effects of siderocalin can be circumvented (483). To compensate for a siderocalin-susceptible siderophore (e.g. a catecholate), bacteria produce other siderocalin immune siderophores (e.g. a hydroxamate-based, citrate-based, or structurally modified siderophore). However, siderocalin evasion is not likely to have been a selective pressure influencing the acquisition of the staphylobactin locus by *S. aureus*, as neither

staphyloferrin incorporates catechol groups (267, 272) and staphylobactin is not a siderocalin-susceptible siderophore.

6.2.2 The staphylobactin locus may be important in different host species

In *B. anthracis,* petrobactin is the more biologically relevant siderophore in mammalian hosts, especially given its capacity to evade the activity of siderocalin (268). Bacillibactin facilitates uptake of iron from ferritin, which suggests that bacillibactin plays an important role *in vivo*, particularly in non-mammalian hosts which lack siderocalin (442). When *S. aureus* strains lacking the ability to synthesise staphyloferrin A, staphyloferrin B and/or staphylobactin where tested in the wax moth larvae infection assay. No difference in virulence was observed compared to the wild-type isolate. This suggests staphylobactin does not play the same role as bacillibactin and is not involved in iron acquisition from ferritin.

The CC130 lineage is able to adapt to a multitude of unrelated hosts. As *S. aureus* possesses a highly conserved core genome, host-specific adaptations are likely to be carried by mobile genetic elements (MGEs). Staphylobactin may allow *S. aureus* to switch host specificities, playing a role in host adaption such as the ability to colonise a particular animal host or a role in its ability to cause disease. Factors influencing the adaption to a host will be determined by the physiological condition of the host, e.g. the immune system, as well as the environmental influences to which the bacteria are exposed, such as antibiotic treatment (21). Adaption mechanisms have already been shown for *S. aureus* isolates of bovine, ovine, equine, poultry, and laboratory mouse origin (464, 467, 476, 484, 485).

6.2.3 The staphylobactin locus may be important during different stages of infection.

In *B. anthracis*, production of petrobactin precedes that of bacillibactin by several hours during outgrowth, suggesting that petrobactin is the sole siderophore mediating iron acquisition during this early intracellular infection stage. Bacillibactin may facilitate iron acquisition late in the infectious process, during the transition from vegetative growth to sporulation (486).

In *Mycobacterium smegmatis*, exochelin can be detected in cell culture medium after 16 hr, whereas carboxymycobactin and mycobactin cannot be detected before 40 hr and their levels only become appreciable after 64 hr. This shows a scavenging role for exochelins, but not carboxymycobactin (487). Whilst attempting to phenotype the staphylobactin locus, the expression of the NRPS genes *stbE* and *stbG* under iron restriction, was assessed using RT-qPCR during the mid-logarithmic phase. When carrying out quantification of siderophore output using the CAS assay, readings were taken at 24 and 48 hours. For detection of siderophores from culture supernatants using mass spectrometry, samples were taken for analysis post exponential phase (approximately 40 h). Although these experiments covered a range of time points, no significant amount of staphylobactin could be detected. Staphylobactin may be expressed very early or late during growth.

6.2.4 The staphylobactin locus may be important in different host niches

The production of multiple siderophores with differential expression within a given pathogen is not unusual, as with bacillibactin and petrobactin in *B. anthracis,* and may be a reflection of niche adaptation and virulence potential (195, 486, 488-491). Conditions that mimic those likely to be encountered within the host, specifically growth at 37 °C and in the presence of CO_2/bicarbonate, constrain bacillibactin production while petrobactin secretion is unencumbered (486, 491).

S. aureus siderophores steal iron from host proteins while the Isd system consumes host haem and releases iron. Both pathways are expressed within the iron-limited host environment. The functional redundancy built into staphylococcal iron acquisition systems guarantees that the pathogen obtains enough iron to successfully colonise a variety of diverse niches within the host. Sheldon and Heinrichs demonstrated that *S. aureus* strains that are unable to synthesise staphyloferrin A, produced smaller abscesses than both the wild-type and staphyloferrin B deficient strains (261). It was shown that the staphyloferrin A transporter in *S. aureus* contributes to bacterial fitness in abscesses and epithelial cells (492). This suggests that staphyloferrin A plays a prominent role in iron acquisition during skin colonisation and infection in both the coagulase-positive (CoPS) and CoNS. Whereas the staphyloferrin B biosynthetic and

transport genes are upregulated in the blood and serum, suggesting a more prominent role in severe and invasive staphylococcal infections (493, 494). Therefore, staphyloferrin A and B are likely to be expressed within specific host niches, and consequently impact the survival and pathogenicity of *S. aureus* within the host. Staphylobactin may be secreted under different environmental conditions encountered in the host.

6.3 Characterising the contribution of staphylobactin in other host niches

In this study the wax moth larvae infection model was used to characterise the contribution of staphylobactin and the staphyloferrins biosynthesis genes towards *S. aureus* virulence. Iron forms complexes with iron binding proteins such as haemoglobin, transferrin, lactoferrin and ferritin. High amounts of ferritin are found in the wax moth haemocoel (495), representing an easily accessible iron-concentrated source for extracellular pathogens. However, wild-type and mutant strains displayed no detectable difference in virulence, suggesting that staphylobactin does not contribute to iron acquisition from ferritin in this host. The ecology and pathology of *S. aureus*, however, is more expansive than could be described by the wax moth larvae infection model, and staphylobactin may play a prominent role in survival in other niches. Beyond colonisation of nares and skin, invasive *S. aureus* can target virtually every organ of the body. Further investigation is required to elucidate the respective roles of staphyloferrins and staphylobactin in staphylococcal pathogenicity within these microenvironments.

S. equi is a host restricted pathogen which invades and causes abscessation of lymph nodes in horses. Deletion of the equibactin locus renders *S. equi* significantly less able to cause acute disease in the natural host (496). *S. equi* is a host restricted pathogen so it is consequently easier to study the effect on virulence in its natural host. Strains containing staphylobactin infect a broad range of host species and cause a wide variety of infections. Identifying the right host species and infection model to use to study the effects of staphylobactin on virulence is made very difficult. In animals, *S. aureus* is the main causative agent of mastitis in cattle, sheep and goats (497, 498). A large proportion of strains in our strain collection containing the staphylobactin locus have been isolated

from bovine hosts. To study the effects of staphylobactin on virulence in the bovine udder, cows could be inoculated with these strains to induce intramammary infections and monitored for inflammation in the inoculated quarters. Mouse models are less costly than cows, however these strains naturally infect cattle and a mastitis model in cows could potentially be a good host to study the contribution of staphylobactin to virulence. Intracellular survival assays in bovine neutrophils or bovine mammary gland epithelial cells could be possible methods to study the involvement of staphylobactin in virulence.

It might be more relevant to use an infection model that has shown a phenotype for the staphyloferrin siderophores. In the skin abscess model, *S. aureus* strains that are unable to synthesise staphyloferrin A are significantly impaired for abscess formation. Disruption of *sstABCD* did not significantly impact staphylococcal burden in the livers and kidneys of mice in a systemic infection model, but it did compromise bacterial survival in the heart (238), suggesting that there are specific niches in which SstABCD plays a role in staphylococcal pathogenesis.

6.4 Siderophores: more than stealing iron

Most of this study focused on the role of staphylobactin as an iron-chelating siderophore. However, recent studies have suggested additional roles for siderophores, including the acquisition of non-iron metals (265, 499) and modulation of host functions (500, 501).

Staphylobactin's ability to act as a chelator of other metals, including copper and zinc, was preliminarily tested by its ability to grow in Chelex-treated minimal media, supplemented with various metal ions. Only slightly reduced growth was observed when supplemented with manganese, and no difference in growth was observed when supplemented with zinc, copper, nickel or cobalt. The ability of staphylobactin to act as a chelator of other metals may be masked by the more dominant metal ion acquisition systems. Staphylobactin may facilitate the absorption of other metals in either the presence or absence of iron. Further analysis would need to be carried out to determine if staphylobactin is involved in the acquisition of non-iron metals.

In addition to numerous siderophores, NRPS enzymes also give rise to antimicrobial peptides (187) and peptide-based antibiotics such as penicillin (188), daptomycin (189), and vancomycin (182). The antimicrobial activity of staphylobactin containing isolates was determined against a variety of bacterial isolates. All strains, including 71277Δ*stb* displayed a zone of inhibition around the paper disc, showing antimicrobial activity towards *S. aureus* strain Newman and RN4220. This activity could be attributed to bacteriocins identified during antiSMASH analysis in *S. aureus* isolates from our collection (360).

6.5 Regulation

Although not statistically significant, MntR is possibly a positive regulator of the staphylobactin operon in an iron-dependent manner. While carrying out antiSMASH analysis of the CC130 genomes, a region encoding an AraC family transcriptional regulator adjacent to the staphylobactin region was highlighted, representing a possible alternative promoter. During this project, from computational analysis, MntR is thought to be the regulator of the staphylobactin operon. Many siderophore systems are regulated at two different levels, therefore it would be of interest to carry out further work to see if *stb* is transcriptionally induced by AraC and if the region upstream of *stbB* contains an AraC-type DNA binding domain. No other candidate regulation genes appear to be encoded on the *S. aureus* chromosome in the vicinity of any of the siderophores.

6.6 Limitations

My work focused primarily on the knockout made in the CC130 strain 71277, however there is some potential to provide more information about these genes if knockouts were generated in *S. lugdunensis* or in more divergent backgrounds. Generating deletion mutants within these strains crippled me in terms of time, allowing less time for a more thorough investigation into function of the staphylobactin locus. Initial studies to assign a function to staphylobactin were hampered by my inability to successfully demonstrate any activity by the gene products in the assays carried out so far. This is likely due to

staphylobactin not being produced, or produced in very small amounts that are undetectable in the conditions tested.

6.7 Future work

Although staphylobactin does not currently appear to be involved in iron acquisition in the conditions tested, further experimental work is necessary to characterise the contribution of the staphylobactin genes to iron acquisition and to assess for its role in staphylococcal survival and virulence.

To ensure that these results were not specific to strain 71277, the experiments should be repeated using *S. aureus* strains from a broad host range and other staphylococcal species, such as *S. lugdunensis*, to see if the equivalent results are obtained.

Elucidating the potential role of staphylobactin may be particularly relevant in CoNS, particularly *S. lugdunensis*, as the vast majority only express staphyloferrin A as a siderophore (210, 357), and while this species is a common constituent of the skin microbiota, it may act as an opportunistic pathogen (502).

As sequence analysis of both the *S. lugdunensis* and *S. aureus stb* genes are predicted to be functionally analogous, it would be interesting to determine whether the *S. aureus stb* genes would complement the *S. lugdunensis* growth defect in serum and whether a siderophore would be made that could be transported. This has been previously carried out in *S. lugdunensis*, which does not produce staphyloferrin A or staphyloferrin B but expresses the transport systems for these siderophores. *S. lugdunensis* grows poorly in iron restricted growth media and the growth defect was complemented when the *S. aureus* staphyloferrin A gene cluster was introduced into *S. lugdunensis* (334).

Using the CAS assay for detection of siderophore and comparative metabolic profiling using LC-MS, Staphylobactin was not identified. This may be due to staphylobactin not being produced in the conditions tested or in small quantities that any difference was not detected. Complete genome sequencing has revealed that many bacteria have the potential to produce specialised metabolites, such as NRPSs and PKSs, however many of these biosynthetic gene clusters are not expressed in laboratory conditions. Approaches to induce a change in staphylobactin expression, such as variation in growth conditions

e.g. temperature, pH, incubation time or other additives, could be carried out. Culture supernatants were tested for the presence of staphylobactin when grown in TMS minimal media. In an attempt to optimise siderophore production, a number of other growth media could be assessed, such as TSB with the addition of iron chelators 2,2'-dipyridyl or EDDHA. Changes in the environment could induce changes in expression, though it is difficult to predict which variables will influence gene expression and activate biosynthesis. Phenotype MicroArrays using the Biolog system (http://www.biolog.com) could be used to provide thousands of growth conditions and allow a very rapid and easy way to look for optimal production conditions. However, I would need an assay for the detection of staphylobactin that could be used in conjunction with the Biolog system. Phenotypic differences between wild-type and Δ*stb* could be analysed using the Biolog system.

The expression of the entire staphylobactin locus in a heterologous host, would simplify the identification of metabolites when the staphylobactin locus is expressed in a host with a limited natural metabolic profile. Reconstitution of the equibactin NRPS was carried out in *E.coli* to determine if all of the genes necessary for production of equibactin were present in the *eqb* locus, and to produce equibactin in a less complex media. However, despite effects on the streptonigrin sensitivity of strain Δ*eqbAE* in cross-feeding assays, LC-MS analysis of culture supernatants failed to identify equibactin (296). I attempted to use the same expression plasmids and *E. coli* strain to carry out the expression of staphylobactin. This has proved difficult with the staphylobactin locus as some genes are too large (*stbE* is 6018bp and *stbG* 2769bp) to use PCR with high fidelity polymerase, as to not introduce SNPs that could affect the function of the gene. Cloning has also proved difficult due to the start codon overlapping at the end of the previous gene within the staphylobactin gene cluster.

Co-culture with other competing microorganisms may be an approach to induce staphylobactin expression as its expression is very low under standard laboratory conditions tested to date and may provide the chemical elicitor needed.

Electrophoretic mobility shift assays were used to analyse recombinant EqbA binding to a DNA fragment containing the upstream region of the *eqb* operon (296). EqbA binds to

the *eqbB* promoter in a Fe^{2+}, Zn^{2+} and Mn^{2+} responsive manner. Binding was not affected by Cu^{2+} or Fe^{3+}. This assay has also been used to demonstrate that SbnI binds DNA upstream of a promoter within the *sbnC* coding region of the staphyloferrin B biosynthesis cluster (411). This procedure could be used to determine if MntR or the predicted AraC transcriptional regulator adjacent to the *stb* locus is capable of binding to DNA regions upstream of *stbB* in the presence of various cations.

In *S. aureus*, reporter gene assays have been used to study promoters and analysis of gene expression in the following ways: to determine whether expression by the promoters of the *mntABC* and *mntH* genes were regulated by manganese or other metal ions and the characterisation of a zinc-responsive regulatory element (zur) (169, 290), to characterise the transcriptional regulation of the *sbn* operon, when grown in iron-replete and iron deplete growth media (202), to characterise *fhuD1* and *fhuD2*, which are regulated by Fur and expressed under conditions of iron limitation (225). I have already made progress using the pMUTIN4 (503) plasmid to create transcriptional *stb::lacZ* fusions which can be tested using β-galactosidase assays. To allow inducible expression of the *stb* operon, a construct could be made by integrating the pMUTIN vector upstream of *stbB,* with transcription of the downstream genes placed under control of an IPTG-inducible promoter. Regulation could be monitored by attaching reporter gene to the predicted upstream regulatory sequence of the *stb* operon and growing these strains in minimal media supplemented with zinc, manganese, or iron or all three metals (metal-replete), and assessing expression by measuring fluorescence.

Virulence was not notably influenced by staphylobactin in the wax moth larvae infection model. Wild-type and staphylobactin deficient strains of both CoPS and CoNS could be tested in a number of mouse models, including skin and nasal colonisation models, to ascertain if staphylobactin contributes to survival on the skin and in the nares. Other mouse models could be tested, including skin abscess and sepsis mouse model. These mouse infection models could be monitored with an *in vivo* imaging system (IVIS). The staphylococcal infections can be assessed non-invasively in real-time using strains labelled with either bioluminescence (e.g. lux) or fluorescence (e.g. red fluorescent protein) (504-506).

Using the mutants generated during this project, I hope to address the role of staphylobactin in iron acquisition in specific host reservoirs, niches, and stages of infection.

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