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CONSTRUCTION OF A RECOMBINATION-BASED REPORTER SYSTEM TO IDENTIFY GENE EXPRESSION IN STAPHYLOCOCCUS AUREUS

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CONSTRUCTION OF A RECOMBINATION-BASED REPORTER SYSTEM TO IDENTIFY GENE EXPRESSION IN *STAPHYLOCOCCUS AUREUS*

(Spine title: A RIVET-based System to Identify Gene Expression in *S.aureus*)

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by

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Abstract

Staphylococcus aureus harbours several iron acquisition systems, allowing for survival within the iron-restricted host environment. Five such systems have been identified in *S. aureus;* it is unknown why *S. aureus* maintains so many of these systems. Studies have shown that some iron transport proteins might be differentially expressed in different organs *in vivo*. The purpose of this study was to construct an *in vivo* expression technology (IVET) system to identify temporal expression patterns of *S. aureus* iron transport systems. An IVET utilizing the Cre*loxP* recombination system was constructed for use in *S. aureus*. Using genome-integrated and multi-copy *cre* constructs driven by the *S. aureus fhuCBG* promoter, resolution of an *in vitro* reporter cassette was determined. Although *fhuCBG* driven *cre* expression was confirmed using the multi-copy *cre* constructs, no detectable Cre was expressed by genome-integrated *cre* constructs.

Keywords

Staphylococcus aureus, RIVET, Cre*loxP* recombination, iron-regulated promoters, iron transport

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List of Abbreviations

α	Alpha
ACME	Arginine catabolic mobile element
Amp^R	Ampicillin resistance
β	Beta
bp	Base pair
CA-SA	Community-associated *Staphylococcus aureus*
Cm^R	Chloramphenicol resistance
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Ery^R	Erythromycin resistance
For	Forward
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVET	*in vivo* expression technology
Kan^R	Kanamycin resistance
Kb	Kilobase
$Linc^R$	Lincomycin resistance
mg	Milligram
mL	millilitre
mM	millimolar
MRSA	Methicillin Resistant *Staphylococcus aureus*
OD_{600}	Optical Density at 600nm
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
pM	picomolar
Rev	Reverse

RIVET	Recombinant *In vivo* expression technology
RPM	Rotations per minute
SDS	Sodium dodecylsulfate
$Spec^R$	Spectinomycin Resistance
Tet^R	Tetracycline Resistance
TSB	Tryptic soy broth
w/v	Weight to volume

Chapter 1: Introduction

1.1 The Staphylococci

The Staphylococci are a group of Gram-positive, facultative anaerobic bacteria, commonly part of the skin and mucosal membrane flora of humans and other mammals (65). Staphylococci are generally split into two groups, based on their ability to produce the clot forming enzyme coagulase. The coagulase-negative *Staphylococcus* (CoNS) group is comprised of staphylococcal species such as *S. epidermidis* and *S. saprophyticus*, while the coagulase-positive *Staphylococcus* (CoPS) group is comprised of staphylococcal species such as *S. aureus*.

1.1.1 *Staphylococcus aureus*

Although several species of staphylococci are capable of causing disease in humans, particularly *S. epidermidis* (84), *S. aureus* has received the most attention as strains have been implicated in a variety of nosocomial- and community-associated infections (61, 76, 108). *S. aureus* is capable of causing such a variety of diseases due to the extensive array of virulence factors it harbours (Table 1). Indeed, *S. aureus* has been implicated as the causative agent of simple skin and soft tissue infections such as impetigo (7), as well as severe invasive diseases such as necrotizing pneumonia (73). More recently, a rise in antibiotic resistant strains of *S. aureus* in both the hospital and community setting have resulted in an increased frequency of difficult to treat *S. aureus* infections. Strains of methicillin resistant *S. aureus* (MRSA), known for their complete resistance to the beta-lactam class of antibiotics, have been frequently associated with such infections, as several MRSA strains have obtained additional genetic elements providing resistance to a variety of other antibiotic

Table 1: A sampling of virulence determinants of *S. aureus*

Virulence Class	Function	Reference
Adhesins		
spa	Binds to antibody Fc region	112
cna	Collagen-binding protein	85
clfA, *clfB*	Fibrinogen-binding proteins	70, 77
Superantigens		
tst, seq, sek	Overstimulation of T-cells, extensive cytokine release	8, 31
Membrane-damaging toxins		
PSMα, PSMβ	Neutrophil lysis	118
hla	Pore-forming toxin	13
Host Defence Evasion		
crtM	Antioxidant; resistance to neutrophil killing	58
cap	Capsule modulation; Resistance to leukocyte opsonophagocytosis	113

classes (29). Due to pathogenic strains such as MRSA, new therapeutic strategies are needed in order to complement, or even replace the current antibiotic-dependent strategies used to treat *S. aureus* and other pathogens. One such strategy involves the interruption of bacterial nutrient acquisition systems critical for the survival of the pathogen. Although many bacterial species (including *S. aureus*) harbour a variety of different nutrient acquisition systems, this thesis will specifically focus on the importance of iron, and bacterial iron acquisition systems.

1.2 Iron: A necessary bacterial nutrient

With the exception of *Borrelia burgdorferi* and some lactobacilli species, iron is an essential nutrient required for bacterial growth (4, 11, 48, 90, 99). However, under physiological conditions, the availability of free iron for use by bacteria is severely restricted. Indeed, in an aerobic, neutral pH environment, concentrations of iron are restricted to a concentration of 10^{-18} M, as iron forms insoluble iron(III) hydroxides in such an environment (14, 24). In addition, iron availability is further restricted for mammalian bacterial pathogens due to the presence of host iron sequestering proteins, such as transferrin and lactoferrin, reducing free iron concentrations to as little as 10^{-24} M (94). In order to survive in such iron restricted environments, a variety of Gram-positive and Gram-negative bacteria have developed specialized iron acquisition systems, some of which will be described below, allowing them to utilize free iron and even 'steal' it from host iron sequestering proteins.

1.3 Bacterial iron acquisition systems

Although there are a variety of iron acquisition systems used by Gram-positive and Gram-negative bacteria, for the purposes of this thesis, only the siderophore-mediated systems will be described in depth here.

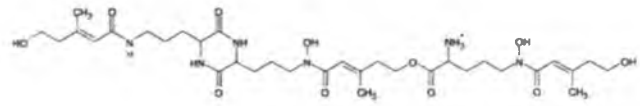
1.3.1 Siderophores

Siderophores are small, highly electronegative low molecular weight molecules with high affinity for free iron(III), which are then brought into the cell through a cognate receptor and transporter. Siderophores are generally classified into one of three groups, each of which are distinguished by the moiety used to coordinate the iron(III) atom (24). Siderophores of the hydroxamate group have hydroxamic acid moieties in which the carboyl and aminohydoxyl groups each occupy an iron coordination site. Catecholates utilize the adjacent hydroxyl groups of catechol moieties in order to coordinate iron. Finally, polycarboxylate motifs coordinate iron(III) atoms using either carboxylic acid moieties (17), or α-hydroxy-carboxylates (1). Interestingly, a variety of siderophores have been identified which utilize combinations of the above three groups (9, 16, 87). Some representative siderophores are depicted in Figure 1.

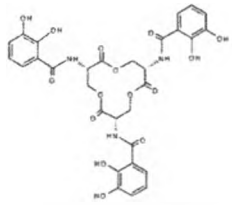
One of the more intriguing aspects of siderophores is that several siderophores can be used by more than one bacterial species, so long as they express the necessary transport apparatus. This has been shown to occur in a variety of bacteria, such as *S. aureus* (109), *E. coli* (119) and *Listeria monocytogenes* (22).

Figure 1: Sample siderophores representing (A) each of the three siderophore groups: Catecolates (enterobactin), hydroxymates (coprogen), polycarboxylates (rhizoferrin), as well a hybrid siderophore (aerobactin: a hydroxamate-carboxylate hybrid), and (B) Siderophores produced by *S. aureus*. Siderophore structures were adapted from (6).

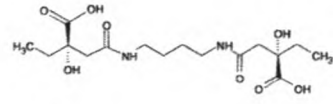
A



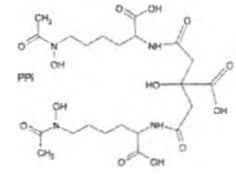
Coprogen B
(*Neurospora crassa*)



Enterobactin
(*Salmonella typhimirium*)

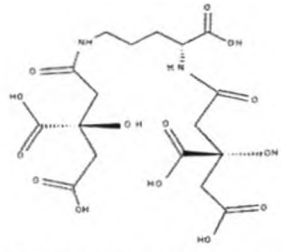


Rhizoferrin
(*Ralstonia pickettii*)

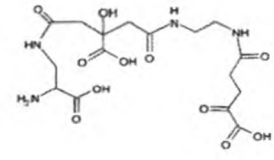


Aerobactin
(*Escherichia coli*)

B



Staphyloferrin A



Staphyloferrin B

1.3.2 Iron uptake ABC transporters

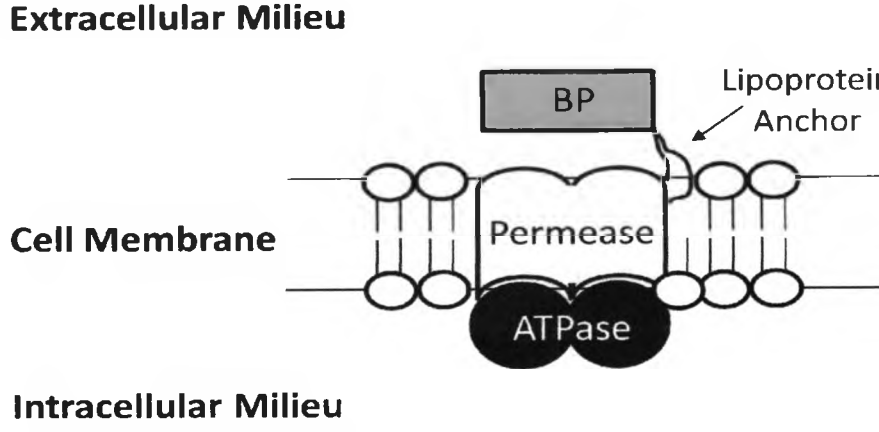
ATP-binding cassette (ABC) transporters are transmembrane proteins which use the energy created by the hydrolysis of adenosine triphosphate (ATP) in order to transport a variety of substrates across cell membranes; many have been shown to be essential for cell viability, as well as bacterial virulence (27). ABC transporters can be used to import, as well as export, substrates across the cell membrane; iron uptake ABC transporters act as importers and consist of a transmembrane permease, an ATP-binding cassette (ABC) protein and a periplasmic binding protein (PBP). The PBP is of particular importance, as it associates with periplasmic iron(III)-siderophore complexes, which are then taken to the appropriate ABC transporter. It is important to note that, due to a lack of an outer membrane in Gram-positive bacteria, there are slight differences between ABC transporters of Gram-positive and Gram-negative bacteria (Figure 2). Gram-positive iron uptake ABC transporters utilize an iron(III)-siderophore binding protein anchored to the cytoplasmic membrane. In general, these proteins have a similar overall protein fold as the Gram-negative PBPs; the binding protein acts similarly to the PBP receptor found in Gram-negative iron uptake ABC transporters (24).

1.4 *Staphylococcus aureus* iron acquisition systems

S. aureus harbours an array of iron scavenging systems, allowing the pathogen to survive in the harsh, iron restricted host environment (Table 2). These iron acquisition systems are regulated by the ferric uptake repressor (Fur).

Figure 2: Cartoon models of a prototypical Gram-positive (A) and Gram-negative (B) ABC intake transporter. While the Gram-positive binding protein (BP) is tethered to the membrane by a lipid anchor, the periplasmic binding protein (PBP) is free in solution. Both systems utilize a permease which transports the ligand across the cellular membrane. An ATPase provides energy for the transport process. Note that various accessory proteins have been left out of the Gram-negative ABC transporter structure to simplify the image. OMR: Outer membrane receptor.

A


Extracellular Milieu
BP
Lipoprotein
Anchor
Cell Membrane
Permease
ATPase
Intracellular Milieu

B

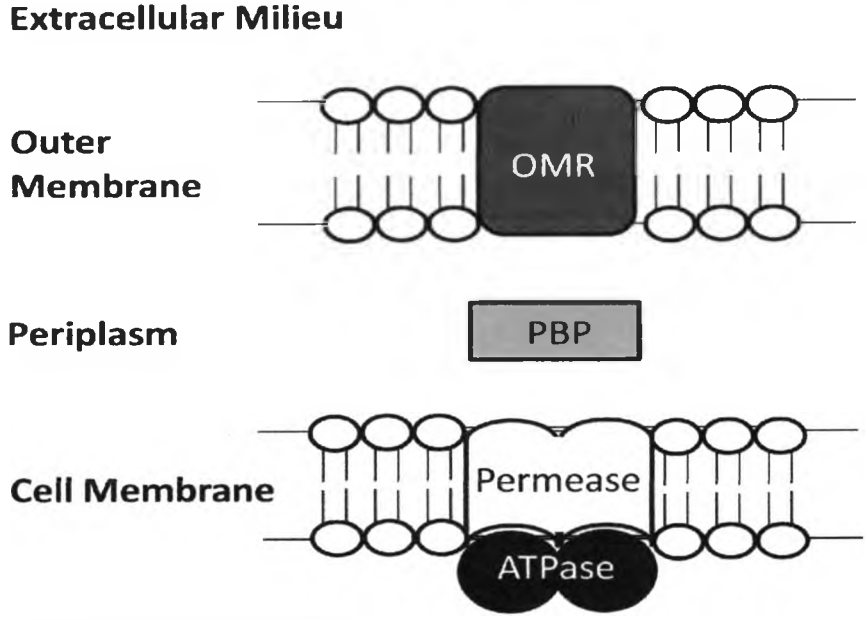

Extracellular Milieu
Outer
Membrane
OMR
Periplasm
PBP
Cell Membrane
Permease
ATPase
Intracellular Milieu

Table 2: *S. aureus* genes associated with iron acquisition

Gene(s)	Function	Reference
fhuCBG	Fe^{3+}-hydroxamate ABC transporter	101
fhuD1, fhuD2	Fe^{3+}-hydroxamate lipoprotein receptor	102, 103
htsABC	Staphyloferrin A ABC transporter; heme transfer (?)	5, 106
sirABC	Staphyloferrin B ABC transporter	25
sstABCD	Siderophore ABC transporter (?)	74
sfaABCD	Staphyloferrin A biosynthesis	21
sbnABCDEFGHI	Staphyloferrin B biosynthesis	26
isdA, *isdC*	Heme binding and transfer	38, 64, 69
isdB, isdH	Hemoglobin/haptoglobin binding	33
isdDEF	Heme transport membrane proteins	38, 89
isdG, isdI	Heme degradation	105

What follows is a brief analysis of these iron acquisition systems (see Figure 3), as well as how they are regulated by Fur.

1.4.1 Regulation of the iron acquisition systems in *S. aureus*

Indeed, *S. aureus* and many other microbes have evolved mechanisms capable of controlling iron uptake. However, it is equally important that these systems are only activated when necessary; too much iron can result in a toxic effect to the bacterial cells (37). All *S. aureus* iron acquisition systems are regulated by a negative repressor protein known as the ferric uptake regulator, or Fur. While Fur is found in an abundant number of bacterial species (92), only *S. aureus* Fur mediated regulation will be discussed here, and is illustrated in Figure 3.

The *S. aureus* Fur protein, combined with Fe^{2+}, the Fur co-repressor, acts to repress the *S. aureus* iron uptake systems when environmental iron concentrations are sufficient for the bacteria to thrive. Repression occurs through the interference of transcription of the iron-regulated genes. The Fur-Fe^{2+} complex binds to a specific sequence of DNA, the Fur box, a 19 bp palindromic sequence (GATAATGATAATCATTATC (28)) located within the promoter region. However, if iron concentrations become limited, then there is a reduced level of Fe^{2+} that can complexed with the Fur protein, resulting in the Fur protein being unable to bind to the Fur box, resulting in de-repression of the iron regulated genes.

Figure 3: Transcriptional control of iron-regulated genes by the Fur protein in *S. aureus*. In the presence of high concentrations of iron, the Fur protein (represented here as solid squares) is associated with ferrous iron, its co-repressor. This Fur-Fe^{2+} complex has a significantly higher DNA binding capacity than that of the Fur protein alone, thereby allowing the Fur-Fe^{2+} complex to bind to the Fur box sequence, resulting in repression of the iron-regulated gene (Top). However, if iron is in limited quantities in the environment, there is too little of the co-repressor to bind to the Fur protein, reducing its ability to bind to the Fur box, resulting in derepression of the iron-regulated gene (bottom).

Iron Rich Environment
Iron-regulated gene is repressed by Fur

Iron restricted Environment
Derepression of Iron-regulated gene

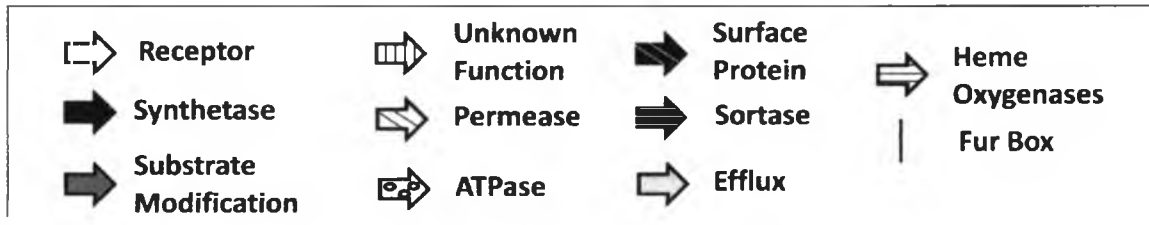
1.4.2 *Staphylococcus aureus* siderophore iron uptake systems

To date, three siderophores have been identified in *S. aureus* supernatants: staphyloferrin A (53), staphyloferrin B (32), and aureochelin (23). The genetic loci and biosynthetic pathways of the polycarboxylates staphyloferrin A and B have been recently identified (Figure 4) (19, 21).

The Staphyloferrin A *sfa* locus is comprised of the *sfaABC* operon, and a divergently transcribed *sfaD*. All four genes are known to be transcriptionally controlled by Fur in *S. aureus* (5), as a consensus Fur box is positioned intergenically between *sfaA* and *sfaD*. Interestingly, deletion of *sfa* does not result in a significant defect in bacterial growth in mammalian serum (5). However, when combined with a staphyloferrin B deletion mutant, bacterial growth in mammalian serum was severely attenuated. Bacterial growth was restored when the *sfa* genes from *S. aureus* were cloned into a complementation vector and introduced into the *sbn-sfa* double mutant (5). The observation that staphyloferrin A-deficient strains of *S. aureus* do not suffer from a reduction in bacterial growth unless combined with a staphyloferrin B deficiency indicates that staphyloferrin A may not play a significant role in iron extraction from transferrin, the primary iron substrate in serum. However, a previous study reported that staphyloferrin A was capable of leaching iron from human transferrin (72); Beasley and Heinrichs have postulated that this may have been caused by the use of a staphyloferrin A concentration which far exceeds that which is typically seen in *S. aureus* supernatants, resulting in transferrin iron extraction by staphyloferrin A (6).

Figure 4: Genetic loci of *S. aureus* involved in iron acquisition. The *sbn-sir* and *sfa-hts* loci are responsible for the biosynthesis and transport of staphyloferrin B, and staphyloferrin A, respectively. The *fhuCBG-D1-D2* locus is responsible for the transport of hydroxamate siderophores, and the *isd* locus encodes components of the heme-scavenging iron-regulated surface determinant system. Finally, the iron-regulated *sstABCD* operon encodes for an ABC transporter, however its substrate has yet to be identified.

Function	Genetic Loci
Staphyloferrin A Transport	
Staphyloferrin B Transport	
Ferric Hydroxamate Uptake	
Unknown	
Heme Scavenging	



The staphyloferrin B siderophore biosynthesis gene cluster, known as *sbn*, is comprised of a nine gene iron-regulated operon (26). Inactivation of the *sbn* operon in *S. aureus* results in attenuated growth in mammalian serum. However, *S. aureus* growth levels are nearly restored to wild-type levels with prolonged incubation; this may be due to the production of staphyloferrin A (5).

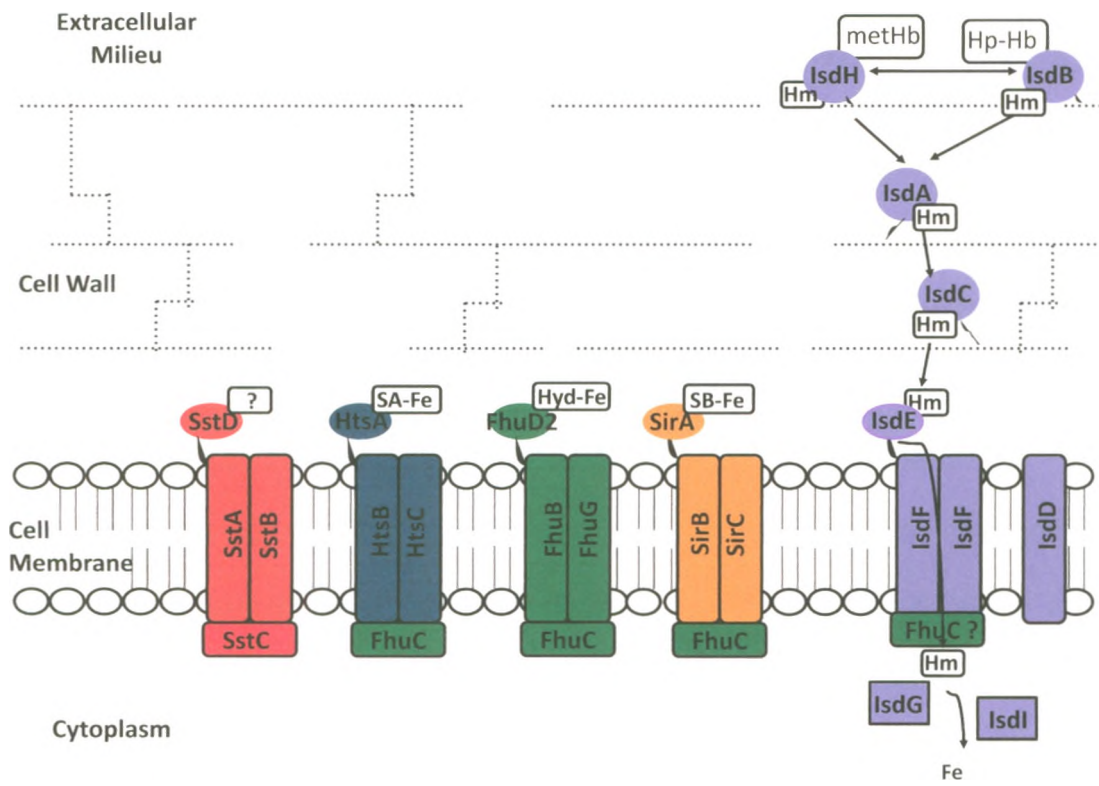
1.4.3 *Staphylococcus aureus* iron(III)-siderophore transport

S. aureus uses an assortment of iron-regulated ABC transporters in order to bring free iron through its cellular membrane and into the cell. Five iron-regulated ABC transporters, depicted in Figure 5, have been identified in *S. aureus*: the ferric hydroxamate uptake (Fhu) system (101), the staphylococcal iron regulated (Sir) system (25), the heme iron transporter (Hts) (106), the staphylococcal siderophore transporter (Sst) (74), and the iron-regulated surface determinant (Isd) heme transporter (69).

1.4.3.1 Ferric hydroxamate uptake system

The ferric hydroxamate uptake (Fhu) system is utilized in the uptake of iron(III)-hydroxamate siderophores. Interestingly, *S. aureus* does not produce hydroxamate siderophores; the Fhu system allows *S. aureus* to utilize exogenous hydroxamate siderophores (101). The iron and Fur-regulated genetic elements of the Fhu system, shown in Figure 4, encode permease components (FhuB and FhuG), an ATPase (FhuC), and dual binding proteins (FhuD1, FhuD2) (103).

Figure 5: A simplified diagram of five iron transport system in *S. aureus*. Arrows in the Isd system represent the paths that heme can take through the system before it is transported across the membrane and degraded. metHB: methemoglobin; Hp-hp: Haptoglobin-hemoglobin; SA-Fe: staphyloferrin A-iron complex; SB-Fe: staphyloferrin B-iron complex; Hyd-Fe: Hydroxamate-iron complex.


Extracellular
Milieu
metHb
Hp-Hb
IsdH
IsdB
Hm
Hm
Hm
IsdA
Hm
Cell Wall
IsdC
Hm
Hm
IsdE
?
SstD
SA-Fe
HtsA
Hyd-Fe
FhuD2
SB-Fe
SirA
SstA
SstB
HtsB
HtsC
FhuB
FhuG
SirB
SirC
IsdF
IsdF
IsdD
Cell
Membrane
SstC
FhuC
FhuC
FhuC
FhuC ?
Hm
IsdG
IsdI
Cytoplasm
Fe

1.4.3.2 Staphylococcal-iron regulated transport system

The staphylococcal iron regulated (Sir) transport system acts as the ABC transporter for the uptake of staphyloferrin B complexed with iron. The three gene *sirABC* operon, which encodes a binding protein (SirA) (42) and permeases (SirB, SirC), is divergently transcribed from the *sbn* operon (Figure 4). Interestingly, the iron and Fur-regulated *sirABC* operon does not encode an ATPase. Studies from our lab have provided evidence that FhuC from the Fhu transport system is utilized as the ATPase required for staphyloferrin B uptake (109).

1.4.3.3 Heme iron transport system

Early studies by Skaar *et al.* originally implicated the heme iron transport system (Hts) in heme transport (106). This study identified the *htsABC* operon, and through the inactivation of *htsB* and *htsC* (each encoding permease components), indirectly showed that the iron preference of *S. aureus* changed from heme to transferrin (106). However, direct involvement of Hts in heme transport has yet to be ascertained. Interestingly, a recent study demonstrated that the Hts transporter is involved in *S. aureus* staphyloferrin A uptake (5). HtsA has been identified as part of the class III family of binding proteins, and has been shown to bind to the iron-staphyloferrin A complex using a highly positively charged binding pocket (40). This pocket contains a patch of six arginine residues which make contact with the siderophore, holding it in place within the pocket. Furthermore, a deletion of the *htsABC* operon resulted in no uptake of staphyloferrin A (5). It should be noted that similar to the *sirABC* operon, no

ATPase-encoding gene is found in the *htsABC* operon. Evidence suggests that the FhuC ATPase is once again used in staphyloferrin A uptake (5).

1.4.3.4 Iron-regulated surface determinant heme transporter

The iron-regulated surface determinant (Isd) heme transporter is one part of the heme-scavenging Isd system (Figure 5). The *S. aureus* genome contains nine iron-regulated *isd* genes (Table 2; Figure 4) which encode nine proteins involved in heme uptake in the isd system. Two additional proteins, sortase A (SrtA) and sortase B (SrtB) are also critical components of the isd system. The IsdA, IsdB, IsdC and IsdH proteins are heme surface receptor proteins anchored to the cell wall (33, 69). With the exception of IsdC, these surface receptors are anchored to the peptidoglycan cross-bridges of the cell wall by SrtA; IsdC is anchored by SrtB (69). While IsdA and IsdC are buried in the cell wall, IsdB and IsdH are exposed to the extracellular milieu, allowing them to bind to heme from methemoglobin or haptoglobin-hemoglobin (32, 69). Heme can then either be transferred bidirectionally between IsdB and IsdH (75), or from either IsdB or IsdH to IsdA, then directly to IsdC. From IsdC, heme is transferred to the Isd ABC transporter. Once heme is bound to IsdC, it is transferred to IsdE, prior to passage through the Isd ABC transporter (IsdF). The heme is then taken through the permease (IsdF), and into the cell. As with the Sir and Hts transporters, there is no obvious ATPase encoded by the *isd* locus. Grigg *et al.* has suggested that the Fhu system ATPase, FhuC, may be used by the Isd ABC transporter (39). Once inside the cell, heme is then degraded by IsdG and IsdI (108), releasing iron which can then be used as a nutrient by the cell.

1.4.3.5 Staphylococcal siderophore transporter

To date, there is limited understanding of the staphylococcal siderophore transporter (Sst). The *sstABCD* operon encodes two putative cytoplasmic membrane proteins (SstA and SstB) believed to form a permease, an ATPase (SstC), and a receptor-binding lipoprotein (SstD) (74). The substrate transported by Sst has not yet been elucidated. However, it is believed that the Sst transporter is involved in iron acquisition in some manner, as the *sstABCD* operon is iron and Fur regulated (74).

1.5 Expression of iron acquisition systems in *Staphylococcus aureus*

While extensive studies of *S. aureus* iron acquisition systems have resulted in the elucidation of a considerable number of iron-regulated genetic elements, little is known about when these genes are expressed *in vivo*. For example, it is unknown why *S. aureus* carries so many different iron acquisition systems in its genome; a recent study by Pishchany *et al.* showed that IsdA and IsdB are differentially expressed in the heart and liver in mice (88). Thus, it could be hypothesized that *S. aureus* may be expressing these different iron acquisition systems in specific organs in order to survive, or even that different systems are expressed at different time points throughout the course of an infection. Typical *in vitro* procedures are unable to ascertain such data; thus, in order to determine what iron acquisition systems are being expressed (or when they are being expressed) *in vivo*, a system capable of accurately identifying genes expressed *in vivo* must be used.

1.6 Identifying genes expressed *in vivo*

An inherent issue with *in vitro* studies is that, despite the variety of growth media used to culture bacteria and mimic *in vivo* growth conditions, they are unable to completely capture the complex nature or cellular interactions of the host environment. Thus, it is possible that select genes that are shown to be active in the *in vitro* environment may not necessarily be active *in vivo*, or vice versa (107). This is of particular importance when studying the role(s) of virulence determinants required for a pathogen to survive during the course of an infection, the ever changing environment of the host to combat the pathogen may result in the different virulence determinants being turned on or off over the course of the infection, something that may not be readily identified using *in vitro* studies (107). In 1987, Osbourne *et al.* developed a system that would later become known as *in vivo* expression technology (IVET), which was designed so as to overcome such problems by selecting for bacterial genes that are highly expressed in the host, but not in laboratory media (82). Since its inception, IVET and similar techniques have been used to identify an assortment of *in vivo* induced (*ivi*) genes, in a variety of microorganisms. Here, several IVET strategies will be described.

1.6.1 *In vivo* expression technology

In vivo expression technology (IVET) is a promoter trap assay capable of monitoring promoter activity through the use of a selectable marker. Although an IVET system was originally designed by Osbourne *et al.* to identify *Xanthomonas campestris* virulence genes induced *in vivo* during an infection with its host the

turnip (82), the term *in vivo* expression technology was not coined until 1993 by Mahan *et al.*, where the system was adapted to a murine model to identify *Salmonella. typhimurium ivi* genes (66). The IVET promoter trap works by identifying active promoters in a specific environment, such as an animal host. Active promoters are identified through the construction of a genomic library in which the bacteria's genomic DNA is randomly cut and cloned upstream of a reporter gene. This gene will endow the cells with a specific phenotype (eg. antibiotic resistance), allowing only those with active promoters to survive in a particular environment. These transcriptional fusions can be placed on a plasmid (82) or integrated into the bacterial chromosome (66). One of the most significant advantages of IVET is its versatility. To date, four different variations of IVET have been established, based on antibiotic resistance, auxotrophy, system specificity, and recombination events involving a group of enzymes known as recombinases, capable of excising a specific genetic marker (95). These modified IVETs have been adapted and applied to a variety of Gram-positive (51, 52, 59, 116), Gram-negative (20, 38, 50, 114,117), Mycobacteria (34) and eukaryotic microorganisms (96,110).

1.6.1.1 Antibiotic resistance selection

For antibiotic resistance based IVET, the reporter gene used is an antibiotic resistance cassette. The most significant aspect of this IVET variant is that it allows for the application of the IVET system in a wide variety of microorganisms, both *in vitro* and *in vivo,* as all that is needed to detect active promoters is to grow the cells in the appropriate antibiotic (82). One significant

downfall of this particular selection method is that the antibiotic itself may impair or modify the microorganism-host interaction when administered to the host organism. For example, the host of *X. campestris*, the turnip, was shown to have inhibited growth and chlorosis after extensive exposure to the antibiotic chloramphenicol (82). This issue can be somewhat alleviated through the administration of differing concentrations of antibiotic, as well as changing the time at which the antibiotic is given (82, 95). This allows for the identification of genes which are expressed at different antibiotic concentrations, as well as at different time points.

1.6.1.2 Auxotrophic selection

The auxotrophy-based IVET strategy employs the use of a mutant strain of the microorganism of study that is incapable of growth in the wild through the mutation of a gene essential to normal growth, typically a biosynthetic gene (95). This mutation can be complemented using the promoter trap; any DNA fragment fused to the essential gene that becomes active will result in the expression of the essential gene, allowing the microorganism to grow normally. The benefit of this strategy is that virtually any biosynthetic gene required for wild-type growth can be mutated so long as there are no metabolites in the environment that the microorganism can use to complement the mutation (95). For example, auxotrophic IVET studies have successfully mutated genes required for purine biosynthesis (Δ*purA*) in *S. enterica* serovar Typhimurium (66), methionine biosynthesis (Δ*metXW*) in *Pseudomonas syringae* (68) and pantothenate biosynthesis (Δ*panB*) in *Pseudomonas fluorescens* (93). Although versatile, one

significant drawback to the auxotrophy-based IVET variant is that, for some microorganisms, the necessary tools have yet to be developed to allow for the construction of an auxotrophic mutant (95). For such microorganisms, another form of IVET could be used to obtain the same results, such as the previously described antibiotic resistance-based IVET.

1.6.1.3 System-specific selection

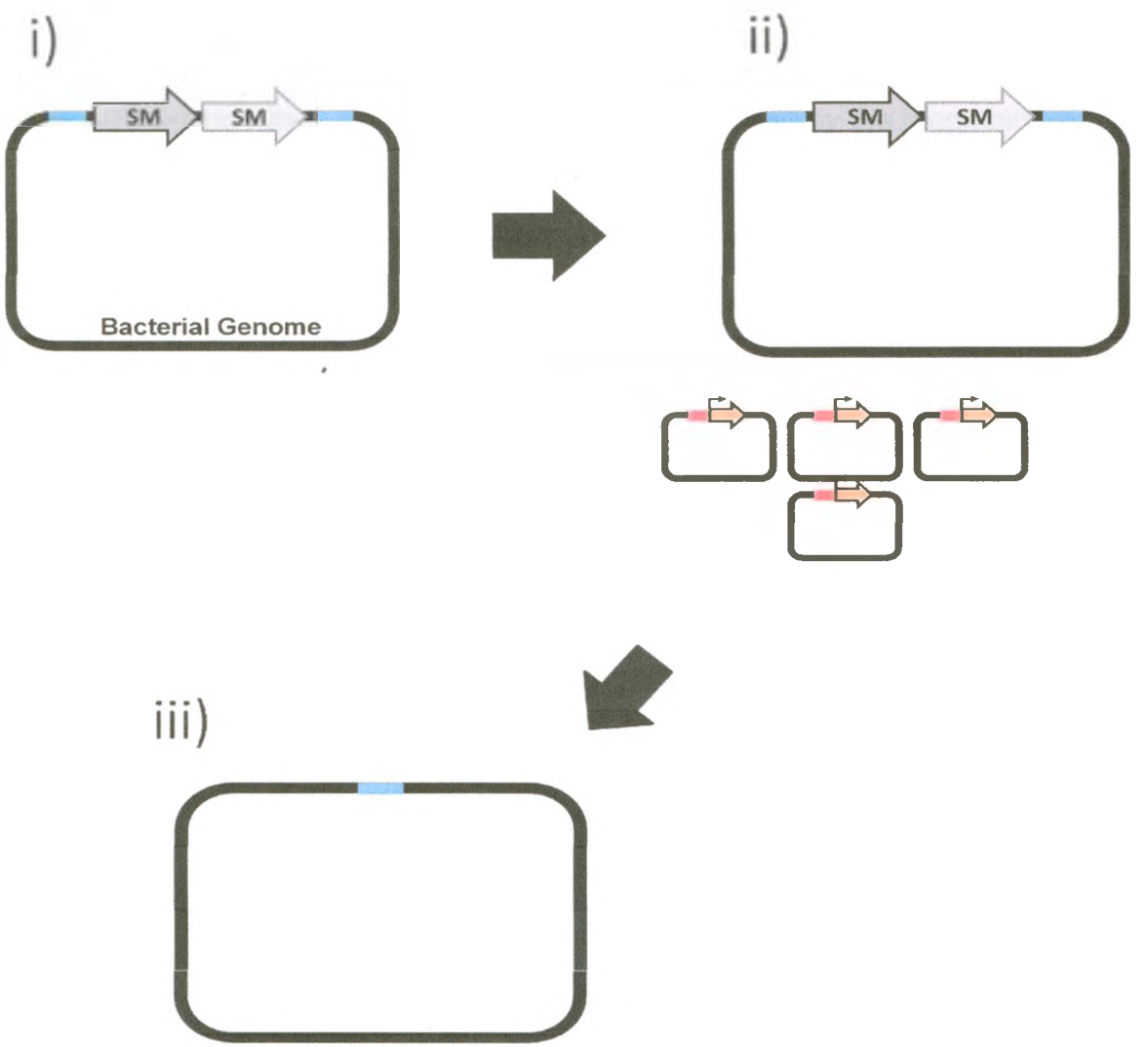
System-specific selection IVET is used to identify promoters which are differentially expressed during a select stage of an interaction between a microorganism and its eukaryotic host (95). It is similar to the auxotrophic IVET system described previously, as it requires the construction of a mutant strain, which can only be complemented with an active promoter region fused to a reporter gene. However, instead of using a reporter gene that is essential for wild-type growth, the reporter gene is one that is required for a specific stage during a microorganism-host interaction. Thus, the microorganism is screened for its ability to establish an interaction with its host (95). In addition to the prototypical promoter trap element, the system-specific IVET variant also measures the effect of the microorganism-host interaction on the host itself *in vitro*, using a scorable host phenotype, such as cell lysis or symbiosis (95). This system was used in symbiosis studies on the plant pathogen *Sinorhizobium meliloti* where *bacA*, required for differentiation of the pathogen into bacteroids (47), was used as a reporter gene for identifying host-induced promoter activity, while *gusA* was used as a reporter gene for *in vitro* promoter activity (81). This allowed the IVET system to screen for and identify genes that were only

expressed before bacteroid differentiation, which only occurs during the beginning stages of symbiosis (81).

1.6.1.4 Recombination *in vivo* expression technology

While the auxotrophic- and antibiotic resistance-based IVET variants have distinct advantages, one of their largest disadvantages is that they are unable to isolate transiently or weakly expressed genes over the course of an infection. This issue is rectified by the recombination *in vivo* expression technology (RIVET) variant (Figure 6). RIVET was designed by Camilli *et al.* in order to identify *Vibrio cholerae ivi* genes in mice during the course of infection (15). With RIVET, a reporter construct is integrated into a non-critical region of the bacterial genome. The construct consists of a positive selection marker, such as an antibiotic resistance cassette, which is flanked by specific recombinase recognition DNA sequences. More recently, counter selective markers, such as *sacB*, have been used in addition to the positive selection marker to create a more robust reporter construct (71, 83). These sequences are specifically bound to by a recombinase, such as Cre (3) or TnpR (59). The recombinase then mediates a recombination event between the two recombinase recognition sites, resulting in the excision of the reporter gene. Thus, RIVET works by constructing a strain of the bacteria containing the reporter construct, and fusions of the promoterless recombinase gene with random genomic DNA as described previously. Fusions with active promoter regions result in expression of the recombinase, leading to an excision event. Thus, cells with active promoters will be deficient of the phenotype that the reporter gene provided. RIVET generally

Figure 6: A general representation of the RIVET system. i) A reporter cassette consisting of one or more reporter markers is flanked by two recombinase recognition regions (blue). ii). A random fragment of genomic DNA (red) is cloned upstream of a recombinase gene on a plasmid. If an active promoter is present in the random DNA, it will drive expression of the recombinase gene (A plasmid carrying a promoterless recombinase gene is used as a negative control). The subsequent recombinase protein product will then bind to the recombinase recognition regions, resulting in iii) excision of the reporter cassette. SM = Selectable marker.


i)
SM
SM
Bacterial Genome
ii)
SM
SM
iii)

consists of two stages. The first involves the construction of the genomic DNA library. Once completed, the library is screened to remove promoter regions activated *in vitro*. For example, if the reporter gene is an antibiotic resistance cassette, then the bacteria will be cultured in media containing the antibiotic to which the cassette endows resistance to. Those harbouring constitutive promoters will be killed, as the cassette has been excised by the recombinase, resulting in antibiotic sensitivity. The second stage involves infecting the animal host to identify active promoter regions. These can be identified using replica plating, once the cells have been recovered from the host looking for colonies which lack the reporter gene phenotype.

While RIVET is a powerful tool, other non-IVET based techniques can be used to identify bacterial *in vivo* expressed genes. One of the most common of these techniques is the microarray. Microarrays are capable of examining the entire genome of a bacterium within a matter of hours, and output a huge quantity of data which can be analyzed by a variety of computer software programs. The benefit of using RIVET over microarray is that a RIVET system can be established in virtually any molecular genetics laboratory using basic molecular biology techniques, whereas microarrays are generally constructed in special laboratories with the established equipment necessary for building and analyzing microarrays, as these components can be quite expensive. Problems can also arise due to the large quantities of RNA that are needed for a microarray; this is particularly a problem with tissue samples, as they tend to have low quantities of RNA that are insufficient for microarray analysis (100).

Additionally, while microarrays do in fact identify when genes are expressed *in vivo*, they can only do so at a specific time frame, specifically, that at which the samples were extracted. RIVET, on the other hand, is capable of identifying temporal gene expression throughout a time course, such as that of an infection.

1.7 Cre/*loxP*

A variety of recombinase systems exist that can be used to design a RIVET system. One such system is the Cre/*loxP* recombination system. Cre/*loxP* was discovered in the P1 bacteriophage, as part of its site-specific recombination system (111). The system is composed of Cre, a type I topoisomerase tyrosine recombinase (80), and two *loxP* (locus of crossing over) sites. A *loxP* site is a 34-bp DNA sequence consisting of two 13-bp inverted repeats that flank an 8-bp asymmetric spacer region (43). A reporter construct, generally consisting of at least one positive selection marker (eg. antibiotic resistance cassette) is cloned in-between the two *loxP* sites.

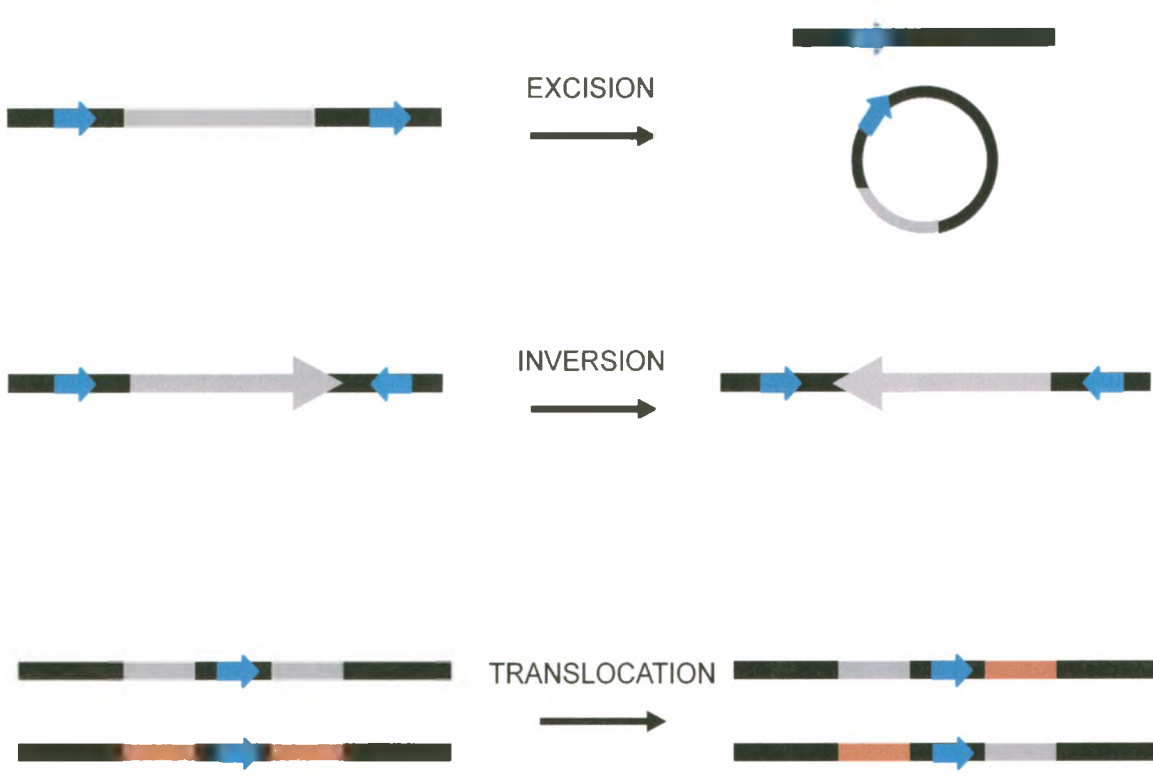
cre encodes a 343-residue protein that has high binding specificity to *loxP* sites. Each *loxP* site has two cre binding domains, each consisting of one of the 13-bp inverted repeats plus the first 4-bps of the spacer region adjacent to the inverted repeat (44). Once the Cre has bound to these domains on two different *loxP* sites (only two molecules of Cre are required per *loxP* site (63)), a recombination event occurs in which one of three outcomes can be observed for the DNA flanked by the *loxP* sites: excision, inversion or translocation (Figure 7). Translocation occurs when recombination is between two *loxP* sites located on

Translocation occurs when recombination is between two *loxP* sites located on separate DNA strands. Excision and inversion occur when the *loxP* sites are on the same strand of DNA; the event that occurs is dictated by the directionality of the *loxP* spacer region (45). If the spacer region of the *loxP* sites are direct repeats in relation to each other, then an excision event will occur. However, if the spacer regions of the *loxP* sites are inverted in relation to each other, then an inversion event will occur.

1.8 The use of an IVET system in *S. aureus*

In 1998, Lowe *et al.* successfully implemented an antibiotic resistance-based RIVET system in *S. aureus* (59). The system successfully identified 65 *ivi* staphylococcal genes in a murine renal abscess model, 45 of which were strongly induced. Only six of these genes were known staphylococcal virulence determinants, including *agrA*, a part of the global regulatory system (79), and *geh*, which encodes the glycerol ester hydrolase gene (49). The remaining 39 genes were either previously unknown *ivi* genes, or had homology to known non-staphylococcal genes. This subsequently led to the identification and sequencing of *murC*, which encodes a UDP-N-acetylmuramoyl-L-alanine synthetase, an essential component for cell wall biosynthesis (60).

Figure 7: The three possible outcomes of Cre/*loxP* recombination. If the orientation of two *loxP* spacer regions (blue arrows) are in the same direction, then all DNA enclosed by the *loxP* sites is excised (Top). If the orientation of two *loxP* spacer regions are inverted in respect to each other, then all DNA enclosed by the *loxP* sites is inverted (Centre). If two *loxP* sites are on separate strands of DNA, then a translocation will occur (Bottom).


EXCISION
INVERSION
TRANSLOCATION

1.9 Thesis and Research Objectives

Although the *S. aureus* based RIVET system designed by Lowe *et al.* proved to be successful in identifying *ivi* genes during a *S. aureus* infection, several modifications can be made in order to improve the system. One such modification would be the addition of a counter selection marker. This, combined with an antibiotic reporter gene could result in a reduction in the number of false positive colonies obtained using the RIVET system. Therefore, an improved *S. aureus* IVET system utilizing the RIVET strategy would be considerably beneficial in the identification of *ivi* genes, as well as determining the temporal expression of these genes. Such data could provide considerable insight into how *S. aureus* functions *in vivo* during an infection.

The goal of this project was to design and construct a RIVET-based system in *S. aureus*, and use it to determine the temporal expression of iron-regulated promoters during growth in an iron-starved environment, both *in vitro* and *in vivo*. To that end, my research objectives consisted of developing a RIVET based system containing a *loxP* reporter cassette with both a positive selection marker and a counter selection marker. This *loxP* cassette would then be integrated into the chromosome of several *S. aureus* strains through the use of a single homologous recombination event. Single- and multi-copy *cre* constructs driven by different promoter constructs were to be built and transformed or integrated into strains of *S. aureus* containing the *loxP* cassette. Expression from promoters would be monitored through *cre* expression resulting in the loss of the *loxP* reporter cassette. My hypothesis states that excision of the *loxP* reporter

cassette would only occur in those cells cultured in conditions where promoter(s) were active. This would represent a valuable laboratory tool for use in gene expression studies, especially *in vivo* during *S. aureus* infection.

Chapter 2: Methods and Materials

2.1 Bacterial strains, plasmids, and growth media

2.1.1 Bacterial cell cultures

All bacterial strains and plasmids used in this study are outlined in Tables 3 and 4. All bacteria were cultured at 37°C. If required, antibiotics were used at the following concentrations: ampicillin (100 µg/mL), chloramphenicol (30 µg/mL) erythromycin (300 µg/mL), kanamycin (50 µg/mL), spectinomycin (50 µg/mL) and tetracycline (10 µg/mL) for *E. coli* cultures; chloramphenicol (5 µg/mL) erythromycin (3 µg/mL), kanamycin (50 µg/mL), lincomycin (50 µg/mL), neomycin (50 µg/mL) and tetracycline (4 µg/mL) for *S. aureus* cultures. All *E. coli* cultures were grown in Luria-Bertani (LB) broth (Difco), while tryptic soy broth (TSB) (EMD Biosciences) was used to culture *S. aureus*. Solid media was obtained by the addition of agar (EMD Biosciences) to 1.5% w/v. In select experiments, *E. coli* was grown on LB agar with 40µg/mL 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) and 0.1mM isopropyl beta-D-thiogalactopyranoside (IPTG).

Iron-restricted media used to culture *S. aureus* was made by adding the divalent cation chelator 2,2'-dipyridyl (Sigma-Aldrich) to a concentration of 1mM. All media and solutions were made with double distilled water.

2.2 DNA preparation

2.2.1 Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* using e.Z.N.A™ plasmid mini prep kits (Omega Bio-Tek), as directed. *S. aureus* plasmid DNA was isolated using Qiaprep mini-prep spin kits (Qiagen). In addition to the standard Qiaprep protocol,

Table 3: Bacterial strains used in this study

Bacterial strains	Description	Reference
Bacteria		
E. coli		
DH5α	F- ϕ80 dL*acZ*ΔM15 *recA1 endA1 gyrA96 thi*-1 *hsdR17*(r_k^- m_k^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169	Promega
XL1-Blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 $proAB^+$ $lacI^q$ Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	Stratagene
S. aureus		
RN4220	r_k^- m_k^+; accepts foreign DNA	54
RN6390	Prophage cured wild type strain	86
Newman	Wild-type clinical isolate	35
SH1000	*rsbU*$^+$ derivative of *S. aureus* strain 8325-4	46
H2067	RN4220::*loxP*; Tet^R	This Study
H2073	RN6390::*loxP*; Tet^R	This Study
H2116	Newman::*loxP*; Tet^R	This Study
H2187	RN4220::*loxP* with integrated promoterless *cre* vector; Ery^R	This Study
H2188	RN4220::*loxP* with integrated P_{FhuCBG}::*cre* transcriptional fusion; Ery^R	This Study
H2219	RN6390::*loxP* with integrated promoterless *cre* construct; Ery^R	This Study
H2218	RN6390::*loxP* with integrated P_{FhuCBG}::*cre* construct; Ery^R	This Study

Table 4: Plasmids used in this study

Plasmids	Description	Reference
pSHE11	*E. coli* Cre expression vector; Amp^R	104
pDG1513	pMTL22 derivative carrying a tetracycline resistance cassette; Amp^R	41
pDG646	pSB119 derivative carrying an erythromycin resistance cassette; Amp^R	41
pLI50	*E. coli*/*S. aureus* shuttle vector; Ap^R Cm^R	56
pAW11	*E. coli*/*S. aureus* shuttle vector; Ery^R	115
pTL2787	Carries ϕ11 *int*	65
pLL29	*S. aureus* single-copy integration vector; Tet^R	65
pLL29-Ery	pLL29 with Tet^R cassette disrupted by an erythromycin cassette	This Study
pSAKO	*S. aureus* gene deletion plasmid containing *sacB*[Bsub]W29; Km^R	30
pBC SK (+)	*E. coli* cloning vector; Cm^R	Stratagene
pCM6	P_{lgt}::*sacB* transcriptional fusion cloned into pLI50; Amp^R Cm^R	This Study
pCM13	Completed *loxP* construct in pBC SK(+); Cm	This Study
pCM14	Promoterless *cre* construct cloned into pAW11; Ery^R	This Study
pCM15	P_{FhuCBG}::*cre* transcriptional fusion cloned into pAW11; Ery^R	This Study
pCM17	P_{FhuCBG}::*cre* transcriptional fusion cloned into pLL29-Ery; $Spec^R$ (*E.coli*) Ery^R (*S. aureus*)	This Study
pCM19	promoterless *cre* cloned into pLL29-Ery; $Spec^R$ (*E.coli*) Ery^R (*S. aureus*)	This Study
pCM21	P_{FhuCBG}::Δ1-27*cre* transcriptional fusion cloned into pAW11; Ery^R	This Study

S. aureus cells were incubated in P1 resuspension buffer containing 50 µg/mL lysostaphin for 30-60min before the addition of lysis buffer P2.

2.2.2 Isolation of *S. aureus* chromosomal DNA

S. aureus chromosomal DNA was obtained using the protocol outlined in (91). Briefly, 5 mL *S. aureus* cultures were grown overnight, with shaking. 500 µL of culture was spun down for 2 minutes at 7000 rpm. The supernatant was decanted, and 200 µL STE (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH 7.5) was used to wash the pellet. The cells were spun down again for 2 minutes at 7000 rpm, and the supernatant decanted. 50 µg lysostaphin was dissolved in 200 µL STE to create a STE/lysostaphin buffer. 25 µL of the STE/lysostaphin buffer was used to resuspend the cell pellet, and incubated for 1 hour at 37°C. 20 µL 10% SDS and 20 µL proteinase K were then added and the entire mixture incubated at 55°C overnight. 80 µL 5 M NaCl was then added; the resulting solution was mixed by inversion. 320 µL 25:24:1 phenol/chloroform/isoamyl alcohol was added and mixed by inversion and the resulting mixture was allowed to incubate at room temperature for 30 min, then spun down at 12000 rpm for 10 min. The aqueous layer of solution was removed using a wide bore tip and placed in a separate eppendorf tube. 300 µL 25:1 chloroform/isoamyl alcohol was mixed with the aqueous layer by inversion and spun down at 12000 rpm for 10 min. The aqueous layer was removed, and 400 µL isopropanol was added and the mixture was incubated at room temperature for 10 min, then spun down at 12000 rpm for 5 min. The supernatant was decanted, and the pellet was washed with 100 µL 95% ethanol. Pellets were dried and resuspended in 100 µL ddH2O.

2.2.3 DNA sequencing

DNA samples sent for sequencing had a minimum DNA concentration of 150ng/μL. Primers used for sequencing had a concentration of 12 pmol. All DNA sequencing was performed at the York University Core Molecular Biology and DNA Sequencing Facility.

2.3 Recombinant DNA methodology

2.3.1 Manipulation of recombinant DNA

All restriction endonuclease digestions, PCR reactions and DNA ligations were performed as outlined by Sambrook *et al.* (97). Restriction endonucleases, rAPid alkaline phosphatase and *Pwol* polymerase were obtained through Roche Diagnostics. T4 DNA ligase and additional restriction endonucleases were obtained from New England Biolabs. T4 polynucleotide kinase was obtained from Fermentas. All oligonucleotides detailed in Table 5 were purchased from Integrated DNA technologies.

2.3.2 Preparation of competent *E. coli* and *S. aureus* cells

For *E. coli* DH5α $CaCl_2$ competent cells, 400 mL LB broth was inoculated to an OD_{600} of 0.01 with an overnight culture of *E. coli* DH5α, and incubated at 37°C with aeration until OD_{600} reached 0.5. Cells were placed on ice for 20 minutes, then centrifugated for 10 minutes at 3000rpm, 4°C. The supernatant was carefully decanted so as to not disturb the pellet. The pellet was resuspended in 100 mL chilled *E. coli* competent cell buffer (0.1 M $CaCl_2$, 15% v/v glycerol), placed on ice for 30 minutes, then spun down for 10 minutes at

Table 5: Oligonucleotides used in this study[a,b]

Name	Sequence (5' → 3')
loxP For	TGAGCTCATAACTTCGTATAGCATACATTATACGA AGTTATGATATCATAACTTCGTATAGCATACATTAT ACGAAGTTATAGGTACCA
loxP Rev	TGGTACCTATAACTTCGTATAATGTATGCTATACG AAGTTATGATATCATAACTTCGTATAATGTATGCT ATACGAAGTTATGAGCTCA
Ery For	CGCGATATCTTACTTATTAAATAATTTATAGC
Ery Rev	CCCGATATCTTTAACTCTGGCAACCC
Promoterless *cre* For	TTGTCTAGAATGTCCAATTTACTGACCGTACACC
cre For	TTGGGTACCATGTCCAATTTACTGACCG
cre Del For	TTGTCTAGAATGGACATGTTCAGGGATCG
cre Rev	ATACTGCAGCTAATCGCCATCTTCCAGC
fhuCBG For	GGGGAATTCGATTTATCAAATTAAGTGC
*fhuCBG Kpn*I Rev	GGGGGTACCAATTTCCCTACTTTCAATAAAATTC
*fhuCBG Xba*I Rev	GGGTCTAGAAATTTCCCTACTTTCAATAAAA
Tet^R For	CCCTCTAGACTCGAGGCAGATAGTGTACGTAAA AAG
Tet^R Rev	GCGTCTAGATTAGAAATCCCTTTGAGAATG
tRNA-Lys For	TTGGAGCTCGCCATTAGCTCAGTTGG
tRNA-Leu Rev	TTCGAGCTCATTTTGAGTCCCGCG
lgt promoter For	GGCGATATCCCCGGGTTTCTTTTCGAAATTC
lgt promoter Rev	CATCTTCATATGGGGTTCACCTCAATT
sacB For	CTCCATATGAACATCAAAAAGTTTGCAA
sacB Rev	CCCGATATCTCTAGATTATTTGTTAACTGTTAA TTGTCC

[a]Restriction sites used for cloning are underlined
[b]Abbreviations: tet^R: tetracycline resistance

3000rpm, 4°C. The supernatant was carefully decanted and the pellet resuspended in 4mL *E. coli* competent cell buffer. 100µL aliquots were placed in sterile 1.5mL eppendorf tubes, flash frozen on dry ice, and stored at -80°C.

For all *S. aureus* competent cells, 200mL TSB was inoculated to an OD_{600} of 0.01 with an overnight culture of the desired *S. aureus* strain and incubated at 37°C with aeration until OD_{600} reached 0.3. Cells were put on ice for 10 minutes, then spun down for 10 minutes at 3000rpm, 4°C. The supernatant was carefully decanted, and the pellet resuspended in 20mL chilled 0.5 M sucrose, then spun down for 10 minutes at 3000rpm, 4°C. The supernatant was carefully decanted and the pellet resuspended in 2.5 mL 0.5 M sucrose. The resuspended cells were incubated on ice for 20 minutes then spun down for 10 minutes at 3000 rpm, 4°C and resuspended in 2 mL 0.5 M sucrose. 60 µL aliquots were placed in sterile 1.5 mL microcentrifuge tubes, flash frozen on dry ice and stored at -80°C.

2.3.3 Transformation of competent *E. coli* and *S. aureus* cells

Competent DH5α *E. coli* cells were thawed on ice. 5 µL of plasmid DNA or 10 µL ligation mixtures were added to the cells and incubated on ice for 40 minutes, heat shocked at 42°C for 2 minutes then cold shocked for 2 minutes. 700 µL LB broth was added to the cells which were then incubated at 37°C for one hour. 100 µL of cells were then plated on selective agar and incubated at 37°C overnight. The remaining 600 µL of cells were spun down and the supernatant decanted. The pellet was resuspended with 100 µL fresh LB and subsequently plated as previously described.

Competent *S. aureus* cells were thawed on ice. 3 µL of plasmid DNA was added to the cells and incubated on ice for 20 minutes, then placed in a 0.2 cm electroporation cuvette and electroporated using a BioRad Gene Pulser (one pulse at 2.5 kV, capacitor at 25 µF, parallel resistor at 100 Ω, time constant of 2.5 ms). 700 µL TSB was immediately added to the cells which were subsequently incubated at 37°C for one hour. The cells were then spun down and the pellet resuspended with 100 µL fresh TSB. The cells were then plated on selective agar and incubated at 37°C overnight.

2.3.4 Construction of P_{lgt}::*sacB* fusion

The *lgt* promoter region was PCR amplified from *S. aureus* Newman chromosomal DNA using primers *lgt* promoter For and *lgt* promoter Rev. *sacB* was PCR amplified from the suicide vector pSAKO using the primers *sacB* For and *sacB* Rev. The *lgt* promoter region and *sacB* were digested with *Nde*I, ligated and PCR amplified resulting in a P_{lgt}::*sacB* fusion. P_{lgt}::*sacB* was phosphorylated, and blunt cloned into a dephosphorylated *Sma*I site in the *E. coli* – *S. aureus* shuttle vector, pLI50.

2.3.5 Construction of pLL29-Ery

pLL29 is a *S. aureus* single-copy integration vector based on the lysogenic bacteriophage L54a and φ11 site-specific recombination systems (62). The plasmid harbours a tet^R cassette, which, for the purposes of this study, needed to be inactivated. To do this, an erythromycin resistance cassette was cloned into pLL29 using a unique *Eco*RV site located within the Tet^R cassette.

The erythromycin cassette was PCR amplified from pDG646 using the Ery For and Ery Rev primers. The PCR product was then cut with *Eco*RV and blunt cloned into the dephosphorylated pLL29 backbone using the aforementioned *Eco*RV site, disrupting the tet^R cassette and resulting in pLL29-Ery.

2.3.6 Construction of P_{FhuCBG}::*cre* fusion

The *fhuCBG* promoter region (Figure 8) was PCR amplified from the *S. aureus* Newman chromosome using the *fhuCBG* For and *fhuCBG Kpn*I Rev primers. *cre* was PCR amplified from pSHE11 using the *cre* For and *cre* Rev primers. The promoter region and *cre* were then digested with *Kpn*I, ligated and PCR amplified resulting in P_{FhuCBG}::*cre*. The resulting fusion was then cloned into either the *E. coli-S. aureus* shuttle vector pAW11, or the single copy *S. aureus* integration vector pLL29-Ery using the unique *Eco*RI and *Pst*I sites. As a control, a promoterless *cre* construct was PCR amplified using the Promoterless *cre* For and *cre* Rev primers, then cloned into pAW11 or pLL29-Ery using unique *Xba*I and *Eco*RI sites.

2.3.7 Construction of P_{FhuCBG}::Δ1-27*cre* fusion

The *fhuCBG* promoter region was PCR amplified as described in 2.3.6 with a single modification: The *fhuCBG Xba*I Rev primer was used to PCR amplify the promoter region instead of the *fhuCBG Kpn*I Rev primer. A truncated *cre* with the first 27 amino acids deleted from the N-terminus was PCR amplified using *cre* Del For and *cre* Rev primers. The *fhuCBG* promoter and truncated *cre*

Figure 8: The *fhuCBG* promoter region of *S. aureus* strain Newman. The Shine-Dalgarno (SD) site, -35 and -10 regions of the promoter region are underlined and the boxed sequence represents the iron-regulated Fur box. The translation-start methionine is depicted in red. Sequence downstream, and including the ATG start-codon is the beginning of *fhuC*.

NWMN_698191 ATAAATGGTATGAGCACACATACTTAAATAGAAGTCCACGG

NWMN_698232 ACAAGTTTTTGAACTATGAAGACTTATCTGTGGGCGTTTTTT

NWMN_698274 ATTTTATAAAAGTAATATACAAGACATGACAAATCGACAATT

NWMN_698316 TAAAAAGTAATGTTAGTCAATAAGATTGAAAAATGTTATAAT

-35 -10

NWMN_698358 GATGTTCATGATAATCATTATCAATTGGGATGTCTTTGAAAA

NWMN_698400 TTGATAATTTAAAAATAGAAATTATTTTTTATAAACAGAAAGA

NWMN_698443 AATTTTATTGAAAGTAGGGAAATTATGAATCGTTTGCATTGG

SD

NWMN_698485 ACAACAAGTTAAAATTGGTTACGGGGATAACACGATTATAA

NWMN_698526 ATAAATTAGATGTTGAAATAC

were digested with *Xba*I, ligated and PCR amplified, resulting in the P_{FhuCBG}::Δ1-27*cre* fusion, which was then cloned into the pAW11 backbone using unique *Eco*RI and *Pst*I sites, creating pCM21.

2.3.8 Construction of the *loxP* cassette

25 µL of primers *loxP* For and *loxP* Rev, consisting of two *loxP* sites separated by an *Eco*RV site, were mixed in a PCR tube and annealed together in a thermocycler by gradually increasing the temperature up to 95°C, at a rate of 0.1°C/sec. The resulting 87 bp PCR product was cloned as a *Sac*I/*Kpn*I fragment into pBC SK+, creating pCM5. The P_{lgt}::*sacB* transcriptional fusion was cut with *Eco*RV and cloned into the aforementioned *Eco*RV site into the dephosphorylated pCM5 backbone, resulting in pCM11. A 2.1 kb fragment containing a tetracycline resistance cassette and its native promoter was PCR amplified from pDG1513 and amplified using the Tet^R For and Tet^R Rev primers, cut with *Xba*I and cloned into a unique *Xba*I site of pCM11, which had been dephosphorylated beforehand, resulting in pCM12. To complete the *loxP* construct, a 2.2 kb fragment flanked by a truncated tRNA-Leu and a tRNA-Lys was PCR amplified from the Newman chromosome (NWMN_tRNA24 to NWMN_tRNA44) using the tRNA-Lys For and tRNA-Leu Rev primers. The PCR product was then digested with *Sac*I and cloned into the unique *Sac*I (dephosphorylated) site outside of the *loxP* cassette in pCM12, resulting in pCM13.

2.4 Integration of the *loxP* cassette into the *S. aureus* chromosome

E. coli harbouring the pCM13 plasmid was cultured overnight. Plasmid preparations of pCM13 were performed until a plasmid concentration of approximately 1 mg/μL was attained. pCM13 was then electroporated into competent *S. aureus* strain RN4220 cells and plated on TSB agar containing tetracycline and incubated overnight at 37°C. Three transformants were obtained. Each was tested to verify *loxP* construct integration by PCR amplification of the tetracycline resistance cassette, as well as *sacB*, using the chromosomal DNA of the transductants as a template (data not shown). Once verified, the *loxP* cassette was transferred from the RN4220 background to other *S. aureus* strains using phage transduction. Transductions, using phage 80α, were carried out as described previously (78).

2.5 Integration of *cre* constructs into the *S. aureus* chromosome

cre constructs were integrated as described (62). Briefly, *E. coli* harbouring a *cre* construct plasmid were grown overnight. The *cre* plasmids were then transformed into competent *S. aureus* strain RN4220 cells and plated on TSB agar containing erythromycin and lincomycin. *cre* construct integration was then verified by PCR amplification of the *cre* gene using the chromosomal DNA of each integration strain as the template. Colonies with verified *cre* constructs were then transduced, using phage 80α, to other *S. aureus* strains as described in 2.4.

2.6 Determining frequency of *loxP* cassette resolution before inoculation of liquid media

Colonies harbouring one of the three *cre* constructs were patched onto two different plates: TSB agar containing erythromycin and lincomycin and TSB agar containing erythromycin, lincomycin and tetracycline. Patched colonies were grown overnight at 37°C, then observed for tetracycline sensitivity.

2.7 Cassette resolution in *S. aureus* strains carrying the *loxP* cassette

To verify the functionality of the Cre/*loxP* system, each multi-copy *cre* construct was electroporated into RN4220. Plasmids were prepared from RN4220 as described above and electroporated into RN6390::*loxP*, plated and grown overnight at 37°C for no more than 18 hours. Three colonies were randomly selected and separately resuspended in 300 µL TSB. 200 mL fresh TSB containing erythromycin and lincomycin were inoculated with the resuspended cultures to an OD_{600}= 0.01. The inoculated 200 mL of TSB was then split in half, with sterilized 2,2'-dipyridyl added to one of these flasks to a concentration of 1mM. Aliquots of each culture were taken every 2 hours and plated on both TSB agar containing erythromycin and lincomycin and TSB agar containing erythromycin, lincomycin and tetracycline, using serial dilutions. Cells were grown for a minimum 18 hours at 37°C, and CFUs were counted. This was repeated using the single-copy promoterless *cre* and $P_{FchuCBG}$::*cre* constructs.

2.8 Bacterial growth curves

S. aureus cultures were grown for 20 hours in TSB with the appropriate antibiotics. Cultures were diluted to an OD_{600} of 0.01 in TSB or TSB + 10%

sucrose and grown at 37°C under constant shaking in a bioscreen C machine (Growth Curves, USA). Optical density was measured at 600nm once every half hour for 48 hours.

Chapter 3: Results

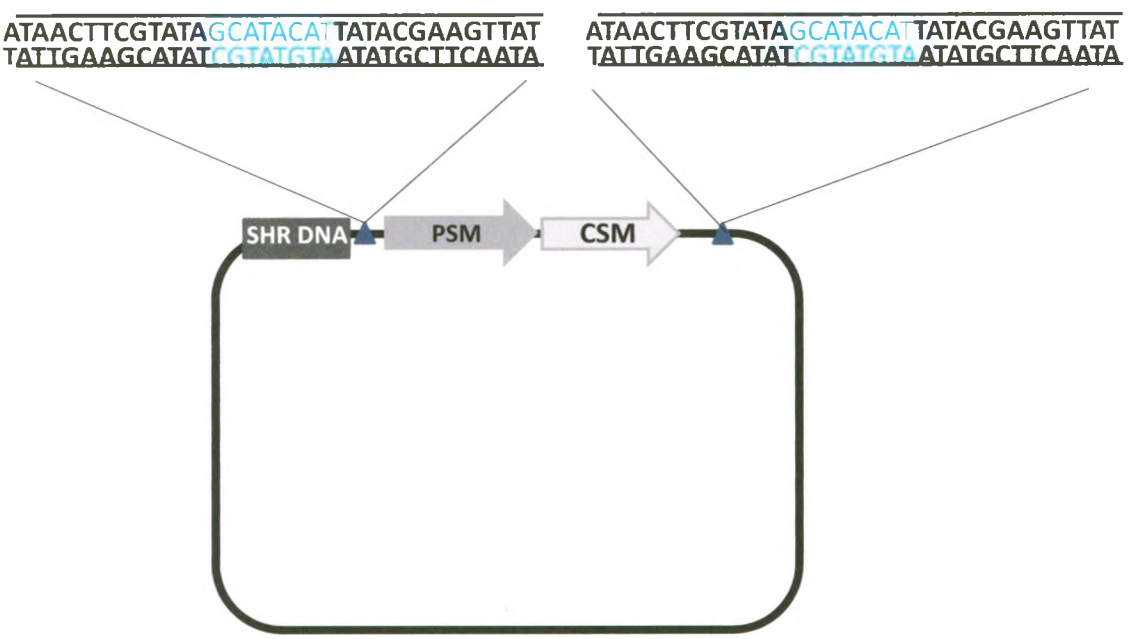
3.1 A Tetracycline-resistance cassette as a suitable positive selection marker

In order to construct a *loxP* reporter cassette (Figure 9), a positive selective marker needed to be chosen. An antibiotic resistance cassette was used, as several of these cassettes encoding for Kan^R, Ery^R/$Linc^R$ and Tet^R have all been used effectively by our laboratory in a variety of *S. aureus* genetic backgrounds (5, 101). Thus, a Tet^R cassette was chosen as the selective marker for use in this *loxP* reporter cassette; both single- and multi-copy vectors carrying a Tet^R cassette (See methods and materials) were tested in several *S. aureus* strains, all of which endowed a Tet^R phenotype to previously Tet^S strains (data not shown).

3.2 *sacB* as a suitable counter selection marker

A counter selective marker was chosen to allow *S. aureus* cells lacking the *loxP* reporter cassette to grow in selective media in order to easily differentiate those in which the reporter cassette had not been excised. Previous studies have used *sacB* from *Bacillus subtilis* as a counter selective marker in a RIVET system (83). *sacB* imparts a sucrose-sensitive phenotype; cells carrying *sacB* grown in the presence of sucrose are killed due to a continued buildup of levan, the end product of levansucrase fructosyl group polymerization and sucrose hydrolysis (10). In addition, the sucrose-sensitive phenotype of *sacB* has been shown to work in *S. aureus* (30); however, it was only tested in the RN4220 genetic background. Thus, it needed to be determined if other *S. aureus* genetic backgrounds could become sucrose-sensitive using *sacB*. *sacB* was fused to the constitutive promoter of the lipoprotein diacylglycerol transferase encoding *lgt*, a

Figure 9: Schematic of a *loxP* reporter cassette integrated into the *S. aureus* chromosome. The cassette consists of a positive selection marker (PSM) and a counter selection marker (CSM) flanked by two *loxP* sites, and is integrated using genomic DNA from *S. aureus* in order to perform a single homologous recombination event (SHR DNA).


ATAACTTCGTATAGCATACATTATACGAAGTTAT
TATTGAAGCATATCGTATGTAATATGCTTCAATA
ATAACTTCGTATAGCATACATTATACGAAGTTAT
TATTGAAGCATATCGTATGTAATATGCTTCAATA
SHR DNA
PSM
CSM

known *S. aureus* virulence determinant (12), creating the P_{lgt}::*sacB* transcriptional fusion. This fusion was then cloned into pLI50 (See methods and materials), creating pCM6. pCM6 was electroporated into four different *S. aureus* strains: RN4220, RN6390, Newman and SH1000. 10µL aliquots of each strain were then grown overnight on TSB agar containing no sucrose, 5%, 10% or 15% sucrose (Figure 10). Sucrose sensitivity was readily apparent in all strains containing pCM6 with the exception of the Newman strain at 5% sucrose (Figure 10A). However, at 10% and 15% sucrose all strains containing pCM6, but not those containing the vehicle control showed sucrose sensitivity. The RN4220, RN6390 and SH1000 strains all maintained a similar level of sucrose sensitivity at both the 10% and 15% sucrose concentrations, with only the Newman strain showing a significant increase in sucrose sensitivity at 15%. In contrast, the same strains containing the pLI50 empty vector displayed no sensitivity to sucrose, regardless of sucrose concentration (Figure 10B), showing that the sucrose sensitivity is due to the P_{lgt}::*sacB* fusion.

Next, three *S. aureus* strains containing a genome-integrated P_{lgt}::*sacB* fusion were tested for a sensitivity to sucrose at the same sucrose concentrations as those used to test the plasmid-based P_{lgt}::*sacB* construct described above (Figure 11). Interestingly, of the three strains tested, only RN6390 appeared to show a slight sensitivity to sucrose against its wild-type counterpart in any of the sucrose concentrations tested. Unlike the plasmid-based P_{lgt}::*sacB* harbouring strains, where sensitivity to sucrose was readily apparent (significantly less cell

Figure 10: Verification of the *sacB* phenotype in *S. aureus* using a plasmid carrying *sacB* expressed from the constitutive *lgt* promoter. The plasmid was electroporated into four different *S. aureus* strains: RN4220, RN6390, Newman and SH1000. The strains were serially diluted, then plated onto varying concentrations of sucrose. All four strains containing pCM6 showed signs of sucrose sensitivity at a sucrose concentration of 10%. A) *S. aureus* strains with the empty vector control. B) *S. aureus* strains with the P_{lgt}::*sacB* carrying plasmid.

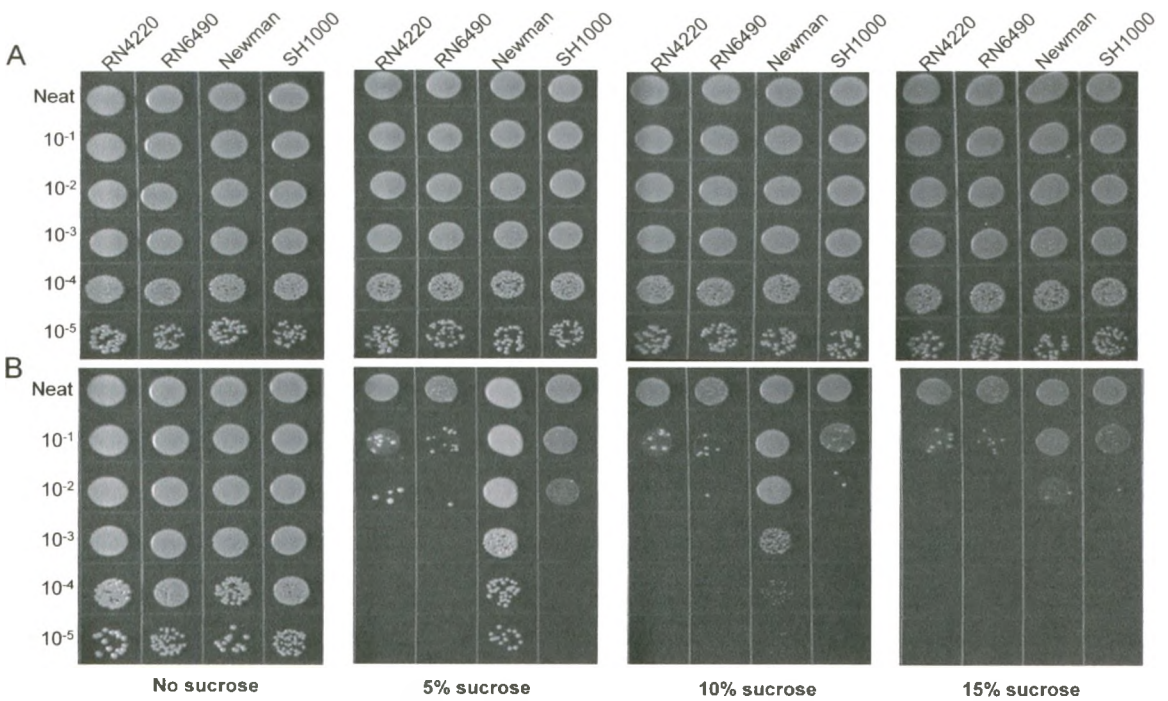
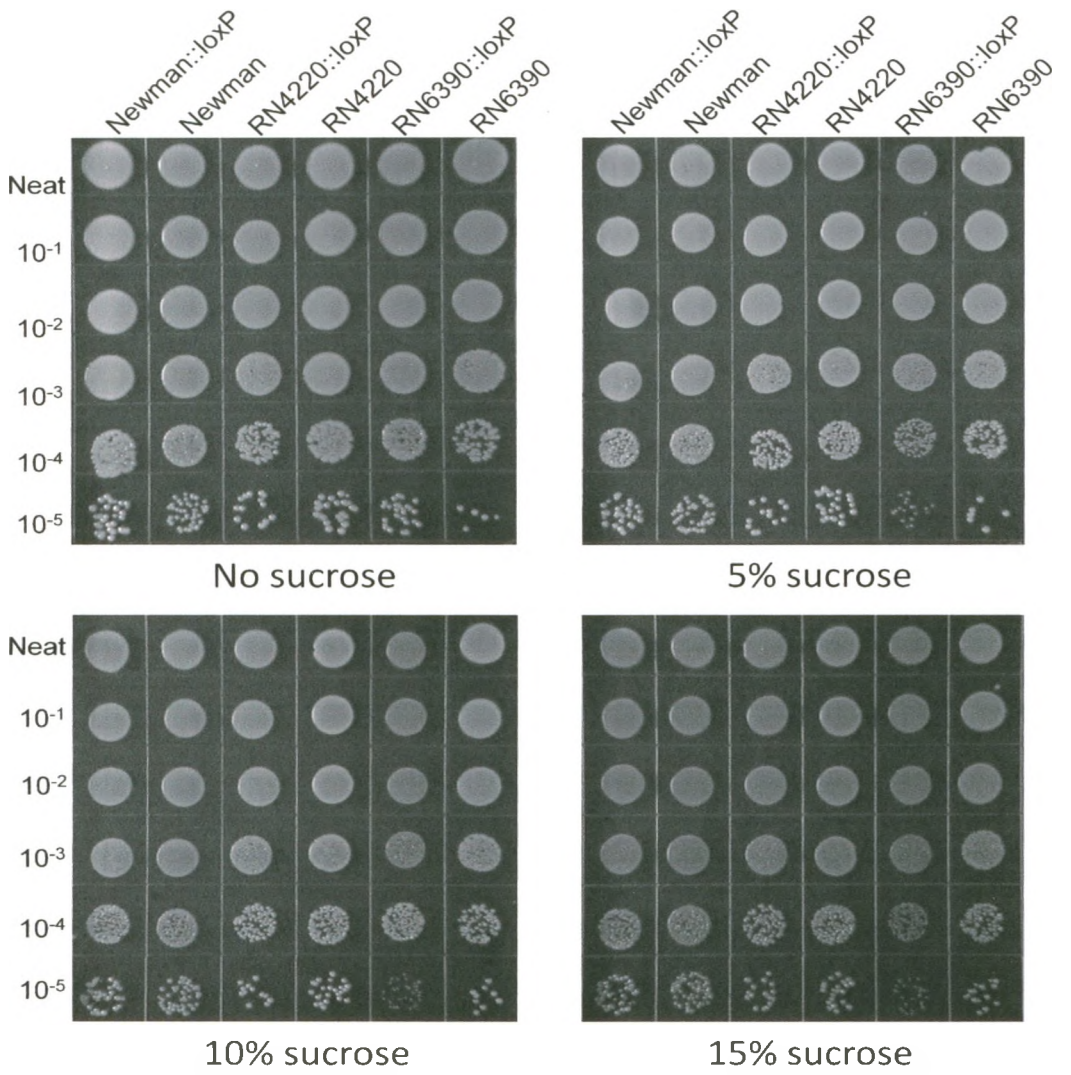
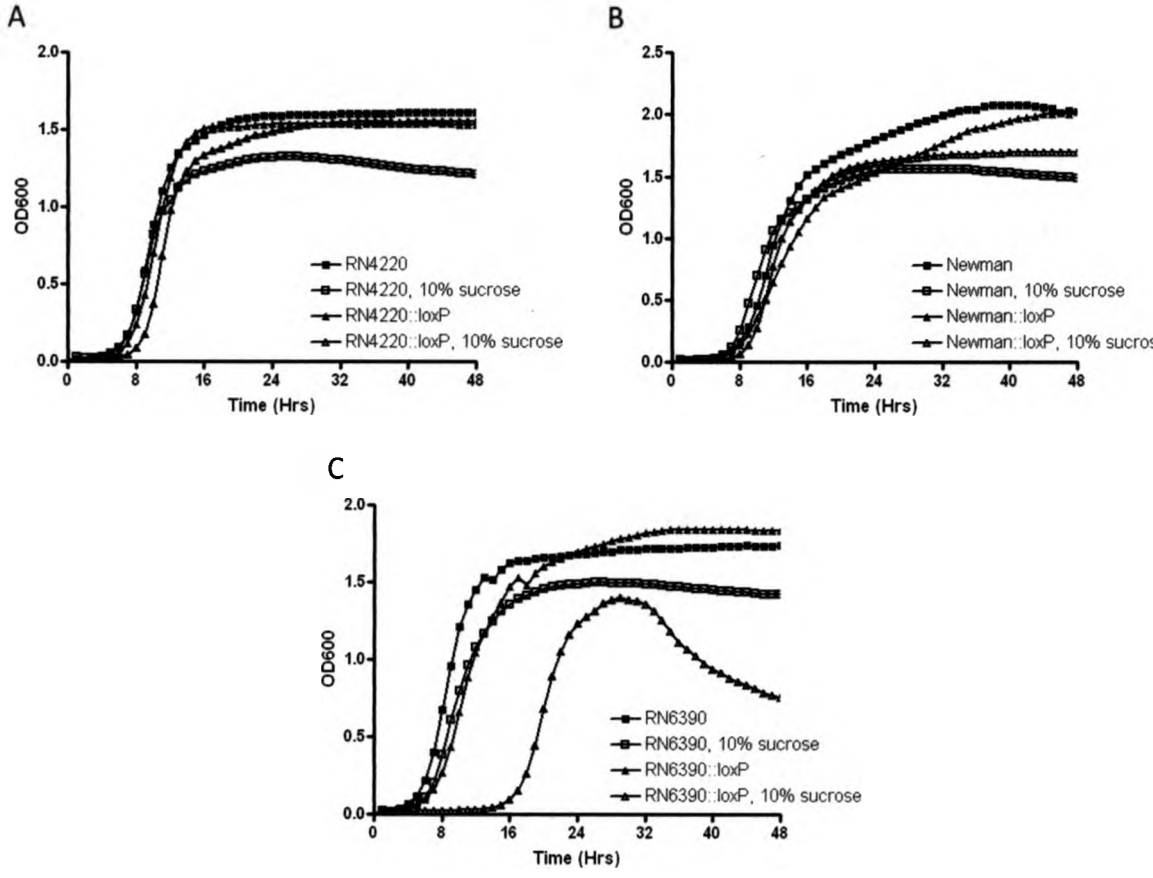

A
B
RN4220
RN6490
Newman
SH1000
Neat
10-1
10-2
10-3
10-4
10-5
No sucrose
5% sucrose
10% sucrose
15% sucrose

Figure 11: Verification of the *sacB* phenotype in *S. aureus* strains with the genome-integrated P_{lgt}::*sacB* fusion. This fusion was tested to evaluate a sucrose sensitive phenotype in RN4220, RN6390 and Newman at varying sucrose concentrations. Of these strains, only the RN6390 carrying the integrated P_{lgt}::*sacB* fusion presented with a minor sucrose sensitive phenotype. The phenotype could be seen on all plates containing sucrose. Wild-type strains without the integrated P_{lgt}::*sacB* fusion were used as controls.


Newman::loxP
Newman
RN4220::loxP
RN4220
RN6390::loxP
RN6390
Neat
10-1
10-2
10-3
10-4
10-5
No sucrose
5% sucrose
10% sucrose
15% sucrose

growth in comparison to strains with the empty vector), sucrose sensitivity in the RN6390 strain with the genome-integrated P_{lgt}::*sacB* strain was poor, represented by limited growth and smaller CFUs versus the wild-type in the final dilution only at all three sucrose concentrations. Due to the limited sucrose sensitivity shown in the three *S. aureus* strains with the genome-integrated P_{lgt}::*sacB* fusion, a different approach was taken to verify the *sacB* phenotype in these strains. Instead of observing sucrose sensitivity on agar plates, I grew each strain for 48 hours in liquid TSB with or without sucrose (Figure 12). Both the RN4220 and Newman strains with the genome-integrated P_{lgt}::*sacB* fusion did not show any sucrose sensitivity (Figure 12A, B). The RN6390 strain harbouring the integrated single-copy P_{lgt}::*sacB* fusion, however, showed significant cell growth impairment in the presence of sucrose, as the OD_{600} was far lower in the presence of sucrose than that seen without sucrose (Figure 12C). Curiously, in the presence of sucrose cell growth of the same strain gradually increased until hour 30, at which point growth began to decrease once again. It is important to note that growth levels of the RN6390 with the genome-integrated P_{lgt}::*sacB* fusion in the presence of sucrose never attained those of the same strain in the absence of sucrose. This indicates that the RN6390::*loxP* strain is sensitive to sucrose. In addition, because the RN6390 wild-type strain showed similar growth curves regardless of the sucrose concentration, the sucrose sensitivity seen in the RN6390 strain is due to the genome-integrated P_{lgt}::*sacB* fusion. Based on these results, only the RN6490::*loxP* strain was used to test for *loxP* cassette

Figure 12: Verification of the *sacB* phenotype in *S. aureus* strains with the genome-integrated P_{lgt}::*sacB* in liquid cultures over 48 hours. Strains were grown in a bioscreen in TSB with or without sucrose. Only the RN6390 strain carrying the P_{lgt}::*sacB* fusion showed a sucrose sensitive phenotype in the presence of sucrose (C). This phenotype was readily apparent until hour 16. Growth then steadily increased until hour 32, at which point cell growth began to deteriorate. Both the RN4220 (A) and Newman (B) strains with the integrated P_{lgt}::*sacB* did not show sucrose induced cell death in the presence of sucrose. Wild-type strains without the genome-integrated P_{lgt}::*sacB* were used as controls for each strain.

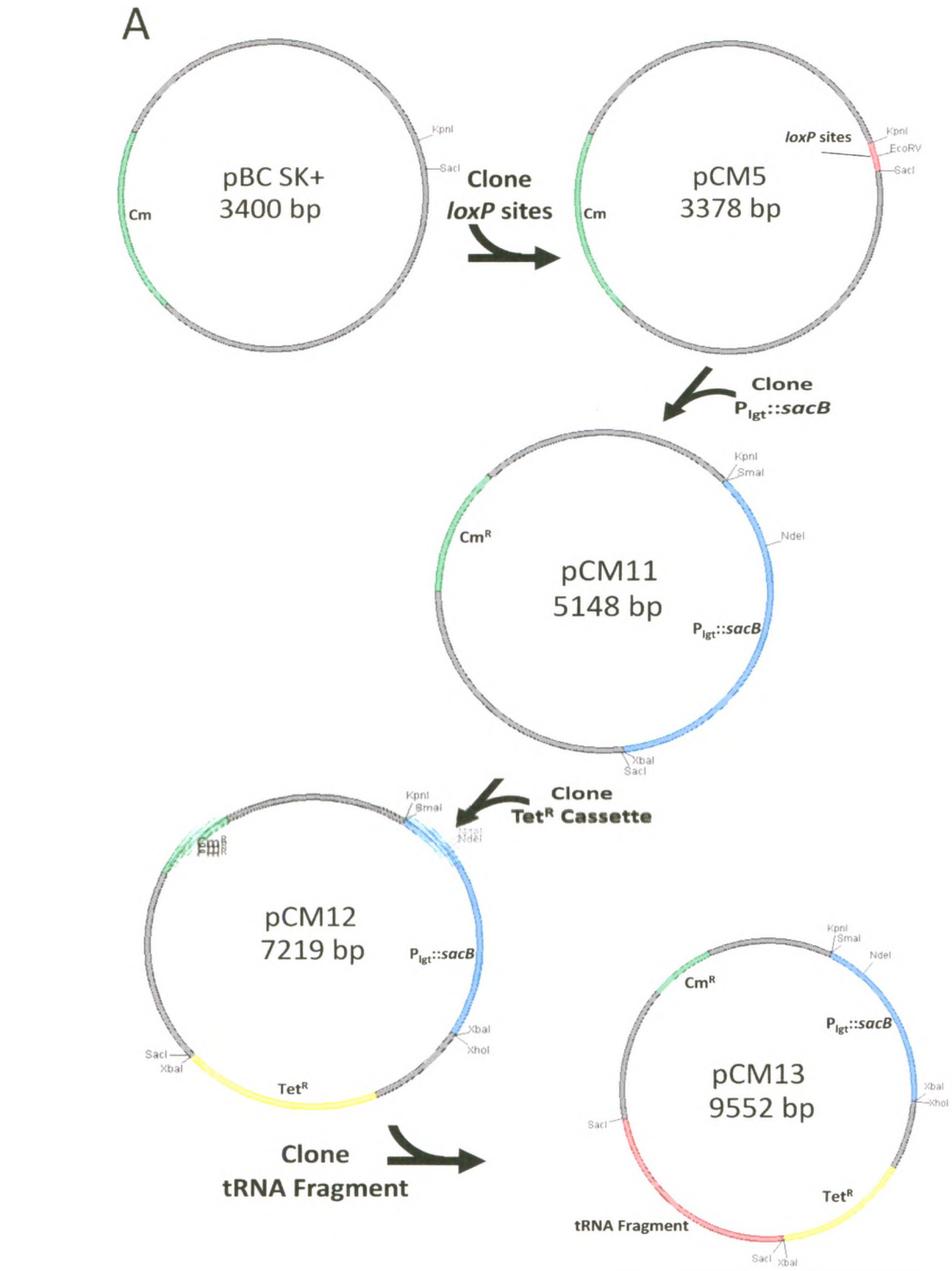

A
OD600
Time (Hrs)
RN4220
RN4220, 10% sucrose
RN4220::loxP
RN4220::loxP, 10% sucrose
B
OD600
Time (Hrs)
Newman
Newman, 10% sucrose
Newman::loxP
Newman::loxP, 10% sucrose
C
OD600
Time (Hrs)
RN6390
RN6390, 10% sucrose
RN6390::loxP
RN6390::loxP, 10% sucrose

resolution with the *cre* expression constructs.

3.3 Construction of the *S. aureus loxP* reporter strain

In order to determine temporal expression of iron-regulated *S. aureus* promoters, a *S. aureus* strain harbouring a *loxP* reporter cassette needed to be constructed (Figure 13). Two *loxP* sites flanking an *Eco*RV site were cloned into pBC SK+, a plasmid containing no Gram-positive origin of replication (ori), resulting in pCM5. The P_{lgt}::*sacB* fusion was then cloned in-between the *loxP* sites of pCM5, creating pCM11. A tetracycline resistance cassette from pDG1513 was then cloned downstream of the P_{lgt}::*sacB* fusion resulting in pCM12. Finally, a tRNA fragment flanked by a truncated tRNA-Leu and a tRNA-Lys from the *S. aureus* Newman chromosome was cloned outside of the *loxP* cassette, resulting in the completed *loxP* reporter cassette, pCM13. This tRNA fragment would allow for a single homologous recombination event to occur, resulting in the integration of pCM13 into the *S. aureus* chromosome. pCM13 was electroporated into *S. aureus* strain RN4220 and grown on TSB agar with tetracycline overnight. Due to pCM13 lacking a Gram-positive ori, only cells that had pCM13 integrated into the chromosome could grow on the tetracycline plates. Three Tet^R colonies were obtained. Verification of a successful integration event was done by PCR amplifying the Tet^R cassette as well as *sacB* using chromosomal DNA as the template; all three colonies contained the integrated *loxP* cassette (Data not shown). The *loxP* cassette was then transduced into the RN6390 and Newman genetic backgrounds, creating RN6390::*loxP* and Newman::*loxP*.

Figure 13: Construction of the *loxP* reporter cassette. A. The *loxP* sites were cloned into pBC SK+ using the *Sac*I *and Kpn*I sites, effectively removing the MCS of pBC SK(+) and creating pCM5. The P_{lgt}::*sacB* transcriptional fusion was then cloned between the *loxP* sites, resulting in pCM11. A Tet^R cassette from the pDG1513 vector was then cloned into pCM11, resulting in pCM12. Finally, a tRNA fragment from *S. aureus* Newman was cloned into pCM12 outside the *loxP* cassette, resulting in pCM13. B. Linear map of pCM13 showing relevant genetic loci; not drawn to scale.


A
pBC SK+
3400 bp
Cm
KpnI
SacI
Clone
loxP sites
pCM5
3378 bp
loxP sites
KpnI
EcoRV
SacI
Clone
P lgt ::sacB
pCM11
5148 bp
CmR
KpnI
SmaI
NdeI
P lgt ::sacB
XbaI
SacI
Clone
TetR Cassette
pCM12
7219 bp
P lgt ::sacB
TetR
SacI
XbaI
XbaI
XhoI
Clone
tRNA Fragment
pCM13
9552 bp
CmR
P lgt ::sacB
TetR
tRNA Fragment
KpnI
SmaI
NdeI
XbaI
XhoI
SacI
XbaI
B
tRNA
loxP
sacB
TetR
loxP
SmaI
XhoI

Successful transductants were verified by PCR as previously described (Data not shown).

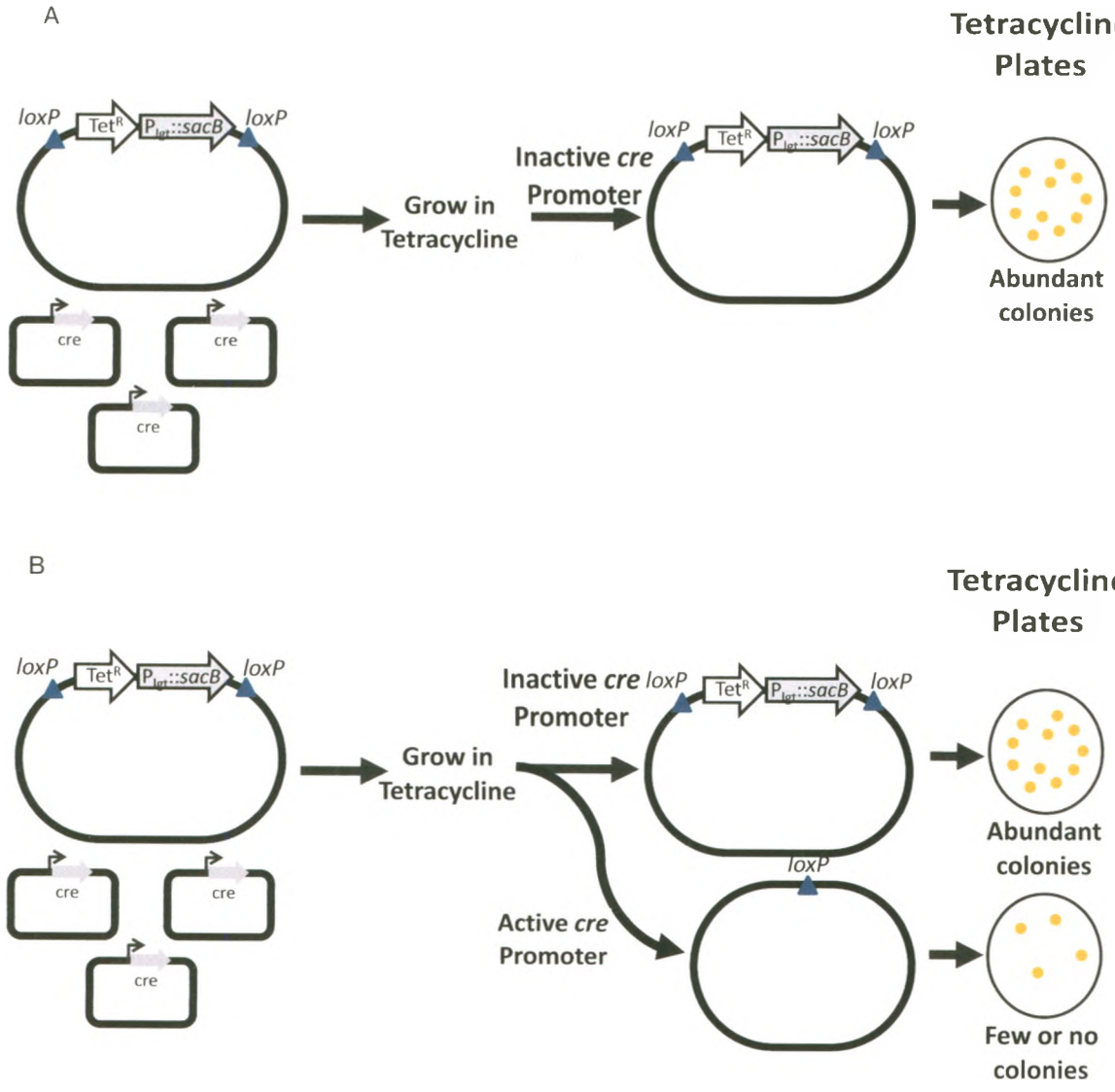
3.4 Construction of the *S. aureus* multi-copy *cre* expression vectors

In addition to the *S. aureus loxP* reporter strains, *cre* expression systems needed to be constructed in order to test for resolution of the *loxP* cassette. To do this, a *S. aureus* iron-regulated promoter needed to be chosen in order to test for successful *cre* expression. The *fhuCBG* promoter was selected, as its ATPase, FhuC, is used in conjunction with several of the *S. aureus* iron acquisition systems (See Figure 5). Thus, *cre* was fused to the *fhuCBG* promoter region, resulting in a P_{FhuCBG}::*cre* fusion. The fusion was then cloned into the *E. coli – S. aureus* shuttle vector pAW11, resulting in pCM15. A promoterless *cre* was also cloned into pAW11 as a negative control, creating pCM14. An additional control using a *cre* in which the first 27 amino acids were deleted was fused to the *fhuCBG* promoter (P_{FhuCBG}::Δ1-27*cre*) and subsequently cloned into the pAW11 backbone, resulting in pCM21.

3.5 Resolution of the *loxP* cassette using multi-copy iron-regulated Cre expression in *S. aureus* strain RN6390::*loxP*

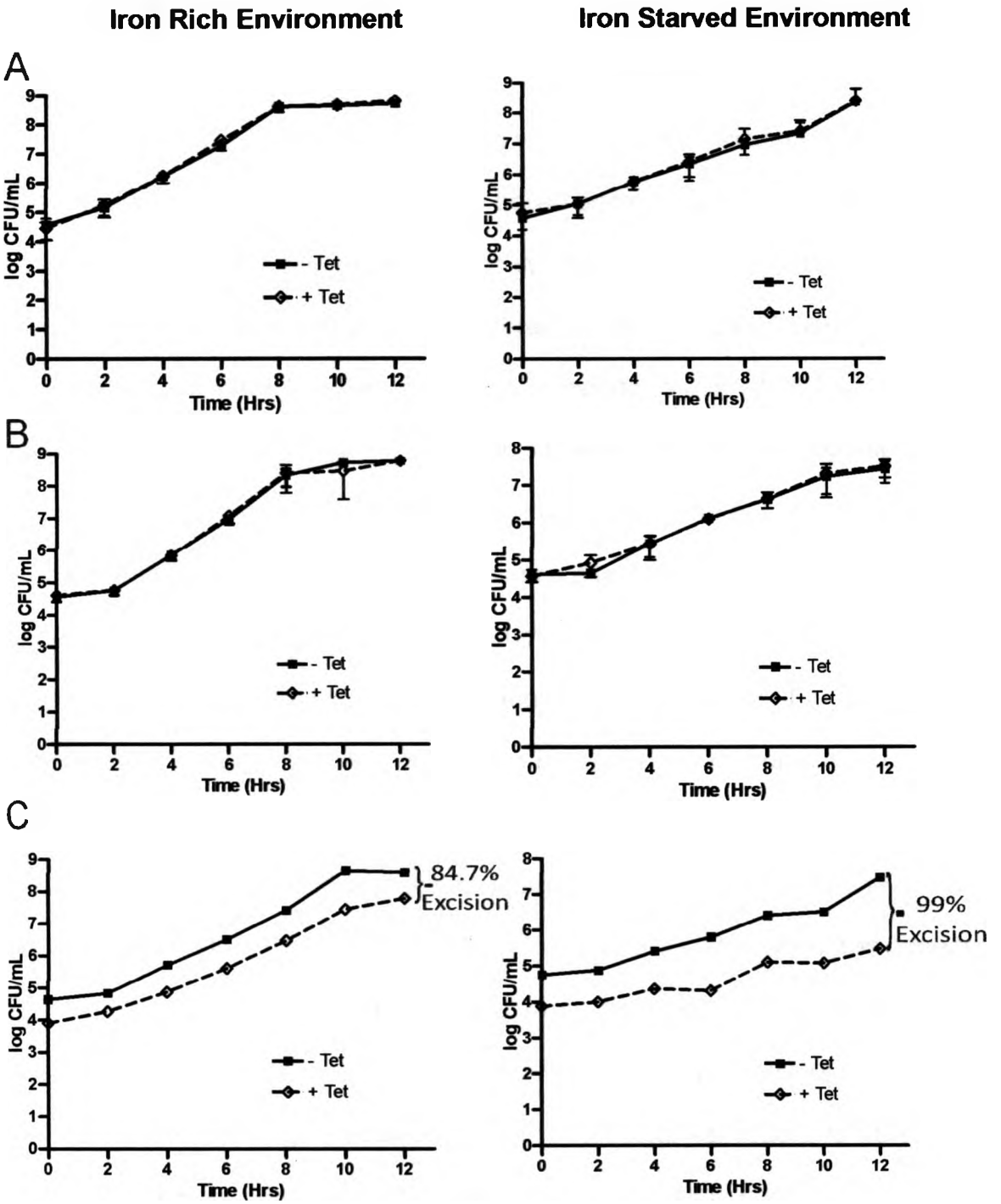
In order to confirm that the P_{FhuCBG}::*cre* construct was expressing Cre, three differen multi-copy *cre* constructs were built: P_{FhuCBG}::*cre*, a promoterless *cre* construct, and P_{FhuCBG}::Δ1-27*cre*, containing an inactive *cre* gene. Each construct was transformed into RN6390::*loxP* then grown n in either iron rich or iron starved conditions over 12 hours (Figure 14). Aliquots from each condition

Figure 14: Sequential diagram of the RIVET system designed to elucidate the temporal expression of *S. aureus* iron-regulated promoters. Plasmids containing *cre* expressed by iron-regulated promoters, are introduced into *S. aureus* strains harbouring the integrated *loxP* cassette. Cells are simultaneously cultured in iron rich (A) and iron starved media (B). Aliquots are serially diluted onto TSB agar with or without tetracycline and grown overnight. In the iron rich conditions, no *cre* expression should be observed, and abundant cell growth should be seen on the tetracycline plates. In the iron starved environment however, the iron-regulated *cre* should express *cre* resulting in excision of the *loxP* cassette, making those cells sensitive to tetracycline, resulting in reduced CFU counts on the tetracycline plates in comparison to the plates containing no tetracycline.


A
Tetracycline Plates
loxP
Tet^R
P_lgt::sacB
loxP
cre
cre
cre
Grow in Tetracycline
Inactive cre Promoter
loxP
Tet^R
P_lgt::sacB
loxP
Abundant colonies
B
Tetracycline Plates
loxP
Tet^R
P_lgt::sacB
loxP
cre
cre
cre
Grow in Tetracycline
Inactive cre Promoter
loxP
Tet^R
P_lgt::sacB
loxP
Abundant colonies
Active cre Promoter
loxP
Few or no colonies

were serially diluted on plates with or without tetracycline in order to verify *loxP* cassette resolution. As expected, both the promoterless *cre* and P_{FhuCBG}::Δ1-27*cre* multi-copy constructs did not show any resolution of the *loxP* cassette in either iron environment, as there was no significant difference between cells grown on the plates with tetracycline in comparison to those grown on plates without tetracycline (Figures 15A, B). The P_{FhuCBG}::*cre* multi-copy construct, however, shows cassette resolution in both the iron rich and iron starved growth environments (Figure 15C). While resolution is observed regardless of the environmental iron available, it is apparent that resolution was higher in the iron starved media due to iron-regulated *cre* expression via the *fhuCBG* promoter. It is important to note that unlike the promoterless *cre* and P_{FhuCBG}::Δ1-27*cre* constructs, data from only one colony is shown for the P_{FhuCBG}::*cre* construct. This is due to the remaining two replicates having no growth on the tetracycline plates at all time points (data not shown), indicating that resolution of the cassette occurred before the colonies were used to inoculate the TSB media. Patching additional colonies onto agar plates with or without tetracycline showed that two out of five colonies (40%) had their cassette resolved during cell growth after electroporation of the P_{FhuCBG}::*cre* plasmid construct into RN6390::*loxP*. Taken together, these results indicate that, while *cre* is being expressed by the P_{FhuCBG}::*cre* construct, its multi-copy nature may be causing artificially high levels of *cre* expression, causing early *loxP* cassette resolution. Therefore, single-copy *cre* constructs needed to be tested to verify this.

Figure 15: Resolution of the *loxP* cassette using multi-copy iron-regulated Cre expression in *S. aureus* strain RN6390::*loxP*. CFUs were counted over a 12 hour time period in either an iron rich environment (TSB media or iron starved media (TSB media + 1mM 2, 2'-dipyridyl) and plated on agar with (+Tet) or without tetracycline (-Tet). Cassette resolution would be observed if a reduced number of colonies were observed on the + tet plates in comparison to the –tet plates. RN6390::*loxP* cells harbouring the promoterless *cre* (A) or P_{FhuCBG}::Δ1-27*cre* (B) constructs did not show any evidence of cassette resolution, regardless of what iron environment the cells were cultured in. However, RN6390::*loxP* cells harbouring the P_{FhuCBG}::*cre* plasmid showed definitive cassette resolution in both the iron rich and iron starved environments, indicating *cre* expression was occurring, and driven by the *fhuCBG* promoter (C). Sample size for each *cre* construct n=3, except for P_{FhuCBG}::*cre* (n=1).


Iron Rich Environment
Iron Starved Environment
A
B
C
log CFU/mL
Time (Hrs)
- Tet
+ Tet
84.7%
Excision
99%
Excision

3.6 The single-copy P_{FhuCBG}::*cre* construct does not resolve the *loxP* cassette in *S. aureus* RN6390::*loxP in vitro*

Single-copy *cre* integration vectors were designed utilizing pLL29 (62), a *S. aureus* single-copy integration vector based on the lysogenic bacteriophage L54a and φ11 site-specific recombination systems. The system allows for fast, reliable chromosomal insertion at two different locations in the *S. aureus* genome, based on the location of each bacteriophage's *attB* site (62). For the purposes of this study, the φ11 integration site was selected, as integration at this site has been shown not to affect the virulence of *S. aureus* (62). One problem with the pLL29 vector is that it carries a tet^R cassette (Figure 16); however, this problem was solved by disrupting the tet^R cassette with an erythromycin resistance cassette. This integration vector, pLL29-Ery, was subsequently used as the backbone for the P_{FhuCBG}::*cre* and promoterless *cre* integration vectors pCM17 and pCM19, respectively. Both vectors were electroporated into RN4220 and grown overnight on TSB agar containing erythromycin and lincomycin. Verification of a successful integration was done by PCR amplifying *cre* using chromosomal DNA as the template (Data not shown). The P_{FhuCBG}::*cre* and promoterless *cre* constructs were then transduced into the RN6390 and Newman genetic backgrounds; successful transductants were verified by PCR as previously described in section 3.2 (Data not shown). Figure 17 shows that both the integrated P_{FhuCBG}::*cre* and promoterless *cre* do not resolve the *loxP* cassette in both the iron rich and iron starved media, indicating that *cre* is not being expressed (or is expressed extremely poorly) from either of

these strains. Table 6 summarizes the *loxP* resolution results of both the multi-copy and single-copy *cre* expression constructs.

Figure 16: Construction of the *cre* expression integration vectors. The tet^R cassette of pLL29 (62) was disrupted using an erythromycin resistance cassette, *ermC*, from the pDG646 vector, creating pLL29-Ery. The P_{FhuCBG}::*cre* transcriptional fusion and promoterless *cre* constructs were then cloned into pLL29-Ery, resulting in pCM17 and pCM19, respectively.

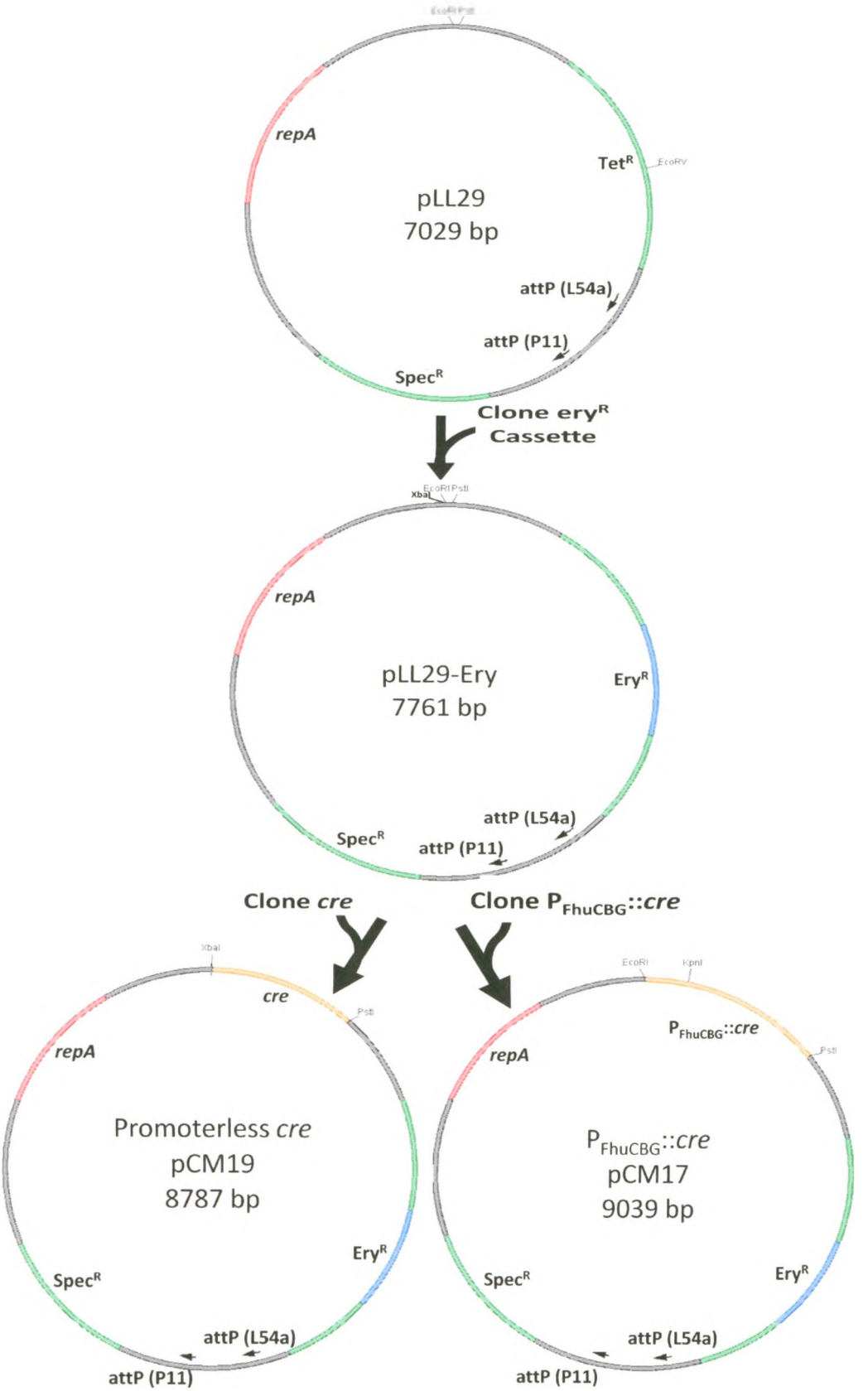

repA
Tet^R
EcoRV
pLL29
7029 bp
attP (L54a)
attP (P11)
Spec^R
Clone ery^R Cassette
XbaI
EcoRI PstI
repA
pLL29-Ery
7761 bp
Ery^R
attP (L54a)
Spec^R
attP (P11)
Clone cre
Clone P_FhuCBG::cre
XbaI
cre
PstI
repA
Promoterless cre
pCM19
8787 bp
Ery^R
Spec^R
attP (L54a)
attP (P11)
EcoRI
KpnI
P_FhuCBG::cre
repA
PstI
P_FhuCBG::cre
pCM17
9039 bp
Spec^R
Ery^R
attP (L54a)
attP (P11)

Figure 17: The integrated *cre* constructs do not resolve the *loxP* cassette in the RN6390::*loxP* strain *in vitro* in either an iron rich or an iron starved environment. CFUs were counted over a 12 hour time period in either an iron rich environment (TSB media; left) or iron starved media (TSB media + 1mM 2, 2-dipyridyl; right) and plated on agar with or without tetracycline. Both the promoterless *cre* (A) and P_{FhuCBG}::*cre* (B) constructs do not show any evidence of cassette resolution, as cell growth on plates containing tetracycline was similar to growth seen on plates with no tetracycline. Sample size, n=3.

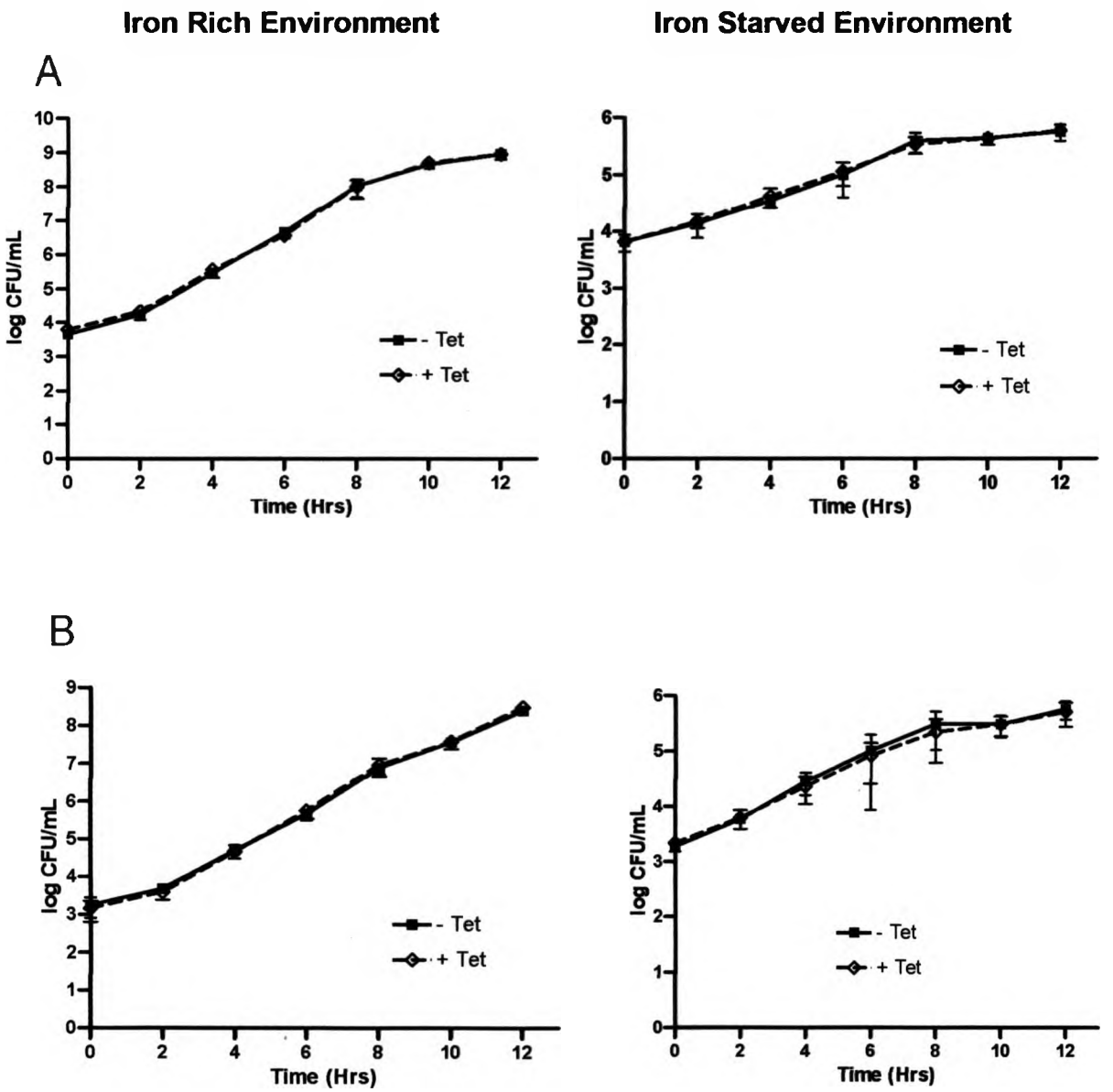

Iron Rich Environment
Iron Starved Environment
A
B
log CFU/mL
Time (Hrs)
- Tet
+ Tet

Table 6: *loxP* cassette resolution after 12 hours in *S. aureus* RN6390::*loxP*

Iron rich media	% Excision (SD)	Iron starved media	% Excision (SD)
Promoterless cre (multi-copy)	0	Promoterless cre (multi-copy)	0
P_{FhuCBG}::cre (multi-copy)	84.7	P_{FhuCBG}::cre (multi-copy)	99
P_{FhuCBG}::Δ1-27*cre*	3.4 (+/- 3.9)	P_{FhuCBG}::Δ1-27*cre*	1.2 (+/- 1.1)
Integrated Promoterless *cre*	17.2 (+/-15.5)	Integrated Promoterless *cre*	9.5 (+/-16.5)
Integrated P_{FhuCBG}::*cre*	0.4 (+/- 0.8)	Integrated P_{FhuCBG}::*cre*	16.7 (+/-16.4)

Chapter 4: Discussion

In the last decade, our understanding of *S. aureus* iron acquisition systems and their mechanisms of action have increased considerably. Critical nutrient systems such as these are essential to the survival of the bacteria, and could play an important role in creating new and innovative methods of treatment against *S. aureus* infections. IsdA and IsdH vaccine trials by Clarke *et al.* have shown promise in reducing *S. aureus* nasal carriage in a cotton rat model (20), while an IsdB vaccine is considered the leading staphylococcal vaccine, and is currently in human clinical trials (55). Such preventative treatments are of particular interest in recent years, especially in relation to *S. aureus* infections. This is due to the continued rise in not only *S. aureus* antibiotic resistant strains such as MRSA, but also in the increased prevalence of antibiotic resistant *S. aureus* strains capable of infecting both the immunocompromised and healthy outside the confines of a hospital. These strains, known as community-associated *Staphylococcus aureus* (CA-SA), in addition to having the variety of iron acquisition systems and virulence determinants found in most *S. aureus* strains, have also recently acquired genetic elements which can provide these strains with a survival advantage in particular environments. A primary example of this is the arginine catabolic mobile element (ACME). While ACME has been found in *Staphylococcus epidermidis*, a known proficient human skin colonizer, it has recently been identified in most CA-MRSA strains (31). The ACME cassette has been suggested to increase the proficiency of skin colonization as it contains the *arcABCD* loci, which has been shown to be important in arginine catabolism, allowing for increased survival in acidic environments such as human skin (31).

The emergence of these CA-SA strains harbouring new genetic elements makes it more imperative to further elucidate *S. aureus*-human host interactions. While extensive *in vitro* techniques have been developed in order to study and culture *S. aureus*, such studies are unable to completely replicate the complexity of the host environment. Thus, the nature of the host-pathogen interaction, particularly during the course of an infection, cannot be elucidated using such techniques alone. In order to study the *in vivo* environment, a variety of techniques, dubbed *in vivo* expression technologies, or IVETs, have been designed. IVET systems utilize a promoter trap strategy to identify bacterial promoters that are activated during an infection. A genomic library is built, and the DNA fragments are fused upstream of a promoterless reporter gene, which is needed in order for the bacteria to grow normally (or at all) under specific conditions. To date, four IVET selection variations have been developed: antibiotic-resistance, auxotrophic, system-specific, and recombination-IVET (RIVET). Due to its high sensitivity, the RIVET system is of particular interest, as it is the only IVET variation capable of identifying bacterial genes that are weakly or transiently expressed during an infection (15); RIVET is also the only IVET system capable of observing and studying temporal gene expression. This strategy utilizes the ability of recombinase proteins to bind to specific DNA sequences that flank at least one reporter gene, resulting in the excision of the reporter gene(s), and therefore a phenotype which can be screened for. Recombinase expression is driven only by bacterial DNA fragments with active

promoters, as these DNA fragments are fused upstream of the recombinase gene.

The purpose of this study was to build a RIVET based IVET system using the Cre recombinase/*loxP* recombination system in order to use it to determine the temporal expression of iron-regulated *S. aureus* promoters, first *in vitro*, then during infection. While an IVET system has already been developed by Lowe *et al.* for *S. aureus*, the system constructed here would be more robust and allow for fewer false positives owing to the incorporation of a counter selection marker in addition to a positive selection marker, an antibiotic resistance cassette. Thus, the *Bacillus subtilis* derived levansucrase-encoding *sacB* was selected as the counter selection marker for use in this RIVET system. *sacB* was selected because it has been used as a counter selective marker in a RIVET system previously (83) and has been shown to function in *S. aureus* (30). Thus, *sacB* was fused to the constitutive *lgt* promoter, creating the P_{lgt}::*sacB* construct. This fusion was used as part of the *loxP* reporter cassette, consisting of the aforementioned P_{lgt}::*sacB* fusion, and a tetracycline antibiotic resistance cassette flanked by two *loxP* sites. The *loxP* cassette was integrated into the *S. aureus* RN4220 chromosome using a single homologous recombination event and subsequently transduced to two other strains: RN6390 and Newman.

D'Elia *et al.* only showed the *sacB* sucrose sensitivity phenotype in a single *S. aureus* strain, RN4220 (30). Thus, it was important to test other *S. aureus* strains for a *sacB* phenotype to see if it could be used as a successful counter selection marker for the RIVET system. All four strains tested (RN4220,

RN6390, SH1000, Newman) that had the *sacB* carrying plasmid pCM6 showed definitive sucrose sensitivity when the media sucrose concentration was at least 10%. However, this was not the case when the *sacB* construct was integrated into the *S. aureus* chromosome. Of the three strains tested, only the RN6390::*loxP* strain showed any sensitivity to sucrose. Thus, a different approach was used in order to verify the *sacB* phenotype in each of these strains. The RN4220::*loxP*, RN6390::*loxP* and Newman::*loxP* and their respective wild-type strains were grown in the presence or absence of sucrose in liquid media over a 48 hour period. Once again, only the RN6390::*loxP* strain showed sensitivity to sucrose. However, the sucrose sensitivity phenotype was much more obvious from the liquid culture results, as little to no RN6390::*loxP* growth was observed within the first 16 hours. This was expected, as *sacB* induced cell death is believed to be reliant on the continued build up of levan, the end product of levansucrase fructosyl group polymerization and sucrose hydrolysis (10). Therefore, as time progresses, levan should continually build up in the cells, resulting in cell death. Unexpectedly, culture optical density (OD) began to increase after the 16 hour mark up until the 32 hour mark, at which point cell growth began to decrease. It is unknown why this increased culture OD is suddenly seen after 16 hours of incubation; further investigation is required in order to elucidate these results. The sudden drop in growth after 32 hours could be attributed to the cells clumping together inside the wells of the bioscreen plate, resulting in an artificial growth curve drop. This could be verified by culturing RN6390::*loxP* cells in liquid media with or without sucrose and plating

aliquots from either environment over a 48 hour period, and observing cell growth on each plate.

Once the *sacB* phenotype was verified in the RN6390 background, plasmid-based *cre* constructs were built in order to determine temporal expression of iron-regulated *S. aureus* promoters in RN6390::*loxP*. The first such promoter chosen for study was that of *fhuCBG*, since *fhuC* is critical to all characterized iron transport systems in *S. aureus* (5, 39). As expected, no *loxP* cassette resolution was seen in cells carrying either negative controls (promoterless *cre* - pCM14; P_{FhuCBG}::*cre*Δ1-27 – pCM21) after 12 hours in either the iron rich or iron starved environment. However, resolution of the *loxP* cassette was readily apparent with cells harbouring the P_{FhuCBG}::*cre* construct (pCM15) in both environments. These data indirectly confirm that cassette resolution is due to *cre* expression, driven by the *fhuCBG* promoter. It should be noted that direct verification of *cre* expression was attempted using western blot analysis, however the anti-cre antibody used was not sensitive enough to identify cre protein (data not shown). Also, while cassette resolution was expected from cells with pCM15 cultured in an iron starved environment (due to the iron-regulated nature of the *fhuCBG* promoter), cassette resolution in the iron rich media was not; this can be attributed to a gene dosage effect due to the multi-copy plasmid carrying the P_{FhuCBG}::*cre* construct. This can result in titration of the Fur protein, as there is an insufficient amount of Fur in the cell in order to properly bind to the multi-copy *fhuCBG* Fur box resulting in an inability to repress all *cre* expression. This may also be due to high basal expression levels of

fhuCBG in the *in vitro* iron rich environment, and due to the highly sensitive nature of RIVET, these expression levels result in resolution of the cassette in the iron rich environment.

In order to eliminate the issue of gene dosage, the promoterless *cre* and P_{FhuCBG}::*cre* fusions were integrated into RN6390::*loxP* and tested for cassette resolution. Unfortunately, colonies with the integrated P_{FhuCBG}::*cre* created no significant cassette excision, regardless of what media they were cultured in over a 12 hour time period. This indicates little to no Cre was expressed by the single-copy P_{FhuCBG}::*cre* construct in the iron starved environment. However, this data may be skewed due to 2, 2'-dipyridyl, the chelator used to create the iron starved media. While the Cre*loxP* system is capable of recombination without any additional reagents, studies have shown that the presence of Mg^{2+} in Cre*loxP* reactions results in a 25% increase in recombination (2). Therefore, chelation of Mg^{2+} from the media may potentially result in a reduction of recombination levels in the iron starved 2,2'-dipyridyl containing environment. To eliminate this problem, future *in vitro* studies should supplement the chelated media with Mg^{2+}, as this may increase the frequency of cassette resolution. Also, EDDHA might have been a better choice of chelator for use in this study, as it is unable to enter the bacterial cell, unlike 2,2'-dipyridyl. Future *in vitro* studies using Cre*loxP* using an iron chelator should take this into account.

While single-copy versions of the *cre* constructs were used in an attempt to eliminate potential gene dosage effects as well as reduce the effect of potentially high basal expression levels of *fhuCBG*, other strategies can be

employed to combat these problems. One such strategy requires that the Shine-Dalgarno sequence of *cre* be modified so as to reduce *cre* transcriptional levels, resulting in less sensitive selection (57); a recent study by Malone *et al.* observed the dramatic affects on gene transcription in *S. aureus* by making slight changes to the Shine-Dalgarno sequence (67). An additional strategy could involve using another iron-regulated promoter (preferably one in which basal expression levels are low) fused to *cre*, and testing it against the P_{FhuCBG}::*cre* construct. Such a construct, a P_{HtsA}::*cre* fusion, has been built, however due to time constraints was not tested.

Future studies using this RIVET system benefit from its versatility. Both reporter genes of the *loxP* cassette can easily be replaced by other reporter genes, given the modular nature of the cassette. The *loxP* cassette also contains two unique restriction sites (*Sma*I and *Xho*I) that can be used to incorporate additional reporter genes. One potential reporter gene that could be implemented into this cassette would be that of a fluorescent gene; recent studies have generated codon-optimized cyan- , yellow- and red fluorescent proteins for *S. aureus* (67, 98). The addition of a fluorescent gene would provide an additional screen for identifying individual cells in which the *loxP* cassette has been excised.

Due to its versatility, a variety of future experiments can be performed using this RIVET system. The most prominent of these experiments would be the identification of *ivi* genes using this system in combination with a genomic DNA library. Bacterial DNA fragments can be fused upstream of *cre*, and

subsequently be screened to first eliminate promoters activated *in vitro*. Those fragments that remain inactive *in vitro* can then be passaged through a variety of hosts, and *ivi* genes can then be identified by screening for a specific phenotype. DNA fragments containing active promoters of unknown function can then be sequenced and characterized. The RIVET system could also be used to develop experiments to determine the temporal expression of specific *ivi* genes over the course of an infection. Such experiments can not only identify potential new virulence determinants, but can also be used to further elucidate host-*S. aureus* interactions through the analysis of when select genes are turned on over the course of an infection.

This thesis describes the construction of what could become a potent and versatile RIVET system that has been successfully shown to work in *S. aureus in vitro*. This system has considerable potential to identify *in vitro* as well as *ivi* genes expressed in a variety of environments. It can also be used to study temporal expression of *ivi* genes over the course of an infection. Such studies could significantly add to our understanding of how *S. aureus* interacts with its host *in vivo*, as well as aiding in the development of new therapeutic treatments against *S. aureus* infections.

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