Exploring the pathways underlying the growth of *Staphylococcus aureus* in acidic stress conditions

Catrin Margaret Beetham

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Department of Infectious Disease

Imperial College London

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Statement of Originality

I certify that, unless stated otherwise, all work presented in this PhD thesis is my own. Work which has been performed by other authors has been appropriately referenced and acknowledged in the text.

Construction and propagation of the Tn libraries for the Tn-Seq experiments and generation of lists of *S. aureus* genes and corresponding ratios were performed by Professor Angelika Gründling and Dr. Christopher Schuster. Circular plots were generated by C. Schuster. Complementation strains were generated by A. Gründling. Whole genome sequencing was performed at the MRC London Institute for Medical Sciences in Hammersmith Hospital. Sequencing analysis for the suppressor strains with increased growth in CDM was performed by A. Gründling. Mass spectroscopy experiments were performed by Dr. Igor Kviatkovski, and Dr. Elena Chekmeneva, Dr. Carolina Sands and Lynn Maslen from the National Phenome Centre. qRT-PCR experiments were performed by I. Kviatkovski.

Some of the figures and text from the results and discussion in Chapters 2, 4, 5, 6 and 7 have been published in Beetham et al., (2024), which was published under a Creative Commons Attribution 4.0 International (CC BY) license (https://creativecommons.org/licenses/by/4.0/). These have been referenced as Beetham et al., (2024).

Catrin Beetham

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Abstract

Staphylococcus aureus is an opportunistic pathogen capable of causing a range of human infections. During infection *S. aureus* encounters several hostile environments, including an acidic environment which is found on the skin, within macrophages and in the vagina. However, little is known about how *S. aureus* detects and responds to low pH. In this thesis, I explore the genes and pathways utilised by *S. aureus* to grow under acidic stress conditions. Previous work has shown that the signalling nucleotide c-di-AMP affects the ability of *S. aureus* to grow at low pH. Here I show that this does not occur due to a dysregulation of the urease enzyme and production of ammonia from urea. Secondly, I utilised a Transposon sequencing approach to identify genes which are either essential or detrimental for the fitness of *S. aureus* under acidic stress conditions. I identified 31 genes which are essential for the fitness of *S. aureus* at low pH, and subsequent follow up experiments revealed that individual inactivation of most of these genes conferred a growth defect at low pH. Finally, the Tn-Seq approach identified several novel genes and pathways which have previously not been associated with the acidic stress response of *S. aureus.* One such gene was *0846*, which I show is a previously uncharacterised histidine transporter. Further experiments determined that histidine and its uptake via 0846 is essential for the growth of *S. aureus* at low pH, which I hypothesise to be because it eliminates the need for the bacteria to synthesise histidine which may be detrimental for growth under acid stress conditions. Taken together, the data presented in this thesis expands our knowledge of the acidic stress response of *S. aureus*.

How does Staph grow in an acidic condition?

To answer this was my PhD ambition.

In the lab for four years,

With blood, sweat and tears,

I now present my thesis submission!

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ADI	Arginine deaminase
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMP	Adenosine monophosphate
Amp	Ampicillin
AMP	Antimicrobial peptide
ATP	Adenosine triphosphate
BCAA	Branched chain amino acid
Cam	Chloramphenicol
CAMP	Cationic antimicrobial peptide
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
c-di-AMP	Cyclic di-adenosine monophosphate
CDM	Chemically defined medium
CFU	Colony forming units
Comp.	Complemented
CPA	Cation proton antiporter
CPM	Counts per minute
CTD	C-terminal domain
D-Ala	D-alanine
$DiOC_2(3)$	3,3'-diethyloxacarbocyanine iodide
DNA	Deoxyribonucleic acid
ENA	European nucleotide archive
Erm	Erythromycin
EV	Empty vector
FACS	Fluorescence activated call sorting
FFA	Free fatty acid
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GDP	Guanosine diphosphate
GlcNAc	N-Acetylglucosamine
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HITS	High-throughput insertion tracking by deep sequencing
IMP	Inosine monophosphate
INSeq	Insertion sequencing
LB	Lysogeny broth
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide

MRSA	Methicillin resistant *Staphylococcus aureus*
MurNAc	N-Acteylmuramic acid
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NGS	Next generation sequencing
NO	Nitric oxide
NTD	N-terminal domain
NTML	Nebraska transposon mutant library
pApA	5'-phosphadenylyl-adenosine
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Potential of hydrogen
PMF	Proton motive force
ppGpp	Guanosine tetraphosphate
pppGpp	Guanosine pentaphosphate
PRPP	Phosphoribosyl pyrophosphate
qRT-PCR	Quantitative reverse transcriptase PCR
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Room temperature
SC	Stratum corneum
SCC*mec*	Staphyloccal cassette chromosome *mec* element
SNP	Single nucleotide polymorphism
SSTI	Skin and soft tissue infections
TCA	Tricarboxylic acid
Tn	Transposon
Tn-Seq	Transposon sequencing
Tra-DIS	Transposon directed insertion sequencing
TSA	Tryptic soy agar
TSB	Tryptic soy broth
V-ATPase	Vacuolar ATPase
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRE	Vancomycin resistance enterococcus
VRSA	Vancomycin resistant *Staphylococcus aureus*
WT	Wild-type
WTA	Wall teichoic acid

Chapter 1 : Introduction

1.1 *Staphylococcus aureus*

1.1.1 General characteristics

Staphylococcus aureus is a member of the *Staphylococcae* family of bacteria in the Bacillota phylum, with the bacterial species characterised by their spherical shape and grape-like cluster formation. There are 50 other species within the *Staphylococcae* genus, all of which are Gram-positive facultative anaerobes. *S. aureus* is the most clinically relevant species in this group, and is differentiated from other *Staphylococcae* species by its production of the coagulase enzyme which causes blood clotting, and its production of staphyloxanthin, the yellow pigment for which *S. aureus* was named.

The genome of *S. aureus*, first sequenced in 2001, is approximately 2.8 mega bases in size (Kuroda et al., 2001). It contains around 2600 open reading frames and has a low G+C content of around 33% (Kuroda et al., 2001). Furthermore, it is estimated that around 44% of the staphylococcal genome is strain variable, mediated by mobile genetic elements such as plasmids, transposons, genomic islands, pathogenicity islands, chromosome cassettes, or phages (Bosi et al., 2016). Mobile genetic elements are estimated to make up to 20% of the genome (reviewed in Copin et al., 2018), and often encode virulence factors and antibiotic resistance genes.

1.1.2 Infections and treatment

S. aureus is capable of colonizing several sites in the human body as a commensal bacterium, including the anterior nares of the nasal passage and the skin, with the former being the more common (Williams, 1963). It is estimated that approximately 20% of the human population are persistent carriers, and a further 30% are transient carriers who carry various strains of *S. aureus* intermittently (reviewed in Kluytmans et al., 1997). Approximately 50% of the human population are non-carriers who never harbour strains of *S. aureus* (reviewed in Kluytmans et al., 1997).

As well as being a commensal bacterium, *S. aureus* is capable of causing a range of infections in humans (reviewed in Lowy, 1998). Carriers of *S. aureus* have a greater risk of developing an *S. aureus* infection, and the strain of *S. aureus* causing the disease often correlates with the carrier strain (von Eiff et al., 2001; Wertheim et al., 2004). This indicates that *S. aureus* causes opportunistic infections, often due to breaches in the skin barrier or immunodeficiencies.

Alternatively, *S. aureus* is commonly transmitted via healthcare settings, and is a leading cause of healthcare-associated infections (Haque et al., 2018). This transmission can occur via intravascular devices directly to the bloodstream (Fitzgerald et al., 2011; Moore et al., 2008), from other patients (Grundmann et al., 2005; Moore et al., 2008), or from healthcare workers (Price et al., 2017).

S. aureus is the most common causative agent of skin and soft tissue infections (SSTIs), accounting for over 80% of cases as reported in some studies (Ray et al., 2013). SSTIs range from the more benign impetigo and cellulitis to the life-threatening necrotizing fasciitis (reviewed in Hatlen & Miller, 2021). In 2019 it was estimated that *S. aureus* SSTIs were responsible for approximately 37,500 deaths worldwide (Collaborators, 2022).

If *S. aureus* breaches the skin barrier, either through an SSTI, an intravascular event such as catheterization, or following surgery, the bacteria can cause bacteraemia. *S. aureus* is one of the most common causes of bacterial bloodstream infections (Anderson et al., 2014; Kern & Rieg, 2020), with a mortality rate ranging from 10% to 35% depending on the length of infection (Bai et al., 2022). There were over 12,000 cases of *S. aureus* bacteraemia in the United Kingdom from 2021 to 2022, an increase of 31% from the same period 10 years ago (UK Health Security Agency, 2022). Furthermore, a recent study has shown that *S. aureus* was the most common organism isolated as a co-infection in patients with COVID-19 (Langford et al., 2022), an observation supported by the increase in hospital associated *S. aureus* bacteraemia cases in both the USA and the UK in 2020 compared to 2019 (UK Health Security Agency, 2022; Weiner-Lastinger et al., 2022). It has been estimated that up to 75% of *S. aureus* bacteraemia cases develop secondary infections, known as metastatic infections (reviewed in Horino & Hori, 2020). These include infective endocarditis, bone and joint infections, and kidney infections, and require longer treatment plans (Liu et al., 2011). *S. aureus* can also cause other infections, such as urinary tract infections, pneumonia, meningitis, ocular infections, nephritis, and toxic shock syndrome (reviewed in Lowy, 1998).

In general, the first-line antibiotics used to treat staphylococcal infections are the β-lactams, a class of antibiotics which work by weakening the bacterial cell wall and leading to cell lysis and death (Fig. 1.1). The bacterial cell wall is made up of the polymer named peptidoglycan, which is comprised of glycan chains of alternating N-acetylglucosamine (GlcNAc) and N-

acetylmuramic acid (MurNAc) residues. These chains are cross-linked by short stem peptides attached to MurNAc. A key class of enzymes in this process are the penicillin-binding proteins (PBPs), which catalyse the polymerisation of the glycan chains (transglycosylation) and the cross-linking of the peptide stems (transpeptidation) (reviewed in Sauvage et al., 2008). *S. aureus* encodes at least four PBPs, all of which possess transpeptidase activity, but only one, PBP2, possesses both transglycosylase and transpeptidase activity. β-lactam antibiotics contain a β-lactam ring which mimics the end of the short peptide stem bound by PBPs for their transpeptidase activity. The PBPs therefore bind to β-lactam antibiotics, and are unable to catalyse the cross-linking of the glycan chains, which leads to a weakened cell wall.

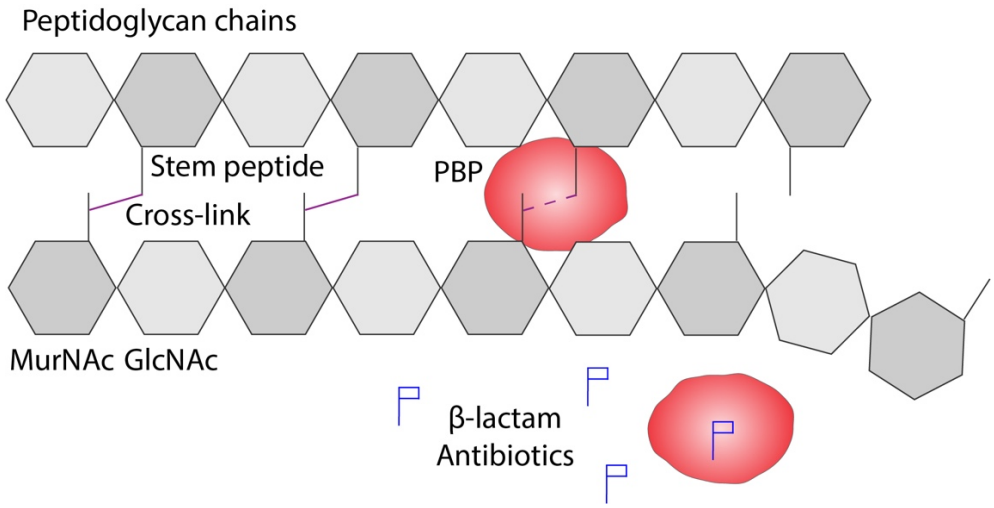


Figure 1.1: Schematic representation of the mechanism of action of β-lactam antibiotics
β-lactam antibiotics work by binding to and inhibiting PBPs. This prevents the formation of cross-links between peptidoglycan chains, weakening the bacterial cell wall.

Despite this, *S. aureus* infections are often associated with high rates of treatment failure. One reason for this failure is the emergence of antibiotic resistance in some strains of *S. aureus*. Resistance was initially caused by the production of penicillinase, an enzyme that cleaves the β-lactam ring of penicillin so that it cannot bind and inactivate penicillin-binding proteins (Barber & Rozwadowska-Dowzenko, 1948; Kirby, 1944). To combat this, penicillinase-resistant penicillin-based antibiotics such as methicillin and oxacillin were developed (Douthwaite et al., 1961). However, a new mechanism of resistance emerged that conferred resistance to almost all β-lactam antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are characterised by the presence of the staphylococcal cassette chromosome *mec* (SCC*mec*) genetic island. This mobile genetic element has been

horizontally-acquired, and often confers resistance to multiple antibiotics (Katayama et al., 2000). The SCC*mec* locus contains the *mecA* gene encoding an alternative penicillin-binding protein, PBP2a, which has a low affinity for β-lactams and enables transpeptidase activity to continue in the presence of β-lactam antibiotics (Hartman & Tomasz, 1984; Song et al., 1987; Utsui & Yokota, 1985). MRSA first emerged and spread within hospitals, where it accounted for more than 50% of staphylococcal isolates in the early 2000s (Klevens et al., 2006). However, in the US and other parts of the world, community-associated MRSA strains have become established, in part due to greater virulence and transmission rates than hospital-associated strains (Rudkin et al., 2012). A major contributor to the rise of community-associated MRSA strains in the USA are the USA300 strains (Diep et al., 2006).

While the β-lactam antibiotics are still used as a first-line antibiotic for the most common *S. aureus* infections such as SSTIs, they are less effective against MRSA strains. In cases where MRSA is proposed to account for the causative strain of *S. aureus*, vancomycin is often used (Fig. 1.2) (Brown et al., 2021). Vancomycin also works by targeting and inhibiting peptidoglycan biosynthesis. This antibiotic binds to the end of the short peptide stem on the MurNAc residues, thus preventing the binding of PBPs to catalyse transpeptidation (reviewed in Muhlberg et al., 2020).

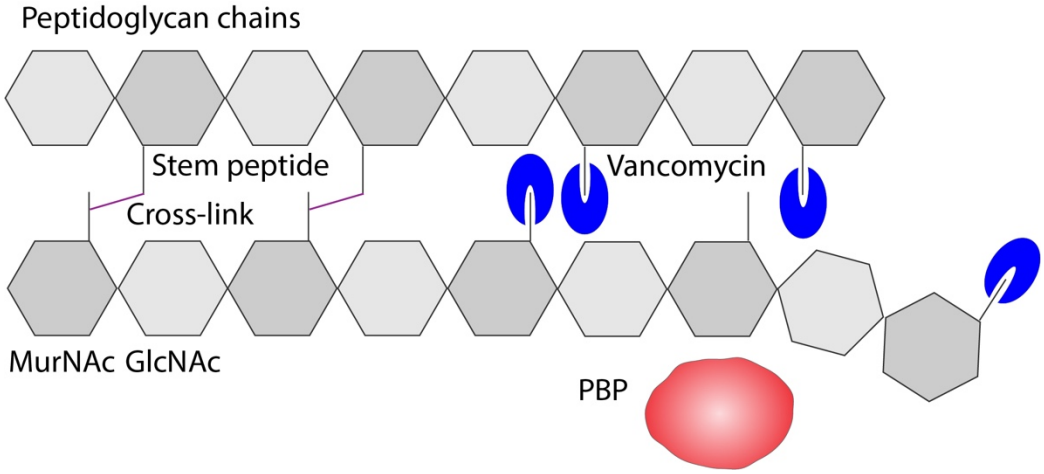


Figure 1.2: Schematic representation of the mechanism of action of vancomycin
Vancomycin binds to the end of the stem peptide which blocks the PBP from binding. This prevents the formation of cross-links between peptidoglycan chains, weakening the bacterial cell wall.

While vancomycin is effective against MRSA, some strains of *S. aureus* have acquired resistance to the antibiotic. Vancomycin-intermediate *Staphylococcus aureus* (VISA) strains

contain multiple mutations whose collective result is a reduced susceptibility to vancomycin (reviewed in McGuinness et al., 2017). Alternatively, vancomycin-resistant *Staphylococcus aureus* (VRSA) strains have complete vancomycin resistance due to the presence of the vancomycin-resistant enterococci (VRE) plasmid (reviewed in McGuinness et al., 2017). This plasmid, which is acquired from enterococcus species, contains the *vanA* operon and allows the incorporation of alternative amino acids onto the short peptide stem (reviewed in McGuinness et al., 2017). The result is that vancomycin can no longer bind to the short peptide stem, therefore allowing transpeptidation and thus peptidoglycan biosynthesis to occur (reviewed in McGuinness et al., 2017).

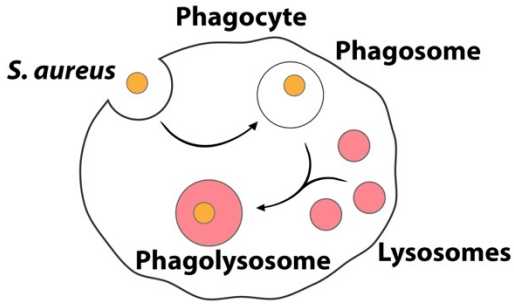
1.1.3 *S. aureus* as a facultative intracellular pathogen

Another major cause of treatment failure of *S. aureus* infections is the prevalence of persistent or recurrent infections. One reason for this is the ability of *S. aureus* to invade and replicate within host cells (reviewed in Garzoni & Kelley, 2011; Tuchscherr et al., 2011). Despite *S. aureus* being traditionally thought of as an extracellular pathogen, more recent evidence has implicated that *S. aureus* is also a facultative intracellular pathogen (reviewed in Horn et al., 2018). Within host cells the bacteria are protected from the host immune system or antibiotic treatment, and thus can act as a reservoir of *S. aureus* for future recurrent infections (reviewed in Horn et al., 2018)

S. aureus has been shown to be internalized by a range of host cells including both non-professional and professional phagocytic cells. The former includes epithelial cells, endothelial cells, osteoblasts, fibroblasts, and keratinocytes (reviewed in Al Kindi et al., 2019; Horn et al., 2018; Rodrigues Lopes et al., 2022; Strobel et al., 2016). Uptake in non-professional phagocytes occurs when bacterial adhesins bind to host cell receptors such as the $\alpha_5\beta_1$ integrin. These receptors are mostly expressed on host cells following tissue damage, such as that which occurs after a wound. However, as *S. aureus* can induce tissue damage itself by producing toxins such as the staphylococcal α-toxin, it implies that the bacteria can regulate their own uptake (reviewed in Horn et al., 2018). Alternatively, *S. aureus* can be phagocytosed by professional phagocytes, including neutrophils and macrophages.

Once phagocytosed, the bacteria are enclosed within a membrane bound vacuole called a phagosome. The phagosome undergoes a maturation process and fuses with lysosomes to form a phagolysosome. During this process the phagolysosome obtains antimicrobial capacity due to its acidification via the vacuolar ATPase (V-ATPase) enzymes, the production of reactive oxygen species (ROS) via a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, the production of nitric oxide (NO) radicals via NO synthetase, the introduction of proteases and hydrolases such as cathepsins and the introduction of cationic peptides (reviewed in Haas, 2007). A further antimicrobial pathway is present in neutrophils, where the phagosomal myeloperoxidase enzyme works with the pathways mentioned above to produce hypochlorous acid, chloramines, hydroxyl radicals and ozone (reviewed in Haas, 2007).

Despite the hostile environment of the phagolysosome, *S. aureus* has been shown to use a range of pathways to survive and replicate within host cells (Fig. 1.3) (reviewed in Horn et al., 2018). Firstly, the bacteria can prevent the fusion of the phagosome with the lysosome, thereby reducing the antimicrobial capacity of the phagolysosome (reviewed in Horn et al., 2018). Secondly, *S. aureus* has been shown to escape from the vacuole and to replicate in the cytoplasm (reviewed in Horn et al., 2018). Finally, *S. aureus* can survive and replicate within the hostile environment of the phagolysosome, despite its antimicrobial capacity (reviewed in Horn et al., 2018). This requires the bacteria to detect and respond to the extreme environmental conditions, including low pH, ROS, NO radicals, and antimicrobial peptides (AMPs). The antimicrobial capacity of macrophages, for example, is reduced over time (Jubrail et al., 2016). Therefore, if enough bacteria are ingested by the macrophage, they only need to survive until the macrophage is exhausted (Jubrail et al., 2016).



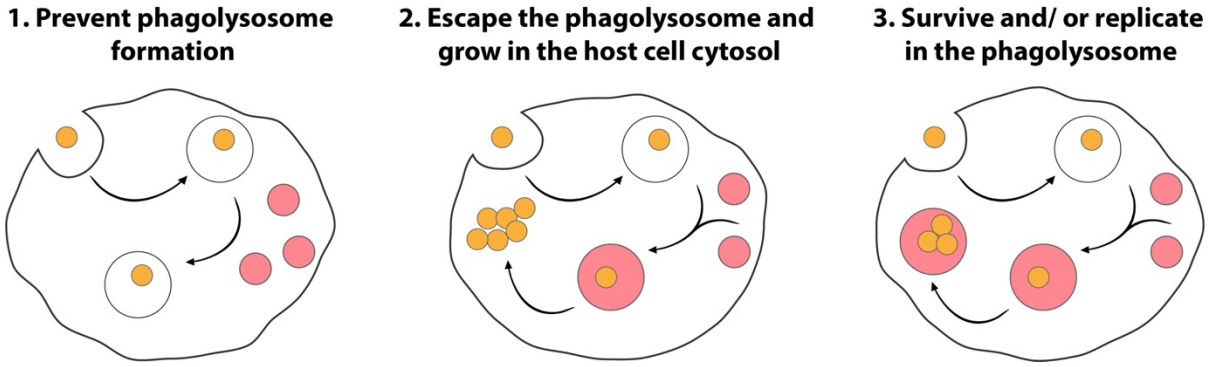


Figure 1.3: Schematic representation of the strategies shown to be used by *S. aureus* to survive and replicate within host cells
Once taken up by a phagocytic cell, *S. aureus* can (1) prevent the fusion of the phagosome and lysosomes, (2) escape the phagolysosome and replicate within the phagocyte cytosol, or (3) survive and/or replicate within the phagolysosome.

As well as acting as a reservoir of bacteria leading to recurrent and persistent infections, the ability of *S. aureus* to survive and replicate within professional phagocytic cells has been suggested to play a role in seeding disseminated infections (Gresham et al., 2000). *S. aureus* containing neutrophils injected into non-infected mice have been shown to induce disseminated *S. aureus* infections (Gresham et al., 2000). Furthermore, mutant mice whose neutrophils have a reduced ability to migrate across endothelial cells show increased survival and a reduced bacterial burden following *S. aureus* infection (Gresham et al., 2000). By contrast these mice display a reduced survival following *Escherichia coli* infection (Lindberg et al., 1996). This shows that bacterial dissemination via neutrophils is not a general consequence of bacterial infection and demonstrates how the facultative intracellular lifestyle of *S. aureus* contributes to its pathogenesis.

1.2 Acidic pH in the human body

As described above, *S. aureus* can cause a wide range of infections. This requires an ability to replicate and cause disease in a variety of conditions found in different host niches. These range from the skin for SSTIs, the blood for bacteraemia, and within phagolysosomes for intracellular replication to seed disseminated infections. One such condition encountered by *S. aureus* in several niches is a low pH. *S. aureus* is capable of growth in a wide range of pH conditions from 4.0 to 10.0 (ICMSF et al., 1996). In this section, I will describe where *S. aureus* encounters a low pH, what type of acids are found within these niches, and how low pH causes bacterial damage.

1.2.1 Acidic niches in the human body

Physiological pH is stated as being 7.35 – 7.45, which is defined as the pH of blood and interstitial fluid. However, there are several niches within the human body which have an acidic pH. The largest in size of these is the skin, which has a pH of 4-6. This pH range can differ depending on either endogenous or exogenous factors (reviewed in Ali & Yosipovitch, 2013; reviewed in Schmid-Wendtner & Korting, 2006). Endogenous factors include age, location, genetics, and ethnicity, and exogenous factors include cosmetics, soaps, or topical antibiotics (reviewed in Ali & Yosipovitch, 2013; reviewed in Schmid-Wendtner & Korting, 2006). The skin has several layers, and it is only the upper layer, or the stratum corneum (SC), which is acidic (Fig. 1.4).

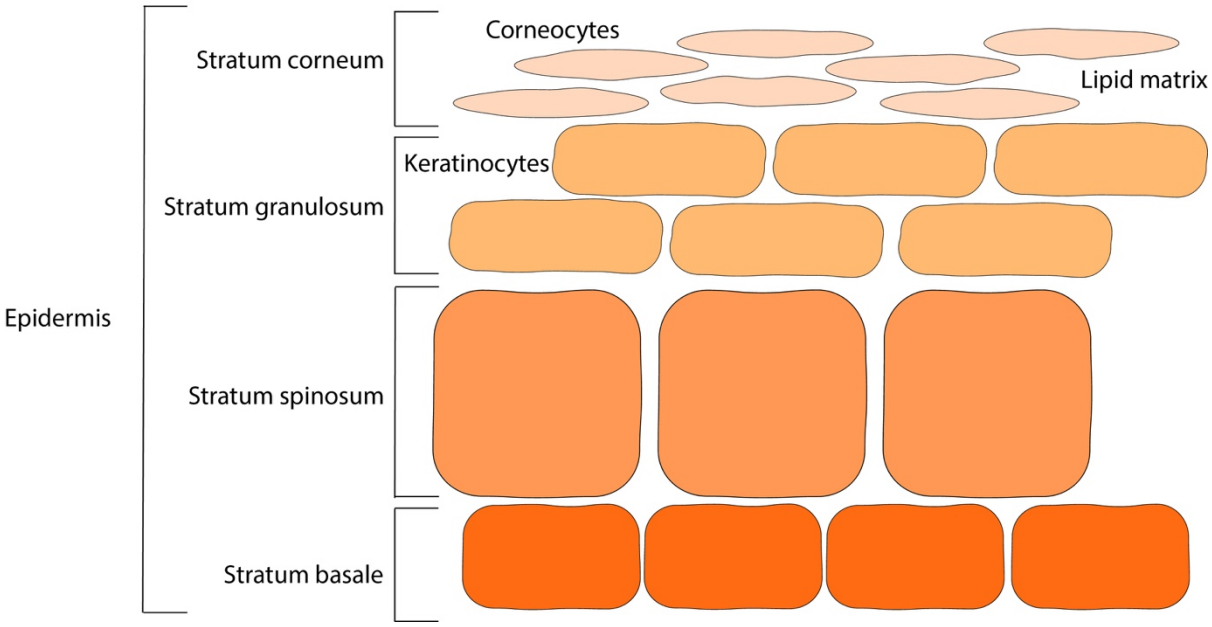


Figure 1.4: Schematic representation of the layers of the skin epidermis
The skin epidermis is composed of the statum basale, statum spinosum, stratum granulosum, and stratum corneum.

The acidic pH of the SC was discovered in 1928 when it was thought to have a solely antimicrobial purpose and the phrase 'acid mantle' was coined (reviewed in Surber et al., 2018). Since then, several other functions have been associated with the SC's acidic pH, including preventing water loss and dampening inflammation (reviewed in Surber et al., 2018).

The SC is built of 'bricks' and 'mortar', with overlapping bricks of dead keratinocyte cells called corneocytes held together by a lipid matrix (Fig. 1.4). This matrix consists of a mixture of cholesterol, ceramides, and free fatty acids (FFAs). Initially, it was thought that the low pH of the SC was maintained by exogenous sources, such as microbial metabolites (reviewed in Chan & Mauro, 2011). However, endogenous sources of acidification in the SC are now known (reviewed in Chan & Mauro, 2011; reviewed in Elias, 2017). The sodium-proton antiporter NHE-1 is important for the acidification of the extracellular niches of the lower SC, with transgenic mice lacking the corresponding gene having more a alkaline SC (Behne et al., 2002). Furthermore, the breakdown of phospholipids into FFAs by secretory phospholipases contributes to the acidic pH of the extracellular lipid matrix. When secretory phospholipase inhibitors were topically applied to mice, their SC displayed an increased pH (Fluhr et al., 2001).

The low pH of the SC favours growth and adhesion of protective bacterial species in the skin microbiome (Lambers et al., 2006), while having an antimicrobial effect on pathogenic bacteria such as *S. aureus* (Del Rosso & Levin, 2011). For example, using a low pH soap cleanser to maintain the acidic pH of the SC resulted in more clearance of *S. aureus* from the skin compared to a neutral pH soap cleanser (Yue et al., 2023). Furthermore, *S. aureus* counts in atopic skin lesions can be reduced by topical application of acid electrolyte water (Sasai-Takedatsu et al., 1997). Despite this, the prevalence of *S. aureus* in SSTIs indicates that enough of the bacteria can cause disease under these conditions. This shows that the acidic niche of the skin is one in which *S. aureus* can grow.

Another niche in the human body which maintains an acidic pH is the vagina, which has a pH of around 4-4.5. This pH changes over the reproductive cycle, as well as during puberty and menopause (reviewed in Godha et al., 2018). The human vagina has a lower pH than other mammals, which have a higher range of 5.4-7.8 (Miller et al., 2016). This is likely due to the presence of *Lactobacillus* species in the human vagina, which make up over 70% of the human vaginal microbiome, while making up less than 1% of other mammalian vaginal microbiomes (Yildirim et al., 2014). Vaginal epithelial cells have a higher glycogen concentration than other cells, which is then released extracellularly (reviewed in Godha et al., 2018). Lactic acid bacteria such as Lactobacilli metabolize the extracellular glycogen and produce lactic acid as a by-product. Furthermore, the vaginal low pH is also maintained by proton secretion out of the vaginal epithelial cells by V-H^+-ATPases (Gorodeski, 2005; Gorodeski et al., 2005). While the function of the low pH of the vagina is unknown, it has been proposed to have a protective role and prevent the growth of unwanted bacteria. This includes *S. aureus*, which is one of the most commonly isolated species from disrupted vaginal microbiomes (Donders et al., 2017; Ghiasi et al., 2014). This shows that *S. aureus* can grow within the acidic niche of the vagina when the normal microbiome is disrupted.

Finally, another important niche in which *S. aureus* can grow and cause disease which has an acidic pH is the phagolysosome. As described previously in this Chapter, phagolysosomes are a mechanism used by both professional and non-professional phagocytes to try and eliminate bacteria. After phagocytosis, the bacterium is enclosed within a phagosome. This phagosome undergoes a maturation process by fusing with endosomes and lysosomes to form a phagolysosome (Fig. 1.3). The phagolysosome is a hostile environment for bacteria, containing

hydrolases, ROS, and AMPs. Furthermore, over the maturation process the pH of the phagosome decreases until it reaches a pH in the range of 4-5 (Lee et al., 2020).

The low pH of phagolysosomes is maintained by V-ATPase enzymes (reviewed in Kissing et al., 2018). V-ATPase is composed of 14 different subunits (reviewed in Cotter et al., 2015). These are organised into the cytoplasmic V_1 sector which hydrolyses adenosine triphosphate (ATP) into adenosine diphosphate (ADP), and the membrane-integral V_0 sector which uses the energy from ATP hydrolysis to pump protons against the electrochemical gradient into the lysosome. As more and more protons are imported, the lysosome becomes more positively charged. Therefore, to allow for further acidification against the charge gradient, the transport of other cations and anions is regulated to counter the increasing positive charge. For example, negatively charged chloride ions are imported via the ClC channels (Mindell, 2012). As well as ion transport, lysosome acidification can be regulated by other factors. These include abundance of V-ATPase, subunit assembly of the V-ATPase, and leakage of protons out of the lysosome (reviewed in Kissing et al., 2018). The importance of the V-ATPase and lysosome acidification is shown by the inability of mouse embryos to survive past early development when lacking a functional enzyme (Sun-Wada et al., 2000).

The acidification of the lysosome and, further, the phagolysosome has several antimicrobial functions (reviewed in Kissing et al., 2018). Firstly, the acidic pH is required for optimal activity of several key hydrolases. For example, it has been shown that fusion of hydrolase-containing lysosomes to phagosomes is reduced upon treatment with V-ATPase inhibitors (Claus et al., 1998). Furthermore, the activity of key hydrolases such as cathepsin-B and β-hexosaminidase is reduced or eliminated upon V-ATPase inhibition (Claus et al., 1998). Secondly, the acidic pH of the phagolysosome promotes the formation of certain ROS elements during the oxidative burst. Once mature, the phagolysosome produces ROS via the NADPH oxidase complex, in particular superoxides ($\bullet O_2^-$). However, due to their charged nature superoxides cannot easily cross the bacterial membrane. Under acidic conditions, the negatively charged superoxide radical binds to positively charged protons to produce H_2O_2. As hydrogen peroxide is uncharged, it can more easily cross membranes and can thus cause more damage inside the bacteria. Finally, the acidic pH of the phagolysosome can itself be antimicrobial. This is supported by the decreased killing of phagocytosed *S. aureus* by alveolar macrophages

treated with a V-ATPase inhibitor (Bidani et al., 2000). However, it is unclear whether the low pH is itself antimicrobial, or whether it induces other antimicrobial effects within the phagolysosome, such as those described above (reviewed in Kissing et al., 2018).

While it is clear that the phagolysosome maintains an acidic pH, and that this has benefits for the phagocyte, what is less clear is whether this is a niche within which *S. aureus* grows. There are many strategies used by intracellular bacteria to avoid or tolerate the antimicrobial defences of the phagolysosome (reviewed in Kissing et al., 2018). For example, *Legionella pneumophila* prevents full phagolysosome maturation or inhibits the V-ATPase enzyme (reviewed in Newton et al., 2010; Xu et al., 2010). *Coxiella burnetti* slows down phagolysosomal maturation so that the bacterium can adapt (reviewed in Voth & Heinzen, 2007). Other bacteria escape from the phagosome to replicate within the phagocyte cytosol. These include *Listeria monocytogenes* (reviewed in Hamon et al., 2006) and *Shigella flexneri* (reviewed in Mellouk & Enninga, 2016). While it is known that *S. aureus* is capable of surviving and growing within host cells, there is contrasting evidence for whether this occurs within an acidic phagolysosome, a non-acidic phagolysosome, or in the phagocyte cytosol as described below.

Several studies have shown that *S. aureus* is found in professional and non-professional phagocytes in vacuoles that associate with markers demonstrating mature, acidic compartments. These include the LAMP-1 glycoprotein, which is a marker of late-stage development of phagolysosomes, and the LysoTracker dye, which fluoresces at low pH. This association has been demonstrated for both clinical and laboratory strains, including USA300 (Flannagan et al., 2016; Grosz et al., 2014; Tranchemontagne et al., 2016), 6850 (Grosz et al., 2014; Schroder et al., 2006), and Novel (Lam et al., 2010) clinical strains; and RN6390 (Jarry & Cheung, 2006; Lam et al., 2010), RN4220 (Lam et al., 2010), and SA113 (Giese et al., 2009) laboratory strains.

However, other studies have shown that *S. aureus* is found within vacuoles that lack either of these two maturation markers. One study showed that only 30% of Newman bacteria existed within acidified vacuoles, and that these vacuoles do not mature into LAMP-1 positive compartments (Jubrail et al., 2016). Furthermore, in another study it was shown that vacuoles containing RN6390 do not all fuse with lysosomes (Kahl et al., 2000). Finally, another study

has shown little co-association between RN4220 bacteria and LysoTracker, implying the bacteria do not exist in acidic compartments (Schnaith et al., 2007). In several of the studies in which it was shown that *S. aureus* exists within acidic compartments, it was shown that the bacteria modify or escape this hostile environment over time. While one study showed that USA300 is found in an acidic 293T phagolysosome, the phagolysosome became less acidic over time as the low pH increased from 2 – 6 h (Grosz et al., 2014). This decrease in acidity was reduced when bacterial protein synthesis was inhibited upon rifampicin treatment, implying that the change in pH is initiated by the bacteria (Grosz et al., 2014). Further studies have demonstrated that while *S. aureus* is found within acidic vacuoles for up to 24 h, bacterial CFU counts decrease, implying that no growth occurs within this niche (Jarry & Cheung, 2006; Schroder et al., 2006). In fact, one study demonstrated increased survival of small colony variants, suggesting *S. aureus* survives the low pH of the phagolysosome, rather than grows within it (Schroder et al., 2006).

In several other studies it has been found that *S. aureus* is capable of escaping the phagosome, either before or after its maturation into a phagolysosome (Bayles et al., 1998; Grosz et al., 2014; Rodrigues Lopes et al., 2022; Shompole et al., 2003). This escape has been shown to occur within as little time as 1 h post-infection (Shompole et al., 2003), implying that *S. aureus* does not grow within the acidic phagolysosome, although it still has to survive it.

On the other hand, two studies have reported that a USA300 *S. aureus* strain can grow within the acidic phagolysosome of professional phagocytic macrophages (Flannagan et al., 2016; Tranchemontagne et al., 2016). In fact, one study demonstrated decreased bacterial growth when phagolysosomal acidification was inhibited (Tranchemontagne et al., 2016). The authors suggest that the low pH is a signal for USA300 to upregulate processes which promote survival and growth within the phagosome, such as modifying the phagolysosome to reduce hydrolase activity. A second study has since supported this by demonstrating that low pH upregulates processes which reduce the susceptibility of USA300 to defensins and cationic antimicrobial peptides (Flannagan et al., 2018). These studies imply that *S. aureus* is capable of growth within the acidic phagolysosome, and that the low pH acts as a signal to reduce bacterial sensitivity to the other antimicrobial strategies within the phagolysosome.

Overall, there are clearly several acidic niches within the human host in which *S. aureus* can grow, which is relevant for the ability of this bacterium to cause disease.

1.2.2 Weak versus strong acid stress

Acids are a group of molecules chemically defined by their ability to donate a proton, or H^+ ion, when in aqueous solution. Solutions of acids are compared by their 'potential of hydrogen', or pH, which is a measure of the concentration of H^+. The pH scale is logarithmic with acids defined as having a pH of below 7.

Acidic stress can be caused by either strong acids or weak acids, both of which are found in different niches within the body. Strong acids, such as hydrochloric acid (HCl), are inorganic and are defined as those that completely ionize in aqueous solution. Strong acids are found in the body in niches such as gastric acid in the stomach, or in acidified phagolysosomes in macrophages. On the other hand, weak acids include organic acids such as acetic acid, lactic acid, or short chain fatty acids, which do not fully ionize in aqueous solutions. Weak acids are often produced as a by-product of bacterial fermentation, and are therefore found in niches with large bacterial communities such as in the oral cavity, on the skin, and in the intestine. The strength of an acid is measured by its pK_a, where the lower the pK_a, the more dissociated the acid is. Strong acids have a pK_a of below 0, while weak acids have pK_a values of above 0.

Due to their different proton dissociation constants strong and weak acids affect bacteria in different ways. Strong acids, which are fully dissociated, exist outside the cell as a positively charged proton and negatively charged anion. In Gram-negative bacteria, the outer membrane is thought to be permeable to protons due to the presence of porins (reviewed in Lund et al., 2014). This is supported by the fact that the periplasm of *E. coli* acidifies very rapidly to the same pH as the growth medium upon acid stress (Wilks & Slonczewski, 2007). By contrast, the inner membrane of Gram-negative bacteria, and the cell membrane of Gram-positive bacteria, is much more impermeable to protons. A range of techniques have been used to measure intracellular pH and, on the whole, bacteria maintain their intracellular pH within a narrow defined range regardless of the extracellular pH within which growth can occur (reviewed in Slonczewski et al., 2009). However, single cell analysis of intracellular pH measurements of *E. coli* has shown that upon strong acid stress the cytoplasm does acidify, although it rapidly returns to a more neutral pH (Wilks & Slonczewski, 2007). Cytoplasmic

acidification was also seen in *B. subtilis* following strong acid stress (Kitko et al., 2009). This clearly demonstrates that protons can cross the membrane, whether in the undissociated form of the strong acid, or through channels (reviewed in Lund et al., 2014).

On the other hand, weak organic acids such as acetic acid or lactic acid do not fully dissociate in water. Despite this, weak acids have been shown to have a more inhibitory effect on bacterial growth than strong acids (Rode et al., 2010). Some molecules of weak acids remain protonated as determined by the pK_a of the acid, whereby the higher the pK_a, the more undissociated they are. These molecules are therefore uncharged and can cross the bacterial membrane to equalise the concentrations of uncharged weak acid inside and outside the cell. As the intracellular pH tends to be higher than outside, the weak acid dissociates in the cytoplasm, releasing the proton and leaving a negatively charged anion. The exact effects of weak acids on bacteria are disputed (reviewed in Hirshfield et al., 2003). Initially it was suggested that the increased ability to acidify the cytoplasm explains their greater inhibitory impact on bacterial growth than strong acids. However, different weak acids can have vastly different effects on bacterial growth despite having the same pH (Young & Foegeding, 1993), showing that the intracellular proton concentration cannot fully account for the effect of weak acids on bacteria (reviewed in Hirshfield et al., 2003). It has also been suggested that weak acids are able to damage membranes by insertion into the membrane in their uncharged forms. Furthermore, the intracellular accumulation of the negatively charged anion has been suggested to inhibit bacterial growth. Finally, the ability of weak acids to carry protons across the membrane can act to dissipate the membrane potential and reduce the ability of the cell to utilise the proton motive force (PMF).

Due to the more complicated nature of weak acid stress, as well as the decreased resistance of bacteria to weak acids, in this study I will now focus on the effects of and responses of bacteria to strong acids.

1.2.3 Effects of low pH on bacteria

Low pH can adversely affect bacteria in multiple ways (Fig. 1.5).

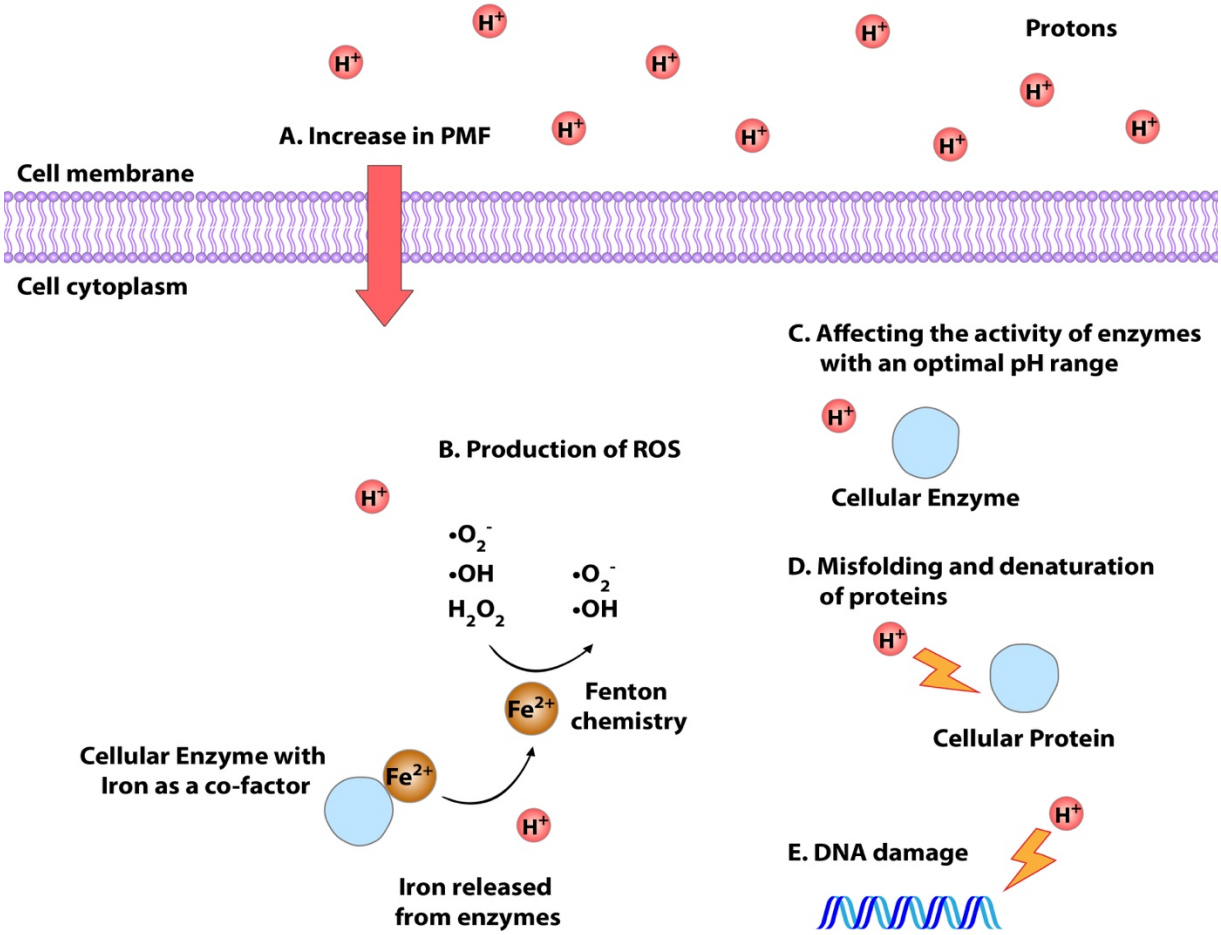


Figure 1.5: Schematic representation of the effects of low pH on bacteria
Low pH can adversely affect bacteria in multiple ways. These include (A) increasing the proton motive force (PMF), (B) increasing the production of reactive oxygen species (ROS), (C) affecting the activity of enzymes which function in an optimal pH range, (D) causing the misfolding and denaturation of proteins, and (E) causing DNA damage.

Firstly, the increased extracellular proton concentration affects the chemical gradient of protons across the membrane (Fig. 1.5A). As mentioned previously in this Chapter, bacteria tend to maintain a more alkaline cytoplasm than the extracellular medium. The higher concentration of protons outside the cell than inside creates a chemical potential across the membrane (ΔpH) (Fig. 1.6). Protons move back into the cell down the proton concentration gradient through various channels such as the ATPase enzymes.

As well as a proton gradient, bacteria also maintain a charge gradient across the membrane, known as the membrane potential (ΔΨ) (Fig. 1.6). Neutralophilic bacteria mostly maintain a negative ΔΨ with a positive outside, negative inside. This charge gradient is the sum of all cations and anions across the membrane, including positively charged protons and potassium

ions, or negatively charged chloride ions. The membrane potential also includes 'captive charges' which remain within the cell, such as negatively charged deoxyribonucleic acid (DNA) (Fig. 1.6).

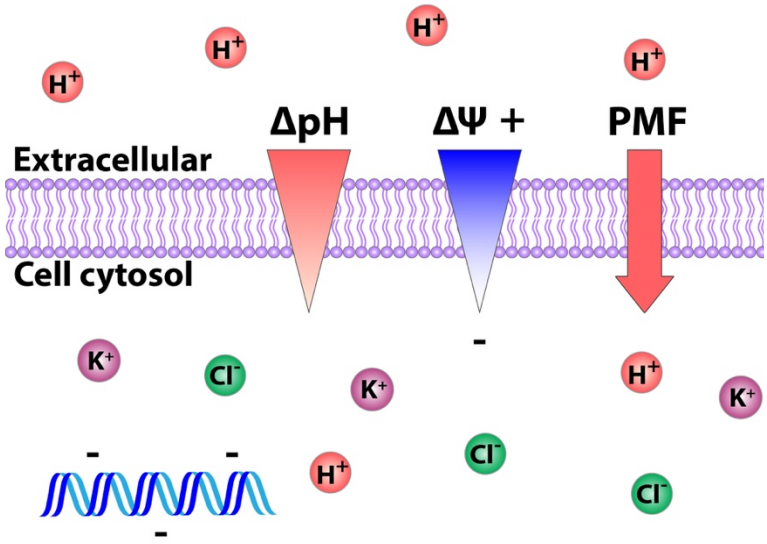


Figure 1.6: Schematic representation of the PMF
The proton motive force (PMF) is the sum of the proton chemical gradient (ΔpH, inside alkaline), and the membrane potential (ΔΨ, inside negative). The membrane potential is the sum of the charges across the membrane, including cations such as potassium ions (K^+), anions such as chloride ions (Cl^-), and 'captive charges' such as negatively charged DNA. Protons move back into the cell down both the ΔpH and ΔΨ gradients through various channels.

The combination of both the ΔΨ and ΔpH is known as the PMF, which determines the movement of protons across the bacterial membrane. The PMF drives a range of processes within the bacterial cell, such as ATP synthesis, active transport of substances, and bacterial motility (reviewed in Yang et al., 2023).

When the extracellular medium is acidified the ΔpH is increased, which will also affect the PMF and the range of processes that the PMF drives (Fig. 1.5A). It has been suggested that the disruption of this homeostasis is a major negative impact of acidic stress conditions on bacteria (Richard & Foster, 2004).

Acidic stress also leads to increased production of ROS (Fig. 1.5B). This can occur by the changing ΔpH affecting the electron transport chain which leads to the increased production of hydrogen peroxide (H_2O_2), superoxides ($\bullet O_2^-$), or hydroxyl radicals ($\bullet OH$) (Clements et al., 1999; Thomas et al., 2014). In addition, the low cytosolic pH increases the free iron

concentration in the cell, which catalyses the breakdown of hydrogen peroxide into superoxides and hydroxyl radicals via the Fenton chemistry (Bruno-Barcena et al., 2010). Due to their extra electron, ROS are highly reactive and can cause damage to a range of macromolecules including membranes, proteins, and DNA.

Protons can also cause damage inside the cytoplasm ranging from affecting the activity of enzymes which have an optimal pH range, causing denaturation and misfolding of proteins, and damaging DNA (Fig. 1.5C,D,E).

Each enzyme in the cell has an optimum pH range within which enzymatic activity occurs. If the pH falls outside this range, the activity decreases. Eventually, the enzyme is denatured and activity stops. pH affects enzyme activity by determining the charge on the amino acids which make up the enzyme. The backbone of an amino acid exists as a zwitterion, meaning that it contains both a positively charged amine group, and a negatively charged carboxyl group (Fig. 1.7). In aqueous solution, most amino acids are therefore uncharged. However, when the pH of the solution changes, this affects the charge of the amino acid backbone. Under alkaline conditions, the amine group loses its additional proton, resulting in the amino acid becoming negatively charged. Under acidic conditions, the carboxyl group gains an additional proton, resulting in the amino acid becoming positively charged.

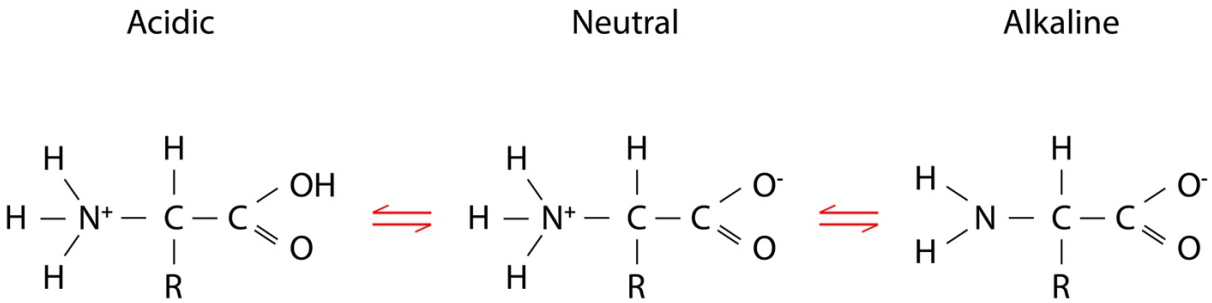


Figure 1.7: Schematic representation of zwitterions
The charge of an amino acid changes depends on the pH of the solution.

As well as the backbone, five amino acids have side chains which can accept or donate a proton, and are therefore charged (Fig. 1.8).

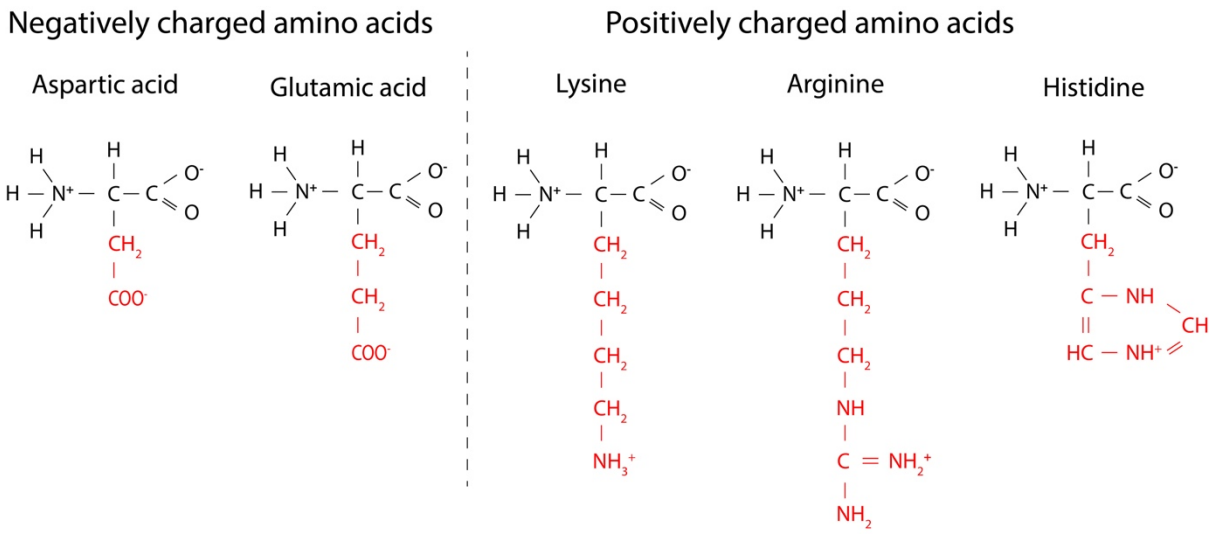


Figure 1.8: Schematic representation of charged amino acids
Some amino acid have additional charges on their side chains. These include the negatively charged aspartic acid and glutamic acid, and the positively charged lysine, arginine, and histidine.

Two amino acids, aspartic acid and glutamic acid, are negatively charged and can donate a proton at a pH value above their pK_a. As described previously in this Chapter, the pK_a is a measure of how much the proton dissociates from the acid in aqueous solution. A higher pK_a indicates a weaker, and thus less dissociated, acid. On the other hand, lysine, arginine, and histidine are positively charged, and can accept a proton at a pH value below their pK_a. This change in amino acid charge can affect the binding of substrates in the active site or the binding of ions required for catalysis, thus reducing enzyme activity. Furthermore, the change in charge of the side chains can affect the interactions which determine the folding and shape of the enzyme. As well as affecting enzyme activity, eventually the interactions weaken so much that the enzyme becomes misfolded or denatured. The changing charge can also affect the interaction of the protein with other proteins or molecules.

Finally, low pH can cause DNA damage (Fig. 1.5E). Chromosomal DNA from *E. coli* cells has been shown to have increased DNA damage such as double strand breaks when exposed to pH 2.0 compared to neutral pH (Jeong et al., 2008). Furthermore, decreasing pH increases the rate at which depurination occurs (An et al., 2014). Depurination occurs when an adenine or guanine base is lost from the phosphate backbone by hydrolysis of the N-glycosydic bond, resulting in an abasic site (Fig. 1.9). This site is more susceptible to damage and can result in mutations (reviewed in Loeb & Preston, 1986). During depurination, a purine nucleotide

becomes monoprotonated on the N7 of the purine base (An et al., 2014) (Fig. 1.9). The charge from this proton becomes redistributed through the ring, and results in the cleavage of the N-glycosydic bond linking the purine base to the phosphate backbone. This results in an uncharged molecule of adenine and a charged abasic ion called an oxocarbenium. This ion then reacts with water to form an uncharged deoxyribose. Under strong acidic conditions the monoprotonated adenine can become doubly protonated with the N3 becoming protonated as well as the N7. Both the released adenine base and oxocarbenium ions are charged, with the resulting deoxyribose becoming uncharged by reaction with water. The resulting abasic site can lead to incorporation of the wrong base during DNA replication or can cause further DNA damage by destabilising the deoxyribose backbone of DNA.

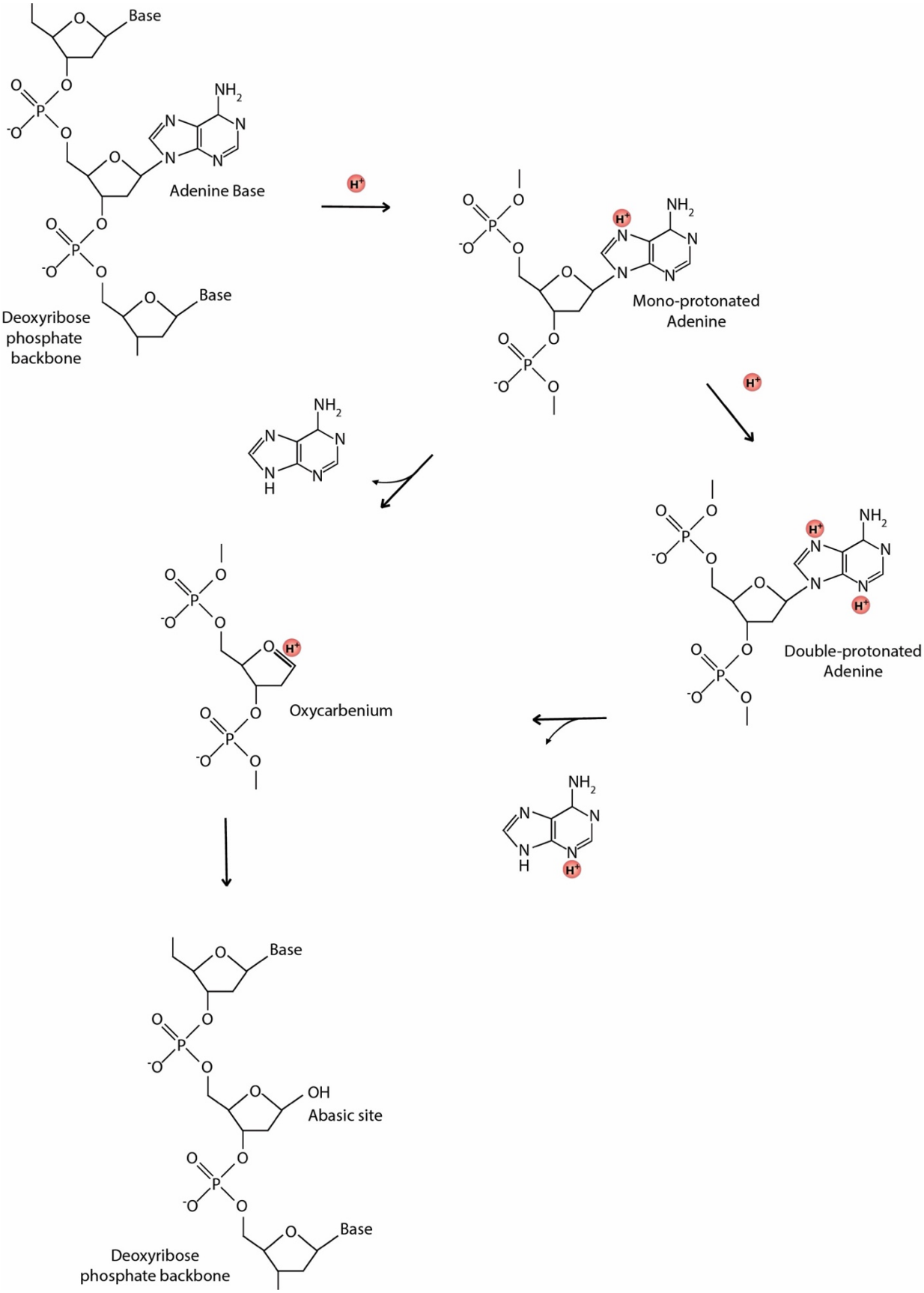


Figure 1.9: Schematic representation of acid induced depurination
During depurination a purine base is lost, resulting in an abasic site. Figure adapted from An et al., 2014 (An et al., 2014).

1.3 Bacterial responses to low pH

Bacteria have a range of mechanisms to deal with low pH (reviewed in Cotter & Hill, 2003; reviewed in Lund et al., 2014) (Fig. 1.10). The four main responses are to:

1. Prevent protons from entering the cell
2. Expel protons out of the cell via proton pumps
3. Sequester or buffer protons in the cytoplasm
4. Activate repair mechanisms to reduce proton damage

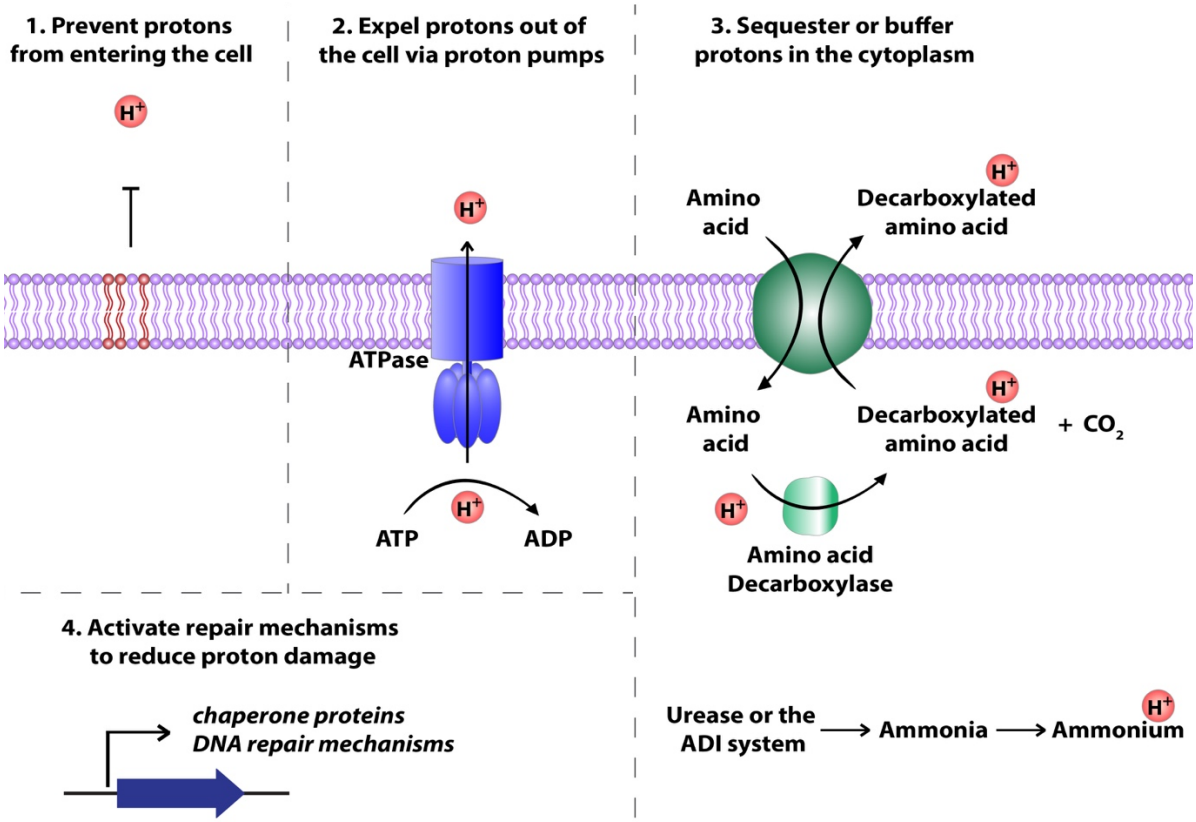


Figure 1.10: Schematic representation of the main bacterial responses to low pH
Bacteria have four main responses to deal with low pH. These are (1) prevent protons from entering the cell, (2) expel protons out of the cell via proton pumps, (3) sequester or buffer protons in the cytoplasm, (4) activate repair mechanisms to reduce proton damage. Figure adapted from Cotter and Hill, 2003 (Cotter & Hill, 2003).

1.3.1 Prevent protons from entering the cell

The first method that can be employed by bacteria to reduce their sensitivity to acid stress is to reduce the permeability of the cell membrane to protons (Fig. 1.10). Differences in cell membrane fatty acid composition upon acid stress have been detected in a range of bacteria.

For example, *L. monocytogenes* produces higher levels of $C_{14:0}$ and $C_{16:0}$ straight-chain fatty acids following adaptation to low pH (van Schaik et al., 1999). An *E. coli* mutant incapable of producing cyclopropane fatty acids was found to have increased membrane permeability to protons and a reduced ability to regulate its intracellular pH (Shabala & Ross, 2008). Furthermore, a *Streptococcus mutans* strain grown at pH 5.5 had increased levels of mono-unsaturated and longer chain fatty acids compared to when grown at pH 7.0 (Quivey et al., 2000). This change in membrane composition was subsequently shown to increase bacterial survival under acidic conditions (Fozo & Quivey, 2004).

As well as the composition, the charge of the cell surface can impact bacterial acid tolerance. In Gram-negative bacteria, changes to the charge of lipopolysaccharides (LPS) on the outer membrane can be induced by acidic pH (reviewed in Groisman et al., 2013; reviewed in Li et al., 2020; Prost et al., 2007). In Gram-positive bacteria, the cell wall charge can be modified by the addition of D-alanine residues onto lipoteichoic acid (LTA) and wall teichoic acids (WTA). The *dlt* operon encoding the enzymes involved in this process has been shown to be important for the acid stress response of *S. mutans* (Boyd et al., 2000; Wu et al., 2021) and *Lactococcus lactis* (Wu et al., 2022). It has been hypothesized that the addition of positive charges onto the cell wall reduces the permeability by repelling the positively charged protons.

1.3.2 Expel protons out of the cell via proton pumps

The major proton pump in bacteria is the F_1F_0ATPase. The ATPase can act as an ATP synthetase, using the movement of protons into the cell by the PMF to synthesise ATP. Alternatively, the ATPase can act as an ATP hydrolase, using ATP to pump protons out of the cell against the PMF (Fig. 1.10). The role of the ATPase in acid stress responses was first discovered in *Enterococcus hirae*, formerly known as *Streptococcus faecalis*. Inhibiting the ATPase by the addition of *N*,*N*'-dicyclohexylcarbodiimide reduced the intracellular pH (Harold et al., 1970). Furthermore, mutant strains of *E. hirae* lacking functional copies of the ATPase showed a reduced ability to grow at pH 6.0 compared to the wild-type (WT), despite having no growth defect at pH 7.8 (Suzuki et al., 1993; Suzuki et al., 1988). As *E. hirae* does not have a respiratory chain and only produces ATP through glycolysis, the importance of the ATPase for acid resistance must be due to proton extrusion rather than ATP generation for use in other processes. The ATPase has been shown to be important for the acid stress responses of other lactic acid bacteria which lack a respiratory chain such as *L. lactis* (Amachi et al., 1998; Carvalho et al., 2013;

O'Sullivan & Condon, 1999), *Lactobacillus* species (Corcoran et al., 2005; Koponen et al., 2012; Kullen & Klaenhammer, 1999), and other *Streptococcus* species (Sekiya et al., 2023).

The ATPase has also been shown to be involved in the acid stress responses of bacterial species which do have a respiratory chain. These include *E. coli* (Sun et al., 2012), *Salmonella enterica* (Foster & Hall, 1990, 1991) and *L. monocytogenes* (Cotter et al., 2000; Datta & Benjamin, 1997; McEntire et al., 2004). In these bacteria, it is unclear whether the ATPase acts as an ATP hydrolase to pump protons out of the cell, or as an ATP synthetase using the movement of protons into the cell to synthetise ATP for use in other acid responses.

1.3.3 Sequester or buffer protons in the cytoplasm

As well as pumping protons out of the cell, bacteria can increase the rate of reactions which consume protons (Fig. 1.10). Well-known reactions for this involve amino acid decarboxylase systems (Gale, 1946). As an overview, during the process an amino acid molecule is decarboxylated, consuming a single proton and producing one molecule of carbon dioxide (Fig. 1.11). The resulting decarboxylated amino acid product is then transported out of the cell by a cognate antiporter in exchange for another molecule of the amino acid, in this manner removing a proton from the cytoplasm. Amino acid decarboxylases are found in a wide range of bacteria, and have been shown to be essential for growth and survival at acid stresses ranging from mild to extreme as described below (reviewed in Cotter & Hill, 2003; reviewed in Kanjee & Houry, 2013; reviewed in Lund et al., 2014).

The most common amino acid decarboxylase system is via glutamate decarboxylase (GAD), which decarboxylates glutamate to γ-aminobutyrate (GABA) (Fig. 1.11). GADs are important for acid tolerance in *E. coli* (Castanie-Cornet et al., 1999; De Biase et al., 1999; Lin et al., 1995; Lin et al., 1996), *L. monocytogenes* (Cotter et al., 2001), *L. lactis* (Sanders et al., 1998) and *Lactobacillus* species (Su et al., 2011).

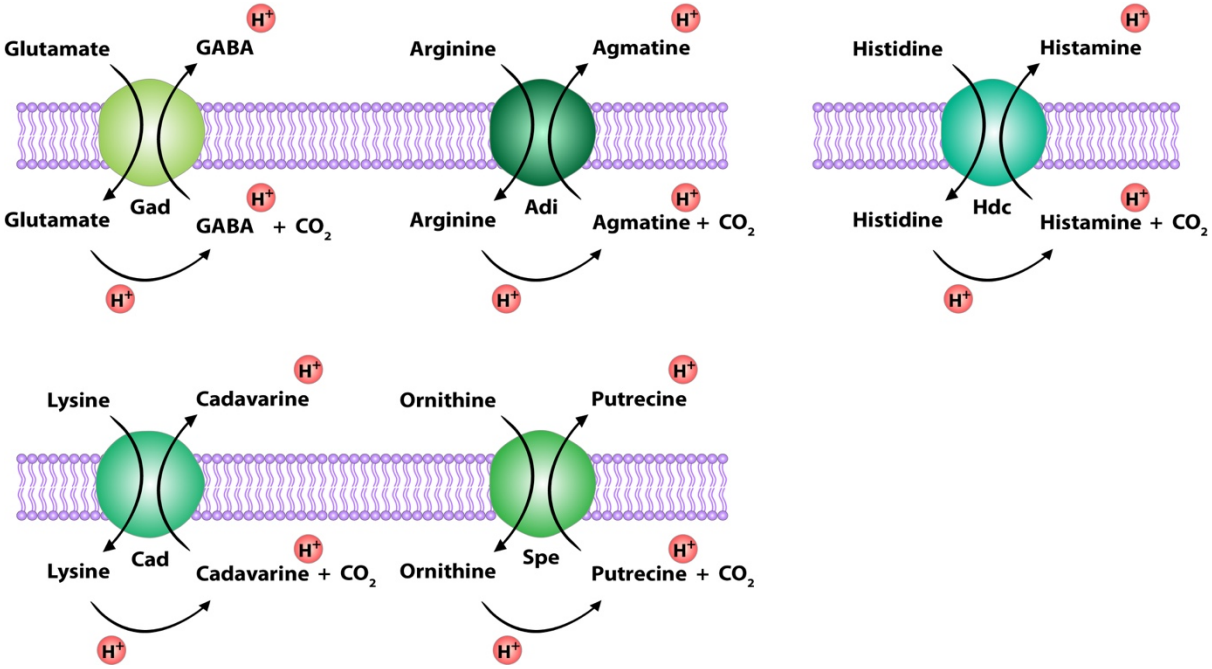


Figure 1.11: Schematic representation of amino acid decarboxylase systems
There are four main amino acid decarboxylase systems which are important for the acid stress responses of Gram-negative bacteria. These are glutamate decarboxylase, arginine decarboxylase, lysine decarboxylase and ornithine decarboxylase. Some lactic acid bacteria contain a histidine decarboxylase system. Figure adapted from Guan and Lui, 2020 (Guan & Liu, 2020).

Other amino acids, which can be decarboxylated include arginine, lysine, and ornithine (Fig. 1.11). These amino acid decarboxylase systems are particularly important for the acid stress responses of Gram-negative bacterial species, especially *E. coli* (Iyer et al., 2003; reviewed in Kanjee & Houry, 2013) and *S. enterica* (Viala et al., 2011). Furthermore, in some lactic acid bacteria a histidine decarboxylase system is present (reviewed in Landete et al., 2008; Molenaar et al., 1993), which has been shown to be important for the acid stress response in this bacterium (Diaz et al., 2020) (Fig. 1.11). Varying evidence exists for the presence of histidine decarboxylases in other bacterial species (reviewed in Landete et al., 2008), including *Staphylococcus epidermidis* (Martin et al., 2006; Yokoi et al., 2011) and *Staphylococcus capitis* (de las Rivas et al., 2008).

As well as amino acids, bacteria can decarboxylate other compounds to the same effect as amino acids. In lactic acid bacteria, the decarboxylation of malate into lactic acid, or malolactic fermentation, consumes a single proton and releases a single molecule of carbon dioxide per reaction (Poolman et al., 1991). As well as removing intracellular protons, this process can be

used to generate ATP. This is because the resulting change in charge of the product due to consumption of a proton, and the subsequent expulsion of the product, generates a membrane potential (reviewed in Konings et al., 1997; Poolman et al., 1991). Whether it is the removal of the intracellular proton or the generation of ATP that is important for the acid stress response is unknown.

Another method used by bacteria to sequester or buffer protons in the cytoplasm is to produce alkaline molecules such as ammonia. Ammonia can be readily protonated to form ammonium ($NH_3 \rightarrow NH_4^+$), which can then be excreted out of the cell. The first of two main pathways used by bacteria to produce ammonia is via the arginine deaminase (ADI) system. Here, arginine is brought into the cell by a transporter where it undergoes a series of reactions and is converted into ornithine (reviewed in Cunin et al., 1986). In the process, two molecules of ammonia are produced. In addition, one molecule of ATP is produced via this reaction, contributing to the energy levels of the cell (reviewed in Cunin et al., 1986). ADI systems are especially important for the acid stress responses of oral bacteria (Casiano-Colon & Marquis, 1988; Curran et al., 1995; Marquis et al., 1987).

Ammonia can also be produced from urea via the urease enzyme. Each molecule of urea results in two molecules of ammonia and one molecule of carbon dioxide (reviewed in Mobley et al., 1995). Urease is an essential virulence factor for stomach colonization of *Helicobacter pylori* (Tsuda et al., 1994). In this case, the low pH of the stomach activates the UreI urea channel, resulting in increased transport of urea into the bacteria (Weeks et al., 2000). The urea is subsequently converted to ammonia by the urease enzyme, which then diffuses into the periplasm of the bacterium. In this way, the periplasm of the bacterium is buffered against the low pH of the stomach (Scott et al., 1998). Besides in *H. pylori*, urease is a virulence factor in other bacteria infecting a range of host niches, including the urinary tract and intestines by *Klebsiella pneumoniae* (Lin et al., 2022; Maroncle et al., 2006). In oral bacteria the regulation of urease has been shown to be pH dependent (Sissons et al., 1990).

1.3.4 Activate repair mechanisms to reduce proton damage

The final mechanism used by bacteria in response to acid stress is to either protect against damage caused by protons, or to activate repair mechanisms once the damage has been caused. The main types of damage caused by protons inside the cell are denaturation of

proteins and DNA damage. One of the major responses of bacteria to acid stress is the upregulation of genes coding for chaperone proteins. In Gram-negative bacteria, the chaperone proteins HdeA and HdeB act to stabilise periplasmic proteins at low pH (reviewed in Hong et al., 2012), and the loss of one or both proteins results in reduced growth at low pH (Gajiwala & Burley, 2000; Waterman & Small, 1996). Other chaperone proteins such as GroELS and DnaK have been shown to be involved in the acid stress responses of a range of bacteria, including *S. enterica* (Bearson et al., 2006), *S. mutans* (Lemos et al., 2001) and *L. lactis* (Frees et al., 2003). Acidic pH also upregulates bacterial expression of genes encoding proteases to remove damaged proteins following acid damage (Frees et al., 2003; Wemekamp-Kamphuis et al., 2004). DNA repair mechanisms have also been shown to be important for the acidic stress response of bacteria. In *S. mutans,* both a *recA* and *uvrA* mutant displayed increased sensitivity to low pH (Hahn et al., 1999; Hanna et al., 2001).

1.3.5 Potassium transport

An additional pathway which has been linked to bacterial acid stress resistance is potassium transport, although the mechanisms for this are unclear. Potassium is one of the most abundant intracellular cations. For example, *S. aureus* maintains a cytosolic K^+ concentration of between 0.5 to 1.5 M, even in conditions of low extracellular K^+ (reviewed in Christian & Waltho, 1964; Graham & Wilkinson, 1992; Gründling, 2013). In several different bacterial species it has been shown that a neutral cytoplasmic pH upon acidic extracellular pH can only be maintained in the presence of potassium (reviewed in Booth, 1985). Furthermore, several potassium transporters have been shown to be involved in the acid stress response of bacteria. In *S. enterica* and *E. coli*, genes encoding the Kdp high-affinity potassium transporter have been shown to be upregulated under acidic conditions (Ryan et al., 2015; Yan et al., 2011). Genes encoding a putative Trk-type potassium transporter have been shown to be upregulated in *S. mutans* at pH 5.5 (Gong et al., 2009). In *Corynebacterium glutamicum*, a mutant lacking a functional copy of the potassium channel gene *cglK* had reduced growth at acidic and neutral pH, but could grow comparable to the WT strain at alkaline pH (Follmann et al., 2009).

One hypothesis for why potassium is important for the ability of bacteria to regulate their cytoplasmic pH upon acidic stress is that K^+ influx dissipates the membrane potential (Bakker & Mangerich, 1981; reviewed in Booth, 1985) (Fig. 1.12). When the extracellular pH is

decreased, the ΔpH increases which in turn increases the PMF. As well as increasing the number of protons entering the cell, this increases the energy required to pump protons out of the cell against the proton gradient. By decreasing the ΔΨ by importing an alternative cation, the PMF is reduced and the intracellular pH can be maintained. This is supported by the fact that in several bacterial species it has been demonstrated that ΔΨ decreases when the ΔpH increases (reviewed in Krulwich et al., 2011).

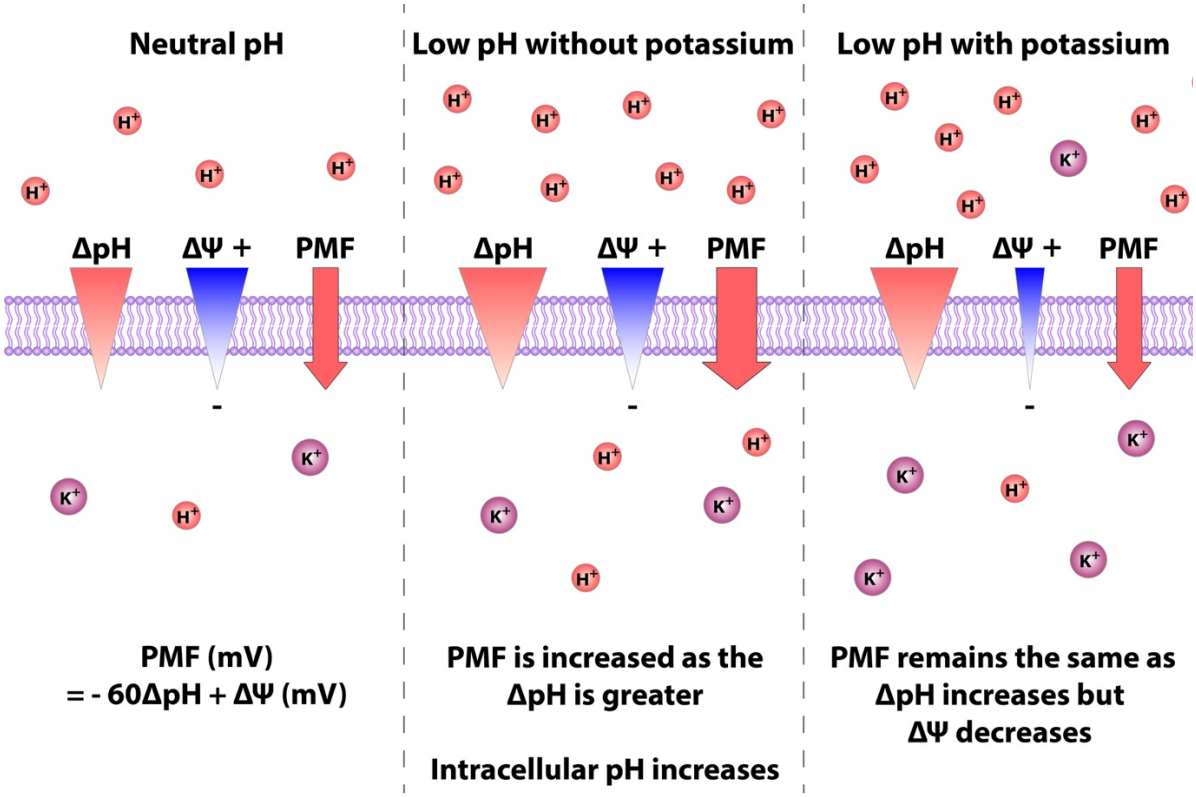


Figure 1.12: Schematic representation of the components of the PMF at neutral pH, low pH without potassium, and low pH with potassium
At neutral pH, the PMF is maintained by the ΔpH (inside alkaline) and ΔΨ (inside negative). At low pH without potassium, the ΔpH increases but the ΔΨ remains the same, resulting in an increased PMF and greater proton influx. At low pH with potassium, the ΔpH increases, but due to potassium influx the ΔΨ decreases, resulting in the PMF staying the same.

In many Gram-positive bacterial species, potassium transporters are regulated by the signalling nucleotide cyclic di-adenosine monophosphate (c-di-AMP) (reviewed in Stülke & Kruger, 2020). For example, in both *Bacillus subtilis* (Gundlach et al., 2019; Rocha et al., 2019) and *L. monocytogenes* (Gibhardt et al., 2019) c-di-AMP has been shown to bind to potassium transporters and inhibit potassium influx. As c-di-AMP has been associated with the acidic

stress responses of a range of bacterial species as described below, this could provide another link between potassium transport and acid stress resistance.

c-di-AMP is produced by diadenylate cyclase enzymes, which convert two molecules of ATP into c-di-AMP (reviewed in Fahmi et al., 2017). The molecule is then degraded by phosphodiesterases into 5'-phosphadenylyl-adenosine (pApA) (reviewed in Fahmi et al., 2017). Several studies have shown that inactivating phosphodiesterases in *L. lactis, B. subtilis* and *L. monocytogenes*, resulting in strains with increased c-di-AMP levels, leads to increased growth and survival at low pH compared to the WT strain (Rallu et al., 2000; Rao et al., 2010; Witte et al., 2013). On the other hand, there have been contradictory results in *Streptococcus.* In *Streptococcus pneumoniae*, a *Δpde* mutant lacking the phosphodiesterase Pde had reduced growth at pH 6 compared to the WT strain (Zarrella et al., 2018), while an *S. mutans* diadenylate cyclase *ΔdacA* mutant with reduced c-di-AMP levels showed no difference in survival at pH 2.8 compared to the WT strain (Cheng et al., 2016). However, c-di-AMP has been shown to bind a range of effector molecules, and thus the effect of c-di-AMP on acidic stress responses cannot be attributed to potassium transport alone.

1.4 Responses of *S. aureus* to low pH

While lots of work has been done in Gram-negative bacteria such as *E. coli* and *S. enterica*, less is known how Gram-positive bacteria counteract acid stress. Despite its wide pH growth range, little is known about the response of *S. aureus* to low pH. Four transcriptional studies have been performed to identify genes with differential expression in *S. aureus* upon acid stress (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010; Weinrick et al., 2004; reviewed in Zhou & Fey, 2020). The first by Weinrick et al., (2004) investigated transcriptional changes following 0, 3 and 6 h growth in Casamino acids-yeast extract-glycerophosphate medium adjusted to pH 4.5 with HCl. The second by Bore et al., (2007) investigated transcriptional changes in *S. aureus* in the initial 0, 10 and 20 min following acidification of the medium to pH 4.5 with HCl. A follow-up study by Rode et al., (2010) explored transcriptional changes following growth for 10 min at pH 4.5 after the acidification of the medium with HCl and 10 min or 180 min at pH 4.5 after the addition of lactic acid. Finally, Anderson et al., (2010) explored transcriptional changes following growth for 30 min at pH 4.0 after adjusting the

medium with HCl. The overall pathways highlighted through these studies, as well as the few phenotypic studies that have been performed, are discussed below.

1.4.1 Prevent protons from entering the cell

Little is known about changes in the *S. aureus* membrane composition in response to acid stress. However, genes involved in capsular polysaccharide synthesis were found to be upregulated in response to acid stress (Weinrick et al., 2004).

Furthermore, the *dltABCD* operon was upregulated in one study in response to strong acid stress, although interestingly these genes were downregulated in response to lactic acid (Rode et al., 2010). As described previously in this Chapter, the proteins coded for by the *dlt* operon catalyse the addition of the positively charged D-Ala residue onto cell wall teichoic acids. This may function to repel the positively charged protons dissociated from strong acids such as HCl, but not uncharged undissociated weak acids such as lactic acid. The *dlt* operon in *S. aureus* is regulated by the GraXRS five component system which is comprised of the GraS and VraFG sensor kinases, a GraX signal transduction protein and the GraR response regulator. The GraXRS system detects and responds to cationic antimicrobial peptides (CAMPs) (Chaili et al., 2016) as well as vancomycin (Meehl et al., 2007). Recent work has shown that this system may also detect and respond to low pH, especially in the context of acidified phagolysosomes (Flannagan et al., 2018; Kuiack et al., 2020; Villanueva et al., 2018).

1.4.2 Expel protons out of the cell via proton pumps

Despite the F_1F_0ATPase being involved in the acid stress responses of a wide range of bacterial species as described above, the genes coding for this proton pump were downregulated or unchanged in all transcriptional studies in *S. aureus* (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010; Weinrick et al., 2004). Recently it has been shown that the ATPase can function as a proton pump during *S. aureus* fermentation, leading to a more alkaline intracellular pH (Grosser et al., 2018), however no link with acid stress has been shown. This does not exclude its importance in the *S. aureus* acid stress response however, as the F_1F_0ATPase is regulated post-transcriptionally, and therefore changes in the activity of F_1F_0ATPase may not be seen in transcriptional studies (Arikado et al., 1999).

1.4.3 Sequester or buffer protons in the cytoplasm

One operon which was highlighted in all four transcriptional studies were the *ure* genes. In *S. aureus*, the urease enzyme is coded for by the *ureABC* operon. In all four studies, these genes were among the most upregulated upon acid stress (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010; Weinrick et al., 2004; reviewed in Zhou & Fey, 2020). Furthermore, in one study the *nixA* gene encoding for a high-affinity nickel transporter necessary for full urease activity was also upregulated (Bore et al., 2007). Urease catalyses the break down of urea into ammonia which can be protonated into ammonium and buffer excess protons. A follow-up study exploring the role of urease in the acid stress response of *S. aureus* found that, under weak acid stress, both a functional urease enzyme and exogenous urea are required for the survival of *S. aureus* (Zhou et al., 2019). In this study it was also shown that the urease enzyme is required for pathogenicity of *S. aureus* in a murine chronic renal infection model (Zhou et al., 2019). However, the importance of urease for acid survival of *S. aureus* in this study depended upon exogenous urea, rather than from endogenous sources such as from arginine metabolism (Zhou et al., 2019). This contrasts to the transcriptional studies, where the urease genes were upregulated despite no exogenous urea being present. This implies that while urease expression is upregulated upon acid stress, it only functions in the acidic stress response in niches within the body which have a high urea concentration, such as urine, blood, sweat, and the intestine (Burne & Chen, 2000).

S. aureus also contains an ADI system, which includes the *arcA*, *arcB*, and *arcC* genes. *arcA* was found to be upregulated in one transcriptional study (Weinrick et al., 2004), while *arcC* was downregulated in three others (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010). The USA300 community-associated MRSA strain also encodes a second ADI system, which contributes to the pathogenicity of the highly successful USA300 strain (Diep et al., 2008). This second ADI system is encoded in the arginine catabolic mobile element, and was shown to facilitate the growth of *S. aureus* at pH 5.0 (Thurlow et al., 2013).

1.4.4 Activate repair mechanisms to reduce proton damage

Upregulation of genes encoding for either chaperone proteins or proteases were seen in several of the transcriptional studies. The chaperone protein encoding genes *groEL* and *groES* were upregulated (Rode et al., 2010), as well as *clpC*, *clpP* and *clpB* encoding subunits of the Clp protease (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010; Weinrick et al., 2004).

Furthermore, genes involved in DNA repair mechanisms were also upregulated, such as *rexAB* (Bore et al., 2007).

Genes involved in ROS detoxification systems were also strongly upregulated. Among the genes highlighted in at least one study were the superoxide dismutases *sodA* and *sodM*, the catalase *katA*, alkyl hydroperoxidase reductases *ahpC* and *ahpF*, and putative thioredoxins *trxA* and *trxB*. Furthermore, inactivating the SodA enzyme has been shown to decrease the survival of *S. aureus* at pH 2.0 (Clements et al., 1999).

1.4.5 Potassium transport

Similarly to other bacterial species, potassium transport has been shown to be necessary for the ability of *S. aureus* to regulate its cytoplasmic pH, with the bacteria unable to maintain a neutral cytoplasmic pH when potassium is not present in the growth medium (Gries et al., 2016). Furthermore *kdpD,* encoding a sensory histidine kinase detecting potassium levels, was upregulated in one transcriptional study (Bore et al., 2007). *S. aureus* encodes two K^+ transporter systems, a Kdp system and a Ktr system (reviewed in Gründling, 2013). Two studies from the same group have investigated the importance of the Ktr system for *S. aureus* pH regulation. In the first study, a *ΔktrA* mutant strain was shown to have a slight growth defect when grown at pH 5.21 compared to the WT strain (Gries et al., 2013). However, in the second study a *ΔktrC* mutant displayed no growth defect compared to the WT strain at pH 6.0 (Gries et al., 2016).

In other bacteria the importance of potassium has been hypothesized to be due to K^+ import dissipating the ΔΨ to allow for proton efflux (Bakker & Mangerich, 1981; reviewed in Booth, 1985). However, the link between potassium transport, membrane potential, and pH regulation is less clear in *S. aureus*. On the one hand, *S. aureus* has been shown to have a reduced ΔΨ at acidic pH (Gries et al., 2016), as has been shown in many different bacterial species (reviewed in Krulwich et al., 2011). Furthermore, a *ΔktrA* mutant strain of *S. aureus* displayed a hyperpolarised membrane at neutral pH compared to the WT strain (Gries et al., 2013), indicating that changes in potassium transport affects the ΔΨ of the bacteria. On the other hand, at an acidic pH of 6.0 a *ΔktrC* mutant displayed no difference in ΔΨ compared to the WT strain, and the dissipation of the ΔΨ in WT bacteria occurred regardless of whether

potassium was present in the medium or not (Gries et al., 2016). The reason for this difference in results is unclear.

As with other bacteria, the signalling nucleotide c-di-AMP which binds to and regulates potassium transporters has been linked to acidic tolerance in *S. aureus*. c-di-AMP is produced in *S. aureus* by the diadenylate cyclase DacA and degraded by the phosphodiesterase GdpP (Corrigan et al., 2011). The levels of c-di-AMP have been shown to correlate with acid sensitivity in *S. aureus*, whereby a low c-di-AMP producing strain had decreased growth at pH 4.5, while a high c-di-AMP producing strain displayed the opposite phenotype (Bowman et al., 2016). Levels of c-di-AMP production were also shown to increase at low pH compared to neutral pH (Bowman et al., 2016). In addition, a Δ*ybbR* mutant strain was found to have increased sensitivity to low pH (Bowman et al., 2016). *ybbR* is found in the same three gene *dacA-ybbR-glmM* operon as *dacA*. In other bacteria, YbbR has been shown to interact with and regulate the activity of DacA (Gibhardt et al., 2020; Gundlach et al., 2015; Mehne et al., 2013; Rismondo et al., 2016; Zhu et al., 2016), and although no interaction has been reported yet in *S. aureus*, the increased sensitivity of the *ΔybbR* mutant to acid further supports a link between c-di-AMP and acid tolerance. However, as with other bacteria, it cannot be definitively stated that the mechanism through which c-di-AMP affects acid tolerance is through potassium transport.

1.4.6 Conclusion

Despite the wealth of evidence from the four transcriptional studies, their results cannot be taken at face value for several reasons. Firstly, transcriptional changes do not take into account post-transcriptional or post-translational modifications. For example, in some bacteria the F_1F_0ATPase has been shown to be mainly regulated by subunit assembly (Arikado et al., 1999). Therefore, changes in the F_1F_0ATPase activity will not be seen in transcriptional studies. Secondly, transcriptional studies do not always correlate with acid sensitivity. For example, while the urease genes are upregulated in *S. aureus* as shown in all four transcriptional studies, it has been shown in *H. pylori* that urease activity depends on urea concentration (Weeks et al., 2000). Therefore, while urease might be upregulated under low pH conditions, that does not necessarily mean that urease activity is increased. Thirdly, most of these transcriptional studies only investigate the transcriptional response in the initial 5-30 minutes following acid shock. Initially following acid stress, the bacterial cytoplasm acidifies (Wilks &

Slonczewski, 2007). However, the cytoplasm rapidly returns to a more neutral pH (Wilks & Slonczewski, 2007). The response of *S. aureus* to initial acid stress to simply de-acidify the cytoplasm may be very different to that used to grow at low pH in response to a more constant stress. Finally, very few phenotypic studies have been performed investigating individual genes and pathways involved in the acid stress response of *S. aureus*.

1.5 Transposon-Sequencing (Tn-Seq)

1.5.1 Transposon mutagenesis

There are several other techniques and tools used by molecular bacteriologists to determine which genes are important for bacterial fitness under particular conditions or stresses. One such technique used is transposon mutagenesis. This is where a transposon inserts into the bacterial genome at select sites, resulting in a library of mutants with each containing a single transposon insertion. These libraries can either be individual where each mutant is separated, or pooled where the mutants are all together (reviewed in Ong et al., 2023). Individual transposon mutant libraries such as the Nebraska Transposon Mutant Library (NTML) (Fey et al., 2013), which have known locations of the transposon insertions, are used to identify genes involved in select phenotypes of interest. While individual mutant libraries are useful for investigating the importance of select genes in pathways of interest, they are often limited in size and may not cover the full genome (reviewed in Ong et al., 2023). Furthermore, screening the impact of each gene on a phenotype of interest is laborious and labour intensive (reviewed in Ong et al., 2023).

On the other hand, using pooled libraries is a much more high-throughput technique, allowing a greater number of mutants and genes to be screened. However, the location of the transposon in resulting mutants is not known, therefore the transposon location and gene of interest has to be determined following the phenotypic assay. Previously, the transposon location was located by inverse PCR or arbitrarily primed PCR to determine the transposon-genome junction, followed by sanger sequencing (McNamara et al., 2000). More recently, however, next-generation sequencing (NGS) methods have been used to identify transposon insertion sites of a much greater number of mutants. Transposon-insertion sequencing techniques such as transposon-sequencing (Tn-Seq) (van Opijnen et al., 2009), transposon-

directed site sequencing (TraDIS) (Langridge et al., 2009), insertion sequencing (INSeq) (Goodman et al., 2009) and high-throughput insertion tracking by deep sequencing (HITS) (Gawronski et al., 2009) have been used to couple transposon mutagenesis with NGS. These techniques have been used in a wide range of bacterial species to identify genes important for several pathways, including the core genome, virulence genes, antibiotic resistance pathways, host adaptation and vaccine candidates (reviewed in Cain et al., 2020; reviewed in van Opijnen & Camilli, 2013). These techniques differ mainly in the method of transposon delivery and preparation of DNA for sequencing to determine the location of the transposon insertion, but for the rest of this thesis all transposon insertion sequencing techniques will be referred to as Tn-Seq.

The basic workflow of Tn-Seq has been reviewed in Cain et al., (2020) (Fig. 1.13). Firstly, a pooled library is generated by introducing a transposon into the bacterial genome by either transformation, conjugation or transduction. The most commonly-used transposable element is the *bursa aurealis* transposon derived from the *Himar1 mariner* element. The transposon also includes an erythromycin resistance cassette and *gfp* (Bae et al., 2004). A good library is determined to have sufficient coverage where every non-essential gene has several transposon insertions at different sites.

Following growth of the pooled library in select conditions, the DNA from the resulting culture is harvested and sequenced to determine the locations of transposon insertions. Genes which do not tolerate transposon insertions in the select conditions compared to neutral conditions are determined to be important or essential for bacterial fitness under these conditions. Alternatively, genes which have increased frequency of transposon insertions following growth in select compared to neutral conditions may be determined to be redundant or even detrimental for bacterial fitness under these conditions. Genes which have a similar number of transposon insertions following growth in select and neutral conditions are assumed to be neutral for bacterial fitness under these conditions.

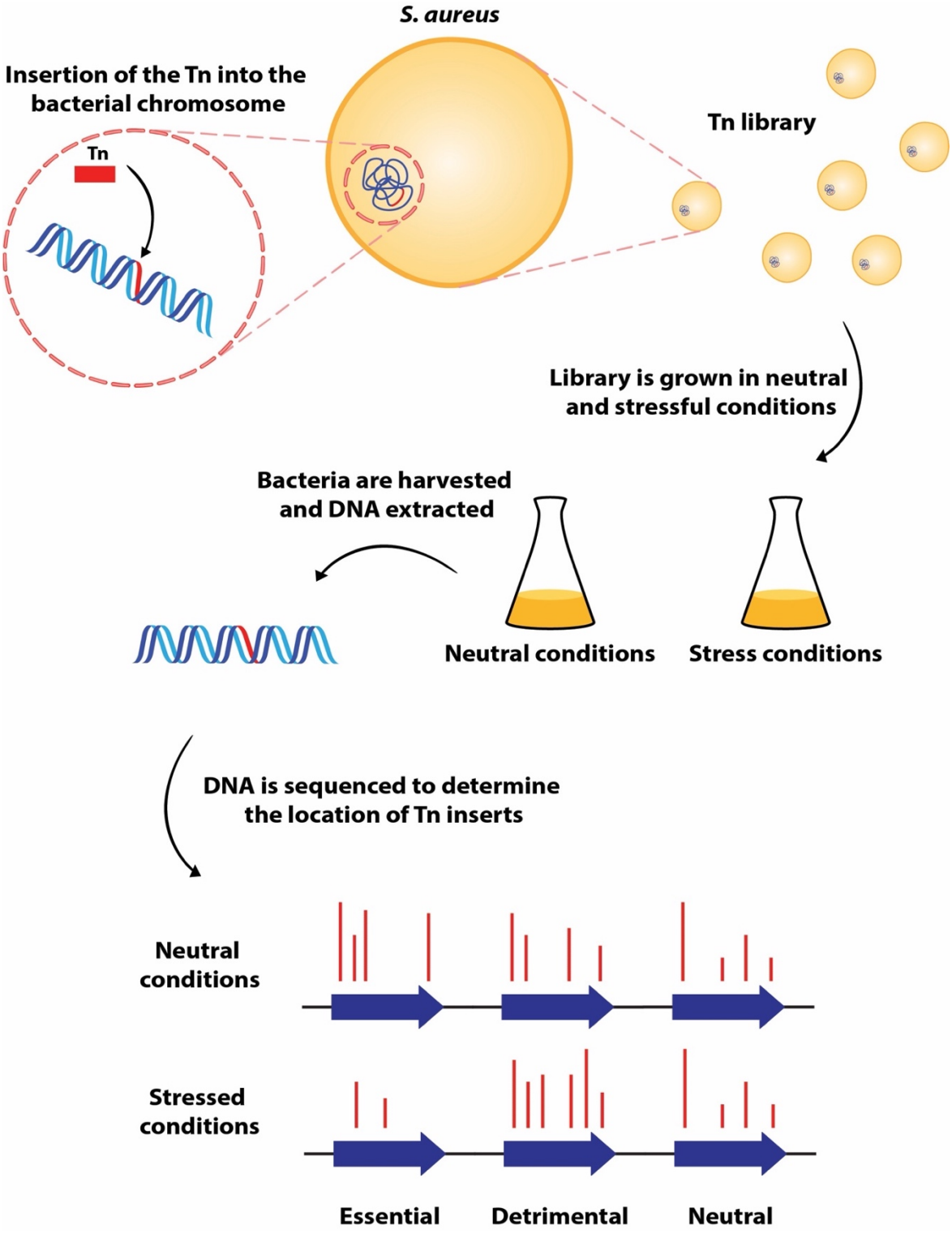


Figure 1.13: Schematic representation of the Tn-Seq workflow

A pooled library of transposon mutants is created by random insertion of the Tn into the bacterial chromosome. The library is then grown in neutral and stress conditions, before the bacteria are harvested, DNA isolated and sequenced to determine the location of Tn insertions. The number of Tn insertions per gene is then compared following growth in neutral and stress conditions to determine whether the gene is essential, detrimental, or neutral for bacterial fitness under the stress condition. Figure adapted from Cain et al., (2020) and Rajagopal et al., (2016).

1.5.2 Tn-Seq in *S. aureus*

Pooled transposon libraries have been used to investigate a range of phenotypes in *S. aureus*. Some of the most common phenotypes investigated using this technique are host-pathogen interactions in various infection models. These include *Caenorhabditis elegans* (Begun et al., 2005), whole blood (Christiansen et al., 2014; Valentino et al., 2014), ocular fluids (Valentino et al., 2014), abscess (Valentino et al., 2014) and mouse infection models (Blattner et al., 2016; Grosser et al., 2018; Ibberson et al., 2017).

Another key area of exploration for the use of Tn-Seq in *S. aureus* has been for the identification of genes involved in antibiotic resistance. Here, the transposon library is grown in the presence and absence of the antibiotic. Any mutants which are able to grow in the presence of the antibiotic are then screened to determine the transposon insertion location to identify genes which convey antibiotic susceptibility. Alternatively, the mutants which are unable to grow in the presence of the antibiotic are screened to determine genes which convey antibiotic resistance. These studies have identified genes involved in the antibiotic susceptibility and resistance pathways of a range of classes of antibiotics, from those targeting the cell wall, DNA replication, protein synthesis and the folate biosynthesis pathway (Coe et al., 2019; Rajagopal et al., 2016; Santiago et al., 2018; Wang et al., 2011).

Other phenotypes which have been investigated using Tn-Seq in *S. aureus* include genes involved in WTA biosynthesis (Santa Maria et al., 2014), bacterial fitness at high temperatures (Santiago et al., 2015), bacterial fitness in the presence of NO (Grosser et al., 2018) and salt stress (Schuster et al., 2020). Together, these studies have identified genes either essential or detrimental for the fitness of *S. aureus* in a wide range of conditions, increasing our understanding of the bacterium's genetic pathways and interactions.

Previous transposon libraries were generated in *S. aureus* using a plasmid-based delivery platform (Bae et al., 2008). Here, the transposon is introduced into *S. aureus* on a temperature-sensitive plasmid. After the transposon has inserted itself into the genome, the plasmid is cured from the bacteria by growth at high temperatures. However, this means that any genes which are essential for fitness at high-temperatures cannot be investigated for their importance in any phenotype as they cannot be inactivated in the transposon library.

One development which has been key to several of the above studies was the use of a phage-based delivery platform (Fig. 1.14) (Meredith et al., 2012; Santiago et al., 2015). Phage-based delivery platforms do not require a plasmid curing stage by growth at high temperatures, and thus a more complete transposon library can be generated (Fig. 1.14) (Meredith et al., 2012; Santiago et al., 2015).

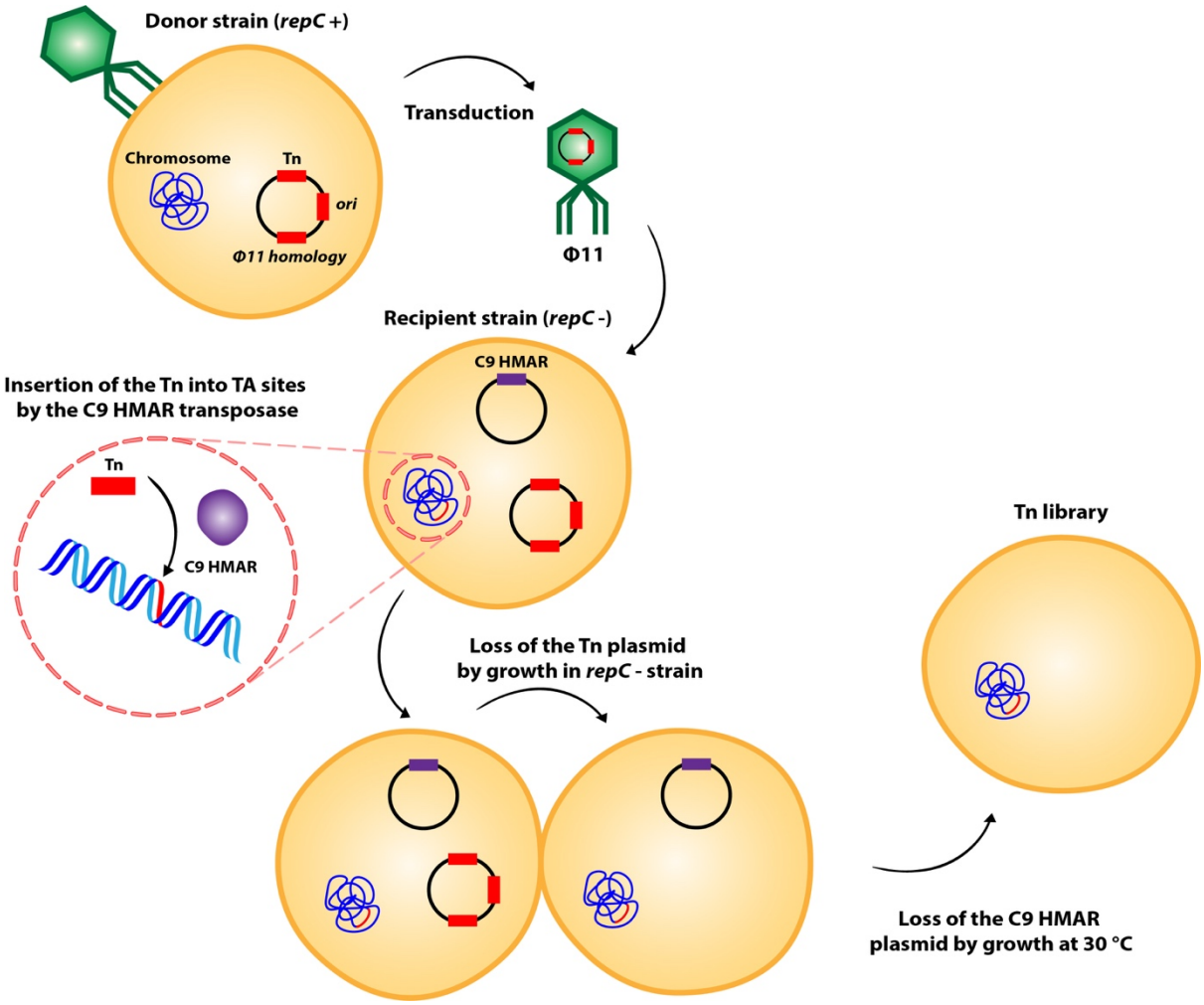


Figure 1.14: Schematic representation of the phage-based delivery system for generating transposon libraries
The transposon is propagated in a *repC*+ donor strain and is transduced into a *repC*- recipient strain via phage transduction. The recipient strain contains the transposase enzyme on a temperature-sensitive plasmid, allowing the insertion of the Tn into the bacterial chromosome. Following insertion the Tn plasmid is lost due to lack of *repC* in the recipient strain, and the transposase plasmid is lost following growth at 30 °C.

In this technique, the transposon is propagated in a donor strain on a plasmid. The plasmid contains an origin (*ori*) which is recognised by a bacterial chromosomally encoded protein

RepC to replicate the plasmid, and thus the transposon (Meredith et al., 2012; Santiago et al., 2015). The plasmid also contains a region with homology to the phage ϕ11, so that when the donor strain is infected with ϕ11 the plasmid is packaged into the phage capsid (Meredith et al., 2012; Santiago et al., 2015). The phage is then used to infect a recipient strain which encodes the C9 HMAR transposase on a separate plasmid (Meredith et al., 2012; Santiago et al., 2015). This transposase catalyses the insertion of the Tn into the bacterial chromosome after TA sites. As the recipient strain is *repC-*, the plasmid containing the Transposon and ϕ11 homology is lost following growth as the plasmid cannot replicate in a *repC-* strain (Meredith et al., 2012; Santiago et al., 2015). The plasmid encoding the HMAR transposase is temperature sensitive and is lost following growth at 30 °C (Meredith et al., 2012; Santiago et al., 2015).

1.6 Work leading up to this project

The overall aim of my PhD project was to explore genes and pathways important for the ability of *S. aureus* to grow at low pH. There were two avenues of work which led up to my PhD project. Firstly, previous published work from the lab showed that a mutant strain of *S. aureus* with low levels of c-di-AMP showed increased sensitivity to low pH, while a high c-di -AMP producing strain showed decreased sensitivity to low pH (Bowman et al., 2016). Furthermore, a Δ*ybbR* mutant strain showed increased sensitivity to low pH (Bowman et al., 2016). Therefore, the first part of my PhD was investigating the involvement of c-di-AMP and YbbR in the acidic stress response of *S. aureus*.

Secondly, Tn-Seq experiments were carried out in the lab prior to the start of my project in order to identify genes which are essential or detrimental for the fitness of *S. aureus* under acidic stress conditions (Beetham et al., 2024). Two individual transposon libraries were generated using the phage-based delivery system described above (Beetham et al., 2024; Coe et al., 2019; Schuster et al., 2020). The libraries were grown in medium at neutral pH, or medium acidified to a low pH by addition of HCl. Following growth, the DNA from the resulting cultures was harvested and sequenced to determine the location of Tn insertions. For each gene, a ratio was then generated comparing the number of Tn insertions present in the gene following growth at low pH, compared to the number of Tn insertions present in the gene

when grown at neutral pH. The above work was carried out by Professor Angelika Gründling and Dr Christopher Schuster prior to the start of my PhD. I started this project by analysing a previously generated list of *S. aureus* genes with their corresponding ratios. The analysis and characterisation of the results from the Tn-Seq experiment formed the basis of the second part of my PhD project.

1.7 Aims of my PhD

Overall, little is known about how *S. aureus* grows under acidic stress conditions. The main aim of my PhD was therefore to explore the genes and pathways used by *S. aureus* to grow under acidic stress conditions. I did this by investigating genes and pathways already known to be important for *S. aureus* and by identifying novel genes and pathways previously unknown for *S. aureus*.

Specifically, I wanted to:

1. Investigate how c-di-AMP regulates the acidic response of *S. aureus.*
    - Based on recent research which showed that the production of ammonia from urea via the urease enzyme was essential for the survival of *S. aureus* under acidic stress, it was hypothesized that: c-di-AMP and YbbR regulate urease activity in response to low pH.
2. Explore whether a Tn-Seq approach is an effective method to identify genes and pathways important for the fitness of *S. aureus* under acidic stress conditions.
    - Using the results of a previously performed Tn-Seq experiment it was hypothesized that: the Tn-Seq experiment would identify genes and pathways which had previously been shown to be upregulated in *S. aureus* in response to low pH.
3. Identify novel genes and pathways that are involved in the acidic stress response of *S. aureus*.
    - Using the results of a previously performed Tn-Seq experiment it was hypothesized that: the Tn-Seq experiment would identify novel genes and pathways important for the fitness of *S. aureus* at low pH.

Chapter 2 : Experimental procedures

2.1 Bacterial strains and growth conditions

Bacterial strains used in this study are shown in Table 2.1. *E. coli* strains were grown in lysogeny broth (LB) (Sigma-Aldrich, 20 g L^{-1}) or on LB agar plates (20 g L^{-1} LB, Sigma-Aldrich + 15 g L^{-1} bactoagar, Thermofisher), and *S. aureus* strains were grown in tryptic soy broth (TSB) (Becton Dickinson, 30 g L^{-1}), TSB with additional glucose (TSB + 31.1 mM glucose), or on TSB agar plates (Becton Dickinson, 40 g L^{-1}). *S. aureus* strains were also grown in chemically defined medium (CDM) made according to a previously described protocol (K_2HPO_4 5.34 g L^{-1}, NaH_2PO_4 1.77 g L^{-1}, $(NH_4)_2SO_4$ 1.0 g L^{-1}, Tris buffer (Sigma-Aldrich) 12.1 g L^{-1}, Glycerol 5.645 g L^{-1}, $MgSO_4.7H_2O$ 0.493 g L^{-1}, L-Arg 50 mg L^{-1}, L-Pro 10 mg L^{-1}, L-Glu 100 mg L^{-1}, L-Val 80 mg L^{-1}, L-Thr 30 mg L^{-1}, L-Phe 40 mg L^{-1}, L-Leu 90 mg L^{-1}, L-Gly 50 mg L^{-1}, L-Ser 30 mg L^{-1}, L-Asp 90 mg L^{-1}, L-Lys 50 mg L^{-1}, L-Ala 60 mg L^{-1}, L-Trp 10 mg L^{-1}, L-Met 10 mg L^{-1}, L-His 20 mg L^{-1}, L-Ile 30 mg L^{-1}, L-Tyr 50 mg L^{-1}, L-Cys 20 mg L^{-1}, Biotin 0.1 mg L^{-1}, Thiamine 2 mg L^{-1}, Nicotinic acid 2 mg L^{-1}, Calcium pantothenate 2 mg L^{-1}, $CaCl_2.2H_2O$ 22 mg L^{-1}, KH_2PO_4 140 mg L^{-1}, $FeSO_4.7H_2O$ 6 mg L^{-1}, $MnSO_4.4H_2O$ 10 mg L^{-1}, Citric acid 6 mg L^{-1}, pH 7.2) (Townsend & Wilkinson, 1992; Zeden et al., 2018), CDM lacking histidine, or CDM lacking leucine. Where specified, the growth medium was acidified to the indicated pH by the addition of HCl prior to autoclaving or filter sterilising. Low pH agar plates were made by adjusting the pH of TSB to the indicated pH with HCl, before the addition of 30 g L^{-1} bactoagar (Thermofisher) before autoclaving as has been described previously (Bowman et al., 2016). If required, the medium was supplemented with antibiotics as described in Table 2.1.

Table 2.1: Bacterial strains used in this study

Unique ID	Strain name and characteristics	Antibiotic resistance	Source
***E. coli* strains**			
ANG243	XL1-Blue pCL55	Ampicillin (Amp) 100 µg/ml	(Chen et al., 2014)
ANG1141	CLG190		Dana Boyd; (Gründling et al., 2003)
ANG6028	CLG190 pCL55-*0846*	Amp 100 µg/ml	This study (strain generated by A. Gründling)
***S. aureus* strains**			

ANG2624	JE2 (WT)		(Fey et al., 2013)
ANG5757	NTML JE2 *ureB::Tn* (NE1642)	Erythromycin (Erm) 10 µg/ml	(Fey et al., 2013)
ANG1575	LAC* (WT)		(Boles et al., 2010)
ANG5951	LAC* *ureB::Tn*	Erm 10 µg/ml	This study
ANG3301	LAC* *ΔybbR*		(Banerjee et al., 2010)
ANG3664	LAC* $dacA_{G206S}$		(Bowman et al., 2016)
ANG3729	TM283 (USA300-TCH1516 without pUSA300HOUMR)		(Coe et al., 2019; Santiago et al., 2015)
ANG4019	NTML JE2 *graS::Tn* (NE1756)	Erm 10 µg/ml	(Fey et al., 2013)
ANG4008	NTML JE2 *vraG::Tn* (NE70)	Erm 10 µg/ml	(Fey et al., 2013)
ANG4223	Strain 923 (WT)		(Boyle-Vavra et al., 2013)
ANG4226	Strain 923 Δ*vraS*	Chloramphenicol (Cam) 5 µg/ml	(Boyle-Vavra et al., 2013)
ANG4227	Strain 923 Δ*vraR*	Cam 5 µg/ml	(Boyle-Vavra et al., 2013)
ANG6295	NTML JE2 *mprF::Tn* (NE1360)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5968	NTML JE2 *fmtA::Tn* (NE1022)	Erm 10 µg/ml	(Fey et al., 2013)
ANG2395	LAC* Δ*dltD*	Erm 10 µg/ml	(Ledger et al., 2022)
ANG3969	NTML JE2 *SAUSA300_0482::Tn* (NE251)	Erm 10 µg/ml	(Fey et al., 2013)
ANG3911	NTML JE2 *SAUSA300_0957::Tn* (NE1384)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5979	NTML JE2 *sagB::Tn* (NE1909)	Erm 10 µg/ml	(Fey et al., 2013)
ANG3909	NTML JE2 *spdC::Tn* (NE1099)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5969	NTML JE2 *srrA::Tn* (NE1309)	Erm 10 µg/ml	(Fey et al., 2013)
ANG3941	NTML JE2 *qoxB::Tn* (NE732)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5957	NTML JE2 *qoxA::Tn* (NE92)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5967	NTML JE2 *SAUSA300_0846::Tn* (NE967)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5970	NTML JE2 *SAUSA300_2389::Tn* (NE1400)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5963	NTML JE2 *SAUSA300_0429::Tn* (NE620)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5964	NTML JE2 *SAUSA300_0543::Tn* (NE802)	Erm 10 µg/ml	(Fey et al., 2013)

ANG5959	NTML JE2 *SAUSA300_0481::Tn* (NE188)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5972	NTML JE2 *SAUSA300_1518::Tn* (NE1474)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5955	NTML JE2 *polA::Tn* (NE22)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5966	NTML JE2 *lepA::Tn* (NE865)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5978	NTML JE2 *pgm::Tn* (NE1891)	Erm 10 µg/ml	(Fey et al., 2013)
ANG2631	NTML JE2 *noc::Tn* (NE486)	Erm 10 µg/ml	(Fey et al., 2013)
ANG4016	NTML JE2 *murA::Tn* (NE1495)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5971	NTML JE2 *mutS2::Tn* (NE1462)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5608	NTML JE2 *lytH::Tn* (NE1369)	Erm 10 µg/ml	(Fey et al., 2013)
ANG6049	LAC* *SAUSA300_0846::Tn*	Erm 10 µg/ml	This study
ANG6197	JE2 *SAUSA300_0846::Tn*	Erm 10 µg/ml	This study
ANG113	RN4220 (WT)		(Kreiswirth et al., 1983)
ANG266	RN4220 pCL55	Cam 7.5 µg/ml	(Corrigan et al., 2013)
ANG6069	RN4220 pCL55-*0846*	Cam 7.5 µg/ml	This study (strain generated by A. Gründling)
ANG4325	JE2 pCL55	Cam 7.5 µg/ml	
ANG6073	NTML JE2 *SAUSA300_0846::Tn* pCL55	Erm 10 µg/ml, Cam 7.5 µg/ml	This study (strain generated by A. Gründling)
ANG6071	NTML JE2 *SAUSA300_0846::Tn* pCL55-*0846*	Erm 10 µg/ml, Cam 7.5 µg/ml	This study (strain generated by A. Gründling)
ANG3795	LAC* pCL55	Cam 7.5 µg/ml	(Schuster et al., 2016)
ANG6078	LAC* *SAUSA300_0846::Tn* pCL55	Erm 10 µg/ml, Cam 7.5 µg/ml	This study (strain generated by A. Gründling)
ANG6076	LAC* *SAUSA300_0846::Tn* pCL55-*0846*	Erm 10 µg/ml, Cam 7.5 µg/ml	This study (strain generated by A. Gründling)
ANG6199	JE2 *SAUSA300_0846::Tn* pCL55	Erm 10 µg/ml, Cam 7.5 µg/ml	This study
ANG6201	JE2 *SAUSA300_0846::Tn* pCL55-*0846*	Erm 10 µg/ml, Cam 7.5 µg/ml	This study
ANG6196	JE2 *SAUSA300_0846::Tn kapB**	Erm 10 µg/ml	This study

ANG6039	NTML JE2 *cpa1-1::Tn* (NE1504)	Erm 10 µg/ml	(Fey et al., 2013)
ANG6040	NTML JE2 *cpa1-2::Tn* (NE366)	Erm 10 µg/ml	(Fey et al., 2013)
ANG4556	NTML JE2 *cpa2::Tn* (NE308)	Erm 10 µg/ml	(Fey et al., 2013)
ANG6037	NTML JE2 *nhaC1::Tn* (NE1470)	Erm 10 µg/ml	(Fey et al., 2013)
ANG6038	NTML JE2 *nhaC2::Tn* (NE1214)	Erm 10 µg/ml	(Fey et al., 2013)
ANG6080	LAC* *SAUSA300_0846::Tn*-S1	Erm 10 µg/ml	This study
ANG6085	LAC* *SAUSA300_0846::Tn*-S2	Erm 10 µg/ml	This study
ANG6087	LAC* *SAUSA300_0846::Tn*-S3	Erm 10 µg/ml	This study
ANG6090	LAC* *SAUSA300_0846::Tn*-S4	Erm 10 µg/ml	This study
ANG6099	LAC* *SAUSA300_0846::Tn*-S5	Erm 10 µg/ml	This study
ANG6111	LAC* *SAUSA300_0846::Tn*-S6	Erm 10 µg/ml	This study
ANG6113	LAC* *SAUSA300_0846::Tn*-S7	Erm 10 µg/ml	This study
ANG6118	LAC* *SAUSA300_0846::Tn*-S8	Erm 10 µg/ml	This study
ANG6058	NTML JE2 *SAUSA300_0846::Tn*-S1	Erm 10 µg/ml	This study
ANG6059	NTML JE2 *SAUSA300_0846::Tn*-S2	Erm 10 µg/ml	This study
ANG6060	NTML JE2 *SAUSA300_0846::Tn*-S3	Erm 10 µg/ml	This study
ANG6061	NTML JE2 *SAUSA300_0846::Tn*-S4	Erm 10 µg/ml	This study
ANG6062	NTML JE2 *SAUSA300_0846::Tn*-S5	Erm 10 µg/ml	This study
ANG6064	NTML JE2 *SAUSA300_0846::Tn*-S6	Erm 10 µg/ml	This study
ANG6065	NTML JE2 *SAUSA300_0846::Tn*-S7	Erm 10 µg/ml	This study
ANG6066	NTML JE2 *SAUSA300_0846::Tn*-S8	Erm 10 µg/ml	This study
ANG6129	NTML JE2 *codY::Tn* (NE1555)	Erm 10 µg/ml	(Fey et al., 2013)
ANG3086	NTML JE2 *rsh::Tn* (NE1714)	Erm 10 µg/ml	(Fey et al., 2013)
ANG5613	NTML JE2 *clpY::Tn* (NE1708)	Erm 10 µg/ml	(Fey et al., 2013)
ANG6293	LAC* *codY::Tn*	Erm 10 µg/ml	This study
ANG6294	LAC* *rsh::Tn*	Erm 10 µg/ml	This study

2.2 Plasmid and bacterial strain construction

2.2.1 Confirmation of correct transposon insertions in NTML strains

Several strains used in this study were derived from the Nebraska transposon mutant library (NTML) library (Fey et al., 2013). The transposon insertion site was confirmed by polymerase chain reaction (PCR) and sequencing for all NTML strains used in this study as described below.

First, chromosomal DNA was extracted from the mutant strains by lysing the bacterial cells with 0.1 mg ml^{-1} lysostaphin in TSM buffer (50 mM Tris pH 7.5, 0.5 M sucrose, 10 mM $MgCl_2$), and DNA was subsequently isolated using the Wizard Genomic DNA purification kit (Promega) according to the manufacturers instructions. The isolated DNA was rehydrated in H_2O and the final concentration measured using a Nano-drop.

Target DNA was amplified from the extracted chromosomal DNA by PCR using *Taq* DNA Polymerase (NEB). 50-100 ng total DNA was used as a template, and primers were designed to amplify the transposon-gene insertion site. The PCRs were carried out in 200 µl PCR tubes as described in Table 2.2 and Table 2.3.

Table 2.2: PCR components for amplifying DNA for confirmation of transposon insertions

Component	**Volume (µl)**
H_2O	16.6
10 X *Taq* Polymerase buffer	2.5
DNA template	0.5 (50-100 ng)
Primer 1 (10 µM)	1.25
Primer 2 (10 µM)	1.25
dNTPs (40 mM)	1.25
Taq DNA Polymerase	0.25
$MgCl_2$ (50 mM)	1.4

Table 2.3: PCR reaction for amplifying DNA for confirmation of transposon insertions

Temperature	Time (s)	Cycle
95 °C	120	
95 °C	30	5 X
45 °C	30	
72 °C	90	
95 °C	30	30 X
54 °C	30	
72 °C	90	
72 °C	600	
10 °C	Hold	

The amplified target DNA regions were checked for their size by mixing 5 µl of the PCR product with 1 µl 6 X DNA loading dye and running the products on a 1% agarose gel (10 g L^{-1} agarose powder in TAE buffer with 10,000 X SYBR™ Safe (Thermofisher)) at 110 V for 35 min. A pre-made 1-kb plus DNA ladder (NEB) was run on the same gel along side the PCR product for size comparison. If a band was seen at the expected size of approximately 1 kb, then the remaining PCR product was cleaned using the Qiagen QIAquick PCR Purification kit (Cat 28104) according to the manufacturer's instructions and eluted in 30 to 50 µl 5 mM TRIS buffer pH 8.0. The DNA concentration was measured on a Nano-drop and the PCR product was sent for Sanger sequencing with the appropriate primers to Eurofins. Correct transposon location insertion was confirmed by comparing the resulting DNA sequence with the target gene-transposon sequence.

2.2.2 Phage transduction of the transposon into a different *S. aureus* background

The *ureB::Tn* transposon region from the original NTML JE2 strain NE1642 (ANG5757), the *0846::Tn* transposon region from the original NTML JE2 NE967 (ANG5967), the *codY::Tn* transposon region from the original NTML JE2 strain NE1555 (ANG6129) and the *rsh::Tn* transposon region from their original NTML JE2 strain NE1714 (ANG3086) were moved by

phage transduction using phage Φ85 into the *S. aureus* LAC* background, yielding strains LAC* *ureB::Tn* (ANG5951), LAC* *0846::Tn* (ANG6049), LAC* *codY::Tn* (ANG6293) and LAC* *rsh::Tn* (ANG6294). The *0846::Tn* transposon region from the original NTML strain NE967 (ANG5967) was also moved by phage transduction using phage Φ85 into the *S. aureus* JE2 background, yielding strain JE2 *0846::Tn* (ANG6197). Correct transposon location insertion was determined by PCR and sequencing as described above.

2.2.3 Generation of complementation strains

Complementation strains were generated by A. Gründling and myself. Firstly, plasmid pCL55-*0846* was constructed. Plasmid pCL55 was isolated from *E. coli* strain XL1-Blue pCL55 (ANG243) using the QIAGEN Plasmid Midi prep kit according to the manufacturer's instructions. The *SAUSA300_0846* gene including its native promoter region was amplified by PCR using primers 3443 (AAAGAATTCGAATTACCGATTACTGCAACCGAACGTGC) and 3444 AAAGGATCCGTCTCTAATAAATGAGTCATATTTTCACC) and the Hercules Fusion Polymerase (Agilent). The PCRs were carried out in 200 µl PCR tubes as described in Table 2.4 and Table 2.5.

Table 2.4: PCR components for amplifying DNA for construction of plasmid pCL55-*0846*

Component	**Volume (µl)**
H_2O	32
5 X Hercules reaction buffer	10
DNA template	1.0 (50-100 ng)
Primer 3443 (10 µM)	2.0
Primer 3444 (10 µM)	2.0
dNTPs (10 mM)	2.0
Hercules Fusion Polymerase	1.0

Table 2.5: PCR reaction for amplifying DNA for construction of plasmid pCL55-*0846*

Temperature	Time (s)	Cycle
95 °C	60	
95 °C	30	5 X
48 °C	30	
72 °C	180	
95 °C	30	25 X
55 °C	30	
72 °C	180	
72 °C	180	
12 °C	Hold	

The resulting PCR product was cleaned using the Qiagen QIAquick PCR Purification kit (Cat 28104) according to the manufacturer's instructions, and 3 µl of the resulting product was run on a 0.8 % agarose gel (8 g L^{-1} agarose powder in TAE buffer with 10,000 X SYBR™ Safe (Thermofisher)) at 110 V for 30 min to confirm the size of the product. The remaining cleaned PCR product and plasmid pCL55 were digested in 100 µl reactions in cut smart buffer with 2 µl EcoRI and 2 µl BamHI (NEB) for 2 at 37°C before being run on a 0.8 % agarose gel (8 g L^{-1} agarose powder in TAE buffer with 10,000 X SYBR™ Safe (Thermofisher)) at 110 V for 1 h. The bands with the correct size were gel extracted using the QIAGEN QIAquick Gel Extraction kit according to the manufacturer's instructions. Cleaned digested pCL55 and *0846* PCR product were ligated overnight at 16°C in a 25 µl reaction in 1 X T4 ligase buffer containing 1 µl T4 Ligase (NEB) and approximately 150 ng cut plasmid DNA and 400 ng cut PCR product. The ligation was heat inactivated for 20 min at 65°C and then dialysed against ddH_2O on a nitrocellulose filter (Millipore) for 30 min to remove any salts.

Electrocompetent *E. coli* strain CLG190 cells for recovery of the pCL55-*0846* plasmid were prepared by diluting an overnight culture 1:100 in LB and growing the culture to an OD_{600} of 0.5. 100 ml of cells were incubated on ice for 15 min, then washed three times by

centrifugation and resuspension in 10 % (v/v) glycerol before being suspended in 1.2 ml ice-cold sterile 10 % (v/v) glycerol.

The pCL55-*0846* plasmid was transformed into electrocompetent *E. coli* strain CLG190 cells by electroporation to generate strain CLG190 pCL55-*0846* (ANG6028). Briefly, 10 µl dialyzed ligation product and 100 µl electrocompetent cells were transferred to a 1 mm electroporation cuvette and electroporated using a Biolabs Gene Pulser with a resistance of 200 Ω, voltage of 1.8 kV and capacitance of 25 µFD. Cells were recovered in 900 ml LB and incubated at 37 °C for 1 h, before 100 µl was plated on LB agar plates supplemented with antibiotics as described in Table 2.1.

Plasmid pCL55-*0846* was subsequently isolated from *E. coli* using a Midi prep as described above and transformed into *S. aureus* strain RN4220 strain. Electrocompetent *S. aureus* strain RN4220 cells were prepared by diluting a bacterial overnight culture 1:100 in TSB and growing at 37°C until an OD_{600} of 0.5 was reached. 200 ml of the resulting culture was then washed 3 x with 100 ml of ice cold 0.5 M sucrose and collected in 2 ml 0.5 M sucrose. The pCL55-*0846* plasmid was dialyzed against water on a nitrocellulose membrane as described above and introduced by electroporation into electrocompetent *S. aureus* strain RN4220 cells, where it integrates into the *geh* gene locus, generating strain RN4220 pCL55-*0846* (ANG6069). For this a 1 mm electroporation cuvette was used and a Biolabs Gene Pulser set to a resistance of 100 Ω, voltage of 2.5kV and capacitance of 25 µFD. The bacteria were recovered in 900 ml TSB with 0.5 M sucrose and subsequently plated on TSB agar plates supplemented with antibiotics as described in Table 2.1.

Finally, the pCL55-*0846* region was transduced with phage Φ85 into either the NTML JE2 *0846::Tn* (ANG5967), LAC* *0846::Tn* (ANG6049), or JE2 *0846::Tn* (ANG6197), resulting in the construction of the complementation strains NTML JE2 *0846::Tn* pCL55-*0846* (ANG6071), LAC* *0846::Tn* pCL55-*0846* (ANG6076), and JE2 *0846::Tn* pCL55-*0846* (ANG6201).

Strains JE2 pCL55 (ANG4325) and LAC* pCL55 (ANG3795) were used as control strains for some experiments, and the empty plasmid pCL55 was also moved using phage Φ85 from *S. aureus* strain RN4220 pCL55 (ANG266) into strain NTML JE2 *0846::Tn* (ANG5967), LAC*

0846::Tn (ANG6049), and JE2 *0846::Tn* (ANG6197) to generate strains NTML JE2 *0846::Tn* pCL55 (ANG6073), LAC* *0846::Tn* pCL55 (ANG6078), and JE2 *0846::Tn* (ANG6199).

Strains ANG6028, ANG6069, ANG5967, ANG6049, ANG6071, ANG6076, ANG6073 and ANG6078 were generated by A. Gründling, while strains ANG6197, ANG6201 and ANG6199 were generated by me.

2.3 Urease assay

To assess the relevance of urease in the acidic stress response of *S. aureus*, growth curves were performed in TSB medium in the presence or absence of 10 mM urea as has been described previously (Zhou et al., 2019). Stationary phase bacteria grown overnight in 3-5 ml TSB medium were inoculated in 20 ml TSB medium with or without 10 mM urea to an OD_{600} value of 0.05. Growth under weak acid stress was investigated by supplementing the medium with 31.1 mM glucose to reach a final glucose concentration of 45 mM, as has been described previously (Zhou et al., 2019). Growth under strong acid stress was investigated by the addition of HCl to the TSB medium prior to autoclaving to reach a pH of 4.5. Cultures were incubated at 37 °C with shaking at 180 revolutions per minute (RPM) until the desired time point.

Bacterial growth was determined by colony forming units (CFU) ml^{-1} counts. At the desired time points, the bacterial culture was diluted in phosphate buffered saline (PBS) in 10-fold dilutions, and 100 µl were plated on TSA plates. Plates were incubated at 37 °C for 16-18 h before colonies were counted.

The pH of the medium was measured during bacterial growth. To this end, 900 µl of culture was removed from the flask at the indicated time points and the bacteria pelleted by centrifugation at 13,000 rpm to remove the cells. The pH of the supernatant was measured using a pH probe.

2.4 Tn-Seq experiment

The generation, propagation, and initial analysis of the TnSeq experiment was performed by A. Gründling and C. Schuster prior to the start of this PhD as has been described previously

(Beetham et al., 2024; Coe et al., 2019; Santiago et al., 2015; Schuster et al., 2020) and as described in the Experimental procedures below.

2.4.1 Generation of Tn mutant libraries

Tn mutant libraries were generated by A. Gründling and C. Schuster prior to the start of this PhD. The TnSeq experiment was repeated using two independent transposon mutant libraries. Both libraries were constructed in the *S. aureus* USA300 TCH1516-derived MRSA strain TM283. The first (library A) was generated using a mix of six different transposons (containing 5 different outward facing promoters and one promoter less blunt transposon), and its construction has been described previously (Coe et al., 2019; Santiago et al., 2015). The library contained around 600,000 mutants. The second library (library B), was generated using a single promoter less blunt transposon, and its construction has been described previously (Schuster et al., 2020). This library contained > 1 million pooled colonies.

2.4.2 Propagation and sample preparation of Tn mutant libraries

The propagation and sample preparation of the Tn mutant libraries was performed by A. Gründling and C. Schuster prior to the start of this PhD. To identify genes essential or detrimental for the fitness of *S. aureus* under acidic conditions, the Tn mutant libraries were propagated in TSB medium at either standard pH (7.3), or TSB medium adjusted to pH 5.5 or pH 4.5 by the addition of HCl prior to autoclaving. Briefly, a vial of the library was thawed on ice, and used to inoculate 20 ml TSB medium supplemented with Erm 5 µg/ml or 10 µg/ml to an OD_{600} value of 0.1. This pre-culture was grown for 37 °C with shaking for 1 h. This pre-culture was subsequently back-diluted and used to inoculate either 25 ml fresh TSB (library A) or 100 ml fresh TSB medium with Erm 5 µg/ml (library B) at either standard pH (7.3), or TSB medium adjusted to pH 5.5 or pH 4.5 with HCl to an OD_{600} of 0.00125. These cultures were grown at 37 °C with shaking at 180 rpm until they reached an OD_{600} value of 1.4 (approx. 10 generations). Following this, bacteria from 12 ml of culture were harvested by centrifugation, washed once in fresh TSB medium, and the cell pellet frozen at -20 °C.

Samples were prepared for sequencing as described previously (Santiago *et al.,* 2015). The genomic DNA was isolated, digested with NotI, then DNA fragments less than 300 bp were selectively removed by precipitation in polyethylene glycol (PEG) 8000. Following this, the remaining DNA fragments were ligated to biotinylated adaptors, before being further digested with MmeI and ligated to Illumina adaptors with barcodes. Samples were then sequenced

using an Illumia HiSeq platform and 100 base single end reads at the TUCF genomics core facility at TUFTS University, USA.

2.4.3 Analysis and mapping of TnSeq data

Analysis and mapping of the TnSeq data was performed by C. Schuster prior to and during the duration of this PhD. Analysis and mapping of the sequencing data was performed as described previously (Coe et al., 2019; Santiago et al., 2015) using the Tufts Galaxy Server. Briefly, reads were trimmed, split by Illumina barcode, and then further split by transposon barcode. Reads were then mapped to a USA300-TCH1516 genome, before Hopcount tables were generated. Statistical analysis was performed using the Mann-Whitney test to find significant differences in the number of reads per gene, and Benjamini-hochberg test to correct the p-value for multiple hypothesis testing. Circular plots were generated using custom R-scripts and circus. Ratios were generated for each gene comparing the number of Tn insertions present in the gene following growth under the low pH stress conditions compared to the number of Tn insertions present in the gene following growth under neutral conditions.

2.4.4 Filtering and comparison of TnSeq results

Further analysis of TnSeq data was performed by me using filtering functions in Excel. Statistically significant genes were identified as those with both ≥ 10 Tn insertions per gene and a Benjamini-Hochberg of ≤ 0.1. Genes were classified as essential if they had a ratio of ≤ 0.5, while detrimental genes were classified if they had a ratio of ≥ 2. While the USA300_TCH1516 genome was used for the Tn-Seq data analysis, USA300 FPR3757 locus tag numbers are shown throughout this study to match up with the NTML strain annotations.

2.5 Agar plate spotting assays

Bacterial growth at low pH was analysed through spotting dilution of bacterial culture on low pH agar plates. Indicated strains of *S. aureus* were grown overnight in 3-5 ml TSB medium at 37 °C with shaking. The following day, bacteria from 1 ml of overnight culture adjusted to an OD_{600} value of 5 were collected by centrifugation and resuspended in 1 ml PBS. Next, 10-fold dilutions down to a dilution of -7 were prepared in PBS, before 5 µl of each dilution were spotted on to standard TSA plates (pH 7.3) or TSA pH 4.5 plates. The plates were incubated at

37 °C for 18-24 h before images were taken. Unless stated otherwise, images are representative of three independent repeats.

2.6 Acid stress survival curves

Bacterial survival at low pH was analysed using an acid stress survival assay. Indicated strains of *S. aureus* were grown overnight in 3-5 ml TSB medium at 37 °C with shaking. Bacteria from the equivalent of 1 ml of overnight culture with an OD_{600} of 8 was washed three times by centrifugation and resuspension in 1 ml fresh TSB medium. The bacterial suspension was then transferred to 20 ml TSB medium with a pH of 2.5. Cultures were incubated at 37 °C with shaking at 180 rpm until the indicated time points. CFU were determined by removing 200 µl of bacterial culture and preparing 10-fold dilutions in PBS. 100 µl of selected dilutions were plated on TSA plates, and the plates were incubated at 37 °C for 16-18 h before colonies were counted. For the T=0 time point, culture aliquots were taken immediately after transfer to the low pH medium. The CFU count at T=0 was set to 100% for each strain, and % survival at the subsequent time points calculated. Unless stated otherwise, the average and standard deviation of the % survival from at least three independent experiments were plotted.

2.7 Bacterial growth curves in a plate reader

The growth of indicated bacterial strains was analysed in various media as stated in the results Chapters and figure legends. For growth curves performed in TSB medium, indicated *S. aureus* strains were grown overnight in 3-5 ml TSB medium at 37 °C with shaking. 1 ml bacterial culture was washed 1 x by centrifugation and resuspension in 1 ml fresh TSB medium. This was used to inoculate 1 ml fresh TSB medium at either standard pH (pH 7.3) or pH 4.5 by to a final OD_{600} value of 0.05. For growth curves performed in CDM, indicated *S. aureus* strains were grown overnight in 3-5 ml TSB medium or CDM pH 7.2 or pH 7.0 medium at 37 °C with shaking as indicated in the results Chapters and figure legends. 1 ml bacterial culture was washed 1 x by centrifugation and resuspension in fresh CDM. This was used to inoculate either 1 ml fresh CDM containing all amino acids, 1 ml fresh CDM without 130 µM histidine, or 1 ml fresh CDM without 686 µM leucine to a final OD_{600} value of 0.05. CDM was made at either pH

7.2, pH 7.0, or pH 4.3 by addition of HCl prior to autoclaving. The amino acid content and pH of the CDM is defined in the results Chapters and figure legends.

For all growth curves, three technical replicates of 200 µl of the washed OD_{600} = 0.05 cultures were transferred to the wells of a 96-well plate. The plates were incubated at 37 °C with shaking at 500 rpm for 300 s every 10 min in a SPECTROstar^{NANO} plate reader, and OD_{600} measurements were taken every 10 min for 24 h. To avoid congestion on the graph, only the readings from every 30 min were plotted. The final OD_{600} values were calculated by averaging the three technical replicates and subtracting the OD_{600} readings of a blank well containing medium only. Unless indicated otherwise, the averages and standard deviations of three independent repeats were calculated and plotted.

2.8 Measurements of membrane potential

Measurements of the membrane potential of indicated strains of *S. aureus* was done using the 3, 3'-diethyloxacarbocyanine iodide ($DiOC_2(3)$) dye using either a fluorescence activated cell sorter (FACS) based method as has been described previously (Shapiro, 2008; Zeden et al., 2018), or a plate-reader based method as has been described previously with modifications (Beetham et al., 2024; Grosser et al., 2018). The dye emits both green and red fluorescence, where green corresponds to cell size, and red corresponds to both cell size and membrane potential. The resulting red / green fluorescence ratio gives a value of membrane potential independent of cell size. As a control, carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added to a second set of samples containing the bacterial cells and dye. CCCP is a protonophore, and thus collapses the membrane potential by allowing the concentration of protons to equalise across the membrane. When CCCP is added, the bacteria will show only green fluorescence, and will thus have a low red / green ratio.

2.8.1 FACS based method to measure the membrane potential of stationary phase cultures

S. aureus strains LAC* WT and LAC* *0846::Tn* were grown overnight in 3-5 ml TSB medium. The following day, 1 ml of overnight culture with an OD_{600} value of 1 was washed three times with alternate centrifugation and resuspension in staining buffer (0.06 M phosphate buffer supplemented with 5mM KCl, 130 mM NaCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM glucose, adjusted to pH 7.2 with NaOH). Following the final wash step, the bacteria were

diluted to an OD_{600} value of 0.2, before 100 µl of this suspension was removed to a FACS tube containing 890 µl staining buffer and 10 µl 3 mM $DiOC_2(3)$ resulting in a final concentration of 30 µM. As a control, 7.5 µl of 2 mM CCCP was added to a separate set of samples containing the bacterial suspension and dye to dissipate the membrane, resulting in a final concentration of 15 µM. The fluorescence of 10,000 events was measured using a FACS Calibur cytometer and a 488 nm or 633 nm laser. Green and red fluorescence was detected in the FL-1 (530/30 nm) and FL-2 (585/42 nm) channels, respectively. The red / green ratio was determined using the FlowJo V10.8 software. A representative histogram of the cell count vs ratio from two independent experiments was plotted, and the mean value of the red / green ratio as determined in the histogram from the same two independent experiments were plotted.

2.8.2 Plate reader based method to measure the membrane potential of log phase cultures

S. aureus strains LAC* WT and LAC* *0846::Tn* were grown overnight in 3-5 ml TSB medium. The following day, 140 µl of the overnight cultures was used to inoculated 20 ml fresh TSB and the cultures grown until reaching an OD_{600} of approx. 0.25. 8 x 1 ml of culture were corrected to an OD_{600} value of 0.25, and washed three times by alternate centrifugation and resuspension in 1 ml staining buffer pH 7.2. After the final wash step, the bacteria were resuspended in 500 µl staining buffer pH 7.2. 5 µl 3 mM $DiOC_2(3)$ was added to the cells to result in a final concentration of 30 µM, before the suspensions were left to incubate in the dark at room temperature (RT) for 10 min. Following this, the bacterial cells were washed once and then resuspended in staining buffer at either pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.2, or 9.0 (the pH of the buffer was adjusted by addition of HCl or NaOH). Next, 200 µl of each culture was transferred to a black-walled 96-well microplate. As a control, 2.25 µl of 2 mM CCCP was added to the remaining 800 µl bacterial culture to result in a final concentration of 15 µM. 200 µl of the depolarised bacterial suspensions were then transferred to a black-walled 96-well microplate. Fluorescence was read on a TECAN plate reader with excitation / emission wavelengths of 488 nm / 528 nm for green and 488 nm / 635 nm for red. Gain values were set at 85 % and 50 %, respectively. Measurements were taken every 6-7 minutes for 30 minutes, with the largest ratio for the WT at pH = 7.2 used to determine which time point to use for plotting the data. The averages and standard deviations of three independent repeats were calculated and plotted.

2.9 Histidine uptake assays

Uptake assays measuring the intracellular accumulation of radioactively labelled amino acid were performed as has been described previously (Zeden et al., 2018). *S. aureus* strains LAC* WT (EV), LAC* *0846::Tn* (EV), and LAC* *0846::Tn* (comp.) were grown overnight in 5 ml CDM at 37 °C with shaking. The following day the cultures were back-diluted to an OD_{600} of 0.05 into 50 ml fresh CDM and grown at 37 °C with shaking until mid-log phase. 2 ml bacterial culture with an OD_{600} equivalent of 8 was harvested by centrifugation at 9,000 rpm for 10 min and suspended in 2 ml CDM without histidine. 1.5 ml of this culture was further washed by centrifugation at 13,000 rpm for 5 min and resuspension in 1 ml CDM without histidine. This culture was subsequently adjusted with CDM lacking histidine to an OD_{600} value of 1. An OD_{600} reading was taken again at this time point, to determine the exact density of this suspension for normalization purposes.

Next, to 450 µl of this washed bacterial culture, 2 µl of radiolabelled histidine, L-[ring-2,5-3H] (ARC UK limited, ART 0234) was added and the suspension mixed by swirling. Immediately afterwards (T=0 time point), 100 µl of culture was removed to a nitrocellulose filter and washed with 32 ml PBS. A vacuum was applied to encourage drainage using a Manifold 1225. The remining bacterial culture was incubated at RT, and further 100 µl samples were taken and processed as above at T=3, 6, and 9 min. The filters were subsequently removed and placed into 9 ml Filter-Count scintillation fluid (PerkinElmer), before using a Wallac 1409 DSA liquid scintillation counter to measure radioactivity in counts per minute (CPM). CPMs were then normalised to the final OD_{600} values of the culture used for these assays. Three independent experiments were performed, and average values and standard deviations calculated and plotted.

2.10 Generation of *0846::Tn* suppressor strains with increased acid resistance

NTML JE2 *0846::Tn* and LAC* *0846::Tn* suppressor strains with increased acid resistance were generated using two slightly different methods. For strain NTML JE2 *0846::Tn*, multiple independent cultures were grown overnight in TSB at 37 °C with shaking. The following day, 50 µl of 10^{-3} dilutions were plated onto TSA pH 4.5 plates. Following incubation at 37 °C for 48

h, colonies obtained on the low pH TSA plates were used to inoculated TSB medium with erm 10 µg/ml and the cultures incubated for 18-24 h at 37 °C with shaking. Following this, the culture was streaked onto TSA pH 7.3 with erm 10 µg/ml and incubated for 24 h at 37 °C. Next, single colonies were picked, grown overnight in TSB with erm 10 µg/ml and aliquots of these suppressor strain cultures stored frozen at -80 °C. For strain LAC* *0846::Tn*, multiple independent cultures were grown overnight in TSB at 37 °C with shaking. The following day, 50 µl of 10^{-2} to 10^{-4} dilutions were plated onto TSA pH 4.4 or pH 4.5 plates. Following incubation at 37 °C for 48 h, colonies obtained on the low pH TSA plates were re-streaked on TSA pH 4.4 or pH 4.5 and the plates incubated again for 48 h at 37 °C. Next, single colonies were picked, grown overnight in TSB medium supplemented with 10 µg/ml erm and aliquots of these suppressor strain cultures stored frozen at -80°C. The increased acid resistance of the NTML JE2 *0846::Tn* and LAC* *0846::Tn* suppressor strains was subsequently confirmed using the agar plate spotting assay described above.

2.11 Whole genome sequencing sample preparation and analysis

Genomic DNA was isolated using the chloroform / isopentanol method described previously (Schuster & Bertram, 2014; Zeden & Gründling, 2023b). Samples were prepared for Illumina sequencing using an Illumina Nextera DNA kit. Samples were sequenced at the MRC London Institute for Medical Sciences in Hammersmith Hospital using a MiSeq machine and an Illumina MiSeq Reagent kit v2 (300 cycles) to generate 150 bp paired-end reads. For genome sequence analysis, a previously described protocol using the CLC workbench Genomics software (Qiagen) was used (Zeden & Gründling, 2023a). Short reads were trimmed, then mapped to a custom *S. aureus* USA300 LAC* reference genome generated in a previous study (Bowman et al., 2016). Single nucleotide polymorphisms (SNPs) were determined based on at least 70% frequency, and strain background SNPs and areas of low coverage were removed by comparing them to SNPs and areas of low coverage also present in the genome sequence of the original NTML JE2 *0846::Tn*, LAC* *0846::Tn*, or LAC* *0846::Tn* (EV) strains. Illumina sequence reads were deposited into the European Nucleotide Archive (ENA) under the project numbers as shown in Table 2.2.

Sequencing analysis for the *0846::Tn* strains with increased acid resistance was performed by me, and sequencing analysis for the LAC* *0846::Tn* strains with increased growth in CDM was performed by A. Gründling.

Table 2.6: Accession numbers for the Illumina sequence reads deposited into the ENA

Accession	Secondary Accession	Title	Submission date
PRJEB62451	ERP147541	Genome sequences of *Staphylococcus aureus* LAC* mutant strains with increased acid resistance	19/05/2023
PRJEB62453	ERP147543	Genome sequences of *Staphylococcus aureus* JE2 mutant strains with increased acid resistance	19/05/2023
PRJEB62454	ERP147544	Genome sequences of *Staphylococcus aureus* LAC* mutant strains with increased growth in CDM	19/05/2023

2.12 Intracellular pH measurements

Measurements of the intracellular pH were performed using the pHrodo™ Red AM intracellular pH indicator dye (Thermofisher, Cat No. P35372) as described previously with modifications (Beetham et al., 2024; Grosser et al., 2018). The dye is amide based and is cleaved intracellularly, preventing loss of the dye once taken up. When in the cell, the dye emits increased fluorescence with decreased pH.

Overnight cultures of LAC* WT, LAC* *0846::Tn* and LAC* *0846::Tn codY*$_{E163*}$ strains were grown in TSB pH 7.3 or TSB pH 5.5 medium at 37 °C with shaking. The following day, 140 µl of the overnight culture was used to inoculate 20 ml fresh TSB medium either TSB pH 7.3 or TSB pH 4.5 medium, respectively. The cultures were grown until the bacteria were in mid-log phase with an OD_{600} value of between 0.4 to 0.7. Cultures were started at different time points to ensure they all reached the desired OD_{600} at the same time. Bacteria from the equivalent of 5 ml culture with an OD_{600} of 0.5 were collected by centrifugation for 20 min at 3700 rpm and subsequently washed 2 x with 5 ml 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.4. Next, 2 ml of each bacterial culture was removed and incubated

with 5 µM pHrodo Red dye and 20 µl PowerLoad™ concentrate for 30 min at RT in the dark according to the manufacturer's instructions. Following this incubation, 350 µl of the stained bacteria were collected by centrifugation and resuspended in 350 µl fresh 50 mM HEPES buffer pH 7.4. Two technical replicates of 150 µl were removed to a black walled 96-well plate, before the fluorescence was read on a TECAN plate reader with excitation / emission wavelengths of 650 / 590 nm at optimal gain. Measurements were taken every 5 min for 25 min, with shaking at 432 rpm for 120 s after each measurement. Intracellular pH values were determined by comparing the average of the two technical replicates of the fluorescence measurements to a standard curve generated independently for each strain and pH growth conditions to correct for differential dye uptake.

For the generation of the standard curve, bacterial cultures were pre-grown to mid-log phase and stained with pHrodo™ Red dye as described above for the experimental sample. Following staining, four x 350 µl of culture per strain and condition were removed, bacteria collected by centrifugation for 3 min at 17,000 g and resuspended in calibration buffers set to pH 4.5, pH 5.5, pH 6.5 or pH 7.5 and containing 10 µM nigericin and 10 µM valinomycin according to the manufacturer's protocol (Intracellular pH Calibration Buffer Kit, ThermoFisher, Cat. No. P35379). The average of two technical replicates per calibration sample was plotted on a graph, and a semi-log linear regression used to calculate a line of best fit. This was used to convert fluorescence readings of the experimental sample into intracellular pH values. Three independent experiments were performed and averages and standard deviations of the intracellular pH at the T=10 min time point were plotted as this is where the pH values fell within the ranges of the standard curve.

Chapter 3 : Exploring whether the signalling nucleotide c-di-AMP affects the activity of the urease enzyme under acid stress conditions

The signalling nucleotide c-di-AMP has been shown to affect the ability of *S. aureus* to grow at low pH. A previous study in the lab showed that a low c-di-AMP producing mutant strain of *S. aureus* shows reduced growth at low pH, while a high c-di-AMP producing mutant strain of *S. aureus* shows increased growth at low pH (Bowman et al., 2016). However, the mechanism behind how c-di-AMP affects the acid stress response of *S. aureus* is unknown. The importance of c-di-AMP could come either directly from its role in regulating potassium uptake, or alternatively through regulating the production of ammonia from urea via the urease pathway which is currently the best studied pathway in the acid stress response of *S. aureus* (Zhou et al., 2019). I therefore next explored whether c-di-AMP affects the activity of the urease enzyme under acid stress conditions, to investigate whether this is the reason for the importance of c-di-AMP for the growth of *S. aureus* under low pH.

3.1 DacA and YbbR do not affect urease activity under weak acid stress

3.1.1 Validating a previously published assay

To investigate whether c-di-AMP affects the activity of the urease enzyme under acid stress conditions, an assay previously described by the Fey group was used (Zhou et al., 2019). This assay involves stressing the bacteria through growth in TSB medium with additional glucose, which results in a lowered pH of the media and reduced bacterial growth. This is because some of the additional glucose will be converted into acetate, resulting in acetic acid stress (Thomas et al., 2014). A previous study showed that under these conditions, the pH of the medium decreases and *S. aureus* has decreased survival over 120 h (Thomas et al., 2014). However, the Fey group showed this decrease in survival and pH could be mitigated in WT bacteria by the addition of exogenous urea, but not in a *ureB::Tn* mutant strain (Zhou et al., 2019).

To validate the assay in our laboratory, the growth and survival of the JE2 WT strain and JE2 *ureB::Tn* mutant bacteria were investigated in TSB medium + 31.1 mM glucose (giving a final concentration of 45 mM glucose in the medium) either with or without the addition of 10 mM

urea by determining colony forming units (CFU ml^{-1}) every 24 h for 96 h. The pH of the medium was also measured at these time points.

As expected, the JE2 WT strain exhibited a 90-fold decrease in survival from 24 h time point to the 96 h time point (Fig. 3.1A). When exogenous urea was added the CFU count for the JE2 WT remained relatively constant between the 24 and 96 time points and only a two-fold drop was observed. The JE2 *ureB::Tn* strain however, had a 75-fold decrease in survival from the 24 h to 96 h time point regardless of whether urea was present. This was further examined through measuring the pH of the growth medium during bacterial growth. While the growth of the JE2 WT strain resulted in an increase of the pH of the medium in the presence of urea from 6.8 to 8.4 over 96 h, indicating urease activity and ammonia production, the growth medium of the JE2 *ureB::Tn* strain remained at an acidic pH of around 5.0 (Fig. 3.1B). This pattern is consistent with the data reported in Zhou et al., (2019), validating the assay.

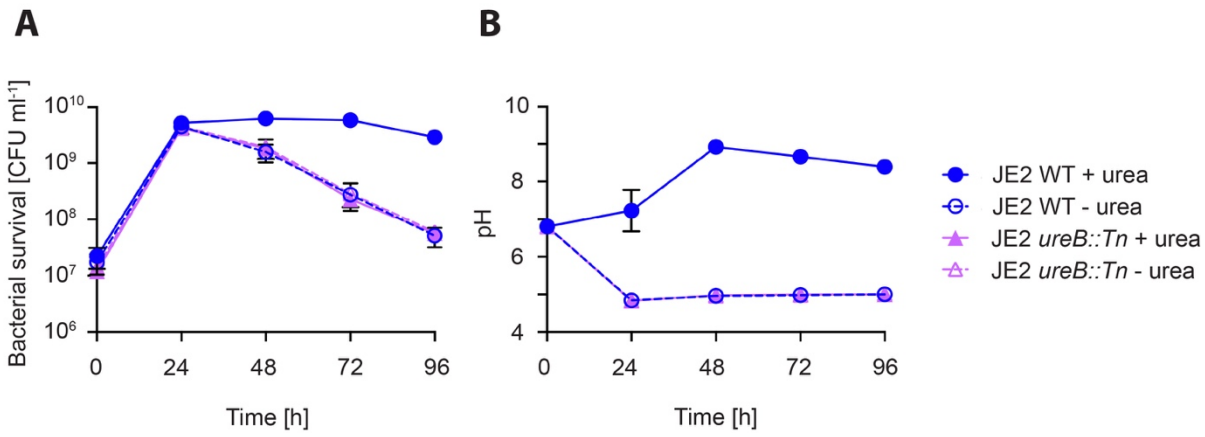


Figure 3.1: Adding exogenous glucose results in weak acid stress and the requirement of the urease enzyme for survival of *S. aureus*
(A) Growth curve showing bacterial growth and survival of the JE2 WT and JE2 *ureB::Tn* strains measured by counting the CFU ml^{-1} every 24 h after growth in TSB + 31.1 mM glucose with or without 10 mM urea for 96 h. (B) pH measurements of the medium following growth of the indicated bacterial strains in the same conditions as (A). The average and standard deviation of three independent experiments were plotted.

3.1.2 Analysis of the growth of the *dacA*$_{G206S}$ and *ΔybbR* strains under weak acid stress

In previous work it has been shown that a LAC* *dacA*$_{G206S}$ mutant strain, which has reduced c-di-AMP levels, has reduced growth at low pH (Bowman et al., 2016). To investigate if the increased acid sensitivity is due to reduced urease activity, the growth and survival, and growth medium pH, of strain LAC* *dacA*$_{G206S}$ was compared with that of the LAC* WT strain.

If, as hypothesized, c-di-AMP stimulates urease activity, then the LAC* *dacA*$_{G206S}$ strain would not be able to utilize the exogenous urea and would show a similar survival regardless of the presence of exogenous urea.

The LAC* *dacA*$_{G206S}$ strain had a slightly lower survival than the LAC* WT from 24 h to 96 h in TSB + glucose, with a 250-fold compared to a 100-fold decrease in CFU ml^{-1}, although this was not statistically significant (Fig. 3.2A). Both strains also maintained an acidic growth medium pH of around 4.9 over the 96 h without urea (Fig. 3.2B). With the addition of urea, both the LAC* WT and LAC* *dacA*$_{G206S}$ strains had a much higher survival, with an only 1.8 and 1.5 fold decrease, respectively from the 24 h to the 96 h time point, and the pH of the growth medium for both strains increased to around 8.3. Clearly, a low c-di-AMP producing strain is still capable of utilizing urea and produce ammonia to increase survival under low pH conditions, indicating that a reduction in c-di-AMP levels does not affect urease activity.

YbbR has also been previously shown to affect the acid stress response of *S. aureus*, although it is unknown how (Bowman et al., 2016). To investigate whether YbbR could affect urease activity, the above assay was repeated with a LAC* *ΔybbR* strain. When grown without urea, both the LAC* WT and the LAC* *ΔybbR* strain had a 100-fold decrease in CFU ml $^{-1}$ count between the 24 h and 96 h, time points and maintained the growth medium pH at around 4.9 (Fig. 3.2). After the addition of urea, both the LAC* WT and LAC* *ΔybbR* strain showed only a 1.8-fold decrease in CFU ml^{-1} count from the 24 h to the 96 h time point, and the pH of the growth medium increased from 6.8 to 8.3 over 96 h.

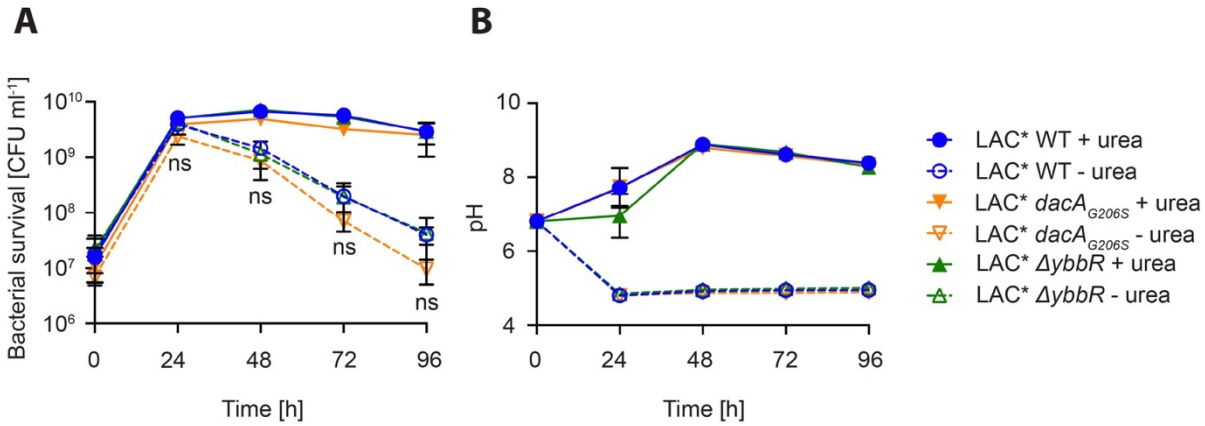


Figure 3.2: Neither c-di-AMP nor YbbR affect urease activity under weak acid stress conditions

(A) Growth curve showing bacterial growth and survival of the LAC* WT, LAC* *dacA*$_{G206S}$, and LAC* *ΔybbR* strains measured by counting the CFU ml^{-1} every 24 h after growth in TSB + 31.1 mM glucose with or without 10 mM urea for 96 h. Lack of statistical significance is shown by ns (P>0.05) performed by one-way ANOVA corrected for multiple-comparisons using Tukey's test. ns demonstrates the statistical significance for comparisons made between LAC* WT and LAC* *dacA*$_{G206S}$, and LAC* WT and LAC* *ΔybbR* strains without urea as both were ns. (B) pH measurements of the medium following growth of the indicated bacterial strains in the same conditions as (A). The average and standard deviation of three independent experiments were plotted.

Taken together, these results show that neither c-di-AMP nor YbbR affect the activity of the urease enzyme under weak acid stress.

3.2 DacA and YbbR do not affect urease activity under strong acid stress

3.2.1 Analysis of the contribution of urease to growth and survival under strong acid stress

The urease assay developed by the Fey group and used above involves stressing the bacteria through long term growth in TSB with additional glucose. This results in acidic stress through the accumulation of pyruvate break down products that do not enter the TCA cycle (Thomas et al., 2014). These include acetate, which when entering the cytoplasm forms acetic acid. Acetic acid is a weak acid due to the fact that it only partially dissociates in water. This contrast to strong acids such as HCl, which fully dissociate in water and therefore release more protons. However, the partial dissociation of weak acids means that they are better able to cross cell membranes, and therefore bacteria have different responses to weak and strong acids (Cherrington et al., 1991; Rode et al., 2010).

Whilst this study and previous studies have shown that urease is important for survival under weak acid stress, it was next investigated whether this was also the case for strong acid stress. Firstly, the Tn insertion in the JE2 *ureB::Tn* strain was transduced into the LAC* strain background by phage transduction. The growth of the LAC* WT and LAC* *ureB::Tn* strains were compared in TSB adjusted to a pH of 4.5 by the addition of HCl with and without 10 mM urea. Both CFU ml^{-1} and growth medium pH were then measured every 24 h for 96 h. When grown without urea, the LAC* WT strain exhibited a 600-fold decrease in CFU ml^{-1} count between the 0 h and 96 h time points, and the medium remained at an acidic pH of around 4.2 (Fig. 3.3). However, when grown with urea the LAC* WT strain had a 250-fold increase in CFU ml^{-1} from 0 h to 96 h, and the growth medium pH increased to 7.8. The LAC* *ureB::Tn* strain, however, had a decrease in CFU ml^{-1} count regardless of whether urea was present or not (250-fold with and 120-fold without), and growth medium remained at an acidic pH of 4.2. Overall, these results show that the utilization of urea through the urease enzyme is an important part of the response of *S. aureus* to both weak and strong acid.

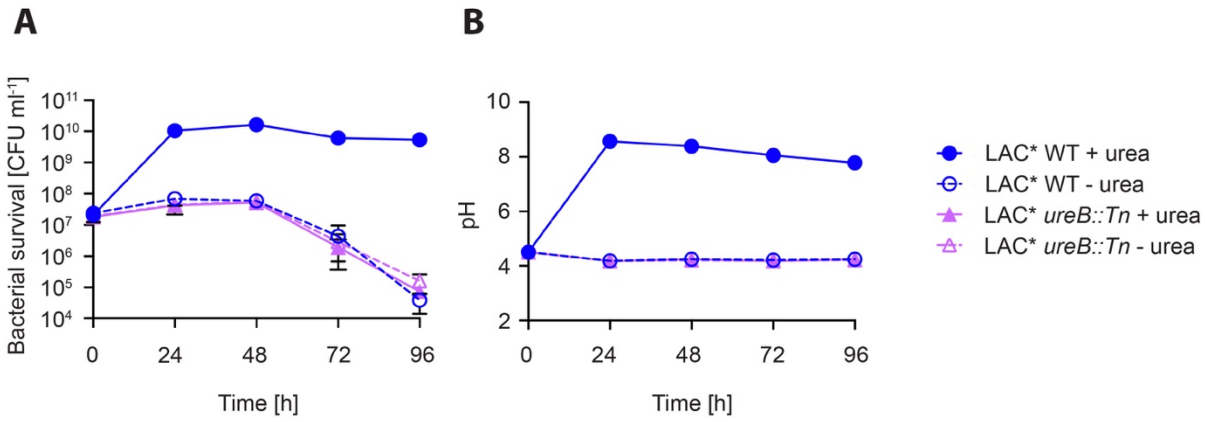


Figure 3.3: Urease is essential for growth and survival under strong acid stress
(A) Growth curve showing bacterial growth and survival of the LAC* WT and LAC* *ureB::Tn* strains measured by counting the CFU ml^{-1} every 24 h after growth in TSB acidified to pH 4.5 by the addition of HCl with or without 10 mM urea. (B) pH measurements of the medium following growth of the bacterial strains in the same conditions as in (A). The average and standard deviations of three independent experiments were plotted.

3.2.2 Analysis of the growth and survival of the *dacA*$_{G206S}$ and *ΔybbR* strains under strong acid stress

To explore whether c-di-AMP regulates urease activity under strong acid conditions, the growth and survival of the LAC* WT, LAC* *dacA*$_{G206S}$, and LAC* *ΔybbR* strains in TSB pH 4.5

medium acidified with HCl was next measured. Without urea the LAC* *dacA*$_{G206S}$ strain showed a slight reduced ability to survive over 96 h in TSB pH 4.5 compared to the LAC* WT strain, although this was not statistically significant (Fig. 3.4A). While the LAC* WT strain showed a 600-fold decrease in CFU ml^{-1} count over 96 h, the LAC* *dacA*$_{G206S}$ strain showed a 7,000-fold decrease in CFU ml^{-1} over 96 h. For both strains, the pH of the medium remained at an acidic pH of 4.3 (Fig. 3.4B). However, when exogenous urea was added, both strains were capable of growth in TSB pH 4.5 medium. The LAC* WT strain showed a 250-fold increase in CFU ml^{-1} count over 96 h, while the LAC* *dacA*$_{G206S}$ strain showed a 230-fold increase in CFU ml^{-1} count over 96 h. For both strains, the pH of the growth medium increased from pH 4.5 to pH 7.8. This therefore shows that c-di-AMP does not regulate urease activity under strong acid stress.

The LAC* *ΔybbR* strain showed a greater decrease in survival in TSB pH 4.5 without urea over 96 h compared to the LAC* *dacA*$_{G206S}$ strain, although this was also not statistically significant (Fig. 3.4A). Over 96 h, the LAC* *ΔybbR* strain showed an approximately 13,000-fold decrease in CFU ml^{-1}, compared to approximately 600-fold for the LAC* WT strain and an approximately 7,000-fold for the LAC* *dacA*$_{G206S}$ strain. Both the LAC* WT and LAC* *ΔybbR* strains maintained an acidic medium pH of 4.3 (Fig. 3.4B). In the presence of exogenous urea, the LAC* WT and LAC* *ΔybbR* strains showed 250-fold and 200-fold increases in CFU ml^{-1} over 96 h, respectively, and the pH of the growth medium rose to 7.8. Overall, these data show that YbbR does not affect urease activity under strong acid stress.

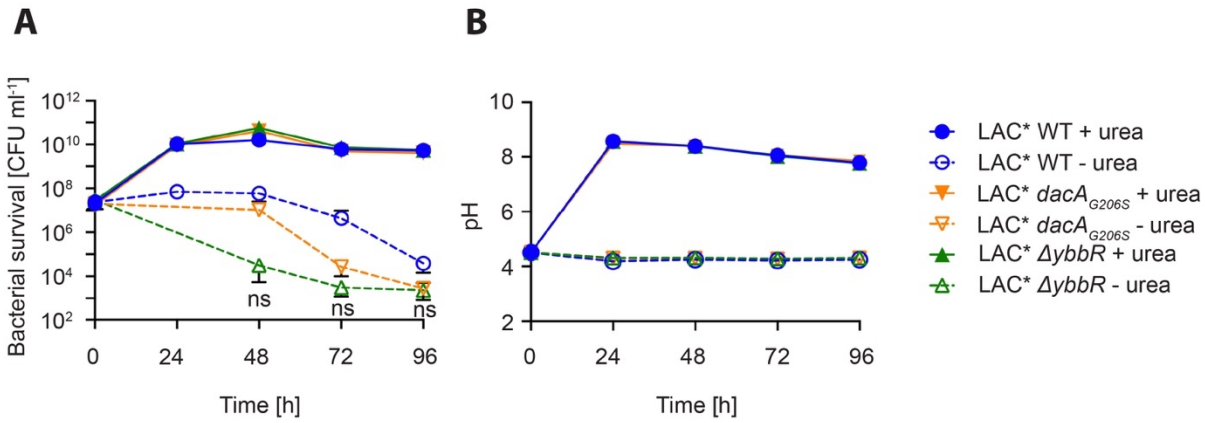


Figure 3.4: Neither c-di-AMP nor YbbR affect urease activity under strong acid stress
(A) Growth curve showing bacterial growth and survival of the LAC* WT, LAC* *ΔybbR* and LAC* *dacA*$_{G206S}$ strains measured by counting the CFU ml^{-1} every 24 h after growth in TSB acidified to pH 4.5 by addition of HCl with or without 10 mM urea. Missing values are due to clumping preventing measurements. Lack of statistical significance is shown by ns ($P<0.05$) performed by one-way ANOVA corrected for multiple-comparisons using Tukey's test. ns demonstrates the statistical significance for comparisons made between LAC* WT and LAC* *dacA*$_{G206S}$, and LAC* WT and LAC* *ΔybbR* strains without urea as both were ns. (B) pH measurements of the medium following growth of the indicated bacterial strains in the same conditions as in (A). The average and standard deviations of at least two independent experiments were plotted. The data for the LAC* WT strain are the same as shown in Fig. 3.3.

3.3 Discussion of Chapter 3

In this Chapter, I explored whether the signalling nucleotide c-di-AMP affected the activity of the urease enzyme under acidic stress conditions. While a previous study demonstrated that a low c-di-AMP producing strain has a growth defect at low pH compared to the WT strain and a high c-di-AMP producing strain has increased growth under the same conditions, the reason for this is not known (Bowman et al., 2016). Previous studies investigating changes in gene expression in *S. aureus* under acidic stress have shown that some of the most upregulated genes at low pH are genes encoding the urease enzyme and accessory proteins (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010; Weinrick et al., 2004; reviewed in Zhou & Fey, 2020). A follow up study demonstrated that urease was essential for the growth of *S. aureus* under weak acid stress due to the production of ammonia, but only when exogenous urea was present (Zhou et al., 2019). I therefore hypothesized that c-di-AMP could potentially regulate the activity of the urease enzyme under acid stress conditions, and that this was the reason for the importance of c-di-AMP for the growth of *S. aureus* under low pH. However, the low c-di-AMP producing strain showed increased growth medium pH and increased

growth and survival under weak and strong acid stress when exogenous urea was present. This suggests that this strain has no difference in urease activity compared to the WT strain under weak and strong acid stress and suggests that c-di-AMP does not regulate urease activity.

In *S. aureus* the *dacA* gene which produces c-di-AMP is found in the *dacA-ybbR-glmM* three gene operon. While the function of YbbR is unknown in *S. aureus*, in some species of bacteria YbbR has been shown to interact with and regulate the activity of DacA. In *B. subtilis*, it has been shown that YbbR positively regulates DacA, resulting in increased c-di-AMP levels (Gundlach et al., 2015; Mehne et al., 2013). On the other hand, in *L. lactis*, YbbR inhibits DacA activity to decrease c-di-AMP production (Zhu et al., 2016). In *L. monocytogenes*, both positive and negative regulation has been shown depending on the assay (Gibhardt et al., 2020; Rismondo et al., 2016). While several studies have investigated the effects of YbbR on DacA in *S. aureus*, no evidence of interaction between the two proteins has been found (Bowman et al., 2016; Tosi et al., 2019). However, the fact that the two genes are found in the same operon, as well as the number of interactions between DacA and YbbR shown in other bacteria, suggests that YbbR could interact with DacA in *S. aureus* as well.

The only phenotype associated with an inactivated *ybbR* gene in *S. aureus* is increased sensitivity to acid. Previously, it has been shown that a LAC* *ΔybbR* strain has a growth defect on TSA acidified to a pH of 4.5 by addition of HCl (Bowman et al., 2016). I therefore also explored whether YbbR affected the activity of the urease enzyme under acidic stress conditions. However, a LAC* *ΔybbR* strain also showed increased growth medium pH and increased growth and survival under weak and strong acid stress when exogenous urea was present, suggesting that YbbR does not affect urease activity under these conditions.

It is therefore still unknown how c-di-AMP affects acid resistance in *S. aureus*. One potential cross-over between phenotypes linked to c-di-AMP and acid stress is through potassium transport. c-di-AMP has been shown to bind and regulate the activity of several potassium transporters in *S. aureus*. This includes the KtrA/C potassium transporter gating component (Corrigan et al., 2013; Kim et al., 2015), and the Kdp two-component system which regulates the expression of the Kdp potassium transport system (Moscoso et al., 2016). A *ΔktrA* mutant *S. aureus* strain has a slight growth defect when grown in pH 5.21 compared to the WT strain

(Gries et al., 2013), and therefore it is possible that the reason for the importance of c-di-AMP for growth under acidic conditions is due to the regulation of potassium transporters.

Another potential reason for the importance of c-di-AMP for the acid stress response of *S. aureus* could be due to DNA damage. In several species of bacteria, c-di-AMP has been shown to be involved in the bacterial response to DNA damage-inducing compounds such as hydrogen peroxide (Gandara & Alonso, 2015) or UV radiation (Bai et al., 2013). Furthermore, in *B. subtilis* production of c-di-AMP has been shown to be affected by DNA damage (Oppenheimer-Shaanan et al., 2011; Witte et al., 2008). In *S. mutans* it has been shown that the expression of DNA repair genes is affected in a low c-di-AMP producing mutant strain (Cheng et al., 2016), which suggests that c-di-AMP affects the susceptibility of the bacteria to DNA damage and DNA damage-inducing compounds by regulating the expression of DNA repair mechanisms. As described in the Introduction Chapter, exposure to low pH induces DNA damage in bacteria. c-di-AMP may therefore be important for the acid stress response of *S. aureus* due to its regulation of DNA repair mechanisms following acid induced DNA damage.

One surprising finding from my study was that neither the low c-di-AMP producing strain nor the *ΔybbR* showed a large decrease in growth and survival over 96 h under strong acid stress, and this was even less under weak acid stress. This contrasts to the previously published study, where both the LAC* $dacA_{G206S}$ and LAC* *ΔybbR* mutant strains show an almost complete inability to grow compared to the LAC* WT strain over 8 h in TSB pH 4.5 (Bowman et al., 2016). This may be because in my study I measured growth and survival under acidic stress conditions over a longer time period of 96 h, whereas the previously published study measured growth over the much shorter time of 8 h. This could suggest that the mechanisms used by *S. aureus* to grow under acidic stress or to survive under acidic stress are different.

Chapter 4 : Using a Transposon Sequencing approach to explore the acidic stress response of *Staphylococcus aureus*

4.1 The Tn-Seq experiment identified genes essential or detrimental for the fitness of *S. aureus* under acidic stress conditions

4.1.1 Construction and growth of two independent Tn mutant libraries

To identify genes essential or detrimental for the fitness of *S. aureus* under acidic stress conditions, the results of the Tn-Seq experiments which had been performed prior to the start of this PhD were analysed. Construction, propagation and generation of lists of *S. aureus* genes and corresponding ratios were performed by Professor Angelika Gründling and Dr. Christopher Schuster and I will briefly describe how the experiment was set up here.

As described in the Introduction Chapter, independent Tn-Seq experiments were performed using two *S. aureus* Tn mutant libraries in the USA300 TCH1516-derived strain background TM283 (Coe et al., 2019; Schuster et al., 2020). The libraries were grown in TSB at standard pH (7.3), or TSB acidified to a pH of either 5.5 or 4.5 by addition of HCl prior to autoclaving. Two different acidic pH conditions were chosen to explore whether the response of *S. aureus* differs under varying levels of stress.

Cultures exhibited a similar growth rate when grown at pH 7.3 and pH 5.5, and reached the target OD_{600} value of 1.4 after around 5 hours (Fig. 4.1). A OD_{600} value of 1.4 was chosen for harvesting the bacteria as it represents 10 generations. However, there was a significant reduction in growth when the cultures were grown at pH 4.5. Library B grown at pH 4.5 reached an OD_{600} value of 1.4 at around 8 h, but Library A grown at pH 4.5 did not reach the desired OD_{600} value due to clumping (Fig. 4.1). This culture was harvested at the lower OD_{600} value of 1.0, but at a time point when it was expected to reach an OD_{600} value of 1.4.

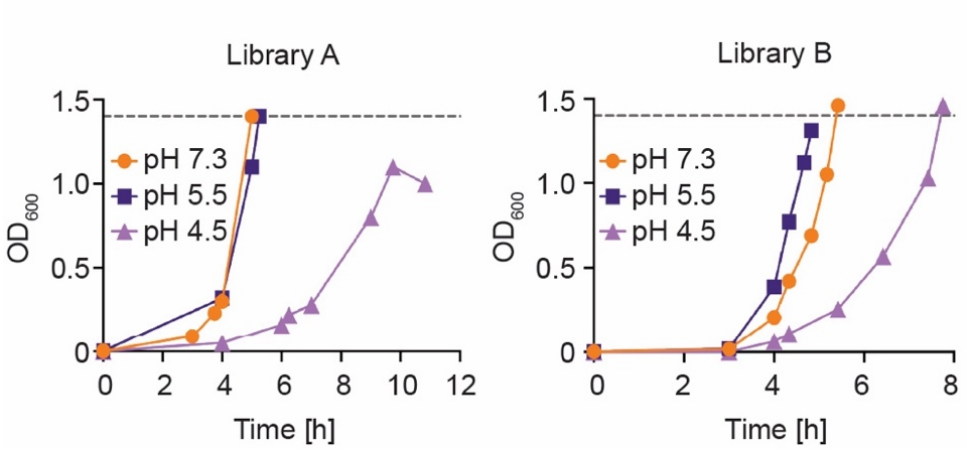


Figure 4.1: Growth curve of two Tn mutant pools (Library A and B)
The growth of Library A and B in TSB medium at standard pH (7.3), or acidified to pH 5.5 or 4.5 by addition of HCl prior to autoclaving was monitored by taking OD_{600} readings at the indicated time points. The dotted line represents the 10-generation cut off point at which the bacteria were harvested. The culture of Library A grown at pH 4.5 did not reach the desired OD_{600} value due to clumping and was instead harvested after 11 h of growth. The results from one independent experiment were plotted. This experiment was performed by A. Gründling and C. Schuster. This figure has been reproduced from Beetham et al., (2024).

Following growth the bacteria were harvested, and the DNA was extracted and samples prepared for next generation sequencing of the transposon junction sites. The location of the Tn insertions was then mapped onto the bacterial genome. This was done separately for both libraries and under each pH condition. As each library had a good distribution of Tn insertions throughout the genome at each pH condition, it indicates good coverage (Fig. 4.2). The aim was to create a library where every possible insertion site was covered at least once. The greater the coverage in a Tn library, the greater the probability that any differences in fitness are due to Tn insertions and not by chance.

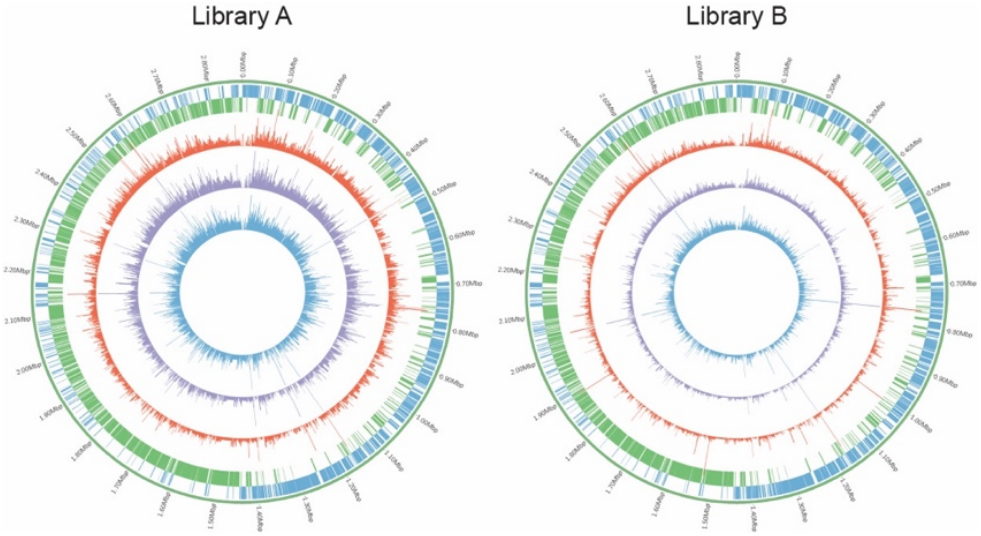


Figure 4.2: Circular plot showing the Tn insertion density along the *S. aureus* genome at different pH conditions
Circular plots for Tn-libraries A and B with the two outer rings depicting genes located on the (+) (blue) or (−) (green) strand in *S. aureus* strain USA300 FPR3757. The inner three rings show the histograms of Tn insertions on a per gene basis after growth of the libraries in TSB pH 7.3 (red), pH 5.5 (purple), or pH 4.5 (blue) medium for 10 generations. This graph was generated by C. Schuster. This figure has been reproduced from Beetham et al., (2024).

For each gene, a ratio was then generated comparing the number of Tn insertions present in the gene following growth at low pH, compared to the number of Tn insertions present in the gene when grown at neutral pH. A separate ratio was generated per gene for growth at pH 4.5 or 5.5. If a gene contained fewer Tn insertions when grown at low pH than when grown at neutral pH, it was assumed that gene inactivation inhibited the fitness of the bacteria at acidic pH (Fig. 4.3). These genes were classified as required for fitness, or 'essential'. The lower the ratio, the more essential the gene was for the fitness of the bacteria at low pH. If a gene contained a greater number of Tn insertions when grown at low pH than when grown at neutral pH, it was assumed that gene inactivation provided a fitness advantage under acidic conditions (Fig. 4.3). These genes were classified as inhibitory for fitness, or 'detrimental'.

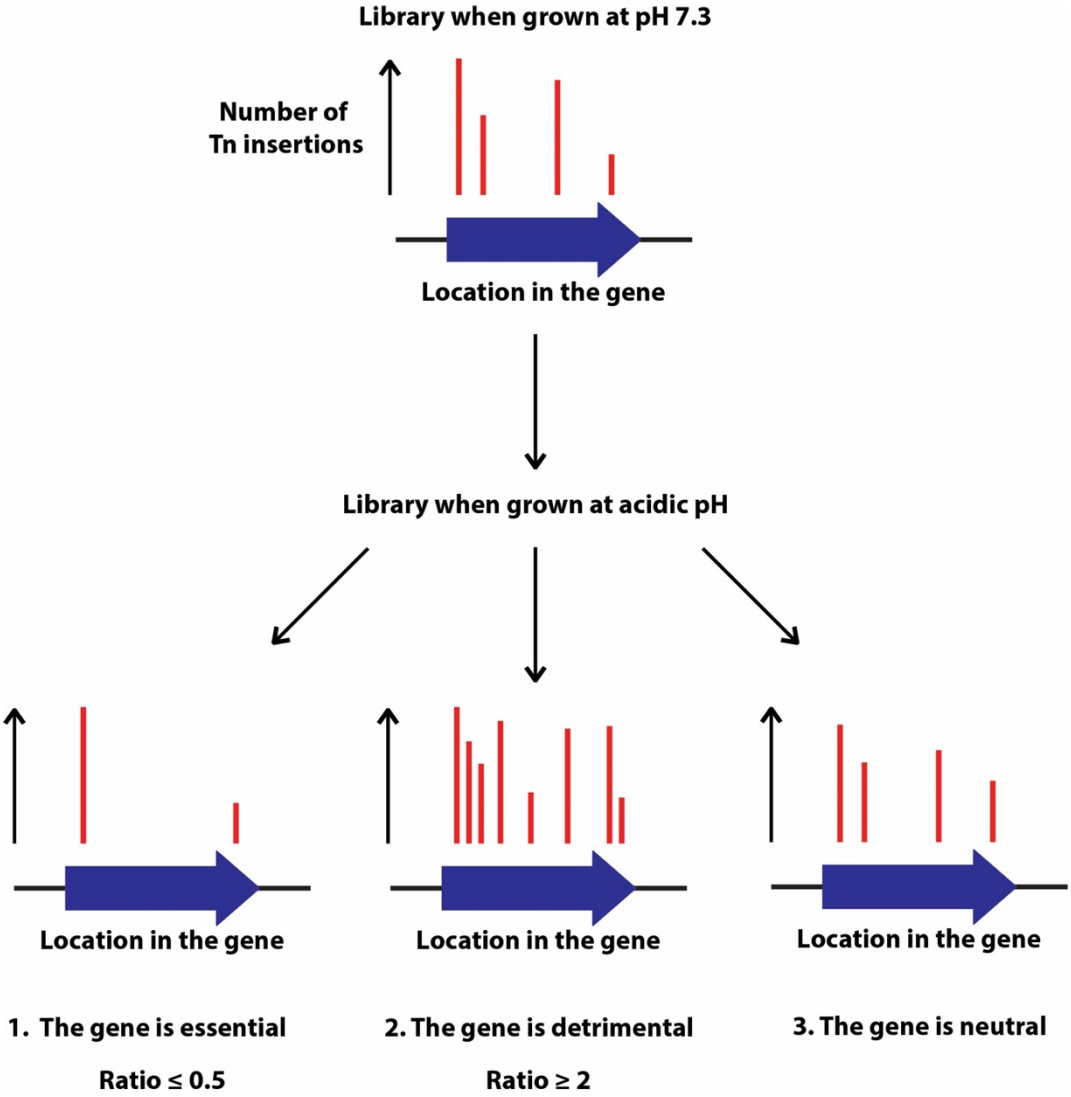


Figure 4.3: Schematic representation of the classification of genes as either essential, detrimental, or neutral

Each gene was classified as essential, detrimental, or neutral for the fitness of *S. aureus* at low pH depending on the ratio of transposon insertions under the different growth conditions. Essential genes were classified as those with at most half as many insertions when grown at low pH than at neutral pH (ratio ≤ 0.5). Detrimental genes were classified as those with at least twice as many insertions when grown at low pH than at neutral pH (ratio ≥ 2). All other genes were considered neutral. Figure adapted from Rajagopal et al., (2016).

4.1.2 Classifying genes as essential or detrimental

I started work on this PhD project by analysing the previously generated lists of *S. aureus* genes and their corresponding ratios. Firstly, I classified genes as either essential, detrimental or neutral by introducing cut-off limits for the ratios (Fig. 4.3). Genes were classified as essential if they had a ratio of less than 0.5, while genes were classified as detrimental if they had a ratio of above 2 (Fig. 4.3). Genes with a ratio of between 0.5 and 2 were classified as neutral,

with limited effect on the fitness of *S. aureus* at low pH (Fig. 4.3). To exclude false-positive hits, only genes with ≥ 10 Tn insertions per gene and a Benjamini-Hochberg corrected p-value of ≤ 0.1 were considered.

As two Tn-Seq experiments were performed using independent libraries, and at two pH conditions, four separate lists of genes considered either essential or detrimental were generated (Table 4.1). For growth at pH 4.5, 60 and 50 genes were classified as essential from libraries A and B, respectively, while 111 and 21 genes were classified as detrimental for fitness. For growth at pH 5.5, 9 and 27 genes were classified as essential from libraries A and B, respectively, while 27 and 17 genes were classified as detrimental. As expected, for both libraries a greater number of genes were classified as either essential or detrimental following growth at pH 4.5 than pH 5.5. I hypothesize that this is because more genes and pathways are required to counter the higher levels of stress encountered by the bacteria at the lower pH.

Table 4.1: Number of genes classified as essential or detrimental for the fitness of *S. aureus* in TSB pH 4.5 or pH 5.5 for Library A and B

	pH 4.5		**pH 5.5**	
	Essential	**Detrimental**	**Essential**	**Detrimental**
Library A	60	111	9	27
Library B	50	21	27	17

4.1.3 Exploring the overlap between the two Tn-Seq experiments

Each Tn-Seq experiment was performed using an independently constructed Tn library. Furthermore, there were slight differences in how the Tn-Seq experiments were performed, as described in the Experimental procedures Chapter. To identify genes which are more likely to be relevant for the acidic stress response of *S. aureus*, any genes common to both Tn-Seq experiments were identified (Fig. 4.4). A significant number of genes were commonly identified as essential for fitness at pH 4.5, with over 50% of the genes identifying as essential in data from both Tn libraries (Fig. 4.4A). Furthermore, a significant number of genes were commonly identified as detrimental for fitness at pH 5.5 (Fig. 4.4B). However, only a small

proportion of genes identified as detrimental at pH 4.5, and essential at pH 5.5, were common to both libraries (Fig. 4.4A,B). This appears to be due to large discrepancies in the total number of genes identified between the libraries under these conditions. An analysis of the initial datasets revealed large discrepancies in the number of false-positive hits identified. The dataset for the ratios generated per gene for library A at pH 4.5, for example, had much fewer genes with significant p-values than library B. However, with the aim of revealing genes which were more likely to be relevant, only the genes identified as being common to both libraries using the stringent condition and using a p≤0.1 were further analysed. These are listed in Tables 4.2 and 4.3.

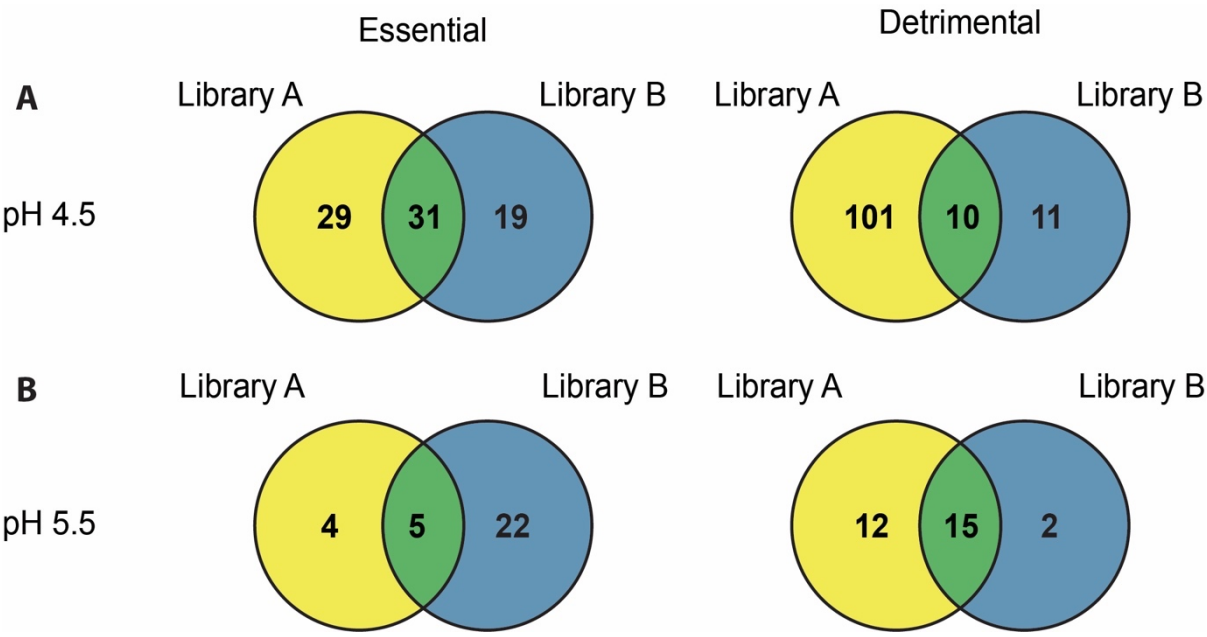


Figure 4.4: Overlap in genes between the two independently constructed Tn libraries
Venn diagrams showing the number of genes classified as either essential or detrimental in Library A, B, and the resulting overlap for pH 4.5 (A) and pH 5.5 (B) stress conditions. The overlapping 31 essential and 10 detrimental genes for the pH 4.5 growth conditions are listed in Table 4.2. The overlapping 5 essential and 15 detrimental genes for the pH 5.5 growth conditions are listed in Table 4.3. This figure has been adapted from Beetham et al., (2024).

Table 4.2: Genes identified by Tn-Seq as essential or detrimental for the fitness of *S. aureus* in TSB pH 4.5 medium in both library A and B

Genes which are identified as essential or detrimental at pH 4.5 and pH 5.5 are shaded grey. This table has been adapted from Beetham et al., (2024).

Locus Tag SAUSA300_	Gene Name	Annotated Gene Function	Phenotype in TnSeq at pH 4.5	Average Ratio	Growth plate assay (pH 4.5) phenotype and other comments
Essential genes: Disruption of these genes should decrease the fitness of *S. aureus* at low pH					
0846		Predicted transporter	Essential	0.01	Acid Sensitive
0646	*graS*	Signal transduction histidine kinase	Essential	0.02	Acid Sensitive
1865	*vraR*	DNA-binding response regulator	Essential	0.02	Acid Sensitive
0648	*vraG*	ABC transporter permease	Essential	0.02	Acid Sensitive
2389		MSF family permease	Essential	0.03	Acid Sensitive
1593	*secDF*	Protein translocase	Essential	0.05	No mutant strain available in NTML collection
1255	*mprF*	Oxacillin resistance related FmtC protein	Essential	0.06	Acid Sensitive
0429			Essential	0.07	No phenotype
0543			Essential	0.07	Acid Sensitive
0959	*fmtA*	Teichoic acid D-Ala esterase	Essential	0.08	Acid Sensitive
0482		Polysaccharide biosynthesis protein	Essential	0.10	No phenotype
2645	*gidA*	tRNA uridine-5-carboxymethylaminomethyl synthesis enzyme	Essential	0.10	No mutant strain available in NTML collection
0481		Transcription-repair coupling factor	Essential	0.11	Acid Sensitive
0836	*dltB*	D-alanyl-lipoteichoic acid biosynthesis protein	Essential	0.11	Acid Sensitive

1866	*vraS*	Two-component sensor histidine kinase	Essential	0.12	Acid Sensitive
0855	*mnhA1*	Monovalent cation/H+ antiporter subunit A	Essential	0.20	No mutant strain available in NTML collection
2646	*trmE*	tRNA modification GTPase	Essential	0.21	Tn insertion site could not be confirmed in NTML strain
1158			Essential	0.22	No mutant strain available in NTML collection
0957			Essential	0.22	No phenotype
1720	*sagB*	Glucosaminidase	Essential	0.24	No phenotype
1518	*cshB*	DEAD-box ATP dependent DNA helicase	Essential	0.24	Acid Sensitive
2506	*isaA*	Immunodominant antigen A	Essential	0.28	No mutant strain available in NTML collection
0672	*mgrA*	MarR family transcription factor	Essential	0.31	No mutant strain available in NTML collection
0730	*gdpS*	GGDEF domain containing protein	Essential	0.31	No mutant strain available in NTML collection
2282	*spdC*	Endopeptidase	Essential	0.31	No phenotype
0726			Essential	0.32	No mutant strain available in NTML collection
1442	*srrA*	Respiratory response protein	Essential	0.33	Acid Sensitive
0962	*qoxB*	Quinol oxidase, subunit I	Essential	0.35	Acid Sensitive
0762	*secG*	Pre-protein translocase subunit SecG	Essential	0.36	No mutant strain available in NTML collection

0963	*qoxA*	Quinol oxidase, subunit II	Essential	0.40	Acid Sensitive
1685			Essential	0.44	No mutant strain available in NTML collection
Detrimental genes: Disruption of these genes should improve the fitness of *S. aureus* at low pH					
1636	*polA*	DNA superfamily I polymerase	Detrimental	2.52	No phenotype
1544	*lepA*	GTP-binding protein	Detrimental	3.21	No phenotype
0759	*pgm*	Phosphoglycermutase	Detrimental	3.35	Acid Sensitive
2174	*ecfT*	Cobalt transport family protein	Detrimental	3.58	Tn insertion site could not be confirmed in NTML strain
0746			Detrimental	3.63	Tn insertion site could not be confirmed in NTML strain
2643	*noc*	ParB family chromosome partitioning family	Detrimental	3.66	No phenotype
2055	*murA*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Detrimental	4.12	No phenotype
1043	*mutS2*	Recombination and DNA strand exchange inhibitor protein	Detrimental	5.10	No phenotype
1509	*actH*	Rhomboid family peptidase	Detrimental	5.52	Tn insertion site could not be confirmed in NTML strain
1588	*lytH*	N-acetylmuramoyl-L-alanine amidase	Detrimental	12.96	No phenotype

Table 4.3: Genes identified by Tn-Seq as essential or detrimental for the fitness of *S. aureus* in TSB pH 5.5 medium in both library A and B

Genes which are identified as essential or detrimental at pH 4.5 and pH 5.5 are shaded grey.

Locus Tag SAUSA300_	Gene Name	Annotated Gene Function	Phenotype in TnSeq at pH 4.5	Average Ratio	Growth plate assay (pH 4.5) phenotype and other comments
Essential genes: Disruption of these genes should decrease the fitness of *S. aureus* at low pH					
0836	*dltB*	D-alanyl-lipoteichoic acid biosynthesis protein	Essential	0.12	Acid sensitive
0846			Essential	0.15	Acid sensitive
0648	*vraG*	Signal transduction histidine kinase	Essential	0.24	Acid sensitive
0387	*pbuX*	Xanthine permease	Essential	0.31	
0650	*pitA*	Putative phosphate transporter	Essential	0.39	
Detrimental genes: Disruption of these genes should improve the fitness of *S. aureus* at low pH					
1043	*mutS2*	Recombination and DNA strand exchange inhibitor protein	Detrimental	2.94	No phenotype
1886	*pcrA*	DNA helicase	Detrimental	3.47	No mutant strain available in NTML collection
1593	*secDF*	Protein translocase	Detrimental	3.71	No mutant strain available in NTML collection
2055	*murA*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Detrimental	3.75	No phenotype
2643	*noc*	ParB family chromosome partitioning family	Detrimental	3.85	No phenotype
2027	*alr*	Alanine racemase	Detrimental	4.73	
1687	*ftsK*	DNA translocase	Detrimental	6.47	
1589	*dtd*	Potential D-tyrosyl-tRNA decyclase	Detrimental	6.73	No mutant strain available in NTML collection

1664	*ezrA*	Septation ring formation regulator	Detrimental	6.97	No mutant strain available in NTML collection
1688	*pheT2*	Phenylanalyl tRNA synthetase subunit	Detrimental	7.57	No mutant strain available in NTML collection
1082			Detrimental	8.95	No mutant strain available in NTML collection
1370	*ebpS*	Cell surface elastin binding protein	Detrimental	9.33	
1509	*actH*	Rhomboid family peptidase	Detrimental	11.04	Tn insertion site could not be confirmed in NTML strain
1083	*sepF*		Detrimental	18.54	No mutant strain available in NTML collection
1588	*lytH*	N-acetylmuramoyl-L-alanine amidase	Detrimental	22.80	No phenotype

To explore similarities in the response of *S. aureus* for fitness at high and low levels of acidic stress, any genes classified as essential or detrimental at both pH 4.5 and pH 5.5 were identified (shaded in grey in Table 4.2, 4.3, Fig. 4.5). Over 50% of the genes classified as essential for fitness at pH 5.5 were also essential at pH 4.5. These were *0846*, *vraG*, and *dltB*. Interestingly, this includes the three genes with the lowest ratio when grown at pH 5.5, and are thus the most essential at this pH condition. This suggests that *S. aureus* has a basal response for fitness in acidic conditions requiring these three genes regardless of the level of stress. However, under higher stress conditions at lower pH, additional pathways are required for fitness.

There was less overlap in genes classified as detrimental for fitness at both pH 4.5 and pH 5.5. The five genes which were commonly identified were *noc*, *murA*, *mutS2*, *actH*, and *lytH*. These genes had the highest ratio when grown at TSB pH 4.5, and are thus the most detrimental for

fitness at the higher stress condition. Furthermore, *lytH* was identified as the gene with the highest ratio for fitness at pH 4.5 and pH 5.5. This further indicates that some of the genes are involved in the acidic stress response of *S. aureus* at both high and low levels of stress. However, the larger number of genes essential for fitness at pH 4.5 alone, and the limited overlap of genes classified as detrimental indicates that the responses of *S. aureus* to high and low levels of stress are distinct.

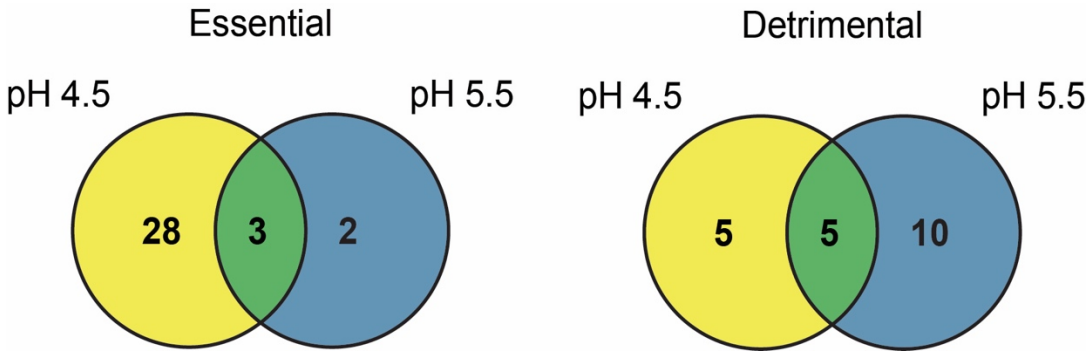


Figure 4.5: Overlap in genes between the high and low acidic stress conditions
Venn diagrams showing the number of genes classified as either essential or detrimental for fitness at pH 4.5 and pH 5.5 stress conditions. The overlapping 3 essential and 5 detrimental genes are shaded in grey in Tables 4.2 and 4.3.

Since few genes were identified as essential under the pH 5.5 condition, suggesting that the stress was not sufficient, further analysis was focussed on the genes identified at pH 4.5 (Table 4.2).

4.2 Many of the genes highlighted as essential for the fitness of *S. aureus* under acidic stress conditions could be grouped by coding for proteins with related functions or in similar pathways

To identify whether any of the genes that are required for the fitness of *S. aureus* at low pH could be grouped according to the functions of their encoded proteins, the list of genes highlighted in the Tn-Seq experiments under pH 4.5 growth conditions was examined in more detail (Table 4.2).

4.2.1 Bacterial cell wall

Several genes which were highlighted as required for the fitness of *S. aureus* under low pH conditions were linked to the bacterial cell wall (Fig. 4.6). Both genes encoding the VraSR two-component system detecting cell-wall stress, *vraS* and *vraR*, were identified as essential with average ratios of 0.12 and 0.02, respectively, in the Tn-Seq experiment (Table 4.2). Furthermore, several genes coding for peptidoglycan hydrolases were also identified as essential, including *spdC* and *sagB* with average ratios of 0.31 and 0.24, respectively (Table 4.2). The SpdC and SagB hydrolases form a protein complex which releases new peptidoglycan during cell division (Schaefer et al., 2021; Wang et al., 2022; Willing et al., 2021). Another gene encoding a peptidoglycan hydrolase identified as essential in the Tn-Seq experiment with an average ratio of 0.28 was IsaA, which is a predicted lytic transglycosylase enzyme (Wang et al., 2022) (Table 4.2).

Other genes linked to the cell wall included those involved in regulating cell surface charge. Cell surface charge can be modulated by the addition of charged residues onto the membrane or peptidoglycan (Fig. 4.6). In *S. aureus*, this can be done for example by the addition of the positively charged D-Ala onto the WTA and LTA present in the cell wall by the Dlt protein complex. A second mechanism is through the production of the positively charged membrane lipid lysyl-phosphatidylglycerol by MprF. Both *dltB* from the *dlt* operon and *mprF* were identified as essential with average ratios of 0.11 and 0.06, respectively (Table 4.2). Both systems are transcriptionally regulated by the GraXRS-VraFG five-component system. The GraS and VraFG histidine signal kinases detect cell wall stressors such as CAMPs (Herbert et al., 2007), and transduce signals through the GraX signal transducer (Fig. 4.6). GraX affects the activity of the GraR transcription factor, which regulates the expression of a range of target genes required for the addition of positive charges to the cell surface (Herbert et al., 2007). *graS* and *vraG,* coding for the two membrane bound components of the GraXRS-VraFG five-component system, were identified as essential with average ratios of 0.02 for both (Table 4.2). Furthermore, *fmtA* was highlighted as essential with an average ratio of 0.08, and codes for a D-amino acid esterase that removes D-Ala from teichoic acids (Table 4.2).

Interestingly, despite several peptidoglycan hydrolases being identified as essential, the two top detrimental genes for the fitness of *S. aureus* at low pH were *lytH* and *actH*, with ratios of 12.96 and 5.52, respectively (Table 4.2). These encode a peptidoglycan amidase and its

partner protein (Do et al., 2020; Page et al., 2022), thus implying that this amidase activity is detrimental for fitness under acidic conditions.

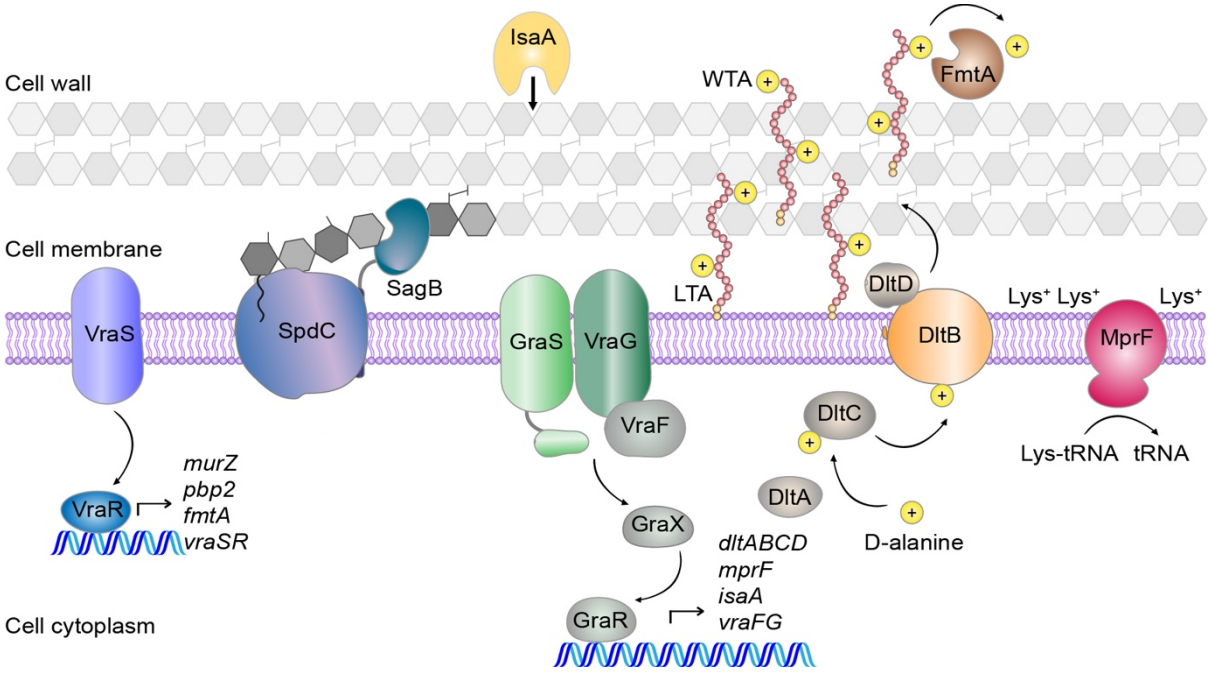


Figure 4.6: Schematic representation of the genes involved in cell wall remodelling which were identified as essential in the TnSeq experiment
S. aureus cell wall proteins highlighted in this study as essential for fitness in TSB pH 4.5 and involved in cell wall synthesis or remodelling are depicted. If the proteins are part of complexes, the proteins identified in the Tn-seq experiment are shown in colour while the others are shown in grey. This figure has been adapted from Beetham et al., (2024).

Taken together, it is clear that pathways which regulate and modify the bacterial cell wall are a key response of *S. aureus* to acidic stress conditions.

4.2.2 Aerobic respiration

Several genes highlighted in the Tn-Seq experiment were linked to aerobic respiration. Firstly, *srrA*, which encodes the response regulator of the SrrAB two-component system, was identified as essential with an average ratio of 0.33 (Table 4.2). SrrAB regulates genes involved in aerobic respiration, such as *qoxA* and *qoxB* (Kinkel et al., 2013), which encode subunits of the Qox cytochrome aa_3-type quinol oxidase, a proton pumping terminal oxidase present in

S. aureus (Hammer et al., 2013). The *qoxA* and *qoxB* genes were also highlighted as essential in the Tn-Seq experiment with average ratios of 0.40 and 0.35, respectively (Table 4.2).

4.2.3 Other genes

Finally, there were several other genes which were highlighted as essential for fitness under acid stress conditions. Firstly, the *SAUSA300_0846* and *mhnA1* genes coding for predicted cation/proton antiporters had average ratios of 0.01 and 0.2 (Table 4.2). Furthermore, *secDF* encoding protein translocase subunits had an average ratio of 0.05, and *gidA* encoding a tRNA uridine 5-carboxymethylaminomethyl modification enzyme had an average ratio of 0.1 (Table 4.2). Several genes with uncharacterized functions were also identified, including *SAUSA300_2389* coding for a predicted MSF family permease with an average ratio of 0.03, *SAUSA300_0429* coding a predicted phospholipid phosphatase with an average ratio of 0.07, and *SAUSA300_0543* coding for a predicted t-RNA adenosine deaminase with an average ratio of 0.07 (Table 4.2).

4.2.4 Conclusion

Several of the pathways highlighted in the Tn-Seq experiment have been previously associated with the acid stress response of *S. aureus*, such as the modification of the cell wall and cell surface charge (Flannagan et al., 2018; Kuiack et al., 2020; Rode et al., 2010; Villanueva et al., 2018). This therefore demonstrates the robustness of using Tn-Seq as an approach to explore the acid stress response of *S. aureus*. In addition, the Tn-Seq approach identified several novel genes highlighted as essential for the fitness of *S. aureus* under low pH, including those linked to aerobic respiration and *SAUSA300_0846*.

4.3 Most of the genes classified as essential result in reduced growth at low pH when inactivated

4.3.1 *S. aureus* mutants with single gene inactivations

To confirm the importance of genes identified as essential or detrimental through the Tn-Seq experiment, their individual impact on the growth of *S. aureus* was investigated. This was done through comparing the growth at low pH of a WT strain of *S. aureus* to a strain with the gene under investigation individually inactivated. For this, the NTML was utilised (Fey et al., 2013).

In this library, most non-essential genes are inactivated by a Tn in the USA300 JE2 genetic background. However, not all genes identified in the Tn-Seq experiment could be further explored using mutant strains from the NTML library (Fig. 4.7). This is because some of these genes are assumed to be essential for the fitness of *S. aureus* under neutral conditions, and therefore cannot be inactivated. Out of 41 genes I was planning to further investigate, 14 did not have a corresponding mutant strain in the NTML collection (Table 4.2). Of these, 3 had corresponding mutant strains in other collections or from other sources. This included a *ΔvraR* and *ΔvraS* mutant strain in the *S. aureus* 923 strain background (Boyle-Vavra et al., 2013). Furthermore, while there was no *dltB* mutant available in the collection, a *ΔdltD* mutant strain was available in the LAC* background strain in our laboratory (Ledger et al., 2022). Each mutant strain was confirmed for the correct location of the Tn insertion using PCR and sequencing of the predicted Tn-genome junction. While the correct Tn insertion site could be confirmed for most of the mutant strains, there were 3 strains for which I could not confirm the insertion site (Table 4.2). In total, out of the 41 genes identified as either essential or detrimental in the Tn-Seq experiment, 27 could be further explored using corresponding mutant strains (Fig. 4.7). This includes 20 genes classified as essential, and 7 genes classified as detrimental.

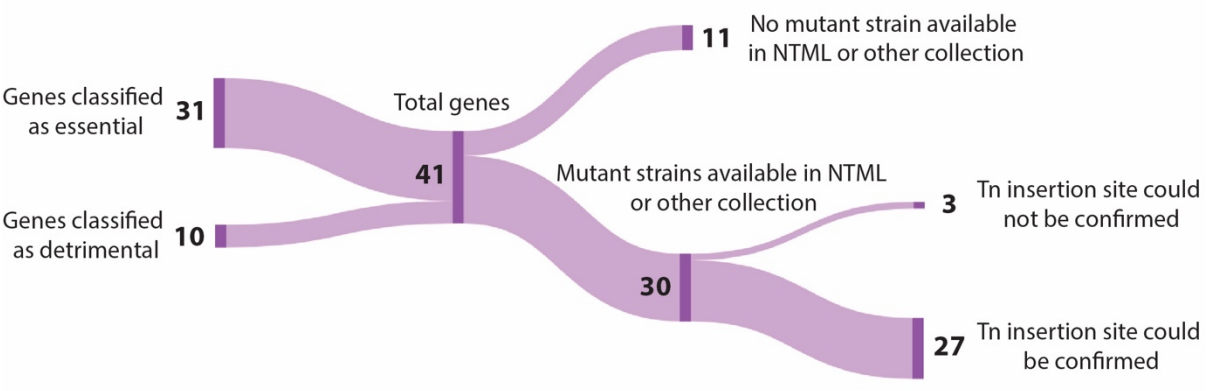


Figure 4.7: Twenty seven genes could be further investigated using corresponding mutant strains

Sankey diagram showing the total number of genes classified as either essential or detrimental, the genes which had a corresponding mutant strain available in the NTML or other collections, and the final number of strains for which the Tn insertion or mutation in specific genes could be confirmed.

4.3.2 Analysis of the genes classified as essential for fitness at low pH

To compare the ability of the 20 strains with mutations in the genes classified as essential for fitness in acidic conditions, a plate spotting assay was used. In this assay, 10-fold dilutions of overnight cultures of the WT and mutant strains were spotted on either standard TSA pH 7.3 plates, or TSA plates acidified to a pH of 4.5 by addition of HCl. As only a limited number of strains could be spotted on one TSA plate, mutant strains were grouped by the predicted function of the gene inactivated by Tn insertion, then spotted in ascending order of their average ratio.

Firstly, the WT and mutant strains corresponding to the genes identified as essential for fitness at low pH were spotted on TSA pH 7.3 plates to see if any of the mutants showed a growth defect at neutral pH. All of the strains grew to the -6 or -7 dilution, and none showed an obvious growth defect compared to the WT strain (Fig. 4.8). As expected, the NTML JE2 *srrA::Tn*, *qoxB::Tn* and *qoxA::Tn* strains had increased staphyloxanthin production as evidenced by their yellow colour (Fig. 4.8E). Increased staphyloxanthin production is an indication of oxidative stress. *srrA*, *qoxA*, and *qoxB* are involved in aerobic respiration, and thus it is clear than inactivating this process conveys a greater oxidative stress on the bacteria. None of the other mutant strains showed increased stress levels.

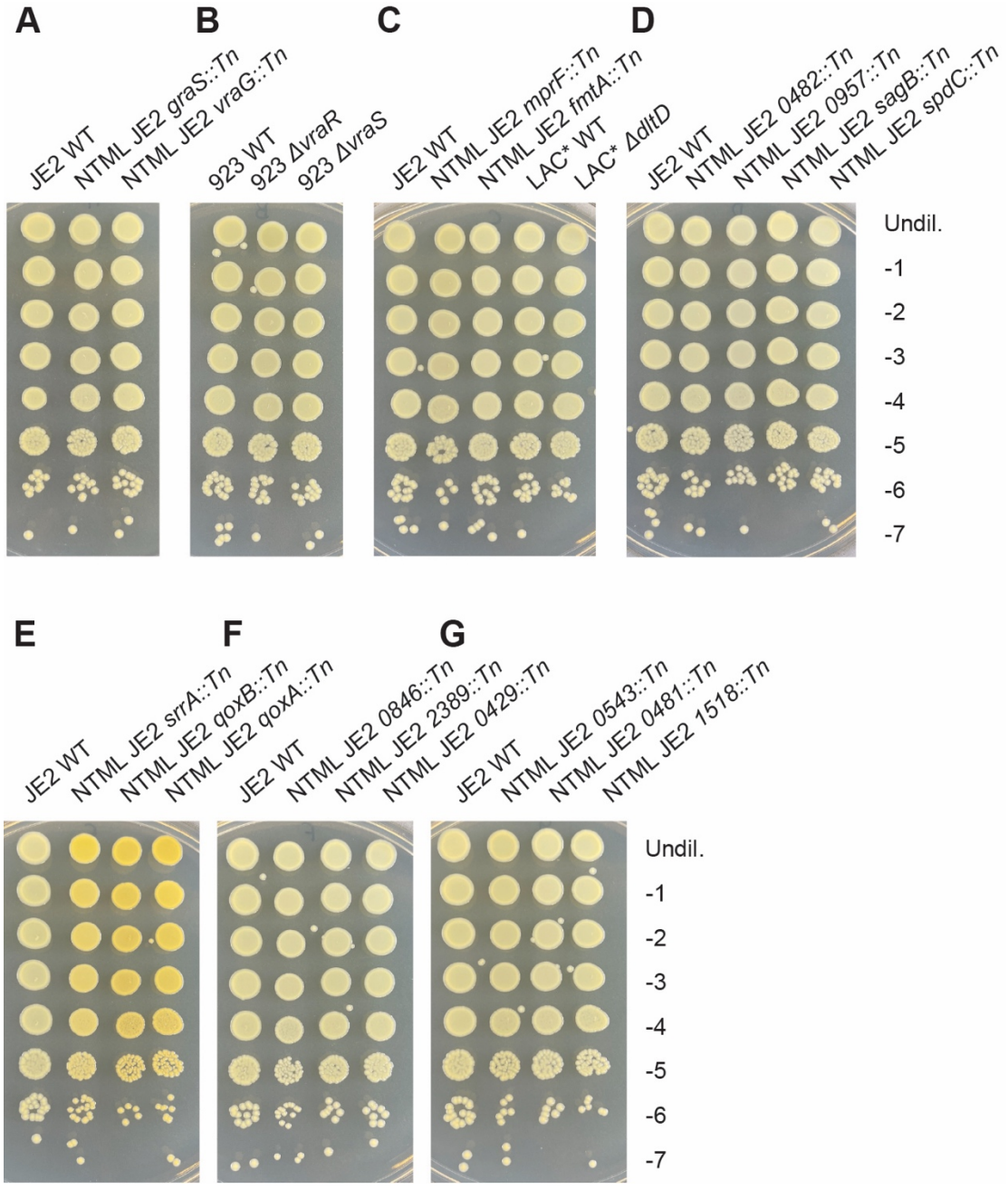


Figure 4.8: Inactivating the genes classified as essential for fitness under acid stress does not confer a growth defect at neutral conditions

Overnight cultures of (A) JE2 WT, NTML JE2 *graS::Tn*, NTML JE2 *vraG::Tn*, (B) 923 WT, 923 *ΔvraR*, 923 *ΔvraS*, (C) JE2 WT, NTML JE2 *mprF::Tn*, NTML JE2 *fmtA::Tn*, LAC* WT, LAC* *ΔdltD*, (D) JE2 WT, NTML JE2 *0482::Tn*, NTML JE2 *0957::Tn*, NTML JE2 *sagB::Tn*, NTML JE2 *spdC::Tn*, (E) JE2 WT, NTML JE2 *srrA::Tn*, NTML JE2 *qoxB::Tn*, NTML JE2 *qoxA::Tn*, (F) JE2 WT, NTML JE2 *0846::Tn*, NTML JE2 *2389::Tn*, NTML JE2 *0429::Tn*, (F) JE2 WT, NTML JE2 *0543::Tn*, NTML JE2 *0481::Tn*, and NTML JE2 *1518::Tn* strains were serially diluted and aliquots spotted onto TSA pH 7.3 plates. Images were taken following 24 h incubation at 37 °C. Each image is a representative of three independent experiments. This figure has been adapted from Beetham et al., (2024).

Next, the WT and mutant strains were spotted on TSA pH 4.5 plates. Several mutant strains showed a growth defect compared to the WT strain at low pH (Fig. 4.9). Both the NTML JE2 *graS::Tn* and *vraG::Tn* mutant strains were almost completely unable to grow on TSA pH 4.5 plates (Fig. 4.9A). Furthermore, the 923 *ΔvraR* and *ΔvraS* mutant strains had an approximately 100,000 fold decrease in growth compared to the 923 WT strain (Fig. 4.9B). Other mutant strains with cell wall genes inactivated that showed a reduced ability to grow on TSA pH 4.5 plates were those involved in the modification of cell surface charge. This included NTML JE2 *mprF::Tn* with an approx. 10-fold decrease, NTML JE2 *fmtA::Tn* with an approx. 100,000 fold decrease, and LAC* *ΔdltD* which was almost completely inhibited in its ability to grow on TSA pH 4.5 plates (Fig. 4.9C). However, strains with mutations in other genes involved in the bacterial cell wall did not show reduced growth on TSA pH 4.5 plates (Fig. 4.9D). These included the NTML JE2 strains *0482::Tn*, *0957::Tn*, *sagB::Tn*, and *spdC::Tn.*

All strains with mutations in genes involved in aerobic respiration showed a growth defect on TSA pH 4.5 plates (Fig. 4.9E). NTML JE2 *srrA::Tn* showed the greatest growth defect, with an approx. 10,000 fold decrease compared to the WT strain. Additionally, both NTML JE2 *qoxB::Tn* and *qoxA::Tn* had an approx. 10-fold decrease compared to the WT strain.

Of the other genes which were classified as essential for fitness under acid stress conditions in the Tn-Seq assay, some showed growth defects at low pH (Fig. 4.9F,G). This included NTML JE2 *0846::Tn* and *2389::Tn*, with an approx. 10,000 and 100,000 fold decrease compared to the WT strain. NTML JE2 *0543::Tn* was almost completely inhibited in its ability to grow on low pH. Additionally, both NTML JE2 *0481::Tn* and *1518::Tn* had a slight growth defect of approx. 10-fold compared to the WT strain. On the other hand, NTML JE2 *0429::Tn* did not show a growth defect at low pH.

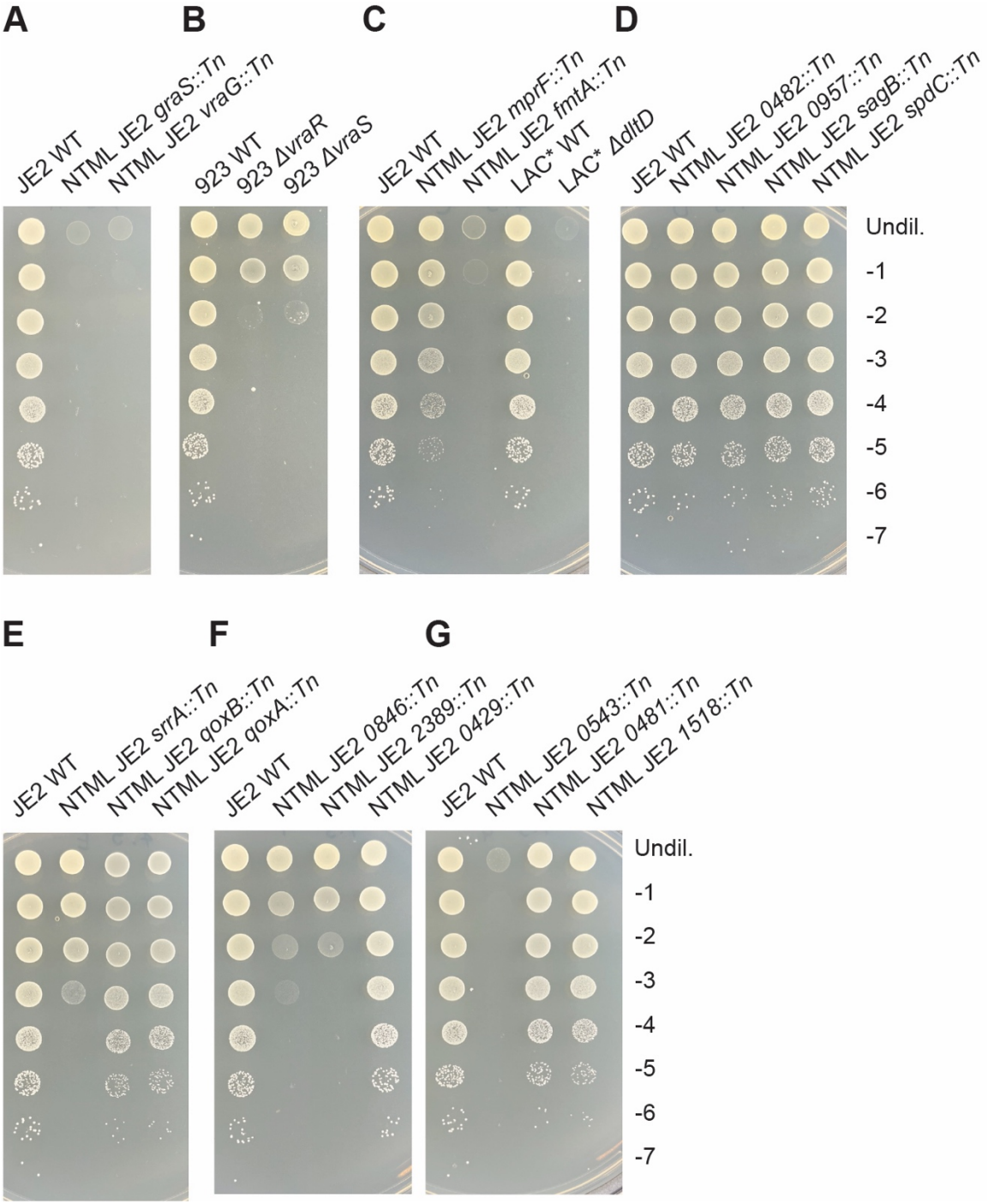


Figure 4.9: Most of the mutant strains with Tn insertions in the genes classified as essential for fitness under acid stress conditions show reduced growth at low pH

Overnight cultures of (A) JE2 WT, NTML JE2 *graS::Tn*, NTML JE2 *vraG::Tn*, (B) 923 WT, 923 *ΔvraR*, 923 *ΔvraS*, (C) JE2 WT, NTML JE2 *mprF::Tn*, NTML JE2 *fmtA::Tn*, LAC* WT, LAC* *ΔdltD*, (D) JE2 WT, NTML JE2 *0482::Tn*, NTML JE2 *0957::Tn*, NTML JE2 *sagB::Tn*, NTML JE2 *spdC::Tn*, (E) JE2 WT, NTML JE2 *srrA::Tn*, NTML JE2 *qoxB::Tn*, NTML JE2 *qoxA::Tn*, (F) JE2 WT, NTML JE2 *0846::Tn*, NTML JE2 *2389::Tn*, NTML JE2 *0429::Tn*, (F) JE2 WT, NTML JE2 *0543::Tn*, NTML JE2 *0481::Tn*, and NTML JE2 *1518::Tn* strains were serially diluted and aliquots spotted onto TSA pH 4.5 plates. Images were taken following 24 h incubation at 37 °C. Each image is a representative of three independent experiments. This figure has been adapted from Beetham et al., (2024).

To summarise, most of the genes identified as essential in the Tn-Seq experiment showed a growth defect on TSA pH 4.5 plates compared to their respective WT strains. Mutant strains with Tn insertions in genes linked to sensory systems detecting cell wall stress, cell surface charge, and aerobic respiration were over represented in showing growth defects on TSA pH 4.5 plates.

4.3.3 Analysis of the genes classified as detrimental for fitness at low pH

To further validate the use of Tn-Seq as a method to identify pathways involved in the acidic stress response of *S. aureus*, the genes identified as detrimental in the Tn-Seq assay were next explored for their individual impact on the ability of *S. aureus* to grow at low pH. It was expected that these mutant strains would grow as well or even better compared to a WT strain on TSA pH 4.5 plates. To investigate this, the plate spotting assay was repeated using the 7 mutant strains which were available in the NTML collection and in which the correct Tn insertion site could be confirmed.

None of the mutant strains showed a reduced ability to grow on TSA pH 7.3 plates compared to the WT strain, indicating that the Tn insertions does not confer a growth defect (Fig. 4.10). Furthermore, none of the strains showed an increased or decreased staphyloxanthin production, suggesting that inactivating the indicated genes does not confer a greater oxidative stress level on the bacteria at neutral conditions.

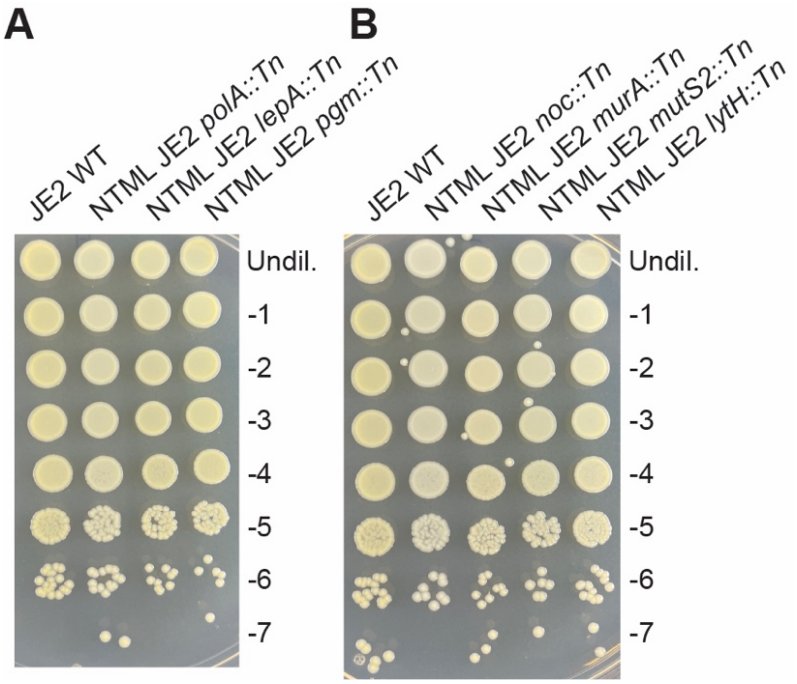


Figure 4.10: Inactivating the genes classified as detrimental for fitness under acid stress conditions does not confer a growth defect at neutral conditions
Overnight cultures of (A) JE2 WT, NTML *JE2 polA::Tn*, NTML JE2 *lepA::Tn*, NTML JE2 *pgm::Tn*, (B) JE2 WT, NTML JE2 *noc::Tn*, NTML JE2 *murA::Tn*, NTML JE2 *mutS2::Tn*, and NTML JE2 *lytH::Tn* strains were serially diluted and aliquots spotted onto TSA pH 7.3 plates. Images were taken following 24 h incubation at 37 °C. Each image is a representative of three independent experiments. This figure has been adapted from Beetham et al., (2024).

Next the WT and mutant strains were spotted onto TSA pH 4.5 plates. As expected, most of the strains grew similar to the WT strain on TSA pH 4.5 plates (Fig. 4.11). These included the NTML JE2 strains *polA::Tn*, *lepA::Tn*, *noc::Tn*, *murA::Tn*, *mutS2::Tn*, and *lytH::Tn*. However, unexpectedly, the NTML JE2 *pgm::Tn* strain showed an approx. 1,000 fold decrease in growth on TSA pH 4.5 plates compared to the WT strain, despite having been classified as detrimental in the Tn-Seq experiment (Fig. 4.11A).

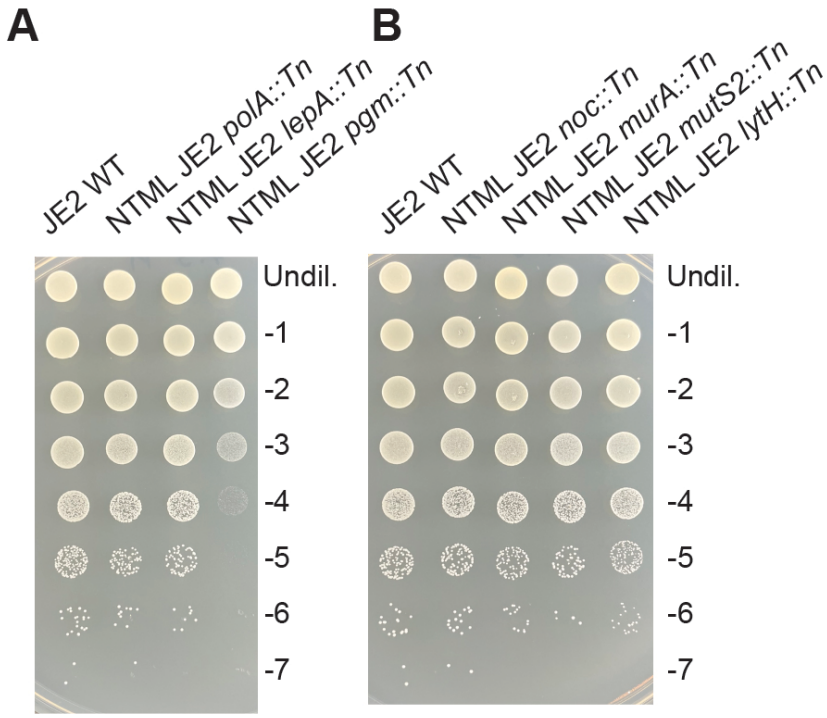


Figure 4.11: Most of the mutant strains with Tn insertions in the genes classified as detrimental for fitness under acid stress conditions do not show reduced growth at low pH Overnight cultures of (A) JE2 WT, NTML JE2 *polA::Tn*, NTML JE2 *lepA::Tn*, NTML JE2 *pgm::Tn*, (B) JE2 WT, NTML JE2 *noc::Tn*, NTML JE2 *murA::Tn*, NTML JE2 *mutS2::Tn*, and NTML JE2 *lytH::Tn* strains were serially diluted and aliquots spotted onto TSA pH 4.5 plates. Images were taken following 24 h incubation at 37 °C. Each image is a representative of three independent experiments. This figure has been adapted from Beetham et al., (2024).

To conclude, these results show that the Tn-Seq is effective at identifying genes essential for the growth of *S. aureus* under low pH conditions. Most of the essential genes identified in the Tn-Seq experiment showed growth defects at low pH when individually inactivated. Furthermore, most of the genes classified as detrimental showed no growth defect on TSA pH 4.5 plates, as expected. However, some of the genes classified as essential did not show reduced growth at low pH when individually inactivated, and one mutant strain with a Tn insertion in a gene classified as detrimental showed reduced growth at low pH.

4.4 There is some overlap between the response of *S. aureus* required for growth or survival in acidic stress conditions

Bacterial stress responses can differ depending on whether the bacteria are capable of growth or just survival in stress conditions. In the former, the stress conditions are lower, but the

bacteria must be able to undergo the metabolic processes required for growth. On the other hand, survival usually indicates a higher stress level, but bacteria can shut down metabolic processes required for growth until the stress has passed. The Tn-Seq experiment specifically investigated the fitness of *S. aureus* under acid stress conditions where growth can occur. Therefore, initially an assay testing bacterial growth (the plate spotting assay on TSA pH 4.5 plates) was chosen to confirm the results of the Tn-Seq experiment. To explore bacterial survival in acidic stress, a separate survival assay was used. The aim was to explore whether the genes classified as essential or detrimental for the fitness of *S. aureus* under acid stress conditions were also involved in the ability of the *S. aureus* to survive acidic conditions.

Bacterial survival under acidic stress conditions was assessed by inoculating overnight cultures into liquid medium with a pH of 2.5 and determining CFU ml^{-1} every 2 h for 8 h. A pH of 2.5 was chosen as it represents a level where the survival of the WT bacteria decreases over the 8 h (Fig. 4.12). This allows for the identification of mutants with both a decreased and increased survival compared to the WT strain.

4.4.1 Exploring the importance of the bacterial cell wall for survival under acid stress

In both the Tn-Seq experiment and the plate spotting assay, genes involved in the bacterial cell wall were over-represented as being essential for the growth of *S. aureus* at low pH. Therefore, it was next investigated whether these same genes were also important for the acid survival response of *S. aureus*.

Of the 11 strains with mutations in genes involved in the bacterial cell wall, 5 had reduced survival compared to their respective WT strains over 8 h in TSB pH 2.5 medium (Fig. 4.12). These were NTML JE2 *vraG::Tn*, NTML JE2 *mprF::Tn*, NTML JE2 *fmtA::Tn*, LAC* *ΔdltD*, and NTML JE2 *0957::Tn* (Fig. 4.12B,E,F,G,I). Both NTML JE2 *vraG::Tn* and *fmtA::Tn* had slight decreases in survival, with a 13-fold and 8-fold decrease in CFU ml^{-1} compared to the JE2 WT strain following 8 h in TSB pH 2.5 medium (Fig. 4.12B,F). NTML JE2 *mprF::Tn* and *0957::Tn* had greater decreases in survival, with both having an approximately 300-fold decrease in CFU ml^{-1} at the 8 h time point compared to the JE2 WT strain (Fig. 4.12E,I). The strain with the greatest decrease in survival in this group was LAC* *ΔdltD*, which had an approximately 7500-fold decrease in CFU ml^{-1} at the 8 h time point compared to the LAC* WT strain (Fig. 4.12G). Unexpectedly, two mutant strains showed an increased survival compared to the JE2 WT

strain over 8 h in TSB pH 2.5 medium. These were NTML JE2 *sagB::Tn* and *spdC::Tn*, which showed a 1400 and 1200-fold increase in CFU ml^{-1} at the 8 h time point compared to the JE2 WT strain (Fig. 4.12J,K).

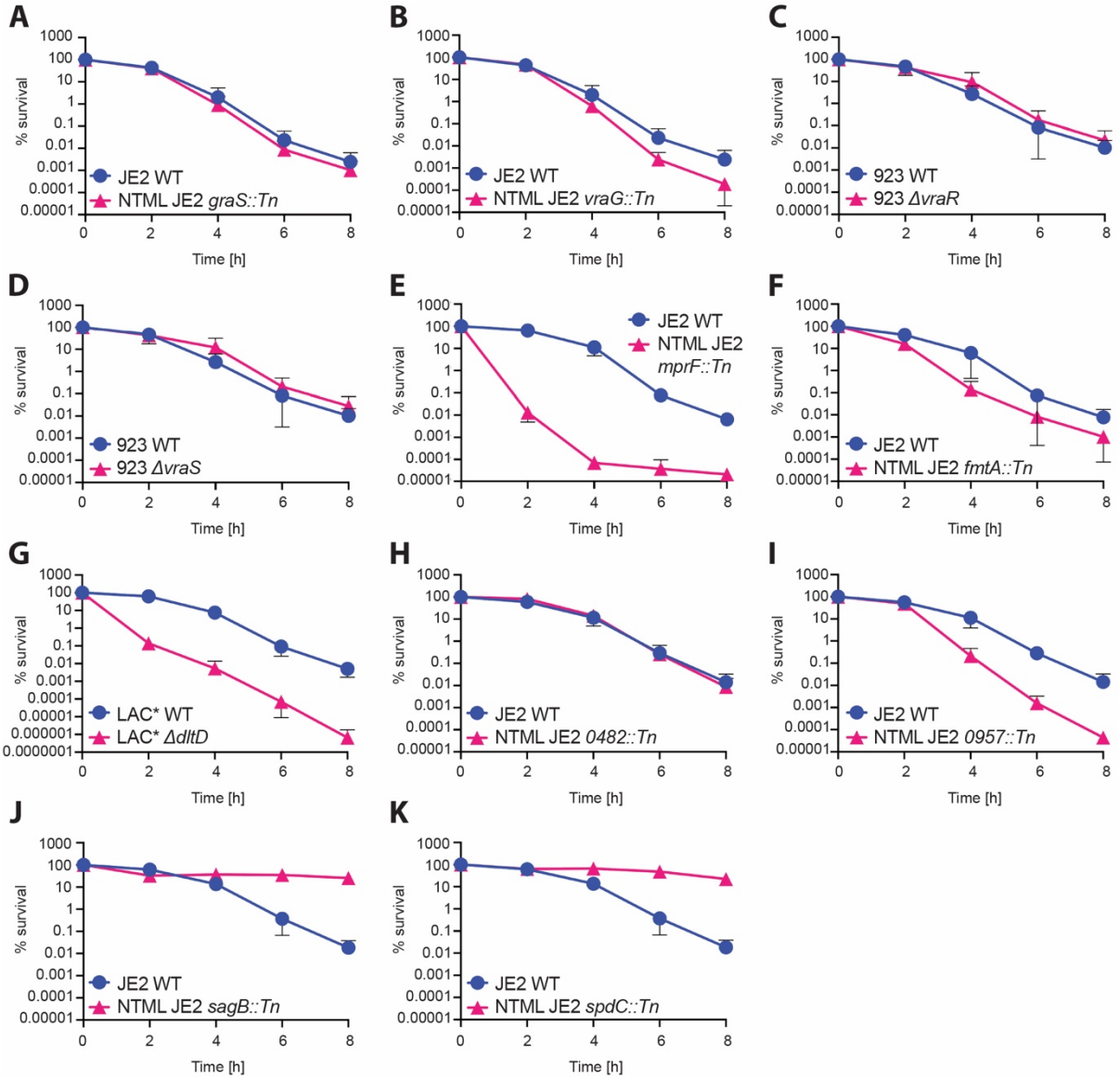


Figure 4.12: Survival analysis of mutant strains with Tn insertions in bacterial cell wall genes classified as essential for fitness under acid stress conditions

Overnight cultures of JE2 WT (A,B,E,F,H,I,J,K), 923 WT (C,D), or LAC* WT (G) as well as NTML JE2 *graS::Tn* (A), NTML JE2 *vraG::Tn* (B), 923 *ΔvraR* (C), 923 *ΔvraS* (D), NTML JE2 *mprF::Tn* (E), NTML JE2 *fmtA::Tn* (F), LAC* *ΔdltD* (G), NTML JE2 *0482::Tn* (H), NTML JE2 *0957::Tn* (I), NTML JE2 *sagB::Tn* (J), or NTML JE2 *spdC::Tn* (K) were washed and diluted into TSB pH 2.5 medium. Immediately afterwards (T = 0 h) and at 2 h intervals up to 8 h aliquots were removed and CFUs determined by plating dilutions onto TSA plates. The CFU count at T = 0 h was set to 100% for each strain and % survival at the subsequent time points calculated. The average and standard deviations of the % survival from at least three independent experiments were plotted.

Mutant strains with the greatest reduction in survival compared to the WT strain were those with a defect in modifying the cell surface charge. On the other hand, mutant strains with Tn insertions in genes encoding the signalling pathways which regulate these processes, including the GraXRS-VraFG five-component system, did not display a reduced ability to survive acidic stress. This is despite these same mutations resulting in an inability to grow on TSA pH 4.5 plates. Furthermore, the NTML JE2 *sagB::Tn* and *spdC::Tn* mutant strains showed an increased survival in TSB pH 2.5 medium, despite these genes having been identified as essential in the Tn-Seq experiment. This shows that while there is some overlap between the genes required to grow or survive in low pH, they are not identical.

4.4.2 Exploring the importance of aerobic respiration for survival under acid stress

All of the mutant strains linked to aerobic respiration had differences in their ability to survive for 8 h in TSB pH 2.5 medium compared to the JE2 WT strain (Fig. 4.13). However, while the NTML JE2 *srrA::Tn* strain showed increased survival, NTML JE2 *qoxB::Tn* and *qoxA::Tn* showed decreased survival. NTML JE2 *srrA::Tn* had an approximately 60-fold increase in CFU ml^{-1} compared to the JE2 WT strain at the 8 h time point (Fig. 4.13A). On the other hand, NTML JE2 *qoxB::Tn* had an approximately 10,000-fold decrease in CFU ml^{-1} at the 6 h time point, and had such a survival defect that the strain was completely killed by the 8 h time point (Fig. 4.13B). NTML JE2 *qoxA::Tn* also had a severe survival defect, with a 4,000-fold decrease in CFU ml^{-1} at the 8 h time point compared to the JE2 WT strain (Fig. 4.13C).

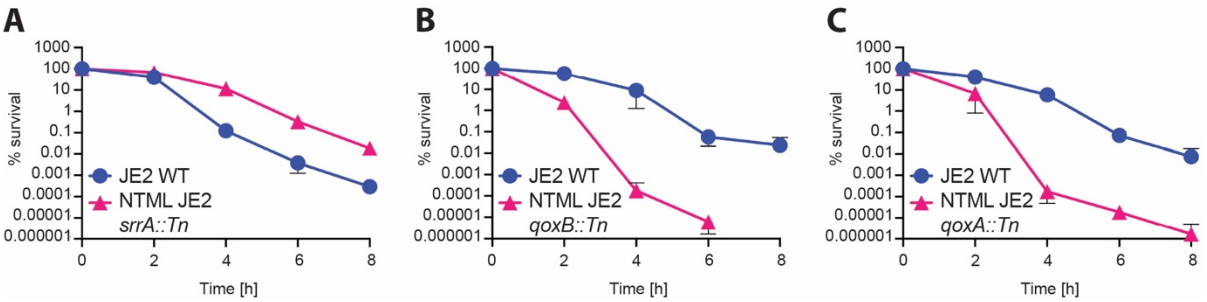


Figure 4.13: Survival analysis of mutant strains with Tn insertions in genes classified as essential for fitness under acid stress conditions and involved in aerobic respiration
Overnight cultures of JE2 WT as well as NTML JE2 *srrA::Tn* (A), NTML JE2 *qoxB::Tn* (B), or NTML JE2 *qoxA::Tn* (C) were washed and diluted into TSB pH 2.5 medium. Immediately afterwards (T = 0 h) and at 2 h intervals up to 8 h aliquots were removed and CFUs determined by plating dilutions onto TSA plates. The CFU count at T = 0 h was set to 100% for each strain and % survival at the subsequent time points calculated. The average and standard deviations of the % survival from at least three independent experiments were plotted.

4.4.3 Exploring the importance of *0846* for acid survival

Of the other genes identified as essential in the Tn-Seq experiment, only one showed a survival defect in TSB pH 2.5 medium when individually inactivated by Tn insertion (Fig. 4.14). This was *0846*, where the NTML JE2 *0846::Tn* strain showed an approx. 60-fold decrease in CFU ml^{-1} compared to the WT strain following 8 h in TSB pH 2.5 medium (Fig. 4.14A). However, this result could not be replicated in a clean transduced mutant strain, as will be described in the next Chapter.

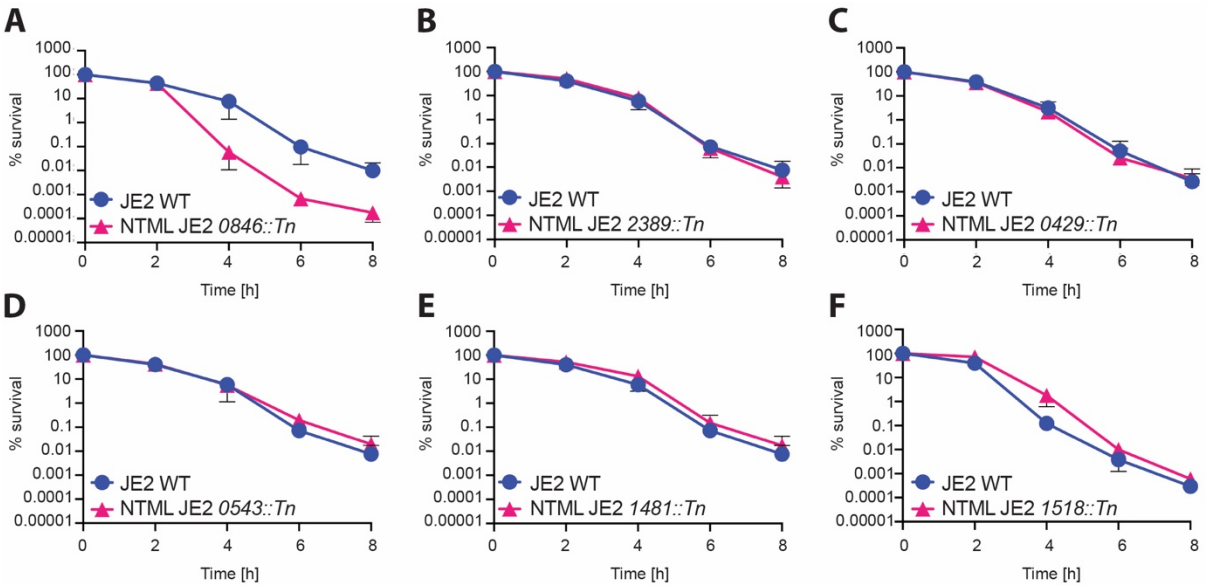


Figure 4.14: Survival analysis of mutant strains with Tn insertions in the remaining genes classified as essential for fitness under acid stress conditions

Overnight cultures of JE2 WT as well as NTML JE2 *0846::Tn* (A), NTML JE2 *2389::Tn* (B), NTML JE2 *0429::Tn* (C), NTML JE2 *0543::Tn* (D), NTML JE2 *1481::Tn* (E), or NTML JE2 *1518::Tn* (F) were washed and diluted into TSB pH 2.5 medium. Immediately afterwards (T = 0 h) and at 2 h intervals up to 8 h aliquots were removed and CFUs determined by plating dilutions onto TSA plates. The CFU count at T = 0 h was set to 100% for each strain and % survival at the subsequent time points calculated. The average and standard deviations of the % survival from at least three independent experiments were plotted.

4.4.4 Exploring the importance of genes classified as detrimental for survival under acid stress conditions

Finally, the genes identified as detrimental in the Tn-Seq experiment were investigated for their ability to survive in TSB pH 2.5 medium when individually inactivated by Tn insertions. Of the 7 mutant strains tested, one showed a reduced survival, and three showed an increased survival compared to the JE2 WT strain (Fig. 4.15). NTML JE2 *murA::Tn* had a 13-fold decrease in CFU ml^{-1} compared to the JE2 WT strain following 8 h in TSB pH 2.5 medium (Fig. 4.15E). On the other hand, the NTML JE2 strains *lepA::Tn* and *noc::Tn* showed slight increases in survival, with an approximately 10 and 18-fold increase in CFU ml^{-1} at the 8 h time point compared to the JE2 WT strain (Fig. 4.15B,D). NTML JE2 *lytH::Tn* showed the highest increase in survival of the strains with a Tn insertion in genes classified as detrimental, with an over 100-fold increase in CFU ml^{-1} compared to the JE2 WT strain following 8 h in TSB pH 2.5 medium (Fig. 4.15G).

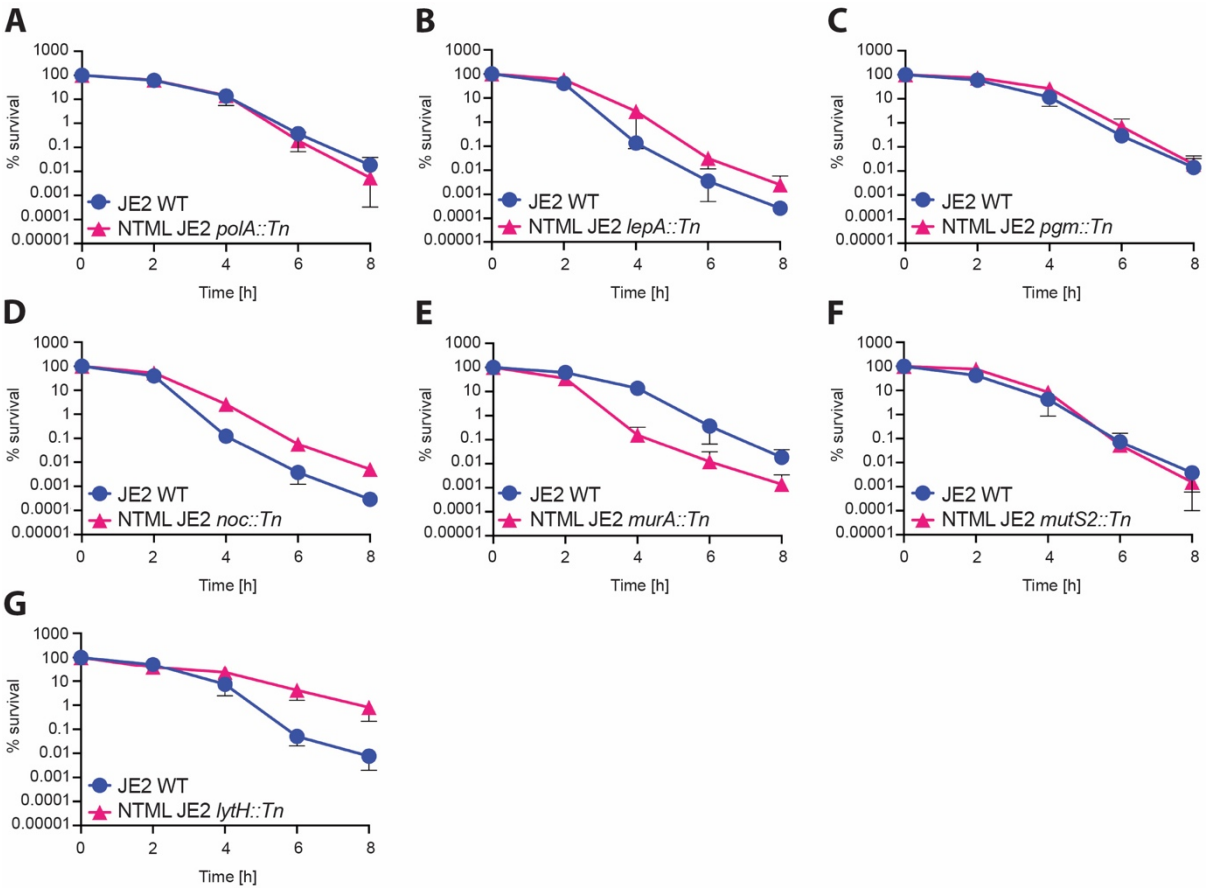


Figure 4.15: Survival analysis of mutant strains with Tn insertions in genes classified as detrimental for fitness under acid stress conditions

Overnight cultures of JE2 WT as well as NTML JE2 *polA::Tn* (A), NTML JE2 *lepA::Tn* (B), NTML JE2 *pgm::Tn* (C), NTML JE2 *noc::Tn* (D), NTML JE2 *murA::Tn* (E), NTML JE2 *mutS2::Tn* (F), or NTML JE2 *lytH::Tn* (G) were washed and diluted into TSB pH 2.5 medium. Immediately afterwards (T = 0 h) and at 2 h intervals up to 8 h aliquots were removed and CFUs determined by plating dilutions onto TSA plates. The CFU count at T = 0 h was set to 100% for each strain and % survival at the subsequent time points calculated. The average and standard deviations of the % survival from at least two independent experiments were plotted. Some of the data in this figure has been adapted from Beetham et al., (2024).

Taken together, this analysis reveals that some of the pathways identified as essential or detrimental for the fitness and growth of *S. aureus* under acidic stress conditions are also important for the survival of *S. aureus* under a greater acidic stress condition. Therefore, there is some overlap between the stress response required for growth or survival in acid.

4.5 Discussion of Chapter 4

A Tn-Seq approach was utilised to identify genes essential or detrimental for the fitness of *S. aureus* at low pH. This work was initiated by A. Gründling and C. Schuster prior to the start of my PhD. Previously, two independently constructed Tn libraries were grown in TSB at standard pH (7.3), or TSB pH 4.5 or 5.5. The resulting location of Tn insertions was determined and used to generate a ratio per gene comparing the number of Tn insertions in the gene when grown at neutral pH to the number of Tn insertions when grown at either pH 4.5 or pH 5.5. My work in this study began with a list of genes in the *S. aureus* genome and their corresponding ratios.

Firstly, genes with a Benjamini-Hochberg corrected p-value of 0.1 or less were classified as either essential or detrimental for fitness at either pH 4.5 or 5.5. Genes were considered essential if they had a ratio of 0.5 or less, and detrimental if they had a ratio of 2 or more. As expected, for each Library more genes were classified as either essential or detrimental for fitness at pH 4.5 than pH 5.5, corresponding with the higher levels of stress encountered by the bacteria at the lower pH. Furthermore, differences in how the libraries were made, grown, or harvested resulted in different sets of genes classified as essential or detrimental for fitness at each pH condition between the libraries. However, with the aim of revealing genes which were more likely to be relevant, only the genes common in both libraries at pH 4.5 were further analysed. This resulted in 31 genes classified as essential, and 10 genes classified as detrimental for the fitness of *S. aureus* at low pH.

Using this Tn-Seq approach, genes were identified as essential which have previously been associated with the acidic stress response of *S. aureus*. For example, the *dltB* gene was one of three genes classified as essential for fitness at both pH 4.5 and pH 5.5. The expression of the *dltABCD* operon has been shown to be upregulated in *S. aureus* in response to low pH (Weinrick et al., 2004). *vraG* was also classified as essential for fitness at both pH 4.5 and pH 5.5, and recent work has shown that the GraXRS-VraFG five-component system may directly detect and respond to low pH (Flannagan et al., 2018; Kuiack et al., 2020; Villanueva et al., 2018). This validates the results of the Tn-Seq experiment as it reproduces the results of the transcriptional studies and confirms that regulating cell surface charge is an important response of *S. aureus* to counter acid stress.

However, the data presented in this Chapter also highlighted other genes involved in the bacterial cell wall as important for the acid stress response of *S. aureus*. For example, several genes coding for proteins with known functions in cell wall assembly and maintenance were highlighted, which indicates that the cell wall as a whole is important for the fitness of *S. aureus* under acid stress conditions.

The Tn-Seq approach identified several novel genes which have previously not been associated with the acidic stress response of *S. aureus*, such as genes linked to aerobic respiration and the QoxAB terminal oxidase. QoxAB accepts electrons from the electron transport chain to reduce oxygen to H_2O. In the process, QoxAB acts as a proton pump to move protons across the membrane and out of the cell. Other bacterial proton pumps, in particular the F_1F_0-ATPase, have been shown to be important for the acidic stress responses of a range of bacteria as described in the Introduction Chapter. These include *E. coli* (Sun et al., 2012), *S. enterica* (Foster & Hall, 1990, 1991), and *L. monocytogenes* (Cotter et al., 2000; Datta & Benjamin, 1997; McEntire et al., 2004). However, it is unclear whether proton pumps act directly to remove protons, or whether they maintain the PMF for ATP generation for use in other acidic stress responses. It is notable however, that *S. aureus* has a second, less active terminal oxidase. While the Cyd oxidase accepts electrons from the electron transport chain to reduce oxygen to H_2O, it does not result in the movement of protons across the membrane. This may explain why it was not identified as essential in the Tn-Seq experiment, unlike the QoxAB terminal oxidase. This may support the hypothesis that the importance of QoxAB for fitness in acid stress conditions is due to its role as a proton pump, rather than ATP generation.

A key component of the acidic stress response of *S. aureus* is the urease enzyme, which produces ammonia from urea to act as a buffer in the cytoplasm. The *ureABCD* genes encoding the urease and supporting enzymes have previously been shown to be highly upregulated in *S. aureus* upon acidic stress (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010; Weinrick et al., 2004). Furthermore, the *ureB* gene has been shown to be essential for the growth of *S. aureus* in weak acid stress conditions (Zhou et al., 2019). In Chapter 3, I demonstrated that strains of *S. aureus* with Tn insertions in the *ureB* gene have reduced growth and survival in TSB under both weak and strong acidic stress conditions when urea is added. However, these genes were not identified as essential in the Tn-Seq experiment. I hypothesize that this is because urease has been shown to be important for growth at low pH

only when exogenous urea is added to the medium, and that the endogenous urea synthesized by *S. aureus* is not sufficient for protection by urease against acid stress (Zhou et al., 2019). As the Tn-Seq assay was performed in TSB without urea added, these genes would not have been identified as essential. By performing future Tn-Seq experiments under different growth conditions, it is likely that additional genes and pathways can be identified which are important for the fitness of *S. aureus* under acid stress in different environmental conditions.

Most of the genes classified as essential through the Tn-Seq experiment could be confirmed for their importance for the growth of *S. aureus* at low pH. The growth of the WT strains on TSA pH 4.5 plates was compared to mutant strains of *S. aureus* with Tn insertions in the highlighted genes from either the NTML or other strain collections. Some genes however could not be further investigated, either because there was no mutant strain available in either the NTML or other strain collection or because the correct location of the Tn insertion could not be confirmed. In total, 27 out of 41 genes could be further explored. In the growth plate assay investigating growth of the individual mutants on TSA pH 4.5, most of the essential genes resulted in a growth defect on TSA pH 4.5 plates when individually inactivated, and most of the detrimental genes had no growth defect on TSA pH 4.5 plates when individually inactivated. This confirms the robustness of the Tn-Seq as an approach to identify genes either essential or detrimental for the fitness and growth of *S. aureus* at low pH.

Finally, in this Chapter I also explored whether the genes identified as essential or detrimental in the Tn-Seq were also important for the ability of *S. aureus* to survive under acidic stress. Bacterial responses to stress conditions differ depending on whether they are growing under a lower stress condition, or surviving at a higher stress condition. However, only some of the genes classified as essential in the Tn-Seq assay resulted in a reduced ability to survive TSB pH 2.5 medium when individually inactivated. For example, while both the NTML JE2 *qoxA::Tn* and *qoxB::Tn* strains had a reduced survival compared to the JE2 WT, the NTML JE2 *srrA::Tn* strain did not, despite all three strains showing a growth defect at pH 4.5. Therefore, the Tn-Seq was not effective at identifying genes required for the ability of *S. aureus* to survive a higher level of acidic stress as there is limited overlap between the response required to grow at pH 4.5 and the response required to survive at pH 2.5.

Chapter 5 : Using a genetic complementation approach to confirm the importance of *0846* for the acid stress response of *Staphylococcus aureus*

The Tn-Seq experiment and subsequent plate spotting assays highlighted several pathways as essential for the fitness and growth of *S. aureus* under acidic stress conditions. Some of these pathways have previously been associated with the acidic stress response of *S. aureus* or other bacteria, such as the modification of the bacterial cell wall and cell surface charge. In addition, several novel genes essential for the fitness of *S. aureus* under low pH were identified in the Tn-Seq experiment, such as aerobic respiration and *SAUSA300_0846* (herein called *0846*).

0846 was chosen for further study as it had the lowest ratio in the Tn-Seq experiment at pH 4.5, and can thus be deemed one of the most important factors for fitness at low pH. The gene was also one of three genes identified as essential in the Tn-Seq experiment at both pH 4.5 and pH 5.5, suggesting that the gene is important for the fitness of *S. aureus* in a range of acidic stress levels. When *0846* was inactivated, the NTML JE2 *0846::Tn* strain showed reduced growth on TSA pH 4.5 plates and reduced survival in TSB pH 2.5 medium, suggesting that the gene is necessary for both the growth and survival of *S. aureus* at low pH. Interestingly, several previous studies have demonstrated that an *0846* mutant strain has reduced virulence in mice, indicating that the gene is important for the pathogenesis of *S. aureus* (Benton et al., 2004; Grosser et al., 2018; Valentino et al., 2014). However, the cellular function of the protein encoded by *0846* is unknown, thus potentially highlighting a novel pathway required for the growth of *S. aureus* under acid stress conditions.

5.1 The growth defect of the *0846::Tn* strain at low pH is due to inactivation of the *0846* gene

5.1.1 Transduction of the Tn insertion into a clean background

To confirm that the reduced growth and survival at low pH of the NTML JE2 *0846::Tn* strain described in Chapter 4 is due to the disruption of the *0846* gene, the *0846::Tn* region was transferred to a clean *S. aureus* background. The assays in Chapter 4 were performed using mutant strains derived from the NTML JE2 collection (Fey et al., 2013). Transferring the Tn insertion from the NTML JE2 *0846::Tn* mutant strain to a clean background by phage transduction should confirm that any phenotypic differences associated with the mutant are

due to the inactivation of *0846*, rather than any additional mutations present in the NTML strain.

The Tn in the NTML JE2 *0846::Tn* strain was transferred by phage transduction to a LAC* background. The LAC* background was chosen as both the JE2 and LAC* strains are derived from the same LAC USA300 lineage. The original LAC strain contains two plasmids, and LAC* has been cured of one plasmid while JE2 has been cured of both. Following the generation of a LAC* *0846::Tn* strain its growth on TSA pH 7.3 and pH 4.5 plates was compared to the LAC* WT strain. As expected, inactivating *0846* in the LAC* background did not confer a growth defect on TSA pH 7.3 plates, but resulted in a 1,000,000 fold decrease in growth on TSA pH 4.5 plates (Fig. 5.1A).

Next, the survival of the LAC* WT and LAC* *0846::Tn* strains were investigated in TSB pH 2.5 medium. Unexpectedly, the LAC* *0846::Tn* strain showed no decrease in CFU ml^{-1} counts compared to the LAC* WT (Fig. 5.1B). This differs to what was shown in Chapter 4 for the NTML JE2 *0846::Tn* strain, which showed a 60-fold decrease in CFU ml^{-1} compared to the JE2 WT strain at the 8 h time point (Fig. 4.14A).

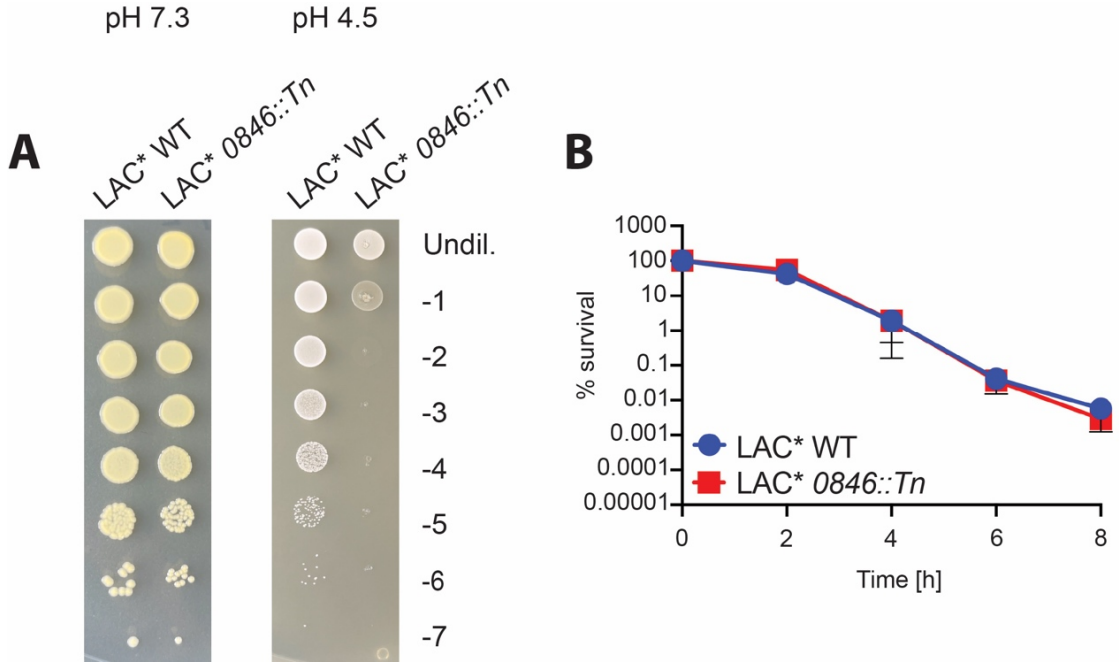


Figure 5.1: A transduced LAC* *0846::Tn* strain shows reduced growth, but not reduced survival, at low pH

(A) Overnight cultures of the LAC* WT and LAC* *0846::Tn* strains were serially diluted and aliquots spotted onto TSA pH 7.3 plates or pH 4.5 plates. Images were taken following 18 h incubation at 37 °C. Each image is a representative of three independent experiments. (B) Overnight cultures of LAC* WT and LAC* *0846::Tn* strains were washed and diluted into TSB pH 2.5 medium. Immediately afterwards (T = 0 h) and at 2 h intervals up to 8 h aliquots were removed and CFUs determined by plating dilutions onto TSA plates. The CFU count at T = 0 h was set to 100% for each strain and % survival at the subsequent time points calculated. The average and standard deviations of the % survival from four independent experiments were plotted.

To investigate whether the difference in survival at low pH between the NTML JE2 *0846::Tn* and LAC* *0846::Tn* strains was due to the different strain backgrounds, the Tn was next transferred from the NTML JE2 *0846::Tn* strain to a clean JE2 background. The resulting strain will hereon be referred to as JE2 *0846::Tn*, while the original strain will be referred to as NTML JE2 *0846::Tn*.

The JE2 *0846::Tn* strain showed an approx. 10,000 fold reduction in growth compared to the JE2 WT strain when spotted on TSA pH 4.5 plates (Fig. 5.2A). On the other hand, the JE2 *0846::Tn* strain did not show any difference in survival at pH 2.5 compared to the JE2 WT strain (Fig. 5.2B). These results mirror those of the LAC* *0846::Tn* strain, and confirm that the difference in survival at pH 2.5 between the NTML JE2 *0846::Tn* and LAC* *0846::Tn* strains was

not due to the different strain backgrounds and is likely due to the presence of additional mutations in the NTML JE2 *0846::Tn* strain.

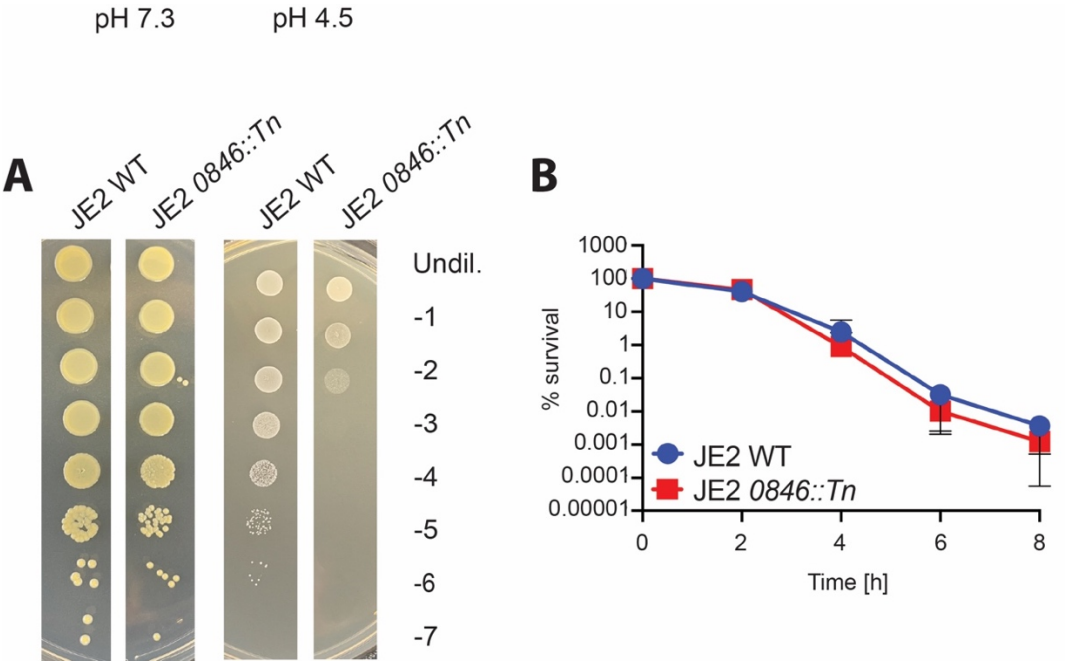


Figure 5.2: A transduced JE2 *0846::Tn* strain shows reduced growth, but not reduced survival, at low pH

(A) Overnight cultures of the JE2 WT and JE2 *0846::Tn* strains were serially diluted and aliquots spotted onto TSA pH 7.3 plates or pH 4.5 plates. Images were taken following 18 h incubation at 37 °C. Each image is a representative of three independent experiments. (B) Overnight cultures of JE2 WT and JE2 *0846::Tn* strains were washed and diluted into TSB pH 2.5 medium. Immediately afterwards (T = 0 h) and at 2 h intervals up to 8 h aliquots were removed and CFUs determined by plating dilutions onto TSA plates. The CFU count at T = 0 h was set to 100% for each strain and % survival at the subsequent time points calculated. The average and standard deviations of the % survival from four independent experiments were plotted.

5.1.2 Generation of complementation strains

To investigate whether the reduced survival of the NTML JE2 *0846::Tn* strain at low pH compared to the JE2 WT strain was due to the presence of additional mutations, a genetic complementation strategy was used. It was expected that if additional mutations are present which are responsible for the reduced survival of the NTML JE2 *0846::Tn* strain, then the addition of *0846* back into the strain would not result in restored survival. Complemented strains were generated by A. Gründling, and I will briefly describe how they were generated below.

The *0846* gene was cloned into the pCL55 plasmid along with 1000 upstream bases to include the native promoter. The resulting pCL55-*0846* plasmid was introduced into the *E. coli* shuttle vector CLG190. Following transformation into the *S. aureus* RN4220 strain, the plasmid integrates into the *geh* locus. The integrated plasmid was then moved to the desired JE2 or LAC* background via phage transduction. As a control, the empty vector pCL55 was also transduced from RN4220 to the same JE2 or LAC* background strains. Complementation strains were generated in the NTML JE2 *0846::Tn*, LAC* *0846::Tn*, and JE2 *0846::Tn* strains resulting in the following strains: NTML JE2 *0846::Tn* (Empty Vector (EV)), NTML JE2 *0846::Tn* (complemented (comp.)), LAC* *0846::Tn* (EV), LAC* *0846::Tn* (comp.), JE2 *0846::Tn* (EV), and JE2 *0846::Tn* (comp.). These were compared to either a JE2 WT (EV) or LAC* WT (EV) strain.

5.1.3 Analysis of the complementation strains

To explore whether the addition of the *0846* gene on an ectopic location on the chromosome could complement the phenotypes observed in the *0846::Tn* mutant strains, I spotted the complementation strains along with the isogenic WT and mutant control strains containing the empty vector on TSA pH 4.5 plates. Both the JE2 and LAC* WT (EV) strains grew to the -6 or -7 dilution, similar to the JE2 WT and LAC* WT strains (Fig. 5.1A, 5.2A, 5.3). This shows that integration of the pCL55 plasmid into the *geh* locus does not confer a growth defect on the WT bacteria.

In all three genetic backgrounds, the *0846::Tn* (EV) strains showed the expected growth defect compared to their respective WT (EV) control strains on TSA pH 4.5 plates. The NTML JE2 *0846::Tn* (EV) and LAC* *0846::Tn* (EV) strains both showed a 10,000 fold reduction in growth compared to the JE2 WT (EV) and LAC* WT (EV) strains, respectively (Fig. 5.3A,B), while the JE2 *0846::Tn* (EV) strain showed a 1,000,000 fold reduction in growth compared to the JE2 WT (EV) strain (Fig. 5.3C). Furthermore, in all three genetic backgrounds the addition of *0846* into the *0846::Tn* mutant bacteria restored growth on TSA pH 4.5 plates (Fig. 5.3). All three *0846::Tn* (comp.) strains grew to the same or similar dilution as their respective WT (EV) strains (Fig. 5.3). Taken together, it is clear that the reduced ability of the *0846::Tn* strains to grow at low pH can be complemented by re-introduction of *0846* into the *0846::Tn* strains.

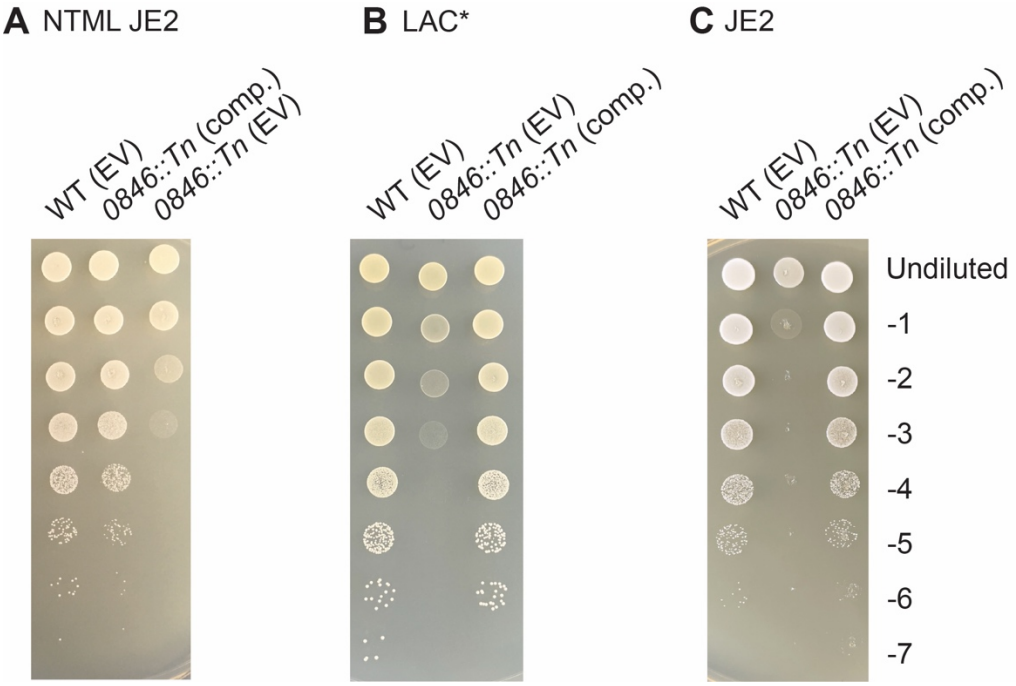


Figure 5.3: Re-introduction of *0846* into the *0846::Tn* strains restores growth on TSA pH 4.5 plates

Overnight cultures of (A) JE2 WT (EV), NTML JE2 *0846::Tn* (comp), and NTML JE2 *0846::Tn* (EV), (B) LAC* WT (EV), LAC* *0846::Tn* (EV), and LAC* *0846::Tn* (comp.), or (C) JE2 WT (EV), JE2 *0846::Tn* (EV), or JE2 *0846::Tn* (comp.) were serially diluted and aliquots spotted onto TSA pH 7.3 plates or pH 4.5 plates. Images were taken following 18-24 h incubation at 37 °C. Each image is a representative of three independent experiments. Some of the data in this figure has been adapted from Beetham et al., (2024).

To confirm that the reduced growth of the *0846::Tn* (EV) bacteria at low pH can be restored in the complemented strains, a growth curve was performed to investigate growth in liquid medium at pH 4.5. To this end, the WT (EV), *0846::Tn* (EV), and *0846::Tn* (comp.) strains in the NTML JE2, LAC*, and JE2 backgrounds were inoculated into TSB pH 7.3 and 4.5 medium, and OD_{600} measurements were taken every 30 min for 24 h. In TSB pH 7.3 medium, all strains showed a similar growth rate and reached a final OD_{600} value of approximately 3 by the 8 h time point (Fig. 5.4). This confirms data from the earlier shown plate spotting assays, which showed that the inactivation of *0846* does not confer a growth defect at neutral pH.

As expected, in TSB pH 4.5 medium the *0846::Tn* (EV) strains showed a reduced growth rate compared to the WT (EV) strains, and reached lower final OD_{600} values in all genetic backgrounds (Fig. 5.4). The NTML JE2 *0846::Tn* (EV) strain showed only a 0.06 decrease in final OD_{600} value compared to the JE2 WT (EV) strain (Fig. 5.4A), while the LAC* and JE2 *0846::Tn*

(EV) strain showed a 0.2 and 0.24 decrease in final OD_{600} value compared to the WT (EV) strains, respectively (Fig. 5.4B,C). In all strains, the re-introduction of *0846* into the *0846::Tn* mutant strains restored the higher initial rate of growth and higher final OD_{600} value, confirming that the reduced growth of the *0846::Tn* strain at low pH compared to the WT strain is due to inactivation of the *0846* gene.

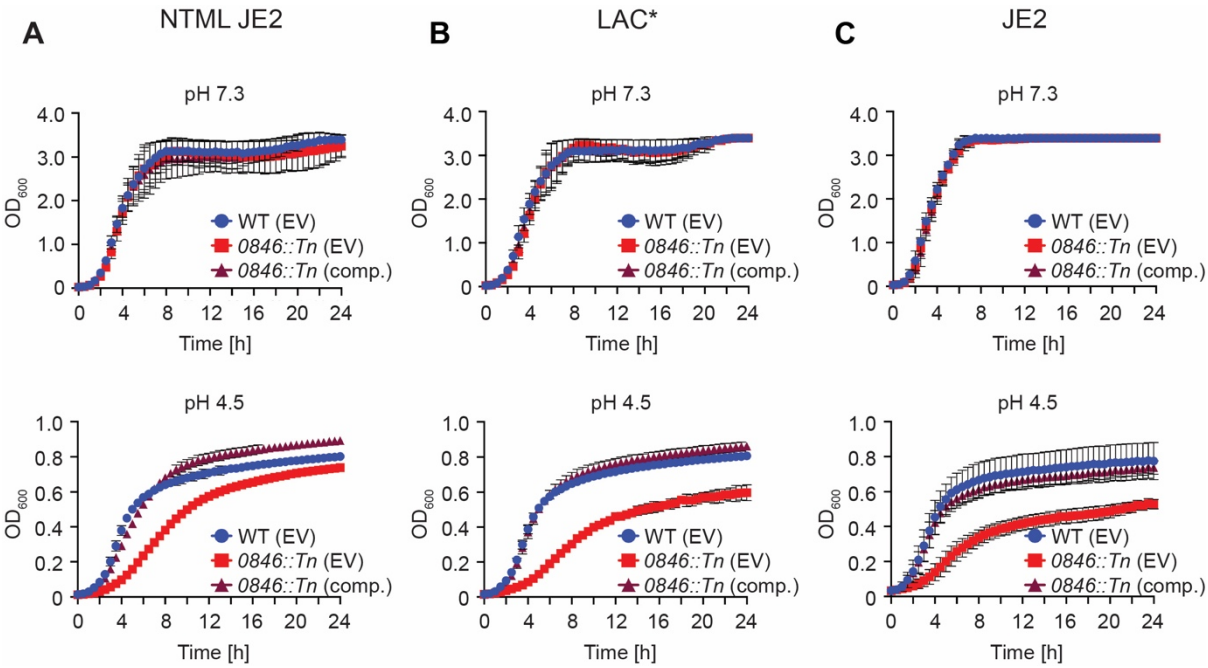


Figure 5.4: Re-introduction of *0846* into the *0846::Tn* mutant strains restores growth in TSB pH 4.5 medium

Overnight cultures of (A) JE2 WT (EV), NTML JE2 *0846::Tn* (EV), and NTML JE2 *0846::Tn* (comp.), (B) LAC* WT (EV), LAC* *0846::Tn* (EV), and LAC* *0846::Tn* (comp.), or (C) JE2 WT (EV), JE2 *0846::Tn* (EV), or JE2 *0846::Tn* (comp.) were inoculated into TSB pH 7.3 or pH 4.5 medium. Strains were grown in 96-well plates with OD_{600} measurements taken at the indicated time points. The average readings and standard deviations of three independent experiments were plotted. Some of the data in this figure has been adapted from Beetham et al., (2024).

On the other hand, the reduced survival in TSB pH 2.5 medium of the NTML JE2 *0846::Tn* mutant strain could not be complemented. As expected, the NTML JE2 *0846::Tn* (EV) showed an approximately 6-fold reduction in CFU ml^{-1} compared to the JE2 WT (EV) strain following 8 h in TSB pH 2.5 medium (Fig. 5.5A). However, the re-introduction of *0846* into the NTML JE2 *0846::Tn* (EV) strain did not restore survival. The NTML JE2 *0846::Tn* (comp.) strain also showed an approximately 8-fold reduced CFU ml^{-1} compared to the JE2 WT (EV) strain, similar to the NTML JE2 *0846::Tn* (EV) strain.

As expected, neither the LAC* *0846::Tn* (EV) and LAC* *0846::Tn* (comp.), nor the JE2 *0846::Tn* (EV) and JE2 *0846::Tn* (comp.) strains showed any decrease in survival over 8 h in TSB pH 2.5 medium compared to their respective WT (EV) strains (Fig. 5.5B,C).

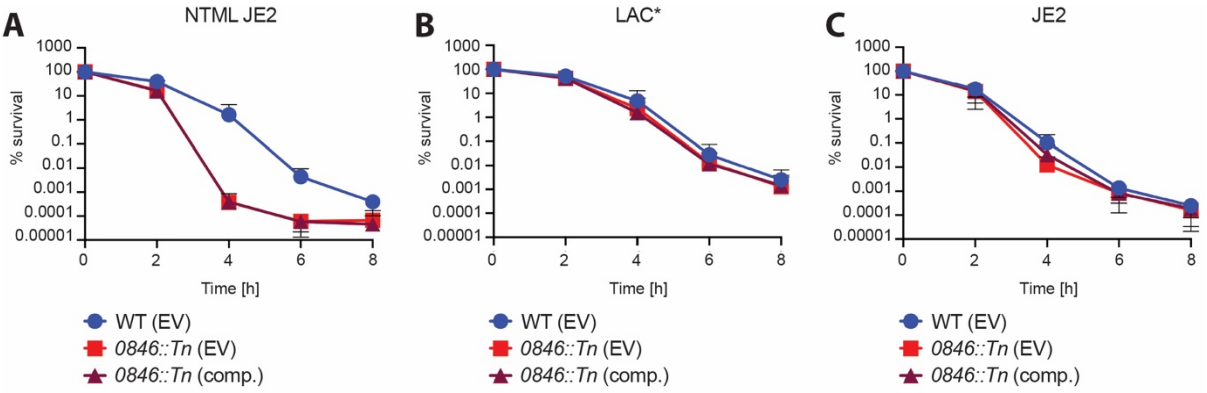


Figure 5.5: Re-integration of *0846* into the NTML JE2 *0846::Tn* mutant strain does not restore survival at low pH
Overnight cultures of (A) JE2 WT (EV), NTML JE2 *0846::Tn* (EV), and NTML JE2 *0846::Tn* (comp.), (B) LAC* WT (EV), LAC* *0846::Tn* (EV), and LAC* *0846::Tn* (comp.), or (C) JE2 WT (EV), JE2 *0846::Tn* (EV), or JE2 *0846::Tn* (comp.) were washed and diluted into TSB pH 2.5 medium. Immediately afterwards (T = 0 h) and at 2 h intervals up to 8 h aliquots were removed and CFUs determined by plating dilutions onto TSA plates. The CFU count at T = 0 h was set to 100% for each strain and % survival at the subsequent time points calculated. The average and standard deviations of the % survival from at least three independent experiments were plotted.

Taken together, these results suggest that the reduced ability of the NTML JE2 *0846::Tn* strain to grow at pH 4.5 is due to the inactivation of the *0846* gene, but that the reduced ability to survive pH 2.5 is not.

5.2 Four additional SNPs are present in the NTML JE2 *0846::Tn* strain compared to the JE2 WT strain

5.2.1 Whole genome analysis of the NTML JE2 *0846::Tn* strain

The data presented thus far in this Chapter imply that the reduced ability of the NTML JE2 *0846::Tn* strain to survive in TSB pH 2.5 medium compared to the JE2 WT strain is not due to the inactivation of the *0846* gene, but rather due to additional mutations present in the NTML JE2 *0846::Tn* strain. Firstly, the survival defect at pH 2.5 is not seen when the *0846::Tn* region

from the original NTML strain is transduced into a LAC* or a new JE2 background strain. Furthermore, the re-introduction of *0846* gene under its native promoter control into the NTML JE2 *0846::Tn* strain does not restore its survival at pH 2.5 to the levels of the JE2 WT strain.

The NTML JE2 *0846::Tn* strain was taken from the NTML collection. Although the correct location of the Tn insertion was confirmed by PCR and sequencing, this does not preclude the presence of additional mutations in the strain. Therefore, a whole-genome sequencing approach was used to compare the genome sequence of the NTML JE2 *0846::Tn* strain with the JE2 WT.

Chromosomal DNA was extracted from the mutant strain and sent for whole genome sequencing using an Illumina platform. A genome sequence was determined following the procedure described in the Experimental procedures Chapter. The resulting whole genome sequence was compared to the whole genome sequence of the JE2 WT strain generated previously by Dr. Cordelia Weiss. Following analysis, the presence of four additional single nucleotide polymorphisms (SNPs) were detected in the NTML JE2 *0846::Tn* genome sequence compared to the WT genome sequence (Table 5.1).

Table 5.1: Four additional SNPs were detected in the NTML JE2 *0846::Tn* strain compared to the JE2 WT strain

Gene locus tag[1]	Annotated gene function	Mutation[2]	Amino Acid Change[3]	Frequency[4]
Upstream of *SAUSA300_0761*		SNP A - T		100
***SAUSA300_0856* (*kapB*)**	Kinase associated protein B	SNP C - T	S12N	100
SAUSA300_1223		SNP T - C	D211G	100
***SAUSA300_1591* (*apt*)**	Adenine phosphoribosyl transferase	SNP C - T	G73D	100

[1]Annotated gene where the mutation occurs. [2]Type of mutation with SNP indicating a single nucleotide polymorphism. Base change for SNP indicated by base in reference genome – base in the same position in the suppressor strain. [3]Amino acid change indicates where applicable the initial amino acid, position of amino acid in the encoded protein, and the resulting amino acid following the mutation. [4]Frequency indicates the frequency that the mutation was found in the suppressor strain in the genome sequence analysis.

The first SNP detected is located 5 bp upstream of *SAUSA300_0761*. This gene encodes a protein with an unknown function but has been putatively labelled as a membrane protein with multiple trans-membrane helices. The second SNP occurs within *SAUSA300_0856* and causes a change in amino acid at residue 12 from serine to asparagine. This gene is predicted to code for a kinase associated protein named KapB, although its function is unknown. *kapB* is found upstream of the *mnh1* operon in the staphylococcal genome. The third SNP is located in *SAUSA300_1223*, a gene encoding a protein with unknown function, but potentially containing a nuclease-related domain. The result of this SNP is a change in amino acid at residue 211 from aspartic acid to glycine. The final SNP is found in gene *SAUSA300_1591*, or *apt*. This gene encodes an adenine phosphoribosyltransferase which catalyses the conversion of adenine to adenosine monophosphate (AMP), and the SNP results in a change in amino acid from glycine to aspartic acid at residue 73.

I was particularly interested in the SNP present in the *kapB* gene due to its location upstream of *mnh1*. The *mnh1* operon contains 7 genes encoding the MnhABCDEFG subunits of the Mnh1 multivalent cation / proton antiporter, and the transporter has been shown to be important for the growth of *S. aureus* under alkaline conditions (Vaish et al., 2018). Furthermore, the Tn-Seq experiment described in Chapter 4 suggested that the *mnh1* genes may be important for the fitness of *S. aureus* at acidic pH, as several genes were identified as essential for fitness at pH 4.5 or 5.5 in one or both libraries (Table 4.2, 5.2). Specifically, *mnhA1* was identified as essential for fitness at pH 4.5 with an average ratio of 0.2 (Table 4.2). Furthermore, the *mnhA1* gene was essential for fitness at pH 5.5 in Library B with a ratio of 0.4, but not in Library A (Table 5.2). Several other *mnh* genes were also identified as essential in the Tn-Seq experiment for fitness at either pH 4.5 of pH 5.5 in one or both libraries. However, as they were not classified as essential in both Libraries, they did not appear in the list of essential genes in Table 4.2 or Table 4.3. This may have been because the ratio was above the cut off of 0.5, or they had a p-value above the cut off of 0.1. For example, *mnhB1* had a ratio of 0.49 in Library A at pH 4.5, but had a p-value above 0.1 in Library B (Table 5.2). *mnhD1* had a ratio of 0.36 in Library A at pH 4.5, however the ratio in Library B was 0.54. Furthermore, *mnhD1* was classified as essential in Library B at pH 5.5 with a ratio of 0.38, but had a p-value above 0.1 in Library A (Table 5.2). Finally, *mnhF1* was essential in Library B at pH

4.5 with a ratio of 0.22, but the ratio in Library A was 0.54 and thus above the cut off (Table 5.2).

Table 5.2: Several genes in the *mnh1* operon were identified in the Tn-Seq experiment as essential for fitness in one or both Libraries

Ratios of individual *mnh1* operon genes following growth of either Tn Library A or B in TSB pH 7.2, pH 5.5 and pH 4.5 medium as described in Chapter 4. Ratios for genes which had a p-value above 0.1 for a particular Library or condition are not shown.

Locus Tag SAUSA300_	**Gene Name**	**Ratio at pH 4.5**		**Ratio at pH 5.5**	
		Library A	**Library B**	**Library A**	**Library B**
0855	*mnhA1*	0.17	0.23		0.40
0854	*mnhB1*	0.49			
0852	*mnhD1*	0.36	0.54		0.38
0850	*mnhF1*	0.54	0.22		

Taken together, this suggests that the *mnh1* operon may be important for the fitness and growth of *S. aureus* under acidic conditions. Therefore, I hypothesized that the reduced survival of the JE2 NTML *0846::Tn* strain in TSB pH 2.5 medium is due to downstream effects on the transcription of the *mnh1* operon due to the presence of the SNP in *kapB*.

5.2.2 Investigation of the *kapB* SNP

To explore whether the SNP in *kapB* is responsible for the reduced survival of the NTML JE2 *0846::Tn* strain in TSB pH 2.5 medium, the SNP was transferred into a clean JE2 background along with the Tn in *0846* by phage transduction. This could occur because the SNP in *kapB* is approx. 8700 base pairs away from the Tn location in *0846* (Fig. 5.6). This is within the approx. 44,000 bp range of DNA that can be packaged by Phage 85 (Kwan et al., 2005), and thus can be transduced together. To ensure the resulting strain contained the *kapB* SNP, the *kapB* gene was amplified by PCR and sequenced. The resulting JE2 *0846::Tn kapB** strain was compared to the NTML JE2 *0846::Tn* strain, and the JE2 *0846::Tn* strain without the *kapB* SNP.

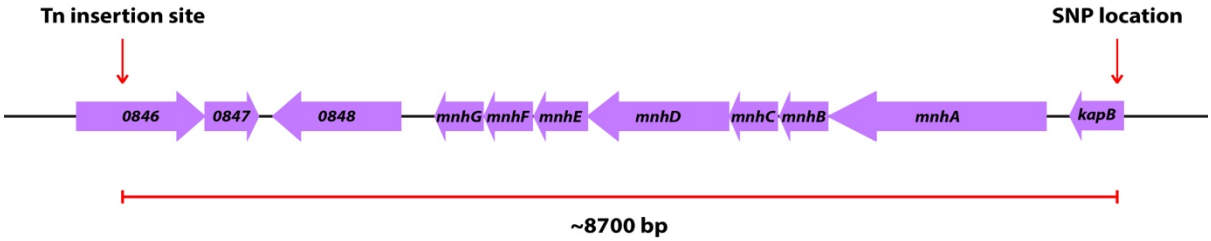


Figure 5.6: Schematic representation of the location of the SNP in *kapB* and the Tn in *0846*
The SNP present in *kapB* was approx. 8700 bp away from the Tn insertions site in *0846*, which is within the approx. 44,000 bp which can be packaged by phage 85.

All four strains showed a similar growth when spotted on TSA pH 7.3 plates, indicating that the presence of the SNP in *kapB* did not confer a growth defect at neutral conditions (Fig. 5.7A). Furthermore, the SNP in *kapB* did not confer a growth defect on TSA pH 4.5 plates. Both the JE2 *0846::Tn* and JE2 *0846::Tn kapB** strains showed a 10,000 fold reduction in growth on TSA pH 4.5 plates compared to the JE2 WT strain. Interestingly, the NTML JE2 *0846::Tn* strain showed a 10 fold increased ability to grow on TSA pH 4.5 plates compared to both the JE2 *0846::Tn* and JE2 *0846::Tn kapB** strains. This could indicate that the presence of the SNPs in the NTML JE2 *0846::Tn* strain helps compensate for its reduced ability to grow at low pH, and may explain their presence. This is supported by data from the growth curves of the complemented strains in the NTML JE2, LAC* and JE2 backgrounds shown previously in this Chapter (Fig. 5.4). The NTML JE2 *0846::Tn* (EV) strain almost reaches the same final OD_{600} value as the JE2 WT (EV) strain when grown in TSB pH 4.5 medium, with only a 0.06 OD_{600} difference following 24 h. However, both the LAC* and JE2 *0846::Tn* (EV) strains have a much lower OD_{600} value, with a difference of 0.2 and 0.24 compared to their respective WT (EV) strains. However, the similar growth on TSA pH 4.5 plates of the JE2 *0846::Tn* and JE2 *0846::Tn kapB** strains shows that the compensatory effect of the additional SNPs is not due to the presence of the *kapB* SNP alone.

Finally, the effect of the *kapB* SNP on the ability of the JE2 *0846::Tn* strain to survive in TSB pH 2.5 medium was investigated. As expected, the JE2 NTML *0846::Tn* strain showed a 23 fold reduction in CFU ml^{-1} compared to the JE2 WT strain at the 8 h time point (Fig. 5.7B). Furthermore, as shown above in Fig 5.2B, the JE2 *0846::Tn* strain did not show a significant reduction in CFU ml^{-1} compared to the JE2 WT strain. Furthermore, the additional *kapB* SNP appeared to have little effect on survival. Whilst a slight reduction in survival of the JE2

*0846::Tn kapB** strain compared to the JE2 *0846::Tn* strain was seen at the 4 h time point, this difference was negated by the 8 h time point. Taken together, these data show that the reduced survival of the NTML JE2 *0846::Tn* strain in TSB pH 2.5 medium is not due to the inactivation of the *0846* gene, nor to the SNP present in *kapB* alone. This phenotype could be due to either of the other three SNPs, a combination of all four, or the presence of additional mutations not detected by the Illumina sequencing platform.

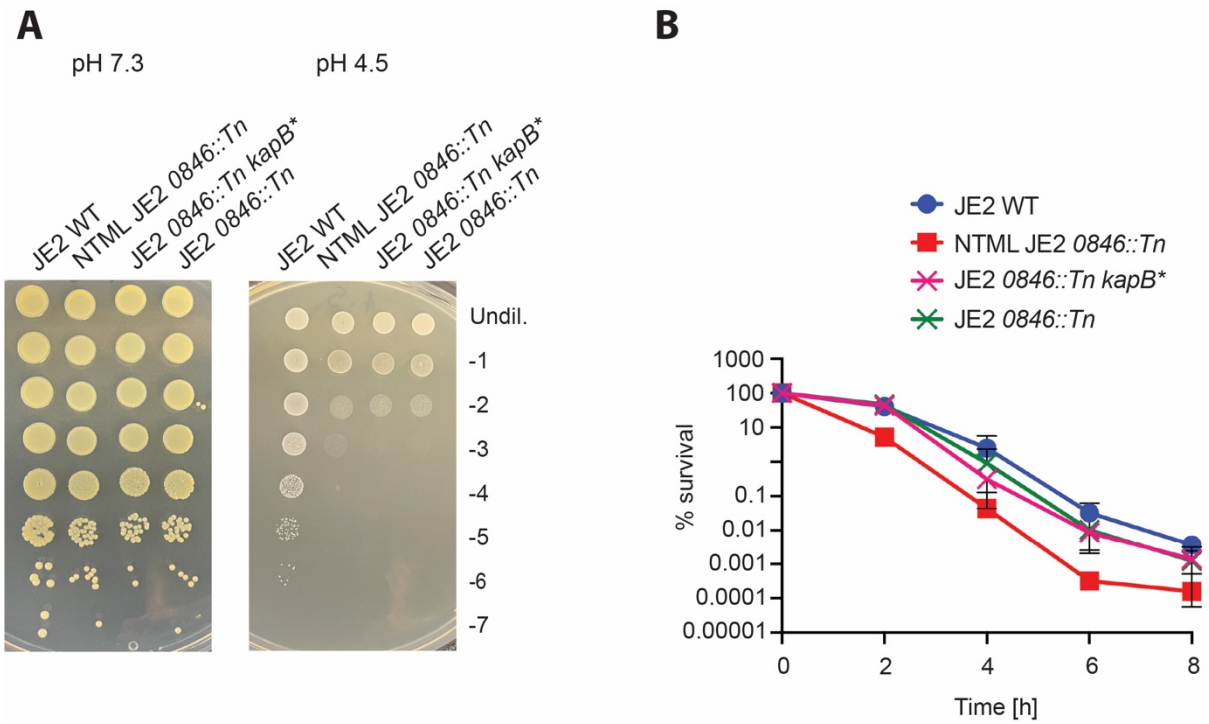


Figure 5.7: The *kapB* SNP does not confer reduced survival at pH 2.5
(A) Overnight cultures of the JE2 WT, NTML JE2 *0846::Tn*, JE2 *0846::Tn kapB**, and JE2 *0846::Tn* strains were serially diluted and aliquots spotted onto TSA pH 7.3 plates or pH 4.5 plates. Images were taken following 18 h incubation at 37 °C. Each image is a representative of three independent experiments. (B) Overnight cultures of JE2 WT, NTML JE2 *0846::Tn*, JE2 *0846::Tn kapB**, and JE2 *0846::Tn* strains were washed and diluted into TSB pH 2.5 medium. Immediately afterwards (T = 0 h) and at 2 h intervals up to 8 h aliquots were removed and CFUs determined by plating dilutions onto TSA plates. The CFU count at T = 0 h was set to 100% for each strain and % survival at the subsequent time points calculated. The average and standard deviations of the % survival from four independent experiments were plotted. (A,B) Data for the JE2 WT and JE2 *0846::Tn* strains are the same as shown in Fig 5.2.

5.3 Discussion of Chapter 5

The data presented in this Chapter demonstrate some of the issues that can arise from using strains derived from large mutant library collections. When the NTML JE2 *0846::Tn* mutant

strain was used directly from the NTML library collection, the strain showed a reduced ability to both grow and survival at low pH compared to the WT strain as shown in Chapter 4. However, further analysis demonstrated that the reduced survival at low pH was not due to the inactivation of the *0846* gene. When the Tn in *0846* was transferred to a fresh LAC* or JE2 background strain, the phenotype was no longer observed. Furthermore, the addition of *0846* back into the genome of the NTML JE2 *0846::Tn* strain did not restore its ability to survive in TSB pH 2.5 medium.

Genetic manipulation in *S. aureus* is difficult due to the low frequency of transformation, and so studies screening a large number of genes often use previously made mutant strains such as those found in the NTML library. The correct presence and location of Tn can be confirmed quickly by designing primers to amplify the predicted Tn-chromosome junction. However, this does not reveal the presence of additional mutations which may be present in the NTML mutant strain. There are two potential approaches to examine whether the NTML mutant strain contains additional mutations. The first is to transduce the Tn into a clean genetic background. The second is to examine the whole genome by sequencing.

Whole genome sequencing of the NTML JE2 *0846::Tn* strain revealed four additional SNPs present compared to the JE2 WT strain. One is located upstream of a gene, two are located within genes coding for proteins with unknown functions, and the fourth is located within *apt* coding for an adenine phosphoribosyltransferase. It is therefore difficult to predict what their likely effect is. One of the SNPs, present within the *kapB* gene, is located upstream of the *mnh1* operon. The *mnh1* operon contains 7 genes encoding the MnhABCDEFH subunits that make up the Mrp family transporter. Many *S. aureus* strains contain two *mnh* operons, however in the LAC, LAC* and JE2 derived strains the Mnh2 transporter is assumed to be non-functional due to a large deletion in the *mnh2* operon (Vaish et al., 2018). The Mnh transporters have been previously associated with cytoplasmic pH regulation in *S. aureus*. For example, the expression of *mnhA1* has been shown to be upregulated at pH 6.0 , and a double *ΔmnhA1ΔmnhA2* mutant in the Newman and SH1000 strains had a severe growth defect at alkaline pH compared to the WT strain, but only a slight growth defect at neutral pH (Vaish et al., 2018). In addition, several genes in the *mnh1* operon were identified as essential for fitness in one or both libraries at low pH in the Tn-Seq experiment described in Chapter 4. This therefore suggests that *mnh1* may have a role in the acidic stress response of *S. aureus*.

However, the Mnh transporters were not assayed since there were no mutant strains available in the NTML or other mutant library collections.

The *kapB* gene ends 131 bp upstream of the *mnh1* operon and could therefore affect the regulation and transcription of *mnh1*. To explore whether the *kapB* SNP is responsible for the reduced survival of the NTML JE2 *0846::Tn* strain in TSB pH 2.5 medium, the SNP was transferred along with the Tn in *0846* to a clean JE2 background. However, the similar growth on TSA pH 4.5 plates and survival in TSB pH 2.5 medium of the JE2 *0846::Tn* strain and JE2 *0846::Tn kapB** strain indicates that the SNP in *kapB* is not responsible for the reduced survival of the NTML JE2 *0846::Tn* strain at low pH. It is possible that one of the other SNPs is responsible, or a combination of all four SNPs, or that another form of mutation is present which was not detected by the Illumina sequencing platform.

In Chapter 4 I showed that while some genes important for the growth of *S. aureus* at low pH in the Tn-Seq experiment and subsequent plate spotting assay were also important for survival at low pH, most genes were not important in both assays. The data presented in this Chapter therefore supports this by showing that *0846* is not important for the survival of *S. aureus* under low pH. However, it is clear that *0846* is important for the growth of *S. aureus* at pH 4.5 on both TSA plates and in TSB medium. Inactivation of the gene by Tn insertion reduces the ability of the bacteria to grow on TSA pH 4.5 plates and in TSB pH 4.5 medium in the NTML JE2, LAC*, and JE2 backgrounds. Furthermore, growth in low pH can be restored on TSA pH 4.5 plates and in TSB pH 4.5 medium by the addition of *0846* in a separate location on the chromosome in the *geh* locus and expressed under its native promoter. Therefore, *0846* represents a novel gene not previously associated with the ability of *S. aureus* to grow at low pH.

Chapter 6 : Determining the function of 0846 and the reason for its importance in the acid stress response of *Staphylococcus aureus*

The genetic complementation approach confirmed that the reason for the reduced growth of the *0846::Tn* strain at low pH is due to the inactivation of the *0846* gene. However, despite the gene having been shown to encode for a virulence factor in several previous studies (Benton et al., 2004; Grosser et al., 2018; Valentino et al., 2014), no study has previously associated *0846* with the acidic stress response of *S. aureus*. Furthermore, the cellular function of 0846 is unknown, and therefore the gene represents a potential novel pathway in the acidic stress response of *S. aureus*.

An analysis of the literature revealed that *0846* is part of the two gene *0846-0847* operon. The function of *0847* is also unknown, although the encoded protein shows homology to a thioesterase protein. On the other hand, an analysis of the amino acid sequence of the 0846 protein sequence revealed homology to two different types of proteins. Firstly, the N- and C-terminal parts of 0846 display homology to Na_H antiport_NhaC-like domains. Secondly, the whole of the 0846 protein has been labelled as a YuiF-type amino acid transporter. Furthermore, the amino acid sequence is predicted to contain 12 transmembrane alpha helices (Fig 6.1).

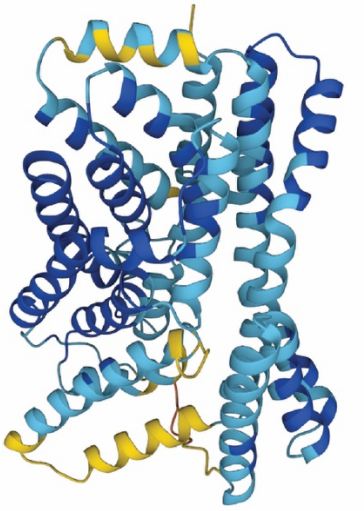


Figure 6.1: *0846* is predicted to encode for a protein with 12 transmembrane helices
Structural prediction of 0846 based on its sequence as determined by AlphaFold (Jumper et al., 2021; Varadi et al., 2022). The colours represent the per-residue confidence metric (pLDDT) as determined by the model, where residues shown in dark blue have a very high confidence metric (pLDDT > 90), residues shown in light blue has a high confidence metric (pLDDT 70 – 90), residues shown in yellow have a low confidence metric (PLDDT 50 – 70), and residues shown in orange have a very low confidence metric (PLDDT < 50).

To explore why 0846 is important for the acidic stress response of *S. aureus*, its potential function was next investigated.

6.1 0846 is the only potential CPA transporter which is important for the acidic stress response of *S. aureus*

A literature and bioinformatics analysis revealed a potential function for 0846 as a cation:proton antiporter (CPA) belonging to the NhaC-type family of transporters. CPAs have a well-established function during growth under alkaline stress conditions in *S. aureus* (reviewed in Krulwich et al., 2009; Vaish et al., 2019; Vaish et al., 2018). Under alkaline conditions and / or high salt conditions, CPAs transport protons into the cell in exchange for sodium or potassium to maintain the intracellular pH and counter salt stress.

Besides 0846, *S. aureus* has 7 additional potential CPAs; two Cpa3-type Mnh transporters, two Cpa1 family transporters, one Cpa2 type transporter, and two additional NhaC family transporters (reviewed in Krulwich et al., 2009; Vaish et al., 2019; Vaish et al., 2018) (Fig. 6.2).

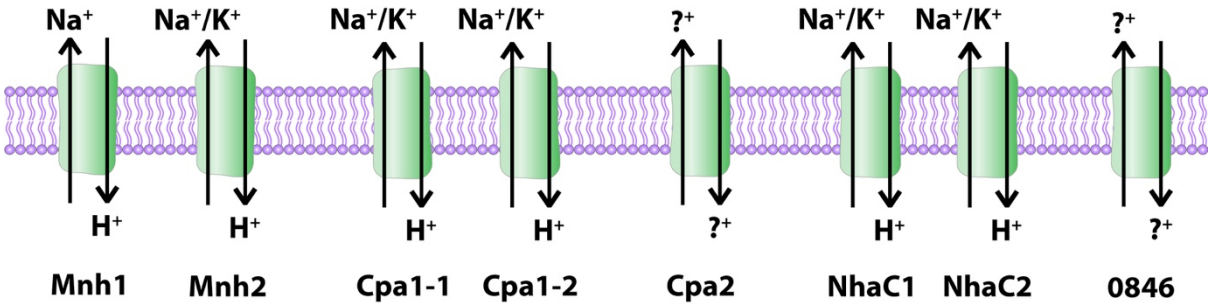


Figure 6.2: Schematic representation of the potential CPA transporters coded for in *S. aureus*
The genome of *S. aureus* is predicted to encode for two Mnh-type transporters, two Cpa1-type transporters, one Cpa2 transporter, and two NhaC-type transporters. Figure adapted from Vaish et al., (2019). The transport of cations is shown as determined by Vaish et al., (2019).

In the LAC, LAC*, and JE2 backgrounds, the Mnh2 transporter is assumed to be non-functional due to a deletion in the *mnh2* operon (Vaish et al., 2018). In a previous study, it was shown that *mnhA1* and *cpa1-1* expression increases at pH 6.0, although the expression of *0846* did not increase at low pH (Vaish et al., 2019). Furthermore, several genes in the *mnh1* operon

were identified as essential for the growth of *S. aureus* under low pH in our Tn-Seq experiments in either one or both Tn libraries (Table 4.2, 5.2). Therefore, I hypothesized that as well as being important for growth under alkaline stress conditions, 0846 and CPAs generally could also potentially be important for growth under acidic stress conditions.

6.1.1 Analysis of the importance of CPA transporters for growth under acid stress

To explore whether CPA activity is required for the acidic stress response of *S. aureus*, the importance of the other potential CPA transporters in *S. aureus* for growth at low pH was investigated. To this end, strains of *S. aureus* with Tn insertions in genes coding for the other potential CPA transporters were obtained from the NTML collection, and the correct Tn insertion confirmed by PCR and sequencing of the Tn-genome junction site. In addition to JE2 *0846::Tn*, five mutant strains were explored. These were NTML JE2 strains *cpa1-1::Tn*, *cpa1-2::Tn*, *cpa2::Tn*, *nhaC1::Tn*, and *nhaC2::Tn*. The *mnh1* transporter was not assayed as there was no corresponding mutant strain available in the NTML or other mutant library.

Firstly, the WT and mutant strains were spotted on TSA pH 7.3 plates to see if any of the mutants displayed a growth defect at neutral pH. All of the strains grew to the 10^{-6} or 10^{-7} dilution, and none showed a growth defect (Fig. 6.3A,B). The NTML JE2 *cpa2::Tn* strain showed an increased staphyloxanthin production as evidenced by the yellow colour of the colonies (Fig. 6.3A). As this was the only strain which showed increased staphyloxanthin production, it indicates that inactivating *cpa2* confers a greater level of oxidative stress than the other potential CPA transporters.

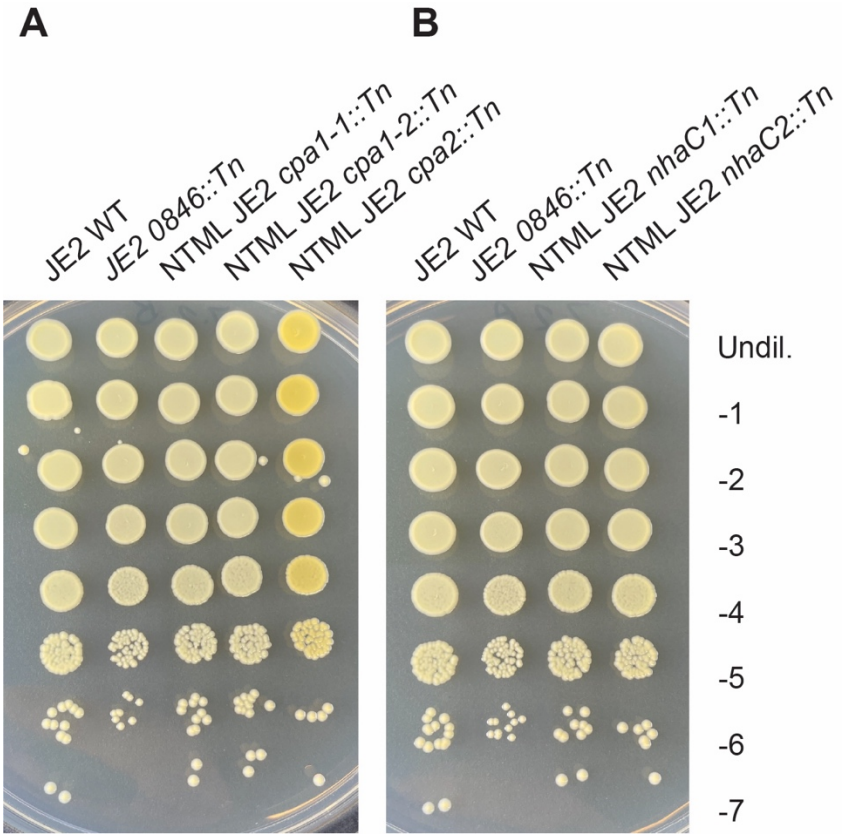


Figure 6.3: Inactivation of individual CPA transporters does not result in a growth defect at neutral pH growth conditions
Overnight cultures of the (A) JE2 WT, JE2 *0846::Tn*, NTML JE2 *cpa1-1::Tn*, NTML JE2 *cpa1-2::Tn*, NTML JE2 *cpa2::Tn*, and (B) JE2 WT, JE2 *0846::Tn*, NTML JE2 *nhaC1::Tn*, and NTML JE2 *nhaC2::Tn* strains were serially diluted and aliquots spotted onto TSA pH 7.3 plates. Images were taken following 24 h incubation at 37 °C. Each image is a representative of three independent experiments. This figure has been adapted from Beetham et al., (2024).

Next, the strains were spotted on TSA pH 4.5 plates. As has been shown earlier, the JE2 *0846::Tn* strain showed a growth defect at low pH, with a 100,000 fold decrease in CFUs compared to the JE2 WT strain (Fig. 6.4). Furthermore, the NTML JE2 *cpa2::Tn* strain showed a slight growth defect compared to the JE2 WT strain (Fig. 6.4A). While both the JE2 WT and NTML JE2 *cpa2::Tn* strains grew to the -7 dilution, the colonies of the NTML JE2 *cpa2::Tn* bacteria were noticeably smaller than those of the JE2 WT bacteria. Overall, however, *0846* is unique among the potential CPA transporters in resulting in a growth defect on TSA pH 4.5 plates when inactivated.

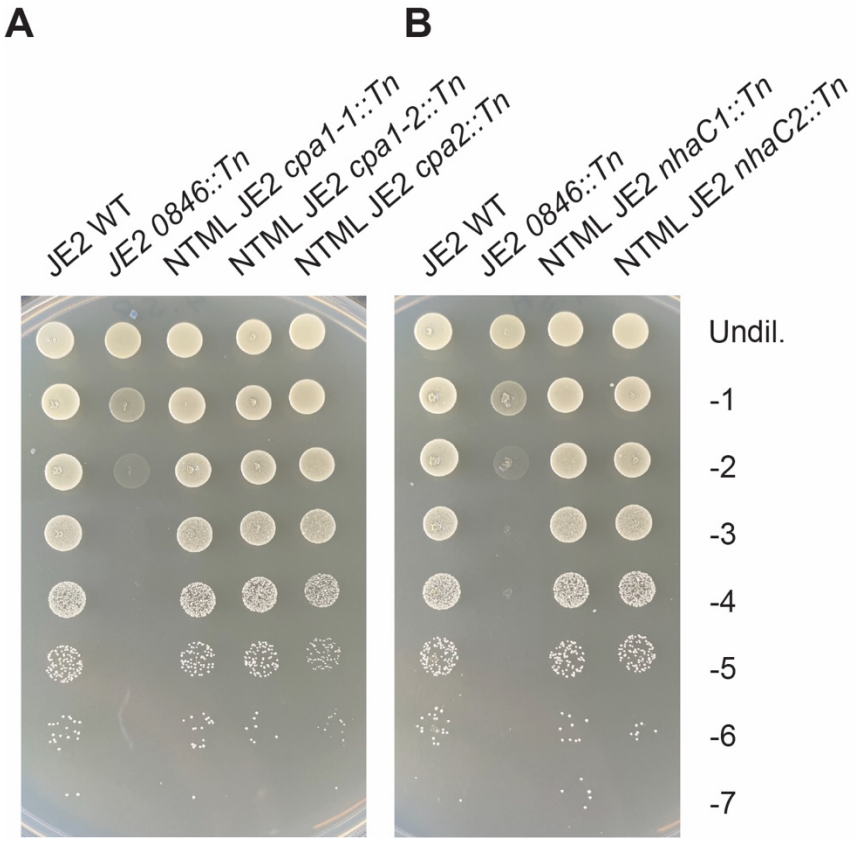


Figure 6.4: Only 0846 but not the other predicted CPA transporters is required for growth at low pH

Overnight cultures of the (A) JE2 WT, JE2 *0846::Tn*, NTML JE2 *cpa1-1::Tn*, NTML JE2 *cpa1-2::Tn*, NTML JE2 *cpa2::Tn*, and (B) JE2 WT, JE2 *0846::Tn*, NTML JE2 *nhaC1::Tn*, and NTML JE2 *nhaC2::Tn* strains were serially diluted and aliquots spotted onto TSA pH 4.5 plates. Images were taken following 24 h incubation at 37 °C. Each image is a representative of three independent experiments. This figure has been adapted from Beetham et al., (2024).

6.1.2 Analysis of the importance of CPA transporters for acid survival

Previously, it has been shown that CPA transporters have CPA activity at different pH conditions. For example, in *S. aureus* Cpa1-1 has been shown to have greater activity at pH 7.0 and 7.5, while Cpa1-2 was shown to have greater activity at pH 8.5 (Vaish et al., 2019). Therefore, I hypothesized that 0846 is important for the acidic stress response of *S. aureus* at pH 4.5, but that the other potential CPA transporters may play a role at other acidic pH conditions.

To explore whether the other potential CPA transporters are important for the acidic stress response of *S. aureus* at a higher level of acidic stress, the survival of the mutant strains in TSB pH 2.5 medium was assayed. None of the strains showed a difference in survival compared to

the JE2 WT, with all strains showing an approx. 4-5 log decrease in CFU ml^{-1} at 8 h compared to 0 h (Fig. 6.5).

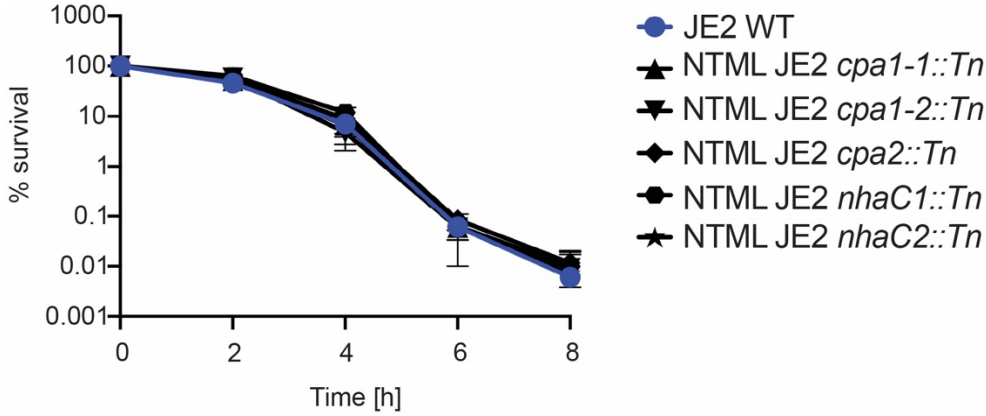


Figure 6.5: Survival analysis of mutant strains with Tn insertions in genes encoding CPA transporters
Overnight cultures of JE2 WT, NTML JE2 *cpa1-1::Tn,* NTML JE2 *cpa1-2::Tn,* NTML JE2 *cpa2::Tn,* NTML JE2 *nhaC1::Tn,* and NTML JE2 *nhaC2::Tn* strains were washed and diluted into TSB pH 2.5 medium. Immediately afterwards (T = 0 h) and at 2 h intervals up to 8 h aliquots were removed and CFUs determined by plating dilutions onto TSA plates. The CFU count at T = 0 h was set to 100% for each strain and % survival at the subsequent time points calculated. The average and standard deviations of the % survival from three independent experiments were plotted.

Taken together, these data show that 0846 is unique among the potential CPA transporters in being important for the acidic stress response of *S. aureus.*

6.2 Inactivating 0846 does not affect the membrane potential of *S. aureus* at neutral or low pH

6.2.1 Measurement of the membrane potential at neutral pH

Previously, 0846 has been shown to export potassium in exchange for proton import (Vaish et al., 2019). However, this was shown in an *in vitro* everted membrane vesicle antiport assay, and it is unknown whether 0846 has potassium export activity *in vivo*. Potassium is one of the most abundant intracellular cations, and is involved in a range of key functions in the bacterial cell (reviewed in Epstein, 2003). As described in the Introduction Chapter, potassium transport has been linked to the acidic stress responses of a range of bacteria including *S. aureus*

(reviewed in Booth, 1985). For example, it has been shown that *S. aureus* is unable to maintain a neutral cytoplasmic pH in extracellular acidic pH conditions when there is no potassium in the medium (Gries et al., 2016). I therefore hypothesized that 0846 is important for the acidic stress response of *S. aureus* due to its potassium transport activity.

To investigate whether 0846 has potassium transport activity *in vivo*, measurements were made of the membrane potential of the LAC* WT and *0846::Tn* bacteria. Previously, it has been shown that a strain of *S. aureus* with an inactivated KtrA potassium transporter has a hyperpolarised membrane (Gries et al., 2013). The membrane potential is the distribution of charge across the membrane, with bacteria mainly maintaining a negative inside, positive outside charge gradient (Fig. 6.6). This charge gradient is the sum of all cations, anions, and 'captive charges' across the membrane. Disrupting this flow of ions, for example by inactivating a potassium transporter, could potentially affect this charge gradient.

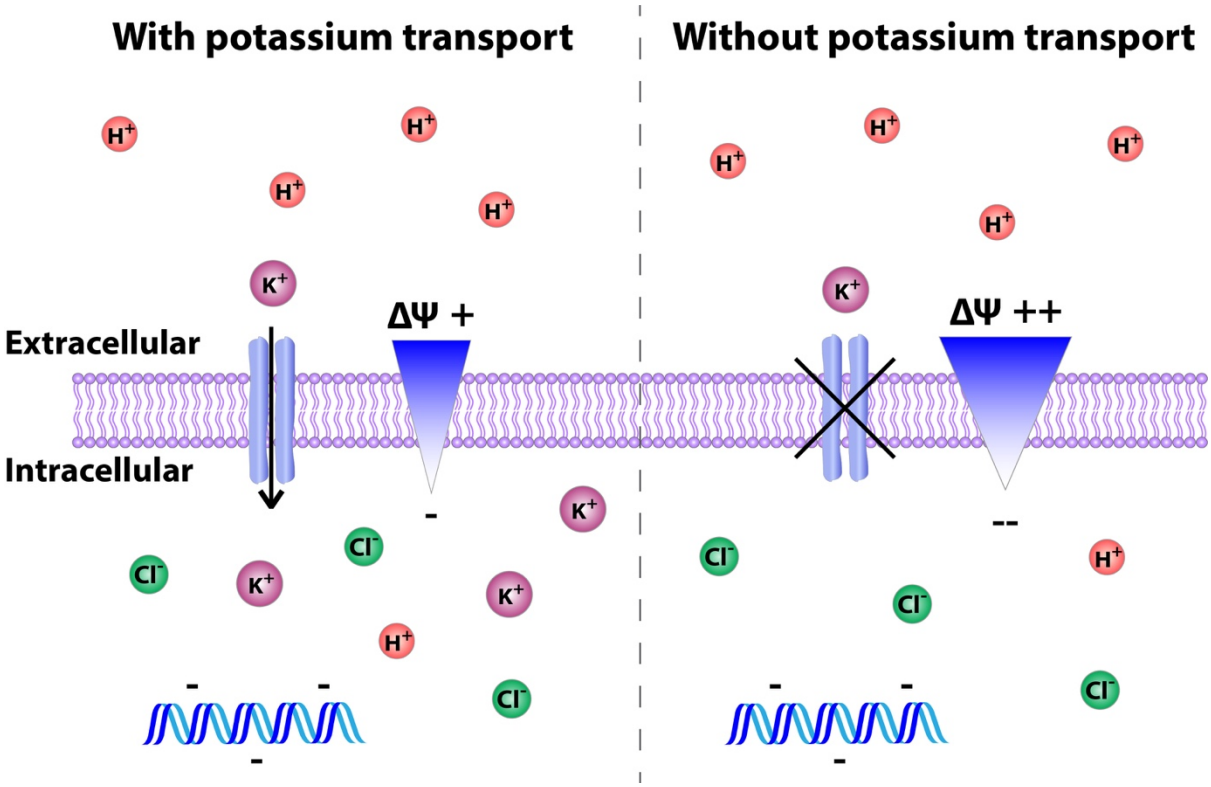


Figure 6.6: Schematic representation of how potassium transport affects the membrane potential
Bacteria maintain a inside negative ΔΨ, which is the sum of all charges across the membrane. This includes cations such as K^+, anions such as Cl^-, and 'captive charges' such as DNA. If the bacteria have a defect in potassium import, the membrane becomes hyperpolarised.

To explore whether inactivating *0846* disrupts the membrane potential of *S. aureus*, measurements of the membrane potential were performed using the fluorescent membrane potential indicator dye 3, 3'-diethyloxacarbocyanine iodide ($DiOC_2(3)$) and a fluorescence-activated cell sorting (FACS) method (Shapiro, 2008; Zeden et al., 2018). $DiOC_2(3)$ emits both green and red fluorescence, where green corresponds to cell size, and red corresponds to both cell size and membrane potential. The resulting red / green fluorescence ratio gives a value of membrane potential independent of cell size. As a control, membrane potential was also measured after the addition of *carbonyl*-cyanide m-chlorophenyl hydrazone (CCCP), a protonophore which depolarizes the membrane potential.

The LAC* WT bacteria showed a red / green fluorescence ratio of 5.4, which is consistent with previous studies which have used the $DiOC_2(3)$ and a FACS-based method for measuring the membrane potential of *S. aureus*, thus demonstrating the validity of the assay (Zeden et al., 2018) (Fig. 6.7A,B). As expected, the addition of CCCP results in a depolarised membrane, with the LAC* WT + CCCP having a red / green fluorescence of 0.6. The LAC* *0846::Tn* bacteria had an identical red / green fluorescence ratio as the LAC* WT bacteria both with and without CCCP. More specifically, the LAC* *0846::Tn* bacteria had a red / green fluorescence of 5.4, and the addition of CCCP depolarised the membrane to result in a ratio of 0.6. Therefore, the LAC* *0846::Tn* bacteria do not have a disrupted membrane potential compared to the LAC* WT bacteria after growth in neutral pH conditions.

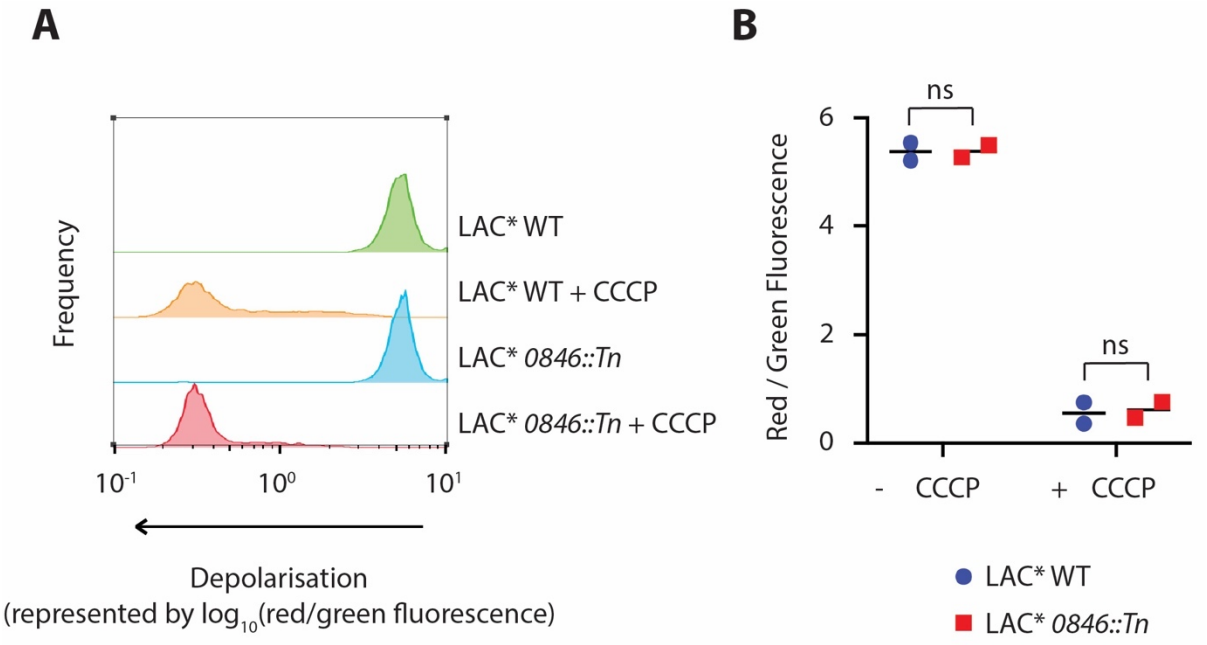


Figure 6.7: Inactivating *0846* does not affect the membrane potential of *S. aureus* at neutral pH

Overnight cultures of LAC* WT and LAC* *0846::Tn* strains were washed 3 x in staining buffer before $DiOC_2(3)$ was added. The red and green fluorescence of 10,000 events was measured using a FACS Calibur cytometer, and the derived log_{10} (red/green fluorescence) was calculated using the FlowJo V10.8 software. A separate set of samples were treated with CCCP. (A) Histogram showing the cell count versus red/green fluorescence. The histogram is representative of two independent experiments. (B) The mean values of the red/green fluorescence as determined in (A) from two independent experiments are plotted. Lack of statistical significance is shown by ns ($P>0.05$) performed by multiple unpaired t-test corrected for multiple-comparisons using the Holm-Sidak method.

6.2.2 Measurement of the membrane potential in a range of pH buffers

Several studies have demonstrated that the membrane potential of bacteria becomes dissipated under acidic conditions (reviewed in Krulwich et al., 2011). It has been hypothesized that this reduces the PMF when the ΔpH is increased, thus reducing the influx of protons and reducing the energy required to expel protons (Bakker & Mangerich, 1981; Booth, 1985) (Fig. 6.8). In some bacteria, it has been shown that ΔΨ dissipation occurs due to potassium influx as an alternative cation during low pH (Tokuda et al., 1981).

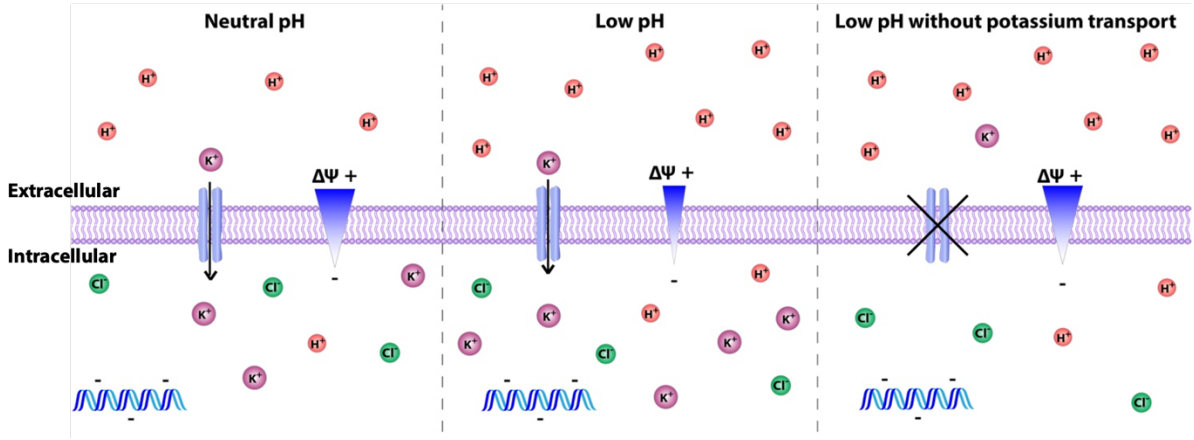


Figure 6.8: Schematic representation of how potassium and potassium transport affects the membrane potential at low pH
At neutral pH, bacteria maintain a ΔΨ which is inside negative. At low pH, the ΔΨ is reduced to reduce the PMF causing proton influx, and reduce the energy required to pump protons out of the cell. I hypothesize that when potassium transporters are inactive, the ΔΨ remains high at low pH due the lack of potassium influx.

I therefore hypothesized that, while disrupting *0846* had no effect on membrane potential at neutral pH, it might affect membrane potential at low pH.

To explore whether *0846* has a role in regulating the membrane potential of *S. aureus* at low pH, measurements of the membrane potential were performed in buffers at pH values ranging from 4.5 to 9.0. Due to the increased number of samples, the measurements were made using the same $DiOC_2(3)$ but using a plate-reader based method as described previously with modifications (Grosser et al., 2018; Hall et al., 2019). As seen for the FACS-based method, the LAC* WT and *0846::Tn* bacteria showed no significant difference in the ratio when suspended in pH 7.2 buffer (Fig. 6.9). Furthermore, when the LAC* WT bacteria were suspended in a buffer with a pH of 4.5, they had a similar membrane potential as when depolarised by the addition of CCCP. When suspended in an alkaline buffer with a pH of 9.0, the LAC* WT bacteria showed a hyperpolarised membrane. This shows that the membrane of *S. aureus* becomes hypopolarised at low pH and hyperpolarised at high pH.

Similar to the LAC* WT bacteria, the LAC* *0846::Tn* bacteria showed a decreased membrane potential when suspended in buffer at low pH, and a hyperpolarised membrane when suspended in buffer at alkaline pH. This shows that at neutral, acidic, and alkaline pH conditions, the inactivation of *0846* does not affect the membrane potential of the bacteria.

Therefore, the reason for the importance of *0846* for the acidic stress response is likely not due to potassium transport and regulation of the membrane potential.

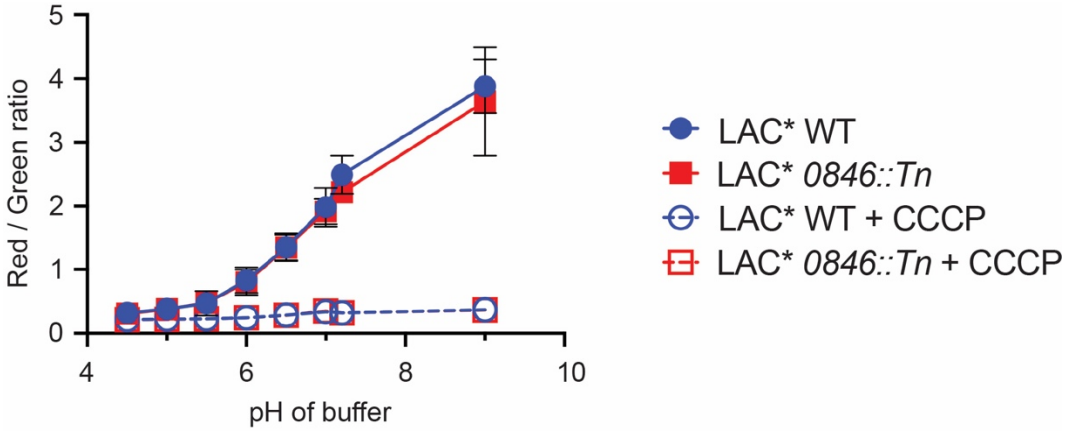


Figure 6.9: Inactivating *0846* does not lead to changes in the membrane potential of *S. aureus* when suspended in buffer with a range of different pHs
Cultures of LAC* WT and LAC* *0846::Tn* grown to mid-log phase were washed 2 x in staining buffer pH 7.2 before $DiOC_2(3)$ was added. Following this, the bacteria were resuspended in staining buffer at either pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.2, or 9.0 (the pH of the buffer was adjusted by addition of NaOH or HCl). Red and green fluorescence was measured using a TECAN plate-reader. A separate set of samples were depolarised using CCCP. The average values and standard deviations of three independent experiments were plotted. This figure has been adapted from Beetham et al., (2024).

To conclude, these data show that the reason for the importance of *0846* for the growth of *S. aureus* at low pH is not due to a potential role in regulating the membrane potential via potassium transport activity as a CPA transporter.

6.3 Inactivating 0846 results in a reduced ability to take up radioactively labelled histidine

6.3.1 Overview of bioinformatics studies

As well as being annotated as a CPA transporter, 0846 has also been annotated as a potential YuiF-type histidine permease. This prediction comes from several bioinformatics studies analysing the *S. aureus* genome. In these studies known *S. aureus* operons were identified, and the upstream sequence scanned for a potential transcription factor binding site. The entire genome was then scanned to find other regions where the predicted transcription

factor binding site occurs to explore other operons or genes potentially regulated by the same transcription factor (Ashniev et al., 2022; Ravcheev et al., 2011).

One of the operons investigated was the histidine biosynthesis operon, and the study identified a potential transcription factor binding site sequence upstream of this operon. A similar sequence was also identified upstream of several other genes in the staphylococcal genome (Fig 6.10). Firstly, a similar sequence was identified upstream of a gene now named *hisR*, which is predicted to code for the HisR transcription factor which regulates transcription of the histidine biosynthesis operon. Secondly, the consensus binding site sequence was identified upstream of *SAUSA300_0432*, a gene encoding a predicted sodium-dependent transporter. Finally, a similar binding sequence was identified upstream of the *0846-0847* operon. This suggests that the expression of *0846* is regulated by the same transcription factor which regulates the histidine biosynthesis operon.

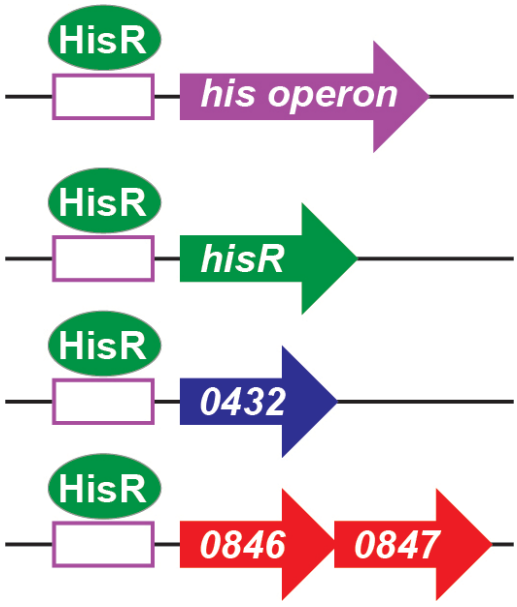


Figure 6.10: Schematic representation of the predicted HisR transcription factor binding sequence throughout the staphylococcal genome
The predicted HisR transcription factor binding site was found upstream of the his biosynthesis operon, *hisR*, *SAUSA300_0432*, and *SAUSA300_0846-SAUSA300_0847*. This figure has been reproduced from Beetham et al., (2024).

6.3.2 Measurement of the uptake of radioactively labelled histidine

To explore whether 0846 is a histidine transporter in *S. aureus*, histidine uptake assays were performed. To this end, the intracellular accumulation of radiolabelled histidine was measured in the LAC* WT (EV), LAC* *0846::Tn* (EV), and LAC* *0846::Tn* (comp.) strains.

The LAC* WT (EV) accumulated radiolabelled histidine intracellularly to a final CPM / OD_{600} value of 8.7 x 10^5 ± 5.3 x 10^4 within 9 minutes (Fig. 6.11). However, the LAC* *0846::Tn* (EV) strain showed a severe defect in histidine uptake and a CPM / OD_{600} value of only 2 x 10^3 ± 4.2 x 10^2 was obtained after 9 min, an approx. 400-fold decrease. Histidine uptake was restored in the LAC* *0846::Tn* (comp.) strain, and a final CPM / OD_{600} value of 8.9 x 10^5 ± 6.0 x 10^4 was obtained for this strain. These data show that 0846 is the main histidine transporter in *S. aureus*.

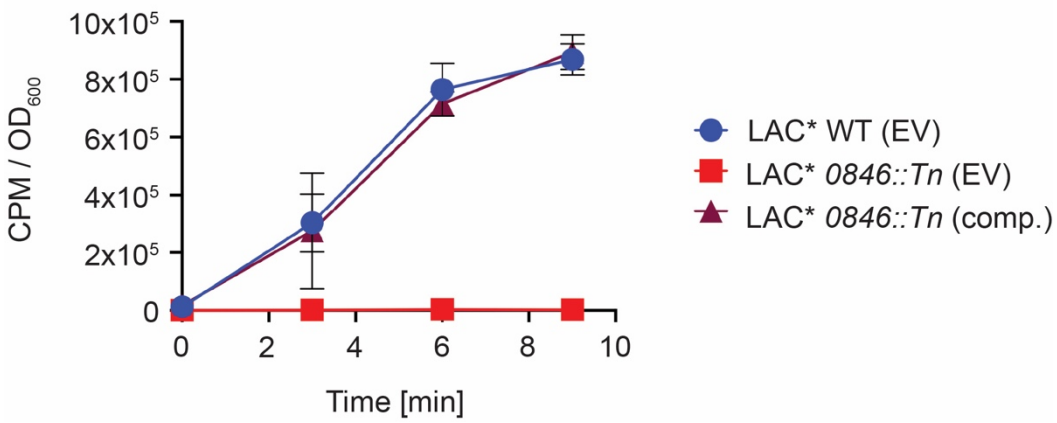


Figure 6.11: 0846 is the main histidine transporter in *S. aureus*
^{3}H radiolabelled L-histidine was added to washed mid-log phase cultures of LAC* WT (EV), LAC* *0846::Tn* (EV), and LAC* *0846::Tn* (comp.). At the indicated time points, culture aliquots were removed, filtered, washed and the accumulated radioactivity in each sample measured as counts per minute (CPM) using a scintillation counter. The CPM values were normalised to OD_{600} values, and the average CPM / OD_{600} and standard deviations of three independent experiments were plotted. This figure has been adapted from Beetham et al., (2024).

6.4 The acid sensitivity of the *0846::Tn* mutant strain can be bypassed by inducing the stringent response

6.4.1 Generation of suppressor strains

To investigate why *0846* is important for the acidic stress response of *S. aureus*, suppressor strains were generated which had improved growth at low pH. To this end, the LAC* and NTML JE2 *0846::Tn* strains were spotted on TSA pH 4.4 or pH 4.5 plates. While most of the bacteria were unable to grow on TSA pH 4.4 or pH 4.5 plates, some colonies were present. When these bacteria were isolated and spotted on TSA pH 4.5 plates, a much greater proportion of the bacteria could grow at the low pH than the original *0846::Tn* strain. It was assumed that the suppressor bacteria have mutations present in their genome which compensates for the acid sensitivity of the original *0846::Tn* strain.

Sixteen suppressor strains were generated across two genetic backgrounds, resulting in eight suppressor strains in the LAC* *0846::Tn* strain, and eight suppressor strains in the NTML JE2 *0846::Tn* strain. When re-spotted on TSA pH 4.5 plates, all 16 strains showed increased growth at low pH compared to the original *0846::Tn* strain (Fig. 6.12A,B). Most suppressor strains showed an approx. 1,000 to 10,000 fold increase in growth on TSA pH 4.5 plates, however three strains showed a noticeably reduced ability to grow at low pH compared to the other suppressor strains. LAC* *0846::Tn*-S1, LAC* *0846::Tn*-S4, and NTML JE2 *0846::Tn*-S15 showed only a 100 to 1,000 fold increase in growth compared to the *0846::Tn* bacteria at low pH.

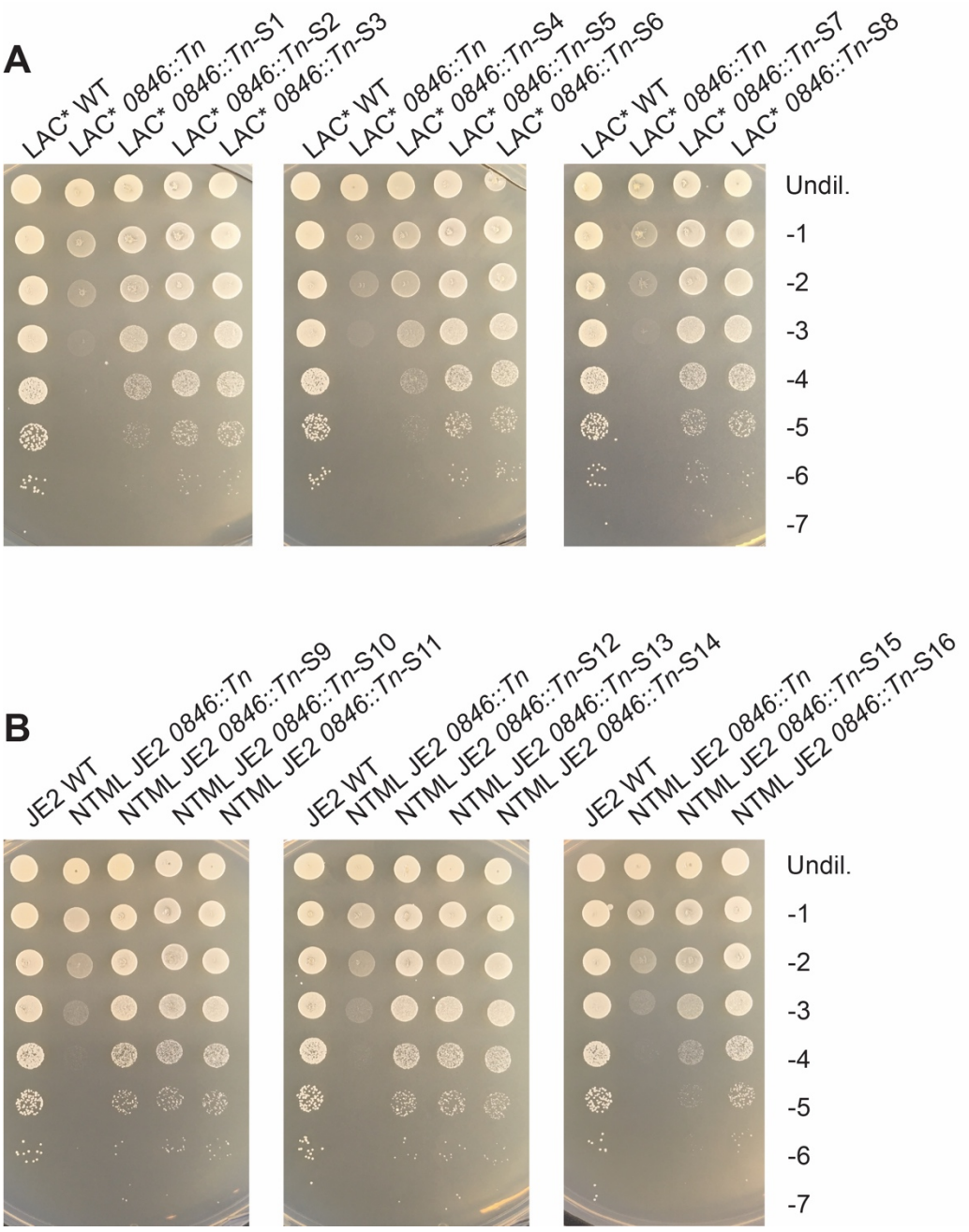


Figure 6.12: Suppressor strains show increased growth at low pH compared to the original *0846::Tn* strains

Overnight cultures of (A) LAC* WT, LAC* *0846::Tn*, LAC* *0846:Tn*-S1, LAC* *0846::Tn*-S2, LAC* *0846::Tn*-S3, LAC* *0846::Tn*-S4, LAC* *0846::Tn*-S5, LAC* *0846::Tn*-S6, LAC* *0846::Tn*-S7, and LAC* *0846::Tn*-S8, and (B) JE2 WT, NTML JE2 *0846::Tn*, NTML JE2 *0846:Tn*-S9, NTML JE2 *0846::Tn*-S10, NTML JE2 *0846::Tn*-S11, NTML JE2 *0846::Tn*-S12, NTML JE2 *0846::Tn*-S13, NTML JE2 *0846::Tn*-S14, NTML JE2 *0846::Tn*-S15, and NTML JE2 *0846::Tn*-S16 strains were serially diluted and aliquots spotted onto TSA pH 4.5 plates. Images were taken following 18 h incubation at 37 °C. Each image is a representative of one experiment. Some of the data in this figure has been adapted from Beetham et al., (2024).

6.4.2 Identification of mutations in the suppressor strains by whole genome sequencing

Following the generation and confirmation of the suppressor strains, the mutations responsible for the increased growth at low pH were identified. To this end, the genome sequence of the original *0846::Tn* mutant strains and suppressor strains was determined. All 18 strains generated sequences with good sequence quality and coverage. The CLC Genomics Workbench programme was used to compare the sequences of the suppressor strains to their respective *0846::Tn* strains.

In all 16 suppressor strains, mutations were identified that were not present in the original *0846::Tn* strain (Table 6.1, 6.2). Most of the suppressor strains generated in the LAC* background had a single SNP with a frequency of 100% (Table 6.1). Six of these SNPs occurred within the *codY* gene, and the remaining two had SNPs in the *rsh* gene. Interestingly, the strains with SNPs present in *rsh* are LAC* *0846::Tn*-S1 and LAC* *0846::Tn*-S4, both of which showed reduced growth on TSA pH 4.5 plates compared to the remaining six suppressor strains which had a SNP in the *codY* gene (Fig. 6.12). One suppressor strain, LAC* *0846::Tn*-S3, had a second SNP in the *SAUSA300_1684* gene as well as a SNP in *codY*. However, the SNP in *SAUSA300_1684* occurred in only 70.97% of reads, and may therefore have only been detected due to a relaxed cut off that was set to 70%, and would not have been detected using a more stringent cut off in frequency.

Most of the suppressor strains in the NTML JE2 background had more than one mutation (Table 6.2). For example, NTML JE2 *0846::Tn*-S11 contained six SNPs when compared to the original NTML JE2 *0846::Tn* strain. All mutations identified in the NTML JE2 *0846::Tn* suppressor strains occurred with a frequency of over 90%. Despite the additional mutations, 7 of the NTML JE2 suppressor strains also had mutations in either the *codY* or *rsh* gene, which is consistent with what I observed for the LAC* suppressor strains. Interestingly, the one suppressor strain with a SNP in *rsh*, NTML JE2 *0846::Tn*-S15, was again one of the three strains which showed reduced growth when spotted on TSA pH 4.5 plates (Fig 6.12). Taken together with the data from the LAC* suppressor strains, it is clear that a SNP in *rsh* was less effective at compensating for the acid sensitivity of the original *0846::Tn* strain than a SNP in *codY*.

One NTML JE2 suppressor strain, NTML JE2 *0846::Tn*-S13, did not have a mutation in either *codY* or *rsh*, but instead had a single SNP in the *clpY* gene which is directly upstream of *codY*.

Furthermore, while all the mutations in the LAC* suppressor strains were SNPs, two of the NTML JE2 suppressor strains contained deletions. NTML JE2 *0846::Tn*-S10 contained a 48 base pair deletion as well as a SNP in the *asp23* gene. Additionally, NTML JE2 *0846::Tn*-S14 contained a single adenine base pair deletion, as well as a SNP in the *secF* gene, both with a frequency of 100%.

Taken together, it is clear that all 16 suppressor strains have mutations present in one of three genes: *codY*, *rsh*, or *clpY*. These three genes code for proteins which interplay with each other through the bacterial stringent response, which is described below.

Table 6.1: Genomic alterations identified in the LAC* *0846::Tn* suppressor strain
This table has been adapted from Beetham et al., (2024).

Strain number and name	Gene[1]	Mutation[2]	Amino acid change[3]	Frequency[4]
LAC* *0846::Tn*-S1	*rsh*	SNP C-A	D615Y	100
LAC* *0846::Tn*-S2	*codY*	SNP G-T	E163*	100
LAC* *0846::Tn*-S3	*codY*	SNP G-T	E163*	100
	SAUSA300_1684	SNP T-C		70.97
LAC* *0846::Tn*-S4	*rsh*	SNP G-T	N53K	100
LAC* *0846::Tn*-S5	*codY*	SNP C-T	L194F	100
LAC* *0846::Tn*-S6	*codY*	SNP G-C	G46A	100
LAC* *0846::Tn*-S7	*codY*	SNP C-A	R156S	100
LAC* *0846::Tn*-S8	*codY*	SNP G-A	G46D	100

[1]Annotated gene where the mutation occurs. [2]Type of mutation with SNP indicating a single nucleotide polymorphism, and DEL indicating a deletion. Base change for SNP indicated by base in reference genome – base in the same position in the suppressor strain. [3]Amino acid change indicates where applicable the initial amino acid, position of amino acid in the encoded protein, and the resulting amino acid following the mutation. AA* denotes an early stop codon. [4]Frequency indicates the frequency that the mutation was found in the suppressor strain in the genome sequence analysis.

Table 6.2: Genomic alterations identified in the NTML JE2 *0846::Tn* suppressor strains

Strain number and name	Gene[1]	Mutation[2]	Amino acid change[3]	Frequency[4]
NTML JE2 *0846::Tn*-S9	*codY*	SNP T-C	V216A	100
NTML JE2 *0846::Tn*-S10	*asp23*	SNP T-C	K41E	98.36
	codY	DEL 48 bp	ΔR167-S182	
NTML JE2 *0846::Tn*-S11		SNP A-G		100
	rrsA	SNP G-T		90
	pabA	SNP T-G	S88A	100
	codY	SNP G-T	G50*	100
		SNP C-T		100
	opuCD	SNP C-G		100
NTML JE2 *0846::Tn*-S12	*codY*	SNP T-A	F24Y	100
	SAUSA300_1327	SNP C-G	A1544P	100
NTML JE2 *0846::Tn*-S13	*clpY*	SNP G-T	A464S	100
NTML JE2 *0846::Tn*-S14	*codY*	DEL A	G118fs	100
	secF	SNP C-T	A61T	100
NTML JE2 *0846::Tn*-S15		SNP G-C		100
	sucA	SNP G-T	Q632K	100
		SNP C-A		100
	rsh	SNP C-G	S409T	100
NTML JE2 *0846::Tn*-S16	*rnhB*	SNP A-T	E64V	100
	codY	SNP A-T	K205I	100

[1]Annotated gene where the mutation occurs. [2]Type of mutation with SNP indicating a single nucleotide polymorphism, and DEL indicating a deletion. Base change for SNP indicated by base in reference genome – base in the same position in the suppressor strain. [3]Amino acid change indicates where applicable the initial amino acid, position of amino acid in the encoded protein, and the resulting amino acid following the mutation. AA* denotes an early stop codon. Fs denotes a frameshift. [4]Frequency indicates the frequency that the mutation was found in the suppressor strain in the genome sequence analysis.

6.4.3 Overview of the stringent response in *S. aureus*

The stringent response is a widely conserved bacterial response to a range of stresses including nutrient and amino acid limitation. It is characterised by the synthesis of guanine pentaphosphate (pppGpp) and guanine tetraphosphase (ppGpp) from guanosine

triphosphate (GTP) and guanosine diphosphate (GDP), respectively. pppGpp and ppGpp are collectively known as (p)ppGpp.

In *S. aureus*, (p)ppGpp is synthesised from GTP / GDP by the Rsh, RelP, and RelQ synthetases (Geiger et al., 2010; Geiger et al., 2014; Gratani et al., 2018; Steinchen et al., 2018; Yang et al., 2019). RelP and RelQ are approximately 200 amino acids in length and belong to the Small Alarmone Synthetase family. These contain only a (p)ppGpp synthetase domain (SYN). On the other hand, Rsh is approximately 700 amino acids in length, and as well as containing the SYN domain also contains a (p)ppGpp hydrolase domain (HYD). Rsh belongs to the RelA/SpoT Homologue (RSH) family (Potrykus & Cashel, 2008), and unlike *relP* and *relQ*, *rsh* is essential in *S. aureus* due to its (p)ppGpp hydrolase activity (Geiger et al., 2010; Gentry et al., 2000). Under conditions of plentiful nutrients and amino acids, Rsh exists in a SYN off / HYD on conformation, resulting in the hydrolysis of (p)ppGpp to GDP / GTP (Gratani et al., 2018) (Fig. 6.13). Under conditions of limited nutrients and amino acids, Rsh exists in a SYN on / HYD off conformation, resulting in net (p)ppGpp synthesis and inducing the stringent response (Gratani et al., 2018).

(p)ppGpp can affect change in the bacterial cell through a range of mechanisms (reviewed in Irving et al., 2021; reviewed in Kanjee et al., 2012), such as through directly binding to and affecting the function of a range of target molecules (reviewed in Kanjee et al., 2012). For example in *E. coli*, (p)ppGpp binds to Ribonucleic acid (RNA) Polymerase at two different binding sites to either induce or inhibit transcription of genes (Ross et al., 2016; Ross et al., 2013). (p)ppGpp can also prevent the formation of mature ribosomes through binding to and inhibiting GTPases (Rojas et al., 1984). Furthermore, (p)ppGpp can also inhibit DNA replication by binding to and inhibiting various enzymes involved in the process, such as the DNA primase DnaG (Maciag et al., 2010).

In Firmicutes, the effects of the stringent response and (p)ppGpp synthesis are often coordinated through a concurrent reduction in cytosolic concentrations of GTP, and the downstream effects on the global gene regulator CodY (Fig. 6.13) (Bittner et al., 2014; reviewed in Geiger & Wolz, 2014; Lopez et al., 1981; Ratnayake-Lecamwasam et al., 2001). This drop in GTP levels can either occur through its use as a reactant for the synthesis of (p)ppGpp, or through (p)ppGpp mediated inhibition of GTP biosynthesis (Gallant et al., 1971;

Lopez et al., 1981; reviewed in Wolz et al., 2010). In *S. aureus*, CodY mostly acts as an inhibitor, where it binds to a DNA binding sequence upstream of target genes and prevents the binding of RNA polymerase, thus preventing gene transcription (reviewed in Geiger & Wolz, 2014). The CodY regulon in *S. aureus* is well described and includes genes coding for proteins with functions ranging from those involved in metabolic pathways to virulence factors (Gao et al., 2023; Majerczyk et al., 2010). The binding of CodY to DNA is facilitated by two activating ligands: GTP and branched chain amino acids (BCAA) (reviewed in Sonenshein, 2005). When CodY is bound by these two activating ligands, its conformation changes to allow it to bind DNA and thus repress gene expression (Hainzl et al., 2023; Levdikov et al., 2009). In the absence of these ligands, CodY can no longer bind DNA, RNA polymerase can now bind to the promoter region, thus leading to transcription of the downstream gene(s) (reviewed in Geiger & Wolz, 2014).

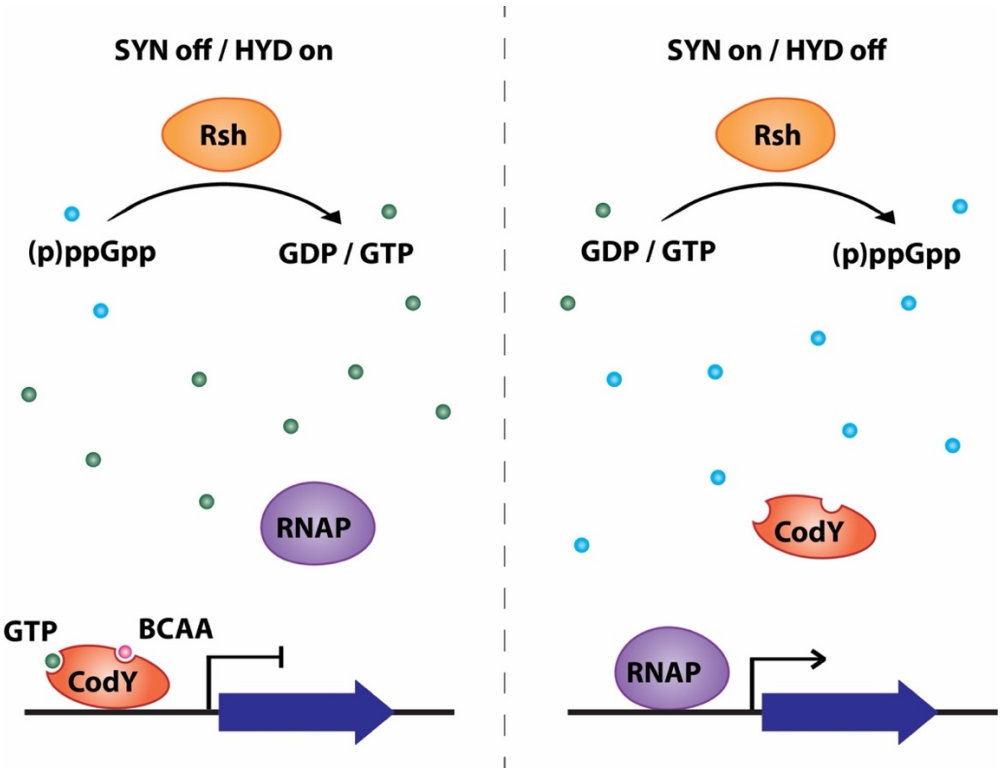


Figure 6.13: Schematic representation of the stringent response in *S. aureus*, and its co-ordination through the CodY global transcription factor
Under conditions with enough nutrients and amino acids, Rsh exists in a SYN off / HYD on conformation, resulting in the hydrolysis of (p)ppGpp to GDP / GTP. CodY binds upstream of genes when bound to GTP and BCAA, preventing the binding of RNA Polymerase and gene transcription. In conditions of limited nutrients and amino acids, Rsh exists in a SYN on / HYD off conformation, resulting in net (p)ppGpp synthesis and reduced cytosolic GTP concentrations. When not bound to GTP and BCAA, CodY no longer binds DNA. This means that RNA Polymerase can bind and initiate gene transcription. This figure has been adapted from Beetham et al., (2024).

6.4.4 Analysis of the mutations in *codY* and *clpY*

Of the 16 suppressor strains, 12 had mutations in *codY* (Table 6.1, 6.2). These were LAC* *0846::Tn*-S2, LAC* *0846::Tn*-S3, LAC* *0846::Tn*-S5, LAC* *0846::Tn*-S6, LAC* *0846::Tn*-S7, LAC* *0846::Tn*-S8, NTML JE2 *0846::Tn*-S9, NTML JE2 *0846::Tn*-S10, NTML JE2 *0846::Tn*-S11, NTML JE2 *0846::Tn*-S12, NTML JE2 *0846::Tn*-S14, and NTML JE2 *0846::Tn*-S16. Two of these suppressor strains contained deletion mutations. The remaining 10 suppressor strains contained SNPs, of which 3 resulted in early stop codons, and 7 resulted in a single amino acid change. The predicted effect of all 12 mutations are discussed below.

The CodY homodimer contains 257 amino acids and consists of two main domains connected by a linker helix (residues 137-178) (Han et al., 2016) (Fig. 6.14). The N-terminal part of the protein (Levdikov et al., 2006) consists of a GAF domain (residues 1-155) named after the enzymes it is commonly found in: cGMP-specific phosphodiesterases, adenylyl cylases, and Fh1A proteins. This domain is where the BCAA and GTP binding sites are located. The C-terminal part of the protein consists of the DNA binding domain from residues 168-257 (Levdikov et al., 2006). The binding of GTP and BCAA to the GAF domain is proposed to reorientate the linker helix, which induces conformational changes in the DNA-binding domain that permits DNA binding (Hainzl et al., 2023; Levdikov et al., 2009). An analysis of the mutations in *codY* indicate that their likely impact is the inactivation of CodY. The effect of the mutations on CodY are grouped into three groups. Firstly some mutations likely result in large structural changes to the CodY protein, such as early stop codons, frameshift mutations, or large deletions. These mutations are shown in pink in Fig. 6.14. Secondly, some mutations likely affect the ability of CodY to bind GTP. These mutations are shown in green in Fig. 6.14. Finally, some mutations likely affect the ability of CodY to bind DNA. These mutations are shown in blue in Fig. 6.14.

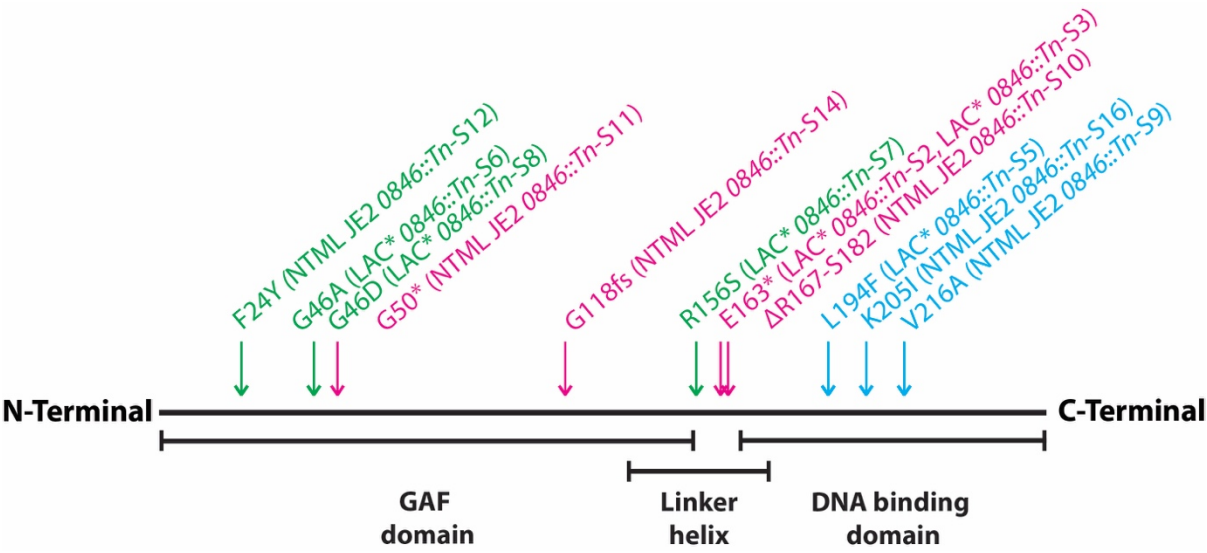


Figure 6.14: Schematic representation of the structure of the CodY amino acid sequence, and the location of mutations in the suppressor strains
CodY contains a GAF domain, linker helix, and DNA binding domain. The arrows represent the location of amino acid changes in CodY observed in the different suppressor strains. The exact amino acid change and suppressor strain in which this change is present is shown above. Amino acid changes likely affecting GTP binding are shown in green. Amino acid changes likely resulting in large structural changes such as early stop codons, frameshift mutations, or large amino acid deletions are shown in pink. Amino acid changes likely affecting DNA binding are shown in blue. Diagram drawn to scale.

Five suppressor strains contained mutations resulting in large structural changes to CodY. These are strains LAC* *0846::Tn*-S2, LAC* *0846::Tn*-S3, NTML JE2 *0846::Tn*-S10, NTML JE2 *0846::Tn*-S11, and NTML JE2 *0846::Tn*-S14 (Table 6.1, 6.2, Fig. 6.14). Strain NTML JE2 *0846::Tn*-S10 contained a 48 base pair deletion, resulting in the deletion of amino acid residues 167 to 182. These residues occur at the end of the linker helix domain and the beginning of the DNA binding domain, and their deletion likely affects the structure of the whole CodY protein (Fig. 6.14). The second suppressor strain containing a deletion, NTML JE2 *0846::Tn*-S14, had a single nucleotide deletion resulting in a frameshift from residue 118 (Fig. 6.14). As this frameshift occurs approx. halfway through the protein sequence, it is likely that it results in a different protein sequence in the C-terminal or even a premature stop codon. Finally, three suppressor strains contained mutations resulting in early stop codons. Both LAC* *0846::Tn*-S2 and LAC* *0846::Tn*-S3 contain the same G-T SNP, resulting in an early stop codon at residue 163 (Fig. 6.14). NTML JE2 *0846::Tn*-S11 contained a G-T SNP resulting in an early stop codon at residue 50 (Fig. 6.14). These mutations therefore result in a truncated version of CodY.

An analysis of the SNPs in the remaining 7 suppressor strains with mutations in *codY* also indicates that their effect is likely to inactivate CodY. Three suppressor strains, LAC* *0846::Tn*-S5, NTML JE2 *0846::Tn*-S9, and NTML JE2 *0846::Tn*-S16, had SNPs resulting in amino acid changes in the DNA binding domain (Table 6.1, 6.2, Fig. 6.14). LAC* *0846::Tn*-S5 had a SNP resulting in a change from leucine to phenylalanine at residue 194. L194 in CodY is highly conserved among bacteria (Fig. 6.15), although this residue does not interact directly with DNA (Hainzl et al., 2023). Secondly, NTML JE2 *0846::Tn*-S9 had a SNP resulting in a change from valine to alanine at residue 216. V216 in CodY is also highly conserved across bacterial species (Fig. 6.15), and the side chain of this valine interacts directly with the DNA by hydrophobic contact (Hainzl et al., 2023). Furthermore, the neighbouring residue 215 is a serine which forms hydrogen bonds with the DNA (Hainzl et al., 2023), and it is possible that a V216A change affects the binding of S215 to the DNA. Finally, NTML JE2 *0846::Tn*-S16 has a SNP resulting in an amino acid change from lysine to isoleucine at residue 205. K205 is less conserved among bacterial species, but the neighbouring residues 203 and 204 are highly conserved and form hydrogen bonds with DNA (Fig. 6.15) (Hainzl et al., 2023). It is possible, therefore, that a K205I change affects the binding of residues 203 and 204 to DNA.

Figure 6.15: Multiple alignment of CodY amino acid sequencies from selected bacterial species

CodY amino acid sequences from *S. aureus* (ABD22108.1), *B. subtilis* (SPY12644.1), *L. monocytogenes* (NP_464805.1), *L. lactis* (WP_003129722.1), *S. pneumoniae* (WP_000940733.1), and *E. coli* (OWC12939.1) were retrieved and aligned using the NCBI COBALT constraint based Multiple Alignment Tool. Amino acids shown in red are conserved among all sequences shown. Asterisks denote amino acids changed due to SNPs in the suppressor strains.

The four remaining suppressor strains with mutations in *codY* had SNPs resulting in amino acid changes in the GTP-binding site (Table 6.1, 6.2, Fig. 6.14). Firstly, NTML JE2 *0846::Tn*-S12 contained a SNP resulting in an amino acid change from phenylalanine to tyrosine at residue 24. F24 interacts directly with GTP via hydrogen bonds and by stacking against the guanine base of GTP via its benzine side chain (Hainzl et al., 2023; Han et al., 2016). The importance of F24 is also highlighted by the fact that both *L. lactis* and *S. pneumoniae*, whose CodY proteins do not respond to GTP, contain a Y24 (Fig. 6.15) (Hendriksen et al., 2008; Petranovic et al., 2004). It has been suggested that a F24Y change affects the stacking of GTP, and therefore reduces the ability of CodY to bind GTP (Han et al., 2016).

Two suppressor strains, LAC* *0846::Tn*-S6 and LAC* *0846::Tn*-S8, have changes to the glycine amino acid at residue 46 (Table 6.1). The mutation in the former results in an alanine, and in the latter in an aspartic acid residue. While G46 has not been shown to interact with GTP, the neighbouring residues S43, R45, and K47 form hydrogen bonds with GTP (Han et al., 2016). Previously it has been shown that a CodY variant with residues S43, R45, K47, H70, and K158 all changed to alanine show a lower GTP-binding affinity than the WT CodY (Han et al., 2016). A G46D change may therefore destabilise these residues and reduce CodY-GTP binding.

Finally, in suppressor strain LAC* *0846::Tn*-S7 the amino acid at position 156 in the linker helix domain of CodY was changed from an arginine to a serine. While R156 does not interact directly with GTP, both the neighbouring R153 and K158 residues form hydrogen bonds with GTP (Han et al., 2016). It is possible that a R156S affects the ability of CodY to bind GTP.

Only one of the 16 suppressor strains did not have a mutation in either *codY* or *rsh*. This was NTML JE2 *0846::Tn*-S13, which had a SNP in *clpY* resulting in an amino acid change at residue 464 from alanine to serine (Table 6.2). *clpY* encodes for a a subunit of the ClpYP ATP-dependent protease. The ClpY protein is 467 residues long, and therefore the amino acid change occurs four residues from the end of the protein (Fig. 6.16). It is therefore unlikely that a single amino acid change affects the activity of the enzyme. However, the *clpY* gene is found in the four gene *xerC-clpQ-clpY-codY* polycistronic operon (Pohl et al., 2009) (Fig. 6.16). As well as a 4.1 kb transcript encompassing the whole operon, *S. aureus* also transcribes a 1.4 kb transcript encompassing *codY* and the last approximately 150bp of *clpY* (Pohl et al., 2009). As the SNP in *clpY* occurs 40 bp upstream of the *codY* start codon, it is possible that this SNP affects the transcription of *codY*.

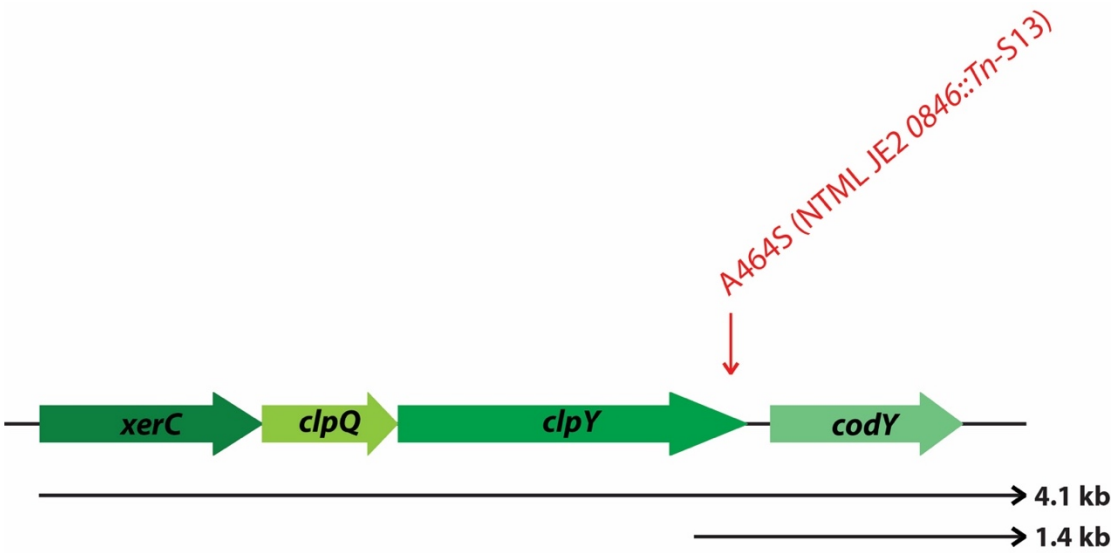


Figure 6.16: Schematic representation of the *xerC-clpQ-clpY-codY* four gene operon
The arrows represent the location of amino acid changes in ClpY observed in the suppressor strain. The exact amino acid change and suppressor strain in which this change is present is shown above. The arrows below the operon schematic represent the two potential transcripts, one 4.1 kb long and one 1.4 kb long. Figure adapted from Pohl et al., 2009 (Pohl et al., 2009). Diagram not drawn to scale.

Overall, the mutations present in the suppressor strains in *codY* and *clpY* are predicted to result in an inactivation of CodY. Five suppressor strains had mutations resulting in large structural changes to the CodY protein such as early stop codons, frameshift mutations, or large deletions. Three suppressor strains had SNPs resulting in changes to amino acid residues involved in binding DNA, likely reducing the DNA-binding ability of CodY in these strains. The remaining four suppressor strains had mutations resulting in changes to amino acids at or near residues involved in GTP binding. These likely reduce the ability of CodY to bind GTP, therefore reducing the DNA binding ability of CodY in these strains. The suppressor strain with a SNP in *clpY* is predicted to potentially have reduced transcription of *codY*. To conclude, the suppressor strains with mutations in *codY* and *clpY* likely have a less active CodY than the original *0846::Tn* strain.

6.4.5 Analysis of the mutations in *rsh*

Of the 16 suppressor strains, three had mutations in the *rsh* gene (Table 6.1, 6.2). These were LAC* *0846::Tn*-S1, LAC* *0846::Tn*-S4, and NTML JE2 *0846::Tn*-S15. All three strains had SNPs resulting in amino acid changes. The predicted effect of these three mutations are discussed below.

The Rsh enzyme is 736 amino acids in length and contains the SYN and HYD domains at the N-terminus (Fig. 6.17). The C-terminus contains three regulatory domains: TGS (named after the ThrRS, GTPase, and SpoT proteins), AH-RIS (α-helical and Ribosome-InterSubunit), and ACT (named after aspartate kinase, chorismite mutase, and TyrA proteins). The TGS domain consists of residues 400-461 and has been shown to interact with the SYN domain to maintain Rsh in a SYN off / HYD on conformation (Gratani et al., 2018; Pausch et al., 2020). The AH-RIS domain consists of residues 475-638 and the ACT domain consists of residues 657 to 736.

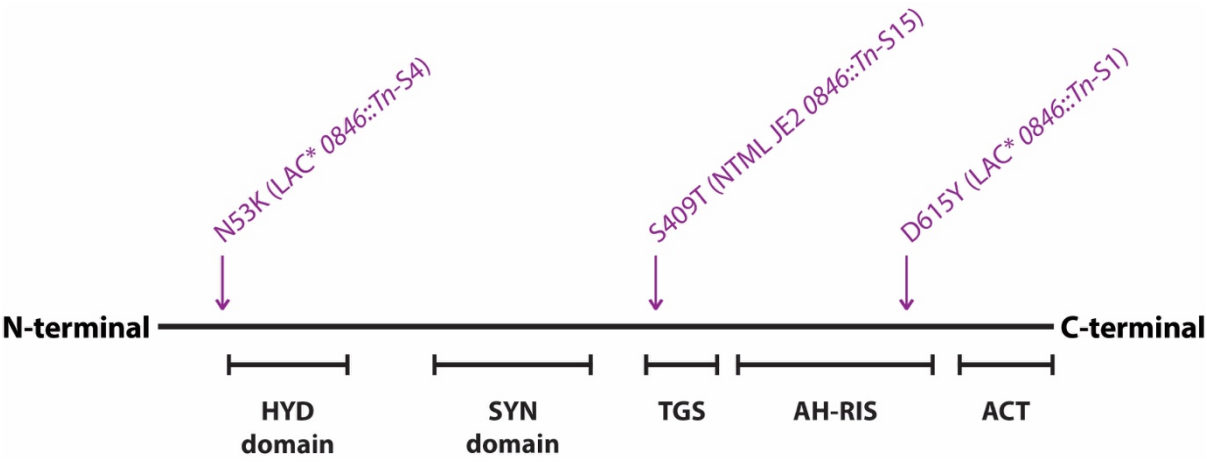


Figure 6.17: Schematic representation of the structure of the Rsh amino acid sequence and the location of mutations in the suppressor strains
Rsh contains a Hyd domain, Syn domain, TGS domain, AH-RIS domain and ACT domain. The arrows represent the location of amino acid changes in Rsh observed in the different suppressor strains. The exact amino acid change and suppressor strain in which this change is present is shown above. Diagram drawn to scale.

One of the suppressor strains, LAC* *0846::Tn*-S4, contained a SNP resulting in an amino acid change from asparagine to lysine at position 53 (Table 6.1, Fig. 6.17). This residue is just before the HYD domain of Rsh, and in *Streptococcus disgalactiae* subspecies *equilimus* it has been shown that the corresponding amino acid is crucial for maintaining the structure of the HYD active site (Hogg et al., 2004). Furthermore, when the residue was changed to a leucine or proline, the hydrolase activity of the enzyme was reduced (Hogg et al., 2004). Interestingly *S. equilimus* and several other bacterial species including *B. subtilis* contain a serine at this residue instead of an asparagine (Fig. 6.18). Both serine and asparagine are polar amino acids, which may explain why this residue can be changed in *S. aureus* Rsh but still maintain its

hydrolase activity. However, changing the amino acid to either a non-polar leucine or proline as seen in *S. equilimus*, or a positively charged lysine as seen in the suppressor strain, may result in an inability to maintain the structure of the HYD active site and therefore a reduced (p)ppGpp hydrolase activity.

Figure 6.18: Multiple alignment of Rsh amino acid sequencies from selected bacterial species

Rsh amino acid sequences from *S. aureus* (CR129444.1), *B. subtilis* (NP_390638.2), and *S. dysgalactiae* subsp. *equisimilis* (CAA51353.1) were retrieved and aligned using the NCBI COBALT constraint based Multiple Alignment Tool. Amino acids shown in red are conserved among all sequences shown. Asterisks denote amino acids changed due to SNPs in the suppressor strains.

The remaining two suppressor strains have SNP mutations resulting in amino acid changes in the CTD. Strain NTML JE2 *0846::Tn*-S15 had a SNP resulting in a change of amino acid residue 409 from a serine to a threonine within the TGS motif (Table 6.2, Fig. 6.17). In *B. subtilis*, where the structure of Rsh and the TGS domains have been solved, it has been shown that the corresponding residue is located in the interface where the TGS domain interacts with the SYN domain to maintain Rsh in a SYN off / HYD on conformation (Pausch et al., 2020). Therefore, a change in amino acid at this residue might reduce the ability of the TGS domain to bind and inhibit the SYN domain. It is difficult to directly compare the *B. subtilis* and *S. aureus* proteins since the *B. subtilis* protein contains a glycine at this residue, while in *S. aureus* it is a serine (Fig. 6.18). However, it is possible that a change at this residue would still increase the (p)ppGpp synthesis and reduce the (p)ppGpp hydrolase activity of Rsh.

The final suppressor strain, LAC* *0846::Tn*-S1, had a SNP resulting in a change in the amino acid at position 615 from aspartic acid to tyrosine (Table 6.1). D615 occurs within the RIS subdomain, and is highly conserved among bacteria (Fig. 6.17, 6.18). In *B. subtilis* and *E. coli*, this subdomain interacts with the 70S ribosome, which allows Rsh to detect stalled ribosomes as a proxy for low cytosolic concentrations of amino acids (Loveland et al., 2016; Pausch et al., 2020). However, in *S. aureus* it has been shown that the TGS domain is important for interacting with ribosomal proteins (Gratani et al., 2018). This might mean that the RIS subdomain does not interact with ribosomal proteins in *S. aureus*, and therefore it is therefore difficult to predict what the effect of the mutation in suppressor strain LAC* *0846::Tn*-S1 is.

Taken together, it is possible that the result of the mutations in *rsh* is to shift the enzyme into a SYN on / HYD off conformation. As discussed above, the HYD domain of Rsh has been shown to be essential for the growth of *S. aureus*, and therefore these three suppressor strains must still have a baseline (p)ppGpp hydrolase activity, but I would predict that this activity is reduced (Geiger et al., 2010; Gentry et al., 2000). Strain LAC* *0846::Tn*-S4 likely has reduced (p)ppGpp hydrolase activity, and strain NTML JE2 *0846::Tn*-S15 likely has increased (p)ppGpp synthesis activity. While the activity of Rsh in strain LAC* *0846::Tn*-S1 is difficult to predict, it is likely that this strain also has increased (p)ppGpp synthesis and reduced hydrolase activity to give the same phenotype as as observed in the other suppressor strains.

6.4.6 Analysis of the other mutations observed in the suppressor strains

There were 13 other SNP mutations present in the suppressor strains, of which 9 occurred within a gene. Out of these 9 mutations, three were silent. LAC* *0846::Tn*-S3 contained a silent SNP in *SAUSA300_1684*, and NTML JE2 *0846::Tn*-S11 contained a silent SNP in *rrsA* and *opuCD*. Strain NTML JE2 *0846::Tn*-S11 also contained a missense mutation in *pabA* resulting in a S88A change. Other genes containing missense mutations included *asp23* in strain NTML JE2 *0846::Tn*-S10 resulting in a K41E change, *SAUSA300_1327* in strain NTML JE2 *0846::Tn*-S12 resulting in a A1544P change, *secF* in strain NTML JE2 *0846::Tn*-S14 resulting in a A61T change, *sucA* in strain NTML JE2 *0846::Tn*-S15 resulting in a Q632K change, and *rnhB* in strain NTML JE2 *0846::Tn*-S16 resulting in a E64V change.

However, it is noticeable that all but one of these additional SNPs occurred in the NTML JE2 background, while only one occurred in the LAC* background. Furthermore, the genes which contain additional mutations do not appear to code for proteins with related functions or shared pathways. Therefore, I hypothesized that these additional mutations do not act to compensate for the acid sensitivity due to the Tn insertion in the *0846* gene, and these additional SNPs will not be discussed further.

6.5 Inducing the stringent response does not confer a growth advantage at low pH independent of 0846

Analysing the mutations present in the suppressor strains revealed that all 16 strains across two different genetic backgrounds had one mutation whose effect could potentially induce the stringent response. As a consequence of the activation of the stringent response, the suppressor *0846::Tn* strains show reduced acid-sensitivity on TSA pH 4.5 plates. However, it is not known whether the activation of the stringent response is a specific response to the *0846::Tn* strain, and thus provides information on why 0846 is important for the acidic stress response of *S. aureus*, or whether activating the stringent response is a general response that is induced to counter acid stress in *S. aureus*.

6.5.1 Exploring the role of the stringent response for growth in low pH medium

To explore whether inducing the stringent response is a general response to acidic stress in *S. aureus,* the ability of the WT strain to grow on TSA pH 4.5 plates was compared to mutant

strains of *S. aureus* with Tn insertions in either *codY, rsh*, or *clpY*. This was compared to the growth of selected suppressor strains and the NTML JE2 *0846::Tn* strain as a control. As the *codY::Tn*, *rsh::Tn*, and *clpY::Tn* strains were derived from the NTML library in the JE2 background, suppressor strains NTML JE2 *0846::Tn*-S10, NTML JE2 *0846::Tn*-S13, and NTML JE2 *0846::Tn*-S15 were chosen for comparison. NTML JE2 *0846::Tn*-S10 contained a 48 bp deletion in *codY*, and will henceforth be named NTML JE2 *0846::Tn codY*$_{\text{DELΔR167-S182}}$. NTML JE2 *0846::Tn*-S13 contained a SNP in *clpY*, and will henceforth be named NTML JE2 *0846::Tn clpY*$_{\text{A464S}}$. NTML JE2 *0846::Tn*-S5 contained a SNP in *rsh*, and will henceforth be named NTML JE2 *0846::Tn rsh*$_{\text{S409T}}$.

Firstly, the WT, mutants with Tn insertions in *0846*, *codY*, *rsh*, *clpY*, and selected suppressor strains were spotted on TSA pH 7.3 plates. None of the strains showed a growth defect at neutral conditions compared to the JE2 WT bacteria (Fig. 6.19A,B). The NTML JE2 *rsh::Tn* bacteria showed an increased staphyloxanthin production as evidenced by the increased yellow colour of the colonies (Fig. 6.19B). This was mirrored by the increased yellow colour of the colonies of the NTML JE2 *0846::Tn rsh*$_{\text{S409T}}$ suppressor strain (Fig. 6.19A). The Tn insertion in NTML JE2 *rsh::Tn* occurs within the AH-RIS domain of *rsh,* and therefore could disrupt the regulatory function of the CTD (Fig. 6.17). As discussed previously in this Chapter, the HYD domain in Rsh is essential in *S. aureus* (Geiger et al., 2010; Gentry et al., 2000). Therefore, the insertion of a Tn in the AH-RIS domain must not fully inactivate the (p)ppGpp hydrolase activity of Rsh. However, the Tn insertion does confer additional oxidative stress onto the bacteria as evidenced by the increased staphyloxanthin production. Despite this, the increased oxidative stress was not sufficient to confer a growth defect, as the *rsh::Tn* strain grew to the same dilution as the JE2 WT strain on TSA pH 7.3 plates.

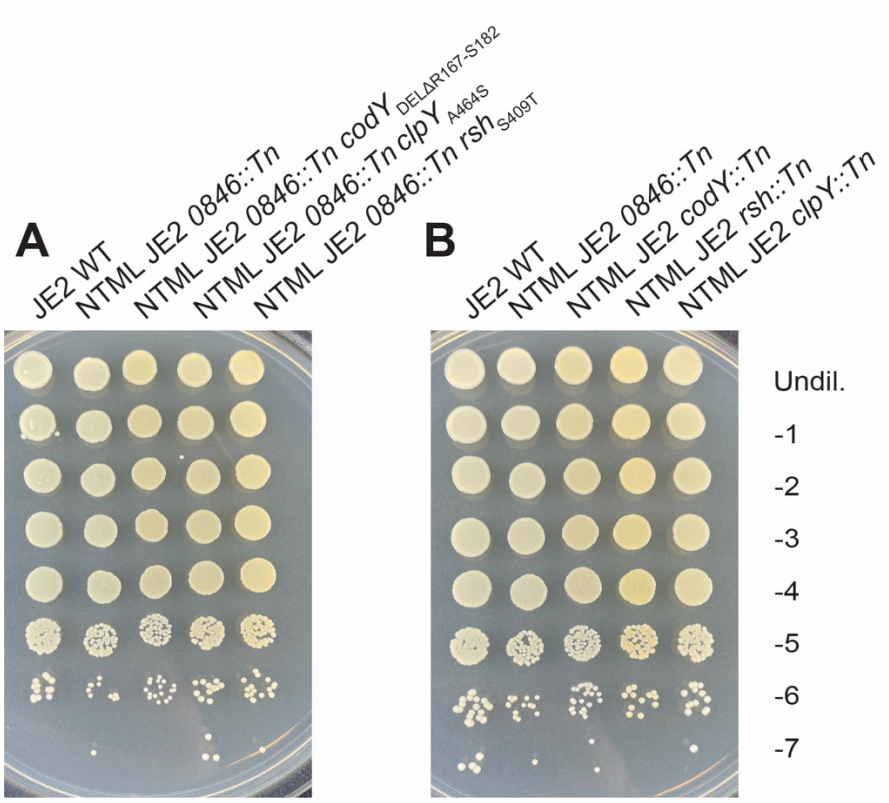


Figure 6.19: Activating the stringent response does not result in a growth defect at neutral pH

Overnight cultures of (A) JE2 WT, NTML JE2 *0846::Tn*, NTML JE2 *0846::Tn codY*$_{DELΔR167\text{-}S182}$, NTML JE2 *0846::Tn clpY*$_{A464S}$, and NTML JE2 *0846::Tn rsh*$_{S409T}$, and (B) JE2 WT, NTML JE2 *0846::Tn*, NTML JE2 *codY::Tn*, NTML JE2 *rsh::Tn* and NTML JE2 *clpY::Tn* strains were serially diluted and aliquots spotted onto TSA pH 7.3 plates. Images were taken following 18 h incubation at 37 °C. Each image is a representative of three independent experiments.

Next the strains were spotted onto TSA pH 4.5 plates. As expected, the NTML JE2 *0846::Tn* strain showed a 10,000-100,000 fold decreased growth compared to the JE2 WT strain (Fig. 6.20A,B). Furthermore, all three suppressor strains showed increased growth compared to the NTML JE2 *0846::Tn* strain (Fig. 6.20A). As described earlier, the NTML JE2 *0846::Tn rsh*$_{S409T}$ strain showed reduced growth on TSA pH 4.5 plates compared to the other suppressor strains. The NTML JE2 *0846::Tn clpY*$_{A464S}$ strain showed the greatest increase in growth compared to the original NTML JE2 *0846::Tn* strain of the three suppressor strains.

However, it was clear that inactivating *codY* and *rsh* did not provide a benefit to *S. aureus*, as the NTML JE2 *codY::Tn* and *rsh::Tn* strains showed reduced growth on TSA pH 4.5 plates compared to the JE2 WT (Fig. 6.20B). The NTML JE2 *clpY::Tn* strain showed no growth defect at low pH. This may be because the Tn insertion, which occurs around residue 920 of *clpY*,

does not disrupt the second *codY* promoter which is located at the end of *clpY*. Therefore using a NTML JE2 *clpY::Tn* strain is likely not an effective method of investigating the role of the stringent response in the acidic stress response of *S. aureus*.

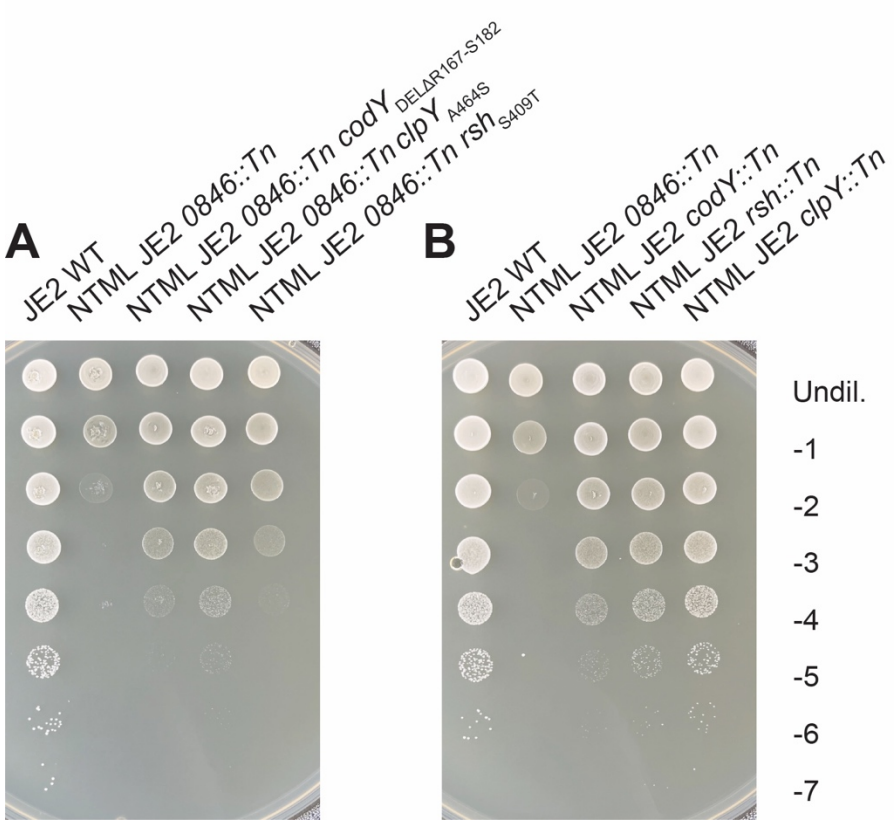


Figure 6.20: Activating the stringent response results in a growth defect at low pH
Overnight cultures of (A) JE2 WT, NTML JE2 *0846::Tn*, NTML JE2 *0846::Tn codY*$_{DEL\Delta R167\text{-}S182}$, NTML JE2 *0846::Tn clpY*$_{A464S}$, and NTML JE2 *0846::Tn rsh*$_{S409T}$, and (B) JE2 WT, NTML JE2 *0846::Tn*, NTML JE2 *codY::Tn*, NTML JE2 *rsh::Tn* and NTML JE2 *clpY::Tn* strains were serially diluted and aliquots spotted onto TSA pH 4.5 plates. Images were taken following 18 h incubation at 37 °C. Each image is a representative of three independent experiments.

To further confirm that inactivating *codY* does not confer a growth advantage at acidic pH, the Tn insertions in the NTML JE2 *codY::Tn* and *rsh::Tn* strains were transferred to a LAC* background strain by phage transduction. As CodY and Rsh affect the transcription of a wide range of genes, it is possible that the NTML JE2 *codY::Tn* and *rsh::Tn* mutants contain additional mutations to moderate gene expression when *codY* and *rsh* are inactivated. Transferring the Tn to a clean background ensures that any phenotypes are due to the presence of the Tn in the individual gene.

The growth of the LAC* WT, LAC* *codY::Tn*, and LAC* *rsh::Tn* strains were first investigated in TSB pH 7.3 medium. This was compared to the growth of the LAC* *0846::Tn* strain as a control,

as well as selected suppressor strains. Strains LAC* *0846::Tn*-S1 containing a D615Y mutation in *rsh*, LAC* *0846::Tn*-S2 containing a E163* mutation in *codY*, LAC* *0846::Tn*-S4 containing a N53K mutation in *rsh* and LAC* *0846::*Tn-S6 containing a G46A in *codY* were chosen to represent a range of different mutations. These strains will henceforth be named LAC* *0846::Tn* rsh_{D615Y}, LAC* *0846::Tn* $codY_{E163*}$, LAC* *0846::Tn*-S4 rsh_{N53K}, and LAC* *0846::Tn* $codY_{G46A}$.

As shown earlier, the LAC* *0846::Tn* strain showed no growth defect compared to the LAC* WT strain in TSB pH 7.3 medium (Fig. 6.21A,B). Furthermore, as seen on TSA plates the LAC* *rsh::Tn* strain also did not show a growth defect in neutral pH liquid medium (Fig. 6.21A). However, the LAC* *codY::Tn* strain showed a growth defect compared to the LAC* WT in TSB pH 7.3 medium, despite showing no growth defect on TSA pH 7.3 plates. This may be because the growth curve assay is more stringent and able to detect smaller differences than the plate spotting assay. Interestingly, when the growth of the suppressor strains was investigated in TSB pH 7.3 medium, both suppressor strains with mutations in *codY* also showed a growth defect compared to the LAC* WT strain (Fig. 6.21B). Strain LAC* *0846::Tn* $codY_{E163*}$ showed a greater growth defect than strain LAC* *0846::Tn* $codY_{G46A}$, which may be because the early stop codon in strain LAC* *0846::Tn* $codY_{E163*}$ confers a full inactivation of CodY, while the missense mutation in strain LAC* *0846::Tn* $codY_{G46A}$ confers only a partial inactivation of CodY. On the other hand, neither strain with a mutation in *rsh* showed a growth defect compared to the LAC* WT strain in TSB pH 7.3 medium.

As expected, the LAC* *0846::Tn* strain showed a significant growth defect when grown in TSB pH 4.5 medium compared to the LAC* WT strain (Fig. 6.21C,D). Furthermore, both the LAC* *codY::Tn* and *rsh::Tn* strains showed a slight growth defect compared to the LAC* WT strain at low pH (Fig. 6.21C). This mirrors the results shown earlier on TSA pH 4.5 plates, where both the NTML JE2 *codY::Tn* and *rsh::Tn* strains were only capable of growth to a lower dilution than the JE2 WT strain. Taken together this shows that inactivating CodY does not confer a general growth advantage to *S. aureus* under low pH conditions.

All four suppressor strains also showed increased growth in TSB pH 4.5 medium compared to the original LAC* *0846::Tn* strain (Fig. 6.21D). Furthermore, as seen on TSA pH 4.5 plates, the suppressor strains with mutations in *rsh* had reduced growth in TSB pH 4.5 medium compared

to the strains with mutations in *codY*. This further indicates that mutations in *rsh* are less able to restore growth at low pH than mutations in *codY*.

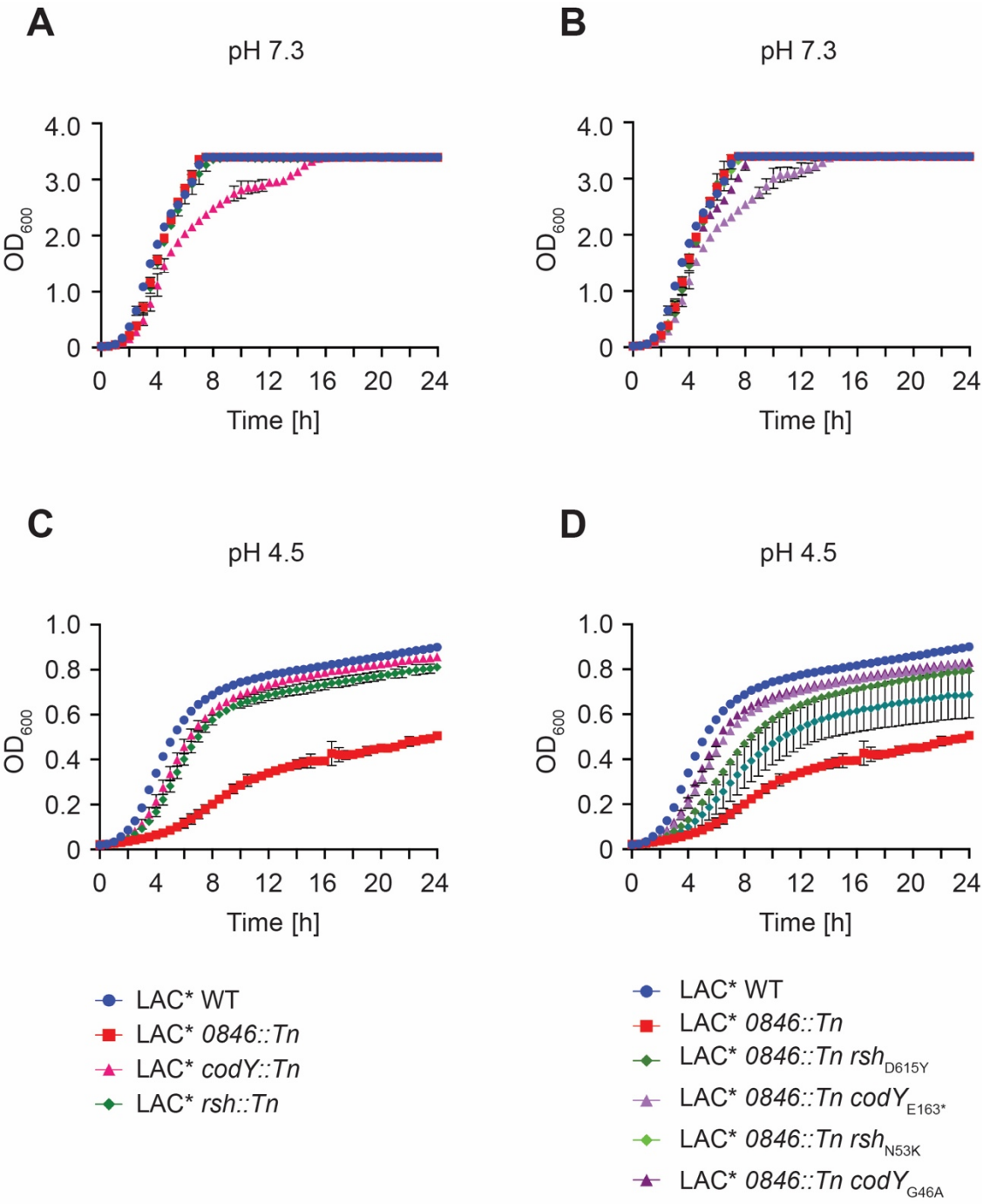


Figure 6.21: Activating the stringent response does not confer a growth advantage at low pH in liquid medium

Overnight cultures of (A,C) LAC* WT, LAC* *0846::Tn*, LAC* *codY::Tn*, and LAC* *rsh::Tn*, or (B,D) LAC* WT, LAC* *0846::Tn*, LAC* *0846::Tn rsh*$_{D615Y,}$ LAC* *0846::Tn codY*$_{E163*}$, LAC* *0846::Tn rsh*$_{N53K}$, and LAC* *0846::Tn codY*$_{G46A}$ were inoculated into (A,B) TSB pH 7.3 or (C,D) TSB pH 4.5 medium. Strains were grown in 96-well plates with OD_{600} measurements taken at the indicated time points. The average readings and standard deviations of three independent experiments were plotted. This figure has been adapted from Beetham et al., (2024).

Taken together, these data show that activating the stringent response does not confer an advantage for the growth of *S. aureus* under low pH independent of *0846*. On both TSA pH 4.5 plates and in TSB pH 4.5 medium, inactivating *codY* and *rsh* by Tn insertion resulted in decreased growth compared to the WT bacteria. This indicates that the suppressor mutations do not result in increased growth of the *0846::Tn* strain by a general acidic stress response, and instead are specific to restore the growth of the *0846::Tn* strain.

6.6 Discussion of Chapter 6

In this Chapter, the function of 0846 and the reason for its importance in the acidic stress response of *S. aureus* were explored. An analysis of the literature and bioinformatic annotation revealed two potential functions for 0846. Firstly, it has been annotated as a cation : proton antiporter with both sodium and potassium export activity. Secondly, it has been annotated as a YuiF-type amino acid permease, with putative histidine transport activity.

A previous study deposited on a preprint server demonstrated that 0846, named NhaC3 in this study, has K^+:H^+ antiport activity at alkaline pH *in vitro* (Vaish et al., 2019). Furthermore, this study showed that the expression of *0846* is increased at pH 9.0 and that removing the *0846* resulted in a slight growth defect compared to the WT bacteria when grown in TSB pH 8.5 and pH 9.5 (Vaish et al., 2019).

The data presented here in Chapter 4 and 5 of this study demonstrate that *0846* is important for growth at acidic pH as well. Despite the role of CPA transporters being well established in the growth of *S. aureus* at alkaline pH, several lines of evidence implied a role for growth at low pH as well. Firstly, a previous study has demonstrated that the expression of both *mnh1* and *cpa1-1* is increased at pH 6.0 compared to neutral pH (Vaish et al., 2019). Secondly both *0846* and *mnh1* were identified as essential for the growth of *S. aureus* at low pH in the Tn-Seq assay in Chapter 4. Taken together, this suggested a role for CPA transporters in the acidic stress response of *S. aureus*. However, when the growth of mutant strains of *S. aureus* with Tn insertions in genes encoding other CPA transporters was investigated on TSA pH 4.5 plates, none showed a growth defect. Furthermore, none of the mutant strains showed a decreased

ability to survive in TSB pH 2.5 medium. This indicates that CPAs do not play a role in the acidic stress response of *S. aureus* at high or low levels of acidic stress.

0846 has been shown to have both sodium and potassium import activity *in vitro* (Vaish et al., 2019). If 0846 functioned to export protons during growth in acidic pH medium, then this must be accompanied by potassium import to balance the corresponding charge movement. Potassium import has been shown to have a role in the acidic stress response of bacteria. During growth in acidic pH medium, the difference in pH across the bacterial membrane is greater than during growth in neutral medium. This affects the dynamics of the PMF, which has consequences for metabolic processes which use energy generated from the PMF. Therefore, to maintain PMF at a constant rate during growth in acidic pH medium, bacteria depolarise their membrane. This depolarisation has been shown to depend on potassium transport, as inactivating potassium transporters can lead to a hyperpolarised membrane (Gries et al., 2013). I hypothesized that if 0846 exports protons and imports potassium during growth in acidic pH medium, then inactivating 0846 would result in a hyperpolarised membrane at low pH. However, no difference in membrane potential was seen in the LAC* *0846::Tn* bacteria compared to the LAC* WT bacteria when suspended in buffers with a range of pHs. Taken with the data from the other potential CPA transporters, this indicates that CPA activity does not explain the importance of *0846* for the growth of *S. aureus* under low pH.

0846 has also been annotated as a YuiF-type amino acid permease with putative histidine transport activity. The *0846-0847* two gene operon has been predicted to be preceded by a potential HisR transcription factor binding sequence. The same sequence was identified upstream of the histidine biosynthesis operon, which in *L. lactis* has been shown to be upregulated in histidine limiting conditions (Delorme et al., 1999). This therefore suggests that the expression of *0846* is also changed during histidine limiting conditions. To test whether *0846* encodes a histidine transporter, I measured the uptake of radiolabelled histidine in LAC* WT (EV), the LAC**0846::Tn* (EV) and the LAC**0846::Tn* (comp.) strains. While the LAC* WT (EV) strain was able to take up histidine, uptake of this amino acid was almost completely eliminated in the LAC* *0846::Tn* (EV) mutant, and restored again to WT-levels in the complementation strain. These data show that under our assay conditions, 0846 functions as the main histidine transporter in *S. aureus*.

As far as I am aware, this is the first *in vivo* evidence of a histidine transporter in *S. aureus*. However, there are several other genes in the staphylococcal genome which have also been annotated as potential histidine transporters. These are *SAUSA300_0319* and *SAUSA300_2265*. The former has been annotated as a YjiH-type histidine transporter, and the latter has been annotated as an alanine glycine permease, but also as a potential histidine transport protein. In a previous study, it was shown that the expression of *SAUSA300_0319* increased upon CodY inactivation, indicating that *SAUSA300_0319* is involved in the response of *S. aureus* to a lack of amino acids (Waters et al., 2016). However, the almost complete lack of accumulation of radiolabelled histidine in the LAC* *0846::Tn* (EV) strain suggests that *0846* encodes the main histidine transporter in *S. aureus*.

To explore why 0846 is important for the growth of *S. aureus* under low pH, suppressor strains were selected which showed improved growth on TSA pH 4.5 plates compared to the original *0846::Tn* strain. Such strains could be readily obtained in both the LAC* and NTML JE2 genetic backgrounds, and the genomic alterations leading to the improved growth of 16 of these suppressor strains were determined by whole genome sequencing. 12 of the suppressor strains contained mutations in the *codY* gene, 3 had mutations in the *rsh* gene, and the final strain had a mutation in the *clpY* gene. Several strains contained additional mutations, however due to the lack of commonality between the genes and gene functions these additional mutations were not analysed further.

CodY is a master gene regulator and together with Rsh an important component of the stringent response system in *S. aureus.* Under nutrient limiting conditions, Rsh produces (p)ppGpp from GTP, leading to a reduction of the intracellular concentration of GTP (reviewed in Geiger & Wolz, 2014). Amongst others, this shifts CodY into a GTP free state, resulting in the transcription of genes involved in nutrient and amino acid biosynthesis. Five of the suppressor strains contained mutations in *codY* which are predicted to cause large structural changes to the protein, three contained mutations in the DNA binding domain, and four contained mutations predicted to affect GTP binding. The predicted effect of these mutations is the inactivation of CodY. Of the suppressor strains with mutations in *rsh*, one contained a mutation in the HYD domain, one contained a mutation in the TGS domain, and one contained a mutation in the AH-RIS domain. The effect these mutations have on Rsh are harder to predict but are likely to inactivate the (p)ppGpp hydrolase and activate the (p)ppGpp synthesis activity

of Rsh. The effect of the mutation in the final suppressor strain in *clpY* I predict will reduce the expression of the downstream gene *codY*. Taken together, it is clear that in order to bypass the acid sensitivity of the *0846:Tn* strain, the suppressor strains have gained mutations resulting in the activation of the stringent response.

It was unclear initially whether activating the stringent response in the suppressor strains was a specific response to bypass the lack of 0846 in the *0846::Tn* strain or whether it represents a general response to increase growth at low pH independent of 0846. The stringent response has been shown to affect the acidic stress response in a range of different bacteria. In *Helicobacter pylori*, the stringent response is activated at low pH (Wells & Gaynor, 2006), and is also required for growth at low pH (Mouery et al., 2006). On the other hand, a *S. mutans codY* mutant showed reduced growth at low pH (Lemos et al., 2008), and for *E. coli* it has been suggested that (p)ppGpp acts to limit its acidic stress response (Kanjee et al., 2011). In *S. aureus*, starvation-adapted bacteria were found to have increased survival at pH 2.0 (Watson et al., 1998). However, the reduced growth of the *codY::Tn* and *rsh::Tn* strains on TSA pH 4.5 plates and in TSB pH 4.5 medium compared to the WT strain indicates that activating the stringent response alone does not increase the ability of *S. aureus* to grow at low pH. Therefore, the beneficial impact of activating the stringent response in the suppressor strains is dependent on the inactivation of *0846*.

In *S. aureus*, the stringent response is activated in response to a lack of amino acids. This leads to the upregulation of genes involved in either amino acid transport or synthesis. For example, it has been shown that the histidine biosynthesis operon is upregulated in a *codY* mutant of *S. aureus* (Pohl et al., 2009; Waters et al., 2016). Therefore, I hypothesized that the effect of the suppressor mutations is to upregulate histidine biosynthesis, which allows the *0846::Tn* strain to compensate for the lack of histidine uptake due to inactivation of the 0846 main histidine transporter. Furthermore, the fact that histidine synthesis can compensate for a lack of histidine transport indicates that maintaining sufficient intracellular histidine concentrations is important for the ability of *S. aureus* to grow at low pH.

Overall, the data presented in this Chapter indicate that *0846* encodes for a main histidine transporter in *S. aureus*. It is likely this transporter is important for the acidic stress response of *S. aureus* due to its histidine import activity, and the suppressor strain analysis indicates

that a defect in histidine uptake can be compensated for by increasing histidine biosynthesis. This suggests that maintaining intracellular histidine concentrations is important for the ability of *S. aureus* to grow at low pH conditions.

Chapter 7 : Exploring the role of histidine in the acidic stress response of *Staphylococcus aureus*

In the previous Chapter, I showed that 0846 is the main histidine transporter in *S. aureus*. Furthermore, the suppressor strain analysis showed that the reduced growth of the *0846::Tn* strain at low pH can be compensated for by inactivating CodY and inducing the stringent response. The reduced growth of the *codY::Tn* and suppressor strains compared to the WT strains at low pH demonstrates that inducing the stringent response does not confer a general growth advantage at low pH. This suggests that activation of the stringent response specifically compensates for the histidine uptake defect observed in the *0846::Tn* mutant strain. I hypothesized that this is because inducing the stringent response activates genes and pathways which respond to a lack of amino acids, such as histidine biosynthesis. To investigate why 0846 and histidine uptake are important for the growth of *S. aureus* at low pH, the role of histidine in the acid stress response of *S. aureus* was next explored.

7.1 Histidine and its uptake via 0846 are important for the growth of *S. aureus* under acid stress conditions

7.1.1 Analysis of the importance of histidine for growth under neutral conditions

To explore whether histidine is important for the growth of *S. aureus* at neutral pH, growth curves were performed in chemically defined medium (CDM) either with or without histidine. To this end, the LAC* WT (EV), LAC* *0846::Tn* (EV) and LAC* *0846::Tn* (comp.) strains were grown overnight in TSB medium, and the following day back-diluted into CDM with or without histidine.

The LAC* WT (EV) strain resumed growth after approximately 3 h in CDM with histidine (Fig. 7.1A). However, when histidine was not present in the medium, the LAC* WT (EV) strain resumed growth following approximately 7 h. In both CDM with and without histidine, the LAC* WT (EV) bacteria exhibited a similar growth rate once log-phase growth was initiated. This suggests that *S. aureus* must adapt to histidine limiting conditions before resuming growth, however once adapted the bacteria are capable of growing as normal.

On the other hand, the LAC* *0846::Tn* (EV) bacteria resumed growth following approximately 8 h in CDM with histidine, and following 9 h in CDM without histidine (Fig. 7.1B). The complementation strain *0846::Tn* (comp.) grew very similar to the WT strain and resumed

growth following 3 h in CDM with histidine, and following 7 h in CDM without histidine (Fig. 7.1C).

When plotted on the same graph, it is clear that the LAC* *0846::Tn* (EV) strain takes longer to resume growth than the LAC* WT (EV) strain in CDM with histidine, however both strains resumed growth following a similar time in CDM without histidine (Fig. 7.1D,E). This suggests that the mutant bacteria do not respond to the presence of histidine in the medium.

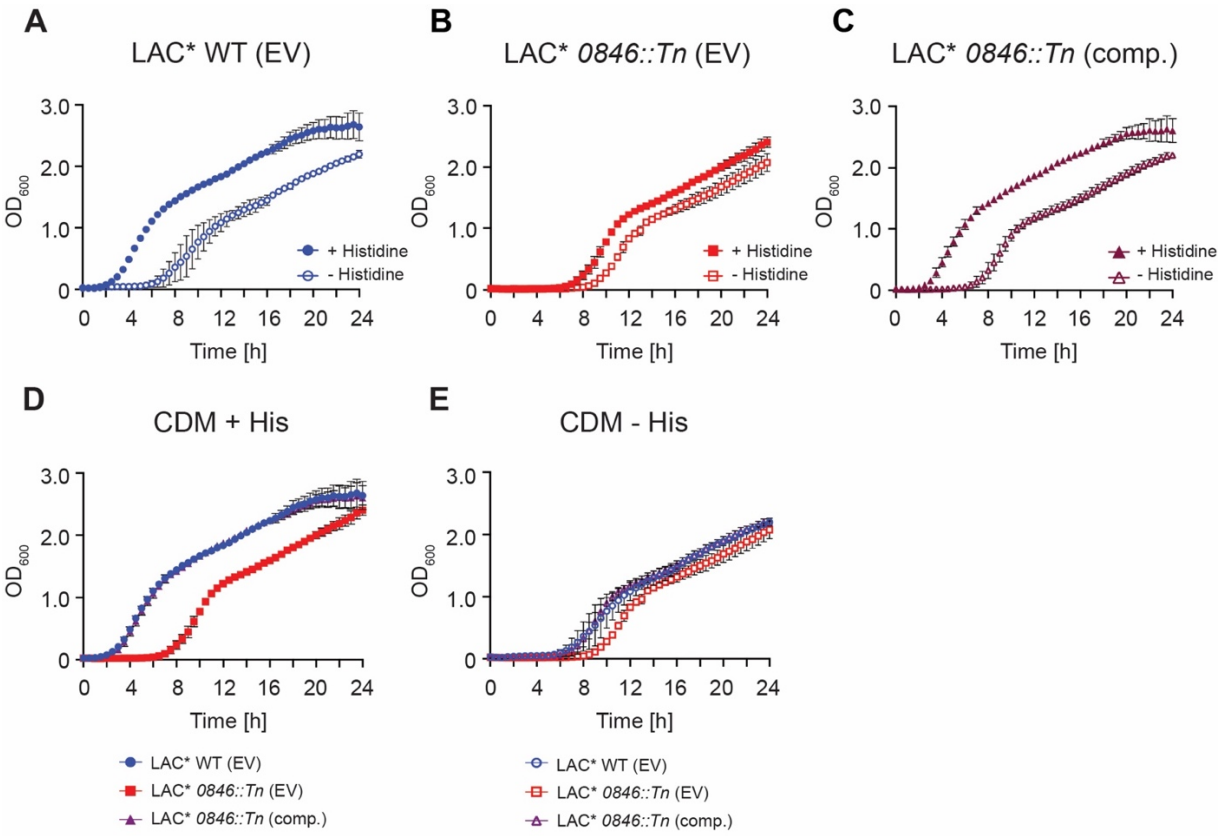


Figure 7.1: The WT bacteria, but not the *0846::Tn* bacteria, are capable of resuming growth quicker when histidine is present in the growth medium
Cultures of (A,D,E) LAC* WT (EV), (B,D,E) LAC* *0846::Tn* (EV), and (C,D,E) LAC* *0846::Tn* (comp.) grown overnight in TSB pH 7.3 medium were inoculated into CDM pH 7.0 either (D) with or (E) without 130 µM histidine. Strains were grown in 96-well plates with OD_{600} measurements taken at the indicated time points. The average readings and standard deviations of three independent experiments were plotted. This figure has been adapted from Beetham et al., (2024).

7.1.2 Analysis of the importance of histidine for growth under acid stress conditions

To explore whether histidine is important for the growth of *S. aureus* under acid stress conditions, growth curves were performed in acidic CDM with or without histidine. To this end, the LAC* WT (EV), LAC* *0846::Tn* (EV) and LAC* *0846::Tn* (comp.) strains were grown overnight in TSB medium before being back-diluted the following day in CDM acidified to pH 4.3 by the addition of HCl prior to filter sterilising. The addition of histidine can act to buffer the medium, and therefore the pH of the medium was measured and adjusted following the addition of histidine.

The LAC* WT (EV) strain resumed growth following 4 h in acidic CDM when histidine was present, but was unable to grow in acidic CDM when histidine was not present (Fig. 7.2A). The LAC* *0846::Tn* (EV) strain was unable to grow in acidic CDM regardless of whether histidine was present or not (Fig. 7.2B). The LAC* *0846::Tn* (comp.) strain again behaved similar to the WT strain, and resumed growth following 4 h in acidic CDM with histidine, but was unable to grow in acidic CDM without histidine (Fig. 7.2C). These data suggest that histidine and its uptake via 0846 are essential for the growth of *S. aureus* under acidic stress conditions.

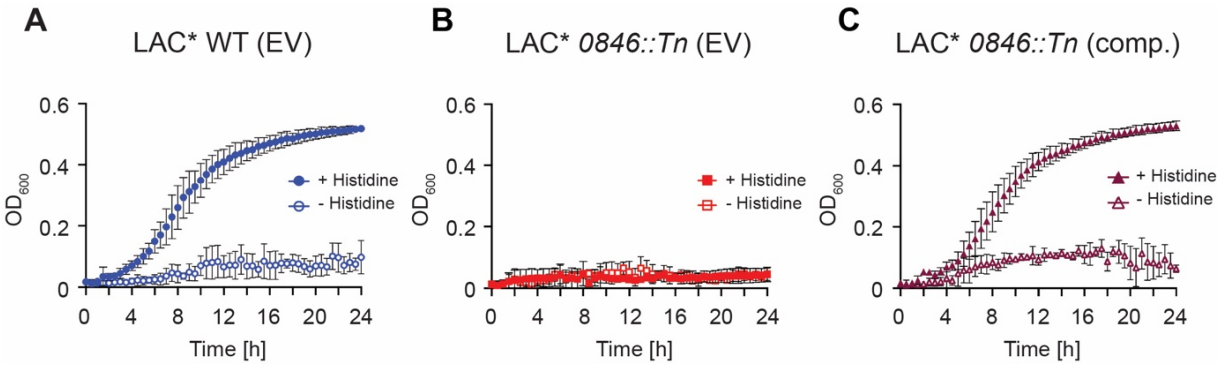


Figure 7.2: The WT bacteria can only grow in acidic CDM if histidine is present in the growth medium, while the *0846::Tn* bacteria cannot grow in acidic CDM with or without histidine
Cultures of (A) LAC* WT (EV), (B) LAC* *0846::Tn* (EV), and (C) LAC* *0846::Tn* (comp.) grown overnight in TSB pH 7.3 medium were inoculated into CDM pH 4.3 with or without 130 µM histidine. Strains were grown in 96-well plates with OD_{600} measurements taken at the indicated time points. The average readings and standard deviations of three independent experiments were plotted. This figure has been adapted from Beetham et al., (2024).

7.2 The *0846::Tn* strain adapts to amino acid limiting conditions following overnight growth in CDM

7.2.1 Exploring the effect of overnight growth in CDM

The data presented above suggests that WT *S. aureus* responds to histidine limiting conditions by undergoing some form of adaptation which allows it to resume growth in CDM without histidine. However, unexpectedly the *0846::Tn* mutant bacteria had not already undergone this adaptation to histidine limiting conditions, as shown by the longer time taken to resume growth in CDM both with and without histidine. This is despite the mutant bacteria seemingly being unable to respond to the presence of histidine in the growth medium, and thus presumably experiencing histidine-limiting conditions in CDM both with and without histidine.

In the growth curves described above, the bacterial strains were first grown overnight in TSB medium, before being back-diluted the following day into CDM. To explore whether the *0846::Tn* bacteria could adapt to histidine-limiting conditions via a pre-growth in CDM, the LAC* WT (EV), LAC* *0846::Tn* (EV) and LAC* *0846::Tn* (comp.) strains were grown overnight in CDM before back-diluting the following day into fresh CDM with or without histidine.

Following overnight growth in CDM, the LAC* WT (EV) strain resumed growth following 3 h in CDM with histidine (Fig. 7.3A). When histidine was not present in the medium, the LAC* WT (EV) strain resumed growth following 7 h. This is very similar to the growth of the LAC* WT (EV) in CDM following overnight growth in TSB medium and suggests that overnight growth in CDM does not not lead to a pre-adaption of the LAC* WT (EV) bacteria to subsequent histidine limiting conditions.

On the other hand, the LAC* *0846::Tn* (EV) mutant bacteria resumed growth following 4 h in CDM both with and without histidine (Fig. 7.3B). The complementation strain behaved more similar to the WT strain, as the LAC* *0846::Tn* (comp.) strain resumed growth following 3 h in CDM with histidine, and following 5 h in CDM without histidine (Fig. 7.3C). However only partial complementation was seen in this assay.

When plotted on the same graph, it is clear that following overnight growth in CDM the *0846::Tn* mutant bacteria resume growth following a similar amount of time as the WT

bacteria in CDM with histidine (Fig. 7.3D). Furthermore, the *0846::Tn* bacteria resume growth faster than the WT bacteria in CDM without histidine (Fig. 7.3E). Therefore it is clear that following overnight growth in CDM the *0846::Tn* strain behaves like the WT strain does in CDM with histidine. This contrasts to what was shown in the growth curves following overnight growth in TSB medium, where the *0846::Tn* strain behaves like the WT strain does in CDM without histidine. Taken together, these data suggest that the *0846::Tn* bacteria adapt to the histidine-limiting conditions they experience in CDM through overnight growth in CDM.

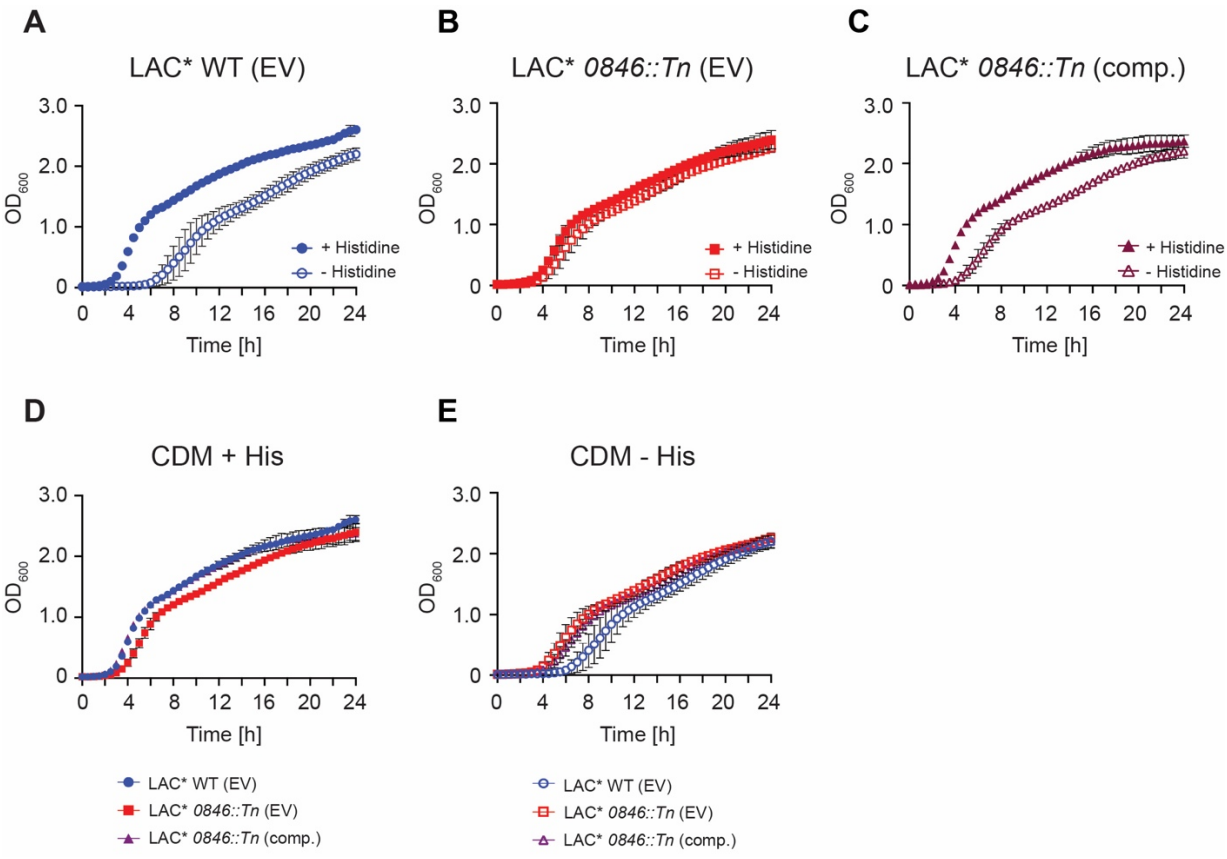


Figure 7.3: The *0846::Tn* bacteria resume growth quicker than the WT bacteria in CDM without histidine following overnight growth in CDM
Cultures of (A,D,E) LAC* WT (EV), (B,D,E) LAC* *0846::Tn* (EV), and (C,D,E) LAC* *0846::Tn* (comp.) grown overnight in CDM pH 7.2 with 130 µM histidine were inoculated into CDM pH 7.2 either (D) with or (E) without 130 µM histidine. Strains were grown in 96-well plates with OD_{600} measurements taken at the indicated time points. The average readings and standard deviations of three independent experiments were plotted.

7.2.2 Whole genome analysis of the *0846::Tn* strain

To explore whether the adaptation of the LAC* *0846::Tn* (EV) strain for growth in histidine-limiting conditions through overnight growth in CDM was due to the generation of suppressor mutations, a whole genome sequencing approach was used. The genome sequence of LAC* *0846::Tn* (EV) following overnight growth in TSB medium, or of 12 cultures of LAC* *0846::Tn* (EV) following overnight growth in CDM were determined. All 13 strains generated sequences with good sequence quality and coverage. The CLC Genomics Workbench programme was used to compare the sequences of the cultures grown overnight in CDM to the culture grown overnight in TSB medium. The sequencing analysis of the suppressor strains was performed by A. Gründling.

However, no additional mutations were detected in any of the genome sequences generated from cultures grown overnight in CDM compared to the sequence generated from the culture grown overnight in TSB medium. This shows that the adaptation of the LAC* *0846::Tn* (EV) for growth in CDM following overnight growth in CDM is not mediated by the generation of compensatory mutations.

7.2.3 Analysis of growth under leucine-limiting conditions following adaptation to histidine-limiting conditions

The data presented thus far in this Chapter show that the *0846::Tn* bacteria adapt to histidine-limiting conditions through pre-growth in CDM. To explore whether this adaptation also conferred an advantage under other amino acid-limiting conditions, growth curves were performed by pre-adapting the bacterial strains through overnight growth in CDM then back-diluting the cultures the following day into fresh CDM with or without leucine. Leucine was chosen as previous data from the group have shown that *S. aureus* has to undergo an adaptation before resuming growth in CDM without leucine.

As expected, the LAC* WT (EV) strain took longer to resume growth in leucine-limiting conditions (Fig. 7.4A). Growth of the WT strain resumed following 3 h in CDM with leucine, and following 9 h in CDM without leucine. The LAC* *0846::Tn* (EV) strain also took longer to resume growth in leucine-limiting conditions (Fig. 7.4B). The mutant strain resumed growth following 4 h in CDM with leucine and following 6 h in CDM without leucine. The

complementation strain behave again very similar to the WT strain and resumed growth following 3 h in CDM with leucine, and following 7 h in CDM without leucine (Fig. 7.4C).

When plotted on the same graph, it is clear that the *0846::Tn* mutant bacteria resume growth quicker than the WT bacteria in CDM without leucine (Fig. 7.4E). This suggests that the adaptation of the *0846::Tn* mutant bacteria to histidine-limiting conditions that occurs through pre-growth in CDM also confers an adaptation to other amino acid-limiting conditions.

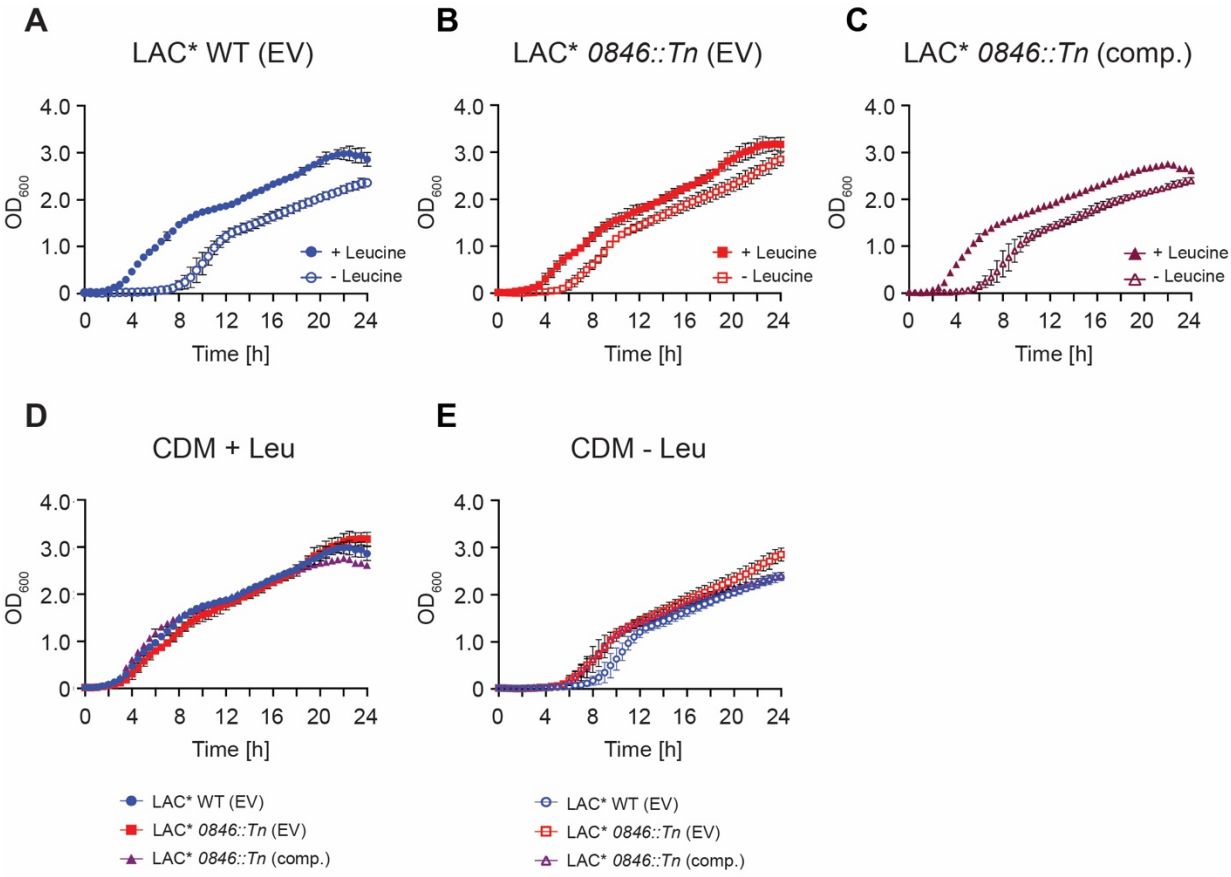


Figure 7.4: Pre-adapted *0846::Tn* bacteria resume growth faster than the WT bacteria in CDM without leucine

Cultures of (A,D,E) LAC* WT (EV), (B,D,E) LAC* *0846::Tn* (EV), and (C,D,E) LAC* *0846::Tn* (comp.) grown overnight in CDM pH 7.2 with 686.1 µM leucine were inoculated into CDM pH 7.2 either (D) with or (E) without 686.1 µM leucine. Strains were grown in 96-well plates with OD_{600} measurements taken at the indicated time points. The average readings and standard deviations of three independent experiments were plotted.

7.3 The adaptation of the *0846::Tn* strain to amino acid-limiting conditions compensates for its growth defect under acid stress conditions

7.3.1 Analysis of growth under acid stress conditions following adaptation to histidine-limiting conditions

The data presented thus far in this Chapter suggests that the LAC* *0846::Tn* (EV) bacteria adapt for growth in amino acid-limiting conditions through pre-growth in CDM. The main pathway used by *S. aureus* to coordinate growth under amino acid-limiting conditions is the stringent response, which is induced in response to amino acid-limiting conditions. In Chapter 6 I have shown that inducing the stringent response in the *0846::Tn* mutant bacteria can compensate for their reduced growth under acid stress conditions.

To explore whether the adaptation of the LAC* *0846::Tn* (EV) strain for growth in amino acid-limiting conditions compensates for its reduced growth under acid stress conditions, the growth of pre-adapted bacterial strains was explored in acidic CDM with or without histidine. To this end, the LAC* WT (EV), LAC* *0846::Tn* (EV) and LAC* *0846::Tn* (comp.) strains were grown overnight in neutral CDM, before being back-diluted the following day into acidic CDM with or without histidine.

Under these experimental conditions, the LAC* WT (EV) strain resumed growth following approximately 4 h in acidic CDM with histidine (Fig. 7.5A). This is similar to what was seen for the LAC* WT (EV) strain in acidic CDM following overnight growth in TSB medium in Fig. 7.2A, and shows that overnight growth in CDM does not affect the ability of the WT bacteria to grow in acidic CDM with histidine. Furthermore, following pre-growth in CDM the LAC* WT (EV) strain took an additional 8 h to resume growth in acidic CDM without histidine than acidic CDM with histidine. This is longer than the additional 4 h taken to resume growth in neutral CDM without histidine than in neutral CDM with histidine (Fig. 7.3B), and shows that a lack of histidine in the growth medium has a greater inhibitory effect on the growth of WT *S. aureus* under acidic than neutral conditions. However, the fact that the WT strain is capable of growth in acidic CDM without histidine following pre-growth in CDM but not following pre-growth in TSB medium shows that pre-growth in CDM induces some form of adaptation in the WT strain.

On the other hand, the LAC* *0846::Tn* (EV) mutant bacteria resumed growth following approximately 4 h in acidic CDM with and without histidine (Fig. 7.5B). The complementation strain behaved more similar to the WT strain, as the LAC* *0846::Tn* (comp.) strain resumed growth following 4 h in acidic CDM with histidine and following 7 h in acidic CDM without histidine (Fig. 7.5C). However, as seen when grown in neutral CDM following overnight growth in CDM, only partial complementation was seen in this assay.

It is clear that the *0846::Tn* mutant strain does not show a growth defect in acidic CDM following pre-growth in CDM. This conclusively shows that the adaptation of the *0846::Tn* strain to histidine-limiting conditions induced through pre-growth in CDM compensates for the growth defect of the mutant bacteria under acid stress conditions.

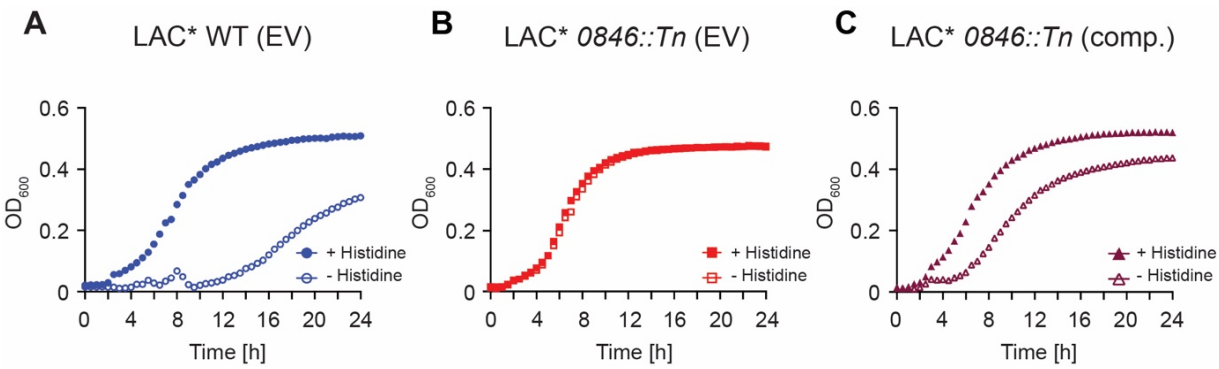


Figure 7.5: The *0846::Tn* strain does not show a growth defect in acidic CDM once adapted for growth in CDM
Cultures of (A) LAC* WT (EV), (B) LAC* *0846::Tn* (EV), and (C) LAC* *0846::Tn* (comp.) grown overnight in CDM + 130 µM histidine were inoculated into CDM pH 4.3 with or without 130 µM histidine. Strains were grown in 96-well plates with OD_{600} measurements taken at the indicated time points. The average readings and standard deviations of one experiment was plotted.

7.4 The catabolism of histidine via the Hut pathway is not important for the growth of *S. aureus* at low pH

7.4.1 Literature analysis for reasons why histidine might be important for growth under acid stress conditions

Following an analysis of the literature, I identified several potential reasons why histidine might be important for the growth of *S. aureus* under acidic conditions. Firstly, as described in the Introduction Chapter, several species of lactic acid bacteria contain a histidine decarboxylase system (reviewed in Landete et al., 2008; Molenaar et al., 1993). Here, a molecule of histidine is brought into the cell before being converted to histamine via a histidine decarboxylase enzyme. In this process, a single proton is consumed. The resulting histamine product is then exported out of the cell. In *Lactobacillus vaginalis* the production of histamine from histidine is important for the survival of the bacteria under acidic stress (Diaz et al., 2020). However, a blast analysis of the *S. aureus* genomes did not reveal any genes with homology to any known histidine decarboxylase genes.

Alternatively, histidine can be broken down into glutamate via the conserved hut pathway, which in *S. aureus* releases two molecules of usable nitrogen per molecule of histidine (reviewed in Bender, 2012). The first molecule of usable nitrogen is released via the first stage of the pathway, whereby ammonia is released as a by-product when histidine is deaminated to urocanate via the HutH histidine deaminase (Fig. 7.6). Urocanate is then hydrolysed by the urocanase HutU to form imidazolone propionate, which undergoes two further hydrolysis reactions to result in the second molecule of usable nitrogen- glutamate. Although this pathway has not been associated with the acid stress response of any bacteria to the best of my knowledge, it is possible that the production of the alkaline ammonia molecule is the reason for the importance of histidine in the acid stress response of *S. aureus*.

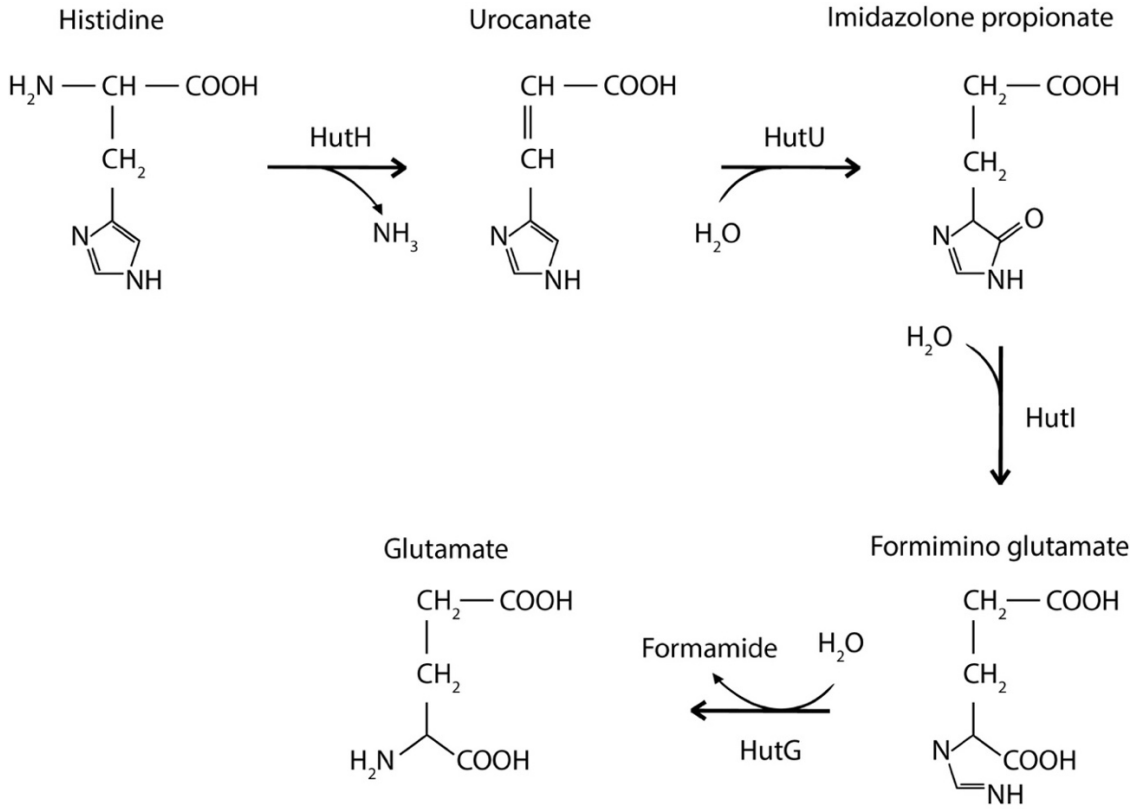


Figure 7.6: Schematic representation of the Hut pathway
Histidine can be catabolised into glutamate via the Hut pathway, resulting in the release of two molecules of usable nitrogen. Firstly, histidine is deaminated by the HutH histidine deaminase to form urocanate, releasing ammonia as a by-product. Secondly, urocanate is hydrolysed by the urocanase HutU to form imidazolone propionate, which undergoes two further hydrolysis reactions to result in glutamate. Figure adapted from Bender, (2012).

7.4.2 Exploring the importance of the Hut pathway for growth under acid stress conditions

To explore whether the catabolism of histidine via the Hut pathway is important for the growth of *S. aureus* under acid stress conditions, the ability of mutant strains of *S. aureus* with Tn insertions in the *hutH* and *hutU* genes to grow under acid stress conditions was investigated. *hutH* and *hutU* encode the enzymes which catalyse the first two steps in the Hut pathway (Fig. 7.6). Firstly, the JE2 WT, JE2 *0846::Tn*, NTML JE2 *hutH::Tn*, and NTML JE2 *hutU::Tn* strains were spotted on TSA pH 7.3 plates to see if any of the strains showed a growth defect at neutral pH. All strains grew to the 10^{-7} dilution, showing that inactivating *hutH* and *hutU* does not confer a growth defect at neutral pH (Fig. 7.7A).

Next, the strains were spotted on TSA pH 4.5 plates. As has been shown previously, the JE2 *0846::Tn* strain showed a 100,000 fold decrease in CFU ml^{-1} compared to the JE2 WT strain (Fig. 7.7B). However, neither the NTML JE2 *hutH::Tn* nor the NTML JE2 *hutU::Tn* strains showed

a growth defect compared to the JE2 WT strain when spotted on TSA pH 4.5 plates. This shows that inactivating the Hut pathway does not inhibit the ability of *S. aureus* to grow at low pH. Taken together, these data shows that the reason for the importance of histidine for the ability of *S. aureus* to grow under acidic conditions is not due to its catabolism and the production of ammonia via the Hut pathway.

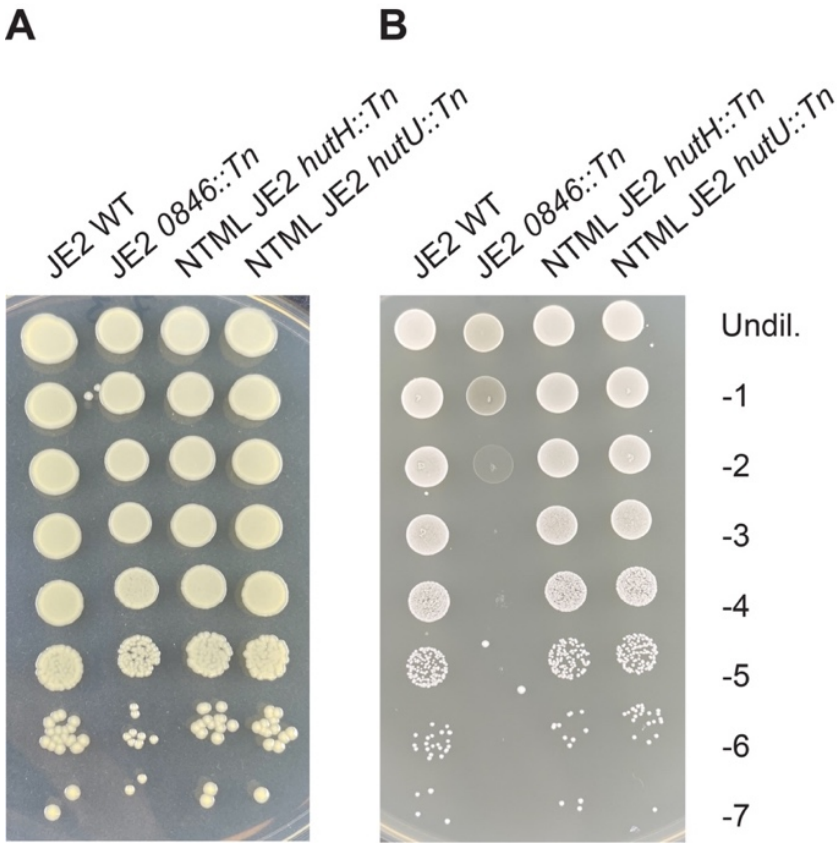


Figure 7.7: Inactivation of enzymes in the Hut pathway does not result in a growth defect at neutral or low pH growth conditions
Overnight cultures of the JE2 WT, JE2 *0846::Tn*, NTML JE2 *hutH::Tn*, and NTML JE2 *hutU::Tn* strains were serially diluted and aliquots spotted onto (A) TSA pH 7.3 plates or (B) TSA pH 4.5 plates. Images were taken following 24 h incubation at 37 °C. Each image is a representative of three independent experiments. This figure has been adapted from Beetham et al., (2024).

7.5 Inactivating *0846* results in a reduced cytoplasmic pH, but inducing the stringent response does not increase the cytoplasmic pH

7.5.1 Measurement of the cytoplasmic pH

One of the characteristics of histidine as an amino acid is that the pK_a value of its side chain is 6.0, and therefore histidine is the only amino acid capable of acting as a buffer within physiological pH range. I therefore next explored whether the reason why histidine is important for the ability of *S. aureus* to grow under acid stress conditions is due to a potential ability to buffer the cytoplasm. To explore this, measurements of the cytoplasmic pH of the LAC* WT and LAC* *0846::Tn* strains were performed using the pHrodo Red intracellular pH dye and a plate-reader based method as has been described previously with modifications (Beetham et al., 2024; Grosser et al., 2018). The pHrodo dye emits increased fluorescence when the pH is decreased. The fluorescence values can be converted into pH values through the generation of a standard curve by measuring the fluorescence of bacterial cultures suspended in buffers with pH 4.5, 5.5, 6.5 and 7.5 with the addition of valinomycin and nigericin.

The LAC* WT bacteria showed a cytoplasmic pH of 7.20 when grown in TSB pH 7.3 medium (Fig. 7.8A). However, when grown in TSB pH 4.5 medium, the cytoplasmic pH of the LAC* WT bacteria decreased to 6.84. The LAC* *0846::Tn* bacteria showed a cytoplasmic pH of 7.01 when grown in TSB pH 7.3 medium, which decreased to 6.43 when grown in TSB pH 4.5 medium. Furthermore, the decrease between the cytoplasmic pH when grown under neutral and acidic conditions was greater for the mutant bacteria, as the Δ pH of the *0846::Tn* bacteria was -0.59 while that of the WT bacteria was -0.36 (Fig. 7.8B). This shows that inactivating the 0846 histidine transporter results in a reduced cytoplasmic pH, especially when grown under acid stress conditions.

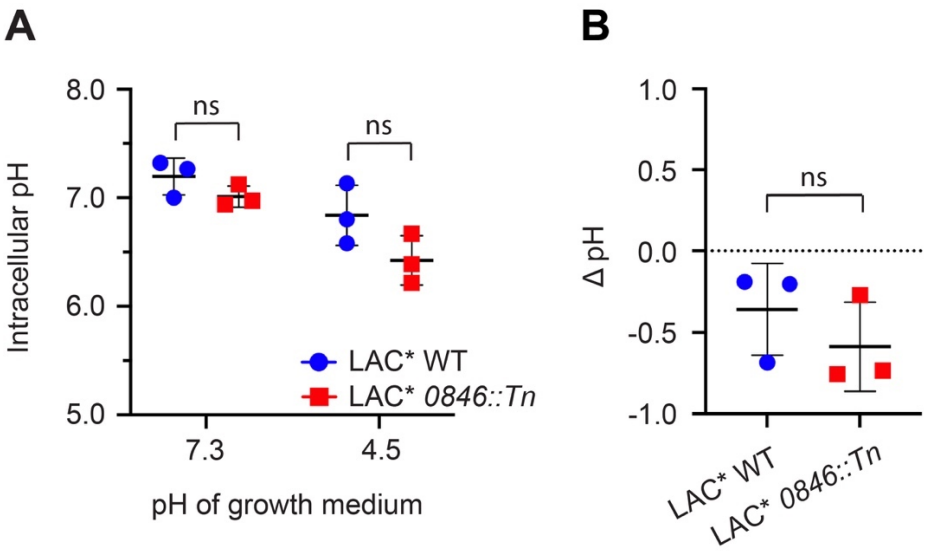


Figure 7.8: Inactivating *0846* reduces the cytoplasmic pH of *S. aureus*
Cultures of LAC* WT and LAC* *0846::Tn* grown overnight in either TSB pH 7.3 medium or TBS pH 5.5 medium were back-diluted the following day into TSB pH 7.3 medium or TSB pH 4.5 medium and grown to mid-log phase. These cultures were washed with HEPES 50 mM pH 7.4 buffer before being stained with pHrodo dye. Fluorescence measurements were converted to pH values by comparison to a standard curve generated by resuspending the bacterial culture in buffer pH 4.5, 5.5, 6.5 and 7.5 with 10 µM valinomycin and 10 µM nigericin. The (A) pH values or (B) Δ pH values from three independent experiments are plotted, along with the average and standard deviation of all three. Values showing statistical significance are marked with asterisks (P ≤ 0.05(*), P ≤ 0.01(**), P ≤ 0.001(***) performed by (A) multiple unpaired t-test corrected for multiple-comparisons using the Holm-Sidak method or (B) unpaired t-test. This figure has been adapted from Beetham et al., (2024).

It is clear that the growth defect of the *0846::Tn* mutant strain under acid stress conditions can be compensated for by inducing the stringent response. To explore whether the effect of inducing the stringent response is an increased cytoplasmic pH, the cytoplasmic pH of the LAC* *0846::Tn* and LAC* *0846::Tn codY*$_{E163*}$ strains were measured following growth in both TSB pH 7.3 and pH 4.5 medium.

As expected, the LAC* *0846::Tn* strain showed a cytoplasmic pH of 6.91 following growth in TSB pH 7.3 medium and 6.36 following growth in TSB pH 4.5 medium (Fig. 7.9A). The suppressor strain showed a cytoplasmic pH of 5.79 following growth in TSB pH 7.3 medium and 6.25 following growth in TSB pH 4.5 medium. While the *0846::Tn* strain showed a decreased Δ pH of -0.55, the suppressor strain showed an increased Δ pH of +0.46 (Fig. 7.9B). Taken together, these data show that while inducing the stringent response does not result in

an increased cytoplasmic pH in the *0846::Tn* strain when grown under acid stress conditions, it does result in an increased Δ pH when comparing cytoplasmic pH following growth under neutral and acidic conditions. These data therefore suggest that it is not the actual value of the cytoplasmic pH that matters, but the change in cytoplasmic pH when comparing growth under neutral and acidic conditions.

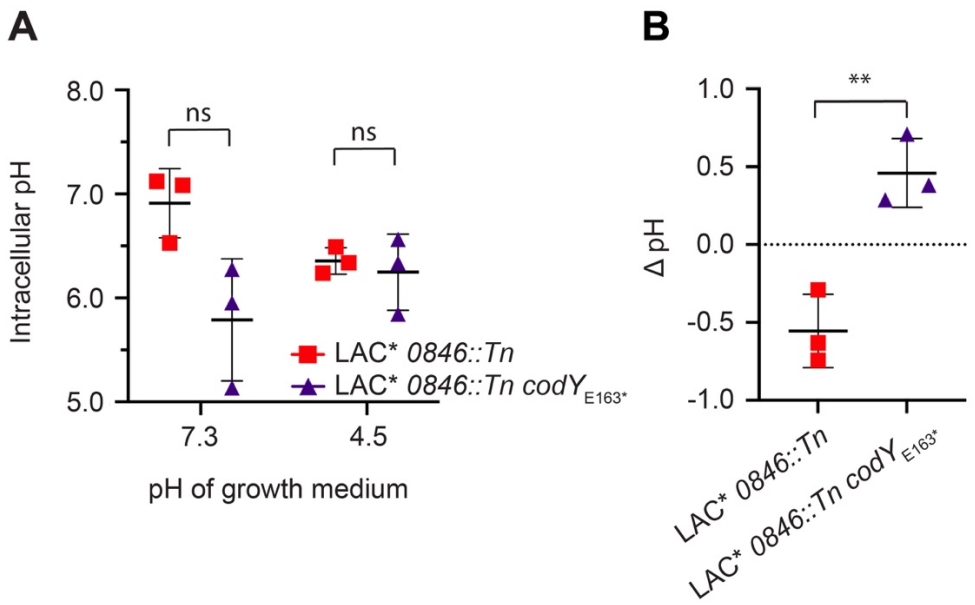


Figure 7.9: Inducing the stringent response does not increase the cytoplasmic pH of the *0846::Tn* strain
Cultures of LAC* *0846::Tn* and LAC* *0846::Tn codY*$_{E163*}$ grown overnight in either TSB pH 7.3 medium or TBS pH 5.5 medium were back-diluted the following day into TSB pH 7.3 medium or TSB pH 4.5 medium and grown to mid-log phase. These cultures were washed with HEPES 50 mM pH 7.4 buffer before being stained with pHrodo dye. Fluorescence measurements were converted to pH values by comparison to a standard curve generated by resuspending the bacterial culture in buffer pH 4.5, 5.5, 6.5 and 7.5 with 10 µM valinomycin and 10 µM nigericin. The (A) pH values and (B) Δ pH values from three independent experiments are plotted, along with the average and standard deviation of all three. Values showing statistical significance are marked with asterisks (P ≤ 0.05(*), P ≤ 0.01(**), P ≤ 0.001(***) performed by (A) multiple unpaired t-test corrected for multiple-comparisons using the Holm-Sidak method or (B) unpaired t-test. This figure has been adapted from Beetham et al., (2024).

7.6 Discussion of Chapter 7

In this Chapter, the importance of histidine in the acid stress response of *S. aureus* was explored. Firstly, growth curves in CDM were performed to investigate whether histidine is an

essential amino acid for the growth of *S. aureus* under neutral pH. As has been described in the group previously, WT *S. aureus* is capable of growth in CDM without histidine at neutral pH, but it takes longer for the bacteria to resume growth when histidine is not present in the growth medium. On the other hand, the LAC* *0846::Tn* (EV) strain resumed growth at the same time as the LAC* WT (EV) did in medium without histidine, regardless of whether histidine was present in the medium or not. This shows that the *0846::Tn* bacteria do not respond to the presence of histidine in the medium, and supports the data presented in Chapter 6 demonstrating that *0846* encodes the main histidine transporter in *S. aureus*.

When the LAC* WT (EV) and LAC**0846::Tn* (EV) strains were grown in CDM under acid stress conditions, it is clear that histidine uptake via 0846 is essential for growth. The LAC* WT (EV) bacteria were only capable of growth under acid stress conditions when histidine was present in the medium, whereas the LAC* *0846::Tn* (EV) bacteria did not grow in acidic CDM regardless of whether histidine was present or not. These data show that histidine and its uptake via 0846 are essential for the growth of *S. aureus* under acid stress conditions and supports the hypothesis that the reason for the importance of 0846 for the growth of *S. aureus* under acid stress conditions is due to its histidine transporting activity.

It is clear that when grown in CDM without histidine, the WT bacteria undergo some form of adaptation to histidine-limiting conditions which allows them to resume growth. However, when grown overnight in TSB medium the *0846::Tn* strain takes the same amount of time to resume growth at the WT strain does in CDM without histidine, which implies that the mutant bacteria have not pre-adapted for growth in histidine-limiting conditions. This is despite them presumably experiencing histidine-limiting conditions during overnight growth in TSB medium. However, when the *0846::Tn* strain was grown overnight in CDM, the mutant strain resumed growth quicker than following overnight growth in TSB medium. This demonstrates that pre-growth in CDM is required for the *0846::Tn* bacteria to adapt to histidine-limiting conditions. TSB is a complex medium with an undefined mixture of compounds and chemicals, whereas CDM is a defined growth medium. It is likely therefore that there is a compound present in TSB medium which allows the *0846::Tn* bacteria to compensate for the lack of histidine uptake.

Further exploration of the adaptation of the *0846::Tn* strain revealed that it is not caused by a suppressor mutation, and that it is not specific to histidine. Pre-adapted LAC* *0846::Tn* (EV) bacteria resumed growth quicker than the LAC* WT (EV) bacteria in CDM without leucine. This implies that the adaptation is a general response to amino acid-limiting conditions. The main mechanism used by *S. aureus* to adapt for growth in amino acid limiting conditions is the induction of the stringent response via the CodY master regulator. As described in Chapter 6, the stringent response results in the transcription of genes such as those involved in amino acid transport and biosynthesis. It has already been shown that inactivating CodY enhances the growth of *S. aureus* in leucine-limiting conditions (Audretsch et al., 2021). I therefore hypothesized that overnight growth in CDM induces the stringent response in the *0846::Tn* mutant strain, which allows it to resume growth quicker in amino acid-limiting conditions.

The data presented in Chapter 6 conclusively shows that the induction of the stringent response in the *0846::Tn* bacteria compensates for their growth defect under acid stress conditions. To explore whether the adaptation of the LAC* *0846::Tn* (EV) bacteria for growth under amino acid-limiting conditions also compensated for their growth defect under acid stress conditions, the LAC* WT (EV) and LAC* *0846::Tn* (EV) strains were pre-grown in CDM before being back-diluted into acidic CDM with or without histidine. These data show that, when adapted to amino acid-limiting conditions, the LAC* *0846::Tn* (EV) bacteria do not show a growth defect under acid stress conditions.

Interestingly, the WT *S. aureus* strain also shows an increased ability to grow in acidic CDM without histidine when pre-grown in CDM, despite pre-growth having no effect on their ability to grow in neutral CDM. The reason for this is unknown, but it is possible that the WT bacteria also undergo some form of adaptation to amino acid-limiting conditions when pre-grown in CDM, and that this adaptation only confers a growth advantage under acid stress conditions. This further supports the hypothesis that histidine is important for the growth of *S. aureus* under acid stress conditions.

There are several hypotheses as to why histidine might be important for the growth of *S. aureus* under acid stress conditions. Several lactic acid bacteria contain a histidine decarboxylation system, where a single molecule of histidine is decarboxylated into histamine and consuming a proton in the process. The resulting histamine is then exported out of the

cell. The importance of this system for bacterial acid stress responses was shown in *L. vaginalis*, where the production of histamine is increased at low pH, and bacterial survival under acid stress conditions depends on the presence of extracellular histidine (Diaz et al., 2020). The authors hypothesise that the decarboxylation of histidine to histamine reduces the intracellular concentration of protons, resulting in increased survival at low pH (Diaz et al., 2020). However, a separate study has shown that in *L. buchneri*, the decarboxylation of histidine to histamine generates a proton motive force due to the movement of protons across the membrane (Molenaar et al., 1993). It is therefore unclear whether histidine decarboxylase acts to buffer the intracellular pH or acts to generate PMF for use in other processes during acid stress.

There are two different groups of histidine decarboxylase enzymes- pyridoxal phosphate-dependent enzymes and pyruvoyl-dependent enzymes (reviewed in Landete et al., 2008). The former are encountered in Gram-negative bacteria, while the latter are encountered in Gram-positive bacteria (reviewed in Landete et al., 2008). However, blast analysis of a range of bacterial histidine decarboxylase enzymes did not reveal any genes with homology to either group of enzymes.

Another potential reason for the importance of histidine for the growth of *S. aureus* under acid stress conditions is through its catabolism to glutamate via the Hut pathway (reviewed in Bender, 2012). The Hut pathway is highly conserved among bacteria, and is well studied as a model for several regulatory pathways (reviewed in Bender, 2012). In the first step of this process, histidine is deaminated by the HutH histidine deaminase to form urocanate (reviewed in Bender, 2012). Secondly, urocanate is hydrolysed by the urocanase HutU to form imidazolone propionate. In the first step a single molecule of ammonia is released (reviewed in Bender, 2012).

The production of ammonia is a key response in the acid stress response of *S. aureus*, as described in the Introduction Chapter. In the four studies which investigated changes in gene transcription in *S. aureus* upon acid stress, the genes encoding the urease enzyme and accessory proteins which catalyse the production of ammonia from urea were among the most highly upregulated (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010; Weinrick et al., 2004; reviewed in Zhou & Fey, 2020). A follow up study demonstrated that the

production of ammonia via urease was essential for the growth and survival of *S. aureus* under weak acid stress (Zhou et al., 2019), and in Chapter 3 I have shown that urease is also important for the growth and survival of *S. aureus* under strong acid stress. However, in both cases the urease enzyme is only important when exogenous urea is added to the growth medium (Zhou et al., 2019). Ammonia can also be produced through the ADI system where a molecule of arginine is deaminated, and a molecule of ammonia is produced as a by-product (reviewed in Cunin et al., 1986). In *S. aureus* the ADI system includes the *arcA*, *arcB* and *arcC* genes. In one transcriptional study *arcA* was upregulated in response to acid stress (Weinrick et al., 2004), although in the three other transcriptional studies *arcC* was downregulated in response to acid stress (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010). Furthermore, the USA300 community-associated MRSA strain encodes a second ADI system which has been shown to facilitate the growth of *S. aureus* at pH 5.0 (Thurlow et al., 2013). Taken together, these studies show that the production of ammonia is important for the acid stress response of *S. aureus*.

However, neither the NTML JE2 *hutH::Tn* nor the NTML JE2 *hutU::Tn* strains showed a growth defect compared to the JE2 WT strain when spotted on TSA pH 4.5 plates. Furthermore, neither gene was classified as essential for the growth of *S. aureus* at low pH in the Tn-Seq experiment. This shows that the reason for the importance of histidine and its uptake via 0846 for the growth of *S. aureus* under low pH is not due to its breakdown via the Hut pathway.

Histidine is unique among amino acids for having a pK_a value of its side chain of 6.0. This means that it is the only amino acid which can act as a buffer within physiological pH range. It is possible therefore that histidine is important for the growth of *S. aureus* at low pH due to its ability to buffer the cytoplasm of *S. aureus*. Previous studies measuring the cytoplasmic pH of neutralophilic bacteria have shown that the cytoplasmic pH stays within a narrow range even in a range of extracellular pH buffers (reviewed in Slonczewski et al., 2009). The data presented in this Chapter supports this by showing that the cytoplasmic pH of *S. aureus* drops by only -0.36 when grown in TSB pH 4.5 medium compared to TSB pH 7.3 medium. On the other hand, the cytoplasmic pH of the LAC* *0846::Tn* (EV) strain drops by -0.59 over the same range, and has a lower cytoplasmic pH under both growth conditions. This suggests that histidine uptake via 0846 is important for the ability of *S. aureus* to buffer its cytoplasm, especially under acid stress conditions.

In Chapter 6 suppressor strains were generated which compensated for the growth defect of the *0846::Tn* strain at low pH by inducing the stringent response. Interestingly, the cytoplasmic pH of the LAC* *0846::Tn codY*$_{E163*}$ bacteria was much lower than that of the *0846::Tn* bacteria when grown in TSB pH 7.3 medium. As both strains had similar cytoplasmic pH measurements when grown in TSB pH 4.5 medium, this meant that the suppressor strain had a positive Δ pH compared to the negative Δ pH of the *0846::Tn* strain. This suggests that it is not the actual value of the cytoplasmic pH that matters, but the change in cytoplasmic pH when comparing growth under neutral and acidic conditions.

To conclude, the data presented in this Chapter show that histidine and its uptake via 0846 are important for the growth of *S. aureus* under acid stress conditions, although the reasons for this are still unclear.

Chapter 8 : Concluding discussion and future directions

S. aureus is capable of growth in a wide range of pH conditions from 4.0 to 10.0 (ICMSF et al., 1996). Despite this, little is known about the mechanisms used by *S. aureus* to tolerate and grow under acidic stress conditions. While four studies have investigated the transcriptional changes in *S. aureus* upon exposure to an acidic pH, very few phenotypic studies have been performed. Therefore, the aim of this PhD was to explore the mechanisms used by *S. aureus* to grow under acid stress conditions.

8.1 Investigation into how c-di-AMP regulates the acidic stress response of *S. aureus*

Previous work from the lab revealed that a low c-di-AMP producing mutant strain of *S. aureus* shows reduced growth at low pH, while a high c-di-AMP producing mutant strain of *S. aureus* shows increased growth at low pH (Bowman et al., 2016). The work described in this PhD project supports these previous results and demonstrates that the low c-di-AMP producing mutant strain shows reduced growth and survival compared to the WT strain under both strong and weak acid stress. However, I show that the reason for this sensitivity to acid is not due to a dysregulation of the urease enzyme and ammonia production from urea. Therefore, the reason for the importance of c-di-AMP in the acid stress response of *S. aureus* is still not known.

As part of my PhD study I found that a *ΔybbR* strain has, as previously reported, reduced growth and survival compared to the WT strain under strong acid stress, but interestingly not under weak acid stress. This supports previous transcriptional studies demonstrating that the response of *S. aureus* to strong and weak acid stress is different (Rode et al., 2010). Again, the importance of YbbR for the growth of *S. aureus* under strong acid stress was, as shown here, not be due to the dysregulation of the urease enzyme and a defect in ammonia production from urea.

The mechanism through which c-di-AMP and YbbR regulate the acid stress response of *S. aureus* remains to be elucidated. One of the most significant pathways that c-di-AMP regulates that may potentially be involved in acid tolerance is potassium transport. As the effect of potassium on the acid stress response of *S. aureus* is less well characterised than in

other bacterial species as described in the Introduction Chapter, it would be interesting to explore the interplay between c-di-AMP, potassium transport, and the acid stress response.

8.2 Exploration of whether a Tn-Seq approach is an effective method to identify genes important for the fitness and growth of *S. aureus* under acid stress conditions

Tn-Seq approaches combine transposon mutant libraries with next generation sequencing techniques to identify genes and pathways essential or detrimental for fitness under select conditions. In *S. aureus*, Tn-Seq approaches have been used to explore the genes and pathways important for fitness in various infection models (Begun et al., 2005; Blattner et al., 2016; Christiansen et al., 2014; Grosser et al., 2018; Ibberson et al., 2017; Valentino et al., 2014), antibiotic susceptibility and resistance pathways (Coe et al., 2019; Rajagopal et al., 2016; Santiago et al., 2018; Wang et al., 2011), WTA synthesis (Santa Maria et al., 2014), fitness at high temperatures (Santiago et al., 2015), fitness in the presence of NO (Grosser et al., 2018) and salt stress (Schuster et al., 2020). In this PhD study, I explored whether Tn-Seq is an effective method to identify pathways important for the fitness of *S. aureus* under acid stress conditions.

Most of the genes which were classified as essential for the fitness of *S. aureus* under acid stress conditions in the Tn-Seq experiment resulted in reduced growth on TSA pH 4.5 plates compared to the WT strain when individually inactivated. Furthermore, several of the genes and pathways which were highlighted as important for the fitness and growth of *S. aureus* under acid stress conditions in the Tn-Seq and subsequent validation assays have previously been associated with the acid stress response of *S. aureus*. This supports the use of Tn-Seq as an approach under these conditions.

However, this study highlighted the importance of considering the environmental conditions under which a Tn-Seq experiment is performed. In this Tn-Seq experiment, the urease genes were not highlighted as essential despite having previously been shown to be some of the most upregulated genes in *S. aureus* upon exposure to acidic stress (Anderson et al., 2010; Bore et al., 2007; Rode et al., 2010; Weinrick et al., 2004; reviewed in Zhou & Fey, 2020). This

demonstrates that different pathways are involved in the acidic stress response of *S. aureus* depending on the environmental conditions. It is likely that performing the Tn-Seq experiment under different environmental conditions will reveal other pathways important for the fitness of *S. aureus* under acidic stress conditions.

8.3 Identification of novel genes and pathways that are involved in the acidic stress response of *S. aureus*

The Tn-Seq experiment also identified several genes which have previously not been associated with the acid stress response of *S. aureus*, such as *0846*. *0846* had the lowest ratio in the Tn-Seq experiment, and hence can be deemed one of the most important factors for fitness under acid stress conditions. Interestingly, several previous studies have demonstrated that an *0846* mutant strain has reduced virulence in mice, indicating that the gene is important for the pathogenesis of *S. aureus* (Benton et al., 2004; Grosser et al., 2018; Valentino et al., 2014). Complementation studies confirmed that the reduced growth of the *0846::Tn* mutant strain at low pH was due to the inactivation of the *0846* gene, however its function was unknown and thus it potentially represented a novel pathway in the acid stress response of *S. aureus*.

In this study I conclusively showed that *0846* codes for the main histidine transporter in *S. aureus*. As far as I know, this is the first *in vivo* evidence of a histidine transporter in *S. aureus*. The importance of histidine and its uptake via 0846 for the growth of *S. aureus* under acid stress conditions was demonstrated when the WT strain could only grow in acidic CDM when histidine was present in the medium, in contrast to the *0846::Tn* strain which could not grow regardless of whether histidine was present or not. As far as I am aware, neither histidine transport nor the 0846 transporter has been associated with the acidic stress response of *S. aureus*. Therefore, the work presented in this PhD thesis has identified a novel pathway in the acidic stress response of *S. aureus*.

Interestingly, the acid sensitivity of the *0846::Tn* strain can be compensated for by the induction of the stringent response through the generation of suppressor mutations, as well as likely through pre-growth in CDM. The stringent response is activated in *S. aureus* in response to amino acid-limiting conditions and results in large-scale reorientation of

metabolism and gene expression in bacteria. Among others, inducing the stringent response increases the expression of amino acid transport and biosynthesis pathways. This might support the hypothesis that it is the lack of histidine uptake via 0846 that results in the acid sensitivity of the *0846::Tn* strain.

An analysis of the literature revealed several potential reasons why histidine might be important for the ability of *S. aureus* to grow under acidic stress conditions. Firstly, some bacterial species contain histidine decarboxylase systems which decarboxylate histidine to histamine and consume a proton in the process. However, an analysis of the genome of *S. aureus* did not reveal any genes with homology to any known groups of histidine decarboxylase enzymes. Secondly, histidine can be catabolized into glutamate via the Hut process, through which ammonia is produced as a by-product. However, inactivating either of the first two enzymes in this process did not confer a growth defect compared to the WT strain on TSA pH 4.5 plates. Finally, histidine is capable of acting as a buffer at physiological pH due to its side chain having a pK_a value of 6.0. Cytoplasmic pH measurements initially appeared to suggest that the reason for the importance of histidine for the growth of *S. aureus* at low pH is due to its ability to buffer the cytoplasm, as the *0846::Tn* bacteria had a lower cytoplasmic pH than the WT bacteria when grown in TSB pH 4.5 medium.

Initially I hypothesized that the *0846::Tn* bacteria had lower concentrations of intracellular histidine due to a lack of histidine uptake. I hypothesized that this results in a reduced ability to buffer the cytoplasm compared to the WT strain, which results in a growth defect under acid stress conditions. However, the similar cytoplasmic pH of the suppressor strain compared to the *0846::Tn* strain when grown in TSB pH 4.5 medium suggests that it is not the actual value of the cytoplasmic pH that causes a growth defect under acid stress conditions, but rather the change in cytoplasmic pH when the bacteria are moved from neutral to acid stress conditions.

Additional work in the lab has further disproven this hypothesis. Measurements of intracellular histidine concentrations via mass-spectroscopy revealed that the *0846::Tn* mutant strain has increased intracellular concentrations of histidine compared to the WT strain (experiment performed by Dr. Igor Kviatkovski and Dr. Elena Chekmeneva, Dr. Carolina Sands and Lynn Maslen from the National Phenome Centre, data from Beetham et al., 2024).

It is clear that the *0846::Tn* mutant strain is capable of synthesizing enough histidine to compensate for the lack of uptake. This is supported by additional quantitative reverse transcription-PCR (qRT-PCR) experiments that were performed in the lab, determining the expression of the histidine biosynthesis genes *hisD* and *hisG*, which showed that the *0846::Tn* strain has greatly increased expression of these genes compared to the WT strain (experiment performed by I. Kviatkovski, data from Beetham et al., 2024). Taken together, these additional experiments suggest that it is not the presence of intracellular histidine that is important for the growth of *S. aureus* at low pH. Instead, these experiments highlight a key difference between the WT and mutant bacteria, whereby the former import histidine while the latter need to synthesise it. This therefore suggests that the process of histidine biosynthesis results in a growth defect when grown under acid stress conditions. The qRT-PCR experiments also revealed that the suppressor strains did not have increased expression of *hisD* and *hisG* compared to the original *0846::Tn* strain (experiment performed by I. Kviatkovski, data from Beetham et al., 2024). This suggests that the reason why inducing the stringent response compensates for the acid sensitivity of the *0846::Tn* strain is likely not due to increased histidine biosynthesis, but could instead result in other metabolic changes which compensate for the increased histidine biosynthesis.

Histidine is synthesized from Phosphoribosyl pyrophosphate (PRPP) through a process involving 10 enzymatic reactions which are identical in all organisms which produce histidine (Fig. 8.1) (reviewed in Alifano et al., 1996). The process is very energetically expensive, using 41 molecules of ATP per molecule of histidine produced (reviewed in Alifano et al., 1996). This explains why *S. aureus* would prefer to import rather than synthesise histidine. Furthermore, due to its imidazole ring, the biosynthesis of histidine is interconnected with both nitrogen metabolism and the *de novo* synthesis of purines, and therefore histidine biosynthesis represents a metabolic cross-road linking several key metabolic pathways in the bacterial cell (reviewed in Alifano et al., 1996).

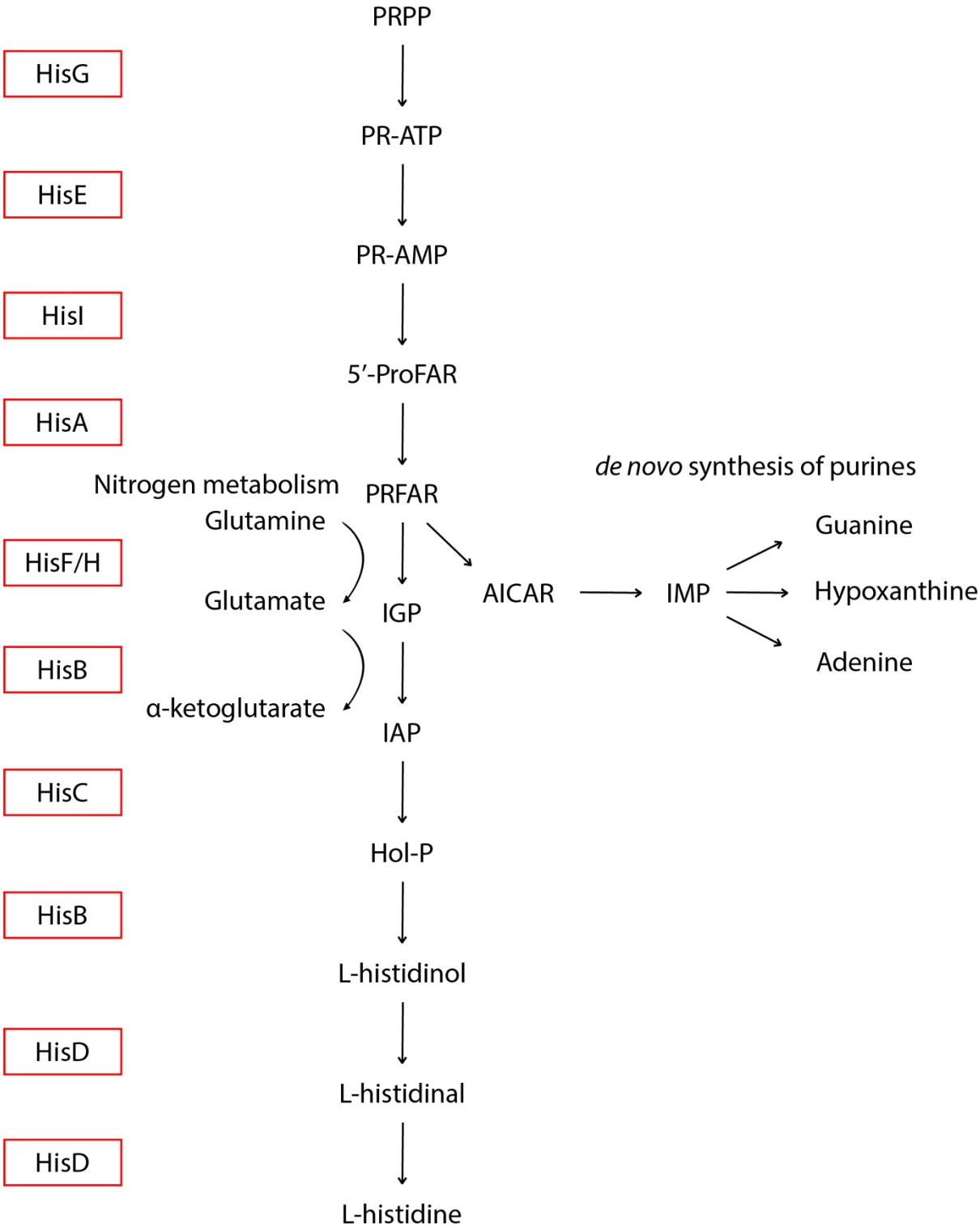


Figure 8.1: Schematic representation of the biosynthesis of histidine
Histidine is synthesised from PRPP in 10 steps. This process is interconnected with both nitrogen metabolism and the de novo synthesis of purines. The enzymes which catalyse each reaction in *S. aureus* are shown in red boxes on the left.

The increased biosynthesis of histidine in the *0846::Tn* strain may therefore affect the metabolic state of the bacterium through either diverting precursor molecules to this pathway from other pathways, or through increased production of by-products in the histidine biosynthesis pathway. Measurements of the intracellular concentration of several compounds by mass-spectroscopy revealed several whose concentration is either higher or lower in the *0846::Tn* strain than the WT strain (experiment performed by I. Kviatkovski, and E. Chekmeneva, C. Sands and L. Maslen from the National Phenome Centre, unpublished data). For example, the concentration of glutamine is higher in the *0846::Tn* strain than in the WT strain (experiment performed by I. Kviatkovski, and E. Chekmeneva, C. Sands and L. Maslen from the National Phenome Centre, unpublished data). During the process of producing histidine, a molecule of glutamine is deaminated to form glutamate, which is further deaminated to alpha-ketoglutarate (Fig. 8.1). The fact that glutamine is increased in the *0846::Tn* strain indicates that the homeostasis of nitrogen metabolism is disrupted in the *0846::Tn* mutant strain compared to the WT strain.

Furthermore, histidine biosynthesis intersects with purine biosynthesis. The molecule 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) is produced as a by-product during histidine biosynthesis (Fig. 8.1), and AICAR can be converted to inosine monophosphate (IMP) which is a central metabolite in purine metabolism. The intracellular concentrations of several metabolites in purine metabolism were shown to be altered in the *0846::Tn* strain compared to the WT strain (experiment performed by I. Kviatkovski, and E. Chekmeneva, C. Sands and L. Maslen from the National Phenome Centre, unpublished data). For example, the concentration of hypoxanthine is higher in the *0846::Tn* strain than the WT strain, while the concentration of guanine is lower (experiment performed by I. Kviatkovski, and E. Chekmeneva, C. Sands and L. Maslen from the National Phenome Centre, unpublished data). Interestingly, one of the mutations which was present in the original NTML JE2 *0846::Tn* strain which showed both decreased growth and survival under acid stress conditions compared to the JE2 WT strain was a SNP in *apt*. Apt is an adenine phosphoribosyltransferase which converts adenine to AMP and is involved in the purine salvage pathway. The original NTML JE2 *0846::Tn* strain showed increased growth on TSA pH 4.5 plates compared to the transduced JE2 *0846::Tn* strain, which could suggest that changing the concentration of purines in the cell compensates for the acid sensitivity of the *0846::Tn* strain. However *apt* is

found in the staphylococcal genome upstream of *rsh*, and therefore it could also be the case that a mutation in *apt* affects *rsh* expression which may result in an activated stringent response.

Taken together, these data show that the *0846::Tn* strain, which has increased histidine biosynthesis compared to the WT strain to compensate for the reduced import of histidine through the 0846 histidine transporter, is in a different metabolic state which affects several key metabolic pathways including amino acid synthesis, nitrogen metabolism, and *de novo* synthesis of purines. It is difficult to isolate one pathway which explains the reason why the *0846::Tn* strain has a growth defect under acid stress conditions, and is likely to be a combination of these pathways.

In conclusion, I hypothesise that the reason why the 0846 histidine transporter is important for the growth of *S. aureus* under acidic stress conditions is that it allows the bacteria to import histidine (Fig. 8.2). When the 0846 histidine transporter is inactivated, the bacteria compensate by increasing the biosynthesis of histidine. I hypothesize that this biosynthesis of histidine affects several metabolic pathways in the bacteria which affects its ability to grow under acid stress conditions.

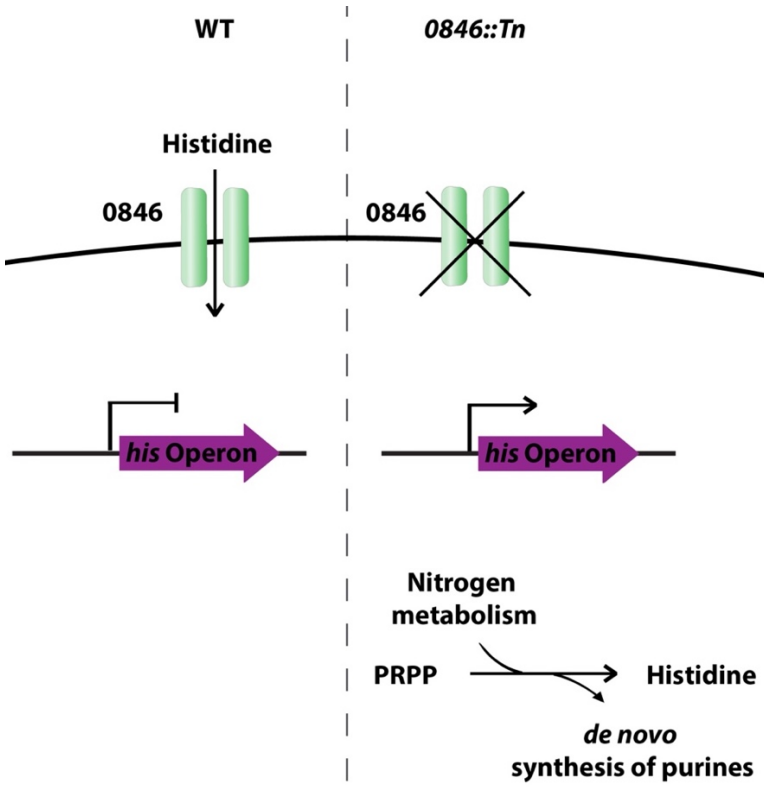


Figure 8.2: Schematic representation of histidine transport versus biosynthesis in the WT and the *0846::Tn* mutant strains
WT *S. aureus* imports histidine via the 0846 histidine transporter. When 0846 is inactivated, the *0846::Tn* mutant *S. aureus* strain increases expression of the *his* operon, resulting in increased histidine biosynthesis. This affects several other metabolic pathways such as nitrogen metabolism and the *de novo* synthesis of purines. It is likely that this reorientation of the bacterial metabolism results in a growth defect at low pH.

8.4 Future directions

To explore why histidine biosynthesis is detrimental for the growth of *S. aureus* under acidic stress conditions, the difference in metabolic pathways between the WT and *0846::Tn* strains could be explored. This could firstly be done by expanding the transcriptional studies to investigate other pathways which are either upregulated or downregulated in the *0846::Tn* strain as a consequence of increased histidine biosynthesis. For example, the expression of genes coding for key enzymes converting the purine precursor molecule AICAR into purine compounds could be measured using qRT-PCR experiments. These genes could include *purH* which converts AICAR into IMP, *guaB* and *guaA* which convert IMP into guanine, *deoD1* and *deoD2* which convert IMP into hypoxanthine, and *purA* and *purB* which convert IMP to

adenine. Exploring the expression of these genes would reveal whether the *de novo* synthesis of purines is affected by increased histidine biosynthesis, which may be the reason for the growth defect of the *0846::Tn* mutant strain under acid stress conditions.

To further investigate whether other metabolic pathways which intersect with histidine biosynthesis are affected in the *0846::Tn* mutant strain, the intracellular concentration of other metabolites could be measured by mass-spectroscopy. For example, it would be interesting to know whether the concentrations of other metabolites that are produced from PRPP differ in the *0846::Tn* mutant compared to the WT strain, as this would indicate whether PRPP is diverted to histidine biosynthesis instead of other processes. Furthermore, the intracellular concentration of other metabolites in nitrogen metabolism or the *de novo* synthesis of purines could be measured to explore whether either of these pathways are affected. It would also be interesting to explore whether either the expression of genes or other metabolites differ in the suppressor strain as well, as this might reveal why inducing the stringent response compensates for the reduced growth of the *0846::Tn* strain under acid stress conditions.

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