Understanding antimicrobial and antiseptic resistance relationships in *Staphylococcus aureus*

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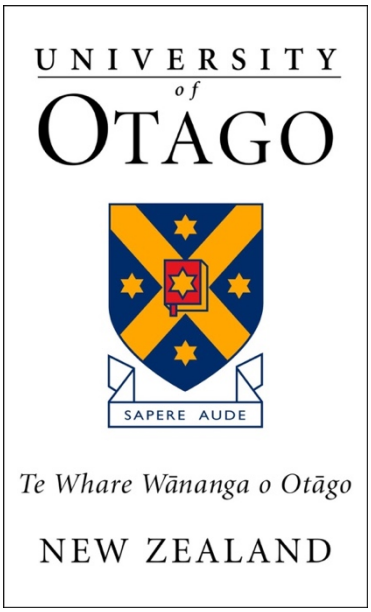
(BSc)

A report submitted in partial fulfilment of the Degree of Master of Science



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Abstract

Staphylococcus aureus is an opportunistic pathogen that causes skin infections, bacteraemia, and endocarditis in humans. In New Zealand, the common lineage of *S. aureus* is the ST1 lineage which harbours a fusidic acid resistance gene (*fusC)* on its chromosome and can carry the multi-drug resistance plasmid pNZAK1. pNZAK1 confers resistance to mupirocin (*mupA)*, ampicillin (*blaZ*), and chlorhexidine (*qacA*). Chlorhexidine is a rapid and effective antiseptic widely used in healthcare to disinfect equipment and patients to prevent bacterial growth. The pNZAK1 plasmid encodes *qacA,* which produces a multi-drug efflux pump that can confer tolerance to chlorhexidine. The aim of this study was to investigate the maintenance of pNZAK1 in *S. aureus* 14487 using a continuous culture system to impose energy limiting conditions and examine the variation in antimicrobial sensitivity and viability. The second objective was to investigate the role of *qacA* using a Δ*qacA* mutant in antimicrobial sensitivity assays against an isogenic $qacA^{+}$ wild-type, specifically looking at tolerance to biocides like chlorhexidine or benzalkonium chloride. Our results indicated that low levels of chlorhexidine can increase gene expression of *qacA, mupA,* and *norA*. Additionally, *qacA* can increase survival of bacteria under antimicrobial pressure from biocide mixtures like Trigene. These data also showed that pNZAK1 was highly stable (>94%) in *S. aureus* 14487 and was maintained even without selection pressure under generalised nutrient limitation. Bacteria that lost pNZAK1 (5-6%) were isolated and showed an increased antimicrobial sensitivity. To investigate key mechanisms of plasmid maintenance we focused on the bacteriocin *lactococcin* 972, and the toxin/antitoxin system *pepA1.* By creating single gene knockouts we observed no change in pNZAK1 plasmid maintenance. These results suggest that pNZAK1 is a highly stable multi-drug resistant plasmid and that plasmid-encoded genes such as *qacA* can be induced by sub-MIC levels of commonly used antiseptics such as chlorhexidine and can be beneficial to survival.

Acknowledgments

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List of Abbreviations

AMR	antimicrobial resistance
Amp	ampicillin
β	Beta
BHIB	brain heart infusion broth
BHIA	brain heart infusion agar
BAC	benzalkonium chloride
bp	Base Pairs
˚C	degrees celsius
CA-MRSA	community acquired – methicillin-resistant *Staphylococcus aureus*
cDNA	complementary DNA (DNA synthesised from RNA)
CFU/mL	colony forming units / millilitre
CHX	chlorhexidine
cm	centimetre
Cm	chloramphenicol
CT	cycle threshold
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EF-G	elongation factor-G
FA	fusidic acid
FA-S	fusidic acid – sensitive
FA- R	fusidic acid– resistant
g	gram
h	hour
HGT	horizontal gene transfer
In	inverse log
LB	Lysogeny (Luria) Broth
OD_{600}	optical density 600 nanometres
PBP	penicillin binding protein

pmol	picomoles
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PVL	Panton-Valentine leukocidin
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
Min	minute
mm	millimetre
nM	nanomolar
nm	nanometre
µg	microgram
µm	micrometre
µL	microlitre
mg	milligram
mL	millilitre
MDR	multidrug resistant
MHB	cation adjusted Mueller-Hinton broth
MHA	cation adjusted Mueller-Hinton agar
MQ-H_2O	milli-Q water
MSSA	methicillin-susceptible *Staphylococcus aureus*
MRSA	methicillin-resistant *Staphylococcus aureus*
RT-qPCR	Reverse transcription polymerase chain reaction
QacA	MDR efflux pump encoded by *qacA*
rpm	revolutions per minute
Rif	rifampicin
Rif^R	rifampicin resistant
RNA	ribonucleic acid
Spa	*Staphylococcus aureus* protein A
s	second
ST	sequence type
TA	Toxin/Antitoxin
TAE	tris-acetate-ethylenediaminetetraacetic acid (EDTA)

TSB	tryptic soy broth
TSA	tryptic soy agar
UV	ultra violet
v	volume
V	volts
v/v	volume to volume
w	weight
WGS	whole genome sequencing
w/v	weight to volume
Δ	gene deletion
× *g*	relative centrifugal force (RCF)

Chapter 1

Introduction

1.1. *Staphylococcus aureus*: life as an opportunistic pathogen

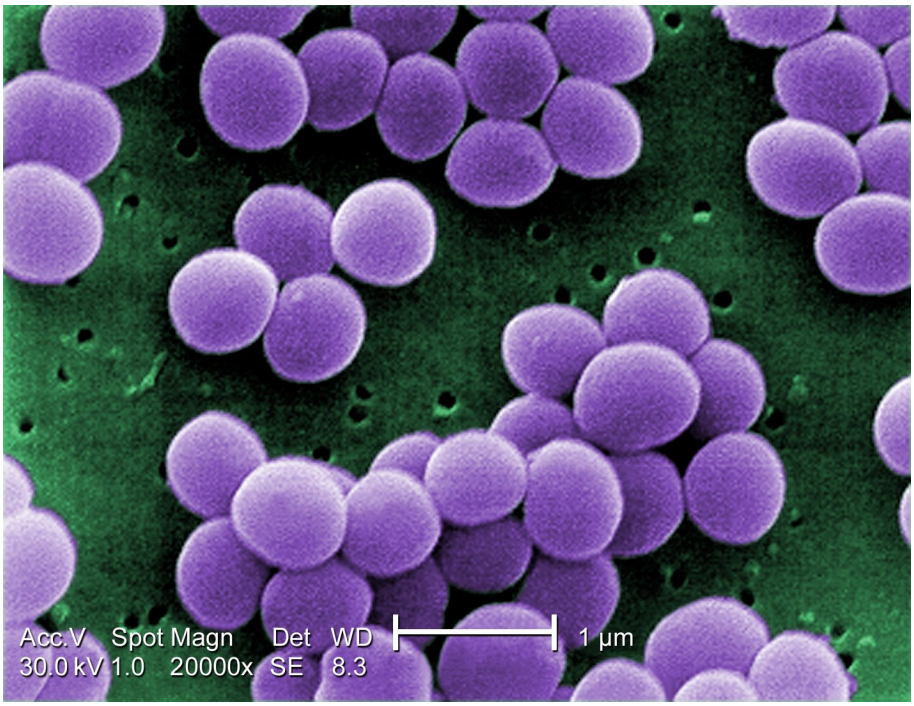


Figure 1.1. Electron microscope image of *Staphylococcus aureus*. (Photo: Janice Haney Carr, Centers for Disease Control (CDC))

The genus *Staphylococcus* are a group of organisms which are members of the *Firmicutes* (1). *Staphylococcus* were discovered in 1880 from surgical pus by Dr. Alexander Ogston and were named *Staphylococcus* because of their grape-like clustered appearance under the microscope (2). The name *Staphylococcus* comes from the Greek *staphyle* (cluster, bunch of grapes) and *kokkos* (seed, grain, berry) (Fig. 1.1). These organisms can colonise the skin, nose and mucus membranes of humans, and animals, such as the teat of cows which can lead to mastitis (3). *Staphylococcus* are normal commensals that can protect the host by producing compounds which limit growth the growth of pathogens (4). *Staphylococcus* can cause fatal soft-tissue infections (including abscesses), known colloquially as '*staph*' infections, respiratory infections, as well as food poisoning. Some strains are also know to produce Panton-Valentine leukocidin (PVL), alphahemolysin (also known as alpha-toxin) as virulence factors (5). PVL lyse white blood cells reducing the body's ability to fight off the infection and alpha-toxin forms pores in human cells for instance; red blood cells or keratinocytes, leading to a more

serious '*staph*' infection (5). Strains of *Staphylococcus* are also known to form biofilms on surgical implants, IVs, or even catheters, which allows the bacteria to evade the host immune response and become resistant to antibiotics as the cells enter stationary phase (5). There are more than 32 different species of *Staphylococcus*. The most significant species and the most well characterised species is *S. aureus*, a catalase- and coagulase- positive bacterium, classified as a facultative anaerobe (6). The facultative nature of this Gram-positive bacterium allows it to proliferate in aerobic and anoxic environments, switching between anaerobic metabolism and aerobic respiration (6). *S. aureus* is able to withstand high sodium chloride concentrations (0.5-13.5%), pH ranges of 4 to 10, and temperature ranges of 7˚C to 48˚C, with a temperature optimum of 37˚C (7). *S. aureus* can contain many antibiotic resistance elements like the SSC*mec* cassette found on the chromosome of MRSA (methicillin-resistant *Staphylococcus aureus)* (8). SSC*mec* encodes the gene *mecA* that confers resistance to methicillin by encoding an alternate penicillin binding protein (PBP) called PBP2a that irreversibly binds to β-lactam antibiotics (9).

1.1.1. Staphylococcus aureus: hospital acquired infections

S. aureus colonises approximately 30% of the human population on skin and nasal mucous membranes without adverse effects, such individuals then act as carriers (5). The ability of *S. aureus* to colonise a high percentage of the human population asymptomatically allows the spread of the organism within a community. However, *S. aureus* is also an opportunistic pathogen and can be highly infectious due to its many virulence factors, such as coagulase, protein A, and fibronectin-binding proteins (6). *S. aureus* is one of the major causes of nosocomial (hospital associated) infections, particularly in immunocompromised patients, where it causes skin infections, bacteraemia, toxic shock syndrome, and endocarditis (10). The ability of *S. aureus* to form biofilms allows it to persist and replicate on medical devices, contributing to hospital-acquired infections. These infections, once readily treatable, are becoming increasingly fatal due to a rise in antimicrobial resistance (AMR) (5). *S. aureus* isolates have become resistant to many clinically-relevant antibiotics, leading to outbreaks of highly resistant clones e.g. MRSA in healthcare facilities and communities (11).

1.1.2. Staphylococcus aureus: community associated infections

Before antibiotics like penicillin were commonly available, minor scratches could potentially lead to a life threatening illness caused by *Staphylococcus* (12). As such, the increase of drug-resistant strains of *S. aureus* circulating within the community pose a serious threat to the immunocompromised who are at risk of infection (12). Resistance elements like SCCmec, which encodes *mecA* that confers methicillin resistance, however, often come with a fitness cost associated with the carriage of these elements (13). Therefore, the limited level of antibiotic selection tends to favour non-MRSA staphylococcal strains in the community (12). The MRSA strain USA300 first came in to the spotlight in 2000 after a CDC investigation into a MRSA outbreak in football players (14). The same strain was also found to be the cause of outbreaks in prison communities around the USA (14). Community-acquired MRSA infections have increased over the past 20 years highlighting the potential health care burden of drug resistant *S. aureus* infections in communities (11,15).

1.1.3. General mechanisms of antimicrobial resistance in *S. aureus*

The rise in AMR observed in *S. aureus* isolates is due to the bacteria's ability to acquire resistance to antimicrobials through horizontal gene transfer (HGT) and chromosomal mutations. Specific chromosomal mutations have been observed which can upregulate the expression of existing resistance mechanisms such as efflux pumps like NorA which encodes resistance to quaternary ammonium compounds like benzalkonium chloride and biguanidines such as chlorhexidine (16).

1.1.4. Acquired resistance in *S. aureus*

Acquired genetic elements can encode alternative genes that can bypass the target of an antibiotic (17). This occurs for example in the development of resistance to β-lactam antibiotics, such as ampicillin. This class of antibiotic targets the DD-transpeptidase activity of penicillin-binding proteins (PBPs) to prevent crosslinking of peptidoglycan and inhibit cell wall synthesis (18). *S. aureus* can acquire *mecA*, which encodes an alternative PBP, PBP-2a,

that has low affinity for β-lactams, or the *blaZ* gene which encodes a β-lactamase, an enzyme which cleaves the β-lactam ring of the antibiotic (19). These resistance mechanisms allows *S. aureus* to proliferate even in the presence of β-lactam antibiotics making it more difficult to treat (16).

1.1.5. Current treatment of *S. aureus* infections

Typically, MSSA infections are treated with oral β-lactam drugs, but MRSA tend to be resistant to β-lactam antibiotics such as methicillin due to the SCC*mec* element (20). The lincosamide antibiotic clindamycin is commonly used in uncomplicated soft-tissue infection (20). For most '*staph*' infections the abscess would be drained and sterilised with an antiseptic while the patient also received oral antibiotics, to reduce the risk of reinfection. For more long-term treatment of invasive *Staphylococcus* infection, IV β-lactam antibiotics for MSSA or IV vancomycin for MRSA are used (20).

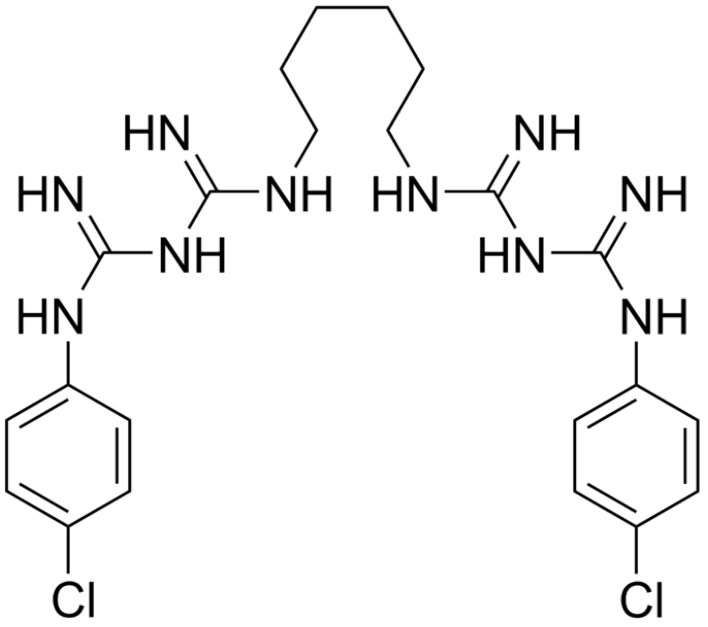
1.2. Biocide tolerance in pathogenic bacteria

Biocides are compounds which have broad spectrum activity against various Gram-positive and Gram-negative bacteria (21). Biocides are used as antiseptics and disinfectants in hospitals, agriculture and the food processing industry (22). Commonly used compounds are biguanides (chlorhexidine) and quaternary ammonium compounds (benzalkonium chloride (BAC)), which are used as antiseptics to disinfect patients and healthcare environments (22). Antiseptic solutions like Trigene, which is a mixture of quaternary ammonium compounds like BAC, polymeric biguanide hydrochloride, and dodecylamine, are also commonly used as disinfectants (23). Given their critical role in human medicine, a number of these compounds are on the World Health Organisation (WHO) list of essential medicines. Unfortunately, due to the widespread and indiscriminate use of these essential biocide compounds it has generated an environment which fosters the growth of bacteria that can tolerate these compounds decreasing their efficacy (22).

1.3. Chlorhexidine and other biocides

Chlorhexidine (Fig. 1.2A) is a highly potent biocide with rapid bactericidal action against both Gram-positive, and Gram-negative bacteria and acts by disrupting the bacterial cell wall by interacting with negatively charged phosphate groups like teichoic acid (Fig. 1.2B) (24). When chlorhexidine is used at low concentrations it has a bacteriostatic effect causing disruptions to membrane integrity (26-27). At high concentrations chlorhexidine affects the cytoplasmic contents of the cell leading to swift bactericidal activity and cell death (26-27). Chlorhexidine is commonly used as an antiseptic on patients and hospital equipment to reduce flora levels and limit infection by pathogens (25,27-28). A study in the UK showed that handwash containing 4% chlorhexidine immediately reduced bacterial levels on the skin by 86% and by 99.2% after 6 applications (6 hours) (29). This highlights the effectiveness of chlorhexidine and its ability to limit the spread of pathogens from person to person (29).

A



B

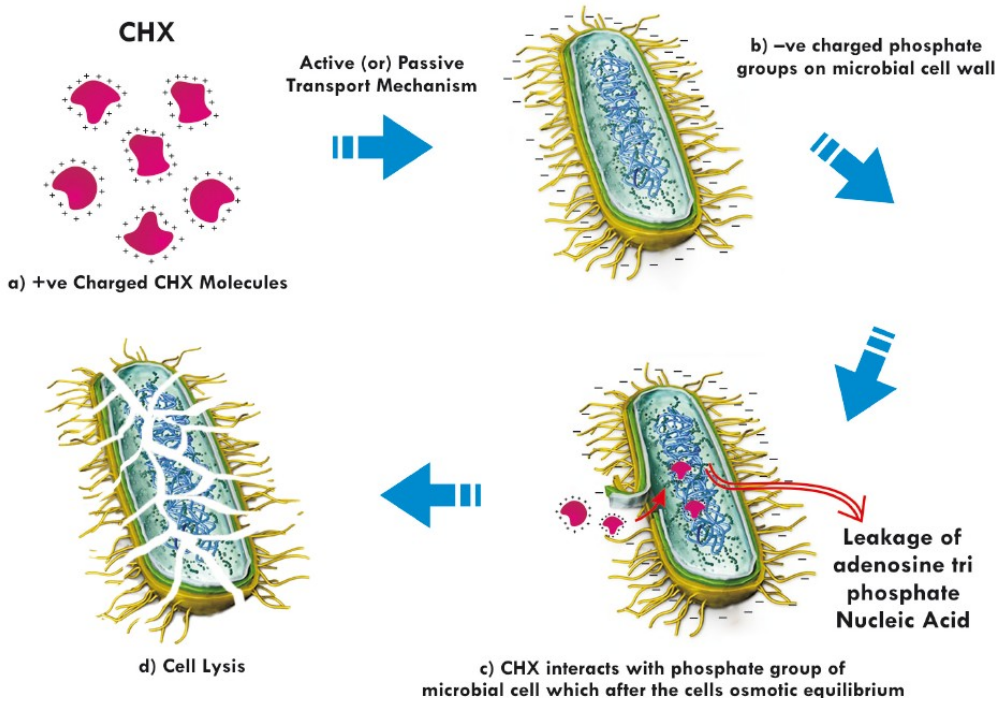


Figure 1.2. Structure and mechanism of chlorhexidine. (**A**) shows the chemical structure of chlorhexidine and is from a study by Chapman *et al.* (30). (**B**) demonstrates the mechanism of action of chlorhexidine. Chlorhexidine binds to negatively charged phosphate groups in the bacterial cell wall triggering leakage of cell contents and cell death. (**B**) is modified from Kandaswamy *et al.* (31)

Chlorhexidine has been used as a disinfectant and antiseptic since the 1950s, and due to its effectiveness, relative low cost and safety it is listed by the WHO as an essential medicine needed in a healthcare setting (32). As such, it is crucial that we increase our understanding about the emergence of chlorhexidine tolerance in bacteria.

1.3.1. Evolution of tolerance and resistance in bacteria

When a bacterium becomes tolerant to a compound it is able to survive longer in the presence of that compound. The intense pressure to survive generates resistant mutants that become more prevalent in the population (33). Tolerance to biocides is a major issue because when a bacterium develops an elevated minimum inhibition concentration (MIC) or minimum bactericidal concentration (MBC). It then has the potential to acquire further genetic elements through horizontal gene transfer or mutations to further evolve its tolerance compound into resistance (Fig. 1.3A-B) (33).

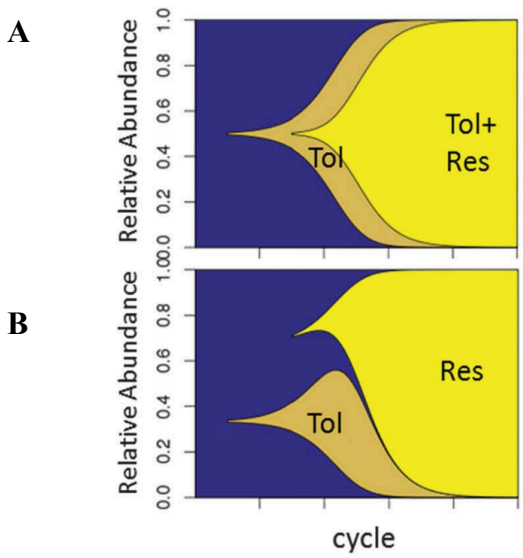


Figure 1.3. Representation of potential ways tolerant and resistant mutants appear in a bacterial population over time. (**A**) Tolerant-mutants (Tol) appear in the population and are positively selected for in the presence of external pressure (e.g. biocides), eventually becoming dominant in the population. These tolerant isolates then acquire secondary mutations leading to the development of resistance (Res). (**B**) Tolerant mutant population appear initially and then dissipate in frequency simultaneously as the resistance mutant population develops and dominates (33).

The increase in antiseptic and biocide tolerance is an issue as biocides are used to disinfect patients and surfaces in healthcare settings to reduce the bacterial load and limit patient infection. Biocide tolerance is not limited to just one organism, as it has been observed in many bacteria including both Gram-negative and Gram-positive pathogens including: *E. coli, S. aureus, Listeria monocytogenes,* and *Enterococcus faecium* (34). However, certain bacteria are more intrinsically resistant to membrane-based sanitisers like chlorhexidine or BAC such as Gram-negative bacteria, mycobacteria and spore-forming bacteria (35). The widespread use of disinfectants, especially in a clinical setting, has selected for resistant bacteria (36).

1.4. The role of biocides in co-selecting for antibiotic resistance

Recently, plasmid-encoded antiseptic tolerance genes such as *qacA*, which encodes the MDR efflux pump QacA, have been described as a mechanism of plasmid maintenance whereby the selective advantage of having a plasmid that carries biocide tolerance genes increases the plasmid carriage in the population (37). These well-maintained plasmids can then acquire antibiotic resistance genes such as *mupA* or *blaZ* and increase the amount of cross-resistance between antibiotics and antiseptics (37).

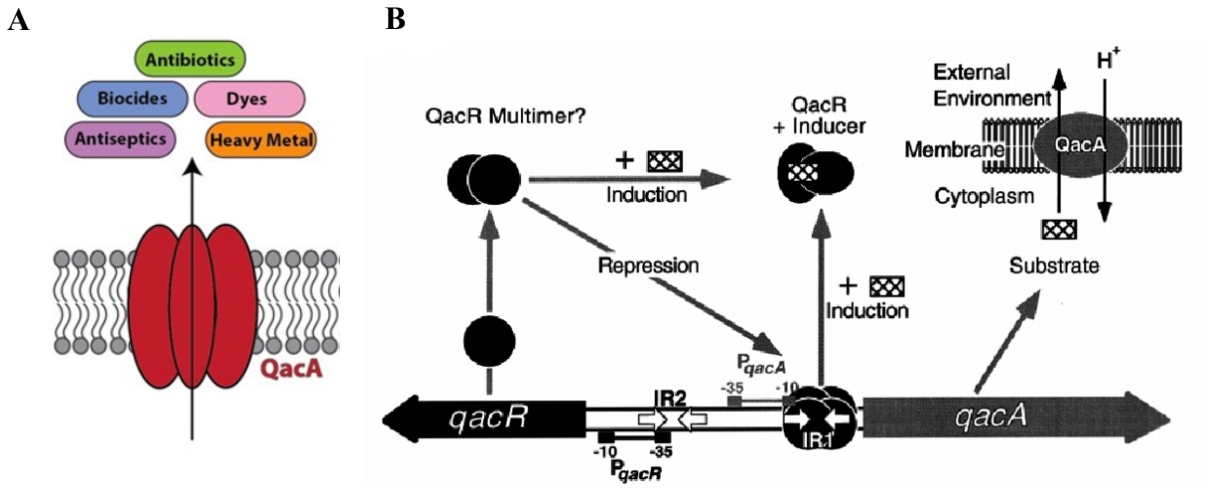


Figure. 1.4. The mechanism and regulation of the QacA multidrug efflux pump. (**A**) Schematic representation of the QacA multidrug efflux pump with its proposed substrate range reproduced from Venter *et al*. (38). (**B**) Schematic overview of the QacA efflux pump and the gene repression by QacR from Brown *et al*. (39).

In *S. aureus*, tolerance to chlorhexidine is mediated by the membrane embedded *qacA*-encoded multidrug efflux pump QacA (Fig. 1.4.) (40). QacA was discovered in the 1980s on the plasmid pSKI in *S. aureus* (41-42). QacA has a large range of potential substrates such as quaternary ammonium compounds like BAC, biguanides like chlorhexidine, diamidines such as pentamidine, and dyes like Ethidium bromide (42). QacA works through using the energy-dependent proton-motive force to pump compounds out of the cell (42). *qacA* is regulated by *qacR,* which produces a gene product that represses *qacA* transcription (Fig. 1.4B) (43). Another important efflux pump NorA was discovered in 1986 in a Japanese hospital from a fluoroquinolone-resistant *S. aureus* strain (41). The MDR efflux pump NorA encoded by *norA* on the chromosome shares similar substrates to QacA, but also confers resistance to fluoroquinolone antibiotics (42). Similar to QacA, NorA is also part of the major facilitator superfamily of drug efflux pumps (41).

Given the clinical importance of MDR *S. aureus*, the potential selection of *qacA*-positive bacteria by hospital antiseptics is a critical area for future investigation. Does the use of chlorhexidine in hospitals co-select for the other resistant genes in MDR *S. aureus* by selecting for bacteria carrying the *qacA* gene? Plasmids conferring antimicrobial resistance tend to be maintained when it is advantageous for the bacteria in that environment, due to the fitness cost of maintaining a plasmid in non-selective conditions (44). Bacteria that are not expending energy maintaining a plasmid have an advantage over those bacteria in the population when growing in an antibiotic-free environment (44).

Decreased susceptibility to disinfectants is a major concern for human healthcare as a number of opportunistic pathogens which are tolerant to the concentrations of sanitisers used clinically, can also be resistant to many clinically-relevant antibiotics (36). A study by Climo *et al.* (2013) showed that daily bathing with 2% chlorhexidine over 6 months in the ICU reduced the amount of bloodstream infections and colonisation of multi-drug resistant organisms (45). However, when bacteria are tolerant to antiseptics like chlorhexidine it means that they could remain present on the patient and then could potentially infect the patient and cause a persistent drug resistant infection (22,45). Increased infection frequency intensifies the burden on the healthcare system, for example, strains of *E. coli* can be resistant to the disinfectant triclosan

(mediated through the AcrAB-TolC efflux pump) as well as the antibiotics chloramphenicol, erythromycin, imipenem, tetracycline, trimethoprim, and others (46). Isolates of *L. monocytogenes* can harbour a mechanism of BAC resistance, encoded by the BAC resistance cassette *bcrABC,* as well as genes mediating resistance to the antibiotics erythromycin, tetracycline, and trimethoprim resistance (47). *P. aeruginosa* has a tripartite efflux system encoded on its chromosome, MexAB-OprM, which plays an important role in the resistance to triclosan and chlorhexidine (38,48). As discussed efflux pumps are one of the main mechanisms that convey resistance to antimicrobial compounds e.g. AcrAB-TolC, MexAB-OprM, QacA, and NorA (Fig 1.5) (38). Unfortunately, the use of chlorhexidine in burn patients to prevent infection has been suggested to select for resistance against last line antibiotics like colistin due to MexAB-OprM and the *pmr* operon, which controls lipopolysaccharide modification (38,48). A study by Pamp (2008) showed that *P. aeruginosa* mutants lacking either the MexAB-OprM or the *pmr* operon were unable to form an effective biofilm that was tolerant to colistin (48).

Broadly, bacteria are able to develop resistance to sanitisers by gene acquisition through HGT, or mutating chromosomal genes involved in the synthesis of the cell membrane. One example of this has been observed in Gram-positive bacteria where increased peptidoglycan crosslinking, leading to a thicker cell wall was found to limit entry of biocides like CHX and phenoxyethanol into the cell (38,49).

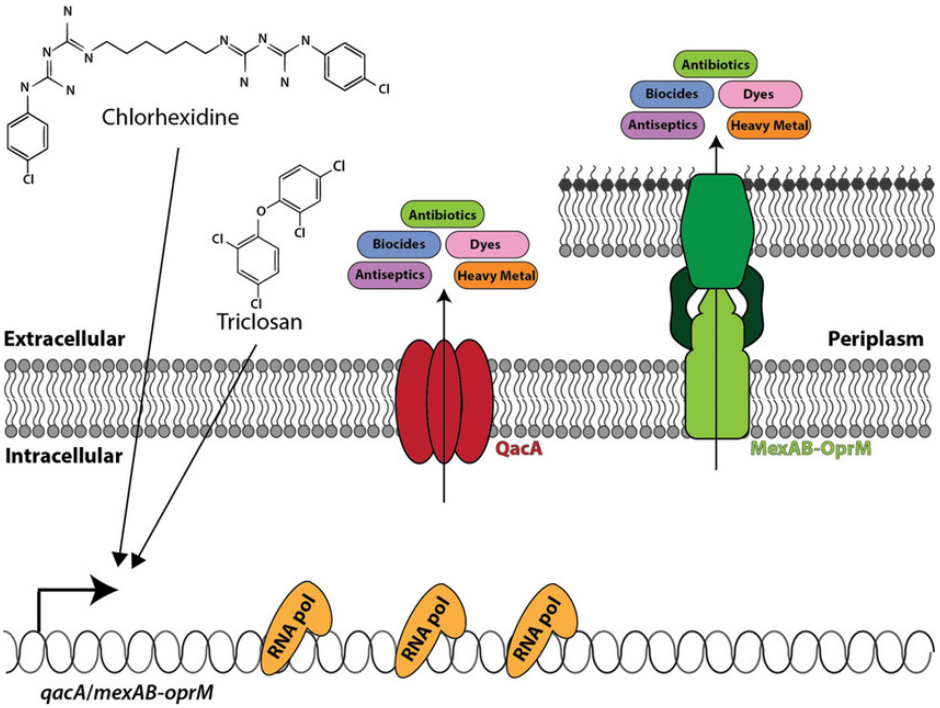


Figure 1.5. Multi-drug efflux pump schematic of QacA and MexAB-OprM, two independent systems which confer tolerance to antiseptics. QacA can be found in the Gram-positive opportunistic pathogen *S. aureus,* while MexAB-OprM is found in the Gram-negative opportunistic pathogen *P. aeruginosa*. Both of these MDR efflux pumps confer resistance to antibiotics as well as antiseptics like chlorhexidine or triclosan. These two systems enable either *S. aureus* or *P. aeruginosa* to tolerate/survive antimicrobial challenge in healthcare settings. This figure is reproduced from Venter *et al.* (38).

1.5. Spread of MRSA in New Zealand

MRSA was first discovered in the Auckland region of New Zealand in 1975 (Fig 1.6) (15,50). Since 1991, the community reservoir of MRSA in New Zealand has been increasing (15), and in the last few decades, MRSA has spread throughout New Zealand (50). The spread of MRSA in New Zealand is due to a number of factors, including the overuse of antibiotics (especially topical antibiotic agents like fusidic acid), exchange of bacteria between community and healthcare settings, and international travel increasing imported resistant bacteria such as USA300 MRSA (50). In New Zealand, MSSA (methicillin-susceptible *S. aureus)* causes the

majority of both hospital and community infections, whereas MRSA infections occur at a lower prevalence. Between 2005 and 2014, the period-prevalence of MRSA in New Zealand increased from 12.9 to 23.8 MRSA-positive cases per 100,000 people. Most of these infections were community-acquired (51). CA-MRSA has started to change over the last decade in the New Zealand setting with an increasing number of clones becoming resistant to antibiotics such as fusidic acid (51).

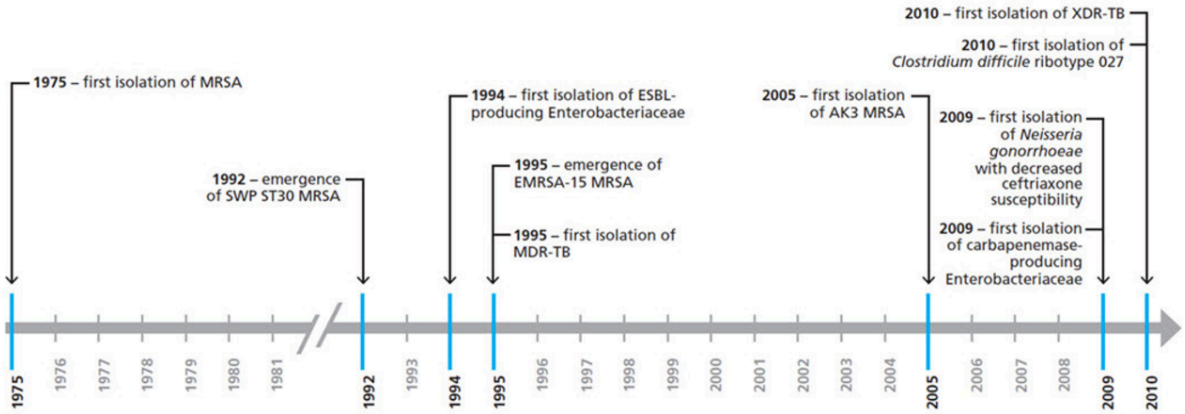


Figure 1.6. A timeline of significant events in antimicrobial resistance in New Zealand. The South West Pacific (SWP) strain of MRSA appeared in New Zealand in 1992. Extended-spectrum β-lactams (ESBL) producing Enterobacteriaceae were first isolated in 1994. Epidemic resistant MRSA (EMRSA-15) first emerged in 1995. Multi-drug resistant tuberculosis (MDR-TB) was first isolated in 1995. Extensively drug resistant tuberculosis (XDR-TB) was first isolated in 2010. The graph is reproduced from a study by Williamson *et al.* (50).

In New Zealand, the ST1 lineages of MSSA and MRSA contain a chromosomal *fusC* gene (Fig. 1.7) (10,11). Fusidic acid inhibits *S. aureus* by binding to elongation factor-G (EF-G), inhibiting protein synthesis. The gene *fusC* encodes the FusC protein which blocks binding of fusidic acid to the drug target site in EF-G (52,53). The widespread use of fusidic acid in topical creams such as Fusidin (LEO) has provided a mechanism for increased selection of the *fusC* resistance gene in the *S. aureus* population (52). To be able to distinguish different sub-types of *S. aureus* into epidemiological lineages spa typing used the sequencing from the variable coding region from protein A in *S. aureus* (54). Spa t127 is highly resistance to fusidic acid in both MSSA and MRSA strains (Fig. 1.7). This is an issue as spa t127 is one of the predominant clonal lineages in New Zealand (55).

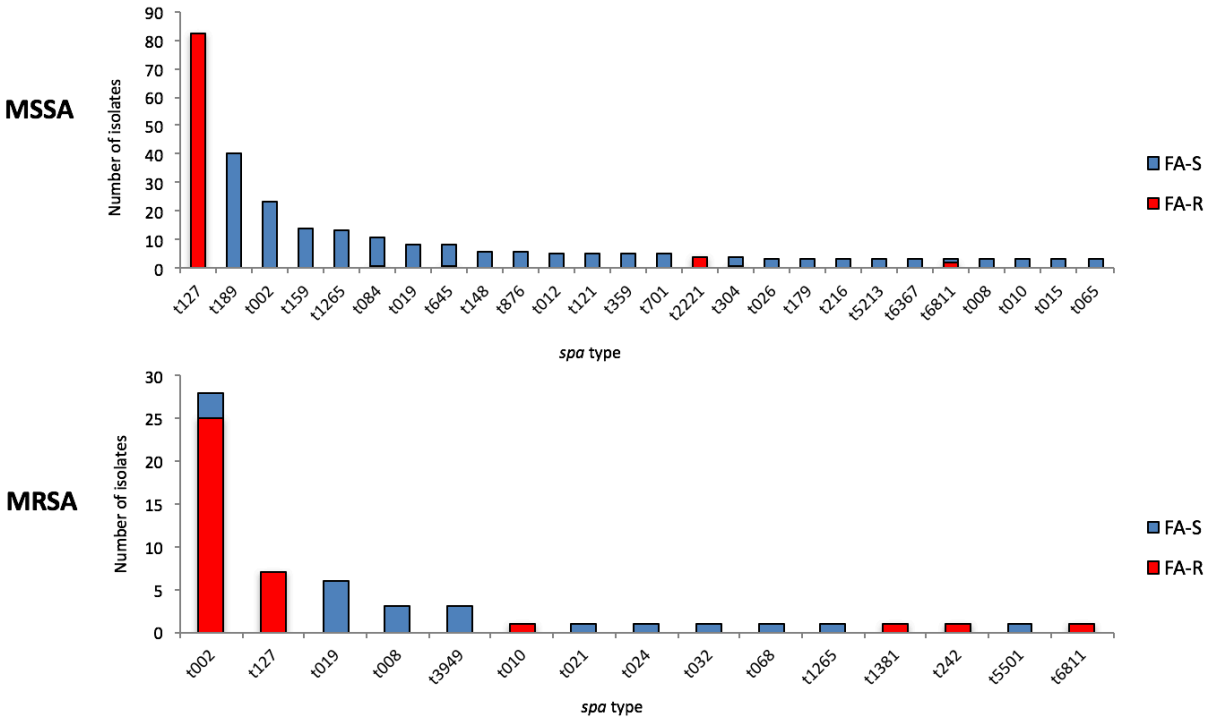


Figure 1.7. Clonal selection of ST 1 lineage MSSA and MRSA fusidic acid resistant clones in New Zealand. *S. aureus* protein A (SPA) was used to differentiate different ST 1 lineage clones. This graph shows FA-S (fusidic acid – sensitive), FA-R (fusidic acid – resistant) MSSA and MRSA. The graph was reproduced from a study by Williamson *et al.* (56).

1.6. Antimicrobial resistance mechanisms encoded on mobile genetic elements

In addition to the chromosomal mediated fusidic acid resistance, the ST1 New Zealand *S. aureus* lineages can harbour a MDR plasmid designated pNZAK1, which contains *blaZ*, *qacA,* and *mupA* genes (Fig. 1.8). As described *qacA* encodes a multi-drug efflux pump QacA that exports a variety of antibacterial compounds and is part of the major facilitator superfamily of transport proteins (57). The gene *mupA* encodes an alternative isoleucyl tRNA synthetase (IleRS-2), conferring resistance to mupirocin (58). Isoleucyl tRNA synthetase helps to link nucleotide triplets to their corresponding amino acid to create a polypeptide chain in protein synthesis. A high amount of multi-locus sequence type ST 1 NZ isolates carry *blaZ*, *qacA*, and *mupA* on a plasmid as well as *fusC* on the chromosome (Fig. 1.9A) (59). An investigation by Carter *et al.* (2018) specifically looked at the genome of *S. aureus* 14487 which harbours pNZAK1 and *fucC.* This *S. aureus* strain is spa type t127 and CC1 (clonal complex 1). This spa type is one of the main clonal lineages in New Zealand, which is why the relationship between *qacA* and pNZAK1 is a major issue (59).

1.7. pNZAK1, general information and genetic features

The ST1 lineage of *S. aureus* commonly harbours the multi-drug resistant plasmid pNZAK1 (Fig. 1.8). This circular plasmid is approximately 28 kb in size with a GC content of 33% (59). pNZAK1 is part of the pMW2-like family of *S. aureus* plasmids that are restriction type 1 (60). pMW2-like plasmids can contain the transposon Tn*552* which encodes the resistance gene *blaZ* as well as *blaR1* and *blaI* (60). The pMW2-like plasmids with Tn*552* insertions are found worldwide including, Australia, New Zealand, the UK, and the USA (60). In New Zealand, isolates with a pMW2-like plasmid containing the Tn*552* insertion were first observed in 1922 (59). pNZAK1 closely resembles pMW2, but has also acquired a 7 kb insertion containing the genes; *qacA, qacR,* and *mupA* (55). The copy number of pNZAK1 is currently unknown, but it can be speculated that due to its large size of 27 kb the copy number would likely be low. Additionally, it is hypothesised that pNZAK1 is not highly conjugative (pers. comm. Dr. Glen Carter, Doherty Institute, Melbourne). *S. aureus* isolates that contained the *qacA* and *mupA* insertion were first recorded in New Zealand in 1991 (59). In New Zealand, the prevalence of *qacA* in *S. aureus* is 7%, however, as there is currently insufficient timescale data detailing

qacA carriage in New Zealand it is unknown if the prevalence of *qacA* is increasing or decreasing (55). The respective prevalence of *fusC, mupA,* and *qacA* in ST1 *S. aureus* lineages is illustrated in Fig. 1.9. Within New Zealand there is a high level of *fusC* carriage in the ST1 lineage isolates, whereby approximately 40% of New Zealand *S. aureus* strains investigated were positive for *fusC, mupA*, and *qacA* (59). The population size of ST1 lineages in New Zealand is increasing, especially after 1991 with the appearance of pNZAK1 as seen in Fig. 1.9. This suggests that the MDR plasmid pNZAK1 may be involved in the rise of the ST1 *S. aureus* lineage within New Zealand.

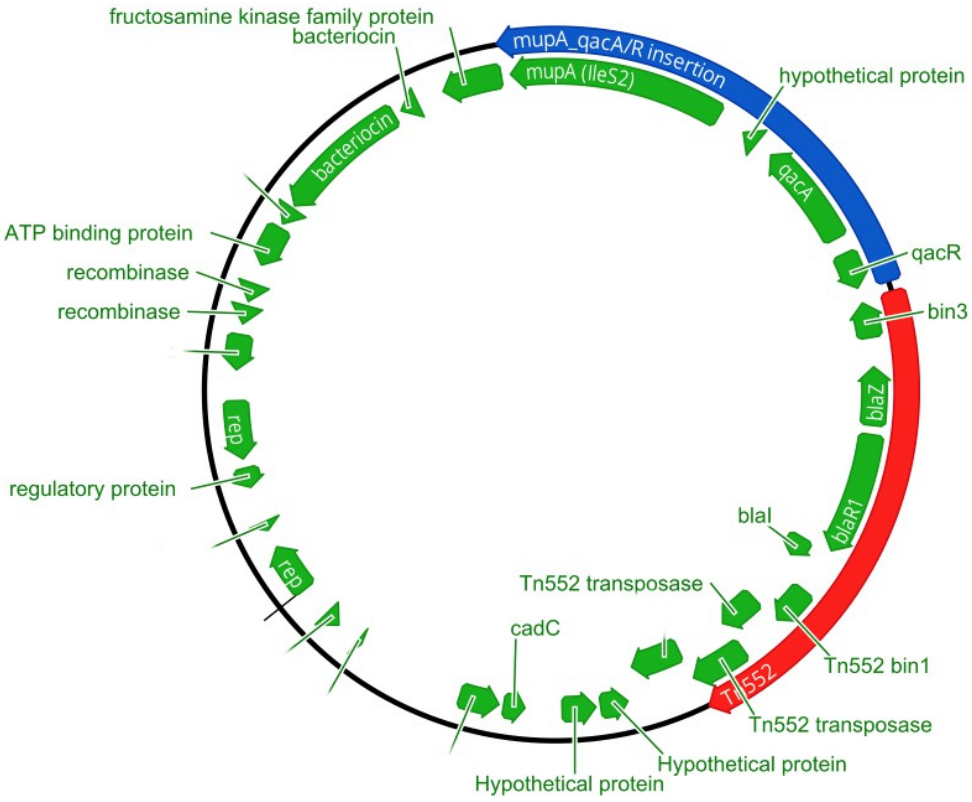


Figure 1.8. Schematic representation of the plasmid pNZAK1. Genes are denoted by coloured arrows. Genes of interest are as follows: the antibiotic resistance genes encoding resistance to mupirocin and β-lactam antibiotics (*mupA* and *blaZ),* the antiseptic resistance protein QacA (*qacA)*, and two potential plasmid addiction mechanisms (*lactococcin 972* bacteriocin and *pepA1*). The *mupA* and *qacA* insertion segment is shown in blue. The transposon 552 insertion segment containing *blaZ* is shown in red (sequence information provided by Dr. Glen Carter, Doherty Institute, Melbourne).

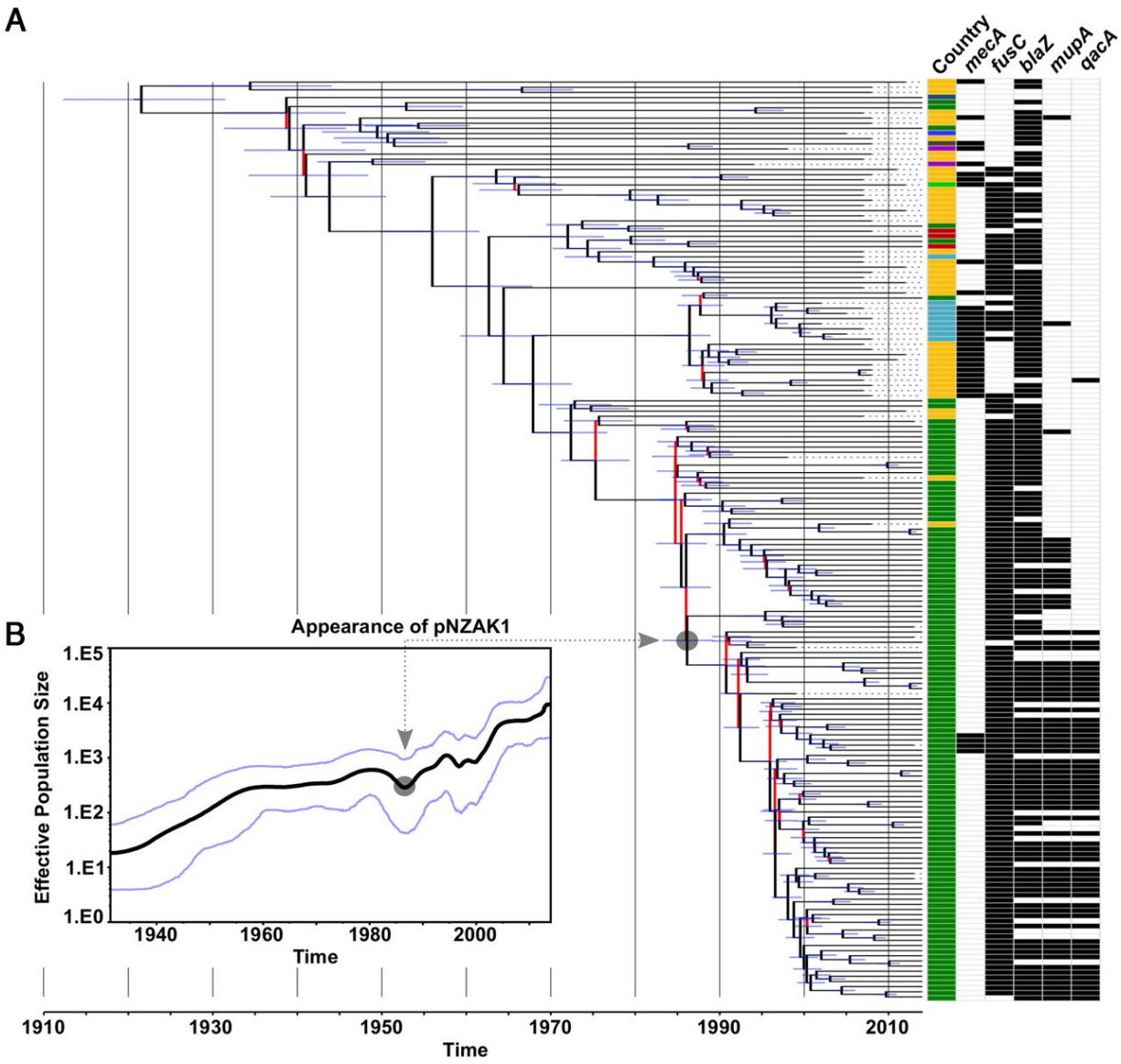


Figure 1.9. History of *fucC*, *mupA,* and *qacA* carriage in New Zealand ST1 *S. aureus* strains. (A) 179 ST1 NZ *S. aureus* genomes were investigated in a study by Carter *et al.* where this figure has been reproduced from (59). The black bar signifies carriage of the gene and the white bar indicates that the gene is not present in that strain. The colours used for each strain indicate the country of isolation dark green; NZ, yellow; Australia, blue; UK, red; Malaysia, light green; India, dark blue; Brazil, grey; Iraq, royal blue; France, purple, USA. **(B)** A bayesian skyline plot of ST1 lineage over time looking at the fluctuation of the effective population size.

1.8. The important role of addiction systems and plasmid maintenance in bacteria

Plasmids are a source of genetic material that can be easily transferred between strains (61). Most bacteria tend to carry plasmid DNA, but the exact size of plasmids can vary dramatically from 1 kb plasmids to >100 kb (61). Small plasmids can be maintained at many hundreds of copies per cell, whereas larger plasmids may be limited to less than 10 copies per cell. Plasmids can encode advantageous genes providing a competitive edge in a niche e.g. encoding antibiotic resistance genes or metabolic genes (17).

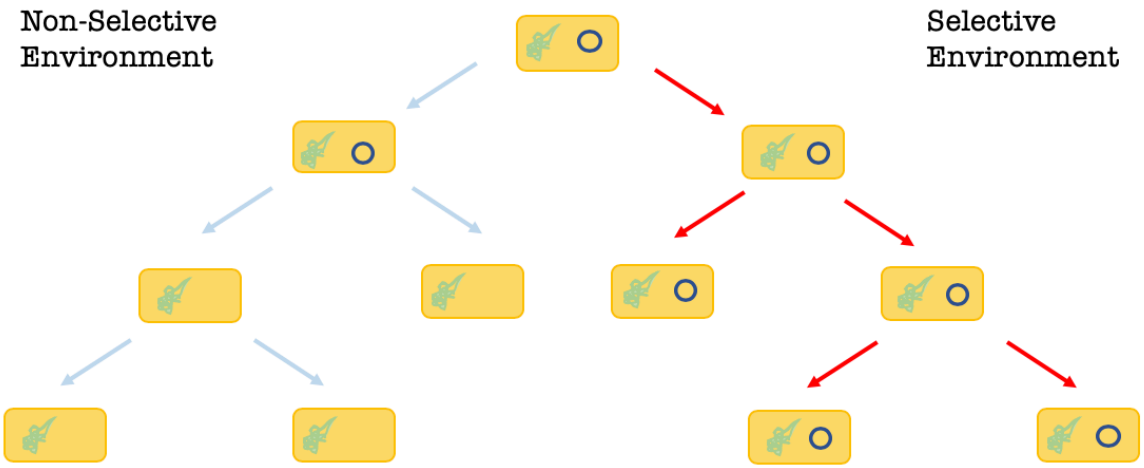


Figure 1.10. Plasmid maintenance in bacteria in selective vs non-selective environments. As bacteria divide, the daughter cells in the presence of a selective environment retain the plasmid, however, in the absence of selective pressure (non-selective environment) daughter cells progressively fail to inherit the plasmid, and it is lost in the population overtime. The diagram of cells shown as orange squares are either in the plasmid-selective environment or non-selective environment. The red arrow signifies selection and the blue arrows show no selection. The plasmid is shown as a blue circle, the genome is green.

For bacteria to maintain plasmid DNA it imposes an energetic burden upon the cell. Therefore, if a plasmid is not useful it will not be maintained, and will be lost from the bacterial population (Fig. 1.10) (61). This process helps bacteria streamline their genome to prevent wasting energy on superfluous genetic elements and allows it to be more competitive metabolically (61).

1.8.1. Plasmid addiction (maintenance) systems (PAS) in bacteria

Plasmid stability can be improved by the presence of resistance elements or addiction systems on the plasmid. There are many types of addiction systems found on bacterial plasmids including toxin/antitoxin (TA) systems, restriction modification systems, and bacteriocin systems (62). TA systems work by encoding a highly stable toxin as well as short-lived antitoxin, thus requiring constant production of the antitoxin to survive (Fig. 1.11) (62). Long-lasting bacteriocin toxins are encoded in the plasmid as well as the immunity gene, which works to inhibit the function of the toxin (63). Restriction modification-based addiction systems use a restriction endonuclease that cleaves unmethylated DNA, the methyltransferase which methylates DNA, is encoded on the plasmid thus making the plasmid indispensable (62).

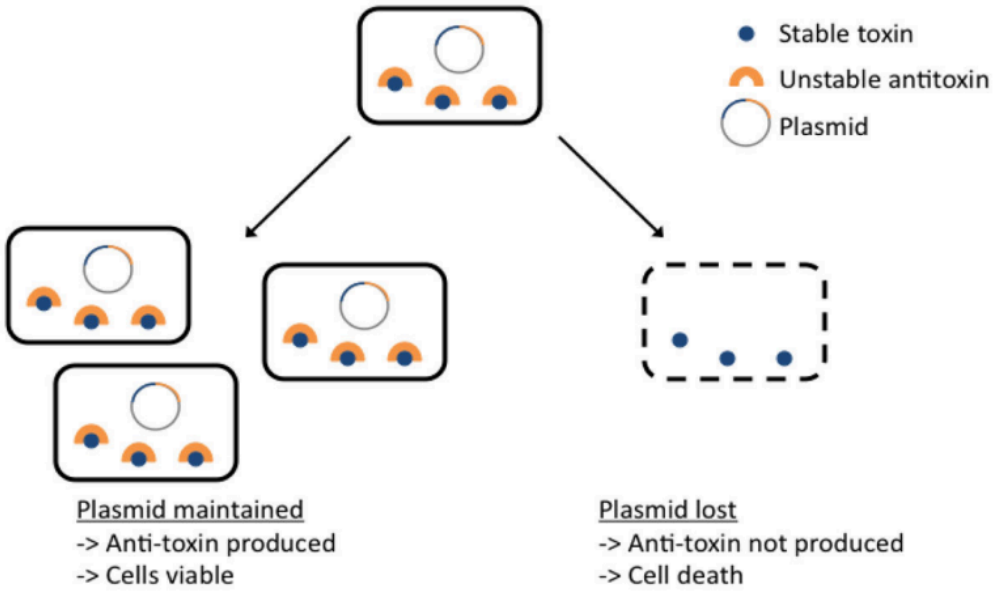


Figure 1.11. Overview of the toxin/antitoxin plasmid addiction mechanism. This diagram highlights the two potential outcomes of the bacterial cell after cell division with and without plasmid maintenance systems. Either the cell maintains the plasmid post-segregation and the plasmid becomes established in the bacterial population and the cell survive; or, the cell loses the plasmid and dies due to accumulation of toxin. The figure is reproduced from Tsang (62).

The are many different types of TA systems in bacteria. A commonly studied TA system is the MazF/MazE system, which is a protein-based inhibitory system (62). MazF is the stable toxin which is inhibited by MazE, the unstable anti-toxin that is degraded by the ClpAP protease, meaning that the cell must continuously generate plasmid-encoded MazE to survive (62). Without MazE, MazF is able to bind to and cleave mRNA inhibiting protein production leading to the death of cells that lack plasmid-encoded MazE (62). Another type of TA system is antisense RNA systems like the *pepA1* system which encodes SprA1AS and SprA1 (64). SprA1 encodes *pepA1*, a cytotoxic peptide, which disrupts the cell membrane leading to host cell death (64). SprA1AS is the antitoxin which bind to the SprA1 RNA and prevents the creation of the deadly peptide *pepA1* (64). When the antitoxin is encoded on the plasmid with the toxin it decreases the frequency of plasmid -ve cells in the population (62).

Bacteriocins are toxic compounds excreted by specific strains of bacteria to hinder closely related strains, but not the host cell (65). The names of bacteriocin stem from the specific producing bacteria such as colicins from *E. coli* or lactococcins from *Lactococcus lactis* (65). Colicins bind to specific surface receptors on susceptible target strains of bacteria triggering cell death (65). Colicins have multiple subtypes with various mechanisms of action, colicin E1 causes cessation of macromolecular generation, colicin E2 triggers degradation of genetic material such as DNA, and colicin E3 inhibits the generation of new proteins in the cell (65). However, the activity of a colicin is inhibited in the presence of a specific protein deemed the immunity protein, this protein allows the producing bacteria to survive.

Another example of a bacteriocin is *lactococcin* 972 which inhibits cell division by preventing the formation of a septum in certain susceptible lactococcal strains by binding lipid II, a cell wall precursor molecule (66,67). This does not kill the cells immediately, but halts the cell cycle so that the cells are no longer growing (66). *lactococcin* 972 was shown to increase plasmid stability when the full *lactococcin* 972 operon is added to plasmids in *Lactococcus lactis* (67). The full operon consisted of *lactococcin* 972, and two other *lactococcin* 972 associated genes which may encode an ABC transporter, and the immunity gene (67).

1.9. Energy limitation in bacteria

Generalised nutrient limitation can impact the growth of a bacterial population, which then influences bacterial fitness. Alongside this the carriage of extrachromosomal elements e.g. plasmid DNA can impose an additional energy burden (68,69). The chemostat first described in 1950 by Novick and Szilard (70) as well as Monod, (71), is a continuous culture vessel which allows precise control of bacterial doubling time, as well as the nutrient concentration, oxygen saturation and temperature of the growth conditions (Fig. 1.12) (68). The residence time (RT) of the chemostat is the time it takes for the reactor to completely exchange the media (RT = V/F) (72). The peristaltic media pump controls the dilution rate (dilution rate (D) = flow rate (F) / volume of reactor (V)). By changing the dilution rate, the bacteria are able to enter steady state at specially controlled growth rates (68). Additionally, when the chemostat has reached steady state the outflow of media and inflow of media are equal, meaning the volume in the chemostat reactor does not change (72).

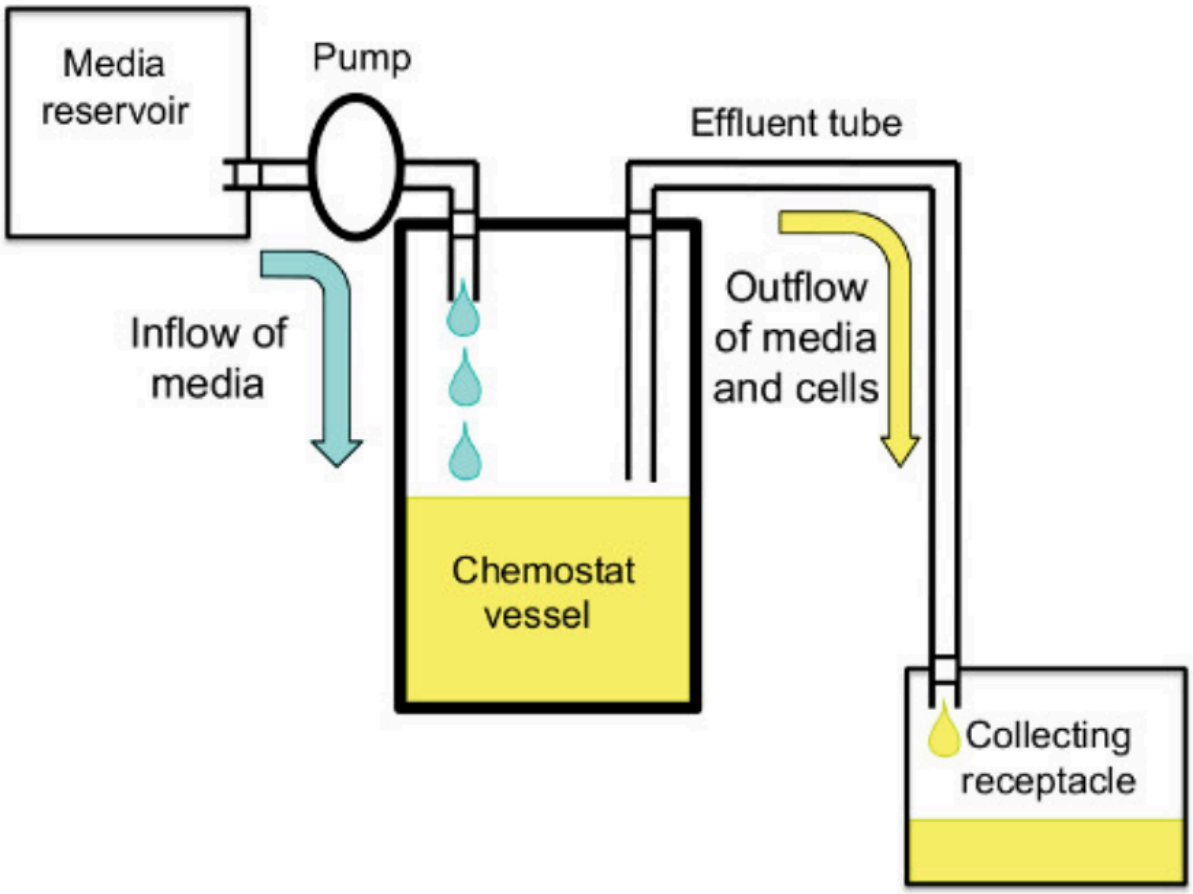


Figure 1.12. Basic organisation of a functioning chemostat setup. A sterile media reservoir is controlled by a peristaltic media pump. The chemostat vessel is then filled with media at a specific dilution rate and waste is pumped through the effluent tube into the waste receptacle. This figure is reproduced from a paper by Ziv *et al.* (68).

Chemostats have previously been used to examine the effect of slow growth rate and hypoxia on the metabolism of *Mycobacterium smegmatis* (73). Additionally, the use of continuous culture has allowed studies to assess the fitness cost of the type I and type IV SCCmec resistance elements in MRSA (13). Earlier studies looking at the effect of bacterial stress responses on antibacterial sensitivity have found that the generalised nutrient limitation can enhance antibiotic resistance levels in bacteria, specifically for β-lactam antibiotics (74).

The chemostat is the ideal tool for studying plasmid maintenance as it can be used to exert energy limiting conditions on bacteria while these organisms compete for limited nutrient sources. Very slow growth rates (imposed by the dilution rate) and competition for limited nutrients can activate stress proteins in the bacteria (75). Theoretically, this activation of stress proteins would then select for bacteria that have lost the plasmid, as it would be a fitness cost to maintain the plasmid under non-selective (e.g. lack of antimicrobials) conditions. The loss of the plasmid from the bacteria can be calculated by examining the increase in antimicrobial sensitivity of the population, suggesting the loss of the drug resistant plasmid.

1.10. Overview of proposed work

The overall aim of this research project was to elucidate the role of *qacA* in *S. aureus* 14487. This strain harbours an MDR resistance plasmid pNZAK1. This research investigated *qacA* specifically in regard to antiseptic tolerance. Additionally, we investigated the fitness cost associated with pNZAK1, and the role of two presumptive plasmid addiction systems, namely, *lactococcin* 927 and *pepA1.*

Objective 1: Determine the role of *S. aureus* 14487 *qacA* in response to the antiseptic; CHX, BAC and Trigene.

We hypothesised that the use of chlorhexidine selected for *qacA* and potentially the expression of other antimicrobial resistance genes (e.g. *blaZ* and *mupA*) encoded on the plasmid pNZAK1. We aim to further elucidate the role of *qacA* in *S. aureus* through the use of an isogenic Δ*qacA* knockout strain of *S. aureus* 14487. Antimicrobial sensitivity testing between wild-type *S. aureus* and Δ*qacA S. aureus* will be used to elucidate the conditions in which QacA is beneficial. To further understand the role of QacA, we examined the effect of antiseptics like chlorhexidine on gene expression of *mupA*, *blaZ*, *qacA,* as well as *norA*.

Objective 2: Investigate the fitness cost associated with the plasmid pNZAK1 in *S. aureus*, under energy limiting conditions imposed by continuous culture (Chemostat)

We propose that in the absence of antimicrobials, under energetically unfavourable growth conditions (e.g. energy limitation), the plasmid pNZAK1 comes with an energetic fitness cost to the host strain that will lead to plasmid loss. To further investigate the fitness cost of pNZAK1, wild-type *S. aureus* and *S. aureus* pNZAK1$^-$ were grown in a mixed culture and the frequency of pNZAK1$^+$/ pNZAK1$^-$ strains was observed over 7 days.

Objective 3: Investigate the role of the presumptive plasmid addiction systems *lactococcin 972* and *pepA1* in pNZAK1 through the generation of double-cross over markerless single gene mutants.

We hypothesis that the plasmid addiction systems *lactococcin* 972 or *pepA1* found on pNZAK1 play an important role in plasmid maintenance. To assess the possible role of *lactococcin* 972 or *pepA1* in plasmid maintenance, we knocked out the genes via homologous recombination.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Media

All commercial media used in this research was purchased from BD chemicals (Sparks, MD, USA), prepared according to the manufacturer's specifications, and sterilised by autoclaving (121°C, 15 min) before storing at room temperature (20°C).

2.2. Solutions

Dimethyl sulfoxide (DMSO) was purchased from Univar and stored at room temperature. Trizol was purchased from Ambion (Life Technologies) and stored at 4˚C.

2.3. Antibiotic stock solutions

All antibiotics, antiseptics and other chemical reagents were purchased from Sigma-Aldrich (New Zealand) unless specified otherwise. Chlorhexidine, fusidic acid, and ampicillin were dissolved in distilled water (dH_2O) to 5.12 mg/mL, filter-sterilised using a 0.2 μm filter (Millipore Corporation, Bedford, MA, USA), and stored at -20˚C. Mupirocin was dissolved in DMSO to 5.12 mg/mL, filter-sterilised using a solvent suitable 0.2 μm filter (Ahlstrom) and stored at -20˚C. Chloramphenicol was dissolved in absolute ethanol to 100 mg/mL or 50 mg/mL as required and stored at -20˚C. Benzalkonium chloride (BAC) was dissolved in filter sterilised dH_2O to 30 mg/mL and stored at -20˚C. Trigene (Advanced), which consists of benzalkonium chloride, didecyldimethylammonium chloride and polyhexanide was purchased from Tristel Solutions limited and stored at room temperature. Rifampicin was dissolved in DMSO to 4 mg/mL and stored at -20˚C in microcentrifuge tubes covered in foil to prevent degradation by UV light.

2.4. Long-term cryo-storage of bacterial cultures

For the long-term storage of bacterial cultures, isolates were grown overnight before diluting with glycerol (final concertation of 87 % [v/v]) and stored in 1 mL volumes in sterile conical cryogenic tubes (NUNC A/S, Roskilde, Denmark) at -80 °C. When required, bacterial cryo-culture stocks were propagated on the appropriate media in the absence of any antimicrobial and grown at 37˚C for 24 h.

2.5. Bacterial strains and growth conditions

The bacterial strains, and plasmids, used or generated in this study are described in Table 2.1. *Staphylococcus aureus* 14487, containing the plasmid pNZAK1, was isolated from Auckland, New Zealand and was kindly provided by Dr. Glen Carter (Doherty Institute, Melbourne). Unless otherwise indicated, *S. aureus*, refers to *S. aureus* 14487 containing pNZAK1. Typically, *S. aureus* was grown aerobically overnight (16 h) in cation-adjusted Mueller Hinton broth (MHB) at 37°C with agitation (200 rpm). For growth on solid media, MHB supplemented with 1.5% (w/v) agar (NZ Seaweed Ltd) was used (MHA). Tryptic soy broth (TSB) and Brain-heart infusion (BHI) media were also used to grow *S. aureus* as required. Liquid broth cultures of *S. aureus* were prepared by inoculating a single colony into 5 mL MHB within a glass universal (30 mL) and incubated at 37˚C (200 rpm) until the desired optical density at 600 nm (OD_{600}) was reached. *S. aureus* strains that lacked either the plasmid pNZAK1, or single gene knockouts of Δ*qacA*, Δ*pepA1*, or Δ*lactococcin 972* (see Table 2.1) were grown under the same conditions as wild-type *S. aureus* 14487.

Escherichia coli strains MC1061 and IMO8 B were used in this study for cloning (Table 2.1). The *E. coli* strains were treated the same as the *S. aureus* strains with the exception that they were propagated in Lysogeny (Luria) broth (LB) and as required solid media was prepared by the addition of 1.5% (w/v) agar (LBA).

Table 2.1. Bacterial strains used in this study

Strain or plasmid	Description	Source or Reference
S. aureus		
14487	Clinical strain isolated from a patient in Auckland, NZ. PacBio sequenced. Contains pNZAK1 plasmid	(59)
IM01 Δ*qacA*	*S. aureus* 14487 strain with pNZAK1 and a *qacA* markerless deletion	Doherty Institute of Melbourne
AK01 pNZAK1^{-}	*S. aureus* 14487 isolated from the chemostat (3 h) that lacks pNZAK1. Confirmed by whole genome sequencing (3-2-162)	This study
14487 rif^{R}	*S. aureus* 14487 that harbours pNZAK1 and spontaneous rif^{R}	This study
ATCC 6538	*S. aureus* strain that is MSSA and is the standard strain used for disinfectant susceptibility testing	(76)
AK02 Δ*pepA1*	*S. aureus* 14487 harbouring pNZAK1 and a Δ*pepA1* markerless deletion	This study
AK03 Δ*bac*	*S. aureus* 14487 harbouring pNZAK1 and a Δ*lactococcin 972* markerless deletion	This study
E. coli		
MC1601	*E. coli* strain used for cloning and plasmid propagation	(77)
IMO8 B	*E. coli* strain with a restriction modification system recognised by *S. aureus* clonal complex 1 (CC1)	(78)
Plasmids		
pNZAK1	MDR plasmid found in *S. aureus*. Contains amp^{R} and mup^{R}	(59)
pKFC	Cloning shuttle vector with BamH1 restriction sites. Contains amp^{R} and cm^{R}	(79)
p*bac*_pKFC	Cloning shuttle vector containing a Δ*lactococcin 972* deletion. Contains amp^{R} and cm^{R}	This study
p*pep*_pKFC	Cloning shuttle vector containing a Δ*pepA1* deletion. Contains amp^{R} and cm^{R}	This study

2.6. Chemostat setup

A 1 L chemostat (New Brunswick Scientific), with a 750 mL working volume containing ¼ strength MHB (to limit nutrients) was inoculated from an overnight culture of *S. aureus* to yield a starting OD_{600} of 0.05 (~2×10^7 CFU/mL). This culture was then grown in batch until an OD_{600} of 1.4 (or ¾ of maximum batch OD_{600} for MHB) before the media pump was turned on to a predetermined dilution rate (*D*) (73). In a more complex media, for example TSB, the maximum OD_{600} can be up to 11 in overnight batch culture of 100 mL (Fig. 3.3). At steady state, the specific growth rate (μ) of the bacterial culture is equal to *D.* The heating blanket was set to 37˚C. Dissolved oxygen was maintained at 50 % oxygen saturation, and the agitation was automatically (and continuously) controlled based on the measured dissolved oxygen content, typically agitation fluctuated between 200-300 rpm.

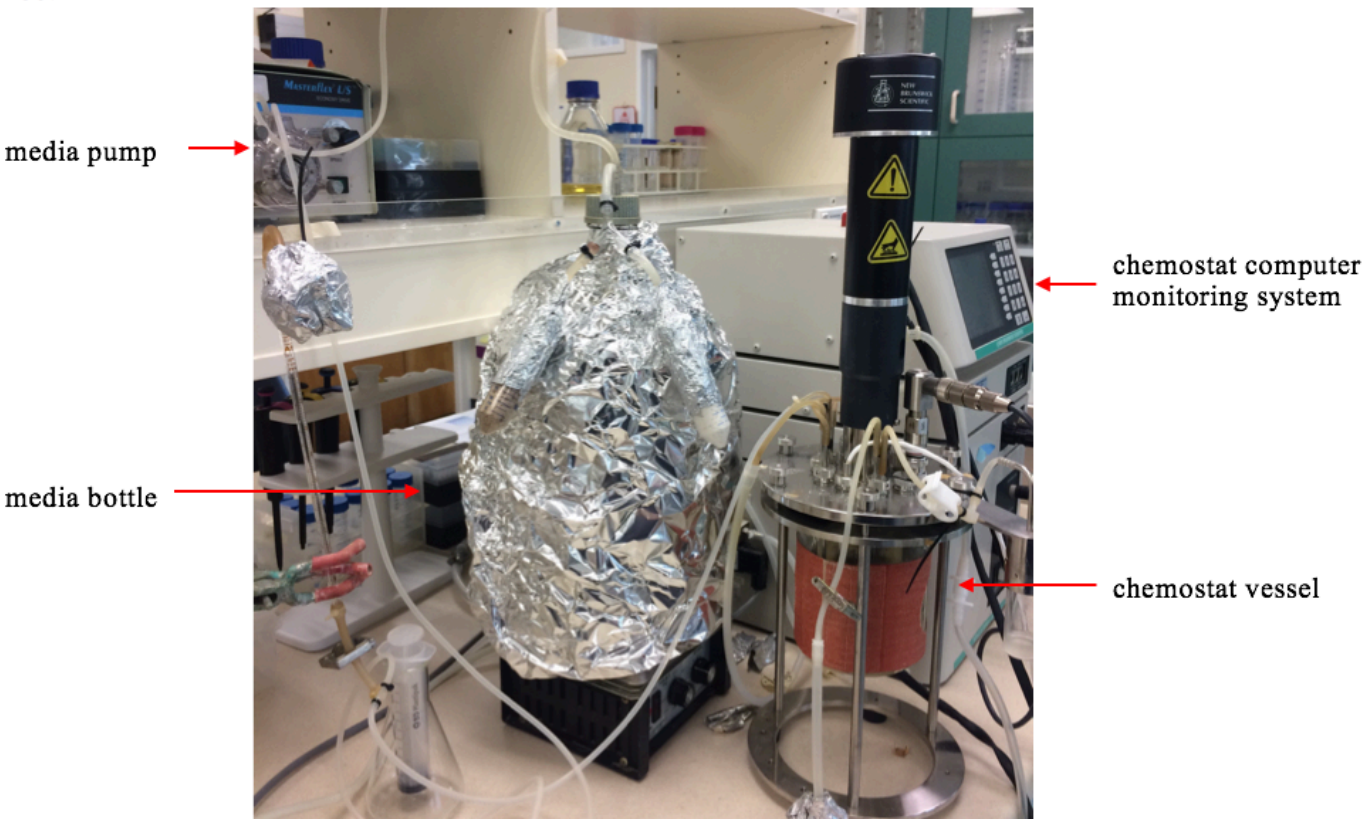


Figure 2.1. A representative chemostat setup, using the chemostat continuous culture system from New Brunswick Scientific. *S. aureus* cells were routinely maintained in steady state at doubling times of 3 h (0.231 h^{-1}), 10 h (0.0693 h^{-1}), and 30 h (0.023 h^{-1}).

To determine the growth rate of the culture following the equation was routinely used:

$$dilution\ time = {\ln(2)}/{growth\ rate\ (D)}$$

Example: 3 h doubling time

$$0.231\ h^{-1} = \frac{0.6931}{3}$$

2.7. Microbial and molecular analysis of the continuous culture (chemostat)

2.7.1. Patch plating

To determine the plasmid loss percentage of pNZAK1 within the *S. aureus* chemostat population, cell samples from the chemostat were withdrawn for each growth rate once steady state conditions had been confirmed. Steady state was confirmed after 3-4 residence times. The samples were then serially diluted 10-fold in sterile PBS. Following this, the 1×10^{-3} to 1×10^{-8} dilutions were then spread plated (50 µL) in technical triplicate onto BHI agar and grown overnight at 37˚C. The CFU/mL for each dilution was recorded. Following this, 300 colonies were patch plated onto MHA, BHI agar, or MHA supplemented with ampicillin (6 µg/mL). The patch plates were then grown overnight at 37˚C. The patching plating was carried out in duplicate for each growth rate. No growth on ampicillin agar indicated loss of the host plasmid pNZAK1.

2.7.2. Whole genome sequencing of presumptive plasmid loss isolates

Isolates from the chemostat that were found to have lost resistance to antibiotics (mupirocin and ampicillin) through MIC and MBC testing were sent for whole genome sequencing at the Doherty Institute. To prepare the isolates for sequencing they were freshly streaked from cryo-stocks onto agar. From this an overnight was prepared from a single colony. A new glycerol stock was then created from the fresh overnight. Glycerol stocks of each isolate were then sent to the Doherty Institute in Melbourne. DNA extraction and next-generation sequencing were performed at the Microbiological Diagnostic Unit Public Health Laboratory of the University of Melbourne. Genomic DNA was prepared from a culture grown from a single colony using a JANUS Chemagic workstation and Chemagic DNA/RNA kit (PerkinElmer, USA). DNA libraries were created using the Nextera XT DNA preparation kit (Illumina, USA). Next-generation sequencing was performed using the Illumina NextSeq platform (80).

2.8. Determination of the minimum inhibitory concentration (MIC) by broth microdilution

To determine the level of sensitivity to the antimicrobials: mupirocin, fusidic acid, ampicillin, and chlorhexidine the minimum inhibitory concentrations (MICs) were measured in biological duplicate and technical triplicate on samples withdrawn from the chemostat. All MICs were carried out in flat-bottom 96-well microtiter plates (ThermoFisher Scientific, New Zealand). At specific time intervals when *S. aureus* cells were in steady state, samples were removed from the chemostat and diluted to an OD_{600} of 0.0007, or ~2×10^5 CFU/mL (0.5 McFarland standard (McF). Bacterial culture (50 µL) was then added to wells of a 96-well microtiter plate containing two-fold serial dilutions of the antibiotic diluted in the appropriate media in a final volume of 100 µL. The compound range tested was 256 µg/mL to 0.125 µg/mL. In addition to the compounds being tested, media and compound vehicle controls were included in each microtiter plate. The microtiter plates were incubated aerobically for 24 h at 37˚C with shaking at 200 rpm. Photographs were taken of each of the MIC plates and the MIC was recorded as the lowest concentration of the test compound where no growth occurred.

2.9. Determination of the minimum bactericidal concentration (MBC)

To determine the bactericidal activity of mupirocin, fusidic acid, ampicillin, and chlorhexidine, the minimum bactericidal concentration (MBC) was determined from samples previously setup in MIC assays. To do so, 10 µL of culture from each well of the 96-well flat bottom microtiter plate was spot-plated onto a MH agar square plate, and the plates were incubated at 37°C for 24 h. All MBCs were carried out in technical triplicate and biological duplicate, and the MBC values were reported as the lowest concentration of the test compounds where no growth occurred.

2.10. Wild-type *Staphylococcus aureus* 14487 versus Δ*qacA Staphylococcus aureus* 14487 cell viability assays

The IMO1 Δ*qacA* strain was kindly provided by Dr. Ian Monk (Doherty Institute of Melbourne) (see Table 2.1). The deletion of *qacA* was confirmed by whole genome sequencing.

Overnight cultures (5 mL) of wild-type *S. aureus* 14487 and Δ*qacA* mutant cells were used to inoculate three 100 mL TSB flasks to an initial starting OD_{600} of 0.05. These flasks were grown to mid-exponential phase (OD_{600} of 0.5 [~$1x10^8$ CFU/mL]) and challenged with 1× the MIC of CHX (2 µg/mL). Samples were withdrawn at time 0 and subsequently at 0.5, 1, 2, 3, 4 and 24 h. At each timepoint, 100 µl samples were withdrawn and diluted into ¼ MHB to determine the OD_{600}. To determine the number of viable cells at each given timepoint, the Miles-Misra technique was followed (81). In brief, 10 µl samples were withdrawn, diluted 10-fold in phosphate buffer saline (PBS) to 1×10^{-8}. Following this, 10 µl of the appropriate dilution were then spot plated onto TSA plates in technical triplicate. These plates were then used to calculate the CFU/mL of the culture at each time point.

2.11. FDA compound screening

S. aureus 14487 and the Δ*qacA* mutant cells were screened for inhibition of growth against the FDA-drug approved library (1399 compounds, Selleckchem, USA). Library compounds, dissolved in DMSO to 1 mM, were added at a final concentration of 25 µM to 96-well polystyrene microtiter plates (ThermoFisher Scientific, New Zealand) inoculated with either *S. aureus* 14487 or the Δ*qacA* mutant at OD_{600} 0.05 (final volume 200 µL). The concentration of DMSO did not exceed 2% (v/v) and control wells containing 2% (v/v) DMSO were included in each plate. Additional control wells included: un-inoculated medium (TSB) and vancomycin (40 µg/mL). All controls were included in technical triplicate. Plates were incubated overnight at 37°C with agitation (200 rpm) and the OD_{600} were recorded using a Thermo Scientific Varioskan Flash plate reader. The inhibition of cells by FDA-compounds was determined by measuring the OD_{600} of treated cells, relative to untreated control cells. Assay performance was assessed by the statistical parameters Z and Z′, which take account of both data variability and signal window (82). Z′ is a measure of the suitability of the assay set up and takes in to account

the separation between negative and positive controls, Z-factor assess the impact of the screening library on the assay (82).

2.12. Investigating the gene expression of specific genes in antiseptic challenged cells.

2.12.1. Glycerol saline quenching preparation of cells for RNA extraction.

S. aureus was grown in 5 mL MHB for 24 h and subsequently used to inoculate a 500 mL MHB flask to a starting OD_{600} of 0.05. To synchronise the cultures for compound challenge, the *S. aureus* culture was grown to an OD_{600} of 0.4 at 37˚C with shaking at 200 rpm. After which time the culture was then split into sterile flasks each containing 100 mL of synchronised culture. The cells were challenged with a given antimicrobial (e.g. 1 µg/mL CHX) for 1 h alongside an untreated control. Following this, an ice cold 3:2 glycerol:saline solution was added directly to the cells (i.e. 50 mL of cells to 100 mL glycerol:saline), which were then harvested by centrifugation at 25,900 × *g* for 20 min at 4˚C. The supernatant was removed and the washed cell pellet was resuspended in 1 mL of a 1:1 glycerol:saline solution. The cell suspension was then aliquoted into Eppendorf tubes (1 mL per tube) and snap frozen in a dry ice ethanol bath. The cell samples were then stored in cryo-culture at -80˚C.

2.12.2. RNA extraction from *S. aureus* cells.

Three washed glycerol:saline samples (1 mL) for each treatment type were centrifuged in a bench-top centrifuge at 16,300 × *g*, 20 min at 4˚C in technical triplicate. The supernatant was discarded, and the cell pellet was resuspended in 1 mL Trizol Ambion (Life Technologies). The cells were then disrupted by zirconium beads (0.1 mm) and a mini-bead beater (Biospec) at 4,800 oscillations per min for 30 s × 3, with a 30 s break between each disruption. Following disruption, the cells were transferred to a RNAse-free 1.5 mL Eppendorf and 200 µL of neat chloroform was added to the cells, followed by ‘hand shaking’ for 15 s. The cells were then incubated for 2 min and then centrifuged at 11,800 × *g*, at 25˚C for 15 min. The clear aqueous phase was then carefully transferred to a new Eppendorf and the RNA was then precipitated with 500 µL isopropanol (100% absolute) and centrifuged at 16,300 × *g* for 30 min at 25 ˚C. The supernatant was removed, and the pellet was washed in absolute ethanol (75 % [v/v]). The

cells were then washed again in absolute ethanol (75 % [v/v]) for 20 min at 8,900 × *g* and the supernatant was discarded. The RNA-containing pellet was air dried for 1 h at 25˚C and dissolved in 50 µL DEPC-treated MQ-H_2O at 55˚C for 10 min. RNA (10 µg) was incubated with Turbo DNase buffer (Invitrogen), 2 µL of Turbo DNase and DEPC-treated MQ-H_2O (50 µL total volume) for 1 h. Following this, DNase inactivation reagent (20 µL) (Invitrogen), was added and the RNA was incubated for 5 min at 65˚C and centrifuged at 16,300 × *g* for 2 min. The supernatant was then transferred into an Eppendorf tube. The RNA concentration was measured via nanodrop (ThermoFisher Scientific, NZ) and was stored at -80˚C until required.

2.12.3. cDNA synthesis

For cDNA synthesis from RNA, 1 µg of RNA was added to an Eppendorf containing 1 µL of a 10 mM dTNP mix (Invitrogen), 250 ng of random primers (Invitrogen), and DEPC-treated MQ-H_2O (final volume, 20 µL). The suspension was then incubated at 65˚C for 5 min, and cooled on ice for 1 min. Following this, 4 µL of 5× First Strand Buffer (Invitrogen by ThermoFisher Scientific), 1 µL of 0.1 M DTT, 1 µL of RNaseOUT (Invitrogen) and 1 µL of SuperScript III Reverse Transcriptase (Invitrogen) was added to each reaction mixture. The cDNA was synthesised under the following conditions: 25˚C for 5 min, 50˚C for 1 h, with a final extension of 70˚C for 15 min. The cDNA was then stored at -20˚C until required.

2.12.4. cDNA purity check

To check for genomic DNA contamination in the RNA samples, a PCR was conducted in triplicate for each treatment group. The PCR contained cDNA, RNA, and a MQ-H_2O control. PCR reactions were performed in 50 µL volumes with 1 µL of Phusion DNA polymerase (ThermoFisher Scientific) in accordance with the manufacturer's instructions. PCRs were performed in a Hybaid PCR express machine, which had been preheated to 72°C. PCR mixes were held at this temperature for a further 2 min before commencing the PCR programme. Amplification consisted of one cycle at 98°C for 2 min and 30 cycles at 98°C for 15 s, 60°C for 30 s and 72°C for 20 s. This was followed by a final cycle consisting of incubation at 72°C

for 5 min. PCR products were then analysed by agarose gel electrophoresis (1% [w/v]), and documented electronically.

2.13. RT-qPCR

2.13.1. RT-qPCR primer efficiency

Before RT-qPCR could be performed it was necessary to first check the efficiency of the primer pairs and the cDNA. Master mixes for each treatment type were created with Platinum SYBR Green qPCR-mix (Thermo Fisher Scientific), cDNA, ROX reference dye (Invitrogen), and MQ-H_2O. The concentrations of cDNA used are listed in Appendix Table 4.4, and state the concentrations tested for: *qacA*, *mupA, norA, blaZ, rho,* and *rrsC* primers on each cDNA isolated from chlorhexidine treated and non-treated cells. The primer efficiency reaction was dispensed in a 96-well qPCR plate and was run in a ViiA 6 qPCR machine (Applied Biosystems). The CT value and cDNA dilutions were plotted to calculate the log of dilution (log 1/10). XY analyses were used to calculate the linear regression (slope) value for each primer pair. The slope value was entered into the ThermoFisher qPCR slope efficacy calculator to generate an efficiency value for each primer pair and cDNA concentration. The primer pair efficiency values are $-1+10^{-1/slope}$, where a value of greater than 90 % indicates good primer efficiency. The qPCR primer pair efficiency values are shown in Table 4.1, and the exact concentrations used for each reaction are found in Appendix B Table 4.5.

2.13.2. RT-qPCR setup

Following primer optimisation, a qPCR was conducted with 3 technical replicates for all treatment conditions. Reactions were carried out in a 96-well qPCR plate (Applied Biosystems by Life Technologies) on a ViiA qPCR machine (Applied Biosystems) with SYBER Green and ROX (ThermoFisher Scientific). The primers used were *qacA, mupA, norA, blaZ,* alongside the housekeeping genes *rho* and *rrsC* at 500 nM. *rho* and *rrsC* were used as control genes to calculate gene expression ratio according to the equation shown below:

$$gene\ expression\ fold-change = \frac{2^{(CT\ control\ gene\ -\ CT\ target\ gene)}\ non-treated}{2^{(CTcontrol\ gene\ -\ CT\ target\ gene)}\ treated}$$

2.13.3. qPCR primer sequences

Table 2.2. qPCR primer sequences

Primer Name	Forward primer sequences	Reverse primer sequences
qacA	5'-GTT GCA TCT GCT CTA ATA ATG-3'	5'-GGC TAC CAA GTA CTG CTA-3'
mupA	5'-ATC AGC ATT TTG GAA TAC TCA AA-3'	5'-TCA TGT GTA CAA TAA GGA GTC ACC T-3'
blaZ	5'-CCT GCT GCT TTC GGT AAG AC-3'	5'-TTC AGA TTG GCC CTT AGG AT-3'
norA	5'-ATG CCT GGT GTG ACA GGT TT-3'	5'- ATC CAC CAA TCC CTG GTC CT-3'
Rho	5'-GGA AGA TAC GAC GTT CAG AC-3'	5'-GAA GCG GGT GGA AGT TTA-3'
rrsC	5'-CAT GCT GAT CTA CGA TTA CT-3'	5'-CCA TAA AGT TGT TCT CAG TT-3'

S. aureus 14487 primer sequences for genes tested in qPCR for gene expression.

Table 2.3. qPCR reagents

cDNA Mix	**Volume**
Platinum SYBR Green qPCR Super Mix-UDG	162.5 µL (1× final)
ROX passive reference dye	0.6 µL
MQ-H_2O	138.9 µL
Forward primer (10 µM)	20 µL (500 nM final)
Reverse primer (10 µM)	20 µL (500 nM final)
cDNA (1/100)	3 µL
Total volume	**25 µL per well**

No RT control Mix	**Volume**
DTT (0.1 M)	7 µL
5× First Strand buffer	28 µL
MQ-H_2O	118 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1µL
DNase treated RNA (80-100 ng/µL)	1 µL
Total volume	**25 µL per well**

No cDNA, H_2O Mix	**Volume**
Platinum SYBR Green qPCR Super Mix-UDG	212.5 µL
ROX passive reference dye	0.85 µL
MQ-H_2O	172.65 µL
Forward primer (10 µM)	20 µL (500 nM final)
Reverse primer (10 µM)	20 µL (500 nM final)
cDNA (1/100)	0 µL
Total volume	**23 µL per well**

2.14. DNA manipulation

2.14.1. Commercial miniprep of plasmid DNA

Small-scale plasmid preparations of plasmids were made using the Zyppy™ Plasmid Isolation Kit (Zymo Research) according to the manufacturer's instructions. Plasmid DNA was eluted in 30 µL MQ-H_2O.

2.14.2. Agarose gel composition and gel electrophoresis

Agarose gels were routinely run at either 100 V for 1 h in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA), using 1% (w/v) agarose (Invitrogen). Agarose gels were viewed on a UV transilluminator and photographed using an Alpha imaging HP system (Protein simple).

2.14.3. Gel extraction

DNA, both plasmid and genomic, was purified from 1% agarose (w/v) (Invitrogen) gels using the Zymo™ Gel Recovery Kit (Zymo Research) following the manufacturer's instructions. DNA was eluted in MQ-H_2O as appropriate.

2.14.4. Sequencing

All DNA and plasmid samples were purified as appropriate and sent to the Massey Genome Service for DNA sequencing. The samples were prepared to the specifications set out by Massey Genome Service. DNA sequences were analysed using the application Snapgene (GSL Biotech), and the programs BLASTN, BLASTP and BLASTX (National Centre for Biotechnology Information, Los Alamos, N. Mex) available via the Internet.

2.14.5. Primers

The oligonucleotides used in this study are described in Table 2.2 and 2.5. Custom DNA oligos were ordered from Integrated DNA technologies (IDT). Primer stock solutions (100 µM) were routinely diluted in MQ-H_2O to prepare either 50 µM or 10 µM working stocks. Primers were stored at -20˚C.

2.14.6. Preparation of chemically competent *E. coli* cells

An overnight culture (5 mL) of *E. coli* (MC1061) (Table 2.1) was grown in LB and 0.1 mL was used to inoculate 100 mL of LB. Cells were grown at 37°C with shaking at 200 rpm until an OD_{600} of 0.6-0.8 was reached. Once the target OD_{600} was reached, the culture was chilled on ice for 15 min and then transferred into 50 mL Falcon tubes, and harvested by centrifugation at 3,000 × *g* at 4˚C for 15 min. The cell pellet was resuspended in 100 mL of 0.1 M $MgCl_2$ and incubated on ice for 5 min. Cells were then washed at 3,000 × *g* at 4˚C for 15 min. The pellet was subsequently resuspended in 50 mL of 0.1 M $CaCl_2$ and incubated on ice for 45 min. Afterwards the solution was centrifuged at 3,000 × *g* at 4˚C for 15 min and the pellet was resuspended in 5 mL of 0.1 M $CaCl_2$ and incubated on ice for 30 min. Sterile glycerol was added to a final concentration of 15% (v/v). The cell suspension was distributed into 100 µL aliquots and snap-frozen in liquid nitrogen. The aliquots were stored cells at -80˚C.

2.14.7. Standard polymerase chain reaction (PCR) for colony screening

In a 1.5 mL Eppendorf tube a single colony was resuspended in 30 µL MQ-H_2O, or 30 µL of InstaGene matrix (Bio Rad) for *S. aureus*. The resuspended colonies were then incubated at 95˚C for 15 min. Following this the cells were centrifuged at 16,300 × *g* in a bench-top centrifuge for 1 min. Standard screening PCR used Dream Taq Polymerase (ThermoFisher Scientific), and PCR components are show in in Table 2.4.

Table 2.4. Standard screening PCR method

Components	Volume
Dream Taq buffer	2 µL
dNTPs 10 mM	0.4 µL
Forward primer 10 µM	1 µL
Reverse primer 10 µM	1 µL
DNA template (~30 ng/µL)	0.5 µL
Dream Taq Polymerase (5 U/µL)	0.2 µL
MQ-H_2O	14.9 µL
Total	20 µL

Temperature (C°)	Duration	
95	3 min	
95	30s	(35 cycles)
60 (increase if non-specific binding)	30s	
72	30s per kb	
72	10 min	
12	Forever	

2.15. Construction of markerless deletion mutant

Markerless deletion of target genes *pepA1* and *lactococcin* 972 was carried out by double cross-over homologous recombination using the shuttle vector pKFC (Fig 2.2 and Table 2.1) as described by Kato *et al,* (79). A diagram outlining the strategy used to generate markerless deletions is shown in Fig. 3.9, and the primer names and sequences used for this process are described in Table 2.1. In brief, using the shuttle vector pKFC, two homologous regions encoding upstream and downstream of the target genes *pepA1* and *lactococcin* 972 were cloned into pKFC generating the two plasmids p*bac*_pKFC and p*pep*_pKFC (Table 2.1, and Section 2.15.2-2.15.3). Following introduction into *S. aureus* (2.15.4) and integration into the host plasmid pNZAK1 (2.15.6), recombination events can occur in either the upstream or downstream region. Excision of target genes by the double-cross over leads to target gene

deletion (see Section 2.15.5). This process generated the isolates AK02 (mutant lacking *pepA1*), and AK03 (mutant lacking *lactococcin* 972).

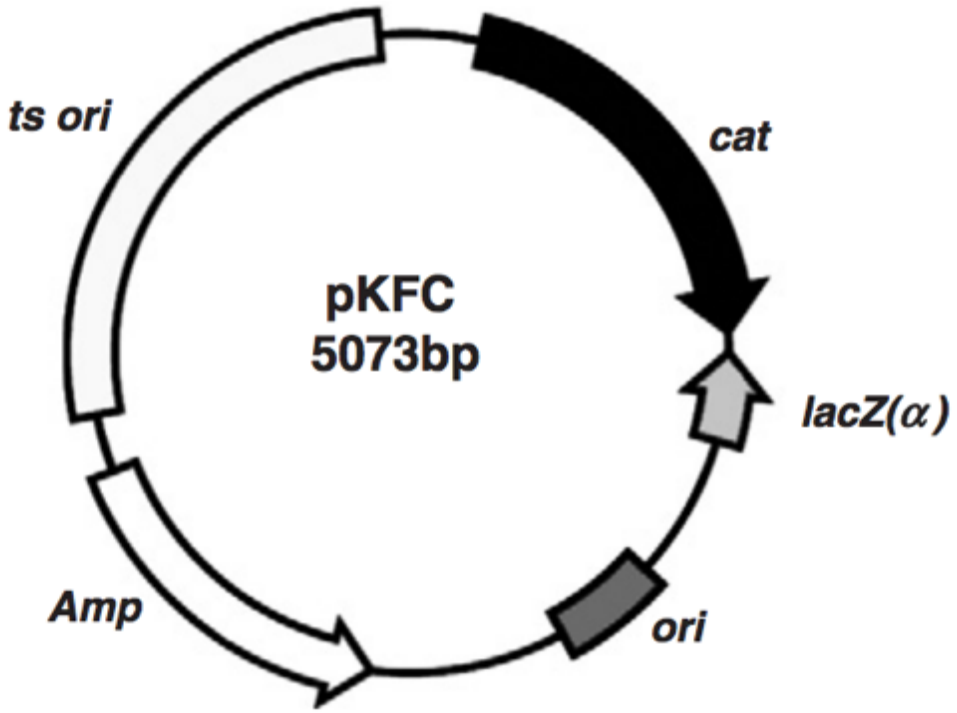


Figure 2.2. pKFC plasmid schematic. The shuttle vector pKFC (79) encodes chloramphenicol resistance (*cat*) in *E. coli* and ampicillin resistance (*Amp*) in *S. aureus*. The plasmid also carries a temperature-sensitive origin (ts ori) of replication derived from pE194ts. The *ts ori* allows straightforward genomic integration of pKFC at high temperatures (42°C). This figure is reproduced from Kato *et al.* (79).

2.15.1. Markerless deletion cloning and gene primers

Table 2.5. Cloning primer sequences

Primer Name & Description	Primer sequences
Bac1 *(Bacteriocin left flank forward)*	5'-AAAAAAGGATCCAAATGGACTTC TTCTTGGGG- 3'
Bac2 *(Bacteriocin left flank reverse)*	5'-TATATGTAATTAGTGAAGACTGA TTGCGTTATAAACGTTCG-3'
Bac3 *(Bacteriocin right flank forward)*	5'-GAACGTTTATAACGCAATCAGTC TTCACTAATTACATATATTAATTAAG GAG-3'
Bac4 *(Bacteriocin right flank reverse)*	5'-AAAAAAGGATCCCGCCTAAGAAT ATGAAAGGC-3'
Bac5 *(Integration left Bacteriocin pNZAK1 forward)*	5'-CAGTTGATGCTTCCAACAAAA-3'
Bac6 *(Integration left Bacteriocin pNZAK1 reverse)*	5'- TTGGTGAAGGTACCCATAAA-3'
Pep1 *(pepA1 left flank forward)*	5'-AAAAAAGGATCCGGCGCCGATAA TATTGGTTT-3'
Pep2 *(pepA1 left flank reverse)*	5'-TCAGGAGGGTTTTTGAAACGGGC TTAGTAA ACGCAACAAATAATTAATA-3'
Pep3 *(pepA1 right flank forward)*	5'-TCAGGAGGGTTTTTGAAACGGGCT AGTAAA CGCAACAAATAATTAATA-3'
Pep4 *(pepA1 right flank reverse)*	5'- AAAAAAGGATCCTTAGACTTTT CCGCGGATTC-3'
Pep5 *(Integration left pepA1 pNZAK1 forward)*	5'-GTTGCTGCTGCTGTTCTTTA-3'

Table 2.5. Cloning primer sequences continued

Pep6 *(Integration left pepA1 pNZAK1 reverse)*	5'-GGTACACTTTGTATTAAAGTCTGAG-3'
pepA1 forward	5'-TACAGGTCGCCTATCTCTCA-3'
pepA1 reverse	5'-CCACTGATGACTGGTGCTAT-3'
Bacteriocin forward	5'-GGTGTTTGGAGCCATGGTAT-3'
Bacteriocin reverse	5'-TGTTTTGTTTCCACCCCAAG-3'

2.15.2. Generation of left and right flank overlap from pNZAK1 for cloning into pKFC

pKFC plasmid DNA was routinely propagated through the use of chemically competent cells. Competent cells (30 µL) were thawed on ice for 10 min, before the addition of 1 µL of plasmid DNA (>30 ng/µL) (section 2.14.1). This mixture was incubated on ice for 10 min and heat shocked at 42˚C for 1 min, after which the cells were immediately cooled on ice for 1 min. After this, 500 µL of LB was added, and the cells were recovered at 37˚C with shaking for 30 min. Cells (100 µL) were then plated onto LB agar containing 100 µg/mL ampicillin. The remaining cells from the initial 500 µL (400 µL) were plated on LB agar containing 100 µg/mL ampicillin. These plates were incubated at 37˚C overnight. To generate the left and right flanks from pNZAK1 homologous flanking regions (approximately 500 bp) adjacent (upstream and downstream) of the target gene were amplified by PCR. The PCR was done using primer pairs Bac1 and Bac2, and Bac3 and Bac4 for *lactococcin* 972, as well as primer pairs Pep1 and Pep2, and Pep3 and Pep4 for *pepA1.* The details for the PCR reaction and PCR cycles are shown in Table 2.6. After the PCR was completed, the samples were analysed by agarose gel electrophoresis and purified using the Zymo Gel DNA recovery kit. The purified DNA was resuspended in MQ-H_2O and stored at -20˚C.

To generate the left and right flank overlap, the upstream left and downstream right DNA flanks generated from PCR were fused together by overlap PCR in a two-step PCR procedure. This PCR involved primer pairs Bac2 and Bac3 for *lactococcin* 972, as we as Pep2 and Pep3 for *pepA*1. The flanks are homologous to pNZAK1 regions upstream and downstream of the target genes either *pepA1* or *lactococcin* 972. The fused overlap is inserted in pKFC to carry out homologous recombination with pNZAK1.The PCR components and steps are shown in Table 2.7.

Table 2.6. Flank PCR reaction reagents and PCR method

Components	**Volume**
5× Phusion HF buffer	10 µL
dNTPs 10 mM	1 µL
Forward primer (50 µM)	0.5 µL
Reverse primer (50 µM)	0.5 µL
DNA template (30 ng/µL)	0.5 µL
Phusion Polymerase (2 U/µL)	0.5 µL
MQ-H_2O	37 µL
Total Volume	50 µL

Temperature (˚C)	**Duration**	
95	3 min	
95	30s	(35 cycles)
60 (increase if non-specific binding)	30s	
72	30s per kb	
72	10 min	
12	Forever	

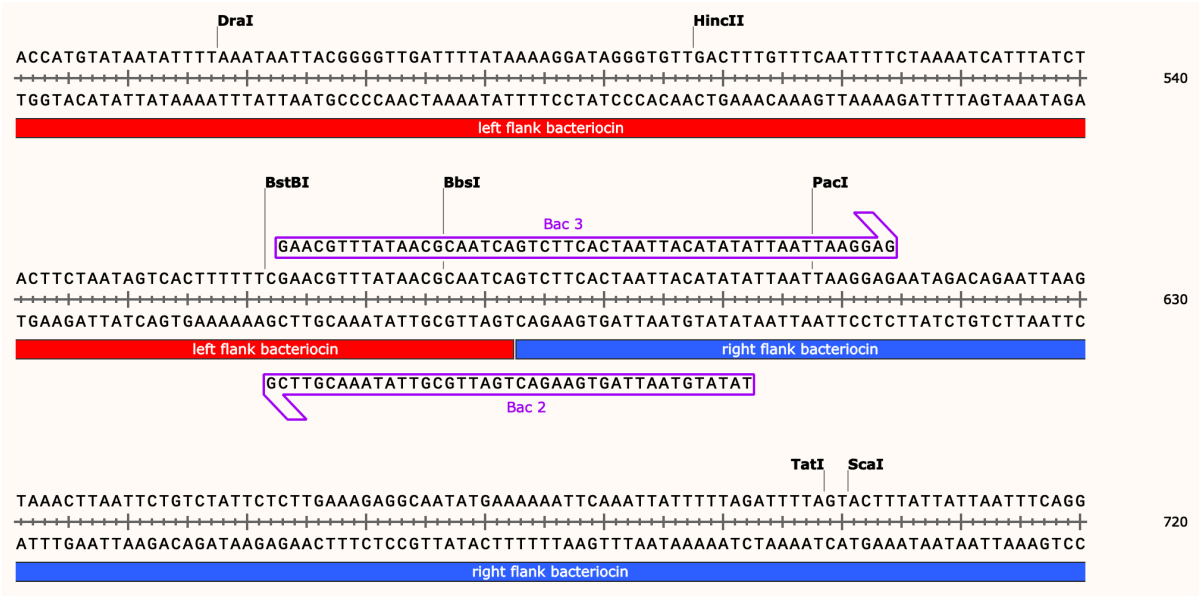


Figure 2.3. Diagram of pNZAK1 overlap fragment (left and right flank) sequence in the bacteriocin gene *lactococcin* 972 on pNZAK1. The red bar shows the left flank of the bacteriocin gene, the blue bar shows the right flank of the bacteriocin gene. The Bac2 and Bac3 primers (Table 2.5) are shown as purple arrows with overlapping sequence in left and right flank sequence of bacteriocin.

Table 2.7. pNZAK1 flank overlap PCR components and method.

Components	**Volume**
5× Phusion HF buffer	10 µL
dNTPs 10 mM	1 µL
Purified PCR product of each flank	1-2 pmol
Phusion Polymerase (2 U/µL)	0.5 µL
MQ-H_2O	37 µL
Total Volume	50 µL

Temperature (C°)	**Duration**	
95	3 min	
95	30s	(15 cycles)
60	30s	
72	30s per kb	
72	10 min	
12	Hold	

At the end of the first PCR 0.5 µL each of 50 µM primer (Bac3 and Bac2) was added to the PCR tube.

Temperature (C°)	**Duration**	
95	3 min	
95	30s	(20 cycles)
72	30s	
72	30s per kb	
72	10 min	
12	Forever	

After the overlap PCR was completed, the samples were run on a 1% agarose gel and gel extracted. The samples were recovered using the Zymo gel DNA recovery kit. The purified overlap fragment needs to be at least >20 ng/µL to be able to continue on to the next step.

2.15.3. Restriction digest and ligation of pKFC and pNZAK1 flank overlap product generating p*bac*_pKFC and p*pep*_pKFC vectors

Restriction digests of DNA were carried out in the enzyme buffer and conditions recommended by the manufacture. The shuttle vector pKFC and the finalised pNZAK1 flank overlap PCR products were digested with BamH1-HF (New England Biolabs). As required, digested vector DNA was treated with alkaline phosphatase (Roche) using 1 U of enzyme, according to the manufacturer's instructions. The digested product was analysed by gel electrophoresis and purified using the Zymo Gel DNA recovery kit. The purified product was stored at -20˚C. The details of the components in the digest mixture are shown in Table 2.8. The purified and digested pKFC (vector) and insert DNA were ligated overnight at 16˚C using T4 DNA ligase (New England Biolabs). The components of the ligation mixture are shown in Table 2.9. The amount of overlap to use was calculated using the nebiocalculator.neb.com using a 1:3 molar ratio of vector:insert. Ligated products (pKFC-insert DNA) were transformed into *E. coli* MC1061 by heat shock for routine plasmid propagation. To alter the methylation state of the plasmid before transformation into *S. aureus* 14887 plasmid DNA was isolated and transformed into *E. coli* IMO8 B.

Table 2.8. Standard restriction digest reaction mixture

Components	Volume / Concentration
10× CutSmart Buffer	5 µL
DNA (plasmid or PCR product)	2-3 µg
Restriction Enzyme (20 U/µL)	1 µL per µg of DNA
Alkaline phosphatase (**plasmid only**)	1 µL (1U) per µg of DNA
MQ-H_2O	Up to 50 µL

Components	Control	Plus insert
10× Ligation Buffer	2 µL	2 µL
Plasmid (20 ng/µL)	1 µL	1 µL
Insert	0 µL	Variable Ratio
Ligase (T4) (400 U/µL)	1 µL	1 µL
MQ-dH_2O	16 µL	Up to 20 µL

Table 2.9. Representative ligation reaction mixture

2.15.4. Preparation of electrocompetent *Staphylococcus aureus* cells and transformation with pKFC

An overnight culture (5 mL) of *S. aureus* in TSB was used to inoculate 50 mL of TSB to a starting OD_{600} of 0.1. Cells were grown to an OD_{600} of 0.6 to 0.8 and harvested by centrifugation at 3,000 × *g* at 4˚C for 10 min. The cell pellet was resuspended in 1 mL MQ-H_2O and centrifuged at 16,300 × *g* at 4˚C for 1 min. The supernatant was removed, and the MQ-H_2O wash step was repeated three times. The washed cell pellet was resuspended in 1 mL sterile 10% (v/v) glycerol, and as before, the cell pellet was washed three times. The cell pellet was resuspended in 300 µL of 10% glycerol and the solution was aliquoted into three Eppendorfs each containing 70 µL of competent cells.

Electrocompetent *S. aureus* cells were thawed on ice for 10 min before the addition of 1 µg of plasmid DNA. Cells were transferred to a pre-chilled 0.2 cm gapped electroporation cuvette (Biorad, USA), and were electrotransformed at 2300V, 25µF, 100Ω using a GenePulser™ (BioRad). Immediately after electroporation of the cells, 500 µL of TSB was added and the cells were allowed to recover by incubating at 28˚C for 2 h. Following this, 400 µL of cells were plated onto TSA containing chloramphenicol (10 µg/mL) and 200 µl of cells were plated TSA plates for each sample. Plates were incubated at 28˚C for 2 days.

2.15.5. Temperature-dependent integration of p*pep*_pKFC and p*bac*_pKFC into host plasmid pNZAK1

Overnight cultures (5 mL) of *S. aureus* 14487 transformed with plasmids p*bac*_pKFC and p*pep*_pKFC were grown in TSB containing chloramphenicol (10 µg/mL) and incubated overnight at 28˚C with shaking at 200 rpm. The following day, 1 mL of sterile TSB medium was aliquoted into three 1.5 mL Eppendorf tubes and heated to 43˚C (labelled 1/100, 1/1000 and 1/10000). The overnight culture of the transformants was diluted 1/100 (e.g. 10 µL of culture to 990 µL of TSB), 1/1000 and 1/10000 into the appropriate Eppendorf tubes and then returned to 43˚C for 10 min. Following heating at 43 ˚C, 100 µl of each dilution was plated onto preheated TSA-chloramphenicol plates (10 µg/mL), and the plates were incubated at 43˚C in a plastic bag to prevent drying out. After 24 h the plates were examined for growth, if colonies were present, then PCR colony screening (see Table 2.4) was carried out to confirm integration. Two sets of primer pairs were used Bac5/Bac4 and Pep5/Pep4, as well as Bac6/Bac1 and Pep6/Pep1 (Table 2.5). Results were analysed by agarose gel electrophoresis.

2.15.6. Excision of integrated vectors and generation of markerless deletion mutants

A (5 mL) TSB universal was inoculated with an upstream integrant and another with a downstream integrant and incubated at 28˚C for 24 h (overnight culture one). Following this, 10 µL of overnight culture was removed and sub-cultured into fresh (5 mL) TSB (Overnight culture two). Overnight culture one was then serially diluted 10-fold in TSB to 1×10^{-8} following this 50 µL aliquots of the 1×10^{-3} to 1×10^{-8} dilutions were spread plated onto TSA and incubated at 37˚C for 24 h. This passage step was repeated with the second overnight culture to generate

overnight culture three. Overnight culture two was serially diluted in the same way as overnight culture one.

Colonies (100) from overnight culture one were patch plated onto TSA and TSA with chloramphenicol (10 µg/mL) and incubated overnight at 37˚C. The passage step was repeated with overnight culture three. The serial dilution step was also repeated with overnight culture three. Patch plate colonies were examined for loss of chloramphenicol resistance indicating excision of pKFC. Colonies that had reduced size or did not grow on the TSA chloramphenicol 10 µg/mL plates were screened for pKFC excision using PCR. PCR primers used in the integration PCR were used again to screen for loss of ~5kb, this size of pKFC. A wild-type colony was included to show the expected product size after pKFC excision. PCR products were analysed by gel electrophoresis.

When PCR screening identified potential colonies that could have lost pKFC and possibly the target gene, the colony was used to inoculate two (5 mL) TSB universals and incubated overnight. The overnight culture was the miniprepped to isolate the mutated pNZAK1 following the protocol shown in section 2.14.1. A glycerol stock for the potential mutant was also generated and stored at -80˚C. The extracted pNZAK1 plasmid DNA was then nanodroped to check DNA quality and concentration and then stored at -20˚C. Plasmid DNA was sent to the Massey genome service for sequencing following their specifications for a sequencing reaction. The sequence was analysed via BLASTn to look for loss of pKFC DNA and target gene.

2.16. Wild-type versus AK02 and AK03 screening for plasmid loss

To investigate the loss of pNZAK1, relative to AK02 (Δ*pepA1*) and AK03 (Δ*lactococcin* 972) (Table 2.1), media-based assays were used. During the excision plating of pKFC, the loss of chloramphenicol resistance was found to coincide with the loss of ampicillin resistance. *S. aureus* 14487 isolates with pKFC integrated were serially passaged in TSB for 7 to 10 days at 37˚C at 200 rpm. Each day, 100 µL of overnight culture was used to inoculate a fresh 5 mL TSB universal. The serially passaged culture (100 µL) was diluted and plated at 1×10^{-5} to 1×10^{6} onto TSA and incubated overnight at 37˚C. Colonies (100) were then patch plated on to

TSA, TSA with chloramphenicol (10 µg/mL), and TSA with ampicillin (100 µg/mL) and incubated overnight at 37˚C.

2.16.1. Plasmid loss assay

Mutants AK02 and AK03, were streaked onto TSA with mupirocin (100 µg/mL) and grown overnight at 37˚C. From this a single colony was used to inoculate 5 mL ¼ MH containing mupirocin (100 µg/mL). The overnights of wild-type *S. aureus* 14487, AK02 and AK03 were used to inoculate (100 mL) ¼ MHB in a 250 mL flask to OD_{600} 0.05 in technical duplicate and biological triplicate. A 50 µL sample of each flask was taken at the time of inoculation as day zero. The sample was serially diluted to 1×10^{-3} and 1×10^{-4} in PBS and 50 µL was plated onto ¼ MHA plates and incubated overnight at 37˚C. Colonies (100) from each sample were patch plated onto ¼ MHA and ¼ MHA with ampicillin (100 µg/mL). The patch plates were then incubated overnight at 37˚C. The plasmid loss percentage was calculated based sensitivity to ampicillin. The serial dilution and patch plated steps were repeated on day three and day seven.

2.17. Batch cultures pNZAK1 competition assay

Wild-type *S. aureus* 14487 was tagged with rifampicin resistance through serial passaging in flat-bottom 96-well microtiter plates (ThermoFisher Scientific, New Zealand) at sub-MIC concentrations until a mutant was generated with rifampicin resistance (based on MIC/MBC values compared to wild-type). This isolate was used in the competition assay to differentiate wild-type *S. aureus* from ΔpNZAK1 *S. aureus* 14487.

Wild-type *S. aureus* 14487 rif^R and AK01 (see Table 2.1) were freshly streaked onto BHI agar, grown overnight and a single colony of each was used to inoculate two 5 mL BHI universals. The volume of culture needed of each strain to inoculate a 10 mL BHI universal to OD_{600} 0.1 (~1×10^8) was calculated. Then the volume of *S. aureus* 14487 pNZAK1$^-$ and Wild-type *S. aureus* 14487 rif^R required to reach OD_{600} 0.1 was added to an Eppendorf tube for each biological replicate. The Eppendorfs were then centrifuged at 16,300 × *g* and the supernatant was discarded. The pellet was washed in 1 mL PBS, and the wash step was repeated twice. The PBS was removed, and the cell pellet was resuspended in BHI in the same volume as previously calculated for the culture to reach OD_{600} 0.1. The mixed culture of both WT rif^R and AK01

strains was then used to inoculate a 10 mL BHI universal. Each biological replicate consisted of 3×10 mL BHI universals. A 50 µL sample was taken from each 10 mL universal and diluted to 1×10^{-3} and 1×10^{-4} and plated onto BHI agar and then incubated overnight at 37˚C. Colonies (100) were then picked and patched onto BHI agar, BHI agar with rifampicin (4 µg/mL) and BHI with ampicillin (100 µg/mL). The serial dilution steps and patch plating steps were repeated on day three and day seven. The results showed the percentage of Rif^R and Amp^R cells in the mixed culture indicating the percentage of wild-type *S. aureus* 14487 and pNZAK1 containing bacteria.

Chapter 3

Plasmid Maintenance of pNZAK1 in *Staphylococcus aureus*

3. Introduction

3.1. Plasmid maintenance overview

Plasmids encode a variety of genes, a number of which under certain environmental stresses can provide the host cell with a selective advantage in these environments including, but not limited to resistance; to antibiotics, heavy metals, or even other bacteria (61). The presence of extrachromosomal DNA e.g. plasmid DNA can impose an 'energetic limitation' on the cells growth properties (growth rate), which in turn can impact plasmid stability (83). A plasmid is therefore generally only maintained long-term in the population (retained in daughter cells after cell division) if it encodes a gene that is beneficial to the bacterial host e.g. antibiotic resistance genes like *blaZ* or *mupA* in an environment under antibiotic selection.

Studies have shown that energy limitation and nutrient deprivation can lead to increased MIC levels for bacteria to antibiotics like β-lactams (74). Additionally, small, slow-growing colonies can appear when bacteria are under energetic limitation, these slow-growing isolates are termed small colony variants (SCV) (84). The slow-growing auxotrophic subpopulation of SCVs lead to increased resistance levels to β-lactams and aminoglycosides from the decreased cell wall turnover and protein synthesis (84). Sigma factors, like SigB, can be activated by nutrient stress and cell envelope stress in *S. aureus* leading to increase resistance levels to β-lactams and other antibiotics (74).

Plasmids can also encode well characterised plasmid addiction mechanisms, and toxin-antitoxin systems are one such example. These systems ensure that only daughter cells, which inherit the plasmid survive. In brief, the plasmid encodes two closely linked genes, one which encodes a 'toxin', the other encodes the 'antitoxin' thus for the cell to survive it must maintain the plasmid. This system is exemplified by the bacteriocin toxin/antitoxin *lactococcin* 972 system, which has been show to increase plasmid maintenance in *Lactococcus lactis* (Fig. 3.1) (67). In a study by Campelo *et al.*, which, utilised four plasmids; pTRL1(a1) and pTRL1(b1) with *lactococcin* 972; and pTRL1(a2) and pTRL1(b2) without *lactococcin* 972. This study monitored by measuring the frequency of m_cherry positive colonies (Fig. 3.1).

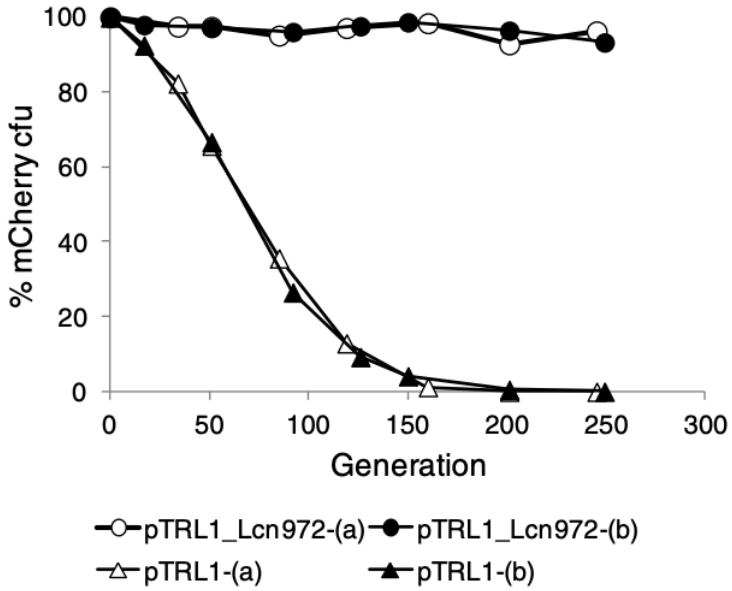


Figure 3.1. pTRL1 plasmid maintenance with and without the *lactococcin* 972 bacteriocin gene cluster in *Lactococcus lactis*. The presence of *lactococcin* 972 on a plasmid can increase the plasmid maintenance over generations in comparison to plasmids that lack the bacteriocin gene. The figure was taken from the study by Campelo *et al.* (67).

The plasmid pNZAK1 found in *S. aureus* 14487 is part of the PW2-like family of *S. aureus* plasmids found worldwide. These plasmids are known to carry antibiotic resistance genes such as *mupA* and *blaZ* as well as the suspected biocide tolerance gene *qacA* (60). pNZAK1 is a large plasmid at 27 kb and it is suggested to have a low copy number (personal communication by Dr. Glen Carter, Doherty institute, Melbourne, Australia.). Genomic analysis of pNZKA1 revealed the presence of a presumptive *lactococcin* 972 bacteriocin cluster as well as a presumptive *pepA1* toxin/anti toxin system. *PepA1* is a type 1 toxin/antitoxin system which encodes $SprA1_{AS}$ antitoxin and SprA1 toxin, they are both encoded as antisense RNA (85). SprA1 is an antisense-RNA, which forms a cytotoxic peptide *pepA1* that enters the plasmid membrane and forms a pour triggering cell death or death of competing bacteria (Fig. 3.2). $SprA1_{AS}$ is a cis-RNA that works to prevent host cell death through inhibiting a build-up of SprA1 in the cell membrane (Fig. 3.2) (85).

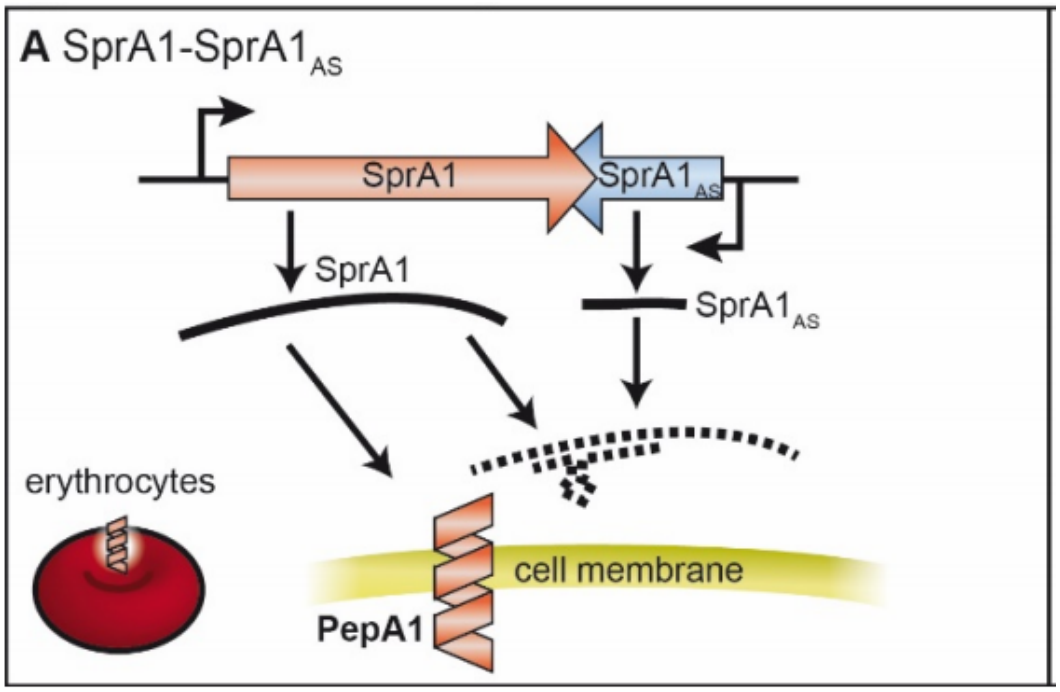


Figure 3.2. *pepA1* system (SprA1$_{AS}$ and SprA1) expression and mechanism. Summary diagram of SprA1$_{AS}$ and SprA1 gene locations and protein products and functions. SprA1 RNA inhibits the function of toxin RNA SprA1$_{AS}$ through binding. The SprA1 toxin RNA is able to express the peptide *pepA1*. Additionally, *pepA1* interacts with the host cell membrane and triggers lysis of red blood cells. The figure was taken from the study by Sayed *et al.* (85).

3.1.1. Chemostat: A tool for investigating plasmid loss

It was proposed that energy limitation in combination with the absence of antibiotic selection would create an environment that would induce plasmid loss of pNZAK1. To investigate the impact of energy limitation on plasmid loss this study used a chemostat, which is a unique tool for investigating energy limitation. The chemostat creates a continuous culture where the nutrient level, oxygen saturation and temperature are controlled. This allows precise control of the doubling time of the bacteria. This allowed *S. aureus* 14487 to be grown at 3, 10 and 30 times slower than its normal growth rate in minimal media exerting an energy limitation on the bacteria.

3.2. Hypothesis and research objectives

Hypothesis 1: We hypothesised that the energy limited state of *S. aureus* NZ14487 cells grown at doubling times of 3 h, 10 h, and 30 h would impact the cells sensitivities to the encoded antimicrobial resistance determinants on plasmid pNZAK1.

Hypothesis 2: We hypothesised that the energetic limitation imposed by continuous culture at slow growth rates would result in the loss of the plasmid pNZAK1 from *S. aureus* 14487.

Hypothesis 3: Additionally, we hypothesised that the two plasmid addiction systems present on pNZAK1; the *lacotcoccin* 972 bacteriocin (accession number: B0I1V8), and the *pepA1* system (accession number: 4B19_A), are involved in plasmid maintenance of pNZAK1 in *S. aureus* 14487.

Research Objective 1: To investigate the impact of energy limitation on antibiotic sensitivity, *S. aureus* NZ14487 was grown in continuous culture allowing steady state growth at fixed dilution rates of 0.231 h^{-1}(3 h dt), 0.0693 h^{-1}(10 h dt), and 0.023 h^{-1}(30 h dt).

Research Objective 2: To investigate the plasmid maintenance of pNZAK1 in *S. aureus* 14487, cells were grown in a chemostat to create energy limiting conditions to stimulate plasmid loss.

Research Objective 3: To understand the fitness cost of pNZAK1 in *S. aureus* 14487, a competition assay was carried out with wild-type *S. aureus* and AK01 Δ*qacA* mutant strain (see Table 2.1).

3.3. Investigating the effect of varied growth rates via batch and continuous culture on the antibiotic susceptibility profile of *Staphylococcus aureus* 14487

3.3.1. S. aureus 14487 growth properties in batch culture

To investigate the growth properties of *S. aureus* 14487, which harbours the plasmid pNZAK1, cells were grown in batch culture with shaking in the absence of any antibiotic or antiseptic treatment (Fig. 3.3). When *S. aureus* was grown in a standard 250 mL flask containing 100 mL of ¼ strength MHB, the organism reached an OD_{600} of approximately 1.0, after ~5 h at 37°C in batch. Under these conditions, *S. aureus* 14487 had a doubling time of 1.64 h, equating to a specific growth rate of 0.421 h^{-1}, and typically reached a maximum OD_{600} of 1.8 (after 24 h). When *S. aureus* was grown in TSB it had a doubling time of 0.830 h , equating to a specific growth rate of 0.834 h^{-1} and reached a maximum OD_{600} of 11 (after 24 h).

3.3.2. S. aureus 14487 growth properties in continuous culture via a chemostat

To examine the growth properties of *S. aureus* 14487 under general energy limited continuous culture conditions a chemostat was utilised. As the MHB broth used in this experiment is not a true minimal medium the concentration was decreased to ¼ strength in order to impose an energy restriction from general nutrient limitation on the *S. aureus* cells. The chemostat peristaltic media pump was turned on and controlled to facilitate cell dilution rates of 0.231 h^{-1} (3 h dt), 0.0693 h^{-1} (10 h dt), and 0.023 h^{-1} (30 h dt). The dilution rate, temperature of 37˚C, oxygen saturation (50%), and nutrient level were precisely controlled. Samples for analysis were removed once the cells had reached steady state. The steady state OD_{600} for the 3 h doubling time was typically around 0.9, whereas for the 10 h and 30 h doubling time the steady state OD_{600} were 1.4, and 1.5 respectively (Fig. 3.4A-B). Steady state was reached after 3-4× resident volumes of media had been exchanged.

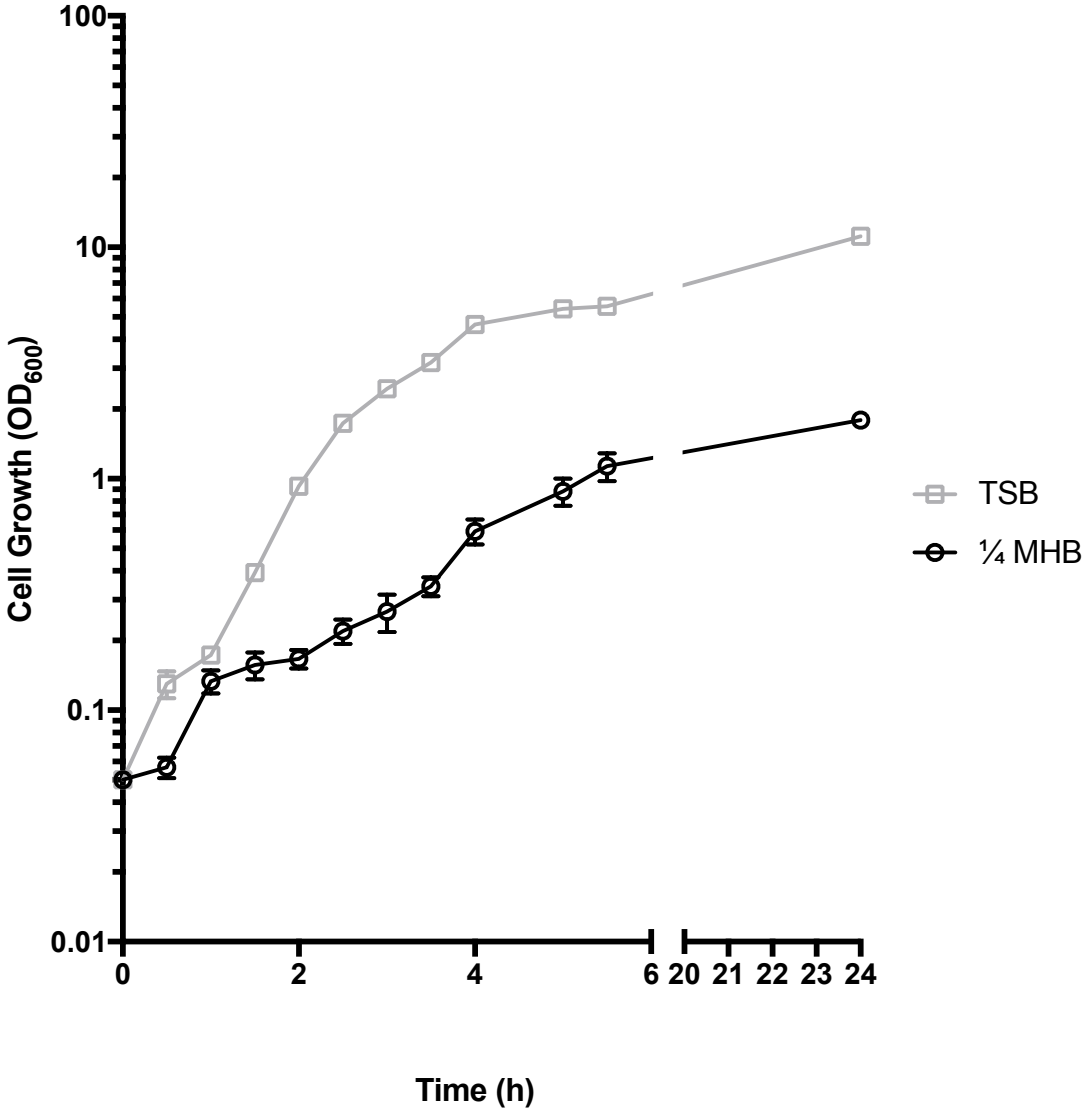


Figure 3.3. Growth of *S. aureus* 14487 in batch culture. Cells of *S. aureus* 14487 were inoculated to an initial OD_{600} of 0.05 and were grown in ¼ strength MHB (black circle) and TSB (grey square). Growth was monitored by the change in optical density (OD_{600}). Under these conditions, *S. aureus* 14487 had a doubling time of 1.64 h in ¼ MHB , equating to a specific growth rate of 0.421 h^{-1}. *S. aureus* 14487 had a doubling time of 0.830 h in TSB, equating to a specific growth rate of 0.834 h^{-1}. The rate was calculated from the 1h to 4 h OD_{600} points at the exponential phase.

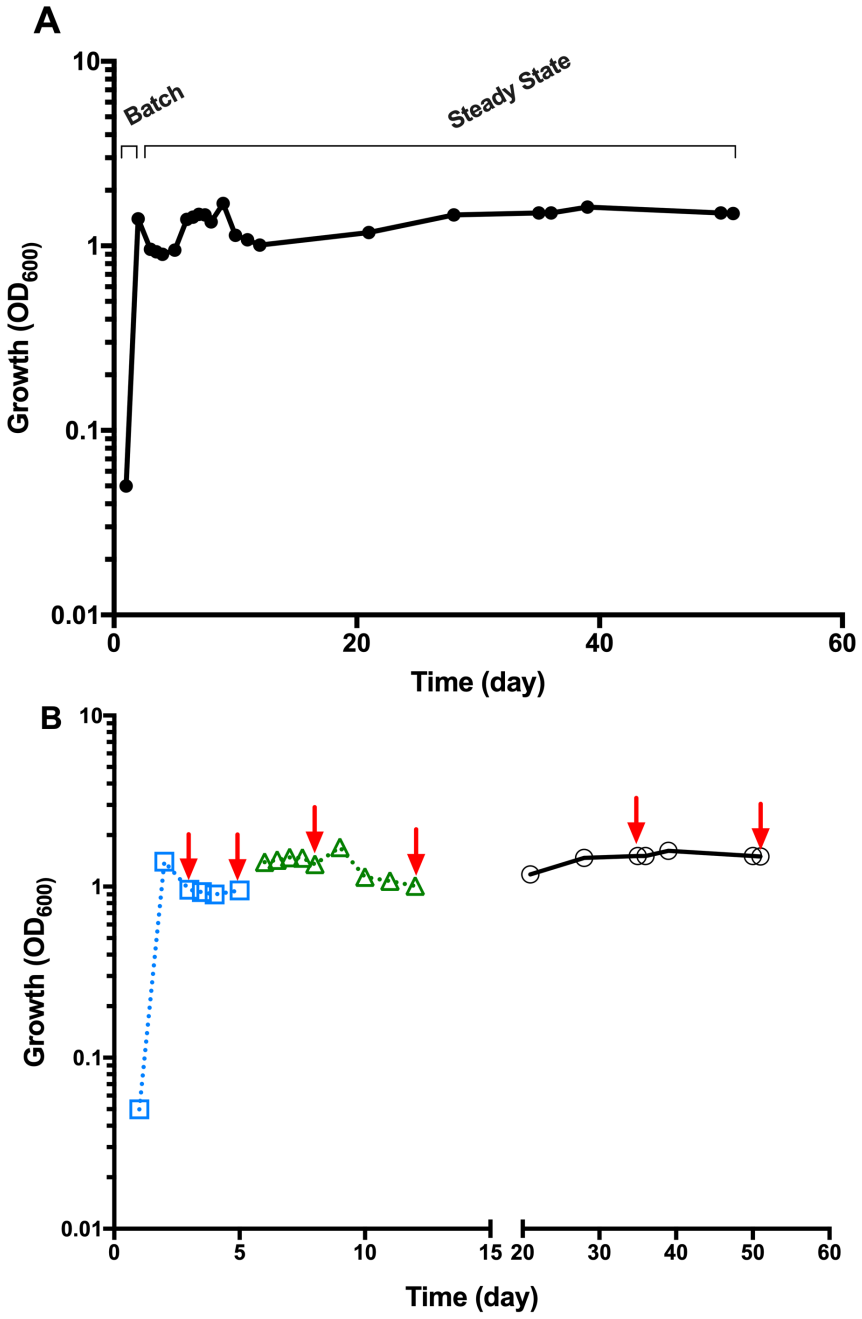


Figure 3.4. Growth of *S. aureus* 14487 in continuous culture. *S. aureus* 14487 cells were inoculated to an initial OD_{600} of 0.05 and were grown in ¼ strength MHB. Growth was monitored by the change in OD_{600}. Red arrows indicate the time points at which samples were withdrawn for MIC, MBC, and plasmid loss assays for the following conditions; 3 h doubling time (0.231 h^{-1}) (⊡),10 h doubling time (0.0693 h^{-1}) (△), and 30 h doubling time (0.023 h^{-1}) (⊖).

3.3.3. The antimicrobial sensitivity of *S. aureus* 14487 is not significantly impacted by the cellular growth rate

To investigate the effect that decreasing growth rates had on the antibiotic susceptibility profile of *S. aureus* 14487, the organism was grown in a continuous culture system, which allowed precise control of the conditions in which the bacteria were grown. The cells were grown at dilution rate of 0.231 h^{-1} (3 h dt), 0.0693 h^{-1} (10 h dt) and 0.023 h^{-1} (30 h dt). To investigate if the antimicrobial sensitivity of *S. aureus* 14487 towards mupirocin, fusidic acid, ampicillin, and chlorhexidine was influenced by the cellular growth rate, we determined the MIC and MBC for each given growth rate (Table 3.1). The MIC and MBC assays were carried out in technical triplicates and biological duplicates.

Table 3.1. Antimicrobial sensitivity of selected antimicrobial compounds against *S. aureus*

Doubling Time	**Compound**	**MIC[a] (µg/mL)**	**MBC[b] (µg/mL)**
3 h	Mupirocin	128-256	256
	Fusidic Acid	16	128
	Ampicillin	32	128
	Chlorhexidine	2	2-4
10 h	Mupirocin	256	>256
	Fusidic Acid	16	256
	Ampicillin	64	128
	Chlorhexidine	2	2-4
30 h	Mupirocin	256	>256
	Fusidic Acid	16-32	128-256
	Ampicillin	32	64-128
	Chlorhexidine	2	2-4

[a]MIC, minimum inhibitory concentration for each test compound, [b]MBC, minimum bactericidal concentration for each test compound. Data is representative of biological duplicates and technical triplicates.

When *S. aureus* 14487 was grown at a 3 h doubling time, the MIC for mupirocin *(mupA* on pNZAK1) was between 128 and 256 µg/mL and remained unchanged when the doubling time was decreased to 10 h, and then 30 h. The MIC for fusidic acid was determined to be 16 µg/mL for the 3 h and 10 h doubling times, but it increased 2-fold to 32 µg/mL when the cells were grown at a 30 h doubling time. The sensitivity of ampicillin increased 2-fold between the 3 h and 10 h doubling time, from 32 µg/mL to 64 µg/mL, however, the MIC reverted to 32 µg/mL when the cells were grown at a 30 h doubling time. The MIC of chlorhexidine did not appear to be affected by the growth rate, and the MIC remained at 2 µg/mL.

As MIC assays only measure the inhibition of bacteria by the compound and not the killing of the bacteria, MBC assays were used to investigate the bactericidal activity of each compound at the three growth rates. The MBC for mupirocin was 256 µg/mL when the *S. aureus* cells were grown at a 3 h doubling time and increased beyond 256 µg/mL for both the 10 h and 30 h doubling times. *S. aureus* 14487 had a stable MBC of 128 µg/ml for ampicillin for the 3 h and 10 h doubling times. The 30 h MBC for ampicillin was between 64 and 128 µg/mL. The MBC for fusidic acid was determined to be 128 µg/mL for both the 3 h and 10 h doubling times, increasing to 128 to 256 µg/mL for the 30 h doubling time. Like the results from the MIC assay for chlorhexidine, the MBC also did not appear to change and stayed constant at µg/mL for all growth rates examined. To confirm that any changes in MIC and MBC values were not influenced by the ratio of viable cells to antibiotic we confirmed that there was no significant change in the cell numbers for each given growth rate by determining the CFU/mL. Overall, the MIC and MBC values for mupirocin, fusidic acid, appeared to increase albeit by one antibiotic dilution as the growth rate decreased, specifically when grown at a 10 h doubling time. The ampicillin MIC and MBC appeared to decrease between the 10 h and 30 h point. Whereas, the MIC and MBC of chlorhexidine was not affected by growth rate.

3.4. Stability of the plasmid pNZAK1 under energy limitation imposed by continuous culture

To investigate if the energy limitation imposed by growth rates up to 30× slower than batch growth impacted the maintenance of plasmid pNZAK1 within the population, we utilised continuous culture in a chemostat. Cultures were grown as previously described (see Section 2.6), and samples were removed at the time points indicated in Fig. 3.4. Plasmid stability assays were conducted by patch plating 100 colonies onto MHA with or without ampicillin (6 μg/mL) and BHI agar. We examined 300 colonies from each growth rate in biological duplicate (Table 3.2). Colonies where no growth was observed on ampicillin were recorded as isolates which had lost the plasmid pNZAK1, as resistance to ampicillin is encoded on the plasmid only.

Table 3.2. Stability of pNZAK1 plasmid from *S. aureus* 14487 under different growth rates imposed by continuous culture

Doubling Time	**pNZAK1 loss (%)**	**CFU/mL[a] of Chemostat sample used for plasmid loss assay**
3 h	284/300 (5.4%) 296/300 (1%)	$3.8x10^8$
10 h	295/300 (1.7%) 296/300 (1.4%)	$2.4x10^8$
30 h	282/300 (6%) 295/300 (1.7%)	$4.4x10^8$

[a] CFU/mL, colony forming units per millilitre. Data is representative of biological duplicates.

From the plasmid stability assays shown in Table 3.2 we observed that for the 3 h doubling time the plasmid loss was 5.4% and 1% from two biological replicates. The 10 h doubling time samples maintained a 1.7% and 1.4% plasmid loss from 300 patch plated colonies. There was 6% and 1.7% plasmid loss from the samples taken from the 30 h doubling time. These data showed that the pNZAK1 plasmid is stable and maintained in the chemostat cell population in

S. aureus 14487 despite the energy limitation of growing at a doubling time 30× slower than that of batch growth and without antibiotic selection. With the exception of experimental variability between biological replicates, decreasing the doubling time of *S. aureus* 14487 cells from 3 h, to 30 h did not have a significant impact on plasmid stability/loss. These data indicate that a proportion of daughter cells within the chemostat culture naturally appeared to have lost the plasmid pNZAK1, independent of any imposed energy limitation. Furthermore, it also indicates that losing the plasmid pNZAK1 provides no additional growth advantage, as these cells failed to accumulate and out compete cells still harbouring pNZAK1 within the chemostat. Interestingly, this small proportion of cells without pNZAK1 is maintained despite the presence of two plasmid addiction systems, see section 3.6 for further information.

3.4.1. Plasmid loss isolates drug sensitivity profile

While it was demonstrated that the plasmid pNZAK1 was highly stable in the majority of cells recovered from the chemostat at decreasing growth rates, we were able to isolate a population of cells that had presumptively lost the plasmid, as determined by patch plating (Table 3.2). To confirm that these results were due to plasmid loss rather than a mutation (inactivation) in the gene conferring resistance, for example to ampicillin (*blaZ*), these presumptive isolates were tested to determine if they had altered MIC's for mupirocin, fusidic acid, ampicillin, and chlorhexidine (Table 3.3).

Table 3.3. Antibiotic sensitivity of plasmid loss isolates as measured by the MIC to selected antimicrobials

Doubling Time	**Plasmid Loss Isolates** [a]	**MIC (μg/mL)** [b]			
		Mupirocin	**Fusidic Acid**	**Ampicillin**	**Chlorhexidine**
Wild-Type *S. aureus* 14487		128-256	16	32-64	2
3 h	**3-2-44**	0.5	8	0.5	1
	3-2-162	0.125	8	0.5	1
10 h	**10-1-189**	0.125	8	0.5	1
	10-1-233	0.25	8	0.5	1
	10-1-234	0.125	8	0.5	1
	10-1-250	0.5	8	1	1
	10-2-186	0.5	8	0.25	1
30 h	**30-1-37**	0.25	8	0.5	1
	30-1-38	0.125	8	0.5	1
	30-1-113	0.125	8	1	1
	30-1-157	0.125	8	0.5	1
	30-1-206	0.25	8	0.5	1
	30-2-103	0.25	8	2	1
	30-2-124	0.25	8	2	1
	30-2-167	0.5	4	2	2
	30-2-176	0.5	8	1	1
	30-2-212	1	16	4	2
	30-2-231	0.25	8	1	4
	30-2-232	0.5	8	2	1
	30-2-276	0.25	8	1	2
	30-2-296	0.25	4	1	1

[a]Isolates identified from plasmid stability experiments which displayed changes in MIC compared to the wild type *S. aureus* 14487. [b] MIC, minimum inhibitory concentration for each test compound was determined by lack of growth in well. This data is representative of technical triplicate and biological duplicates.

The results indicated that the isolates that lost the plasmid showed an overall decrease in MIC (increased susceptibility) for all tested compounds. The MIC for mupirocin in some isolates decreased 2048-fold from 256 µg/mL to 0.125 µg/mL - 1 µg/mL. MIC values for fusidic acid decreased to 8 µg/mL, where two 30 h isolates had an MIC of 4 µg/mL and one 30 h isolate having and MIC of 16 µg/mL. For all the growth rate isolates collected the MIC for ampicillin decreased at least 16-fold to be between 0.25 µg/mL - 2 µg/mL. The MIC for chlorhexidine was predominantly 1 µg/mL with three isolates at 2 µg/mL and one at 4 µg/mL. These results show that there was a dramatic increase in antibiotic sensitivity for the isolates that had presumptively lost the plasmid pNZAK1 compared to the parental *S. aureus* 14487 isolate.

3.4.2. Genomic analysis of plasmid loss isolates

To identify any significant mutations which may have occurred due to growth up to 30× slower than batch, selected isolated which had presumptively lost the plasmid were analysed by whole genome sequencing (Table 3.4).

Table 3.4. Chromosomal sequencing analysis of plasmid loss isolates

Doubling time	Isolates	Whole genome sequencing results
3 h	**3-2-44**	**1.** ACG →→ AGG, Threonine to Arginine at amino acid 1,210 of a 'DUF1542 containing-protein' (similar to FmtB or SasC)
	3-2-162	
10 h	**10-1-189**	**1.** ACG →→ AGG, Threonine to Arginine at amino acid 1,210 of a 'DUF1542 containing-protein' (similar to FmtB or SasC)
	10-1-233	**1.** ACG →→ AGG, Threonine to Arginine at amino acid 1,210 of a 'DUF1542 containing-protein' (similar to FmtB or SasC) **2.** TTA →→ TCA, Leucine to Serine at amino acid 83 of an adenine phosphoribosyltransferase
	10-1-234	**1.** ACG →→ AGG, Threonine to Arginine at amino acid 1,210 of a 'DUF1542 containing-protein' (similar to FmtB or SasC) **2.** Frameshift in a DNA-binding response regulator, with +TG following amino acid 129 of the normally 220 amino acid protein
	10-1-250	**1.** ACG →→ AGG, Threonine to Arginine at amino acid 1,210 of a 'DUF1542 containing-protein' (similar to FmtB or SasC) **2.** GGA →→ AGA, Glycine to Arginine at amino acid 209 of YSIRK signal domain/LPXTG anchor domain surface protein **3.** AAA →→ GAA, Lysine to Glutamic Acid at amino acid 82 of an adenine phosphoribosyltransferase, the same gene as in DMG1701341 **4.** CAT →→ CGT, Histidine to Arginine at amino acid 220 of an ATP-binding protein
	10-2-186	**1.** ACG →→ AGG, Threonine to Arginine at amino acid 1,210 of a 'DUF1542 containing-protein' (similar to FmtB or SasC) **2.** GGA →→ AGA, Glycine to Arginine at amino acid 209 of YSIRK signal domain/LPXTG anchor domain surface protein

		3. AAA →→ GAA, Lysine to Glutamic Acid at amino acid 82 of an adenine phosphoribosyltransferase, the same gene as in DMG1701341 **4.** CAT →→ CGT, Histidine to Arginine at amino acid 220 of an ATP-binding protein
30 h	**30-1-37**	**1.** ACG → AGG, Threonine to Arginine at amino acid 1,210 of a 'DUF1542 containing-protein' (similar to FmtB or SasC) **2.** TCA→TTA, Serine to Leucine at amino acid 213 of the transcriptional regulator of the lactose metabolism operon lacA-lacG **3.** Deletion of 22 genes including: ▪ A cation transporter ▪ An aldehyde dehydrogenase ▪ A bifunctional acetaldehyde-CoA/alcohol dehydrogenase ▪ Six genes involved in capsular biosynthesis **4.** AAA → GAA, Lysine to Glutamic Acid at amino acid 82 of an adenine phosphoribosyltransferase, **5.** AAG → AAT, Lysine to Asparagine at amino acid 302 of a hydroxyacid dehydrogenase **6.** TTT→TCT, Phenylalanine to Serine at amino acid 182 of a DNA-binding response regulator, immediately downstream from the ATP-binding protein that was mutated in 10 h isolates
	30-2-296	**1.** ACG → AGG, Threonine to Arginine at amino acid 1,210 of a 'DUF1542 containing-protein' (similar to FmtB or SasC) **2.** TCA→TTA, Serine to Leucine at amino acid 213 of the transcriptional regulator of the lactose metabolism operon lacA-lacG **3**. Deletion of 22 genes including: ▪ A cation transporter ▪ An aldehyde dehydrogenase

- A bifunctional acetaldehyde-CoA/alcohol dehydrogenase
- Six genes involved in capsular biosynthesis

4. AAA → GAA, Lysine to Glutamic Acid at amino acid 82 of an adenine phosphoribosyltransferase

5. CAG → TAG, Glutamine to STOP at amino acid 67 of a two-component sensor histidine kinase

6. TTT→TCT, Phenylalanine to Serine at amino acid 182 of a DNA-binding response regulator, immediately downstream from the ATP-binding protein that was mutated in 10 h isolates.

Through whole genome sequencing we confirmed that plasmid loss isolates all lost the pNZAK1 plasmid (Table 3.4). The 3 h isolates, 3-2-44 and 3-2-162 both had mutations in the DUF1542 protein that is similar to FmtB, which encodes methicillin resistance and SasC and is associated with biofilm formation, and cell aggregation (86,87). 10 h isolates all showed the same mutation in DUF1542 as the early isolates. Isolates 10-1-233, 10-1-250, and 10-2-186 all had mutations leading to an amino acid change in adenine phosphoribosyltransferase, which aids in the uptake of adenine, a vital purine needed for DNA replication (88). Isolate 10-1-234 had frameshift in a DNA response regulator leading to a truncated polypeptide. DNA response regulators help bacteria respond to generalised nutrient limitations and other environmental stresses (89). Isolates 10-1-250 and 10-2-186 also had an amino acid change in the YSIRK signal domain/LPXTG anchor domain surface protein which is involved in many surface proteins e.g. protein A (90). Isolates 10-1-250 and 10-2-186 showed an amino acid change in an ATP-binding protein. The end-point isolates from the 30 h doubling time both shared the DUF1542 mutation, the alteration in adenine phosphoribosyltransferase as well as a mutation in the DNA response regulator of an ATP-binding protein. Additionally, these isolates had a mutation in the transcription regulator of the *lacA-lacG* operon which is involved in the utilisation of lactose and galactose (91). Both isolates had 22 gene deletions which included a cation transporter, an aldehyde dehydrogenase, an acetaldehyde-CoA/alcohol dehydrogenase and 6 capsular biosynthesis genes (92).

These deletions could significantly hinder the metabolism of the bacteria as well as decrease the virulence with the loss of capsular biosynthesis genes. Overall, the isolates taken at the 3 h doubling time had less phenotypically significant mutations compared to those isolates taken from the 30 h doubling time. These gene deletions were thought to be potentially phenotypically important, but it is uncertain if they play a role in plasmid maintenance.

3.5. pNZAK1 competition assay: Wild-type *Staphylococcus aureus* 14487 versus AK01 pNZAK1$^-$ strain

To investigate the fitness cost of harbouring pNZAK1, we compare the survival of wild-type cells to that of isolates which have lost the plasmid pNZAK1$^-$ (Fig. 3.5). For this experiment, we selected the isolate 3-2-162 from the 3 hour doubling time, and designated this isolate AK01 pNZAK1$^-$ (Table 2.1). In order to differentiate the two isolates from each other, we tagged the wild-type *S. aureus* 14487 with rifampicin resistance, generating the strain *S. aureus* 14487 rif^R. The ratio of wild-type *S. aureus* to AK01 pNZAK1$^-$ strain was measured using rifampicin and ampicillin resistance as an indicator of wild-type *S. aureus* in the mixed culture.

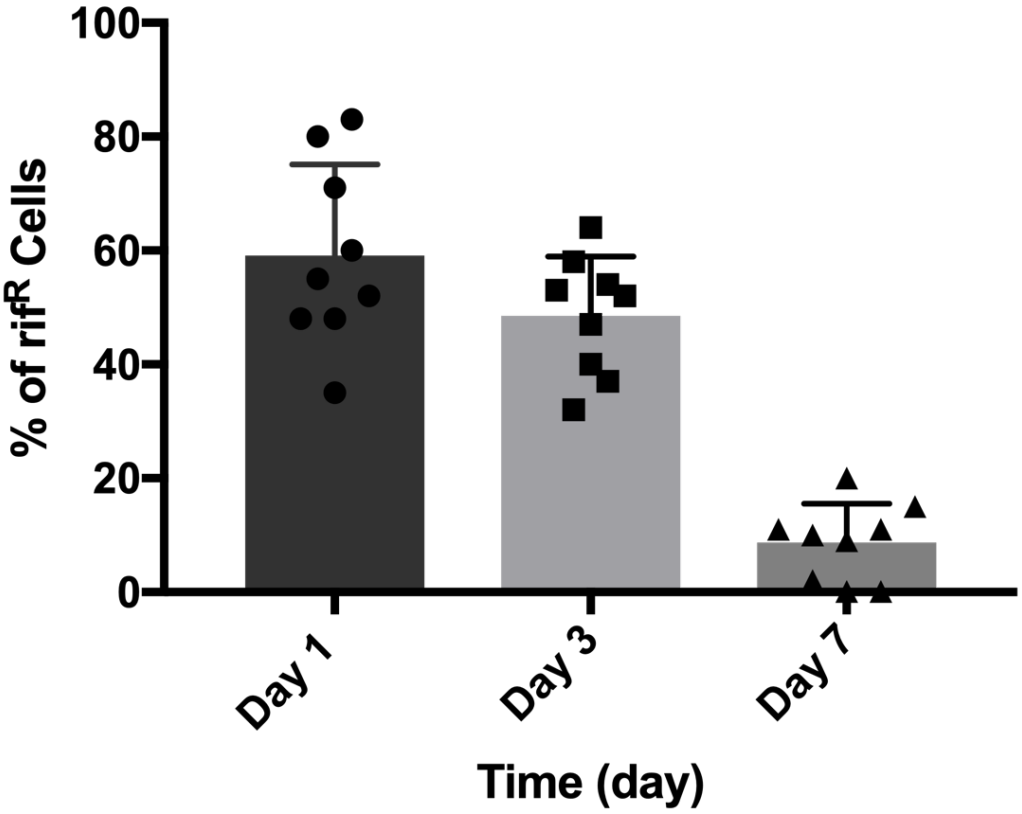


Figure 3.5. Competition assay showing the proportion of wild-type *S. aureus* 14487 rif^R in the presence of AK01 pNZAK1$^-$ following non-selective growth. Wild-type *S. aureus* 14487 rif^R and AK01 pNZAK1$^-$ cells were grown in BHI over 7 days at 37˚C with shaking at 200 rpm. Wild-type *S. aureus* 14487 were tagged with rifampicin resistance (rif^R). Day 1 sample was taken 24 h after inoculation. At the indicated times samples were withdrawn, plated on BHI agar, and then patch plated onto BHI agar, BHI agar with rifampicin (4 µg/mL), and BHI agar with ampicillin (100 µg/mL) to calculate the percentage of wild-type *S. aureus* 14487 in a mixed culture. Data represents the results of 3 independent experiments, with error bars showing the standard deviation of the mean. Individual data points are also shown.

Given the lack of fitness cost imparted on the cells by pNZAK1 when grown in continuous culture we investigated the effect of the pNZAK1^{+}/pNZAK1^{-} phenotype in competition experimets. To facilitate this experiment, we generated a wild-type rifampicin resistant strain (rif^{R}) by serial passaging. We confirmed that this mutant had no observable fitness cost through a growth curve in BHI (Fig. 3.6).

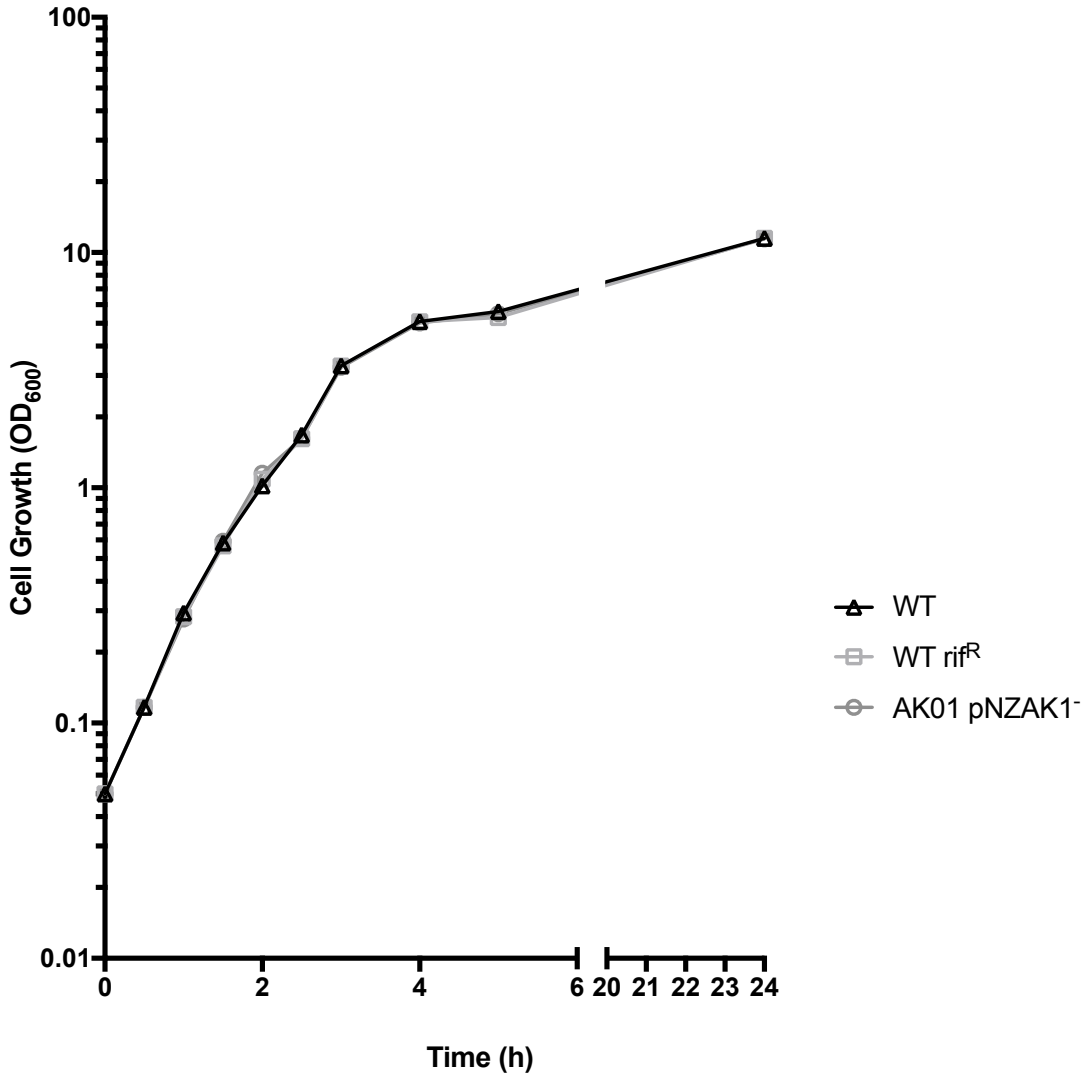


Figure 3.6. Growth of S. aureus 14487 in batch culture in BHI. Cells of wild-type (black triangle), rif^{R} strain (grey square) and AK01 (grey circle) were inoculated to an initial OD_{600} of 0.05 and were grown in BHI Growth was monitored by the change in optical density (OD_{600}). Under these conditions, all *S. aureus* 14487 strains had a doubling time of 0.7264 h in BHI,

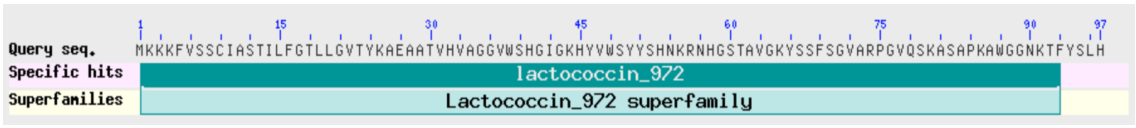
equating to a specific growth rate of 0.954 h^{-1}. The rate was calculated from the 1h to 4 h OD_{600} points at the exponential phase.

Our results showed that on Day 1 (24 h after inoculation) there was approximately 60% wild-type *S. aureus* 14487 present in the mixed culture, and approximately 40% AK01 pNZAK1^{-}. By Day 3 the percentage of wild-type *S. aureus* 14487 decreases to approximately 50%. However, by Day 7 the proportion of wild-type *S. aureus* 14487 decreased to approximately 15%. These data indicate that either pNZAK1 imposes a fitness cost on *S. aureus* 14487, as the wild-type strain can be outcompeted in batch culture in the absence of selective pressure, or that a plasmid addiction system is expressed. This system could kill wild-type cells over the course of the experiment while the plasmid-loss isolate has already mutated to be able to survive in the presence of the toxin. These data also suggest that under the experimental conditions reported, there was no observable transfer of pNZAK1 into the cured strains, given the proportion of cells observed (pers. comm. Dr Glen Carter, Doherty Institute, Melbourne).

3.6. Identification of plasmid addiction systems in pNZAK1

The maintenance of pNZAK1 in *S. aureus* cells is stable in the absence of antibiotic selective pressure (Table 3.2), stable under energy limiting conditions, and pNZAK1^{+} cells are outcompeted by pNZAK1^{-} in rich media at stationary phase (Fig. 3.5). These data suggest that there could be a functioning plasmid addiction mechanism encoded on pNZAK1.

A



B

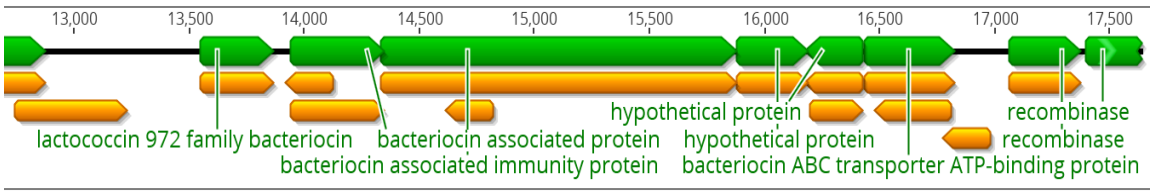


Figure 3.7. Analysis of putative bacteriocin gene, *lactococcin* 972, in pNZAK1.

(A) The BLASTp comparison of the query pNZAK1 amino acid sequence compared to the *lactococcin* 972 bacteriocin sequence. **(B)** The gene cluster of the *lactococcin* 972 bacteriocin, immunity protein and other associated genes on pNZAK1 taken from the program Geneious.

A

B

Chain A, PEPA1

Sequence ID: 4B19_A **Length:** 30 **Number of Matches:** 1

Range 1: 1 to 28 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
53.9 bits(128)	6e-11	Compositional matrix adjust.	21/28(75%)	28/28(100%)	0/28(0%)

```
Query  21  LIIFVHIIAPVISGCAVAYYTYWLSKRN  48
          ++IFVHIIAPVISGCA+A+++YWLS+RN
Sbjct  1   MLIFVHIIAPVISGCAIAFFSYWLSRRN  28
```

Figure 3.8. Amino acid sequence of *pepA1* and BLASTn search.

(A) The amino acid sequence of *pepA1* from pNZAK1 taken from the program Geneious.

(B) The BLASTn search of the amino acid sequence highlighting *pepA1* as a potential match to the sequence found on the plasmid pNZAK1.

Previously, pMW2-like plasmids have been shown to have genes encoding a putative bacteriocin, and analysis of pNZAK1 indicate that the putative bacteriocin is *lactococcin* 972 (Fig. 3.7) (60). Our analysis also revealed that pNZAK1 putatively encode *pepA1*, a type 1 antitoxin system (Fig. 3.8).

3.7. Development of PCR products to generate markerless deletion mutants in the plasmid pNZAK1 by homologous recombination

As described, two presumptive plasmid addiction systems, *lactococcin* 972 and *pepA1*, were identified to be encoded on pNZAK1 and were hypothesised to be involved in the maintenance of pNZAK1. To investigate the role of these two plasmid addiction systems, we therefore undertook experiments to individually delete the *lactococcin* 972 system, and the *pepA1* toxin/antitoxin system. The knockouts were generated via markerless gene deletion method as described in Section 2.15.2 and in Kato *et al.* (79).

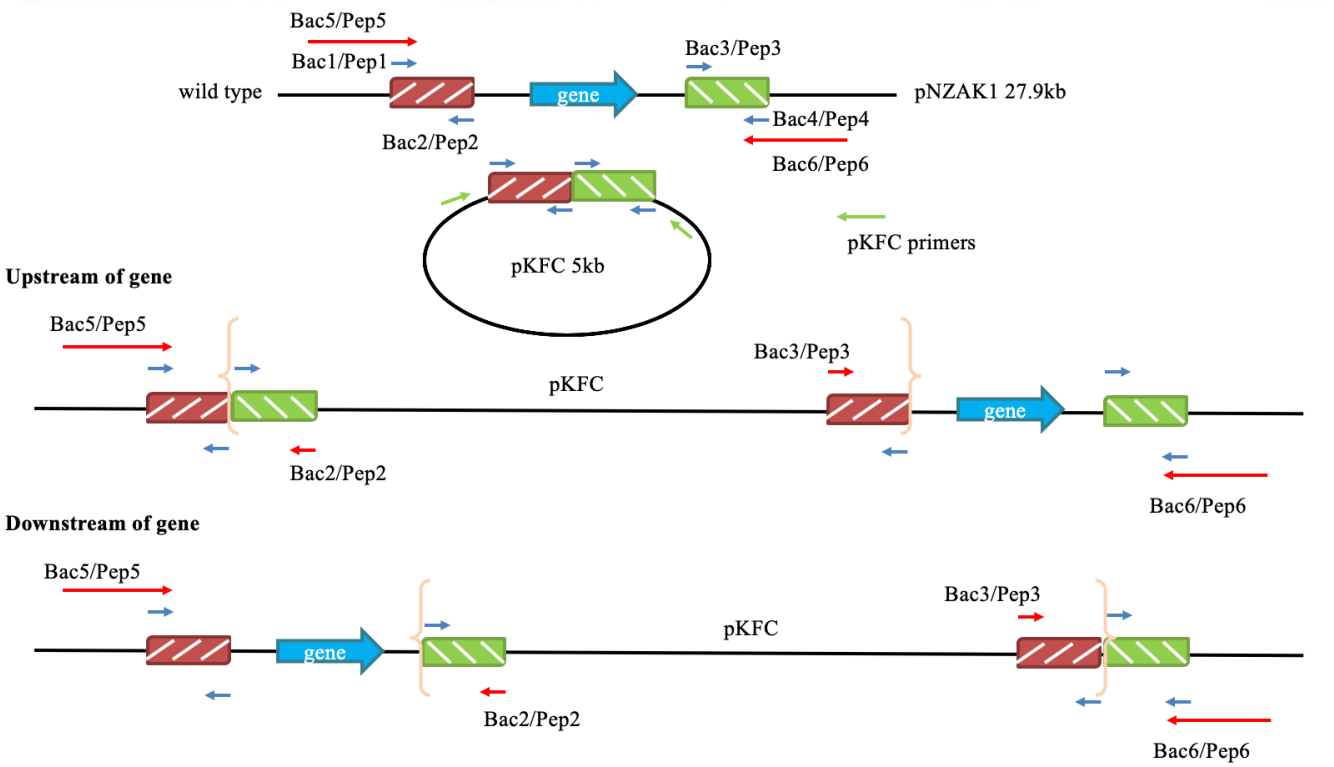


Figure 3.9. Schematic representation of markerless deletion of target genes (*lactococcin* 972 and *pepA1*) in pNZAK1 mediated by the shuttle vector pKFC. To selectively target a gene for deletion in pNZAK1 e.g. *lactococcin 972,* two homologous fragments upstream and downstream of the target gene were amplified using the primer pairs Bac1/Bac2 and Bac3/4 (Table 2.5). These homologous DNA flanks were fused together by PCR overlap extension using primer pair Bac2/Bac3. The fused overlaps for both *lactococcin* 972 and *pepA1* were separately cloned into pKFC generating the markerlesss deletion vectors; p*pep*_pKFC and p*bac*_pKFC (Table 2.1).

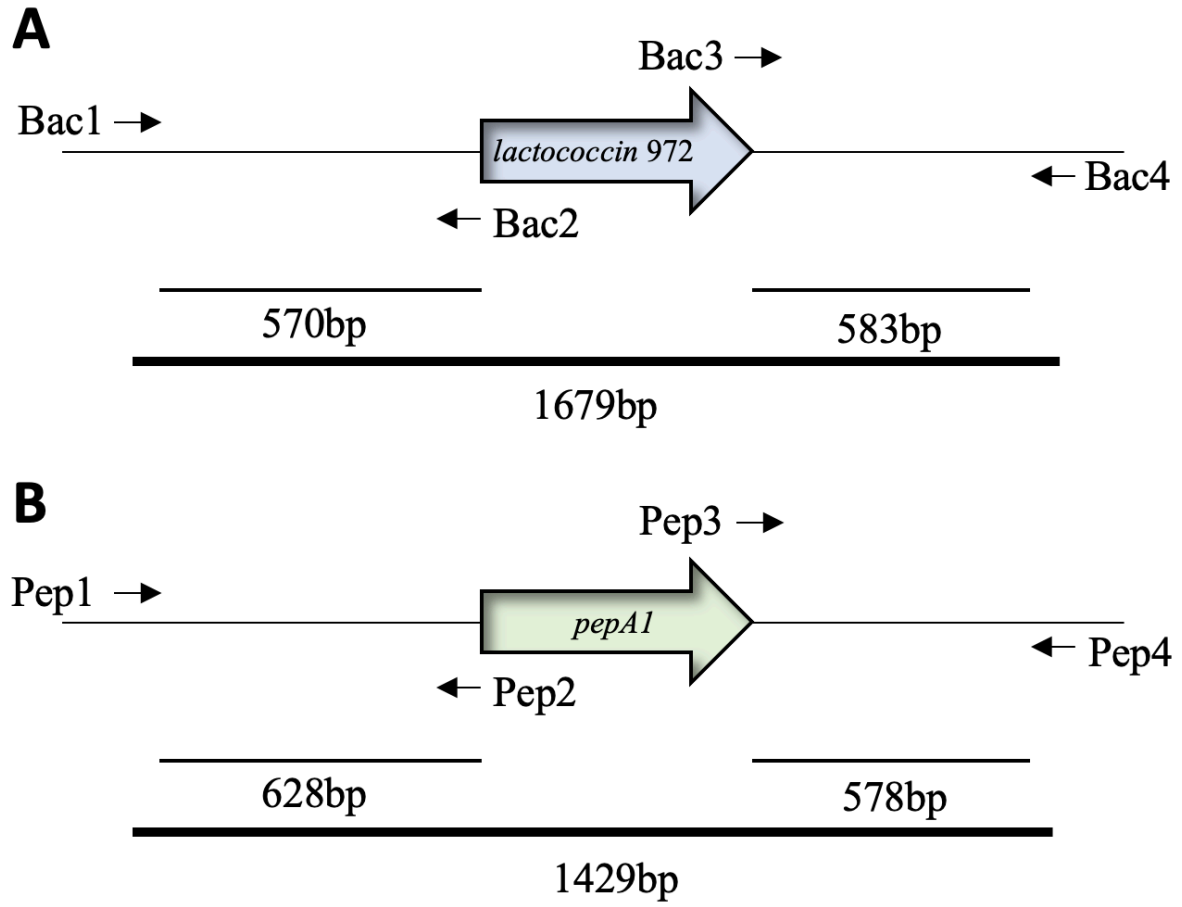


Figure 3.10. Representative diagram of left and right flanks of *lactococcin* 972 and *pepA1* primers and fragment size on pNZAK1. (A) *lactococcin 972* left flank of 570 bp with Bac1 and Bac2 primers and right flank of 583 bp with Bac3 and Bac4 primers. **(B)** *pepA1* left flank of 628 bp with Pep1 and Pep2 primers and right flank of 578 bp with Pep3 and Pep4 primers. These fragments were generated from pNZAK1.

The first step in the deletion process was to generate by PCR homologous flank regions (approximately 500-600 bp) just upstream and downstream of the target genes amplified from pNZAK1 (*lactococcin* 972 or *pepA1*) (Fig. 3.10). For each gene the primer pair Bac1/Pep1 and Bac2/Pep2 were used to generate the left flank and to create the right flank primer pair Bac3/Pep3 and Bac4/Pep4 were used. The generated PCR pairs; *lactococcin* 972 Left and Right and *pepA1* Left and Right flanks (Fig. 3.11 A) were then fused together through a two-step overlap PCR process (Fig. 3.12 B-C). All PCR products as indicated were analysed by agarose gel electrophoresis.

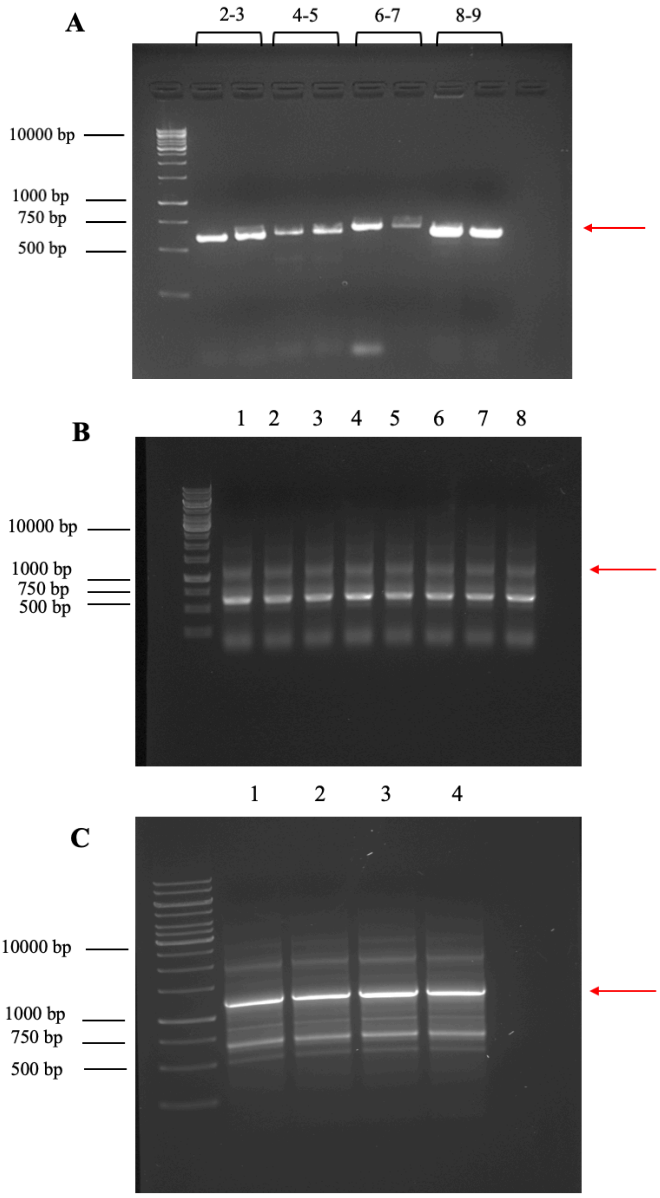


Figure 3.11. Generation of flanking regions and flank overlaps by PCR to facilitate the markerless deletion of *lactococcin* 972 and *pepA1*. Lane 1 (from left) contains the DNA Ladder GeneRuler 1 kb. (**A**) Generation of the *lactococcin* 972 bacteriocin left (Lane 2-3) and right flanks (Lane 4-5), and generation of the *pepA1* left (Lane 6-7) and right flanks (Lane 8-9) by PCR. (**B**) Generation of the flank overlap PCR products from the *lactococcin* 972 bacteriocin flanks, PCR product is indicated by the red arrow (1153 bp) in 8 replicates (Lane 1-8) from (A lane 2-5). (**C**) Generation of the flank overlap PCR from the *pepA1* flanks (from (A lane 6-9)). The PCR product is indicated by the red arrow (1206 bp) in 4 replicates (Lane 1-4) and gel purified.

After the overlap PCR products for each gene were generated, the overlap product was cloned into the pKFC vector (see section 2.15.7-8) and maintained on agar containing chloramphenicol (10 µg/mL), thereby generating modified shuttle vectors p*pep*_pKFC and p*bac*_pKFC (Table 2.1).

3.7.1. Integration screening for temperature dependent allelic replacement via double-crossover recombination

After the modified pKFC vectors were transformed into *S. aureus* 14487, the cultures were heated to 43˚C to induce integration of the plasmids p*pep*_pKFC and p*bac*_pKFC into plasmid pNZAK1. This temperature is non-permissive for plasmid replication, but the isolate retains chloramphenicol resistance. Colonies that grew on agar plates with chloramphenicol (100 µg/ml) at 43˚C were screened for pKFC integration through PCR, using primers pairs Bac5/Bac2 and Pep5/Pep2 for the upstream integration screen and primers pairs Bac3/Bac4 and Pep3/Pep4 for the downstream integration screen. PCR products were analysed by agarose gel electrophoresis (Fig. 3.12 A-B).

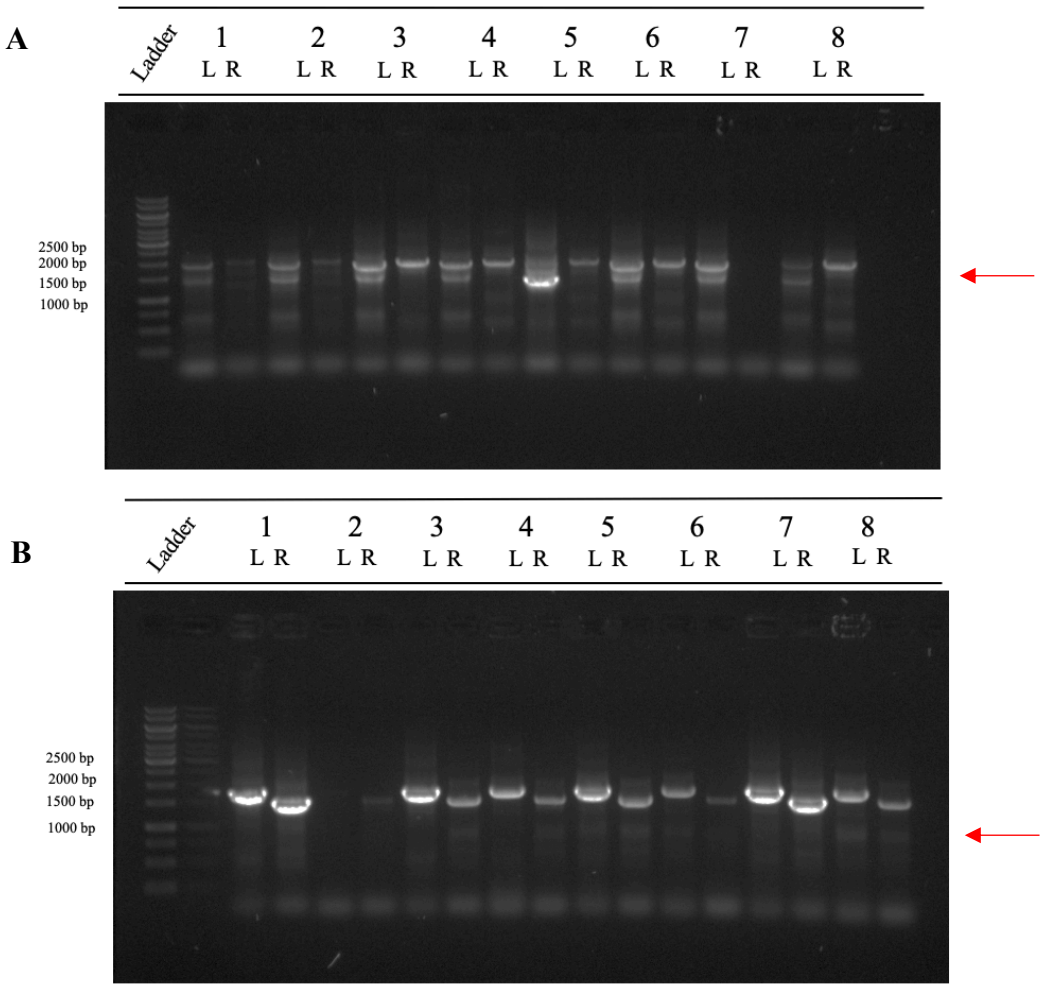


Figure 3.12. PCR validation of pKFC (p*pep*_pKFC and p*bac*_pKFC) integration into pNZAK1 within *S. aureus* 14487. (**A**) The *pepA1* presumptive integrants were verified by PCR using primers Pep5/2 (L) and Pep3/6 (R) from chloramphenicol-resistant colonies (10 µg/mL). (**B**) The *lactococcin* 972 putative integrants were verified by PCR using primers Bac5/Bac2 (L) and Bac3/Bac6 (R) from chloramphenicol-resistant colonies (10 µg/mL). Lane 1 (from left) contains the DNA Ladder GeneRuler 1 kb. Lanes 1-8 (as indicated on agarose gel image) contain putative integrants.

Screening revealed that lane 1- 5 in Fig. 3.12A were potential *pepA1* pKFC integration isolates as the product size varied from the expected wild-type *S. aureus* size of 1.7 kb left product and 1.5 kb right product. Putative integrants from lane 2 and 6 were sequenced.

Lane 1, 7, and 8 in Fig. 3.12B were possible *lactococcin* 972 bacteriocin integration isolates as the product size varied from the expected 1.7 kb left and right wild-type flank size. Putative integrants from lane 1 and 8 were sequenced.

To confirm that the presumptive integration isolates were correct, we sequenced using primers Bac5/Pep5 and Bac2/Pep2 for the upstream integration screen and primers Bac3/Pep3 and Bac4/Pep4 for the downstream integration screen. The sequencing results found one *pepA1* pKFC integration isolates and two *lactococcin* 972 bacteriocin integration isolates.

3.7.2. Excision of plasmid p*pep*_pKFC or p*bac*_pKFC in pNZAK1 by double-crossover

As DNA sequencing indicated that we had successfully integrated one p*pep*_pKFC and two p*bac*_pKFC,we proceeded with excision plating to excise the integrated p*pep*_pKFC or p*bac*_pKFC from pNZAK1 through a double crossover recombination event. The integration p*pep*_pKFC isolate and two p*bac*_pKFC integration isolates were grown on TSA at 28˚C and serially passaged into TSB broths without chloramphenicol selective pressure. After each passage the colonies were plated onto TSA with and without chloramphenicol. Colonies that were found to be sensitive to chloramphenicol were screened for pKFC excision using PCR with Bac5/Bac6 or Pep5/Pep6 primers. The PCR products were then analysed by agarose gel electrophoresis (Fig. 3.13 A,B and C)

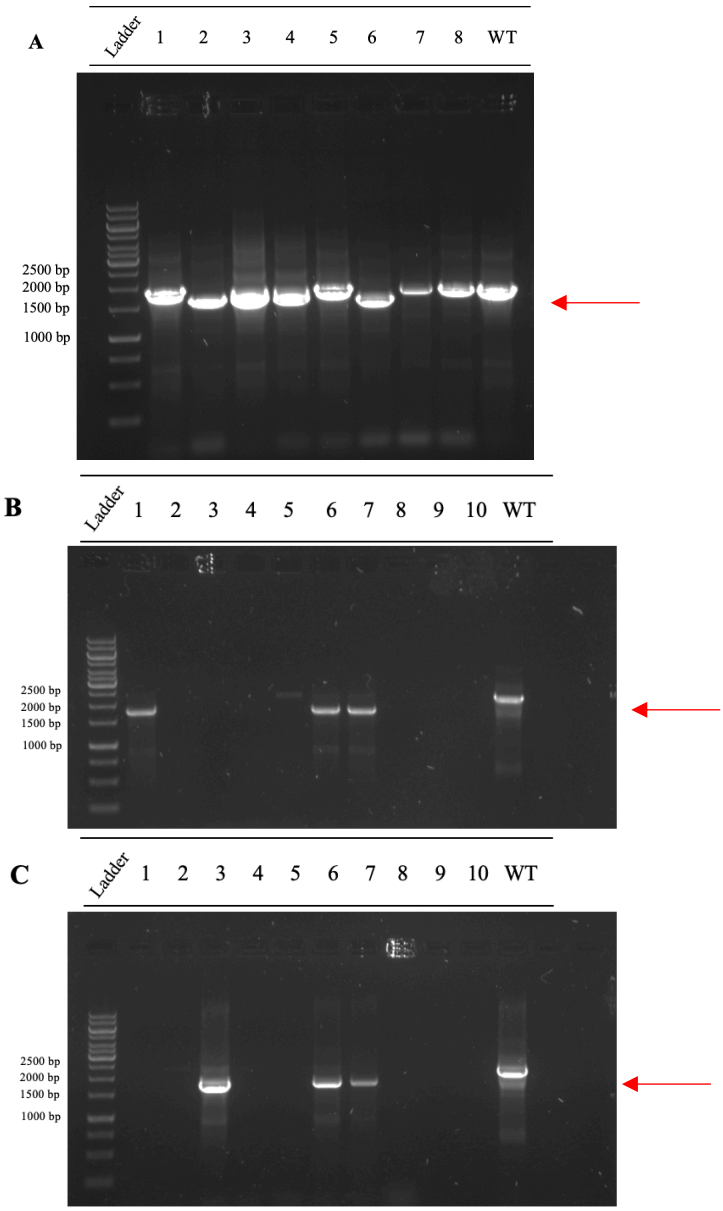


Figure 3.13. PCR validation of excision screening of p*pep*_pKFC or p*bac*_pKFC from pNZAK1 in *S. aureus* 14487. (**A**) p*pep*_pKFC excision screening of sequence confirmed integration isolate, replicates in lanes 1-8 (from left) with Pep5/Pep6 integration primer pair. (**B**) sequence confirmed *pbac*_pKFC integration isolate one excision screening of *pbac*_pKFC with Bac5/Bac6 integration primer pair, (replicates in lanes 1-10) as indicated on agarose gel image. (**C**) DNA sequence confirmed *pbac*_pKFC integration isolate 2 excision screening with Bac5/Bac6 integration primer pair (replicates in lane 1-10). Wild-type control shown in lane 11 in all agarose gels (*lactococcin* 972; 2373 bp, *pepA1*; 1878 bp). The ladder used was GeneRuler 1 kb ladder. The red arrow indicates the expected product.

The excision screening identified two presumptive isolates; AK02 (Fig. 3.13A, lane 1) and AK02.1 (Fig. 3.13A, lane 6), which appeared to have excised pKFC resulting in the deletion of the *pepA1* gene. Likewise, we were able to isolate AK03 (Fig. 3.13B, lane 1) which appeared to have excised pKFC resulting in the deletion of the *lactococcin* 972 gene. To confirm the presumptive mutants, plasmid DNA was isolated, and analysed by DNA sequencing using integration primers pairs Bac5/Bac6 and Pep5/Pep6. The expected deletion sizes shown in Table 3.5.

Target gene for deletion	**Length of deletion region (bp) in pNZAK1**	**Upstream DNA fragment (bp)**	**Downstream DNA fragment (bp)**	**Inserted Overlap flank fragment size (bp)**
Lactococcin 972 bacteriocin	318	570	583	1153
pepA1 system	150	628	578	1206

Table 3.5. Markerless deletion genes and related fragment sizes in modified pKFC vector

3.7.3. Gene deletion screening

To further validate the presumptive mutants; AK02, AK02.1, and AK03 then isolates were sequenced using integration primers Pep5 and Pep6 for *pepA1* (Fig. 3.14A), and integration primers Bac5 and Bac5 for *lactococcin* 972 (Fig. 3.14B). Samples were analysed by agarose gel electrophoresis, and compared to the wild-type *S. aureus* genes also generated by PCR.

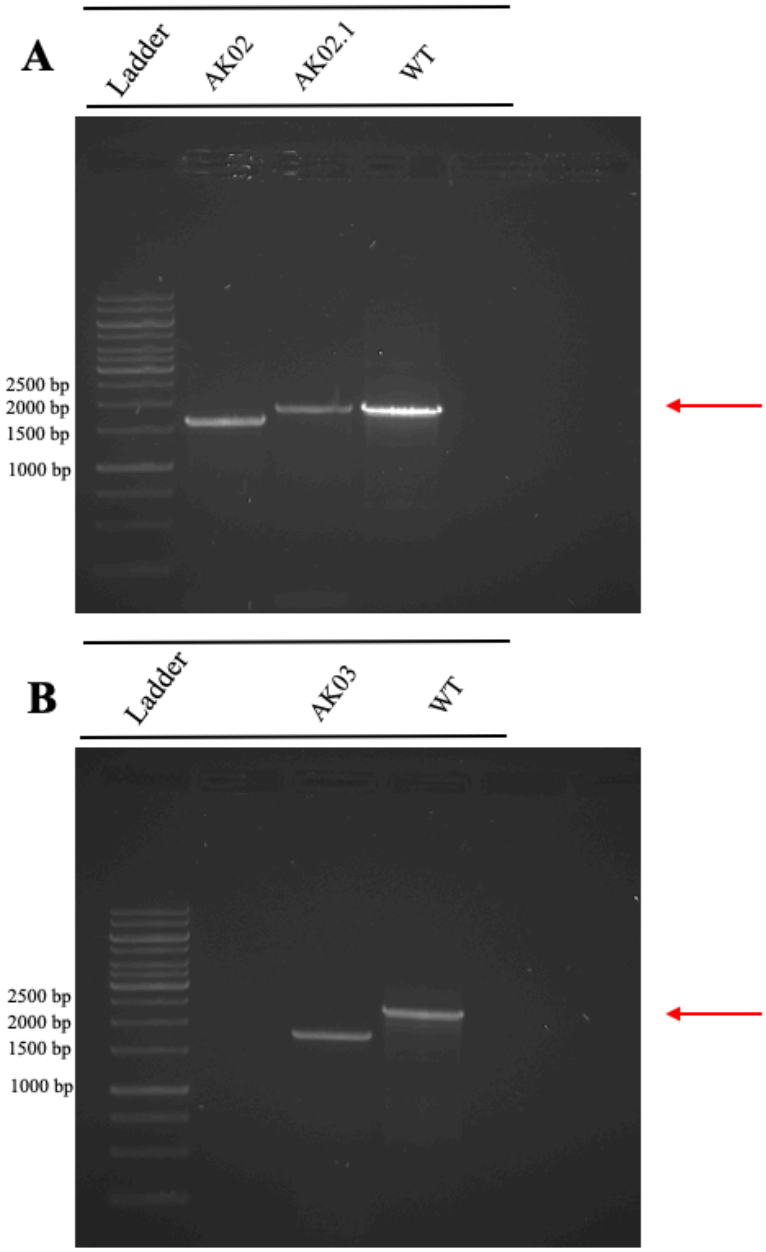


Figure 3.14. PCR validation of deletion of *pepA1* or *lactococcin* 972 from pNZAK1 in *S. aureus* 14487. (**A**) PCR product of AK02 (1728 bp) and AK02.1 with Pep5/Pep6 integration primers with wild-type control (1878 bp). (**B**) PCR product of AK03 (2055 bp) with Bac5/Bac6 integration primer with wild-type control (2373 bp). The ladder used was GeneRuler 1 kb ladder. Red arrows indicated expected wild-type size. AK02.1 was shown to have not undergone gene deletion and we did not carry on with this strain

Analysis by agarose gel electrophoresis revealed a 150 bp deletion for the *pepA1* mutant, AK02 (Fig. 3.14A), and a 318 bp deletion for the *lactococcin 972* bacteriocin isolate, AK03 (Fig. 3.14B) (Table 2.1). Taken with the DNA sequencing data we had successfully introduced the two independent single deletions of *pepA1* and *lactococcin 972* bacteriocin in the plasmid pNZAK1. No attempts in this study were made to generate a double mutant isolate, lacking both *pepA1* and *lactococcin 972* bacteriocin. To ensure that no secondary mutations had occurred in this process, which may impact our analysis, we sent the two isolates for whole genome sequencing.

3.8. Characterisation of mutants *S. aureus* AK02 and AK03

3.8.1. Excision plating: chloramphenicol and ampicillin screening

As part of the process to generate the two mutants we periodically observed that the loss of chloramphenicol resistance (Cm^S) (indicating loss of pKFC) coincided with the loss of ampicillin resistance (Amp^S), which is encoded only on pNZAK1. This data suggested that the excision of pKFC and potential generation of a deletion mutant might stimulate the loss of pNZAK1 from *S. aureus* 14487. To investigate this observation further we went back to the serially passaged excision patch plates containing colonies with the integrated plasmids; p*pep*_pKFC and p*bac*_pKFC, and transferred Cm^s colonies (that have lost pKFC) from TSA plates onto TSA plates containing ampicillin (100 µg/mL) (Fig. 3.15). Of the colonies which were Cm^S indicating loss of pKFC, 30-40% were also observed to be Amp^S for AK03 whereas 10- 20% of the Cm^S AK02 were Amp^S . This was the first instance where we had observed a significant loss of plasmid pNZAK1 and suggests a decrease in plasmid stability in both gene deletion mutants.

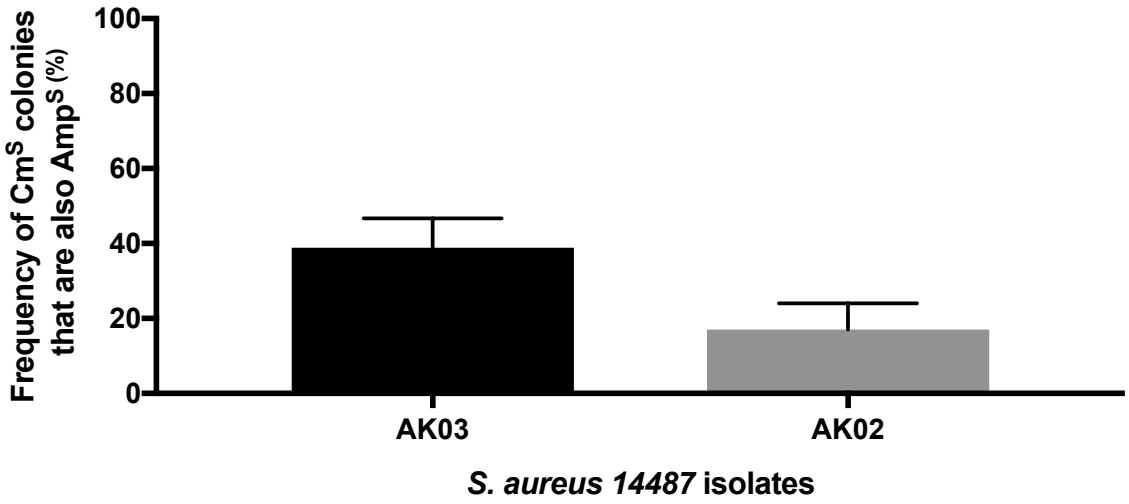


Figure 3.15. Ampicillin sensitivity of integration colonies, AK03 and AK02, that have excised pKFC. Integration colonies that were shown to have lost pKFC ($Cm^{S)}$ were patch plated on to TSA supplemented with ampicillin (100 µg/mL) to look for loss of pNZAK1. The frequencies of Cm^S *S. aureus* colonies also being Amp^S is shown above for both the AK03 isolate (Δ*bac*) and the AK02 isolate (Δ*pepA1)*.

3.8.2. Investigating the plasmid stability of the pNZAK1 mutants AK02 and AK03

To investigate plasmid loss of pNZAK1 in the AK02 and AK03 mutants the strains were grown in ¼ strength MH medium to limit nutrients in 100 mL volumes in flasks.

The plasmid loss assays in ¼ MH flasks shown in Table 3.6 showed that pNZAK1 is stable in *S. aureus* 14487 and that the single deletions of either plasmid addiction systems, *pepA1* or *lactococcin* 972 results in marginally increased levels of plasmid loss of 1.75% and 2.25% respectively, up from 0.5%. These data suggest that the *pepA1* system and *lactococcin* 972 bacteriocin may not be involved in the maintenance of pNZAK1 in *S. aureus* 14487, or that in the single mutants we have generated here, the presence of one system is sufficient to maintain pNZAK1 in daughter cells.

Table 3.6. Plasmid loss of pNZAK1 in the mutant isolates AK02 and AK03 over 7 days.

Strains	**Day 0 average plasmid loss (%)**	**Day 3 average plasmid loss (%)**	**Day 7 average plasmid loss (%)**
Wild-type *S. aureus* 14487	0%	0%	0.5%
AK02	0.5%	1.5%	1.75%
AK03	0.75%	2.25%	2.25%

This data is representative of technical and biological triplicate.

3.9. Discussion

3.9.1. Antimicrobial sensitivity of *S. aureus* 14487 under energy limitation

This study investigated the effect of energy limitation on the ability of pNZAK1 to confer drug resistance after being grown in a nutrient limited environment without antimicrobial selection. This phenomenon has previously been observed in *S. aureus* before where generalised nutrient limitation triggered increased antibiotic resistance (74). We therefore hypothesised that chemostat imposed energy limitation may alter the gene expression levels of pNZAK1 encoded resistance genes leading to altered antimicrobial resistance. However, despite growing cells up to 30× slower than their maximum growth rates to mimic severe nutrient limitations, we observed no significant differences in either the MIC or MBC values for ampicillin, fusidic acid, mupirocin, and chlorhexidine. This indicates that under our experimental conditions the imposition of energy limiting conditions did not impact the cells drug resistance profile to the tested antibiotics.

3.9.2 pNZAK1 is maintained in *S. aureus* 14487 in the absence of antimicrobial selective pressure, and in the presence of energy limitation

It is well established that the maintenance of extrachromosomal DNA in the absence of selective pressure imposes an energy limitation on the cell (83). There are a number of mechanisms by which a plasmid can be maintained in a bacterial host, making it a complex system to investigate, these include 1) the plasmid conferring resistance to an antibiotic or heavy metal, 2) active partitioning of plasmids randomly in to daughter cells 3) addiction systems like toxin/antitoxin systems or bacteriocin system (93,94). In the well characterised example of plasmids like pEC156 plasmid derivatives (pIB8) in *E. coli* with ColE1 toxin, the plasmid loss rates do not change in the absence of selective pressure (93). However when the addiction system is removed or inhibited e.g. the deletion of gene *pcnB* which, decreases ColE1 plasmid maintenance, the plasmid stability is lost (95).

To investigate if *S. aureus* plasmid pNZAK1 would be lost under energy limiting conditions, this study used continuous culture. Despite energy limiting conditions and growth rates up to 30× slower than standard batch growth, pNZAK1 was maintained in *S. aureus* 14487, even

without antibiotic selection pressure. While a small population of cells were recovered which appeared to have lost plasmid pNZAK1, as determined by whole genome sequencing, at no point were these cells able to out compete cells retaining pNZAK1 in the chemostat. However, when in stationary phase competition experiments between wild-type cells (pNZAK1^{+}) and pNZAK1^{-} cells in rich media (BHI) were carried out, wild-type cells did exhibits a fitness cost. This phenomenon previously been shown with this plasmid pNZAK1 in a study by Carter *et al*. (59)

We explored the possibility that if pNZAK1 imposes an energetic burden on *S. aureus* 14487 in non-antimicrobial conditions and it is not lost under energy limited conditions, there could be an addiction mechanism that is stimulating pNZAK1 maintenance. The *pepA1* system and *lactococcin 972* bacteriocin encoded in pNZAK1 were proposed to be potential plasmid maintenance mechanisms. To further elucidate the role of *pepA1* and *lactococcin* 972 in plasmid maintenance, both of the genes were deleted using homologous recombination. During the excision of modified pKFC vectors; p*pep*_pKFC and p*bac*_pKFC, it was shown that Cm^{S} colonies, colonies that had lost pKFC and potentially the target gene, were periodically also Amp^{S}, meaning that they lost pNZAK1. The coinciding loss of pKFC and highly stable pNZAK1 suggested that the loss of pKFC and potentially the target gene could decrease pNZAK1 stability. However, once both genes were separately deleted and tested for plasmid loss the results showed no major increase in plasmid loss in the AK02 or AK03 colonies in comparison to wild-type *S. aureus* 14487. This suggested that the *pepA1* system and *lactococcin 972* bacteriocin system do not play a major role in plasmid maintenance of pNZAK1 in *S. aureus* 14487 in isolation. There could be another mechanism contributing to maintenance of pNZAK1 that could work solely or in tandem with the *pepA1* and *lactococcin 972* bacteriocin systems.

Further work is required to elucidate the long term stability of pNZAK1 in the population in the absence of selective pressure. We propose that there could be an alternative plasmid maintenance mechanism present on the plasmid that is yet to be discovered. Potentially, the *pepA1* system and *lactococcin 972* bacteriocin system could play some role, perhaps in functional redundancy, but the conditions tested in this study were not able to demonstrate it.

Chapter 4

Investigating the Role of QacA in Biocide Tolerance

4. Introduction

4.1. pNZAK1 and Biocides

In New Zealand, the ST1 lineage of *Staphylococcus aureus* can contain the plasmid pNZAK1, which harbours genes conferring resistance to a number of clinically-important antimicrobials. pNZAK1 harbours the *mupA* and *blaZ* genes, which confer resistance to the antibiotic mupirocin, frequently used as a topical antimicrobial in community and hospital settings, as well as β-lactam antibiotics, e.g. ampicillin (59). pNZAK1 also carries the gene *qacA,* which encodes for the multi drug efflux pump QacA. The primary role of QacA is the export of antiseptic and disinfectant compounds including quaternary ammonium salts. Given the frequent use of sanitisers e.g. chlorhexidine or benzalkonium chloride (BAC) in hospitals it is likely that the use of biocides co-selects for the maintenance of pNZAK1 plasmid, and therefore the maintenance of antibiotic resistance genes (40,96). The chromosomally-encoded efflux pump NorA has also been shown to increase tolerance to chlorhexidine and other biocides (97). The increased tolerance of *S. aureus* isolates to antiseptics like chlorhexidine worldwide has been recorded, however, the exact importance and clinical impact of this emergence is unknown (40,55). Elevated MICs from efflux pumps is concerning as it allows bacteria to become tolerant to biocides and remain on the skin of patients or surfaces and potentially cause infection even after being sanitised (97). In New Zealand *S. aureus* strains the prevalence of *qacA* is 7% and is associated with the main clonal lineage of *S. aureus* strains (*spa* type 127) in New Zealand (55).

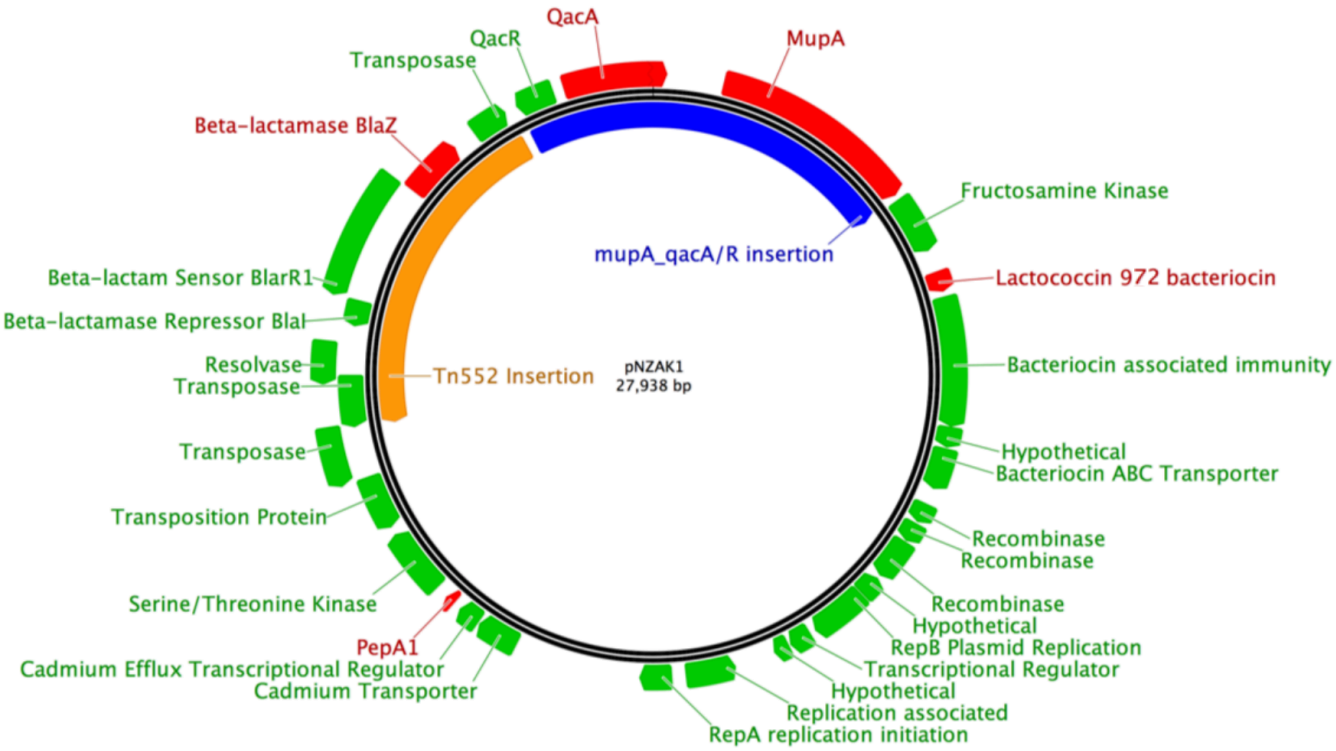


Figure 4.1. Schematic representation of the plasmid pNZAK1.

Genes are denoted by coloured arrows, and the red genes indicate genes of specific interest to this investigation. These are as follows: the antibiotic resistance genes encoding resistance to mupirocin and β-lactam antibiotics (*mupA* and *blaZ),* the antiseptic resistance protein QacA (*qacA)*, and two potential plasmid addiction mechanisms (*lactococcin 972* bacteriocin and *pepA1*). The *mupA* and *qacA* (59) insertion segment is shown in blue. The transposon 552 insertion segment containing *blaZ* is shown in orange (sequence information provided by Dr. Glen Carter, Doherty Institute, Melbourne) (59).

4.2. QacA genetic regulation and proposed mechanism of action

In *S. aureus*, tolerance to chlorhexidine can be mediated by the *qacA*-encoded multidrug efflux pump QacA (7). The gene *qacA* is regulated by *qacR,* which produces a gene product that represses *qacA* transcription (98). QacA is part of the major facilitator superfamily of efflux pumps. QacA functions by utilising the proton motive force (pmf) to pump out substrates like quaternary ammonium compounds e.g. CHX (39). QacA confers resistance to biocides like

BAC and CHX as well as a number of dyes, including ethidium bromide (39). QacB is closely related to QacA and also found in *S. aureus*, but has a reduced substrate range (99). However, presence of *qacA* does not necessarily confer resistance to biocides like CHX or BAC (96).

4.3. Hypothesis and Research Objectives

Hypothesis 1: That treatment of *S. aureus* 14487 with biocides (e.g. chlorhexidine) will alter the expression of antimicrobial resistance genes on the plasmid pNZAK1.

Hypothesis 2: That the isogenic *S. aureus* Δ*qacA* mutant IM01, which lacks the QacA MDR efflux pump, will have increased sensitivity to chlorhexidine and other biocides compared to the wild-type *S. aureus* NZ14487.

These hypotheses will be investigated through the completion of the following objectives:

Research objective 1: RT-qPCR was used to investigate changes in the gene expression of *qacA, mupA, blaZ,* and *norA* following cell challenge with sub-inhibitory concentrations of chlorhexidine.

Research objective 2: To further characterise the role of *qacA*, antimicrobial sensitivity (MIC) and cell-killing assays were conducted to compare the properties of wild-type *S. aureus* NZ14487 cells and the Δ*qacA* isogenic mutant IM01.

4. Results

4.4. Analysis of gene expression in response to sub-inhibitory chlorhexidine challenge

To investigate the role that key genes found on pNZAK1 may have in antibiotic resistance, we examined their expression profiles in response to challenge with sub-inhibitory concentrations of chlorhexidine. To do so, RNA was extracted from cells challenged with 0.5× the MIC of chlorhexidine and the gene expression of *qacA, mupA, blaZ*, and *norA* was examined by RT-qPCR (Fig. 4.2). As previously reported the *S. aureus* housekeeping genes *rho* (transcription termination factor) and *rrsC* (16s ribosomal RNA) were included in the RT-qPCR as control genes to monitor the level of gene expression for genes unaffected by the test compound, and were used to calculate the fold change in gene expression of *qacA, mup. blaZ,* and *norA* (100). All primer pairs were optimised for their binding efficiency assay, and their respective efficiencies are shown in Table 4.1. All RT-qPCR primer binding efficiencies were > 90% indicating that they have suitable binding efficiencies to the synthesised cDNA.

Table 4.1. RT-qPCR primer efficiency

Primer Name[a]	Slope Value	Efficiency
qacA	-3.4 ± 0.1414	96.84%
mupA	-3.5 ± 0.5916	93.07%
blaZ	-3.1 ± 0.2646	110.17 %
norA	-3.4 ± 0.3464	96.84 %
rho	-3.3 ± 1.852	100.5 %
rrsC	-3.7 ± 0.1732	86.32 %

[a] see Table 2.2 for qPCR primer sequences

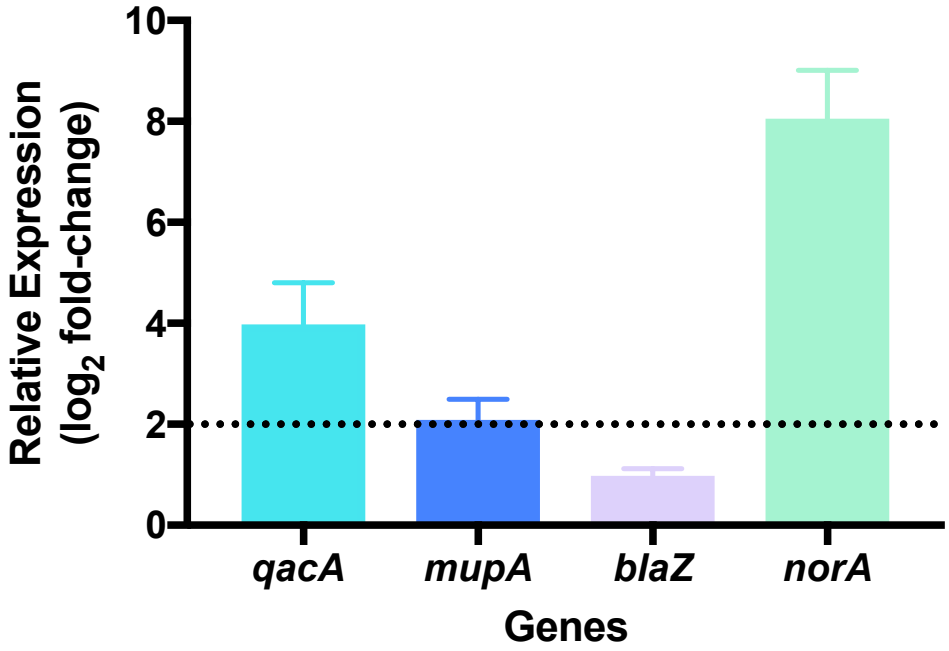


Figure 4.2. Gene expression profile of the *qacA*, *mupA*, *blaZ*, and *norA* genes from *S. aureus* 14487 after challenge with sub-inhibitory concentrations of chlorhexidine. *S. aureus* 14487 cells were grown to mid-exponential phase (OD_{600} 0.4) and challenged with 0.5× the MIC of chlorhexidine (1 µg/mL) for 1 h. cDNA synthesised from treated and non-treated cells was used to determine the transcript levels for the selected genes by RT-qPCR. Log_2 fold-changes were calculated relative to untreated controls, and the data was normalised relative to a reference gene using the $\Delta\Delta C_T$ method (101). The dotted line represents the value at which the genes are two-fold overexpressed in the treated cells compared to non-treated. The error bars correspond to the standard deviation of the mean from three biological replicates in technical triplicate.

RT-qPCR demonstrated that *S. aureus* 14487 cells challenged with a sub-inhibitory concentration of chlorhexidine (0.5× MIC) for 1 h, had a 4-fold increase in gene expression of *qacA*, relative to the control genes (Fig. 4.2). The *mupA* gene expression also increased 2-fold in response to chlorhexidine challenge, but no changes were observed in the expression of *blaZ*. Interestingly, an 8-fold increase in the expression of the chromosomally encoded *norA* efflux pump was also observed. These results demonstrate the *qacA* gene located on plasmid pNZAK1 is induced by chlorhexidine at sub lethal concentrations as are *mupA* and *norA*.

4.5. Time-dependent cell killing by chlorhexidine against wild-type *Staphylococcus aureus* 14487 and the IM01 Δ*qacA* mutant

To investigate the possible advantageous nature of *qacA* carriage, wild-type *S. aureus* 14487 cells and IM01 Δ*qacA* cells were challenged with 1× the MIC of chlorhexidine to examine the differences in killing kinetics between the two strains. Cells were grown to mid exponential phase and challenged with chlorhexidine, samples were withdrawn, and the cell viability was determined by measuring the CFU/mL at each given time point (Fig. 4.3). However, when exponentially growing cells at a starting concentration of 1×10^8 CFU/mL were challenged with a known inhibitory concentration of chlorhexidine, no significant differences between wild-type *S. aureus* cells and the IM01 Δ*qacA* mutant were observed.

4.6. Drug sensitivity of wild-type *Staphylococcus aureus* 14487 versus IM01 to hospital disinfectants

To understand the role which QacA may play in tolerance to the quaternary ammonium compound BAC, and the blended disinfectant Trigene, MIC and MBC assays were conducted with the wild-type *S. aureus* 14487, IM01 Δ*qacA* mutant, and the ATCC strain *S. aureus* 6528 which naturally lacks *qacA* (Table 2.1). The MIC for BAC in wild type *S. aureus* 14487 was 2.5 µg/mL, five times higher than the MIC observed in the IM01 strain, and ATCC 6538 where the MIC was 0.5 µg/mL (Fig. 4.4A). Likewise, the MBC for the wild-type strain was 5.0 µg/mL and the MBC for the IM01 strain and ATCC 6538 strain was 1.0 µg/mL (Fig. 4.4B).

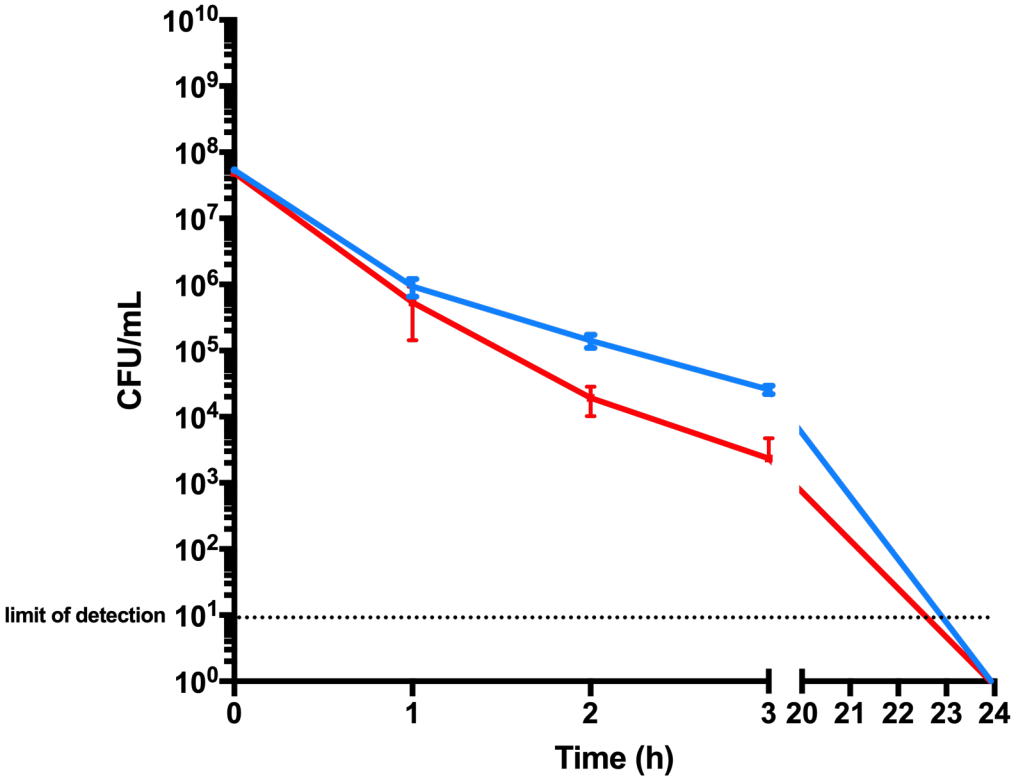


Figure 4.3. Time-dependent killing in wild-type *S. aureus* 14487 and the IM01 Δ*qacA* mutant when challenged with chlorhexidine (1× MIC, 2 µg/mL) over 24 h. *S. aureus* cells were grown to an OD_{600} of 0.15 and then challenged with chlorhexidine (2 µg/mL). Samples were withdrawn at 0, 1, 2, 3, and 24 h, diluted in PBS, and spotted in 20 µl volumes onto TSA to determine the CFU/mL. The experiment was carried out in 100 mL TSB flasks at 37˚C with shaking at 200 rpm. Error bars represent the standard deviation of the mean from a biological triplicate.

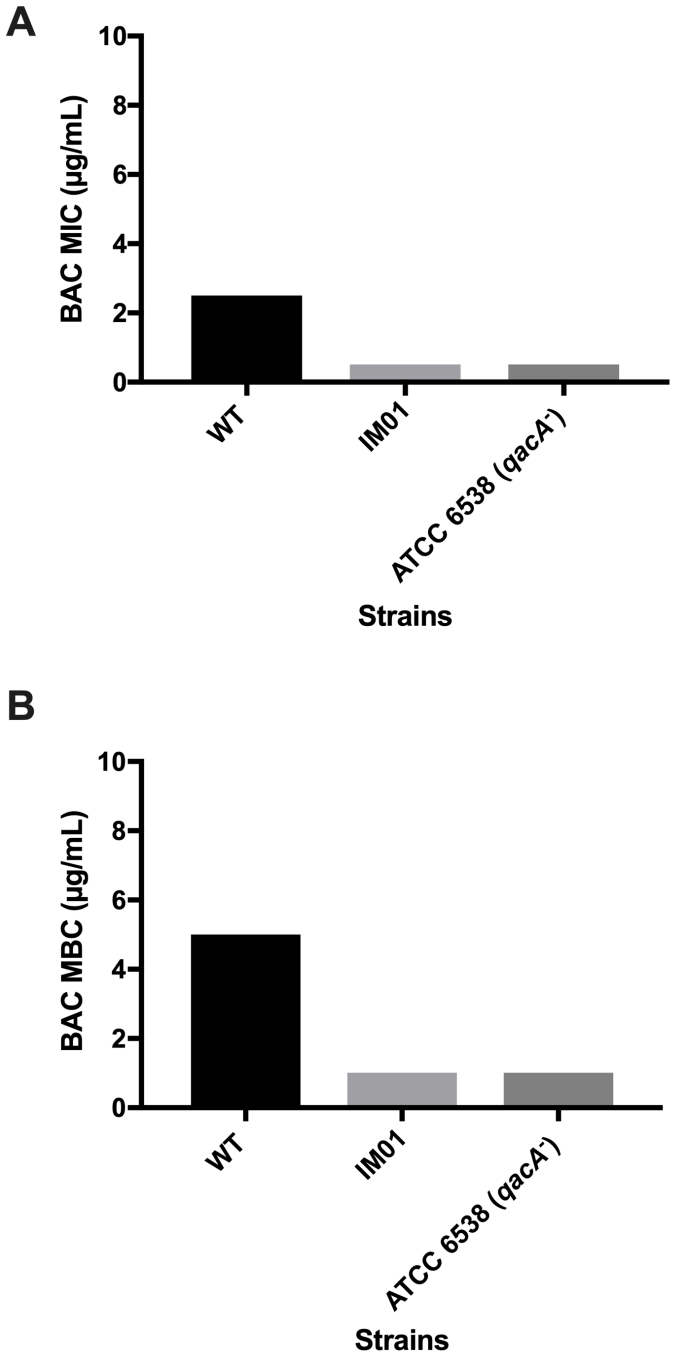


Figure 4.4. Susceptibility of *S. aureus* strains to benzalkonium chloride (BAC). (**A**) To investigate the susceptibility to BAC the minimum inhibitory concentration (MIC) was determined in TSB after 24 h against the following strains: wild-type, IM01, and ATCC 6538. (**B**) Following MIC assays samples where inhibition was observed were further analysed to determine the minimum bactericidal concentration (MBC). This data is representative of a biological and technical triplicate.

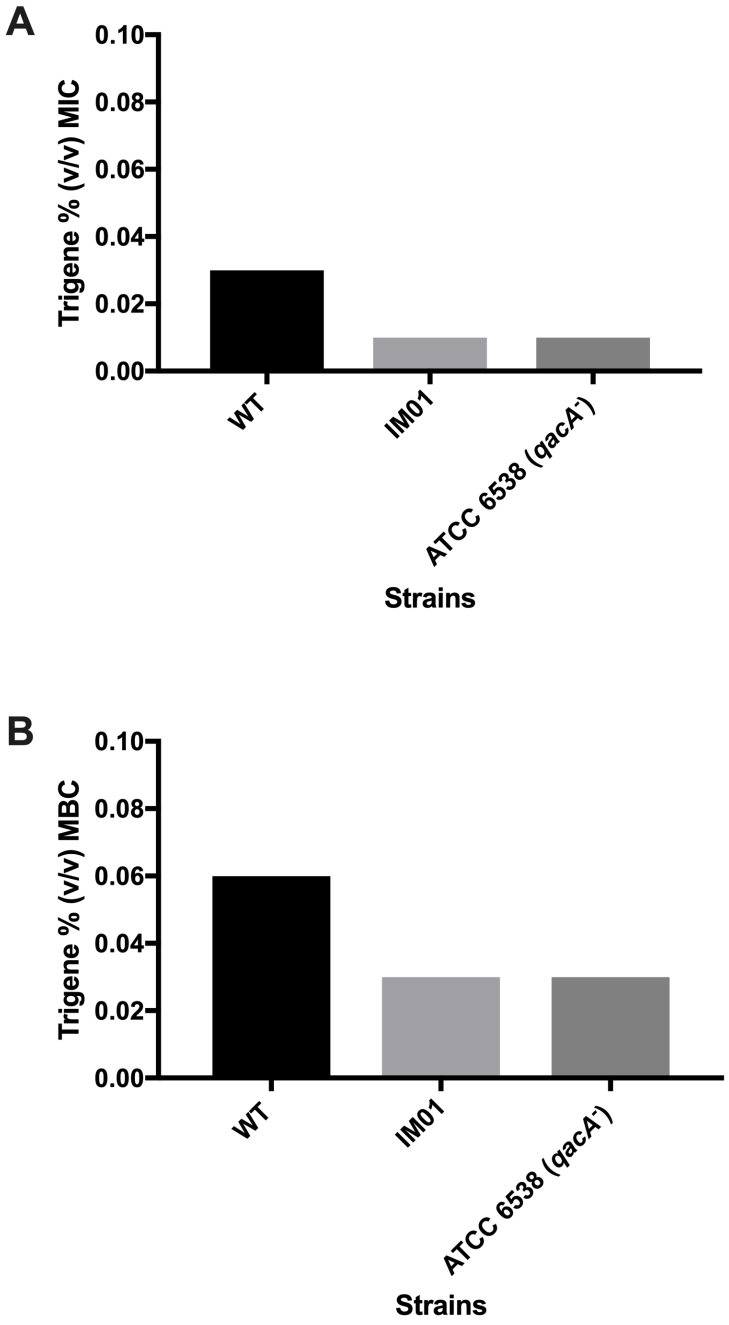


Figure 4.5. Susceptibility of selected *S. aureus* strains to the disinfectant Trigene. (**A**) To investigate the susceptibility to Trigene the minimum inhibitory concentration (MIC) was determined in TSB after 24 hours exposure against the following strains*;* wild-type, IM01, and ATCC 6538 (**B**) Following MIC assays samples where inhibition was observed were further analysed to determine the minimum bactericidal concentration (MBC). This data is representative of a biological and technical triplicate.

The MIC for Trigene against the wild type *S. aureus* 14487 was 0.03 % (v/v), while the MIC for Trigene for both IM01 Δ*qacA* mutant and ATCC 6538 strain was 0.01 % (v/v), or three times lower (Fig. 4.5A). The Trigene MBC for the wild-type *S. aureus* cells was 0.06% (v/v) two-fold higher than the MBC observed for both the IM01 Δ*qacA* mutant and *S. aureus* ATCC 6538 (Fig. 4.5B). Overall, there appears to be a considerable difference in MIC and MBC of BAC and Trigene in strains of *S. aureus* with and without *qacA*.

4.7. Time-dependent killing of *Staphylococcus aureus* 14487 versus IM01 Δ*qacA* in the presence of benzalkonium chloride and Trigene

To further elucidate the importance of *qacA* in *S. aureus*, the wild-type strain and the IM01 Δ*qacA* mutant were grown to mid-exponential phase (OD_{600} of 0.5) and challenged with BAC (1× MIC, 2.5 μg/mL) or Trigene (1× MIC, 0.03%[v/v]). Samples were analysed at 0, 0.5, 1, 2, 3, 4, and 24 h and the cell viability was determined by measuring the CFU/mL (Fig. 4.6A-B).

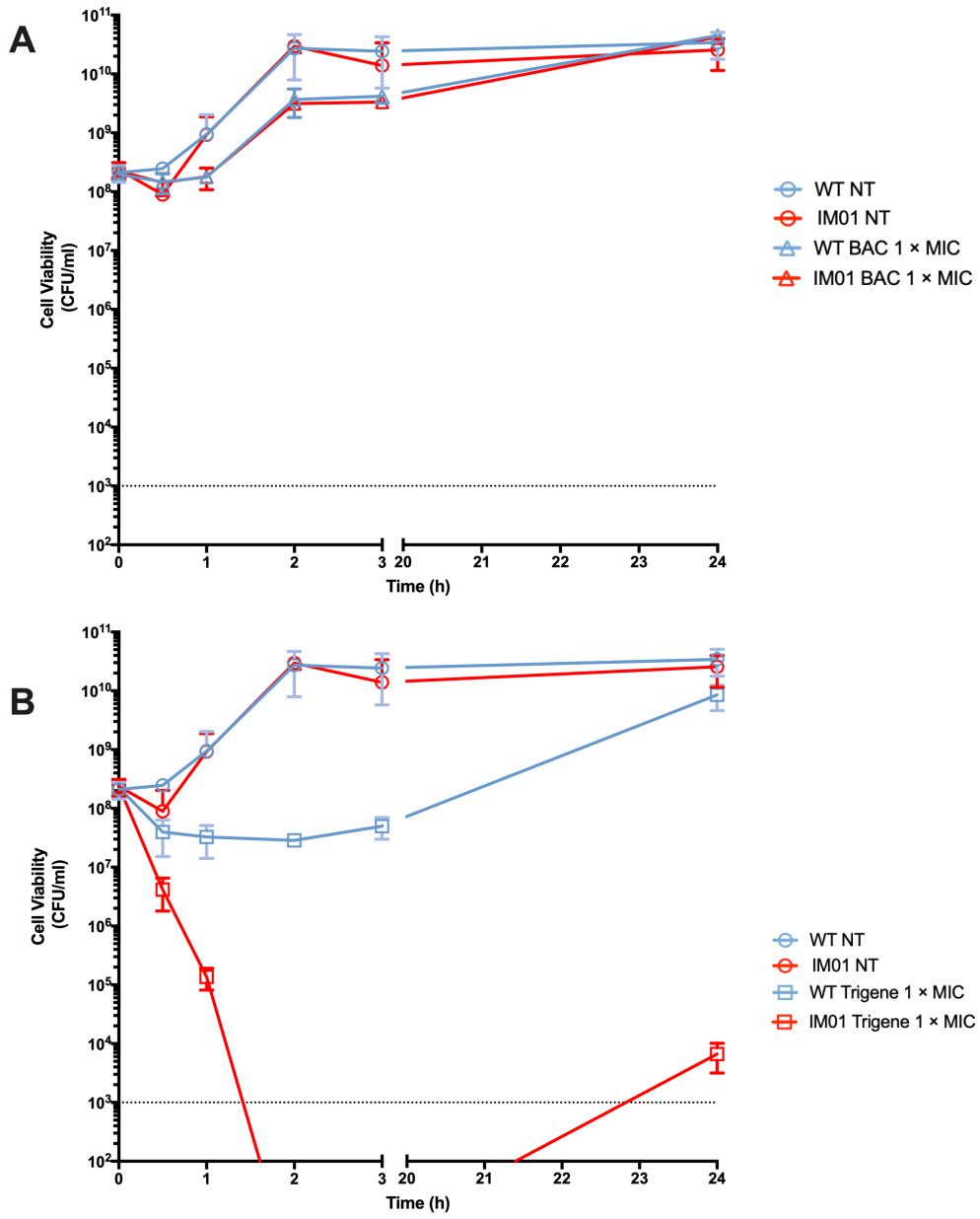


Figure 4.6. Time-dependent killing of wild-type *S. aureus* 14487 and IM01 Δ*qacA* mutant against BAC and Trigene over 24 h. Wild-type cells (blue line), and IM01 Δ*qac* mutant (red line), were grown to mid-exponential phase (OD_{600} 0.5) in TSB media and challenged with: (**A**) BAC (2.5 µg/mL) (triangle) or (**B**) Trigene (0.03%[v/v]) (square), untreated cells (circles). Samples were taken at times 0, 0.5, 1, 2, 3, and 24 h, diluted in PBS, and spotted in 10 µl volume on TSA to determine the CFU/mL. This data is representative of three biological replicates and the error bars represent the standard error of the mean. The dotted line represents the limit of detection at 10^3 CFU/mL.

When *S. aureus* was challenged with 1× MIC BAC (Fig. 4.6A) for 1 h, the wild-type *S. aureus* and IM01 mutant had the same CFU/mL of ~1 x 10^8 1 h after challenge whereas the untreated cells were 2.6×10^8. After 24 h the CFU/ml of non-treated cells and cells challenged with 1× MIC BAC were around $2\text{-}4\times10^{10}$. This suggests that there is a slight difference between non-treated cells and cells treated with 1×BAC. However, there is not a significant difference between wild-type strains with $QacA^+$ and the IM01 $QacA^-$ mutant in response to challenge with BAC (1× MIC).

There was a substantial difference between wild-type CFU/mL and IM01 mutant strain CFU/mL when challenged with 1× MIC Trigene (0.03% v/v) (Fig. 4.6B). After 1 h, the CFU/mL of the IM01 mutant dropped below the limit of detection, while the wild-type *S. aureus* CFU/mL remained fixed between 1×10^7 and 1×10^8 between the 1 h and 4 h timepoints. At 24 h, the CFU/mL of 1× MIC Trigene wild-type S. *aureus* had increased to 1×10^9 CFU/mL, close to that of the non-treated wild-type *S. aureus* at 1×10^{10} CFU/mL. Conversely, at the 24 h timepoint the CFU/mL of 1× MIC Trigene IM01 mutant increased above the limit of detection to ~ 1×10^3 CFU/mL. This highlights the difference between *S. aureus* strains that lack *qacA* and how they tolerate some antimicrobial compounds.

4.8. Chemical compound library screening to further investigate the gene function of *qacA*

To further understand the gene function of *qacA*, we screened the FDA-approved Drug Library (Selleckchem), which contains 1399 FDA-approved drugs (including antibiotics and antiseptics). As QacA has many possible substrates, by identifying compounds which work more effectively against the Δ*qacA* mutant relative to the wild type *S. aureus* cells provides insight into compounds which are recognised by QacA. Additionally, to further investigate the effect of these compounds on *S. aureus* 14487, an isogenic $pNZAK1^-$ mutant, AK01, was also challenged with selected inhibitory compounds (see section 3.1 for the generation of this ΔpNZAK1 strain isolate). Chemical library screening was carried out as specified in section 2.11 in 96-well plates with 25 μM of each FDA compound used. OD_{600} values were recorded for each strain after challenge, and were compared to non-treated control cells. The antibiotic vancomycin was used a positive control. When *S. aureus* was screened against the FDA-

compound library at 25 μM, excellent mean Z′ and Z-factors were returned (e.g. *S. aureus* Z′ = 0.657-0.923, Z-factor = 0.648-0.977). Whereby Z-factor values 1 > Z > 0.5 indicates 'an excellent assay (82).

4.8.1. Screening of FDA compound library against IM01 Δ*qacA* mutant and wild-type *S. aureus*

To identify potent substrates of QacA, the FDA compound library was screened against wild-type *S. aureus* and the IM01 mutant (Table 2.1). Nine compounds were identified from the FDA compound library that inhibited growth (OD_{600}) of the Δ*qacA* mutant by more that 50% relative to wild-type cells. These compounds are as follows:

- **Cephalothin Sodium** is a first generation cephalosporin and is a bactericidal β-lactam antibiotic that works in the presence of penicillinase-producing bacteria (102).
- **Cefamandole** is a cephalosporin β-lactam antibiotic active against Gram-positive and Gram-negative bacteria (103).
- **Carmofur (1-hexylcarbamoyl-5-fluorouracil)** is a derivative of fluorouracil used to treat colorectal cancer (104).
- **Bexarotene** is a retinoid X receptor agonist used in cancer treatment (105).
- **Pentamidine** is a derivative of amidine and is shown to have antiprotozoal and antifungal properties (106,107).
- **Nifuroxazide** is a nitrofuran antibiotic and member of benzoic acids. It has been shown to have anti-diarrhoeal properties and can help treat colitis (108).
- **Benzbromarone** is a uricosuric compound used in treating gout (109).
- **Clarithromycin** is a macrolide antibiotic that binds to the 50S subunit of the ribosome inhibiting protein synthesis (110).
- **Rufloxacin hydrochloride** is a fluoroquinolone antibiotic which targets the topoisomerase II and interferes with DNA synthesis (111,112).

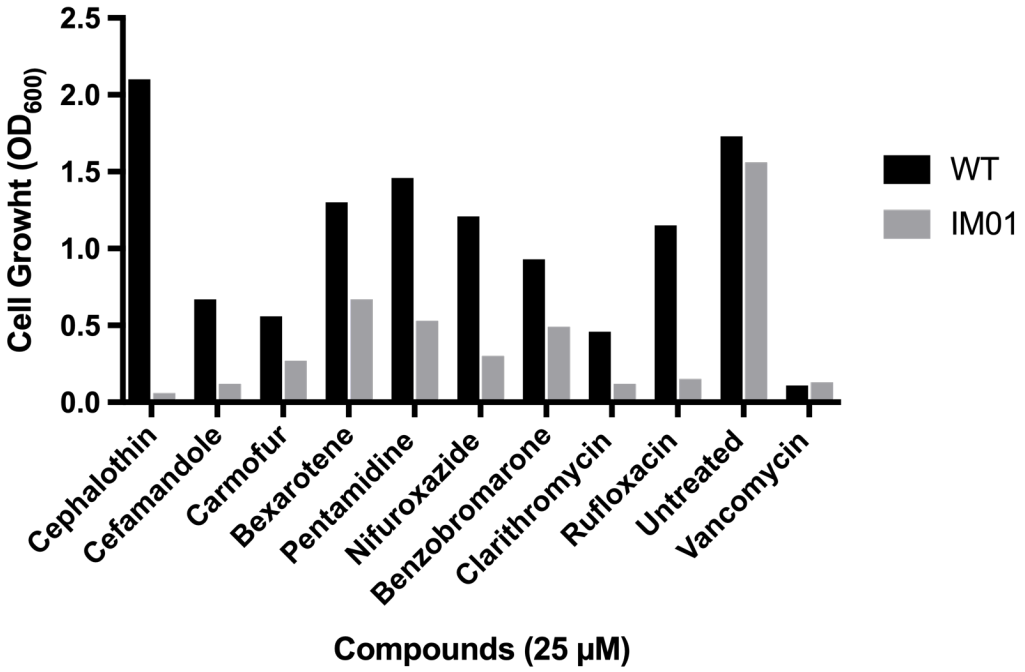


Figure 4.7. Screening of FDA-drug approved compounds against *S. aureus* 14487 wild-type and IM01 Δ*qacA* mutant cells. To investigate the susceptibility of the strains; *S. aureus* 14487 and the IM01 mutant to FDA compounds, the OD_{600} of each strain was recorded after challenge with 25 µM of each compound. The starting OD_{600} was 0.05 in TSB, the cells were then challenged and grown at 37˚C and 200 rpm for 18h. The OD_{600} values were recorded using a Thermo multiscan FC platereader. On the right-hand side, the OD_{600} of untreated and vancomycin treated cells are shown. Vancomycin was used at a concentrations of 27.6 µM (40 µg/mL). The results are representative of a technical triplicate in 96-well plates.

Nine compounds (Tables 4.2 and 4.3) inhibited growth of the IM01 mutant relative to the wild-type *S. aureus* cells by greater than 50%. Data from the chemical library screening at a fixed compound concentration of 25 μM is shown in Fig. 4.7 At 25 μM cephalothin and rufloxacin showed the biggest difference in OD_{600} between wild-type and the IM01 mutant. To confirm how many compounds were more active against IM01 compared to wild-type, we determined the MIC for all nine compounds against wild-type *S. aureus* cells and the IM01 mutant (Table 4.2).

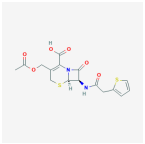
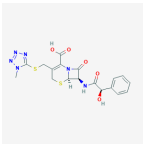
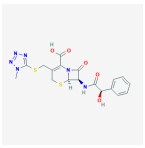
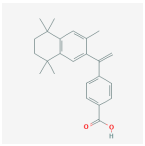
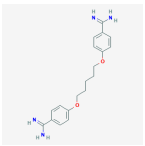
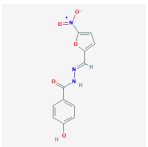
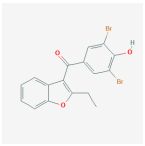
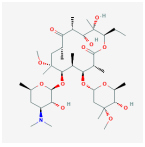
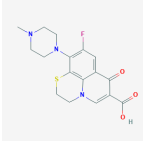
Table 4.2. Antimicrobial sensitivity (MIC) of FDA compounds against *S. aureus* 14487 and IM01 mutant.

	MIC (μg/mL) [a]	
Compound	**Wild-Type**	**IM01**
Cephalothin	0.25 (0.598 μM)	0.25 (0.598 μM)
Cefamandole	8 (15 μM)	8 (15 μM)
Carmofur	0.25 (0.972 μM)	0.25 (0.972 μM)
Pentamidine	128 (216 μM)	16 (27 μM)
Nifuroxazide	2 (7.27 μM)	2 (7.27 μM)
Clarithromycin	0.125 (0.167 μM)	0.125 (0.167 μM)
Rufloxacin	1 (2.5 μM)	1 (2.5 μM)
Chlorhexidine [b]	2 (2.2 μM)	1-2 (~1.1-2.2 μM)

[a]The MIC values shown are representative of technical and biological triplicates. Minimum inhibitory concentration testing was carried out in Mueller Hinton broth medium, at 37˚C. and 200 rpm. The initial inoculation OD_{600} was 0.0007. The compound range tested was 0.125-256 μg/ml which is relative to 0.15-640 μM for each compound.

[b] Chlorhexidine was included as a positive control to insure the MIC assay was accurate

Table 4.3. Properties of the selected FDA compounds from library tested against the IM01 mutant

Compound	Structure	Description	Reference
Cephalothin Sodium		Cephalosporin β-lactam antibiotic	(102,113)
Cefamandole		Cephalosporin β-lactam antibiotic	(103,114)
Carmofur (1-hexylcarbamoyl-5-fluorouracil)		Derivative of fluorouracil	(104,115)
Bexarotene		Retinoid X receptor agonist	(105,116)
Pentamidine		Derivative of amidine and is shown to have antiprotozoal and antifungal properties	(106,107,117).
Nifuroxazide		Nitrofuran antibiotic	(108,118)
Benzbromarone		Uricosuric compound	(109,119)
Clarithromycin		Macrolide antibiotic	(110,120)
Rufloxacin hydrochloride		Fluoroquinolone antibiotic	(111,112,121)

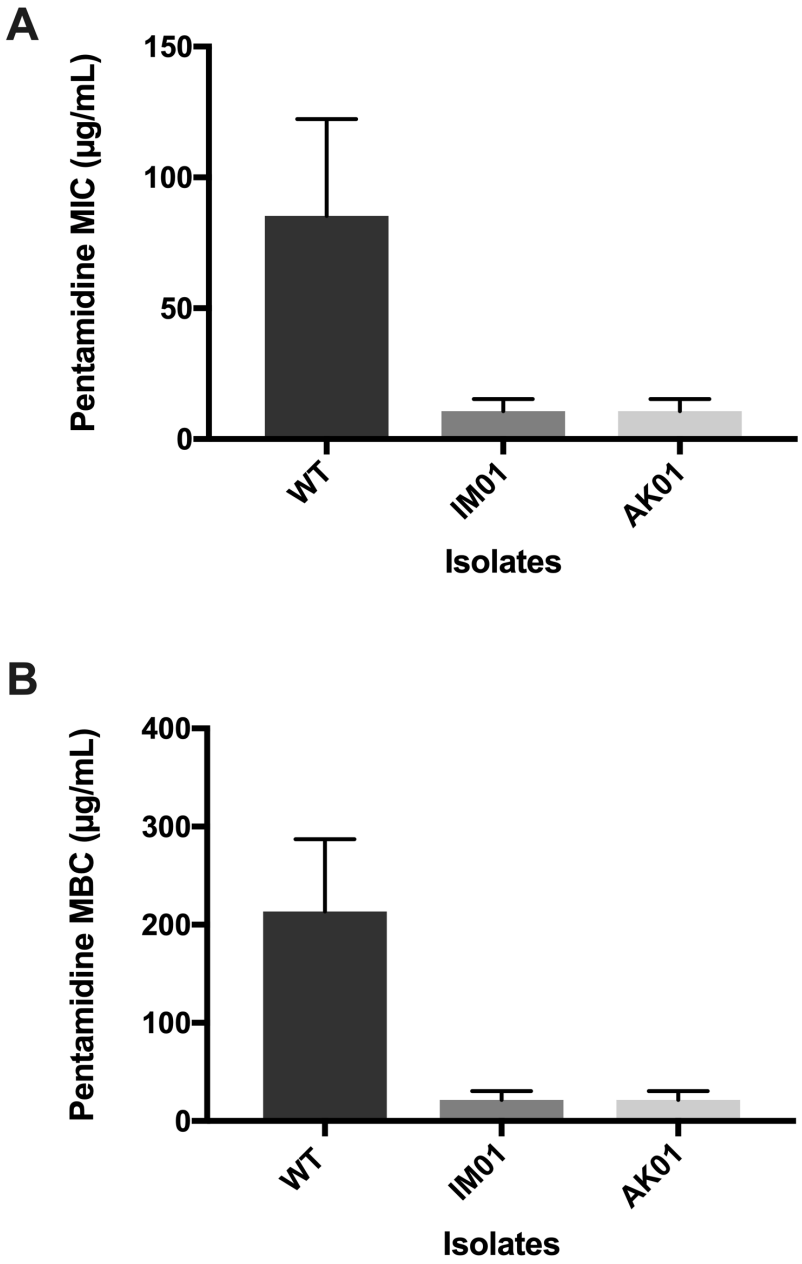


Figure 4.8. Susceptibility of *S. aureus* 14487 isolates to pentamidine. (**A**)To investigate the susceptibility of the strains; *S. aureus* 14487, IM01 Δ*qacA* mutant, and AK01 pNZAK1^{-} mutant to pentamidine the minimum inhibitory concentration (MIC) was determined in TSB after 24 h. (**B**) Following the MIC assays samples where inhibition was observed were further analysed to determine the minimum bactericidal concentration (MBC). This data is representative of a biological and technical triplicate.

The drug sensitivity of *S. aureus* 14487 was measured using MIC and MBC assays (Fig. 4.8). The MIC of pentamidine in wild type *S. aureus* 14487 cells was between 64 and 128 µg/mL and the MBC was between 128 and 256 µg/mL. The MIC of pentamidine in *S. aureus* 14487 IM01 and AK01 isolates was between 8 to 16 µg/mL and the MBC was between 16 and 32 µg/mL. There was a considerable difference in MIC and MBC between the wild type *S. aureus* 14487 cells that have *qacA* in comparison to the IM01 and AK01 cells.

There was a difference between the OD_{600} values from the high throughput FDA compound screening as it uses one standard concentration of 25 µM therefore the result is yes or no for activity whereas MIC is carried out with a range of concentrations. The screening is also carried out in TSB and not MHB, MHB is more suitable for antimicrobial sensitivity testing than TSB. The screening was also done with a higher starting OD_{600} of 0.05 compared to 0.0007 leading to decreased sensitivity of the assay.

4.9. Discussion

4.9.1. Gene expression of *qacA*, *mupA,* and *norA* increased by sub-inhibitory chlorhexidine challenge

Typically antimicrobial resistance genes are upregulated in response to antibiotics or antiseptics, as the fitness cost of constitutively expressing antimicrobial genes is not necessarily advantageous (122). To examine gene expression of the plasmid-encoded antimicrobial resistance genes *qacA, mupA, blaZ*, and *norA. S. aureus* 14487 cells were challenged with sub-lethal concentrations of chlorhexidine (Fig. 4.2). Chlorhexidine exposure induced a 4-fold increase in the expression of *qacA*, a 2-fold increase *mupA* expression, as well as an 8-fold increase in the gene expression of *norA.* The gene *norA* has previously be shown to be overexpressed after treatment with biocides like chlorhexidine or BAC (123). These data indicate that the *qacA, mupA*, and *norA* promoters are inducible by chlorhexidine. However, chlorhexidine did not induce gene expression of *blaZ.* This could be due to the post-transcriptional regulation of *blaZ* by *blaRI* meaning that a change in RNA levels would not be observed after the addition of chlorhexidine (124). These data suggest that antibiotic resistance genes like *qacA, mupA* and *norA* are inducible by compounds like chlorhexidine even at sub-

lethal concentrations. Our data highlights that the use of chlorhexidine sanitisers in healthcare settings could potentially select for the survival of $QacA^+$ isolates over $QacA^-$ isolates. This selection pressure in healthcare settings is concerning as selection of *S. aureus* isolates containing *qacA* on pNZAK1 also selects for mupirocin and β-lactam resistance as well. Studies have previously shown the $qacA^+$ strains outcompete $qacA^-$ strains in decolonisation studies and that MRSA clone CC22 carrying *qacA* were more likely to have elevated MICs to CHX (125).

4.9.2. The loss of QacA increases drug susceptibility

The objective of this research was to further understand the properties of *qacA* with a focus on investigating the function of *qacA* in antimicrobial sensitivity. To do so we compared the growth properties of wild-type *S. aureus* 14487 with that of an isogenic mutant IM01 that lacks *qacA*. When we examined the growth of wild-type *S. aureus* 14487 compared to the IM01 mutant in the presence of chlorhexidine (Fig. 4.3) we observed that there was no significant difference between the two isolates ability to grow, despite chlorhexidine being a known substrate for QacA (57,126). This could be due to the overexpression of the NorA efflux pump which has also been shown to efflux similar substrates to QacA like chlorhexidine (96,97). To further explore the role of QacA. We examined two biocides typically used in hospital settings, these being Trigene and BAC. Additionally, on the basis of results from high-throughput screening of the FDA-approved drug library we also investigated the antimicrobial properties of pentamidine in both the wild-type and IM01 mutant cells. Where no difference was observed for chlorhexidine, the BAC and Trigene MIC and MBC values were markedly higher in wild-type *S. aureus* 14487 cells in comparison to the IM01 mutant (Fig. 4.4 and 4.5). However, time-dependent cell killing assays showed no significant difference between wild-type *S. aureus* 14487 and IM01 after treatment with 1× MIC BAC (Fig. 4.6). Conversely, after treatment with 1× MIC Trigene there was a noteworthy difference between *S. aureus* 14487 cells with *qacA* and those without. The CFU/mL of cells lacking *qacA* was reduced after 1 h while the wild-type *S. aureus* 14487 cells were only inhibited. Furthermore, wild-type *S. aureus* 14487 was able to grow up to non-treated CFU/mL levels after 24 h while the IM01 mutant only grew slightly about the limit of detection. This data supported the idea that QacA

conferred a survival benefit in environments with extensive selective pressure of antimicrobial sanitiser.

The third compound appeared to be more effective against cells that lack *qacA* was pentamidine. Pentamidine is a FDA compound used to treat the parasite *Trypanosoma brucei* and the fungus *Pneumocystis jirovecii* (127). Pentamidine is traditionally not associated with *S. aureus*, however, the fact that it is a substrate for QacA is of interest. The mechanism of pentamidine has not been confirmed for all species, but it is speculated to target the DNA synthesis pathway (128). The MIC of pentamidine increased from 8-16 µg/mL in the IM01 mutant to 64-128 µg/mL in wild-type cells. The MBC of pentamidine increased from 16-32 µg/mL in the IM01 mutant to 128-256 µg/mL in wild-type cells. The difference in MIC and MBC between wild-type *S. aureus* 14487 and the IM01 mutant emphasises that the sensitivity of pentamidine is reduced by the presence of *qacA* (Fig. 4.8). This data demonstrates the extensive range of substrate that QacA has and why it is such a problematic multi-drug efflux pump as it exhibits resistance to even non-bactericidal compounds like pentamidine, which is on the WHO list of essential medicines. This emphasises how vital the investigation of QacA is, as it is located on a mobile genetic element (pNZAK1) and can be easily transferred between isolates and with the added selection pressure of antimicrobial sanitisers. As such, the prevalence of *qacA* in bacteria will most likely only increase (59,96). The spread of antiseptic resistance could become more of a burden to the healthcare system, increase hospital acquired infections and potentially even fatalities from drug resistance infections (59,96).

4.10. General Conclusions

This study has illustrated the need to further understand *qacA* and its role in antiseptic resistance. The fact that *qacA* is encoded on a plasmid with additional antibiotic resistance genes such as *mupA* and *blaZ* only increases the need for further research. This study has shown that sub-MIC levels of chlorhexidine are able to stimulate gene expression of *qacA, mupA,* and *norA*. By allowing the potential selection of $QacA^+$ strains over $QacA^-$ strain in healthcare settings, where antiseptic sanitisers are widely used, we are fostering an environment for tolerance to occur to essential compounds. Moreover, we have highlighted that carriage of

qacA. can increase survival over isolates that lack *qacA.* Antiseptic selection pressure creates an environment that selects for *qacA* isolates to become more frequent in *S. aureus* strains.

The multi-drug efflux pump QacA is encoded in pNZAK1, a MDR plasmid found in *S. aureus* worldwide. This study demonstrates that despite the fitness costed imposed by pNZAK1 the plasmid is still highly stable in *S. aureus* 14487 even in energy limiting conditions, and in the absence of antibiotic selection. Additionally, that even though pNZAK1 was shown to confer a fitness cost it is very stable in *S. aureus* 14487. Further clarifying the pNZAK1 maintenance mechanism in *S. aureus* is vital to be able to reduce the spread of multi-drug resistance plasmids in healthcare settings.

4.11. Future directions

The role of *qacA* in antiseptic tolerance is still not fully understood. The effect of efflux pump inhibitors like verapamil is undetermined and could possibly be used in synergy with compounds that are more effective against Δ*qacA* isolates. Antiseptic solutions could be developed that contain efflux pump inhibitors to resensitise $QacA^{+}$ isolates. To investigate potential synergy between BAC/Trigene and other potential compounds we could carry out another screen with wild-type and IM01. Additionally, we could generate IMV and test fluorescent ethidium bromide in a quenching assays to look for compounds that inhibit QacA.

The maintenance of pNZAK1 is still unknown. Other possible plasmid maintenance mechanisms need to be identified. There could be multiple mechanisms working tandem to generate a highly stable plasmid even though it confers a fitness cost. Possible genes could be identified through the use of RNAseq or Tn mutagenesis, where cells from the chemostat are sequenced and genes with high expression unrelated to metabolism or general cell stress could be analysed as potential candidates. Potentially, a double knockout could also be generated to explore the idea of both addiction mechanisms working in together. Another possibility is to rerun the chemostat using true minimal media and observe any changes in plasmid stability.

Appendix A: Media

A.1 Luria Broth for growth of *E. coli*

To 1 L of distilled water add:

10 g/L tryptone

5 g/L yeast extract 10 g/L of $NaCl_2$

For LB agar add 15 g/L of bacto agar (Sigma-Aldrich)

A.2 ¼ Mueller Hinton Broth for growth of *S. aureus*

To 1 L of dH_2O add:

¼ of recommend amount of powder for Mueller Hinton cation-adjusted broth (Becton Dickinson) and diluted into dH_2O.

For ¼ MH agar add 15 g/L of bacto agar (Sigma-Aldrich).

Appendix B: qPCR plate layout

Table 4.4. Layout of RT-qPCR primer efficiency

	Primer Name				
	1	**2**	**3**	**4**	**5**
cDNA Dilution	1/10	1/100	1/1000	1/10000	1/100000
A	*norA* NT 500 nM	*norA* NT 500 nM	*norA* NT 500 nM	*norA* NT 500 nM	*norA* NT 500 nM
B	*norA* CHX 500 nM	*norA* CHX 500 nM	*norA* CHX 500 nM	*norA* CHX 500 nM	*norA* CHX 500 nM
C	*qacA* NT 500 nM	*qacA* NT 500 nM	*qacA* NT 500 nM	*qacA* NT 500 nM	*qacA* NT 500 nM
D	*qacA* CHX 500 nM	*qacA* CHX 500 nM	*qacA* CHX 500 nM	*qacA* CHX 500 nM	*qacA* CHX 500 nM
E	*rho* NT 500 nM	*rho* NT 500 nM	*rho* NT 500 nM	*rho* NT 500 nM	*rho* NT 500 nM
F	*rrsC* NT 500 nM	*rrsC* NT 500 nM	*rrsC* NT 500 nM	*rrsC* NT 500 nM	*rrsC* NT 500 nM

Table 4.5. Representative RT-qPCR 96-well plate layout

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Primer Name	***NorA* 500 nM**	***NorA* 500 nM**	**QacA 500 nM**	***QacA* 500 nM**	***Rho* 500 nM**	***Rho* 500 nM**	***RrsC* 500 nM**	***RrsC* 500 nM**	**No RT control 500 nM**
A	NT1	NT1	NT1	NT1	NT1	NT1	NT1	NT1	NT1
B	“	“	“	“	“	“	“	“	“
C	“	“	“	“	“	“	“	“	“
D	CHX1	CHX1	CHX1	CHX1	CHX1	CHX1	CHX1	CHX1	CHX1
E	“	“	“	“	“	“	“	“	“
F	“	“	“	“	“	“	“	“	“
G	H_2O	“	“	“	“	“	“	“	
H	H_2O	“	“	“	“	“	“	“	

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