Investigating the prevalence and genetic basis of resistance to the antimicrobial agents: bacitracin, nitrofurantoin, and silver in key pathogenic bacteria

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Resistance to certain antimicrobial agents is poorly understood, with knowledge gaps regarding both the prevalence and genetic basis of resistance. In this thesis, these aspects were examined for three antimicrobial agents (silver, bacitracin, and nitrofurantoin) that are used in the prevention and treatment of bacterial infections.

Overt silver resistance was not prevalent in a cross-section of clinical Gram-negative isolates. A large proportion of *Klebsiella* spp. and *Enterobacter* spp. isolates have the potential to readily acquire silver resistance, with resistance arising as a consequence of single point mutations in *silS* that led to the activation of the Sil system. The development of silver-resistant strains is likely in clinical settings, and this could affect the clinical utility of silver.

Resistance to bacitracin was identified in ~3% of *Staphylococcus aureus* isolates tested, and an epidemiological cut-off (ECOFF) point was proposed for 'true' bacitracin resistance of <512 mg/L. All such strains carried the *bcrABRS/bacA* locus that encodes bacitracin resistance determinants. This locus is on a plasmid which suggests that bacitracin usage could induce the dissemination of this resistance in the future.

After examining the nitrofurantoin susceptibility profile in a group of *S. aureus* isolates; it was apparent that MICs for nitrofurantoin naturally span a broad range. No nitrofurantoin resistance was detected, and a suggested ECOFF point for nitrofurantoin resistance in *S. aureus* was therefore 64 µg/mL. No evidence for nitrofurantoin resistance determinants was obtained among the isolates exhibiting reduced susceptibility to nitrofurantoin. Nitrofurantoin seems to be of value in the treatment of *S. aureus,* and it could be the drug of choice for urinary tract infections caused by *S. aureus* strains.

In conclusion, this study provides insights that could help to inform the clinical application of these antimicrobial agents and can underpin future work to further dissect the biochemical and structural basis for resistance.

Table of Contents

List of Tables

List of Figures

Abbreviations

ABC	Adenosine triphosphate binding cassette
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BHI	Brain Heart Infusion
CA	Catalytic/ATP-binding domain
CDC	Centres for Disease Control and Prevention
CFU	Colony-forming unit
CLSI	Clinical Laboratory Standards Institute
ºC	Degrees Celsius
CC	Clonal complex
D	Day(s)
DHp	Dimerization and histidine phosphotransfer domain
ECOFF	Epidemiological cut-off
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia: a Latin phrase that means "for example"
G	Gram(s)
G	Gravity
GC	Guanine-cytosine
H	Hour(s)
HAMP	Histidine kinase, adenylate cyclase, methyl accepting protein and phosphatase domain
ICE	Integrative conjugative element
ISS	*i*ntegration *s*ite *s*equence
I-TASSER	Iterative threading assembly refinement

Kb	Kilo base pair
KV	Kilovolt
LB	Lysogeny broth
LBA	Lysogeny broth agar
Mb	Megabase
MDR	Multi-drug resistance
MF	Mutation frequency
MGE	Mobile genetic element
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
min	Minute(s)
µg	Microgram(s)
µF	Microfarad
µL	Microlitre(s)
µM	Micromolar
mg	Milligram(s)
mL	Millilitre(s)
MLST	Multilocus sequence typing
mM	Millimolar
mm	Millimetre
MRSA	Methicillin-resistant *Staphylococcus aureus*
NARSA	Network on Antimicrobial Resistance in *Staphylococcus aureus*
NCBI	National centre for biotechnology information
NCTC	National collection of type cultures
NGS	Next-generation sequencing
OD	Optical density
ORFs	Open reading frames
PBP	Penicillin-binding protein

PCR	Polymerase chain reaction
RAST	Rapid annotation and subsystem technology
RGP	Rhamnose-glucose polysaccharide
Rpm	Revolutions per minute
S	Second(s)
SaPI	*Staphylococcus aureus* pathogenicity island
SCC	Staphylococcal cassette chromosome
SLV	Single locus variant
SNP	Single nucleotide polymorphism
SSD	Silver sulfadiazine
ST	Sequence type
TAE	Tris-acetate-EDTA
TCS	Two-component system
TM	Transmembrane domain
TSA	Tryptone soya agar
TSB	Tryptone soya broth
V	Volt
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization
WT	Wild-type

Chapter 1 Introduction

1.1 A brief history of antimicrobial chemotherapy

Chemotherapy is the treatment of disease through chemical means. Historically, the Indians from ancient Peru were believed to be first in performing successful chemotherapy by treating malaria using the bark of the cinchona tree (Achan et al., 2011, Gould, 2016). It is also known that primitive people used poultices containing antimicrobial agents, and the ancient Chinese are believed to have used antimicrobial substances to treat infections (Aminov, 2010, Jesman et al., 2011).

However, the actual concept of antimicrobial chemotherapy was not introduced until the beginning of the 20th century by Paul Ehrlich. He discovered two antimicrobial agents; p-rosaniline for treating trypanosomiasis, and arsphenamine for treating syphilis. He thereby pioneered the concept of the “magic bullet” - the idea that certain chemicals could be used to specifically attack invading microorganisms in the body without being toxic or harming the host (Ehrlich and Hata, 1910, Bosch and Rosich, 2008, Kaufmann, 2008). In the 1930s, Gerhard Domagk discovered synthetic sulphonamide drugs, which were the first antibacterial drug class to be widely used (Otten, 1986, Jesman et al., 2011). Though Alexander Fleming’s discovery of the first natural product antibiotic (penicillin G) in 1929 predated the discovery of the sulphonamides by several years, it was not until 1939 that penicillin was successfully isolated by Florey and colleagues and shown to be effective in treating bacterial infections in patients (Chain et al., 2005, Aminov, 2010, Ventola, 2015a, Durand et al., 2019). The success of penicillin prompted Waksman to search

for further antibiotics for environmental microorganisms (notably, the streptomycetes), and he was responsible for discovering erythromycin and chloramphenicol (Jesman et al., 2011, Sengupta et al., 2013). By the 1960s, in part driven by a reduction in the discovery of novel antibiotic classes from natural sources, medicinal chemistry approaches were extensively used to generate analogues of known antibiotic classes to improve their properties. This effort resulted in important antibacterial drugs that include ampicillin, carbenicillin, methicillin, and doxycycline (Martens and Demain, 2017, Durand et al., 2019). Figure 1.1 summarises the timeline of antibiotic discovery.

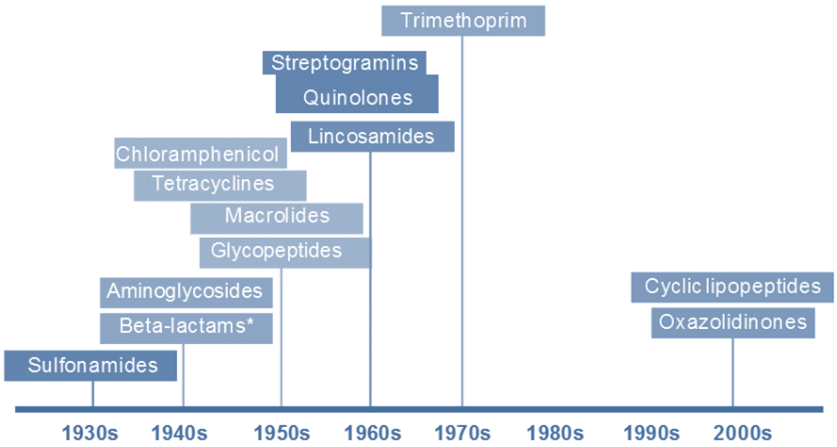

Figure 1.1 Timeline showing discovery of new antibiotics. Adapted from Silver (2011).

1.2 Antimicrobial resistance

The importance of antimicrobial agents comes from their role in saving countless cases of serious infectious diseases either by treating or preventing (vaccination) the infection (Gould and Bal, 2013, Sengupta et al., 2013). But because they have been used extensively, this has engendered a strong evolutionary selection pressure that has led to the emergence of antibiotic

resistance, which in turn reduces the clinical efficacy of these agents (Figure 1.2) (WHO, 2017, Tacconelli et al., 2018). Antibiotic resistance is now a global threat requiring significant measures to avoid the emergence of further resistance and to limit the spread of existing resistance (Golkar et al., 2014, Akova, 2016). According to O'Neill's report on antimicrobial resistance, nearly 700,000 deaths occur every year as a result of infections caused by drug-resistant microorganisms without appropriate intervention. That figure is predicted to increase to 10 million by 2050 (O'Neill, 2016). In the United States, about two millions people get infected every year with antibiotic-resistant bacteria, of which nearly 23,000 people die (CDC, 2019). Antibiotic-resistant infections are a threat to all, but specific groups of individuals are at a greater risk, which includes the immunocompromised, neonates and the elderly (O'Neill, 2016, Martens and Demain, 2017).

Globally, antibiotic-resistant bacteria are spreading at an alarming rate, and the antibiotic discovery pipeline lacks novel drug candidates potentially capable of addressing the problem (Silver, 2011, Frieri et al., 2017). Various health organisations have warned about the danger of antibiotic resistance and indicated that the fast emergence of resistant bacterial species is a crisis which could have catastrophic consequences (Viswanathan, 2014). The Infectious Diseases Society of America (IDSA) conducted a survey of infectious disease specialists that revealed that, within a single year, >60% of the participants had seen a pan-drug resistant (resistant to all available antimicrobial agents) or untreatable bacterial infection (Magiorakos et al., 2012, Spellberg and Gilbert, 2014).

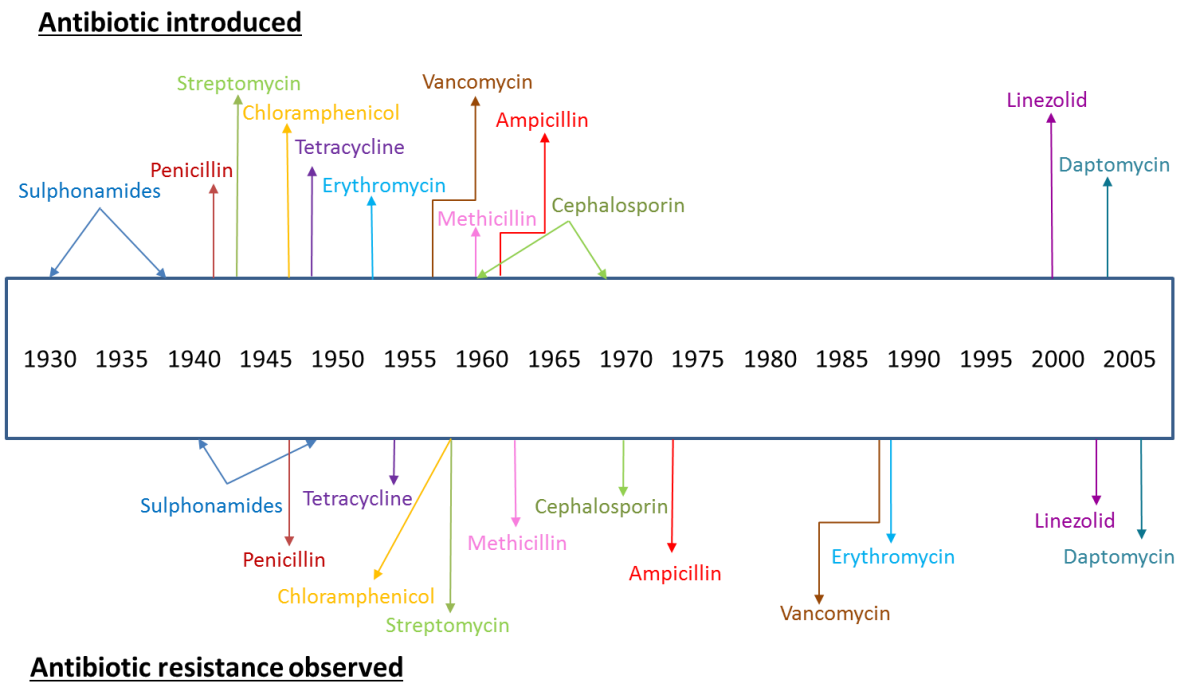

Figure 1.2 Timeline of developing antibiotic resistance. The top arrows show when the antibiotic was introduced, and the bottom arrows show when antibiotic resistance was observed. Adapted from Ventola (2015).

Whilst multidrug-resistant (MDR) Gram-positive bacteria (e.g. *Staphylococcus aureus* and enterococcus species) (Gross, 2013, Golkar et al., 2014, Rossolini et al., 2014) have long been a concern, infections caused by multi-drug resistant Gram-negative pathogens are currently the most pressing area of unmet medical need. Enterobacteriaceae (especially *Klebsiella pneumoniae*), *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are the most prevalent pathogens causing serious infections in health care settings (Li and Webster, 2018, Tacconelli et al., 2018). Antibiotic-resistant bacterial infections have been categorised by the WHO based on several factors as urgent, serious or concerning threat (Table 1.1) (WHO, 2017).

Table 1.1 Assessment of Antibacterial Resistance Threats (WHO, 2017)

Degree of Threat	Bacterial Genera
Urgent Threats	*P. aeruginosa,* carbapenem-resistant
	A. baumannii, carbapenem-resistant
	Enterobacteriaceae*,* carbapenem-resistant, ESBL-producing
Serious Threats	*S. aureus*, methicillin-resistant, vancomycin-intermediate and resistant
	Enterococcus faecium, vancomycin-resistant
	Helicobacter pylori, clarithromycin-resistant
	Salmonellae, fluoroquinolone-resistant
	Campylobacter spp*.,* fluoroquinolone-resistant
	Neisseria gonorrhoeae, cephalosporin-resistant, fluoroquinolone-resistant
Concerning Threats	*Haemophilus influenzae,* ampicillin-resistant
	Streptococcus pneumoniae, penicillin-non-susceptible
	Shigella spp*.,* fluoroquinolone-resistant

1.3 Genetic routes to antibiotic resistance

Resistance to an antibiotic can be innate, which means that bacteria are inheritably resistant to a certain antibiotic. This is characteristic of specific bacterial species such as *Escherichia coli* which has an innate resistance to vancomycin (Tenover, 2001, Giedraitiene et al., 2011, Cohen, 2013). Another possible basis of resistance is acquired resistance which can occur as a result of mutational events or by horizontal gene transfer (Martinez et al., 2009, Frieri et al., 2017). For instance, *Mycobacterium tuberculosis* primarily acquires

resistance through mutation, whereas other bacterial pathogens such as members the Enterobacteriaceae can acquire resistance determinants through horizontal gene transfer (Paterson, 2006, Palmer and Kishony, 2013). These two routes to resistance – often referred to as endogenous and exogenous development of antibiotic resistance (Courvalin, 2008) – are described in further detail below.

1.3.1 Mutational (Endogenous) Resistance

Mutational resistance usually plays a major role in the evolution of antibiotic resistance in most bacterial pathogens and is of particular importance in those organisms that do not routinely engage in genetic exchange. Resistance of this type usually results in target alteration or affects antibiotic accumulation (Anderson and O'Toole, 2008, Blair et al., 2015, Munita and Arias, 2016). Although mutational events in bacteria are relatively rare (1 in 10^6 to 10^8 of the bacterial population), they can occur as a result of errors in the DNA replication process, or as a result of inappropriate repair of damaged DNA (Giedraitiene et al., 2011, Palmer and Kishony, 2013). A single-point mutation can occur in the genes encoding certain antibiotic targets, which in turn cause resistance to that antibiotic. During a bacterial infection, there are different populations of microbes, and if a mutation in an antibiotic target occurs, those strains carrying that mutation will proliferate in the presence of an antibiotic rather than the other strains with no mutation (Courvalin, 2008, Blair et al., 2015, Peterson and Kaur, 2018). It was shown that *E. coli* have the genetic ability to gain mutations in order to select for antibiotic resistance (LeClerc et al., 1998, Tanabe et al., 1999, Miller et al., 2002).

In many occasions, resistance results from mutation usually affect the fitness and the homeostasis of the cell, and this kind of resistance is mostly preserved when it is required (i.e. in the presence of a specific antibiotic) (Palmer and Kishony, 2013). Also, it was long assumed that acquisition of resistance could cause an energy cost to the bacterial cells. Furthermore, it was speculated that under laboratory conditions, resistant mutants usually have fitness cost and low growth rates when compared to the wild-type (WT). As a consequence, it was hypothesised that in the absence of selection an (antibiotic) multidrug-resistant mutant would exhibit instability and a short lifespan (Andersson, 2006, Davies and Davies, 2010). However, as regularly indicated, laboratory conditions do not mimic the real life environment, and evidence assumes that microorganisms with a number of mutations and combinations of resistance genes can adapt and survive well *in vivo* (Mwangi et al., 2007).

1.3.2 Antibiotic resistance resulting from horizontal gene transfer

The majority of antibiotics used in clinical practice derive from natural sources and those bacterial species that ordinarily exist in natural environments containing antibiotics usually exhibit intrinsic resistance to those antibiotics (Davies, 1997, Marti et al., 2014). It has been suggested that this ‘environmental resistome’ is an important source for the ultimate emergence of antibiotic resistance through horizontal gene transfer in clinically-relevant bacterial species (Thomas and Nielsen, 2005, Munita and Arias, 2016). In general, acquisition of genetic material in bacterial cells occurs via three major mechanisms: conjugation (bacterial mating), transformation (the uptake of naked DNA) and transduction (by means of bacteriophages) (Marti et al.,

2014). These processes are shown in Figure 1.3 and more details about them are mentioned below.

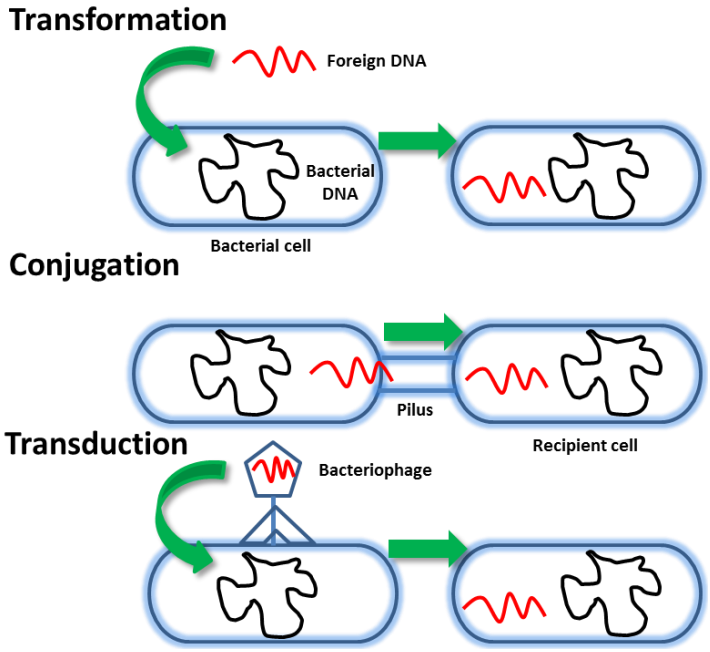

Figure 1.3 The mechanisms of genetic acquisition in bacterial cells occur via three major mechanisms: conjugation (bacterial mating), transformation (uptake of naked DNA) and transduction (by bacteriophages).

While transformation is in principle the simplest type of horizontal gene transfer, only a small proportion of clinically important bacterial species have been shown to spontaneously uptake naked DNA and thereby gain antibiotic resistance. By contrast, conjugation is believed to occur at high frequency in certain environments (Thomas and Nielsen, 2005, Manson et al., 2010). Several studies suggest that genetic transfer among the gut microbiome occurs at diverse and high rates (Shoemaker et al., 2001, Sommer et al., 2009). Interestingly, genetic transfer by conjugation has been reported widely under laboratory conditions and under experimental ecosystem (microcosm) that

mimic the environmental conditions. However, it has been hypothesised that the transmission frequency of conjugation in the natural environment is higher than what is seen in the laboratory (Sørensen et al., 2005, Davies and Davies, 2010).

Plasmids and transposons are common mobile genetic elements (MGE) carrying antibiotic resistance genes, and both are known to participate in the evolution and distribution of antibiotic resistance in clinically important bacterial species (Partridge et al., 2018). MGE-encoded antibiotic resistance is particularly important since, in contrast to chromosomally-encoded resistance, it exhibits increased stability and transmissibility, and has the benefit (from the bacterium's perspective) of allowing the simultaneous transfer of multiple resistances to new hosts (Rodríguez-Rojas et al., 2013, Marti et al., 2014).

Transduction is another route of bacterial genetic exchange, and it is mediated solely by bacteriophages (independently replicating bacterial viruses). Some bacteriophages have the ability to transfer bacterial genes from an infected cell to a recipient bacterium, predominantly as a result of errors in DNA packaging or prophage excision (Frost et al., 2005, Penadés et al., 2015). In nature, bacteriophages play a crucial in the spread of antimicrobial resistance determinants among different bacterial species, and this phenomenon has also been demonstrated in clinically-relevant bacterial species (Balcazar, 2014, Haaber et al., 2017). There are two types of transduction, firstly is the generalised transduction in which any of the host genes can be transduced (Penadés et al., 2015). In generalised transduction during the lytic cycle, the

phage breaks the bacterial DNA of the donor cell into small fragments and then unwittingly the newly forming phage particles incorporate a fragment of the bacterial DNA into the phage head instead of the phage DNA (Salmond and Fineran, 2015, Tzipilevich et al., 2017). After that, the transducing phage particles attack another bacterial cell (recipient cell) and inject their contents (which might be the donor bacterial genes) into the recipient cell and incorporate into the genome of this cell by recombination (Malachowa and DeLeo, 2010). Secondly is the specialised transduction where only certain host genes can be transduced. The same scenario as generalised transduction occurs again in specialised transduction apart from the specific transfer of bacterial DNA located adjacent to the integrated prophage instead of random DNA excision (Suttle, 2007, Salmond and Fineran, 2015).

1.4 Horizontal gene transfer in *S. aureus*

The purpose of this section is to provide further insights into horizontal gene transfer in *S. aureus* with a focus on its relation to antibiotic resistance because *S. aureus* is a major focus of this thesis.

S. aureus is an opportunistic pathogen that has the ability to adapt to different environmental conditions and to cause a wide variety of life-threatening infections. This organism carries a diverse array of multidrug-resistance genes on plasmids which help the distribution of antibiotic resistance among different species (Lowy 1998, Lowy, 2003, McCarthy et al., 2014, Cafini et al., 2017b). Moreover, horizontal gene transfer has substantially participated in the evolution of *S. aureus* in nature. However, under laboratory conditions strong restriction barriers are present in *S. aureus,* but it has been shown that *S.*

aureus can accept and transfer DNA derived from foreign bacteria by electroporation following established transformation protocols (Monk et al., 2012, Jones et al., 2015). Mobile genetic elements represent about 15–20% of the genome of *S. aureus* including staphylococcal chromosome cassettes, bacteriophages, plasmids, *S. aureus* pathogenicity islands, insertion sequences, and transposons (Lindsay, 2014, Haaber et al., 2017).

Methicillin-resistant *S. aureus* (MRSA) is a common cause of hospital-acquired infections which are difficult to treat with current antibiotics (Grundmann et al., 2006, Lerminiaux and Cameron, 2019). MRSA strains carry the methicillin resistance gene (*mecA*) on a mobile genetic element named staphylococcal cassette chromosome (SCC). This gene encodes an alternative penicillin-binding protein (PBP2a) that has low affinity for all β-lactams and can maintain cell-wall synthesis even in their presence (Alibayov et al., 2014, Lindsay, 2014). The first hospital-associated MRSA strain, reported in the UK in 1961, carries SCC*mec* type I, whereas the first community-associated MRSA strains that were reported during the 1990s and early 2000s in USA and Australia have SCC*mec* types IV and V (Ito et al., 2001, Ito et al., 2004). SCC*mec* elements have only been detected in staphylococci and range in size from 20 kb to >60 kb; along with the methicillin resistance genes, they carry other elements such as other antibiotic resistance genes, transposons, insertion elements, or plasmids (Katayama et al., 2000, IWG-SCC, 2009). Generally, these elements have similar characteristics such as the presence of the *mec* gene complex that carries *mecA, mecR1* (encoding the beta-lactam sensor/ signal transducer protein, MecR1) and *mecI* (encoding the transcriptional repressor, MecI) (Katayama et al.,

2000, IWG-SCC, 2009, Wang and Archer, 2010). Furthermore, SCC*mec* elements carry another gene complex (*ccr*) that encodes for unique site-specific serine recombinases known as cassette chromosome recombinases (Ccr) such as CcrAB or CcrC, and which mediate integration into (and excision from) the chromosome via recombination at specific *i*ntegration *s*ite *s*equence (ISS) for SCC*mec* known as *att* (Ito et al., 2001, Ito et al., 2004, Ray et al., 2016). The *attS* site is located on the SCC*mec* element, whereas the *attB* site is located on the staphylococcal chromosome and comprises the 3′ end of *rlmH* gene (previously called *orfX*), a highly conserved gene of unknown function located near the *S. aureus* origin of replication (Purta et al, 2008, Ero et al, 2010, Wang and Archer, 2010). When the Ccr recombinase binds to *attB* and *attS* sites, it triggers DNA cleavage and mediates integration, which results in the incorporation of SCC*mec* into the staphylococcal genome and generates repeated copies of the *att* sites at each end of the inserted SCC*mec* that are termed *attL* and *attR* (Ito et al., 1999, Katayama et al., 2000, Bitrus et al., 2018). SCC*mec* elements can also undergo excision from this site; during excision, the *attL* and *attR* sites recombine together to excise the SCC*mec* element from the staphylococcal chromosome and reconstitute the *attB* site on the chromosome and *attS* site on the circular form of the SCC*mec* element. These sites also contain a conserved 8-base core region (required for SCC*mec* binding), and variable flanking direct repeat (DR) sequences that determine the rate and the efficiency of the insertion of the SCC*mec* element (Wang and Archer, 2010, Wang et al., 2012, Ray et al., 2016).

It remains to be established how SCC*mec* elements transfer from one strain to another. These elements contain one to four copies of the insertion

sequence IS*257*/*431*, a sequence that is also frequently found on staphylococcal plasmids, and which is known to allow recombination-mediated capture of antibiotic resistance genes and other genetic sequences (Berg et al, 1998, Caryl and O'Neill, 2009, IWG-SCC, 2009). It has been hypothesised therefore that the presence of IS*257*/*431* elements in every SCC*mec* type would allow integration of SCC*mec* into common conjugative plasmids such as pGO1/ pSK41, with the plasmid providing the vehicle for transfer to another host (Berg et al, 1998, Caryl and O'Neill, 2009, Ray et al., 2016). This transfer route has been demonstrated in laboratory studies. SCC*mec* was successfully integrated into a conjugative plasmid (pGO400, a derivative of the pGO1/pSK41) by recombination at an IS*257*/*431* site, allowing subsequent dissemination by conjugation between several *S. aureus* sequence types (Ray et al., 2016).

Bacteriophage may also participate in the horizontal transfer of *SCCmec.* Smaller SCC*mec* elements (<45 Kb) in particular should be amenable to transduction, e.g. SCC*mec* type IV, which has a size of 20 to 25 kb. This acquisition of SCC*mec* could occur as a result of homologous/ site-specific (a common mechanism for phage integration) or *ccr*-mediated recombination (Scharn et al., 2013, Sansevere and Robinson, 2017).

As these elements can transfer successfully and have the ability to replicate or integrate into the chromosome or plasmids efficiently, this could lead to the dissemination of the resistance determinants they are carrying (McCarthy et al., 2014, Cafini et al., 2017b, Langhanki et al., 2018). It has been reported that SCC*mec* elements accumulate antibiotic resistance genes from the

insertion of several mobile genetic elements like Tn4001, Tn554, pUB110 and pT181 (Zong et al., 2011, Liu et al., 2016, Sansevere and Robinson, 2017).For instance, TetK is an efflux pump with 14 transmembrane helices that confers resistance to tetracyclines, and it is encoded by plasmid pT181 which is located within the SCC*mec* type III element of MRSA strains (Jensen and Lyon, 2009). Furthermore, resistance to aminoglycosides conferred by the acquisition of aminoglycoside modifying enzymes (adenyltransferase aadD) is encoded by plasmid pUB110, which is integrated within the SCC*mec* type II element in some MRSA strains (Wright, 1999, Jensen and Lyon, 2009, Ramirez and Tolmasky, 2010, Foster, 2017).

Phage-mediated horizontal gene transfer is thought to have participated substantially in the evolution of *S. aureus* (Xia and Wolz, 2014). Several bacteriophages are prevalent in *S. aureus* populations (e.g. ϕ11, ϕ80), and generalised transduction is usually the main type of transduction in *S. aureus* (Deghorain and Van Melderen, 2012, Xia and Wolz, 2014). Bacteriophages encode unique integrases that mediate the integration of the phage DNA into the bacterial chromosome; to date, eight different families of integrases have been defined in *S. aureus* bacteriophages, with each one associated with a corresponding insertion site (Maiques et al., 2007, McCarthy et al., 2012). These insertion (target) sites are characterized by *att* flanking regions and the phage DNA integrate with them in a site-specific way. Most bacteriophages enter a long-term relationship with the bacterial cell, whereby the DNA of the phage integrates and replicates as part of the staphylococcal genome as a prophage (lysogenic cycle) without inducing particle formation or cell lysis (Mir-Sanchis et al., 2012, Lindsay, 2014). Nevertheless, prophage induction under

certain conditions can induce the activation of the lytic cycle where the bacteriophage DNA starts to replicate and manufacture new bacteriophage particles that are released and lyse the bacterial cell completely (Penadés et al., 2015, Oliveira et al., 2019). Towards the end of the lytic cycle, phages generally employ several mechanisms to distinguish their own phage DNA from the host's during DNA packaging of new phage particles (Black, 1989, Rao and Feiss, 2015). One of these mechanisms is the headful (*pac*) packaging process characterised by the presence of the phage-specific packaging site *pac*, where the phage terminase enzyme (a hetero-oligomer of small and large terminase proteins) cuts the DNA to initiate packaging the phage DNA into phage heads (Chiang et al., 2019, Oliveira et al., 2019). Then, when the phage head has reached its capacity (typically not more than 45 kb), the terminase makes a nonspecific subsequent cut to terminate the packaging process (Streisinger et al., 1964, Streisinger et al., 1967, Chiang et al., 2019). For instance, ϕ11 is a temperate phage that is capable of both lytic and lysogenic growth. During the lysogenic life cycle of ϕ11 the phage DNA integrates and resides as prophages using a mechanism of site-specific recombination (headful *pac* mechanism) mediated by integrases and occurring between the phage attachment site (*attP*) and the bacterial attachment site (*attB*) (Ye and Lee, 1993, Oliveira et al., 2013, Penadés et al., 2015, Oliveira et al., 2019). DNA mispackaging could occur when bacterial DNA is packaged into the phage capsids instead of phage DNA resulting in transducing phage particles that carry variable DNA sequences of the bacterial cell (donor) and subsequently inject this DNA into a another bacterial cell (recipient) (Penadés et al., 2015, Oliveira et al., 2019). These mispacking

events occur at relatively low frequency, but are sufficiently common to make transduction a useful laboratory tool that allows transfer of effectively any chromosomal marker to another staphylococcal strain.

Staphylococcal phages have been shown to transfer *S. aureus* pathogenicity islands (SaPIs) that carry toxin-encoding genes, including toxic shock syndrome toxin 1 and other superantigens (Malachowa and DeLeo, 2010, McCarthy et al., 2014, Kali, 2015). Also, it has been documented that the transduction of phages and other genetic elements is possible between multiple staphylococcal species and even to other Gram-positive genera. For instance, SaPIbov5, which encodes several virulence genes, can be transferred at high frequency by ϕ12 bacteriophage between different *S. aureus* isolates and also from *S. aureus* to *Listeria monocytogenes* (Chen et al., 2015). Additionally, SCC*mec* elements that belong to clonal complex 8 (CC8) backgrounds have been reported to be transduced by ϕ80α and ϕ29 bacteriophages using a mechanism that is similar to generalised transduction, which might increase the risk of dissemination of antibiotic resistance genes that are already on the SCC*mec* elements (Scharn et al., 2013, Sansevere and Robinson, 2017). Also, it was documented that ϕ80α and ϕJB bacteriophages can perform efficient transduction for penicillin and tetracycline resistance plasmids between clinical isolates belonging to the USA300 clone and could participate in the spread of this kind of resistance (penicillin and tetracycline resistance) (Varga et al., 2012, Langhanki et al., 2018).

Meanwhile, staphylococci cells usually have one or more plasmids that are different in their gene content. Also, most of the intercellular transfer of staphylococcal plasmids happens by transduction or conjugation because *S. aureus* has limited ability to uptake DNA from the environment when compared to *E. coli* or *Bacillus subtilis.* When the plasmid enters the bacterial cell, it can remain as free circularised DNA or integrate into the staphylococcal chromosome (Morikawa et al., 2003, Malachowa and DeLeo, 2010). Generally, conjugative plasmids represent about 5% of all known *S. aureus* plasmids. They carry *tra* genes that are required for the autonomous conjugative transfer process including those responsible for pore formation, DNA processing and replication (Smillie et al., 2010, Pollet et al., 2016, Ramsay et al., 2016). One of the enzymes encoded by these genes is the relaxase enzyme that is responsible for the binding to a specific recognition sequence named the origin of transfer (*oriT*), and for the cleavage of one strand of the double-stranded plasmid to begin the transfer and then ligate it back to complete the transfer process (Smillie et al., 2010, Pollet et al., 2016, Ramsay et al., 2016).

However, non-conjugative *S. aureus* plasmids are classified as mobilizable or non-mobilizable plasmids. Mobilizable plasmids usually carry *mob* genes which facilitate DNA processing and replication but lack the genes required for mating and pore formation (Ramsay et al., 2016, LaBreck et al., 2018). Therefore, they recruit the conjugative plasmid's machinery to be able to be transferred from one cell to another. The vast majority of documented mobilizable plasmids exploit conjugative plasmids for horizontal dissemination through three mechanisms: the classical relaxase-*in cis*, relaxase-*in trans* and

replicative relaxase (Grohmann et al., 2003, Pollet et al., 2016). In addition to that, staphylococcal strains are hosts to various antimicrobial-resistance plasmids, which contribute to the dissemination of antimicrobial resistance among staphylococci (Ramsay et al., 2016). For instance, the emergence of gentamicin resistance was reported to be caused by members of the pSK41/pGO1 plasmid family (Liu et al., 2013). Also, these plasmids have been documented to be responsible for conferring resistance to several antibiotics (penicillin, bleomycin, mupirocin, tetracycline, trimethoprim, macrolides, lincosamides and streptogramin B), antiseptics and disinfectants along with the aminoglycoside resistance genes. These various resistance determinants are mainly encoded by several small plasmids that are located between IS*257*/IS*431* within the pSK41/pGO1 plasmids (Firth and Skurray, 2006, Cafini et al., 2017a). Of note, linezolid and high-level vancomycin resistance have been reported to be associated with plasmids of this type (Weigel et al., 2003, Bender et al., 2015). Vancomycin is the antibiotic of choice for treating MRSA and other antibiotic-resistant infections, but vancomycin-resistant *S. aureus* (VRSA), which emerged from MRSA strains by the acquisition of a plasmid from *Enterococcus faecalis,* might participate to the development of pandrug-resistant bacteria (resistant to nearly all current antibiotics) where no effective antibiotic treatments are available (Weigel et al., 2003, Gardete and Tomasz, 2014). Furthermore, linezolid is one of the effective resources for MRSA treatment, though dissemination of plasmids-mediated linezolid resistance is also predictable (Cafini et al., 2017a).

Nonetheless, integrative conjugative elements (ICEs) are another component of conjugation machinery that are infrequently considered in the

staphylococcal literature. ICEs usually combine the conjugation characteristics of plasmids with the recombination characteristics of phages and transposons. In most cases, ICEs encode a phage-like tyrosine recombinase for the purpose of integration/excision and a relaxase which is involved in the DNA transfer and replication (Cury et al., 2017, Sansevere and Robinson, 2017). ICEs have been mainly overlooked, and only two families have been studied so far in staphylococci (Tn*916*, and ICE*6013*), which are mostly found in clinical isolates of *S. aureus*. Tn*5801* is a well-studied element of the Tn*916* family that was initially found in the vancomycin-intermediate *S. aureus* (VISA) strain (Mu50). The biological consequences of hosting Tn*5801* consist of the acquisition of tetracycline resistance, alteration in bacterial growth rates, and inhibitions of restriction barriers to acquiring mobile genetic elements (Kuroda et al., 2001, Mingoia et al., 2013, Sansevere and Robinson, 2017).

1.5 Molecular basis of antibiotic resistance

Bacteria can become resistant through a variety of cellular mechanisms. These can be grouped according to mechanistic type, with the most common being (i) reduced antibiotic accumulation, (ii) alteration of the antibiotic, and (iii) alteration of the drug target (Figure 1.4) (Hawkey, 1998, Shaikh et al., 2015). These different types of resistance mechanism are described in more detail below.

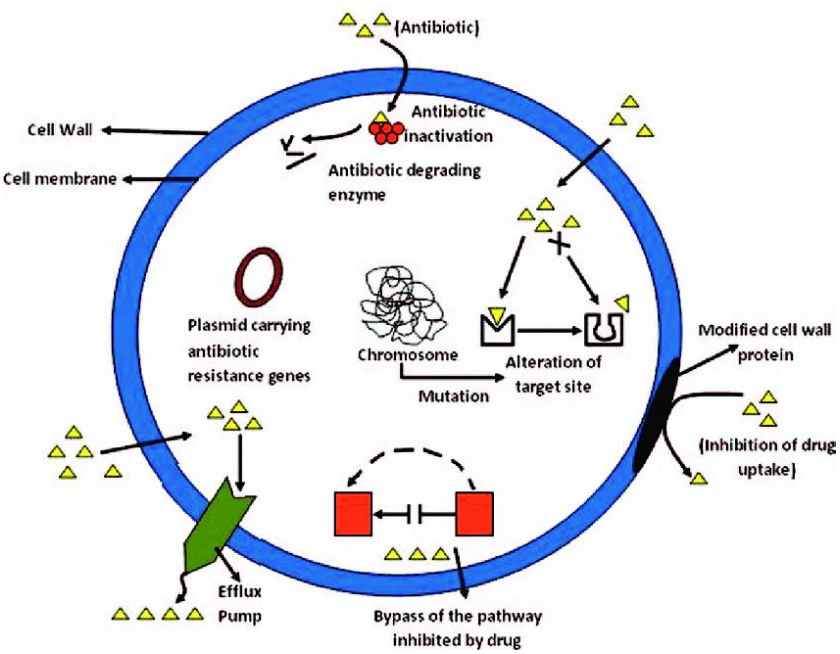

Figure 1.4 Molecular mechanisms of antibiotic resistance. This diagram shows the different mechanisms of resistance which might occur as a result of a mutation in the chromosome or by means of horizontal gene transfer (i.e. plasmid). The main mechanisms are prevention of antibiotic penetration and efflux, inactivation of the antibiotic molecule, modification of the target site and bypassing the pathway inhibited by the antibiotic. Taken from (Singh et al., 2014).

1.5.1 Prevention of antibiotic penetration and efflux

1.5.1.1 Reduced permeability

A substantial number of clinically used antibiotics have targets located inside bacteria. Consequently, the antibiotic must penetrate the outer and/or inner membrane to reach its target and exert its antibacterial effect (Kapoor et al., 2017). Reducing antibiotic uptake is one of the mechanisms employed by different bacterial species to prevent (or reduce) antibiotic from reaching its periplasmic or intracellular target (Blair et al., 2015). Restriction of the influx of molecules from the external milieu is particularly common in Gram-negative bacteria, in which the outer membrane acts as a dedicated diffusion barrier and the first line of defence to prevent the ingress of toxic substances (Munita and Arias, 2016).

A basic example that demonstrates the efficacy of this membrane is antibacterial drugs exhibiting potent antibacterial activity against Gram-positive pathogens (e.g. vancomycin) but lack useful activity against Gram-negative bacteria because they are too large to fit through the protein channels (‘porins’) of the outer membrane, and are therefore unable to traverse the latter (Quintiliani Jr, 1995, Nicolosi et al., 2010, Fernandes et al., 2017).

Furthermore, several antibiotics ordinarily effective against Gram-negative bacteria are adversely affected by alterations in - or reduced expression of - the porins, e.g. tetracyclines, β-lactams and fluoroquinolones (Pagès et al., 2008). *Pseudomonas* spp. and *A. baumannii* have innate low susceptibility against β-lactams when compared to Enterobacteriaceae species, which is likely due to the difference in the number and expression of porins between those genera (Hancock and Brinkman, 2002).

1.5.1.2 Efflux

Bacterial cells are able to produce complex machinery to excrete toxic molecules out of the cell and can use these as a resistance mechanism against antibiotics (Kapoor et al., 2017). The first antibiotic efflux system was described in the 1980s when it was discovered that *E. coli* employs a transporter protein to pump tetracycline out of the cytoplasm (McMurry et al., 1980). Several different types of antibiotic efflux system have since been discovered in Gram-positive and Gram-negative bacteria, though this mechanism of resistance has a substantially greater clinical impact in the case of the latter.

These efflux pumps can be either substrate-specific (like the *tet* determinants that are specific for tetracycline), or exhibit a broad substrate profile (like those

found in multidrug-resistant pathogens) (Poole, 2005). Efflux pumps are often encoded by native genes located on the chromosome but can also occur on mobile genetic elements that undergo horizontal transfer. A large number of antibiotic classes are affected by efflux, including the β-lactams, fluoroquinolones, macrolides, polymyxins and carbapenems (Wise, 1999, Kapoor et al., 2017).

1.5.2 Alteration of the antibiotic molecule

Chemical alterations to the antibiotic molecule can reduce its ability to interact with its cognate target, and can thereby overcome its inhibitory effect (Wilson, 2013). Bacteria can catalyse such chemical alterations either by breaking key chemical bonds within the molecule, or by adding chemical moieties (‘modification’). A number of antibiotics that inhibit protein synthesis at the ribosomal level are affected by antibiotic modifying enzymes including those that catalyse adenylation (lincosamides, aminoglycosides), phosphorylation (chloramphenicol, aminoglycosides) and acetylation (aminoglycosides, streptogramins, chloramphenicol) (Wise, 1999, Wilson, 2013). Although there are various biochemical reactions that lead to enzymatic modification of different antibiotics, they usually lead to steric hindrance (reduced affinity) between the antibiotic and its target (Wilson, 2013, Kapoor et al., 2017). The most clinically-significant example of antibiotic resistance caused by modification of the drug involves the aminoglycoside modifying enzymes, which attach amino or hydroxyl moieties to aminoglycoside compounds, and are the predominant mechanism of aminoglycoside resistance worldwide (Ramirez and Tolmasky, 2010).

The predominant resistance mechanism against β-lactam antibiotics involves hydrolysis by β-lactamase enzymes. β-lactam antibiotics lose their antimicrobial activity when β-lactamase enzymes break the crucial amide bond of the β-lactam ring (Abraham and Chain, 1988, D'Costa et al., 2011). The first β-lactamase enzyme was discovered in the early 1940s, while β-lactamases are thought to have existed in bacteria for millions of years (Abraham and Chain, 1988, Bush, 2018). Of note, when the clinical use of penicillin began, β-lactamase-mediated resistance rapidly emerged in *S. aureus*. This resistance was caused by a plasmid-encoded penicillinase, and was rapidly disseminated between *S. aureus* strains (Bush, 2013). In order to combat this problem, more β-lactam antibiotics were synthesised and developed with greater stability to penicillinases and with a broader spectrum of activity.

On the other hand, in Gram-negative bacteria, TEM-1 β-lactamase (that has the ability to destroy ampicillin) was discovered in the 1960s (Paterson and Bonomo, 2005). Since that discovery, nearly every new generation of β-lactams developed and introduced to the market has been followed by the rapid emergence of resistance (Munita and Arias, 2016).

1.5.3 Alteration of the antibiotic target site

Many antibiotics have a high affinity for - and selectively bind to - their targets, thereby causing loss of the normal functioning of the target. Therefore, structural changes in the target that enable the target to perform its normal role but prevent efficient antibiotic binding can lead to resistance against that antibiotic (Lambert, 2005, Shaikh et al., 2015).

One of the predominant examples of target mutation is methicillin resistance in *S. aureus* (MRSA), in which resistance is conferred by the acquisition of genes encoding proteins homologous to the methicillin target but with some changes (Katayama et al., 2000). Methicillin resistance (as mentioned above) occurs as a consequence of acquiring the SCC*mec* element, which has *mecA* gene that encodes PBP2a, which is a β-lactam insensitive protein. By the presence of this protein, the cell wall biosynthesis process will continue even if the antibiotic inhibits the native PBP (Katayama et al., 2000, Shore et al., 2011, Hiramatsu et al., 2013). There is evidence that there are several SCC*mec* elements found in various staphylococcus bacterial species and that they have been mobilised widely across those species (Shore et al., 2011).

Another example of antibiotic resistance caused by enzymatic alteration of the antibiotic target is erythromycin resistance through ribosomal methylation (Weisblum, 1995). This resistance mechanism involves an enzyme encoded by the *erm* genes that promotes the methylation of a specific nucleotide of the 23S ribosomal RNA, which alters the nature of the macrolide binding site on the ribosome. Because of this chemical modification, the interaction between the antibiotic and its target is impaired, and macrolide resistance results (Weisblum, 1995, Leclercq, 2002, Ghanbari et al., 2016). Interestingly, expression of *erm* genes results in cross-resistance towards other MLSB group antibiotics (macrolides, lincosamides, and streptogramin B), because all these shares overlapping binding sites on the ribosome. Various *erm* genes (about 30) have been found in aerobic and anaerobic Gram-negative and Gram-positive bacteria (Roberts, 2008, Ghanbari et al., 2016, Yao et al., 2019).

One of the well-known examples relates to this mechanism is the ribosomal protection proteins that are involved in tetracycline resistance such as Tet(M) and Tet(O) determinants (Chopra and Roberts, 2001, Dönhöfer et al., 2012, Li et al., 2013b). Tetracycline mechanism of action depends on its binding to the ribosome and preventing the elongation phase of protein synthesis. Tet(M) and Tet(O) work by interacting with the ribosome and changing the geometry of the tetracycline binding site on the ribosome, which in turn results in ribosomal conformation (structural changes). These changes in the ribosome dissociate tetracycline from its binding site in the ribosome, enabling the protein synthesis process to resume in the presence of tetracycline (Burdett, 1996, Connell et al., 2003, Grossman, 2016).

1.6 Introduction to the present study

There is a worldwide challenge to combat antimicrobial resistance in pathogenic bacteria, which causes high mortality and morbidity rates among different populations. Conventional antimicrobial agents have failed to treat infections caused by multidrug resistant Gram-positive and Gram-negative bacteria (Akova, 2016, Velez and Sloand, 2016). The resistance patterns deployed by various bacterial pathogens resulted in difficult to treat or untreatable bacterial infections. When coupling the dramatic rises in the emergence of resistance with the poor standards of infection prevention and control, bacterial resistance can readily be distributed in hospitals and in communities (Frieri et al., 2017, Aslam et al., 2018).

Therefore firstly, understanding the genetic basis of bacterial resistance (which was investigated here in this study) is crucial for the future usage of an antimicrobial agent which could help to improve treatment strategies and guide

the development of new combinations of antimicrobial agents with improved or expanded activity against important species (Ventola, 2015b, Frieri et al., 2017). Also, it is important to understand the potential for resistance to become disseminated and spread rapidly, especially during the medical administration of an antimicrobial agent which could impose a selection pressure on the resistance to arise.

Secondly, it is important to set up breakpoints for antibiotics (susceptible, intermediate, and resistant) which can help in identifying if this antibiotic remains effective for the treatment of a specific bacterial infection or not. The establishment of clinical susceptibility breakpoints mainly relies on the knowledge of the WT distribution of minimum inhibitory concentrations (MIC) (*in vitro* microbiological data), *in vitro* resistance markers (both phenotypic and genotypic), evaluation of the pharmacokinetics/ pharmacodynamics of the antibiotic, and assessment of the clinical outcome of infections when the antibiotic is used (Kahlmeter et al., 2003, Turnidge and Paterson, 2007, EUCAST, 2017). No single set of data can be used alone to make decisions, but all these data collectively assist breakpoint-setting organisations in selecting breakpoints. Determination of MIC distributions and WT cut-off values (which was investigated here in this study) for a bacterial species against specific antibiotics is the first step in the development of breakpoints for that antibiotic. Construction of histograms or bar charts of the broth and/or agar dilution MICs for all organisms of interest provides an immediate overview of whether or not only WT strains are present or strains with elevated MICs are also available (Turnidge and Paterson, 2007, Hombach et al., 2014).

On that account, the overarching idea in this project was that the mechanisms of resistance for certain antimicrobial agents are poorly understood, and information about the clinical breakpoints for some antimicrobial agents are lacking. Hence, there are important gaps in our understanding of the complete picture of the prevalence and genetic basis of this resistance. That is why this project focused on studying the prevalence and genetic basis of resistance to three antimicrobial agents: bacitracin, nitrofurantoin, and silver in key pathogenic bacteria, which would help collectively on optimising the future clinical applications of these antimicrobial agents. Those agents were chosen because there is limited information in the literature about their MIC breakpoints, their mechanism of resistance and how prevalent it is in a specific group of bacteria. Because of their importance as key pathogenic bacteria in different settings; common skin and soft tissue Gram-negative pathogens were selected to investigate silver and *S. aureus* was chosen to study bacitracin and nitrofurantoin. More details about every antimicrobial agent and why a test pathogen was chosen will be mentioned individually in later chapters.

Briefly, the main objectives of this study were:

- The epidemiological susceptibility for each antimicrobial agent was investigated in a specific group of bacteria to provide an insight into the efficacy of the antimicrobial agents in treating infections caused by those species of bacteria.
- Also, the propensity of certain bacterial species to develop resistance to the antimicrobial agents under study and the genetic basis of this resistance was studied.

Chapter 2 Materials and Methods

2.1 Bacterial strains, phages, and plasmids

2.1.1. Clinical isolates

Gram-negative isolates (*n*=444) used in this study were obtained from hospitals across the UK and abroad, as follows: Leeds General Infirmary; Leeds (*n*=71), Royal Liverpool University Hospital; Liverpool (*n*=130), Northampton General Hospital; Northampton (*n*=106), Temple Street Children's University Hospital; Dublin (*n*=92) and from sites across the USA (*n*=45). These clinical isolates were collected over the period 2012 – 2015, and the overall distribution of different bacterial species/genera was as follows*:* *E. coli*; *n*=135, *Enterobacter* spp.; *n*=75, *Klebsiella* spp.; *n*=105, *P. aeruginosa*; *n*=91, *Acinetobacter* spp.*;* *n*=27, *Proteus* spp.; *n*=6 and *Citrobacter* spp.*;* *n*=5.

S. aureus isolates used in this study were collected during the period 1995 – 2015, and totalled 1138 isolates. They were obtained from clinical samples collected from hospitals across the UK and abroad, as follows: Royal Veterinary College; London (*n*=217), London Imperial College; London (*n*=55), Beaumont Hospital Reference Laboratory; Dublin (*n*=237), St. James's Hospital; Dublin (*n*=38), different sites across Australia (*n*=24), the collections of The Faculty of Biological Sciences, School of Molecular and Cellular Biology; University of Leeds (*n*=465) and from The Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA); BEI Resources https://www.beiresources.org/ (*n*=102) (Appendix 2).

2.1.2. Laboratory strains, plasmids, and phages

The laboratory strains, plasmids and bacteriophages used in this study are shown in Table 2.1.

Table 2.1 Summary of laboratory strains, plasmids and phages used in this study

Strain/ Plasmid or Phage Name	Description/ Genotype	Reference/Source
***E. coli* BW25113**	Derivative of *E. coli* K-12 strain BD792 (*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$*hsdR514* Δ*araBAD*$_{AH33}$Δ*rhaBAD*$_{LD78}$)	(Datsenko and Wanner, 2000)
***E. coli* 50110 J53-(pMG101)**	Silver-resistant *E. coli* K-12-J53 strain carrying plasmid pMG101	NCTC
***Enterobacter cloacae* ATCC 13047**	Strain harbouring the *sil* operon on the chromosome	ATCC
***S. aureus* SH1000**	Derivative of 8325-4 strain, with functional *rsbU* gene reinstated	(Horsburgh et al., 2002)
***S. aureus* RN42220**	Restriction-deficient cloning host	(Kreiswirth et al., 1983)
***S. aureus* VRS1, VRS2, VRS3a and VRS4**	Vancomycin-resistant *S. aureus*	O'Neill Lab, University of Leeds
***E. coli* XL10-Gold**	Tet^{r}Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r}) Amy Cam^{r}].	Agilent Technologies, California, USA
Plasmid pSK5487	*E. coli*/ *S. aureus* pSK41-based shuttle vector engineered to have BstBI restriction site, carrying the constitutive promoter P$_{qacR}$	(Grkovic et al., 1998, Caryl and O'Neill, 2009)
Φ11 and Φ80 bacteriophage	Bacteriophage of serological group B documented to be able to transduce plasmids and chromosomal genes in *S. aureus*	(Novick, 1967, Kayser et al., 1972, Iandolo et al., 2002, Mašlaňová et al., 2013)

2.2 Antimicrobial agents and chemicals

All antimicrobial agents and chemicals used in this study were dissolved either in water, ethanol, or dimethyl sulfoxide (Sigma-Aldrich, St. Louis, USA). A list of the antimicrobial agents and chemicals used in this study and their manufacturers is provided in Appendix 1.

2.3 Routine culture and storage of bacteria

All media used in this study was obtained from Oxoid (Hampshire, England) and prepared according to the manufacturer's instructions. Usually, bacterial strains were cultured on tryptone soya agar (TSA) or broth (TSB), lysogeny broth (LB) or agar (LBA), and incubated aerobically at 37°C for 18 h. Broth cultures were incubated under the same conditions in a shaking incubator with vigorous aeration (180 rpm). Glycerol stocks were made for storage of the strains at -80°C by adding 900 µL culture to 100 µL of 80% glycerol (Fisher Chemicals, Loughborough, UK). BHI (Brain Heart Infusion) media was used to culture clinical isolates that could not be recovered using LB or TS media.

2.4 General microbiology techniques

2.4.1. Determination of susceptibilities to antimicrobial agents

Determination of the MIC of antimicrobial agents against the clinical isolates in this study was carried out according to CLSI guidelines by agar dilution in MHA (CLSI, 2012). Cultures were grown in Mueller Hinton broth (MHB) and agar (MHA) for MIC determination.

2.4.2. Selection of spontaneous antimicrobial-resistant mutants and determination of mutation frequencies to antimicrobial resistance

To select spontaneous mutants resistant to antimicrobial agents, an aliquot (1 mL) of saturated culture (OD_{600} ≈2, 10^9 cells/mL) was centrifuged and resuspended in 100 µL of MHB. This was then plated onto agar containing 8X MIC of the antimicrobial agent, and plates incubated at 37°C for 24 to 48 h to recover resistant mutants.

Mutation frequencies to antimicrobial resistance were determined according to established methods (O'Neill and Chopra, 2004, Randall, 2013). Briefly, three independent saturated cultures were plated onto MHA containing antimicrobial agent, and onto non-selective agar to determine viable cell numbers. After 24 h of incubation at 37°C, the total number of colonies in all the non-selective plates (total cell number) and the antimicrobial-selection plates (resistant mutants) were counted and represented as CFU/mL (colony-forming units/mL) and the mutation frequency (MF) was determined.

2.4.3. Selection of resistant mutants via extended gradient MIC

An overnight culture was used to select antimicrobial resistance by means of continuous incubation in subinhibitory concentrations of the antimicrobial agent, as described previously (Friedman et al., 2006). To achieve this, a broth microdilution MIC in MHB was performed according to the CLSI guidelines (CLSI, 2012). After an overnight incubation, the well containing the highest concentration of the antibiotic that permitted bacterial growth was diluted 1:100 in MHB and used as inoculum for the next MIC ‘passage’. This process was

repeated for up to 21 d or until resistant mutants were detected by an increase in the MIC (>4 fold). Daptomycin was used as a control antibiotic in this experiment with calcium ions (50 µg/mL) in the form of $CaCl_2.6H_2O$ added to all culture media.

2.5 Molecular biology techniques

2.5.1. Genomic DNA extraction

DNA was extracted using the Bacterial Genomic Purification kit from Edge Biosystems (Maryland, USA). An aliquot (3 mL) of overnight culture in LB or TSB was harvested and processed according to the manufacturer's instructions. For *S. aureus* isolates, 100 µg/mL lysostaphin was added to the cells after their resuspension in Spheroplast buffer and incubated at 37°C for 30 min to facilitate cell lysis. Lysates were then processed according to the manufacturer's instructions, and genomic DNA was stored at -20°C.

2.5.2. Plasmid DNA extraction

Plasmid DNA was extracted using the QIAprep Miniprep kit from Qiagen (Hilden, Germany). Aliquots (9 mL) of overnight culture in LB or TSB were harvested and processed according to the manufacturer's instructions. As above, *S. aureus* cultures required the addition of 100 µg/mL lysostaphin to the cells once in P1 buffer, and incubated at 37°C for 30 min, to facilitate cell lysis. Purified plasmid DNA was stored at -20°C.

2.5.3. DNA purification

DNA from PCR reactions, restriction digests and ligation reactions were purified using QIAquick PCR purification kit or MinElute Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

When necessary, DNA was concentrated by ethanol precipitation (Sambrook et al., 2001).

2.5.4. Measuring DNA concentration

DNA concentration was determined using a NanoPhotometer™ Pearl Nano Spectrophotometer (Implen GmbH, Munich, Germany). The concentration was quantified spectrophotometrically at 260 nm, and the purity was evaluated based on the ratios of 260/280 and 260/230 (Sambrook et al., 2001).

2.5.5. Polymerase chain reaction

Oligonucleotide primers were designed using Oligo software (Colorado, USA) and synthesised by Eurofins Genomics (Ebersberg, Germany) (Appendix 3). Polymerase chain reaction (PCR) was carried out using T100 Thermal Cycler (Bio-Rad, Hertfordshire, UK). Phusion high-fidelity DNA polymerase (New England Biolabs, Hertfordshire, UK) was used in PCR reactions with a total volume of 25 µL. Each reaction contained 0.25 µL Phusion high-fidelity DNA polymerase, 0.5 µL 10 mM dNTPs (New England Biolabs, Hertfordshire, UK), 5 µL 5X Phusion HF buffer, 1.25 µL of each primer (100 pmol/µL), 1 µL template DNA (50-100 ng) and 15.75 µL of nuclease-free water (Promega, Madison, USA). The thermal cycling conditions were: initial denaturation at 98°C for 1 min, 30 cycles of denaturation (at 98°C for 10 s), annealing (at primer's Tm for 30 s), and extension (at 72°C for 30 s/kb), followed by a final extension cycle at 72°C for 7 min. Tm was optimised for each reaction based on the primer using Tm calculator from New England Biolabs at https://tmcalculator.neb.com/#!/ [last accessed 15.10.2017]. PCR reactions were stored at 4°C until needed.

In the case of colony PCR, fresh colonies were suspended in 20 µL of nuclease-free water to make a colony suspension, and 1.5 µL of this suspension was used for each PCR reaction. GoTaq PCR master mix (Promega, Madison, USA) was used for amplification. Each reaction contained 12.5 µL GoTaq PCR master mix, 1.25 µL of each primer (100 pmol/µL), 1.5 µL colony suspension and 8.5 µL of nuclease-free water. PCR conditions included initial denaturation at 95°C for 10 min, 35 cycles of denaturation (at 95°C for 10 s), annealing (at primer's Tm for 30 s) and extension (at 72°C for 1 min/kb), followed by a final extension cycle at 72°C for 7 min. PCR reactions were stored at 4°C until needed.

2.5.6. Agarose gel electrophoresis

Agarose gel electrophoresis was performed in a Bio-Rad electrophoresis cell (Bio-Rad, Hemel Hempstead, UK) to determine the presence and size of PCR products and DNA samples. Agarose gels were composed of 0.8% (w/v) agarose (Geneflow LTD, Fradley, UK) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing SYBR safe DNA stain (Molecular Probes, Invitrogen; Paisley, UK). Hyper Ladder 1 kb was used as a size marker, and samples were mixed with 6X DNA loading dye (New England Biolabs, Hertfordshire, UK) in a 6:1 ratio. Electrophoresis of the DNA samples was for 30 min at 90 V and gel images captured by the Gene-Genius Bio-Imaging-System (Syngene, Frederick, MD) using the GeneSnap software (Syngene).

2.5.7. Transformation of *E. coli* with DNA

After the required DNA fragment was amplified and purified, it was phosphorylated using T4 Polynucleotide Kinase (New England Biolabs, Hertfordshire, UK) according to the manufacturer's instructions. Plasmid

pSK5487 was used as a vector for cloning and was digested with *BstBI* restriction enzyme (New England Biolabs, Hertfordshire, UK) according to the manufacturers' instructions in 50 µL volumes. Plasmid was then subjected to dephosphorylation using Antarctic Phosphatase (New England Biolabs, Hertfordshire, UK) according to the manufacturer's instructions. Both the plasmid and the DNA fragment were incubated with T4 DNA Ligase (New England Biolabs, Hertfordshire, UK) overnight to achieve ligation according to the manufacturer's instructions.

An aliquot (2-4 µL) of the ligation reaction was used for transformation of XL10-Gold competent cells (Agilent Technologies, California, USA) following the manufacturer's transformation protocol. Volumes (100 µL) of the transformed cells were spread onto LBA with 100 µL/mL ampicillin, and incubated at 37°C. After overnight incubation, several colonies were screened for the presence of the insert using colony PCR. Once the colonies with the right insert were found, these were picked for plasmid extraction followed by transformation into *S. aureus* RN4220 competent cells by electroporation.

2.5.8. Preparation and transformation of *S. aureus* competent cells

S. aureus RN4220 electrocompetent cells were prepared and transformed according to a previously defined method with some modifications (Lofblom et al., 2007). Briefly, overnight cultures in 10 mL TSB supplemented with 2.5% yeast extract was diluted to an optical density of ~0.5 at OD_{578} in 250 mL fresh TSB media (2.5% yeast extract), then incubated at 37°C to an OD_{578} of ~0.6. Once the required OD was reached, the culture was chilled on ice for 10 min and the cells harvested at 4000 x *g* for 10 min and resuspended in an equal

volume of ice-cold water. Cells were then centrifuged as previously and resuspended in 1/10th, then in 1/25th and finally in 1/200th of the original volume of ice-cold sterile 10% (w/v) glycerol. Finally, aliquots (50 µL) of competent cells were stored at -80°C.

The electroporation experiment required the competent cells to be thawed on ice for 5 min and left at room temperature for another 5 min. Then, the cells were centrifuged at 5000 x *g* for 1 min and resuspended in 50 µL of 10% (w/v) glycerol (500 mM sucrose solution). After that 5 µL of the required DNA (≥1 µg/mL) was added to the cells and transferred into a 1-mm electroporation cuvette (Geneflow, Elmhurst, UK), and followed by pulsing at 21 kV/cm, 100 Ω, and 25 µF in a GenePulser XCell Electroporation System (Bio-Rad). Finally, cells were mixed with 1 mL TSB (500 mM sucrose) and incubated at 37°C for 1 h followed by plating out onto TSA plates containing the appropriate antibiotic overnight at 37°C. Putative transformants were then subjected to a confirmation process to check the presence and expression of the correct construct, which included: PCR for genes of interest or diagnostic restriction digest, MIC to confirm the resistance phenotype, and DNA sequencing. For the transformation using total genomic DNA from strain NRS384, 5 µL (≥1 µg/mL) of the genomic DNA (prepared according to section 2.5.1.) of NRS384 was used to transform RN4220 electrocompetent cells following the same electroporation protocol.

2.5.9. Bacteriophage propagation and transduction

Bacteriophage propagation and transduction were performed using either Φ11 or Φ80 bacteriophage according to previously published protocols with some modifications (Foster, 1998, Olson, 2016). First bacteriophage was

propagated in the donor (resistant) strain by growing an overnight culture in phage broth (20g/L Oxoid nutrient broth No. 2). Culture was diluted by adding 20 µL culture to 2 mL phage broth supplemented with 10 mM calcium chloride ($CaCl_2$). An aliquot (200 µL) of bacteriophage particle solution (neat, 10^{-1},10^{-2}, 10^{-3}) was added to 300 µL cells and left at room temperature for 30 min. The mixture was then mixed with 10 mL molten phage top agar (phage broth and 3.5 g/L oxoid agar No.1) at 45-50°C supplemented with 10 mM $CaCl_2$, and immediately poured over two phage base agar plates (phage broth and 7g/L oxoid agar No.1) supplemented with 10 mM $CaCl_2$. The plates were then incubated at 37°C overnight base down in a sealed bag. After the incubation, the surface of the plates with the highest dilution that gave confluent lysis was scraped to collect the phage top agar layer into a centrifuge tube and centrifuged at 5000 x *g* for 10 min. The phage containing supernatant was removed and filtered twice through 0.45-micron filters (Millex-GV) and then stored at 4°C.

The bacteriophage was transduced into recipient bacteria (RN4220) by growing culture of RN4220 in 20 mL TSB in 250 mL flask overnight at 37°C. Cells were then harvested at 5000 x *g* for 10 min and resuspended in 1 mL TSB. Samples (500 µL) of cells and 500 µL bacteriophage particles were incubated at 37°C in 1 mL LBB supplemented with 10 mM $CaCl_2$ for 25 min in a static water bath and then at 200 rpm in an orbital shaker with vigorous aeration for 15 min. Cells were then placed on ice and mixed with 1mL cold 0.02 M fresh (filtered) sodium citrate, followed by centrifugation at 5000 x *g* for 10 min. Then, cells were resuspended in 1mL cold 0.02 M sodium citrate and left on ice for 2 h. An aliquot (100 µL) of the cells was spread onto TSA

supplemented with 0.05 % sodium citrate with appropriate antibiotic selection and incubated overnight at 37°C. Thereafter, the putative transductants were re-streaked on TSA plates supplemented with sodium citrate and appropriate antibiotic to ensure the complete loss of phage. Successful transductants were checked for acquisition of the anticipated plasmid/chromosomal marker by standard methods, including PCR for genes of interest and MIC to confirm the transfer of the resistant phenotype.

Here in chapter 4 and 5, the transduction experiment was attempted more than one time with a longer period of antibiotic exposure which might induce the expression of resistance determinants (inducible resistance genes). Therefore, the transduction experiment was conducted as mentioned above, but this time before performing the last step of plating out onto antibiotic selection plates, some conditions were changed. In more detail, when the recipient cells (RN4220) were resuspended in 1ml cold 0.02 M sodium citrate and left on ice for 2 h, the cells were then centrifuged, resuspended and exposed to subinhibitory concentration of antibiotic (16 µg/ml bacitracin or 8µg/mL nitrofurantoin in 1 ml TSB) for 30 min at 37°C. This was followed by centrifugation and resuspension in 1 ml TSB with no antibiotic and incubation for 2 h, then the cells were plated out onto antibiotic selection plates.

2.6 DNA sequencing and bioinformatics analysis

2.6.1. DNA sequencing of PCR products and plasmid constructs

Purified DNA samples were sequenced by Beckman Coulter Genomics (Buckinghamshire, UK) using the appropriate oligonucleotide primers. The

sequencing data was then analysed using Sequencher software (Gene Codes, Michigan, USA).

2.6.2. Whole genome sequencing and bioinformatics

Whole genome sequencing was performed using next-generation sequencing (NGS) technology (Illumina sequencing-by-synthesis method 2x250 paired-end reactions on a MiSeq platform) at Leeds Clinical Molecular Genetic Centre (St. James's Hospital, University of Leeds). The sequences for each strain were obtained, and a *de-novo* assembly was performed to make a scaffold for each genome using CLC Genomic Workbench (CLC Bio., Cambridge, MA, USA). The WGS (Whole genome sequencing) of Dr Louise Kime's *S. aureus* isolates was performed at the Welcome Sanger Institute, UK. The DNA libraries (450 bp insert size) were generated and sequenced using 100 bp paired-end reads on the Illumina HiSeq2000 platform, and then *De novo* genome assembly was performed as previously described (Page et al., 2016, Reuter et al., 2016).

The whole genome for each strain was annotated on RAST (Rapid Annotation using Subsystem Technology) online software [http://rast.nmpdr.org/], and also the closest neighbouring strains for each sequenced strain were identified using RAST (Aziz et al., 2008, Overbeek et al., 2014, Brettin et al., 2015). Furthermore, MAUVE software (The Darling lab, University of Technology, Sydney) was used for multiple alignments of the obtained DNA sequences; in order to identify the differences and similarities between different genomes (Darling et al., 2004, Darling et al., 2010).

Also, the MLST (Multilocus Sequence Typing) approach [https://pubmlst.org/] was conducted to identify the sequence type and the clonal complex for each strain and to determine how closely related they are (Maiden et al., 1998, Urwin and Maiden, 2003, Ibarz Pavón and Maiden, 2009, Patiño et al., 2018). Phyloviz software (Francisco et al., 2009, Ribeiro-Gonçalves et al., 2016) was employed to assess phylogenetic relationships between sequenced isolates using the eBURST algorithm – goeBURST. MEGA software (Tamura et al., 2013, Kumar et al., 2018) was used to construct a neighbour-joining phylogenetic tree based on the sequence identity of 16S RNA. Pfam [https://pfam.xfam.org/] (Finn et al., 2016) and I-Tasser [https://zhanglab.ccmb.med.umich.edu/I-TASSER/] (Zhang, 2008, Yang et al., 2015) searches were used for the prediction of protein domains of silS, while the Clustal Omega online tool [https://www.ebi.ac.uk/Tools/msa/clustalo/] was employed to perform amino acid sequence alignment (Sievers et al., 2011, Li et al., 2015).

Chapter 3 Investigating the prevalence and mechanism of silver resistance in Gram-negative clinical isolates

3.1 Abstract

Silver is used extensively in the prevention of bacterial infection, and there are concerns that its growing use could fuel the emergence of silver resistance, thereby compromising its therapeutic utility. Therefore, this study sought to assess the prevalence of cryptic (unexpressed) silver resistance amongst clinical isolates of Gram-negative bacteria and examine how overt (apparent) silver resistance becomes activated in such strains. Overt silver resistance was not detected in a recent collection of clinical Gram-negative isolates (*n*=444), but a considerable proportion of *Klebsiella* spp. (~58%) and *Enterobacter* spp. (76%) isolates became silver resistant (MIC >128 μg/mL) at high frequency (~10^{-8}) upon silver challenge. This resistance arose as a consequence of single point mutations in *silS* that led to the activation of the Sil system. Conversely, silver resistance was not selected in the remainder of the isolates that lacked the sil operon. In conclusion, while overt silver resistance is uncommon amongst Gram-negative bacteria, cryptic silver resistance is prevalent and can be easily activated in some Gram-negative genera.

3.2 Introduction

3.2.1 History of silver usage in human health

Silver has been employed for millennia for numerous medical applications. It exhibits antimicrobial activity against a broad range of microorganisms and has been an effective agent in the treatment of a variety of infectious and non-infectious conditions (Klasen, 2000a, Alexander, 2009). Furthermore, silver has contributed substantially to the management of wound healing and the treatment of burns (Klasen, 2000a, Alexander, 2009).

Historical records indicate that ancient people used silver preparations to aid the healing of wounds and ulcers (Alexander, 2009). Silver nitrate was also employed medically by the Romans and was referred to in a Roman pharmacopoeia in 69 B.C.E (Hill, 1939, Alexander, 2009). Since then, the medical use of silver has been reported many times (C.E to 1800), primarily to promote wound healing but also to treat epilepsy. In 1852, Doctor James Sims developed a new strategy for the treatment of vesico-vaginal fistulas by introducing silver sutures, and he also employed silver in urinary catheters (Sims, 1884). After that, a German obstetrician, Doctor Carl Crede, made a seminal contribution to the medical use of silver by using silver nitrate eye drops to prevent and treat ophthalmia neonatorium (a chlamydial or gonococcal eye infection known to cause blindness in neonates), which led to a significant (~80%) decrease in blindness among neonates (Schneider, 1984, Klasen, 2000b).

In the 1920s, the US Food and Drug Administration (FDA) approved colloidal silver as an agent for the management of wound healing (Hugo and Russell, 1982, Demling and Desanti, 2001). The use of silver foil and silver nitrate in

burn and wound dressings increased in the early 20th century to promote healing and prevent infections (Shillito, 1929, Kissmeyer, 1936, Lansdown, 2002). However, following the introduction of penicillin in the early 1940s, the usage of silver as an antimicrobial agent diminished (Moyer et al., 1965). This remained the case until the re-emergence of silver nitrate in the 1960s as a cheaper alternative to antibiotics in the prevention of burn wound infections. Moyer *et al.* (1965) found that the use of 0.5% silver nitrate in burn wounds reduced the development of sepsis from 81% to 33% and decreased mortality significantly.

In 1968, silver sulfadiazine (SSD) cream was produced by combining silver nitrate with sulphonamide antibiotics and became the drug of choice for burn management because of its broad-spectrum antibacterial activity and reduced side effects (Fox, 1968, George et al., 1997, Chopra, 2007). SSD has activity against many Gram-positive bacteria, such *S. aureus*, and Gram-negative bacteria such as *P. aeruginosa* (Fox, 1968, Modak and Fox Jr, 1973, Fox, 1977, Wright et al., 1998). It was thought that SSD was the most appropriate therapy for wound infection, as it has the antibacterial action of silver ions without having several complications associated with silver nitrate (Fox, 1977, Lansdown, 2002). However, another study suggested that SSD may not prevent wound infections when applied to burns (Barajas-Nava et al., 2013). In summary, silver has played a major role in medicine and was one of the most effective antimicrobial agents available before the discovery of antibiotics.

3.2.2 Current clinical applications of silver as an antimicrobial agent

Silver is used today for several medical applications, such as burn and wound management, coating catheters and in dental clinics. These applications exploit the agent's antimicrobial activity against Gram-positive and Gram-negative bacteria, viruses and fungi (Slawson et al., 1992, Silver and Phung, 1996). Burn and wound treatment are a critical issue in the healthcare sector because they involve a loss of skin which is the physical barrier that acts to protect the internal tissues from infections (Lansdown, 2002, Edwards-Jones, 2009). Wounds and burns are usually moist and susceptible to infections particularly with bacteria, such as Gram-negative rods (*E. cloacae* and *P. aeruginosa*), *Staphylococci* and *Streptococci* spp. (Lowbury, 1977, Lansdown, 2002). Silver is considered to be the agent of choice for topical treatment of skin infections and wounds (Edwards-Jones, 2009), and the incorporation of silver nitrate in wound dressings has greatly contributed to the effective management of Pseudomonas infections (Moyer et al., 1965, Klasen, 2000b).

There has been increased focus on the improvement of wound dressings over the last 20 years, particularly those used in the treatment of extensive burns, difficult-to-heal wounds, and chronic ulcers. There are various factors to be taken into account, including the patient's comfort, convenience of usage and reduction of the bacterial burden, odour and wound exudate (Lansdown, 2002). Silver dressings (containing silver nanoparticles) have shown improved activity compared to other dressings, as they provide a sustained and slow release of silver ions to the wound bed for up to seven days (Lansdown, 2002, Edwards-Jones, 2009). It has been proven that the application of

nanocrystalline silver dressings decreased the cost of treatment in a burns ward in Australia when compared to silver sulfadiazine/chlorhexidine cream (Fong et al., 2005). The reduction in treatment expenses was due to the decrease in nursing time, use of antibiotics and dressing changes. Therefore, it was suggested that nanocrystalline silver could be applied to large surface area burns with efficacy in reducing infection at a low cost (Fong et al., 2005). Another study showed comparable results for silver nanoparticles when compared to silver sulfadiazine in terms of their antibacterial efficacy and percutaneous absorption (Brandt et al., 2012). The Public Health England (previously known as the Health Protection Agency) rapid review panel awarded a level 1 recommendation for the use of silver coated catheters to prevent urinary tract infections and reduce the duration of hospital stays in patients with short-term catheterization (Seymour, 2006, Edwards-Jones, 2009).

3.2.3 Silver activity and mode of action

Silver has four different oxidative forms: Ag^0, Ag^+, Ag^{++}, Ag^{+++} and it must be in the ionised form to have antimicrobial activity. The most clinically important form is Ag^+; the other states produce complexes that are insoluble or have limited bactericidal activity (Edwards-Jones, 2009). Silver is inert in its non-ionised form and becomes silver cation (Ag^+) once it comes into contact with moisture on the skin surface or interacts with wound fluids. Therefore, all silver-containing products that have biocidal properties provide a source of silver ions, including organic and inorganic silver compounds and metallic silver (Dibrov et al., 2002, Lok et al., 2008, Edwards-Jones, 2009). Silver ions (Ag^+) have a strong binding affinity for sulphur-containing compounds (e.g.

cysteine) and to halides (e.g. Cl, Br). It has been suggested that the antibacterial activity of silver is directly proportional to it is binding affinity to nucleophiles (Liau et al., 1997, Nies, 1999).

The hypothesised major effect related to the antimicrobial action of silver is the perturbation of the cell membrane leading to the loss of cellular integrity and functional capacity, which is associated with leakage of electrolytes, metabolites and nutrients (Woodward, 1963, Jung et al., 2008). Cell membranes play an essential role in maintaining intracellular homeostasis and limiting the influx of toxic substances from the environment (Lansdown, 2010). The increase in cell membrane permeability can lead to the leakage of ions from the cell and imbalances in the exchange and uptake of nutrients, such as glutamine, succinate, mannitol and phosphates (Schreurs and Rosenberg, 1982). Large increases in cell wall permeability and severe pitting in the membrane of *E. coli* were identified upon exposure to silver nanoparticles (Sondi and Salopek-Sondi, 2004). There was a 70% inhibition of bacterial growth due to the penetration of silver ions (Ag^+) through the cell wall and the incorporation of Ag^+ into the cell membrane.

It has been suggested that the loss of membrane integrity is the major and primary bactericidal effect of silver on the bacterial cell (Randall et al., 2013b). Silver can cause 97% membrane damage after just 10 minutes of exposure, which consequently leads to the loss of intracellular components. Thus, the interaction of silver with other cellular components will occur as a result of the internalisation of silver after damaging the membrane. (Randall et al., 2013b).

3.2.4 Bacterial resistance to silver compounds

Silver resistance has been reported in Gram-negative bacteria but not in Gram-positive bacteria. In fact, there are numerous reports of silver resistance in Gram-negative bacteria with several silver resistant bacterial isolates found in clinical and environmental settings (Rosenkranz et al., 1974, McHugh et al., 1975, Annear et al., 1976, Gayle et al., 1978, Bridges et al., 1979, Hendry and Stewart, 1979, Haefeli et al., 1984, Vasishta et al., 1989, Starodub and Trevors, 1989, Deshpande and Chopade, 1994, Ip et al., 2006, Lansdown and Williams, 2007). The exact mechanism of silver resistance was not investigated in most of these cases but was proposed to involve the horizontal acquisition of resistance determinants (exogenous resistance) which might be located on plasmids. On the other hand, it is unlikely for silver resistance to arise in Gram-positive bacteria. This hypothesis was supported by work carried out by Randall *et al.* (2013) where a large collection of *S. aureus* isolates were uniformly found to be susceptible to silver and silver-resistant mutants could not be selected from staphylococcal isolates after 42 d of continuous exposure to silver.

Silver resistance in *Salmonella* Typhimurium isolated from a burns ward was attributed to the 180 kb conjugative plasmid pMG101 that encodes resistance to several antibacterial agents including ampicillin, sulphonamides, streptomycin and mercury (McHugh et al., 1975, Gupta et al., 1999, Silver, 2003). This plasmid confers resistance to silver by reducing the concentration of silver in the periplasmic space through a condition of sequestration and efflux (McHugh et al., 1975, Gupta et al., 1999, Silver, 2003). Another study proposed that a 75 kb non-conjugative plasmid, pKK1, was responsible for

silver resistance in *P. stutzeri* obtained from a silver mine. The plasmid does not confer resistance to any other antibacterial agents, and it encodes a mechanism of intracellular detoxification of silver through enhanced production of sulphur-rich compounds which increases silver sequestration (Haefeli et al., 1984). There are another two plasmids that were identified in Gram-negative bacteria that are believed to confer silver resistance via a detoxification mechanism. These findings need to be further investigated to determine exactly how the genes presented on those plasmids confer silver resistance (Starodub and Trevors, 1989, Deshpande and Chopade, 1994). These plasmids are pUPI199, a 54 kb conjugative plasmid obtained from an environmental isolate of *A. baumannii*, and pJT1, which was identified in a burn wound isolate of *E. coli* (Starodub and Trevors, 1989, Deshpande and Chopade, 1994).

3.2.4.1 Molecular and genetic basis of exogenous (horizontally acquired) resistance to silver

Of the four plasmids recognised to confer resistance to silver, only pMG101 in Gram-negative bacteria has been investigated at a molecular level. The silver resistance determinants inhabit 14.2 kb of that plasmid (Gupta et al., 1999, Silver et al., 2006). Sequencing and functional studies of the genomic components of this plasmid led to the identification of nine ORFs collectively known as the *sil* operon. The *sil* operon is comprised of seven structural genes (*silE*, *silC*, *silF*, *silB*, *silA*, silG and *silP*) and two regulatory genes (*silR* and *silS*) that contribute a two-component regulatory system (Figure 3.1) (Gupta et al., 1999, Silver et al., 2006, Randall et al., 2015, Blanco Massani et al., 2018).

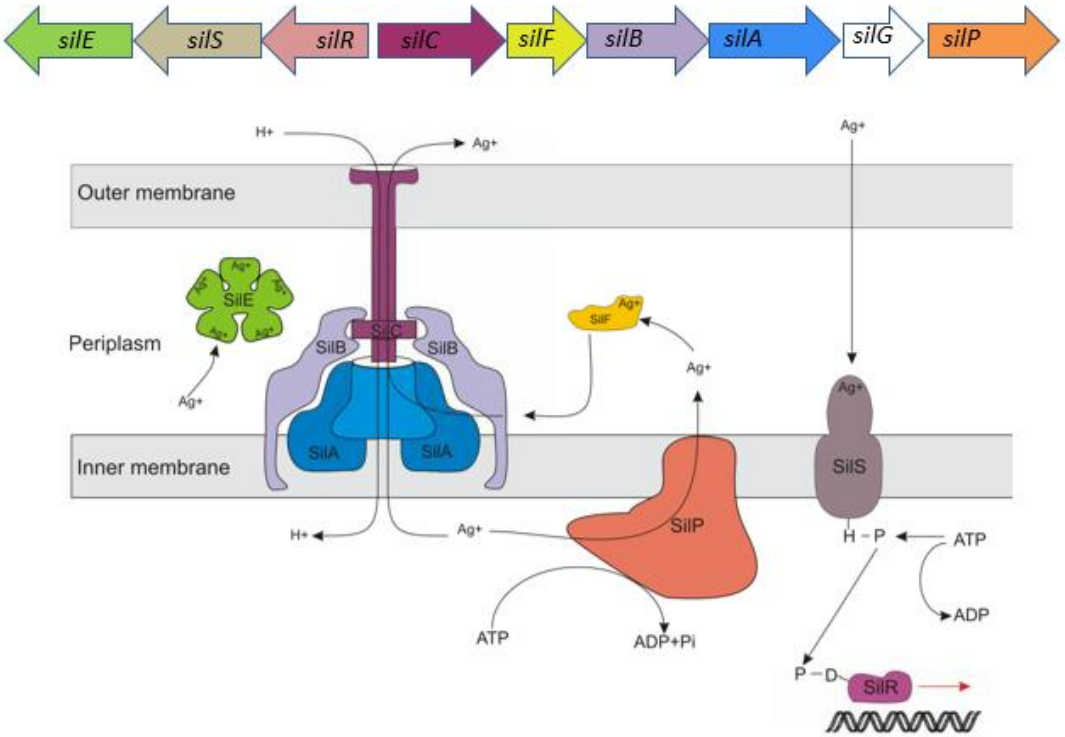

Figure 3.1 The proposed genes and proteins of the Sil system, which are involved in silver resistance in Gram-negative bacteria. Taken from Randall et al. (2015).

Until now, functional studies at the protein level have only been conducted on SilE (Gupta et al., 1999). The functions of the other components of the Sil system are based on amino acid sequence similarity with other heavy metal resistance genes (the Czc and Pco systems). Generally, the proteins of the Sil system are hypothesised to mediate silver resistance through limiting the accumulation of silver in the cell as a result of a combination of active efflux (via SilCBA and SilP transporters) and sequestration of silver (by binding to SilE, SilF and SilG) (Figure 3.1) (Gupta et al., 1999, Silver et al., 2006, Randall et al., 2015). According to a recent study on exogenous silver resistance only SilE, SilF, SilA, SilB and SilC were indispensable for the silver resistance phenotype whereas SilP and SilG were not required (Randall et al., 2015).

The *silR* and *silS* genes encode a two-component regulatory system consisting of a sensor kinase (SilS) and response regulator (SilR) protein (Figure 3.1) (Silver et al., 2006). Although there are no studies on the structure and function of SilRS, the function of these proteins has been allocated depending on their sequence identity with the regulatory systems of other operons conferring resistance to copper, cobalt, zinc and cadmium (Gupta et al., 1999). This sensor-response system is assumed to positively control the expression of the whole *sil* operon in order to regulate silver resistance (Gupta et al., 1999). In addition to that, it has been shown that silver resistance can be easily activated via a single missense mutation in *silS* that induces the constitutive transcription of the *sil* operon (Gupta et al., 1999, Randall et al., 2015).

SilE is a small periplasmic metal-binding protein with the ability to bind to up to 5 silver ions (Ag+) (Figure 3.1). Studies show its specificity in binding to silver ions rather than other heavy metal ions (Gupta et al., 1999, Silver, 2003). SilE likely prevents silver ions from reaching the antibacterial target (the cytoplasmic membrane) by sequestering them as they enter the periplasm (Silver, 2003). The *silF* gene encodes a periplasmic binding protein (SilF), which has sequence similarity to CusF (periplasmic silver and copper-binding protein) (Silver et al., 2006). Therefore, it is likely that both proteins act in the same manner by binding and sequestering silver ions as they reach the periplasm (Silver et al., 2006, Xue et al., 2007, Randall et al., 2015).

The *silCBA* genes are believed to code for a three-protein complex (SilCBA), which acts as a cation antiporter belonging to resistance-nodulation-division (RND)-type efflux transporter (Figure 3.1). SilA has a similar sequence to

AcrB, which is part of the AcrAB-Tol multidrug efflux transporter in *E. coli* (Gupta et al., 1999, Nies, 1999, Randall et al., 2015). SilA is considered to be the inner membrane component of this system, and it acts as a proton/cation antiporter (Silver, 2003, Nikaido, 2009). Furthermore, SilB is the adaptor protein that helps to attach SilA to the outer membrane porin (SilC), which then transports Ag^+ to the outside of the cell (Gupta et al., 1999, Silver, 2003).

3.2.4.2 Endogenous silver resistance via spontaneous mutation

Endogenous bacterial resistance to silver can arise in Gram-negative bacteria from down-regulation or the loss of outer membrane porins and up-regulation of silver ion efflux via the copper/silver transporter, CusCFBA (Liau et al., 1997, Lok et al., 2008, Randall et al., 2015). This kind of resistance was studied in *E. coli,* and it was discovered that in the resistant strains there was a deficiency in the OmpF and OmpC outer membrane porins in addition to overexpression of the CusCFBA transporter (Liau et al., 1997, Lok et al., 2008, Randall et al., 2015). It was shown that endogenous silver resistance could arise through missense mutations in *ompR* and *cusS*, leading to the prevention of the expression of OmpF and OmpC and the constitutive expression of CusCFBA, respectively (Randall et al., 2015). Loss of OmpC and OmpF porins reduces outer membrane permeability, which in turn leads to restricted silver ingress into the cell (Li et al., 1997, Randall et al., 2015). Also, overexpression of the CusCFBA efflux transporter was found to mediate active efflux of silver out of the cell. These phenotypic changes lead to reduced intracellular accumulation of silver and reduced silver susceptibility (confer silver resistance) (Randall et al., 2015).

3.2.5 Introduction to the current study

Taking the extensive use of silver into consideration, questions/assumptions have been raised here about how this can increase the potential for silver resistance of clinical relevance to emerge in bacteria and compromise the therapeutic utility of silver (Chopra, 2007, Randall et al., 2015). As mentioned earlier, there is no evidence of silver resistance in Gram-positive pathogens whereas several studies have already found a low prevalence of silver resistance in Gram-negative pathogens (Davis et al., 2005, Ip et al., 2006, Sutterlin et al., 2012, Randall et al., 2013b, Finley et al., 2015). However, to date, there have been no large-scale studies on the prevalence of silver resistance in clinical isolates of Gram-negative bacteria.

Meanwhile, there are a number of studies that have reported the presence of genes encoding the Sil system components in Gram-negative species that are nonetheless susceptible to silver (Kremer and Hoffmann, 2012, Sutterlin et al., 2014, Finley et al., 2015, Randall et al., 2015). For instance, before the start of this study isolated strains of *K. pneumoniae* and *E. cloacae* were studied in the O'Neill's laboratory (the place where the author conducted this study), and it was found that although they carry the *sil* operon, they do not demonstrate phenotypic silver resistance. However, it was found that cryptic (hidden potential to develop resistance through mutations in the regulatory factors) silver resistance could be readily selected in the laboratory upon silver challenge (Randall et al., 2015). Therefore, the present investigation into the phenomenon of cryptic silver resistance was motivated by the hypothesis that the *sil* operon is not ordinarily expressed in the pathogens that harbour it and

that mutational activation of this system is essential for cryptic silver resistance to manifest.

Here, a large-scale study on the prevalence of silver resistance was conducted using a recent collection of Gram-negative pathogens (representing the skin and soft tissue pathogens where silver is usually applied) isolated from patients from the UK and abroad. Additionally, the potential of Gram-negative pathogens to confer silver resistance (spontaneous resistant mutants) was investigated. Furthermore, the genetic basis of silver resistance in Gram-negative pathogens was studied to confirm the previous suggestion about the role of the Sil system in sliver resistance.

3.3 Results

3.3.1 The nature of the clinical isolates used in this study

All the clinical isolates used here were Gram-negative bacteria obtained from hospitals across the UK and abroad (detailed information provided in Chapter 2) in the period 2012 - 2015. When collecting this panel of strains, the aim was to have a collection representative of the common skin and soft tissue Gram-negative pathogens. Figure 3.2 provides details about the distribution of the 444 bacterial isolates used in this study.

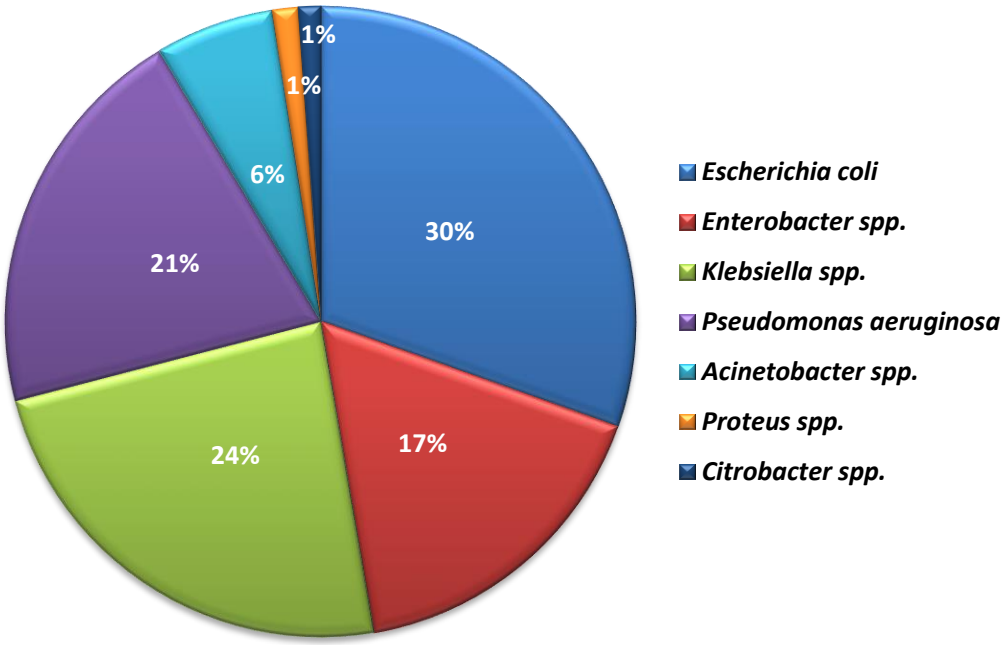

Figure 3.2 Distribution of the clinical isolates used in this study. The numbers shown in each section of the pie chart are the percentage for the presence of each bacterial genera in relation to the total number of isolates (*n*=444).

3.3.2 Detecting the susceptibility of this panel of strains to silver

All the Gram-negative bacterial isolates used in this study (*n*=444) were examined for their susceptibility to silver nitrate by agar dilution. The MIC value for each isolate represented an average (mode) of the MIC of three replicates. The negative control used in this susceptibility determination was the silver susceptible strain *E. coli* BW25113 (MIC 4 µg/mL), and the positive controls were the silver resistant strains *E. cloacae* ATCC 13047 and *E. coli* 50110 J53-(pMG101) (MIC >128 µg/mL). The MIC of silver for all isolates ranged from 1-8 µg/mL (MIC_{50} of 4 µg/mL, MIC_{90} of 8 µg/mL) (Table 3.1).

Table 3.1 Summary of silver nitrate MIC ranges against test organisms used in this study (*n*=444)

Bacterial Strain	MIC Range (µg/mL)
***Enterobacter* spp.**	2-8
***Klebsiella* spp.**	4-8
***Acinetobacter* spp.**	1-4
P. aeruginosa	1-8
E. coli	2-8
***Citrobacter* spp.**	1-4
***Proteus* spp.**	1-4

3.3.3 Attempts to select for silver resistance

While all tested strains were susceptible to silver, the ability to evolve resistance under silver selection was investigated. The selection of resistant mutants does not always indicate a cryptic resistance mechanism but here the ability to yield silver resistant colonies upon silver challenge was investigated to detect cryptic silver resistance. The whole panel of strains was screened to select for silver resistance by plating saturated cultures onto agar containing silver nitrate at 128 µg/mL. A total of 119 isolates (76% of isolates of *Enterobacter* spp., ~58% of isolates of *Klebsiella* spp. and ~0.7% of isolates of *E. coli*) selected silver resistant mutants after being exposed to silver (Figure 3.3). In contrast, resistance to silver could not be selected under these conditions in any of the other genera tested. All the silver resistant mutant isolates (*n*=119) were confirmed as resistant to silver by performing susceptibility tests against silver nitrate for three resistant colonies for each isolate. In each case, MICs were > 128 µg/mL.

To identify how easily silver resistant mutants can emerge and compromise the silver treatment regimen, the frequency with which silver resistant mutants arose was subsequently measured in a subset ($n = 60$) of strains that were able to develop resistance to silver. Mutation frequencies to silver resistance in all 60 isolates were similar (ranging from $\sim 1.5 \times 10^{-8}$ to $\sim 5.2 \times 10^{-7}$). Again, all the mutant isolates were found to be resistant to silver nitrate concentrations >128 µg/mL by picking three resistant colonies for each strain and determining MICs for silver nitrate.

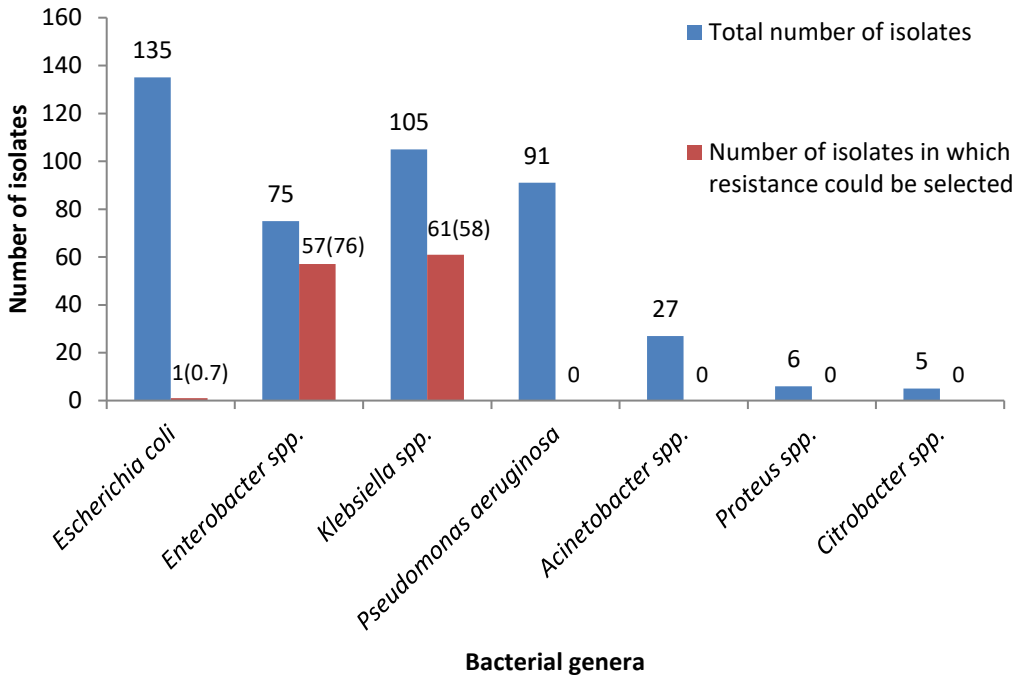

Figure 3.3 Overview of the distribution of the isolates which were able to develop resistance to silver across different bacterial species. The red bars show the number of strains that developed resistance in each bacterial genus and the number in brackets next to it is the percentage (%) in relation to the total number of isolates in that bacterial genera.

3.3.4 Determining the genetic basis of silver resistance in resistant mutants with MIC of >128 µg/mL

High-level silver resistance in these isolates was considered to likely result from mutational activation of a cryptic Sil system. To test this, colony PCR was used to screen for *silS* in the first instance, as a marker for the presence of the Sil system. In all 119 isolates in which spontaneous resistance to silver could be selected, *silS* was detected. Moreover, the parental strains for all 119 isolates were found to carry *silS*. Figure 3.4 gives an example of the gel electrophoresis photos obtained after performing PCR to detect *silS* for a number of the resistant mutants and their parental strains. The negative

control is *E. coli* BW25113 (silver susceptible strain), and the positive control is *E. cloacae* ATCC 13047 (silver resistant strain).

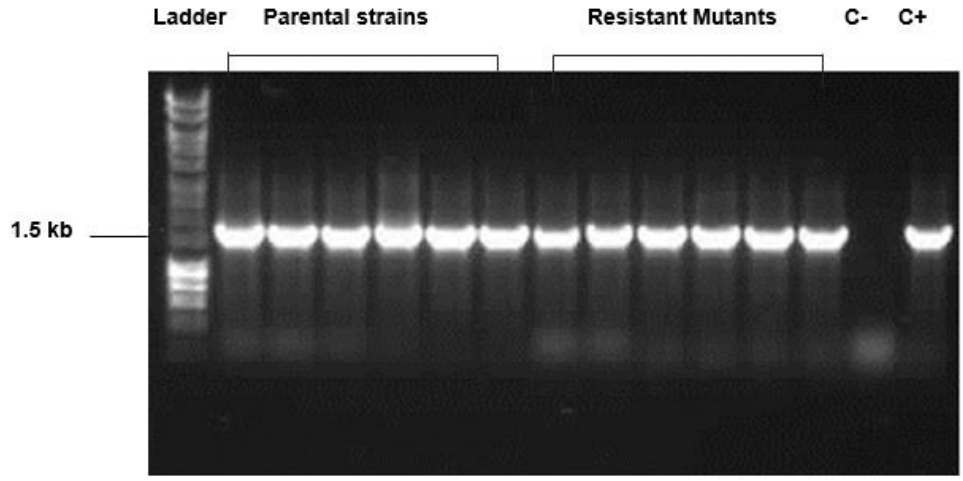

Figure 3.4 Photo of gel electrophoresis of colony PCR to detect *silS* (1.5 kb) performed for six parental and six silver resistant mutant strains of *K. pneumonia* and *E. cloacae* (C+ and C- are positive and negative controls, respectively).

To establish if the presence of *silS* in an isolate is synonymous with the ability to evolve silver resistance by spontaneous mutation, a cross section (*n*=30) of the remaining 325 isolates for which silver resistance could not be selected was screened for the presence of *silS, silR* and *silE* by colony PCR. The detection of more than one *sil* genes was employed in this experiment just to rule out that the Sil system would be harboured in those isolates but not expressed. None of these *sil* components was found in this panel of isolates from which silver resistant mutants could be not recovered.

To assess whether silver resistance phenotype in these isolates was likely the result of mutational activation of a cryptic Sil system, *silS* sequences were analysed to check for mutations. DNA sequence of purified PCR fragments of

the entire *silS* gene from a selection of *silS*$^{+}$ isolates (*n* = 22) and from a single silver resistant mutant recovered from each isolate were sent for sequencing to Beckman Coulter Genomics. The *silS* sequencing data for each susceptible parental strain was aligned with the *silS* sequence of the corresponding resistant mutant using Sequencher 4.8 software. Comparison of *silS* sequences from silver resistant mutants and their respective silver susceptible parent (*n*=22) revealed nonsynonymous mutations in each mutant suggesting that in all isolates, silver resistance has arisen as a consequence of nonsynonymous mutations in *silS* leading to amino acids substitutions in SilS (Figure 3.5).

The SilS protein is a sensor histidine kinase (SHK) of a two-component regulatory system that works to positively regulate the *sil* operon via phosphorylation of the transcription factor, SilR (Silver, 2003). Although mutations in *silS* are known to confer silver resistance by activation of Sil system expression, the mechanism by which this occurs is unclear. Therefore, PFAM and I-TASERR searches were employed to predict the location and extent in SilS of functional domains that are common to SHK proteins, and this information was used to produce a schematic of SilS onto which all of the substitutions identified in this study were mapped (Figure 3.5) (I-TASSER, Pfam, Zhang, 2008, Yang et al., 2015, Finn et al., 2016). This analysis revealed that the substitutions leading to activation of SilS are clustered within three functionally distinct regions: the transmembrane domains (TM), the dimerization/histidine phosphotransfer (DHp) domain, and the catalytic/ATP-binding (CA) domain (Figure 3.5). The amino acid substitutions shown were

all detected in single mutants, except for A13V and G210E, which were found in mutants selected from two and three independent isolates, respectively.

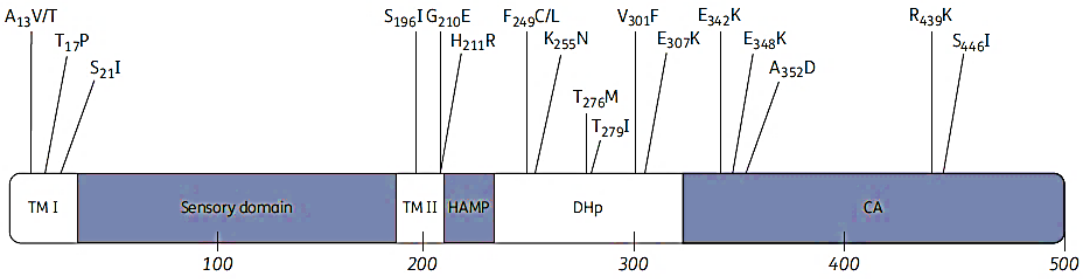

Figure 3.5 Schematic of the SilS protein showing the predicted domains architecture by comparison with other SHK proteins and indicating the sites of amino acid substitutions that activate the cryptic Sil system. TM, transmembrane domain; HAMP, histidine kinase/ adenylyl cyclase/ methyl-accepting/phosphatase domain; CA, catalytic/ ATP-binding domain.

With exception to their sensory domain, sensor histidine kinases (SHKs) share a highly conserved structure. Therefore, comparison of SilS with more extensively studied SKs could provide some insight into how these mutations lead to activation of the Sil system. Though SilS is itself not well studied, SHK proteins as a class have been extensively investigated, offering the opportunity to understand the effect of mutational changes in the SilS protein by analogy. Sequence alignment of SilS with other sensor kinases (EnvZ and NtrB) using Clustal Omega revealed that several mutations identified in this study match or are close to the mutations found in these sensor kinases (Figure 3.6) (EMBL-EBI, Sievers et al., 2011, Li et al., 2015). Substitutions identified in the DHp and CA domains are located at similar positions to those found in mutants of the SHKs EnvZ and NtrB that exhibit constitutive activation as a consequence of being unable to dephosphorylate their cognate response

regulator; suggesting that this is also the case for these SilS protein mutants (Huynh et al., 2013). By contrast, the substitutions found in the transmembrane regions adjacent to the sensory domain likely prompt constitutive activation of the Sil system by uncoupling signalling from sensing (Hsing et al., 1998).

Figure 3.6 Alignment of DHp domain sequences of SilS, EnvZ and NtrB. DHp domain has the H box and X box (boxed in black), residue boxed in red has a catalytic activity and residue boxed in green has a role in determining the specificity of DHp domain interaction. These two residues are the ones that match those in EnvZ and NtrB.

3.3.5 Selection of silver resistant mutants via extended gradient MIC

Failure to select for spontaneous silver resistance mutants in some of the *Klebsiella* spp. and *Enterobacter* spp. isolates led to the adoption of an alternative method to attempt to select for silver resistance. To further examine the potential for the development of silver resistance, 40 isolates (*Klebsiella* spp. and *Enterobacter* spp. isolates in which silver resistance was not identified by spontaneous mutation), were continuously exposed to silver nitrate in the form of serially-repeated broth microdilution experiments. The repeated exposure to silver nitrate was continued for 21 d or until resistant mutants were detected by an increase in their silver MIC (≥ 4X original MIC). Only 7 isolates of 40 strains tested produced silver resistant mutants after 3 d of passaging, and these exhibited an increase in silver MIC from 4 or 8 µg/mL to 32 µg/mL. For the rest of the isolates, no change in silver MIC was detected by 21 d of passaging. For those 7 isolates; silver MICs did not increase any

further (32 µg/mL) even after plating on agar plates containing a higher concentration of silver nitrate (128 µg/mL).

The possibility that the Sil system was involved in this kind of resistance was first investigated. Colony PCR was employed to screen for the *silS* (1.5 kb) gene, an amplification target was chosen as a representative marker for the presence of the Sil system. In all seven isolates, *silS* was not detected (Figure 3.7). To further confirm that the Sil system is not present in these isolates, the presence of other components of the *sil* operon (*silR* and *silE*) was also checked by colony PCR; again, none of these genes was detected.

Figure 3.7 Photo for gel electrophoresis of colony PCR for detecting *silS* (1.5 kb) performed for seven silver resistant mutants selected by extended gradient MIC (MIC 32 µg/ml) and four silver resistant spontaneous mutants (MIC 128 µg/ml) of *Klebsiella* spp. and *Enterobacter* spp. *silS* was not detected in those mutants with MIC of 32 µg/ml (C+ and C- are positive and negative controls).

3.4 Discussion

Given the increasing use of antimicrobial silver over the past years, concerns have been raised over the potential for silver resistance to arise and persist in bacterial species of clinical relevance. Therefore, it was important to assess the prevalence of silver resistance in a collection of clinical isolates comprising the most significant Gram-negative genera associated with skin and soft tissue infections, a condition where silver is commonly applied (Figure 3.2) (Dryden, 2010, Fernandes and Prudêncio, 2010).

The MIC values presented here were similar to those seen for silver-susceptible laboratory strains of *E. coli* (MICs of 4–8 µg/mL) and are substantially lower than those observed for known silver-resistant strains (MICs of >128 µg/mL) (Randall et al., 2015). In the absence of clinical breakpoints and when using the susceptibility testing methodology employed here, an ECOFF point of ≤ 8 µg/mL can be proposed to distinguish Gram-negative strains whose response to silver is wild type from those that express a silver resistance mechanism (Kahlmeter et al., 2003). Consequently, all the isolates in this study were considered to be silver-susceptible. These data corroborate previous reports that overt (apparent) silver resistance is rare amongst Gram-negative pathogens (Ip et al., 2006, Finley et al., 2015).

Although all tested strains were susceptible to silver, the ability for resistance to develop upon silver challenge was investigated (Figure 3.3). The frequency with which silver resistant mutants arose reveals that resistant mutants can be easily recovered at a rate sufficiently high to imply that resistance was the result of a single mutational event in each case (O'Neill and Chopra, 2004).

Within the same context, spontaneous mutants to silver resistance have been observed previously in Enterobacteriaceae harbouring the Sil system (Randall et al., 2015). This suggests that for *Klebsiella* spp*.* and *Enterobacter* spp*.* infections, silver resistance could easily arise during treatment and may cause treatment failure in patients. However, it is reassuring that silver resistance could not be selected under these conditions in any of the other genera tested (Figure 3.3). These observations are in line with findings in the literature, which have reported how difficult it is to select mutants resistant to silver in some bacterial pathogens (Randall et al., 2013b).

A proportion of *Klebsiella* spp*.* and *Enterobacter* spp*.* failed to become silver resistant upon silver challenge. To assess whether these strains nonetheless possess the potential to become silver resistant, an alternative method was employed in an attempt to select resistance, which resulted in the development of mutant strains with slightly reduced susceptibility to silver (MIC of 32 µg/mL). PCR-based detection revealed the absence of the *sil* operon in those *Klebsiella* spp*.* and *Enterobacter* spp*.* strains, implying that reduced susceptibility is mediated through other means. Potentially, and as reported for *E. coli*, reduced susceptibility can arise through upregulation of native metal transport systems (Randall et al., 2015). Therefore, whole genome sequencing would provide an option to investigate the genetic basis for this phenotype change in future studies. Conclusively, the failure to select for silver resistance in a number of *Klebsiella* spp*.* and *Enterobacter* spp*.* strains after prolonged exposure to silver indicates that the emergence of silver resistance in some *Klebsiella* spp*.* and *Enterobacter* spp*.* by an endogenous mechanism

in clinical settings can occur, but less readily than resistance through activation of the Sil system.

Silver resistance has previously been reported to arise in two *sil*+ isolates as a consequence of nonsynonymous mutations in *silS* (Randall et al., 2015). This gene encodes a putative sensor kinase and is part of the SilRS two-component system that regulates expression of SilCFBAGPE. The observed mutations result in constitutive activation of SilCFBAGPE expression, which confers a silver resistance phenotype (Gupta et al., 1999, Randall et al., 2015). Therefore, to determine the genetic basis for silver resistance in the resistant mutants recovered, this research proceeded on the basis that the silver resistance phenotype in these strains was likely the result of mutational activation of a cryptic Sil system. Accordingly, *silS* was chosen to identify the presence of the *sil* operon in the isolates under study. Both *silS* and *silR* are assumed to control the expression of the whole *sil* operon in order to regulate silver resistance (Gupta et al., 1999, Randall et al., 2015). The *silS* gene was detected in all 119 isolates in which spontaneous resistance to silver could be selected (Figure 3.4). However, the components of the *sil* operon was reported to be absent in non-resistant *Klebsiella* spp. and *Enterobacter* spp. strains. Consequently, it appears that silver resistance in the resistant mutants is attributable to the activation of the Sil system, which could occur as a result of mutational change in *silS* as previously reported.

After that, the next step was to ascertain if the presence of *silS* in an isolate is actually synonymous with its ability to evolve silver resistance by spontaneous mutation. Analysis of the sequencing data of *silS* revealed a single missense mutation in each resistant mutant at a variety of sites along *silS*, when the

sequences of the parental and silver resistant mutant strains were aligned to each other to find out if there was any mismatch among them. Although mutations in *silS* are known to confer silver resistance by dysregulation of the Sil system expression, the mechanism of dysregulation is not exactly known (Gupta et al., 1999). The hypothesis is that following a sensory stimulus, SHKs undergo a conformational change that enables phosphotransfer through a conserved histidine residue onto a conserved aspartate on the cognate response regulator (RR, i.e. SilR in the case of SilS) (Keener and Kustu, 1988, Stewart, 2010). The phosphorylated RR (phos-RR) then acts as a transcription factor modulating the expression of associated regulons. In the absence of sensory stimulus, a number of SHKs switch to a 'phosphatase' state and dephosphorylate any phosphorylated RR (Keener and Kustu, 1988, Stewart, 2010). An important next step in this work will be to generate a reporter construct in *E*. *coli* using the first part of the sil operon and clone the *silS*, which has the mutation, into it to further confirm that changes in the Sil system are responsible for silver resistance in Gram-negative pathogens.

The polymorphisms identified in SilS from silver resistant strains appear to fall within three putative domains of SilS (Figure 3.5). Mutations found in the transmembrane (TM) domain adjacent to the sensory domain may prompt constitutive activation of the Sil system by uncoupling signalling from sensing (Hsing et al., 1998). In contrast, there were no substitutions found in the sensory domain. If there were any, these substitutions were likely lead to conformational changes that are similar to those that occur in this domain (sensory domain) following stimulus, presumably leaving the SilS stuck in a 'kinase' state and enabling constitutive phosphorylation of SilR (Hsing et al.,

1998, Salvi et al., 2017). In the dimerization and histidine phosphotransfer (DHp) domain, several mutations are clustered around the conserved H- and X-boxes. The H-box contains a conserved threonine or asparagine essential for catalysing dephosphorylation of the RR, whereas the X-box is required for the interaction between the SHK and phos-RR prior to dephosphorylation (Willett and Kirby, 2012, Huynh et al., 2013). In the SHKs of EnvZ, NtrB and PhoB, several mutations conferring a 'phosphatase minus' phenotype have been identified either as a consequence of catalytic inactivation (e.g. H-box associated) or deficiencies in SK and phos-RR interaction (X-box associated) (Willett and Kirby, 2012, Huynh et al., 2013). Sequence alignment of SilS with EnvZ and NtrB using Clustal Omega revealed several mutations identified in this study match or are close to these previously identified mutations. Indeed, in one mutant, the putative catalytic residue for dephosphorylation ($E_{307}K$) underwent substitution, strongly suggesting that loss of phosphatase activity in SilS can lead to Sil system activation and silver resistance. Furthermore, the CA domain substitutions $R_{439}K$ and $S_{446}I$ are located close to substitutions found in the SK PhoQ, known to affect the binding of ADP required to accept phosphate during dephosphorylation, again leading to a phosphatase-minus phenotype (Yeo et al., 2012, Huynh et al., 2013).

It is not clear why cryptic silver resistance is far more prevalent than overt resistance - is it perhaps a consequence of fitness costs *in vivo* that limit the ability of strains to arise, spread and persist? To address this question, future studies should examine the fitness costs of silver resistance. It is known that fitness costs in bacteria can play a pivotal role in the dissemination of antibiotic resistance, especially under trace or no antibiotic conditions (Andersson,

2006, Palmer and Kishony, 2013). Resistance conferred by spontaneous mutation can have pleiotropic effects (such as a reduction in growth, virulence, or transmission) which in turn impart a fitness cost (Melnyk et al., 2015). Thus, the fitness cost of the resistant strains identified here could be measured through growth rate determinations or via competitive fitness experiments that put the resistant organism against the susceptible strain in mixed culture. Sensitive strains that do not have to compensate the expense of resistance should therefore substitute the resistant strains at a rate corresponding to the extent of the expense imposed by resistance (Schulz zur Wiesch et al., 2010, Lin et al., 2018).

Chapter 4 Bacitracin resistance in *S. aureus*: definition, prevalence, and genetic basis

4.1. Abstract

Bacitracin is used to treat superficial staphylococcal infections, though little information exists regarding the prevalence and genetic basis for bacitracin resistance. This study investigated bacitracin susceptibility among clinical isolates of *S. aureus* (*n*=1138) and sought to identify and further characterise instances of bacitracin resistance. About 97% of *S. aureus* strains tested had MICs of ≤256 µg/mL towards bacitracin, with the remainder (~3%) associated with bacitracin MICs of ≥512 µg/mL. Whole genome sequencing of representative strains belonging to the latter group identified the presence of the *bcrABRS/bacA* locus, a bacitracin resistance determinant previously found in enterococci. This locus was shown to be responsible for resistance in those *S. aureus* isolates belonging to the same group. An ECOFF point was proposed for 'true' bacitracin resistance of 512 mg/L, indicating that only ~3% of the isolates tested here were resistant. Although bacitracin resistance is not prevalent in *S. aureus* at present, the fact that resistance is conferred by a genetic cluster that is located on a mobile genetic element suggests that further dissemination of this resistance is likely in the future.

4.1 Introduction

Bacitracin is a branched cyclic dodecyl peptide antibiotic that belongs to a group of related cyclic polypeptides produced by *Bacillus licheniformis* and some strains of *B. subtilis* (Figure 4.1) (Ming and Epperson, 2002, Economou et al., 2013). Bacitracin is an effective antibiotic when used topically and has been used for years to prevent and treat wound and soft tissue infections including skin as well as ophthalmic infections (Johnson et al., 1945, Azevedo et al., 1993, Ishihara et al., 2002, Butaye et al., 2003). Bacitracin is considered to be toxic if used systemically, and its absorption from the gastrointestinal tract is not appreciable. Consequently, this antibiotic is deployed mainly as a topical agent for treating superficial infections (Bywater et al., 2005, Pavli and Kmetec, 2006, Howard, 2007).

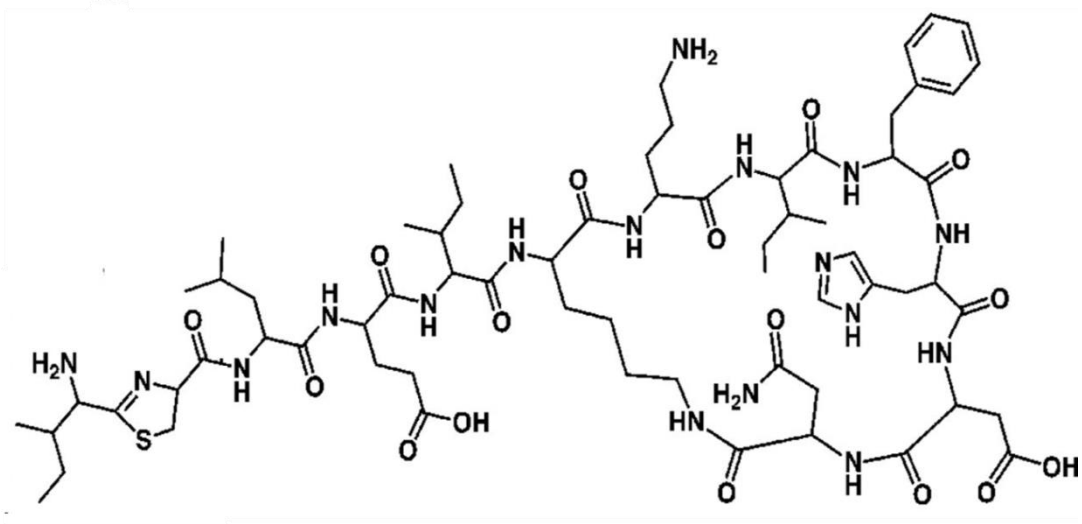

Figure 4.1 Chemical structure of bacitracin. Taken from (Economou et al., 2013)

Bacitracin biosynthesis occurs non-ribosomally through a large multienzyme complex (peptide synthetase) named bacitracin synthetase ABC (BacABC), which is encoded by a biosynthetic operon comprising of three genes: *bacA*, *bacB*, and *bacC* (Frøyshov, 1977, Adimpong et al., 2012). A number of pharmacologically active peptides including siderophores, penicillin and vancomycin are produced non-ribosomally similarly to bacitracin, and they all represent a large part of the bioactive secondary metabolites produced by bacteria and fungi (Eppelmann et al., 2001). The biosynthesis process involves the incorporation of substrates like amino acids, hydroxyl and carboxylic compounds into the peptide chain that results in extraordinary structural diversity, which leads to the enormous variety of biological properties of this group of natural products. Additionally, further modification can happen to the intermediates produced through the process of biosynthesis by epimerisation, glycosylation, acylation, heterocyclization, or *N*-methylation (Marahiel et al., 1997, Doekel and Marahiel, 2001). In the case of bacitracin, the multienzyme complex BacABC activates the incorporation of its substrate amino acids (isoleucine and cysteine) using ATP hydrolysis. It also, promotes ring formation between isoleucine and cysteine, chain elongation through peptide bond formation and liberation of the peptide chain (Konz et al., 1997, Murphy et al., 2007).

4.1.1 Antibacterial mechanism of action of bacitracin

Bacitracin is actually a mixture of constituents with varying degrees of antimicrobial activity (A, A1, B, B1, B2, C, D, E, F, G, and X), of which bacitracin A is the most clinically significant form. It is known that bacitracin B and B2 are both potent and roughly 90% as active as bacitracin A, but the

other compounds have not been thoroughly studied (Drablos et al., 1999, Butaye et al., 2003, Economou et al., 2013).

The best-characterised biological effect of bacitracin is the inhibition of bacterial cell wall biosynthesis. The mechanism of action involves bacitracin binding to the C55-isoprenyl pyrophosphate IPP (undecaprenyl pyrophosphate), which is mediated by metal ions such as Zn (II) (Storm and Strominger, 1974, Kaemmerer and Kietzmann, 1980). C55-isoprenyl pyrophosphate IPP is the lipid carrier for the peptidoglycan precursors from the cytosol to the extracellular surface of the cytoplasmic membrane. During the transport process, this lipid carrier acquires an extra phosphate group, and dephosphorylation of this carrier is required to revert it to its original state and allow a new cycle of transport (Storm and Strominger, 1974, Drablos et al., 1999). Bacitracin binds to C55-isoprenyl pyrophosphate IPP and inhibits its dephosphorylation, thereby inhibiting recycling of the carrier and eventually inhibiting peptidoglycan biosynthesis (Siewert and Strominger, 1967, Stone and Strominger, 1971, Toscano and Storm, 1982).

4.1.2 Medical applications of bacitracin

Bacitracin has a relatively limited human therapeutic application given that its usage is largely restricted to topical application. However, it has also been used extensively as a feed supplement and growth promoter for livestock and poultry to prevent and treat bacterial disease. In Europe, such bacitracin usage has now been banned, and deployment of this antibiotic is restricted to humans (Barton, 2007, Phillips, 2007, McNamee et al., 2013).

Bacitracin exerts antibacterial action *in vitro* against a range of Gram-positive and some Gram-negative bacteria. Bacitracin has a pronounced activity against staphylococci and also it has limited success eliminating vancomycin-resistant enterococci, but bacitracin is generally used to treat superficial staphylococcal infection (Armstrong-Evans et al., 1999, Husain, 2004, Zhang et al., 2012). Bacitracin, in the form of zinc salt, is usually used in combination with polymyxin B, neomycin and some corticosteroids for the treatment of various skin and eye infections, and for the control of wound infections. It is commonly available as an ointment and also as eye drops for ophthalmic use (Jacob MD and James MD, 2004, Sheikh et al., 2012, Pass and Rattner, 2014, Gografe et al., 2015).

4.1.3 Resistance to bacitracin

Several mechanisms of bacitracin resistance have been reported in bacteria, especially amongst bacitracin producer species (Cain et al., 1993, Chalker et al., 2000, Bernard et al., 2005, Charlebois et al., 2012, Chen et al., 2016, Radeck et al., 2016). Generally, resistance to this antibiotic is based on the presence of ABC (ATP-binding cassette) transporters which are believed to mediate active efflux of bacitracin out of the cell. The best-studied of these transporters is the *B. subtilis* BceAB system (Ohki et al., 2003, Bernard et al., 2007, Radeck et al., 2016). Another system has been detected in *S. aureus* with a two-component system (TCS) named BceRS that senses bacitracin, and regulates the expression of BceAB (ABC transporters) in response (Rietkötter et al., 2008, Yoshida et al., 2011). BceA compromises the nucleotide binding domain, and BceB is the membrane spanning domain of

the ABC transporter (Ohki et al., 2003, Ouyang et al., 2010, Yoshida et al., 2011).

Interestingly, another ABC transporter mediating bacitracin resistance found in *B. licheniformis* is encoded by the *bcrABC* genes (Podlesek et al., 1995). A homologue of this transporter encoded by the *bcrABD* genes is also found among bacitracin-resistant strains of *E. faecalis* (Manson et al., 2004). The genes encoding this transporter have been found both on the chromosome and on plasmids (Podlesek et al., 2000, Charlebois et al., 2012, Han et al., 2015). Based on the amino acid sequence homology BcrA (nucleotide binding domain) and BcrB (membrane spanning domain) constitute an ABC transporter. Expression of the genes encoding this transporter is regulated by the proteins encoded by *bcrS* and *bcrR,* which are predicted to constitute a two component regulatory system (Podlesek et al., 1997, Neumuller et al., 2001, Manson et al., 2004, Han et al., 2015). An additional gene found in the same locus is *bacA* (namely *bcrD* or *bcrC* according to some studies), exhibits homology to undecaprenol kinases and was first reported to mediate bacitracin resistance in *E. coli* (Cain et al., 1993). The BacA protein leads to increased abundance of the cellular amount of the lipid carrier, causing a reduction in susceptibility to bacitracin in *S. aureus* and *S. pneumoniae* (Chalker et al., 2000).

Similar transporters have been found to confer resistance to bacitracin in *S. mutans*, encoded by the *mbrABCD* genes (Tsuda et al., 2002, Kitagawa et al., 2011). Interruption of *mbrA* and *mbrB* genes, which encode the nucleotide binding domain (MbrA) and membrane spanning domain (MbrB) of the ABC transporter, respectively, led to an almost 100-fold increase in bacitracin

susceptibility (Tsuda et al., 2002). The two other genes (*mbrC* and *mbrD*), which encode the response regulator (MbrC) and the sensor kinase (MbrD) of the two-component regulatory system, respectively, are usually present immediately downstream of *mbrAB*. Likewise, interruption of *mbrC* or *mbrD* genes resulted in a reduction in bacitracin resistance comparable to that observed in *mbrA* and *mbrB* mutants (Tsuda et al., 2002).

Another mechanism of bacitracin resistance has been reported in bacteria that secrete exopolysaccharides. Polysaccharide synthesis employs the same lipid carrier that is required for peptidoglycan synthesis and consequently by blocking the synthesis of exopolysaccharides, there is more lipid carrier available for cell wall synthesis, and the cell becomes less susceptible to bacitracin (Pollock et al., 1994, Tsuda et al., 2002). It has been shown that in *S. mutans,* the presence of RGP (rhamnose-glucose polysaccharide) in the cell wall is associated with resistance to bacitracin. Furthermore, inactivation of the *rgp* locus (responsible for the RGP synthesis) leads to enhanced sensitivity to bacitracin (Tsuda et al., 2002).

4.1.4 Introduction to the present study

While the existence of bacitracin resistance in *S. aureus* was first reported in 1949 (Stone, 1949), information is limited regarding the nature of bacitracin resistance in *S. aureus*. Of note, *S. aureus* usually colonises and causes antibiotic-resistant wound and soft tissue infections, and because bacitracin is mostly administered topically, the possibility of development and spread of bacitracin resistance is concerning (Anderson and Kaye, 2009, Rezaei et al., 2011). Additionally, *S. aureus* is an important Gram-positive human pathogen and one of the leading causes of hospital and community-acquired infections.

This is in large part because of its ability to adapt to a wide variety of environments and because it harbours an array of virulence genes that enable it to cause a wide range of infections. These include pneumonia, endocarditis, skin abscesses, meningitis, septicaemia and toxic shock syndrome (Lowy 1998). The difficulty in treating staphylococcal infections has increased the demand to further investigate the genetic basis of antimicrobial resistance in *S. aureus* (Lowy, 2003).

Of note, to date, no large-scale study has assessed the prevalence of bacitracin resistance in *S. aureus* in order to set up an ECOFF point for bacitracin in this organism. Although some studies have been conducted on clinical isolates of *S. aureus*, the number of isolates studied was small, and the genetic basis for resistance was not properly established (Baker et al., 1986, Maple et al., 1989, Suzuki et al., 2011, Bessa et al., 2016). For example, in one study where susceptibility to bacitracin was studied, bacitracin resistance was reported in 100% (*n*=68) of *S. aureus* isolates tested based on historical MIC breakpoints (>256 µg/ml) obtained from (SFM, 2003), which is not a commonly used source to obtain MIC breakpoints from and is also not clear on how this MIC was detected (Bessa et al., 2016). In terms of the mechanism of resistance studies, there is a lack in the information about bacitracin resistance in a number of reports and the *bcr* genes were mentioned but only as genes present in some *S. aureus* strains; whether they confer resistance to bacitracin in this setting has not been established. In those studies, MIC determination for bacitracin was performed sometimes for a couple of strains under study and those strains were considered resistant at different MIC levels (MIC >128 µg/ml and MIC >256 µg/ml) in each study but

again no further investigations were performed (Highlander et al., 2007, Kennedy et al., 2010, Sabat et al., 2017).

Thus, since information about bacitracin resistance in *S. aureus* is limited, it was selected to be one of the antimicrobial agents to be studied in this thesis. Consequently, in this part of the study, the aims were:

- Identify the susceptibility profile for bacitracin in a panel of *S. aureus* clinical isolates which could then be used to establish an ECOFF value for resistance surveillance studies.
- Ascertain the propensity for bacitracin resistance to arise by mutation in *S. aureus* under bacitracin selection.
- Characterise the genes responsible for bacitracin resistance among those clinical isolates and to identify the possibility of the dissemination of this resistance by means of horizontal gene transfer.

4.2 Results

4.2.1 Bacitracin susceptibility testing

This study initially sought to investigate bacitracin susceptibility in a panel of *S. aureus* clinical isolates (*n*=1138) collected from the UK and abroad with a view to providing a comprehensive evaluation of bacitracin susceptibility in this organism. Approximately 97% (*n*=1104) of the isolates tested had a bacitracin MIC of 256 µg/mL or less, with the reminder (~3%, *n*=34) exhibiting lower levels of susceptibility towards bacitracin (MIC range from 512 µg/mL to > 2048 µg/mL) (Figure 4.2).

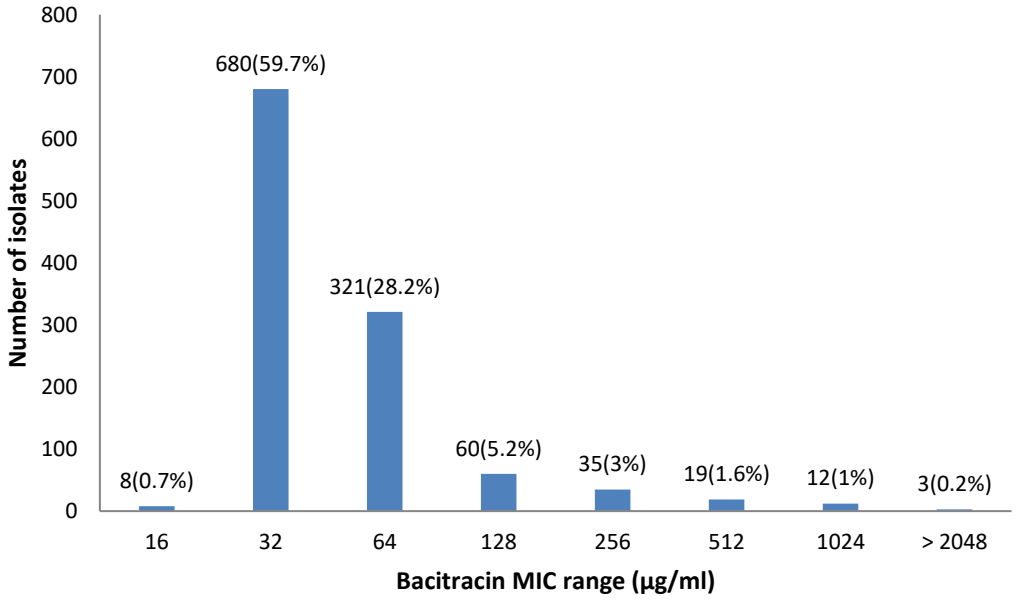

Figure 4.2 Bacitracin susceptibility of 1138 clinical isolates of *S. aureus*. The number above each bar is the total number of isolates associated with that bacitracin MIC and the number in brackets is the percentage of the total number of isolates.

4.2.2 Attempts to select for bacitracin resistance *in vitro*

Bacitracin susceptibility testing of *S. aureus* revealed the apparent presence of bacitracin resistance in this bacterial species. In subsequent studies, the author sought to determine the genetic basis for bacitracin resistance in these strains. Antibiotic resistance in staphylococci can result either from mutational change (the endogenous route to resistance), or as a consequence of horizontal acquisition of resistance determinants (the exogenous route). In preliminary laboratory resistance studies, the author examined whether *S. aureus* has the potential to evolve bacitracin resistance via the endogenous route.

This was investigated by performing mutation frequency determinations and extended gradient MIC experiments for *S. aureus* SH1000 (bacitracin MIC 64 µg/mL) to select for bacitracin resistant mutants. No resistant mutants were recovered (limit of detection $<1.41 \times 10^{-9}$ cfu/mL) upon plating of saturated cultures onto agar containing bacitracin at 4x MIC (256 µg/mL). Subsequently, the author sought to select bacitracin resistance through mutation by serial passage of SH1000 for 21 d in the presence of a range of concentrations of bacitracin. Daptomycin was used as an antibiotic control in this experiment since it has been shown that resistance to daptomycin in *S. aureus* can be selected using such a method (Friedman et al., 2006). Susceptibility to bacitracin did not change over the whole experimental period. In contrast, SH1000 developed a clinically-significant level of increase in the original MIC of daptomycin (MIC 16 µg/mL) following 21 d of exposure to daptomycin, as

previously reported (Figure 4.3) (Friedman et al., 2006, Randall et al., 2013a).

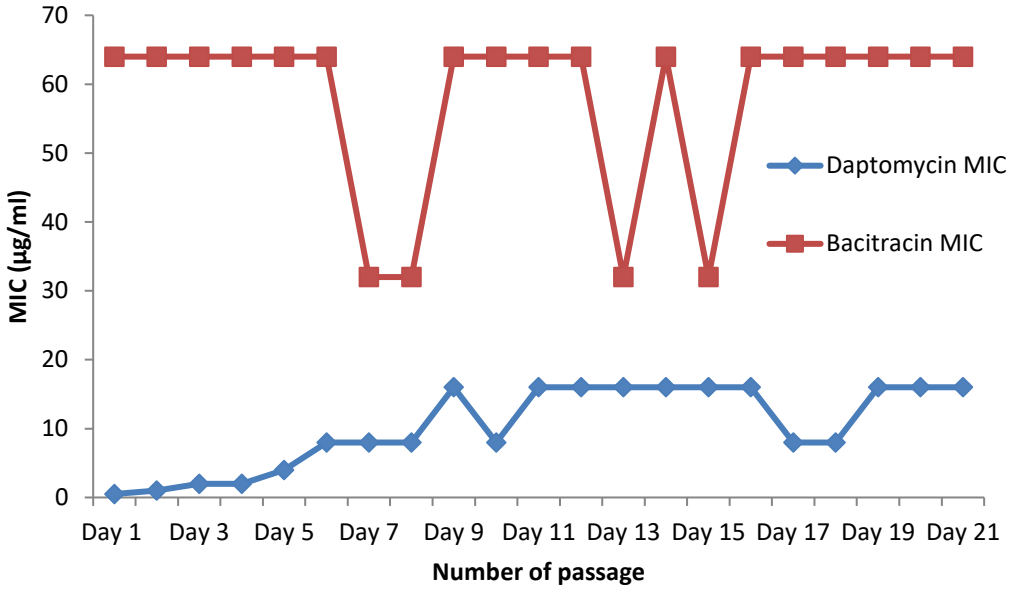

Figure 4.3 Failure to select resistance to bacitracin following passaging of SH1000 (*n*=3) using the extended gradient MIC method. Daptomycin was used as a control antibiotic for the selection of resistance. The MIC value was recorded for each antibiotic over 21 d.

4.2.3 Identification of the genetic basis of bacitracin resistance

Having established that bacitracin resistance does not readily evolve via the endogenous route, it seemed more likely that the genetic basis for bacitracin resistance in clinical isolates would involve horizontally acquired resistance determinants. Three isolates (NRS19, NRS384 and NRS386) for which bacitracin has an MIC of 2048 µg/mL were subjected to whole genome sequencing. Sequence reads were imported into CLC genomics workbench 8.0, trimmed to remove low-quality bases (using standard settings) and subjected to *de novo* assembly (which also provided an estimate of genome size) (Table 4.1). The GC content of all three sequenced strains was within the expected percentage for *S. aureus* strains, though genome sizes for NRS19

and NRS384 strains were slightly larger than typical for *S. aureus* strains (2.8Mb), but this could due to the presence of plasmids and phages in the genomes of those isolates. Another noticeable thing in the data is that the NRS19 strain has a large number of contigs compared to the other two strains, which is most likely the result of sample degradation (Kwong et al., 2015, Van Walle et al., 2019).

Table 4.1 Summary of the quality measurements for the three sequenced strains

Measurements criteria	NRS19	NRS384	NRS386
Size (bp)	3,835,136	3,309,667	2,880,404
GC Content (%)	36.9	34.7	33.3
N75	3979	55658	100085
N50	203394	202442	176788
N25	443406	864209	315458
Minimum Contig Length	982	1009	1005
Maximum Contig Length	606366	864209	578675
Average Contig Length	5599	9649	26426
L50	7	5	5
Number of Contigs (with PEGs)	685	343	109
Number of Reads	9000000	9000000	9000000
Matched Reads	8739196	8798834	8837753
Not matched Reads	260804	201166	162247

NRS19, NRS384 and NRS386 are the bacitracin resistant strains. N75 is the minimum contig length needed to cover 75% of the genome. N50 is the minimum contig length needed to cover 50% of the genome. N25 is the minimum contig length needed to cover 25% of the genome. L50 is the number of contigs whose summed length is N50. PEGs stand for "protein-encoding genes".

After that, a gene library was compiled in CLC Genomic Workbench containing known bacitracin resistance determinants (mentioned in the introduction section of this chapter), identified both from the published literature and from the NCBI database. The sequences of these resistance determinants were used as the search terms to check for the presence of any of these genes in the DNA sequences of the three isolates. The presence of the *bcr* genes was identified in all three strains, and included the same component genes in all cases (*bcrA* [ABX30722.1], *bcrB* [ABX30721.1] and *bacA* [AOH60243.1], *bcrR* [CP000731.1] and *bcrS* [CP000732.1]) (Figure 4.4) (Highlander et al., 2007). Furthermore, in all cases, the *bcr* genes were flanked on one side by a transposase belonging to the IS*6* family (IS*431* mec) (Figure 4.5). Thus, the *bcr* locus represents a likely cause of bacitracin resistance in these strains and appears to be closely associated with a potential mobile genetic element.

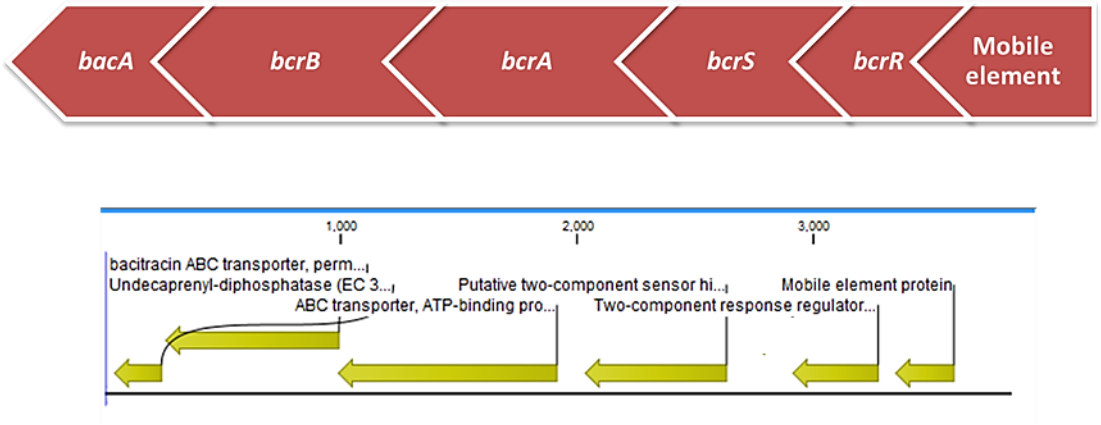

Figure 4.4 A diagram of the contig that contains the *bcr* genes from the NRS384 strain. The mobile element appears at the right-hand side of this figure, followed by the *bcr* cluster. The top diagram was created manually using PowerPoint based on the bottom diagram, which was obtained from CLC Genomic Workbench.

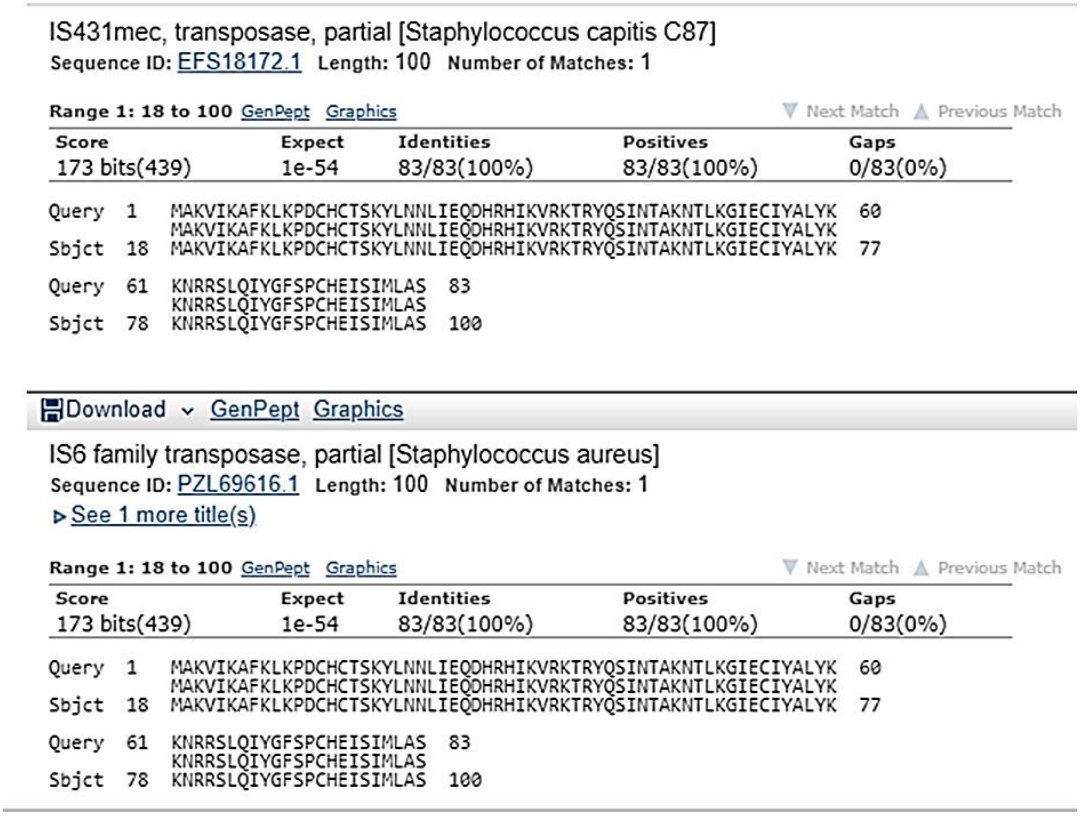

Figure 4.5 A snapshot from the NCBI website for a BLAST search for the mobile element protein which was found next to the *bcr* genes. It has a close identity to the IS*6* family (IS*431* mec).

Subsequently, the remainder of the strains for which bacitracin had an MIC of ≥512 µg/mL were screened by PCR for the presence of the *bcr* genes. Three *bcr* genes were picked for screening as representatives of the *bcr* cluster: *bcrA*, *bcrB* and *bacA*. All three genes were present by PCR in all isolates with MICs of ≥512 µg/mL. A selection of isolates in each MIC range below 512 µg/mL was also screened for the presence of the *bcr* genes (just to rule out the presence of *bcr* genes), though were not found to be present in any case (Table 4.2). In each case, a positive control (one of the three screened resistant strains) and a negative control (SH1000) for the *bcr* genes were used.

Table 4.2 Presence of the *bcr* genes in *S. aureus* isolates as determined by PCR

MIC µg/mL	Total No. of Isolates	No. of Isolates Tested	*bcrA*	*bcrB*	*bacA*
16	8	8	8 (-)	8 (-)	NT
32	680	10	10 (-)	10 (-)	NT
64	321	10	10 (-)	10 (-)	NT
128	60	10	10 (-)	10 (-)	NT
256	35	20	20 (-)	20 (-)	NT
512	19	19	19 (+)	19 (+)	19 (+)
1024	12	12	12 (+)	12 (+)	12 (+)
> 2048	3	3	3 (+)	3 (+)	3 (+)

(+) indicates that the gene was detected, (-) indicates that the gene was not detected and (NT) indicates none tested.

4.2.4 Bioinformatics analysis of bacitracin resistance in *bcr*+ strains

RAST was used to annotate the genomes of the three sequenced bacitracin resistant strains described above. During the analysis, the software provided a read-out of the 'closest neighbouring strains' for each of these sequenced strains, i.e. closely related strains whose genomes have been sequenced and are publicly available (Aziz et al., 2008, Overbeek et al., 2014, Brettin et al., 2015). The author recognised that some of these closest neighbouring strains were already stocked in the O'Neill laboratory (where the present study was undertaken) and considered that it would be of interest to evaluate the bacitracin susceptibility of these latter strains. Ahead of undertaking that analysis, the RAST output was further investigated using an MLST approach

to identify the sequence type and the clonal complex for each strain (three bacitracin resistant strains and three of their closest neighbouring strains), thereby determining how closely related they are so they can be compared to each other in further analysis. MLST is a commonly used technique for the analysis of clonal relationships in several clinically relevant microorganisms and utilises the DNA sequence of a number of housekeeping genes to produce a unique identifier which is referred to as the sequence type (ST). Strains can then be further analysed by investigating the relatedness between their STs to find out which clonal complex (CC) they belong to (Maiden et al., 1998, Urwin and Maiden, 2003, Ibarz Pavón and Maiden, 2009, Patiño et al., 2018). Among the six strains (three bacitracin resistant strains and three of their closest neighbouring strains) tested, the MLST analysis identified four different sequence types (ST5, ST8, ST72 and ST371) that belonged to two clonal complexes (CC5 and CC8) (Table 4.3).

Table 4.3 Summary of the results of MLST analysis for the three bacitracin resistant strains and three of their closest neighbouring strains

Strain ID	Sequence Type (ST)	Clonal Complex (CC)
NRS19	5	CC5
NRS384	8	CC8
NRS386	72	CC8
VRSA1	5	CC5
VRSA2	5	CC5
VRSA3a	371	CC5

NRS19, NRS384 and NRS386 are the bacitracin resistant strains. VRSA1, VRSA2 and VRSA3a are their closest neighbouring strains

Then the data of the MLST analysis of the six strains was analysed against a global ST profile for *S. aureus* (which were obtained from https://pubmlst.org/general.shtml) in Phyloviz software in order to obtain a hypothetical phylogenetic relationship using the eBURST algorithm – goeBURST (Figure 4.6). Here, the diversification of the clonal complexes is reflected in the appearance of sequence types (STs) differing only in one housekeeping gene sequence – single locus variants (SLVs). When only considering SLV links, the eBURST graph aims at identifies different clonal complexes rather than linking the entire population (Francisco et al., 2009). Figure 4.6 shows the phylogenetic relationship between the strains in this study (three bacitracin resistant strains and three of their closest neighbouring strains) and the global database. The graph shows each ST as a node and they are clustered with respect to their number of SLVs. The lineage (SLV path) between ST5 and ST8 in Figure 4.6 make the linkage between CC5 and CC8. The three bacitracin resistant strains and their three closest neighbouring (reference) strains belong to CC5 and CC8 which are linked. In fact, CC5 and CC8 are among the largest and most important *S. aureus* clonal complexes and they are both considered to be a source of a pandemic MRSA. It was found that these two clonal complexes are closely linked to each other and that their different lineages of sequence types (especially ST239) are forming the connection between these two clonal complexes (Figure 4.6) (Deurenberg et al., 2005, Shambat et al., 2012, Dabul and Camargo, 2014). That means the closest neighbouring strains could be considered as reference strains for the three bacitracin resistant strains.

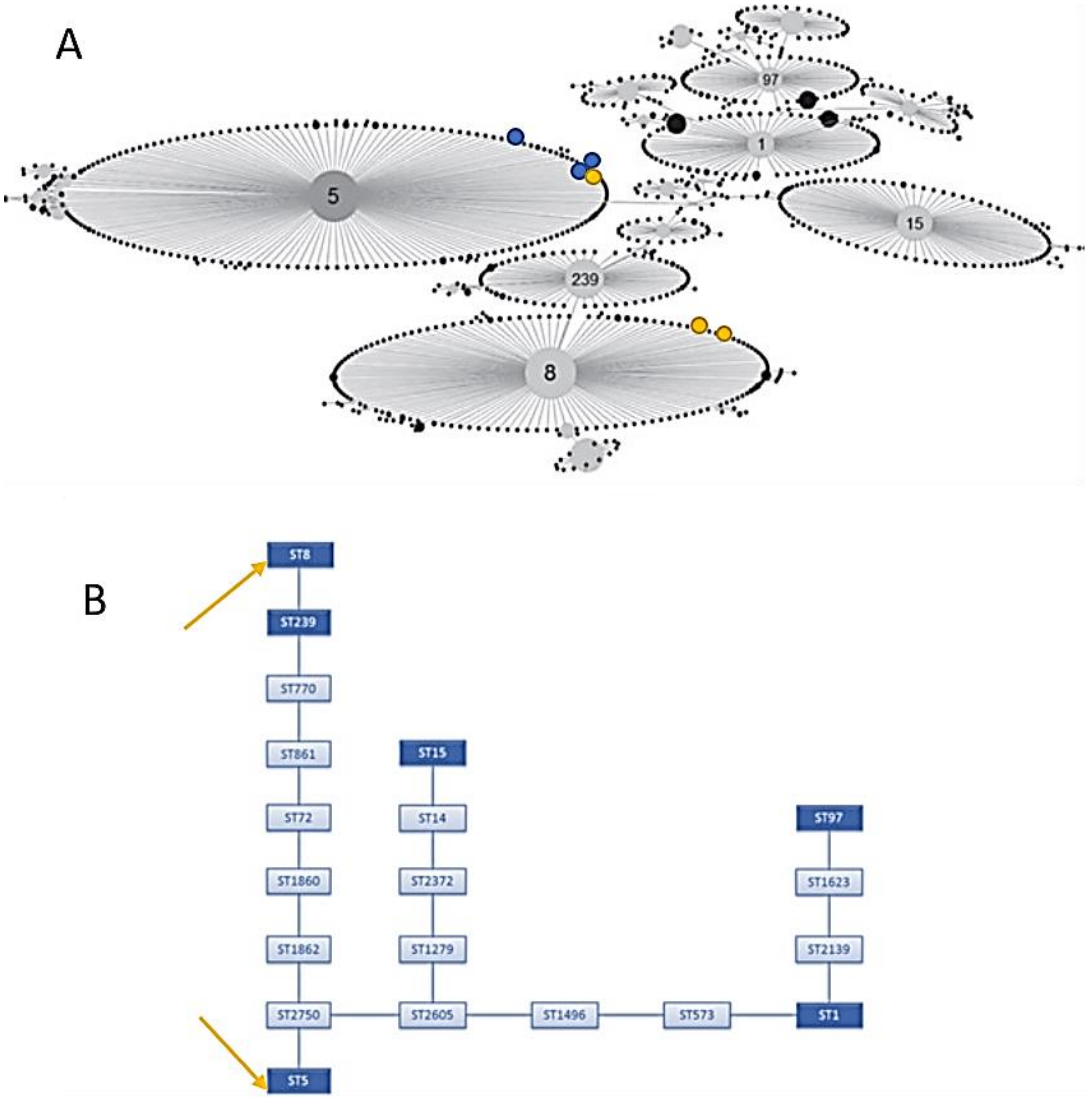

Figure 4.6 The phylogeny and genetic relationship of the three bacitracin resistant strains and the global *S. aureus* database. A) Snapshot from EBURST for *S. aureus* MLST database against the three bacitracin resistant strains (indicated by yellow circles) and their three closest neighbouring (reference) strains (marked with blue circles). B) The relationship between the main *S. aureus* STs (sequence type) which make the connections between the different *S. aureus* CC (clonal complexes). The lineage between ST5 and ST8 (marked with yellow arrows) makes the linkage between CC5 and CC8. The three bacitracin resistant strains and their three reference strains belong to CC5 and CC8 which are linked.

Interestingly, for those three strains (that were the closest neighbouring strains to the bacitracin isolates identified in this study) the MIC for bacitracin was found to be 128 µg/mL, indicating that they were susceptible to bacitracin. PCR

analysis established that these strains lacked the *bcr* genes, indicating that these are unique to the resistant isolates, and further strengthening the suggestion that these genes are likely responsible for bacitracin resistance.

After that, a comparative genome analysis was needed to identify any genes or regions which are unique to the resistant strains (which was proposed to be the *bcr* genes) but not the susceptible ones and that they might be responsible for conferring bacitracin resistance phenotype in the resistant strains. MAUVE software was used to do multiple alignments for the obtained DNA sequences to identify the differences and similarities between different genomes of the resistant and susceptible strains (Darling et al., 2004, Darling et al., 2010).

Firstly, the sequences of the three resistant isolates and three of their susceptible closest neighbouring strains were downloaded on MAUVE. Secondly, multiple alignments were performed for all six strains using MAUVE. Thirdly, the alignment results were analyzed visually by looking for unique regions or genes, which were only present in the resistant strains (represented by similar coloured blocks on MAUVE) and could be considered as a candidate gene for causing resistance (Figure 4.7). Based on analysis of the bioinformatics data, it was revealed that these regions are in fact the *bcr* genes. The presence of the *bcr* cluster was found to be unique to the resistance strains, implying that the *bcr* genes are the likely source of bacitracin resistance in those isolates.

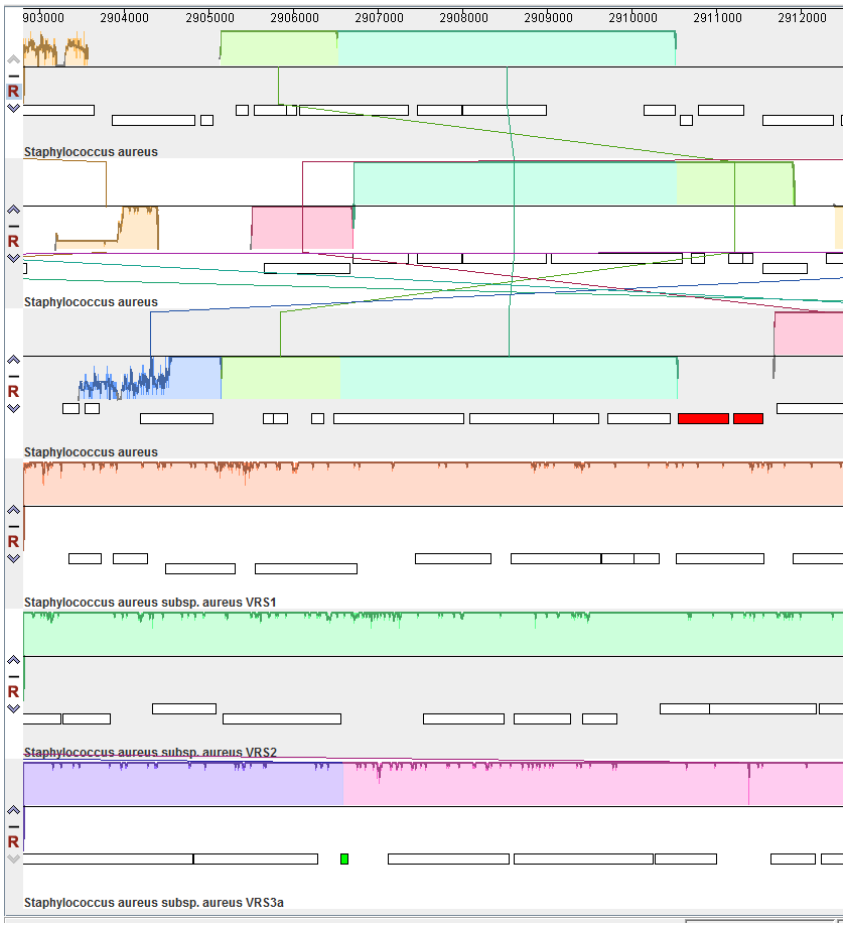

Figure 4.7 A screenshot from MAUVE for the alignment of the three bacitracin resistant strains(upper three sequences) with the three susceptible strains (lower three sequences). The six horizontal sequences of blocks showing ~8 kb regions of the genomes of NRS19, NRS384, NRS386 (bacitracin-resistant isolates), and VRSA1, VRSA2 and VRSA3a (closest neighbouring [reference] strains that are bacitracin susceptible). The green blocks in sequences of the resistant strains represent the *bcr* cluster that is not present in the susceptible strains, suggesting the *bcr* genes are likely source of bacitracin resistance in the resistant isolates. The coloured blocks in the upper three sequences (resistant strains) represent different groups of genes involved in antibiotic resistance (lime green), replication (pink), phage-related genes (orange), and virulence (blue). The large blocks (orange for VRSA1, green for VRSA2, and light/ dark purple for VRSA3a) on the bottom three sequences (reference strains) represent different parts of *S. aureus* genome including genes associated with metabolism, virulence, translation, and cell wall biosynthesis, which are of no relevance to bacitracin resistance.

While this work was in progress, a lab colleague (Dr Louise Kime) independently generated a whole genome sequence for NRS384 (one of the

bacitracin resistant strains found in this study) that was of higher quality than those the author generated (Table 4.4). The nucleotide sequences determined here used the Illumina a MiSeq platform with 2x250 paired-end reactions, while Dr Kime's NRS384 sequence used 100 bp paired-end reads on the Illumina HiSeq2000 platform. It is therefore potentially the case that the differences in the sequencing method led to a larger number of contigs for the three resistant strains when sequenced by the method employed here in this study.

Table 4.4 Summary of the quality measurements for the two WGS of NRS384 obtained from this study and from Dr Kime

Measurement Criteria	NRS384 From Dr Kime	NRS384 From This Study
Size (bp)	2,892,086	3,309,667
GC Content (%)	32.6	34.7
N50	1088350	202442
L50	2	5
Number of Contigs (with PEGs)	13	343

N50 is the minimum contig length needed to cover 50% of the genome. L50 is the number of contigs whose summed length is N50. PEGs stand for "protein-encoding genes".

Analysis of this obtained sequence of NRS384 identified a ~32 kb contig carrying the *bcr* bacitracin resistance determinants, along with several other antibiotic resistance genes (Appendix 4). BLAST analysis using this entire contig detected extensive regions of similarity with other sequenced plasmids

from *S. aureus,* but the complete identity was not observed (Figure 4.8). It, therefore, appears that *the bcr* genes in these strains reside on a multi-drug resistance plasmid, and one that is distinct from those published to date. It was, therefore, important to obtain a complete sequence of this plasmid.

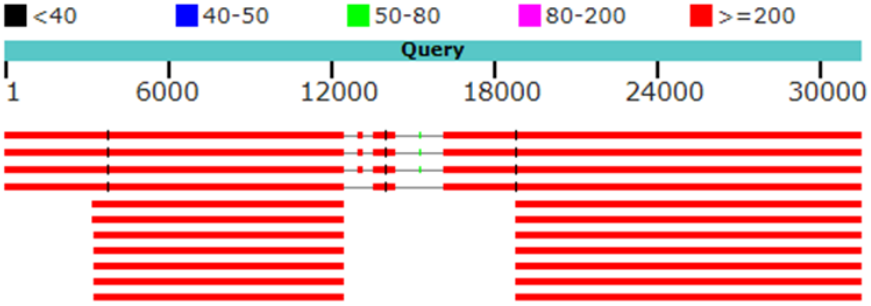

Figure 4.8 A screenshot showing part of the results of a BLAST search for the contig containing the *bcr* genes from NRS384 against the database from NCBI. There was no complete identity with other sequences.

The contig hypothesised to be a plasmid (according to the above BLAST search) is likely lacking sequence at its ends since it could not be circularised *in silico*. To obtain a complete sequence of the plasmid, the missing sequence was generated by using outward-facing oligonucleotide primers targeted to the ends of this contig to generate PCR products that could be sequenced to fill the gap between the two ends (oligonucleotides NRS384 plasmid U and L and NRS384 plasmid extension U and L, Appendix 3). A PCR amplicon of ~700bp was generated, that upon sequencing, enabled the plasmid sequence to be circularised *in silico* (Figure 4.9). The size of the entire plasmid (named pNRS384) is 32,033 bp and encodes resistance to heavy metals, aminoglycosides, macrolides, and penicillin (Appendix 4).

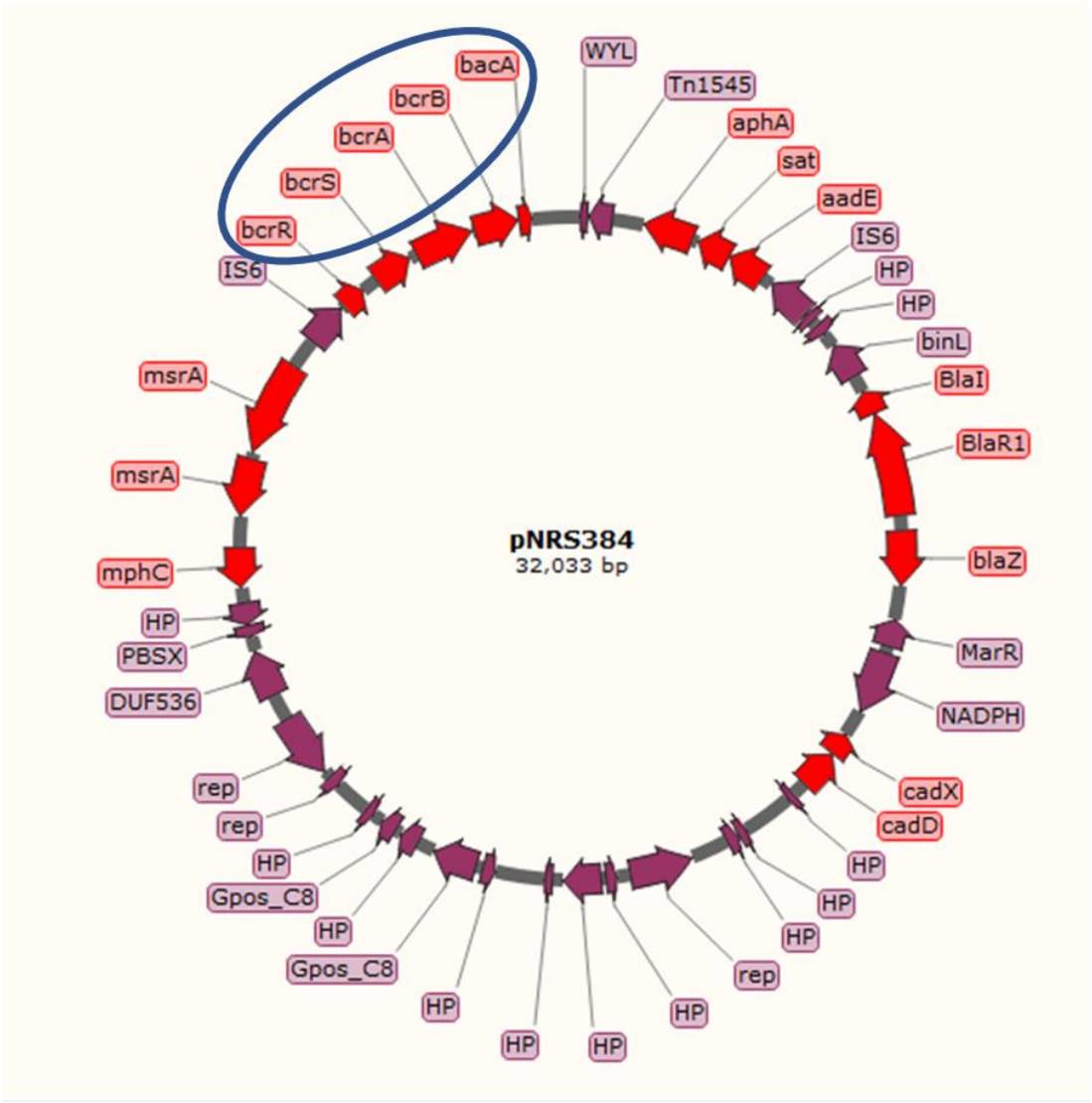

Figure 4.9 Schematic of the plasmid found in NRS384 (pNRS384) obtained from SnapGene software. Red arrows indicate resistance genes and purple arrows refer to other genes and ORFs. HP refers to a hypothetical protein, for which no function has yet been defined. *bcrR, bcrS, bcrA, bcrB* and *bacA* are the bacitracin resistance determinants (surrounded by a blue circle). *aphA*, *sat* and *aadE* are the aminoglycosides resistance genes. *blaZ*, *BlaR1* and *BlaI* are the β-lactams resistance determinants. *cadD* and *cadX* are the cadmium efflux genes. *msrA* and *mphC* are the macrolides resistance genes. A list of all the genes found here is mentioned in Appendix 4.

4.2.5 Is the *bcr* cluster responsible for bacitracin resistance?

After the identification of the presence of the *bcr* cluster in all the isolates with MICs of ≥512 µg/mL, the next step was to establish if this cluster is responsible

for conferring bacitracin resistance in *S. aureus*. A ~5 kb fragment from the genome of bacitracin resistant strain NRS384 containing the entire *bcr* cluster (bacitracin resistance cluster) was amplified using oligonucleotide primers (*5'-* TATATAAATTCGAACATACAGAAGACTCCTTTTTG and *5'-* ATATATATTTCGAAATTAAGAGAACGTGGTGTAAA [engineered *BstBI* restriction sites shown underlined]). The resulting amplicon was ligated into the single *BstBI* site on cloning vector pSK5487 and propagated in *E. coli* XL10 Gold. Once the colonies with the right insert (*bcr* cluster) were detected on the transformation plate by PCR amplification of the MCS (multiple cloning site) of pSK5487 plasmid and sequencing, they were used to transform *S. aureus* RN4220 (bacitracin susceptible strain) competent cells by electroporation. The resulting transformants (16 colonies tested) exhibited bacitracin resistance, showing the same level of susceptibility as strain NRS384 (>1024 µg/mL) and PCR identification of the *bcr* genes (*bcrA, bcrB* and *bacA*) showed that the transformants RN4220 (pSK5487:*bcr* locus) carried these genes. By contrast, RN4220 transformed with empty pSK5487 plasmid showed no change in bacitracin susceptibility (MIC of 64 mg/L). These results suggest that the *bcr* genes are likely responsible for the bacitracin resistance phenotype associated with strain NRS384.

4.2.6 Transduction of the bacitracin resistance determinants into *S. aureus* RN4220

To further link the presence of the *bcr* genes in the clinical isolates to bacitracin resistance, the author sought to examine whether transduction of the bacitracin resistance phenotype from NRS384 into a bacitracin susceptible host strain was associated with the transfer of the *bcr* genes. This experiment

was conducted by following the protocol mentioned in Chapter 2/ section 2.5.9. Briefly, Φ11 bacteriophage was propagated in the donor strain NRS384 (bacitracin resistant) by growing an overnight culture in phage broth. After incubation, the surfaces of the plates with the highest dilution that gave confluent lysis were scraped to collect the phage top agar layer and centrifuged. Then the phage containing supernatant was removed and filtered. Φ11 bacteriophage particles (which were propagated in NRS384) were used to infect the recipient strain RN4220 (bacitracin susceptible) by adding the phage particles to the RN4220 culture and the cells were then plated on bacitracin selection plates (1024 µg/mL).

Putative transductants were recovered at a frequency of $2.1X10^{-7}$ and tested for the presence of the *bcr* genes by PCR. The *bcr* genes were present in all tested cases (*n*=16), and all transductants exhibited levels of bacitracin resistance comparable to that seen for NRS384 (MIC of >1024 µg/mL). Furthermore, these transductants were also resistant to other antibiotics (penicillin, erythromycin and kanamycin) against which pNRS384 encodes resistance (Table 4.5). No increase in susceptibility to other antibiotics (tetracycline and cefoxitin) was observed in transductants, which was anticipated since the resistance genes responsible (though present in NRS384) are not resident on plasmid pNRS384. These findings corroborate the idea that the bacitracin resistance phenotype in NRS384 is attributable to the presence of the *bcr* genes. Conversely, the bacitracin MIC of the RN4220 infected with Φ11 bacteriophage alone was 64 µg/mL, which is the same as the original MIC (MIC 64 µg/mL) of the recipient strain RN4220.

Table 4.5 Summary of the MIC results against different antibiotics for the transductants (T1, T2), NRS384 and RN4220

Strain	Tetracycline	Cefoxitin	Bacitracin	Penicillin G	Erythromycin	Kanamycin
T1	1 µg/mL	2 µg/mL	>1024 µg/mL	>128 µg/mL	16 µg/mL	>128 µg/mL
T2	1 µg/mL	2 µg/mL	>1024 µg/mL	>128 µg/mL	16 µg/mL	>128 µg/mL
NRS384	32 µg/mL	16 µg/mL	>1024 µg/mL	>128 µg/mL	8 µg/mL	>128 µg/mL
RN4220	1 µg/mL	2 µg/mL	64 µg/mL	0.0625µg/mL	0.5 µg/mL	2 µg/mL

T1 and T2 were two of the RN4220 transductants that were infected with Φ11 phage propagated in NRS384, NRS384 was the donor strain, and RN4220 was the recipient strain. The resistance determinants for tetracycline and cefoxitin were not found on the same identified plasmid (contig) with the bcr genes but they were identified elsewhere in the genome of NRS384 during the analysis of the genome in CLC Workbench.

4.2.7 Further studies into the transfer of bacitracin resistance

Since the *bcr* genes in NRS384 are located on a plasmid and bacitracin resistance could be transferred by transduction, the author examined whether resistance could also be transferred by means of transformation. The whole genome extract from NRS384 was used to successfully transform the bacitracin-susceptible strain RN4220 to resistance. Transformants were associated with a bacitracin MIC of >1024 µg/mL, compared to the original MIC for the recipient strain RN4220 (MIC 64 µg/mL). The *bcr* genes (*bcrA, bcrB* and *bacA*) were detected in the transformants by PCR.

4.2.8 Investigating the genetic basis of low-level bacitracin resistance

A proportion of the isolate panel originally tested for bacitracin susceptibility was associated with bacitracin MICs of 256 µg/mL. This figure is substantially higher than that seen for susceptible laboratory strains, but the *bcr* genes could not be detected in such strains, potentially suggesting the existence of another determinant (or determinants) conferring low-level bacitracin resistance. However, *in silico* analysis failed to identify any other bacitracin resistance genes (mentioned in the introduction of this chapter) in these strains. To further explore this idea, the author used the approach described above of attempting to transfer bacitracin resistance from two low-level resistant strains that could be infected with Φ11 bacteriophage to a susceptible host (RN4220) by transduction.

No transductants were recovered in initial experiments. Therefore, the transduction experiment was re-attempted with a longer period of bacitracin exposure that might better allow for the expression of bacitracin resistance determinants. This experiment was conducted by following the modified protocol mentioned at the end of Section 2.5.9/ Chapter 2. Control experiments were performed to ensure that transduction was capable of transferring other antibiotic resistance phenotypes from these strains into RN4220, which is susceptible to both bacitracin (MIC 64 µg/mL) and erythromycin (MIC 0.5 µg/mL). In respect of the latter, erythromycin resistance could be successfully transferred at a frequency of $3.2X10^{-6}$ transductants per plaque forming unit on erythromycin plates (8 µg/mL). On the basis that this type of bacitracin resistance might require some degree of induction to manifest, recipient

RN4220 cells were exposed to a sub-inhibitory concentration of bacitracin (16 µg/mL) before plating cells onto bacitracin selection plates. However, even under these conditions, no transductants exhibiting reduced susceptibility to bacitracin could be recovered on bacitracin plates (256 µg/mL).

4.3 Discussion

This study investigated bacitracin susceptibility among clinical isolates of *S. aureus*, with a particular emphasis on identifying and further characterising instances of bacitracin resistance. Resistance to bacitracin (defined below) was identified in ~3% of *S. aureus* isolates tested, and all such strains carried the *bcrABRS/bacA* locus that was confirmed in molecular cloning experiments to confer bacitracin resistance.

The identification of the susceptibility profile for bacitracin can be used to establish ECOFF values for resistance surveillance. That would be useful since no precise clinical breakpoints or ECOFF values have been published to date to allow investigators to establish at what point reduced bacitracin susceptibility in *S. aureus* should be considered true resistance. The ECOFF point is defined as the concentration of an antibiotic which separates microorganisms of a given species without and with resistance mechanisms, enabling one to distinguish WT susceptible strains from those harbouring/expressing a resistance determinant (EUCAST, 2017). Additionally, it can help to set clinical breakpoints, optimise antibacterial agent selection and dosing, guide clinicians in their decisions for everyday use of antibacterial agents in patient therapy and assist future surveillance (Turnidge and Paterson, 2007, Hombach et al., 2014).

According to a number of studies, *S. aureus* strains have arbitrarily been considered clinically susceptible to bacitracin if their MICs were <256 µg/mL (SFM, 2003, Highlander et al., 2007, Bessa et al., 2016, Sabat et al., 2017). The present study supports a value of 512 µg/mL as the ECOFF point since

only isolates with that level of bacitracin resistance or above were found to carry a bacitracin resistance determinant (Table 4.2). Based on this ECOFF, only a small percentage (~3%) of the large collection of isolates tested exhibited bacitracin resistance. The ECOFF for bacitracin could be defined here because of the large sample size, the wide geographical distribution of the isolates, the fact that the majority of the strains had an MIC of ≤256 µg/ml (*in vitro* microbiological data based on the bar chart overview) (Figure 4.2) and also because the bacitracin MIC for both the *S. aureus* laboratory strains, SH1000 and RN4220, was 64 µg/mL, which is close to the majority of the clinical population in this study (Kahlmeter et al., 2003, Turnidge and Paterson, 2007, EUCAST, 2017). Taking all that into consideration, *S. aureus* strains could be considered resistant if their MICs to bacitracin were ≥512 µg/ml, but more clinical studies are still needed to set up a clinical breakpoint for bacitracin.

At first glance, this prevalence figure (~3%) appears reassuring, suggesting that bacitracin resistance is not currently common in the clinical settings. This is especially the case in light of the observations made here that bacitracin does not readily arise by the endogenous route, implying that resistance prevalence will not increase as a result of mutation (in contrast to the situation seen for many other antibiotic classes) (Woodford and Ellington, 2007, Fernández and Hancock, 2012). An alternative view is that a prevalence figure of ~3% does not lie far from the 5% resistance prevalence threshold suggested by the WHO as the cut-off point beyond which an antibacterial drug should no longer be used empirically (WHO, 2017).

The whole genome sequencing data was very useful in understanding the genetic basis of bacitracin resistance in the resistant strains, although there was a large number of short contigs in the sequencing data of the genome of NRS19 strain (Table 4.1), probably because of sample degradation (Bradnam et al., 2013, Kwong et al., 2015). This was supported by looking at the contig and read measurements criteria such as the contig average size which was lower than the other two sequenced strains with lower numbers of contigs (NRS384 and NRS386) (Table 4.1). Nonetheless, this does not affect the use of the sequencing data for further analysis in this study, especially because the GC content and genome size of NRS19 are within the expected value for *S. aureus* and the results of the MLST and Phylogenetic relationship analysis further confirm this (Bradnam et al., 2013, Kwong et al., 2015, Van Walle et al., 2019). Meanwhile, regarding the sequence of the NRS384 strain, large numbers of contigs were not seen on re-sequencing the strain using another Illumina-based method (the data obtained from Dr Kime), implying that it is not an issue with the strain *per se* or with using Illumina to sequence it (Table 4.4). In fact, the newly obtained data had longer contigs which helped to identify the pNRS384 plasmid (Figure 4.9 and Appendix 4).

Furthermore, horizontally acquired bacitracin resistance is associated with mobile genetic elements, including plasmids that could in principle facilitate rapid dissemination of the *bcrABRS/bacA* locus throughout staphylococcal populations. Indeed, in this study, it has been demonstrated that plasmids harbouring the *bcr* genes can be readily transferred by transduction and transformation. While it does not seem likely that pNRS384 (Figure 4.9 and Appendix 4) could transfer by conjugation (it lacks both the *tra* and *mob* genes

enabling conjugation and mobilisation respectively), this does not exclude the possibility that other *bcr*-containing plasmids harbour these functions. Especially since about 5% of all known *S. aureus* plasmids are conjugative and carry the genes (*tra* genes) required for autonomous conjugative transfer (Smillie et al., 2010, Pollet et al., 2016, Ramsay et al., 2016).

Since no *tra* genes were identified on this plasmid, filter-mating experiments could be performed to ensure the necessary cell-to-cell contact as transconjugants can be detected, when the donor and recipient cells are separated by a filter. Also, solid and liquid mating techniques could be employed here, taking into consideration the optimal conditions for gene transfer, including donor-to-recipient ratio. Moreover, mobilizable plasmids usually carry *mob* genes which facilitate DNA processing and replication but lack the genes required for mating and pore formation (Grohmann et al., 2003, Pollet et al., 2016, Ramsay et al., 2016, LaBreck et al., 2018). Therefore, since no *mob* genes were detected on pNRS384, *In Silico* analysis to identify genes typically associated with mobilizable plasmids could be an option to check if pNRS384 is able to be mobilised in the presence of a specific conjugative plasmid.

The *bcrABRS/bacA* determinant has previously been detected in *S. aureus*, but has not previously been definitively linked to bacitracin resistance in this organism (Highlander et al., 2007, Kennedy et al., 2010, Sabat et al., 2017). Several lines of evidence suggest that this determinant is both necessary and sufficient for the bacitracin resistance observed in this study. Thus, (i) the *bcr* genes were found in all isolates with a bacitracin MIC of ≥512 µg/ml (and not in any of the bacitracin susceptible isolates), (ii) molecular cloning of the *bcr*

genes conferred bacitracin resistance on a susceptible *S. aureus* host strain, and (iii) transduction of the bacitracin resistance phenotype from a bacitracin-resistant isolate to a susceptible one was associated in each case with transfer of the *bcr* genes.

How do the *bcr* genes confer resistance to bacitracin? It has been speculated that the Bcr complex is an efflux transporter that works similarly to the P-glycoprotein-related multidrug resistance transporter to eliminate bacitracin from the lipid bilayer directly to the extracellular medium (Bolhuis et al., 1996, Podlesek et al., 2000, Ohki et al., 2003, Charlebois et al., 2012). In the case of bacitracin, the target is buried inside the membrane, and the suggested membrane-oriented transport activity of bacitracin could be through the 'hydrophobic vacuum cleaner' model of P-glycoprotein activity, which is the same hypothesised resistance mechanism for the membrane acting polyether-ionophore antibiotic tetronasin (Linton et al., 1994, Podlesek et al., 2000, Charlebois et al., 2012). Based on the P-glycoprotein model, access to membrane-associated transporters is acquired directly from the lipid phase, which would provide an explanation of the mechanism of bacitracin resistance. The drug is either 'flipped' from the inner leaflet to the outer leaflet of the cytoplasmic membrane (flippase-like mode of action) or pumped into the external medium (Higgins and Gottesman, 1992, Gottesman and Pastan, 1993). That said, the mode of action of a similar bacitracin transporter (BceAB) of *B. subtilis* was investigated very recently and evidence provided to suggest that it acts by displacing bacitracin from lipid II cycle intermediates (cellular target of bacitracin), thereby imparting resistance via a target protection mechanism (Kobras et al., 2019). A similar mechanism is plausible for

BcrABD, though further experimentation will be required to definitively establish how this system mediates bacitracin resistance.

Moreover, based on the transduction experiment performed here, the findings confirmed that in addition to bacitracin resistance determinants, other antibiotic resistance determinants located in the same region can be transferred by transduction to a susceptible strain and confer resistance for their specific antibiotic (Table 4.5). In the literature, transduction in *S. aureus* had been considered as one of the important mechanisms of horizontal gene transfer, and it has been employed for studying the transfer of resistance determinants and in genome modifications (Malachowa and DeLeo, 2010, McCarthy et al., 2014, Kali, 2015). In one study, the efficiency of transduction of pUSA300-HOUMR-like penicillinase plasmid was proved in *S. aureus*. This plasmid had been reported to carry the *bcr* genes along with other resistance determinants and has a close identity to the plasmid found in NRS384, but no susceptibility testing had been performed for any antibiotic in that study to confirm the expression of the resistance phenotype (Mašlaňová et al., 2013). This all supports the suggestion that bacitracin resistance determinants are carried on a mobile genetic element and that there is a risk not just for the dissemination of bacitracin resistance, but also resistance to other antibiotics that are carried on the same region being disseminated in a similar manner. Of note, SOS response can be induced when some antibiotics interfere with bacterial DNA replication which leads to a high frequency of horizontal gene transfer. This induces the excision and transduction of some mobile genetic elements in the bacterial genome including prophages and staphylococcal pathogenicity islands (Maiques et al., 2006, Malachowa and DeLeo, 2010,

Haaber et al., 2017). Within this context, another explanation for the dissemination of the bacitracin resistance cluster is the mobile genetic elements which reside on the *S. aureus* genome and mediate their own transfer and integration, but again this cannot be confirmed without further investigation.

Chapter 5 Investigating the prevalence and the mechanism of nitrofurantoin resistance in *S. aureus*

5.1 Abstract

Nitrofurantoin resistance is used for the treatment of uncomplicated urinary tract infections caused by different bacterial species that include *S. aureus*. The prevalence of nitrofurantoin resistance has traditionally been low, but that may change as clinical nitrofurantoin use increases. Therefore, in this study, it was important to investigate the clinical prevalence and genetic basis of resistance to nitrofurantoin in *S. aureus.* MICs for nitrofurantoin spanned the range from 4 µg/mL to 64 µg/mL among a panel of clinical isolates of *S. aureus* (*n*=1138), and the MIC_{90} was 16 µg/mL. Strains exhibiting reduced susceptibility to nitrofurantoin at the upper end of this range did not appear to possess a discrete resistance determinant that could be transferred to a susceptible host by transduction. Whole genome sequencing and genetic comparison with fully nitrofurantoin-susceptible isolates identified two genetic regions (5 and 15 kb in size) only present in the strains with reduced nitrofurantoin susceptibility. However, the transfer of these regions into a susceptible host did not transfer resistance. Nitrofurantoin susceptibility in *S. aureus* naturally spans a broad range and implying that nitrofurantoin resistance was not prevalent among a group of *S. aureus* and suggested an ECOFF for nitrofurantoin in *S. aureus* of 64 µg/mL. Thus, the universal susceptibility of staphylococci to nitrofurantoin and failure to select for nitrofurantoin resistance suggests that nitrofurantoin remains a viable option for the prevention and treatment of staphylococcal infections.

5.2 Introduction

Nitrofurantoin is an antibacterial agent belonging to the nitrofurans, a class characterised by the presence of a furan ring (a five-membered aromatic ring with four carbon atoms and one oxygen) and a nitro group (Figure 5.1) (Vass et al., 2008). Furazolidone and nitrofurazone are other antibiotics belonging to this group, and all display antibacterial activity and are employed clinically to treat several types of infections.

Nitrofurantoin is mainly restricted to the treatment of acute uncomplicated urinary tract infections (because at therapeutic doses nitrofurantoin only reaches the bactericidal concentrations in the urinary tract) caused by various species of bacteria (Cunha, 1989, Vass et al., 2008, Cunha et al., 2011, Munoz-Davila, 2014). It has proven efficacy against *E. coli*, *S. aureus*, *Enterobacter* spp., *Enterococcus* spp., and *Klebsiella* spp., but is not active against strains of *Pseudomonas.* spp., *Proteus.* spp. or *Serratia* spp. (Spencer et al., 1994, Brumfitt and Hamilton-Miller, 1998, Sandegren et al., 2008).

Nitrofurantoin is taken orally, and the most frequently reported side effects of nitrofurantoin in clinical trials are nausea, headache, urine discolouration and flatulence. Rare hepatotoxic and hypersensitivity reactions have occurred. Also, superinfections caused by resistant microorganisms and *Clostridium difficile*-associated diarrhoea can occur during or after treatment with nitrofurantoin (Koulaouzidis et al., 2007, Kiang et al., 2011).

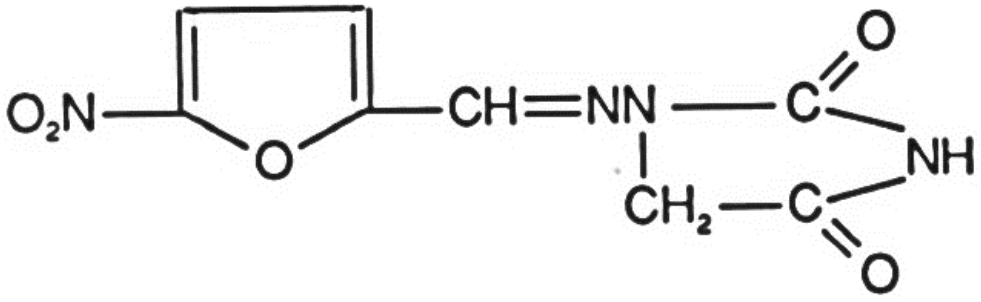

Figure 5.1 Chemical structure of nitrofurantoin. Taken from Munoz-Davila (2014).

5.2.1 Antibacterial mechanism of action of nitrofurantoin

Nitrofurantoin has a unique and complex mechanism of action when compared to other antibacterial agents. Nitrofurantoin is a prodrug which means it is inactive until becoming reductively activated inside the bacterial cell (McOsker and Fitzpatrick, 1994, Finch et al., 2003). This is achieved by the rapid reduction of nitrofurantoin with nitroreductases (NtrA, NfrA) to produce highly reactive intermediates. These intermediates are thought to disrupt and damage bacterial proteins, cell wall synthesis, DNA and other macromolecules within bacteria and lead to inhibition of growth or cell death (McOsker et al., 1989, Guay, 2001, Giske, 2015).

It has been reported that *E. coli* possesses two groups of nitroreductases: type I is insensitive to oxygen and type II which is sensitive to oxygen (Bryant et al., 1981, Sandegren et al., 2008). Based on chemical analysis, type I nitroreductases reduce the nitro-moiety of the prodrug to a biologically inactive end-product via a sequence of intermediates. Those intermediates that include a nitroso and hydroxylamine state are thought to be responsible for the antibacterial activity (McCalla et al., 1970, Peterson et al., 1979). It is believed that hydroxylamine is the bio-reactive intermediate, which binds and disrupts

bacterial proteins and DNA, but still, the specific identity of this toxic intermediate is unknown (McCalla et al., 1970, Tu and McCalla, 1975, Peterson et al., 1979). It is hypothesised that the broad spectrum of targets inhibited as a consequence of the mechanism of action of nitrofurantoin is likely to be responsible for the slow development of bacterial resistance to this compound (McOsker and Fitzpatrick, 1994, Bandow et al., 2003).

5.2.2 Medical applications of nitrofurantoin

Historically, nitrofurantoin was infrequently used in the treatment of uncomplicated urinary tract infections because multiple doses are required (up to 4 times per day), however, in recent years macrocrystalline forms have become more widely available, enabling twice-daily dosing (Gleckman et al., 1979, Shah and Wade, 1989, McKinnell et al., 2011, Giske, 2015). There is more than one form of nitrofurantoin available: Macrodantin; a macrocrystalline form, Furadantin; a microcrystalline form, and Macrobid; a sustained-release form of the macrocrystalline form. Usually, the macrocrystalline form is prescribed for patients who cannot tolerate the microcrystalline form. It also has a slower absorption rate than the microcrystalline form (Guay, 2001, McKinnell et al., 2011).

According to a randomised trial study focused on treating women with acute urinary tract infections, nitrofurantoin was as effective as trimethoprim /sulfamethoxazole (also known as co-trimoxazole) (Gupta et al., 2007). Another study that investigated ranges of uropathogens resistance considered nitrofurantoin a reasonable alternative to fluoroquinolones and trimethoprim/sulfamethoxazole (McKinnell et al., 2011). This idea is supported by IDSA (Infectious Diseases Society of America) recommendations that

nitrofurantoin is an appropriate choice and first-line empirical therapy for uncomplicated urinary tract infections because of its efficacy and minimal propensity for resistance and collateral damage (Gupta et al., 2011).

5.2.3 Mechanism of bacterial resistance to nitrofurantoin

Despite the availability and the usage of nitrofurantoin for more than six decades, resistance to nitrofurantoin has not been a substantial challenge among common urinary tract pathogens. No cross-resistance with other antibiotics has been detected, and transferable resistance is uncommon (Sandegren et al., 2008, McKinnell et al., 2011). This picture is uncommon amongst antibiotics used in this setting. For instance, the prevalence of nitrofurantoin resistance among urinary tract infection pathogens is very low in comparison with fluoroquinolones and trimethoprim/ sulfamethoxazole (Brumfitt and Hamilton-Miller, 1998, Karlowsky et al., 2002, McKinnell et al., 2011).

However, plasmid-mediated resistance to nitrofurantoin has been recognised since 1983, and it was characterised by a moderate increase in MIC (Breeze and Obaseiki-Ebor, 1983). There is a small number of studies regarding this kind of resistance, but one example is the efflux pump OqxAB which was found in a multidrug-resistant *E*. *coli* isolate (Ho et al., 2016). OqxAB is a RND-type efflux pump which has a wide substrate specificity including chloramphenicol, nalidixic acid, trimethoprim, ciprofloxacin, and benzalkonium (Hansen et al., 2007, Ho et al., 2016). The *oqxAB* genes were flanked with IS*26* elements and were carried by plasmids found in *E. coli* and *Salmonella* Enterica and also located in the chromosome of *Klebsiella* spp*.,* but without the IS*26* elements

(Li et al., 2013a, Guillard et al., 2015). It was suggested that plasmid-mediated OqxAB efflux pump is a crucial nitrofurantoin resistance mechanism, and it is sufficient to confer nitrofurantoin resistance (Ho et al., 2016).

Meanwhile, nitrofurantoin resistance has been reported in *E. coli* with resistance attributable to a decline in the activity of the nitroreductase enzymes (Sandegren et al., 2008). This resistance was attributed specifically to mutations in the *nfsA* and *nfsB* genes (oxygen-insensitive nitroreductase genes) which are responsible for the conversion of nitrofurantoin into its active form (Figure 5.2) (McCalla et al., 1978, Bryant et al., 1981, Giske, 2015).

It was demonstrated that there was a difference in the ability to reduce nitrofurantoin between nitrofurantoin-resistant and susceptible *E. coli* strains which suggests that if there is impairment in the bacterial reducing activity, it would lead to a decline in the nitrofurantoin antibacterial effect and hence reduce nitrofurantoin susceptibility (Asnis, 1957, Sandegren et al., 2008).

Several studies have linked this decline in the reductive capacity of nitrofuran-resistant mutants to step-wise mutations in the genes encoding the nitroreductase enzymes (Bryant et al., 1981, Breeze and Obaseiki-Ebor, 1983, Whiteway et al., 1998). Sequencing of resistant strains (clinical isolates and *in vitro*-isolated) revealed that first-step mutations occurred only in *nfsA* and the two-step mutants contained a second mutation in *nfsB* with a decrease in nitrofurantoin susceptibility (Sandegren et al., 2008). Collectively the main mechanism for nitrofurantoin resistance is suggested to be mediated by inactivation of *nfsA* followed by inactivation of *nfsB* (Sandegren et al., 2008). In contrast, it has been reported that under anaerobic conditions, increased

nitrofurantoin susceptibility of nitrofurantoin-resistant strains is observed, and it was thought to be due to the activity of the oxygen-sensitive reduction system (McCalla et al., 1978, Breeze and Obaseiki-Ebor, 1983, Whiteway et al., 1998).

Recently it has been shown in *E. coli* that mutations in *ribE* (a gene encoding lumazine synthase which is an important enzyme involved in the biosynthesis of bacterial nitroreductases) led to nitrofurantoin resistance, but this mechanism of nitrofurantoin resistance has so far not been described clinically (Figure 5.2) (Vervoort et al., 2014).

Additionally, nitrofurantoin resistance has been detected in *Clostridium* spp. Isolates and these Clostridium mutant strains exhibiting nitrofurantoin resistance were selected in the laboratory. They appeared to inactivate nitrofurantoin but without nitrofurantoin having any antibacterial activity on the cell, implying that mechanisms other than reduced nitroreductases activity can mediate nitrofurantoin resistance in bacteria (Rafii and Hansen, 1998).

Moreover, while nitrofurantoin is considered to be a key antibiotic for the treatment of uncomplicated urinary tract infections caused by different bacterial species including *S. aureus* (Gardiner et al., 2019), it is clear that there is a lack of information about nitrofurantoin resistance in *S. aureus.* Therefore, the objectives of this part of the study were set to provide new insights about nitrofurantoin resistance in *S. aureus.*

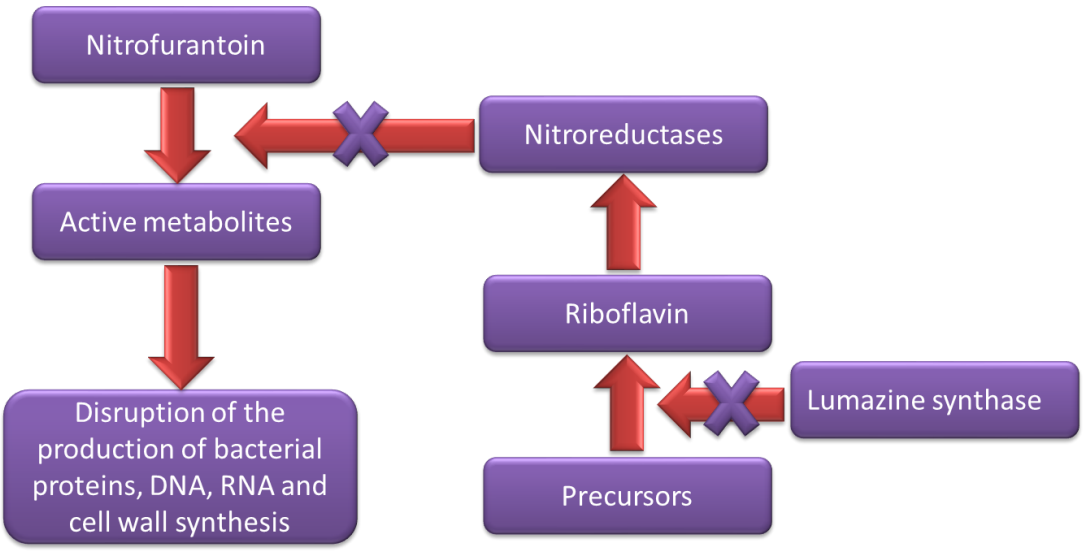

Figure 5.2 Diagram illustrating the mechanism of action of nitrofurantoin and the possible mutations in some genes which could lead to the development of resistance. Resistance can arise as a result of (1) mutations in the *nfsA* and *nfsB* genes which are responsible for the conversion of nitrofurantoin into its active form and (2) mutations in *ribE* that encode lumazine synthase which is an important enzyme involved in the biosynthesis of bacterial nitroreductases. Adapted from Giske (2015).

5.2.4 Prevalence of urinary tract infections

Because nitrofurantoin is commonly used for the treatment of urinary tract infections; it is important to provide an overview about the prevalence of urinary tract infections.

Urinary tract infections are a frequent cause of disease in clinical settings and the community and can be caused by a wide range of pathogens. The development of antibiotic resistant pathogens and the risk of microbial colonisation of urinary catheters complicates the control and treatment of these infections (Orenstein and Wong, 1999, Khan and Ahmed, 2001, Tandogdu and Wagenlehner, 2016). Urinary tract infection are among the most common bacterial infections in women and the elderly with symptoms ranging from mild self-limiting illness to severe sepsis that is associated with

a mortality rate of 20% to 40% especially in the older patient population (Gajamer et al., 2018, Gharbi et al., 2019). Also, the frequency of hospital-acquired urinary tract infections among all hospital-acquired infections is increasing significantly, with the prevalence ranging from 13% to 24% worldwide. It is estimated that in England 60% of urinary tract infections are associated with catheter insertions (Tandogdu and Wagenlehner, 2016, Thornley et al., 2019).

It is important to identify the causative pathogen of urinary tract infections, especially those associated with resistant strains to avoid treatment failure and bloodstream infections. The resistance rate to almost all available antibiotics in hospital-acquired urinary tract infections is more than 20% globally, and this rate varied significantly depending on the geographical location (Tandogdu and Wagenlehner, 2016). Urinary tract infection is the second condition where empirical antibiotics are being prescribed with about 50% of prescribed antibiotics considered to be unnecessary, especially in older patients (Crnich et al., 2017, Gharbi et al., 2019). As a result, the prevalence of antibiotic resistance and its associated threat to public health is increasing substantially (trimethoprim resistance has increased to 30% among *E. coli* urinary isolates), therefore it is prudent to have current clinical data to help choose the appropriate empirical therapy (Gajamer et al., 2018, Gharbi et al., 2019, Thornley et al., 2019).

5.2.5 Introduction to the current study

While the prevalence of nitrofurantoin resistance remained low, and the acquisition of resistance to nitrofurantoin is still relatively rare, an enormous

increase in the consumption of nitrofurantoin highlights the need to maintain vigilance in resistance surveillance. Additionally, the clinical information supporting the efficacy of nitrofurantoin in the treatment of bacterial infections that are commonly associated with antimicrobial resistance is very limited. Systematic surveillance of nitrofurantoin resistance is important particularly because it is considered to be a key antibiotic for the treatment of uncomplicated urinary tract infections caused by different species including *S. aureus* (Gardiner et al., 2019). In fact, a small number of studies have reported that nitrofurantoin resistance exists in clinical samples of *S. aureus,* but none of these studies investigated the mechanism of this resistance and also most of these studies did not screen a large number of isolates, which means that their findings cannot be representative for the clinical population of *S. aureus* (Brown and Ngeno, 2007, Eksi et al., 2008, Babakir-Mina et al., 2012).

Moreover, the most common urinary tract infection pathogens are *E. coli*, *Klebsiella* spp., *P. mirabilis*, *S. saprophyticus*, and *Enterobacter* spp. (Orenstein and Wong, 1999, Khan and Ahmed, 2001, Tandogdu and Wagenlehner, 2016), though it has been reported that the prevalence of *S. aureus* in urinary tract infections has increased (Aboderin et al., 2009, Looney et al., 2017). Although, *S. aureus* accounts for small cases of urinary tract infections and mainly affects hospitalised patients, the emergence of antibiotic resistant *S. aureus* is a global problem in clinical medicine (Flores-Mireles et al., 2015).

As mentioned earlier, *S. aureus* is considered to be one of the five most common pathogens of nosocomial infections, and there is no approved vaccine for *S. aureus* (Goetghebeur et al., 2007, Gordon and Lowy, 2008). It

is an opportunistic pathogen with the ability to cause a wide variety of life-threatening infections, and it carries a several multidrug-resistance genes on plasmids which help the distribution of antibiotic resistance among different species (Lowy 1998, Lowy, 2003). Although several effective antimicrobial agents are available, the mortality rate due to *S. aureus* infections is alarming. As quickly as a new antimicrobial agent is introduced for clinical use, *S. aureus* elaborates an effective mechanism to resist it (Mylotte et al., 1987, Lowy, 2003).

Based on the above, the overarching idea here was that there remain important gaps in our understanding of nitrofurantoin resistance in *S. aureus*, including prevalence and mechanism. Consequently, this part of the study first sought to examine the clinical prevalence of nitrofurantoin resistance in *S. aureus* isolates obtained from different locations around the UK and abroad by determining their susceptibility to nitrofurantoin. Second, it looked at the possibility for spontaneous nitrofurantoin-resistant mutants to arise among *S. aureus* to identify the ease of emergence of nitrofurantoin resistance in *S. aureus*. Thirdly, this part of the study searched for the genetic basis of nitrofurantoin resistance in *S. aureus* and if it can spread among *S. aureus* isolates.

5.3 Results

5.3.1 Nitrofurantoin susceptibility testing

A collection of *S. aureus* clinical isolates (*n*=1138), collected from around the UK and abroad, were screened for their susceptibility towards nitrofurantoin. Nearly 99% of the isolates (*n*=1130) were associated with nitrofurantoin MICs of 16 µg/mL or less (i.e. MIC_{90} = 16 µg/mL), while a small number of isolates (*n*=8) exhibited a degree of reduced susceptibility to nitrofurantoin (MICs ranging from 32 µg/mL to 64 µg/mL) (Figure 5.3).

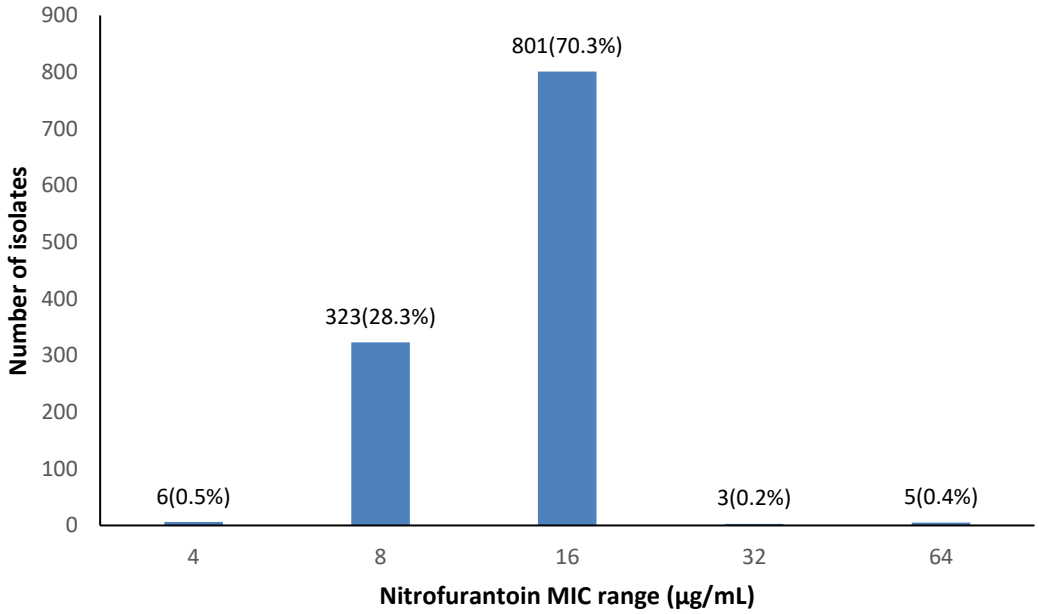

Figure 5.3 Nitrofurantoin susceptibility of 1138 clinical isolates of *S. aureus*. The number above each bar is the total number of isolates which had that specific MIC, and the number in brackets is the percentage for that number of isolates in relation to the total number of isolates.

5.3.2 Attempts to select for nitrofurantoin resistance *in vitro*

In initial studies, the author assessed - in laboratory studies - whether it is possible for nitrofurantoin resistance to develop through the endogenous route (by mutation) since failure to generate resistance by this route would suggest that nitrofurantoin resistance in clinical isolates is more likely the result of horizontally-acquired resistance determinants. Selection for nitrofurantoin resistance was approached in two ways, including determination of mutation frequency for nitrofurantoin resistance and extended passage in the presence of the drug.

No nitrofurantoin-resistant mutants were identified at the limit of detection of $<1.41 \times 10^{-9}$ cfu/mL when plating saturated cultures of the nitrofurantoin susceptible strain, *S. aureus* SH1000, onto agar containing nitrofurantoin at 4x MIC (64 µg/mL). Furthermore, no resistance to nitrofurantoin was selected during a 21 d extended gradient MIC selection experiment. Daptomycin was used as a control antibiotic in this latter experiment, and after 21 passages, the daptomycin MIC increased 32-fold to 16 µg/mL (Figure 5.4).

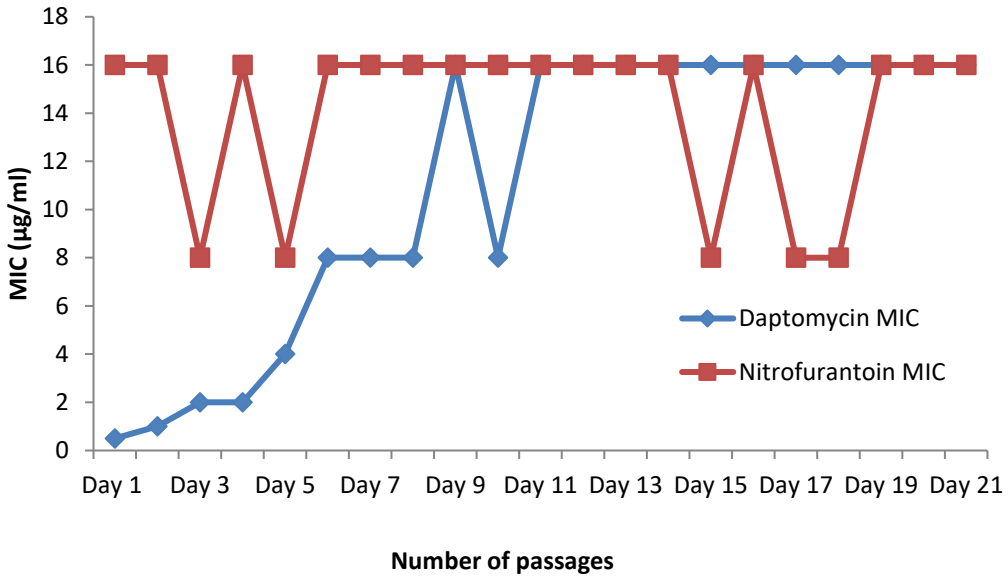

Figure 5.4 Failure to select resistance to nitrofurantoin following passaging of SH1000 (*n*=3) using the extended gradient MIC method. Daptomycin was used as a control antibiotic for the selection of resistance. The MIC value was recorded for each antibiotic over 21 d.

5.3.3 Investigating the genetic basis for reduced nitrofurantoin susceptibility

Since it was established that nitrofurantoin does not readily evolve via the endogenous route, it seemed more likely that the genetic basis for reduced nitrofurantoin susceptibility in these clinical isolates would involve horizontally acquired determinants. Therefore, in order to establish the genetic basis of reduced nitrofurantoin susceptibility, whole genome sequences for three strains exhibiting reduced susceptibility to nitrofurantoin (MIC 64 µg/mL) were generated (W80, W97 and W99). DNA genomes were sequenced using NGS technology, and sequence reads were imported into CLC Genomics Workbench 8.0, trimmed to remove low-quality bases (using standard settings) and subjected to de novo assembly (which also provided an estimate

of genome size) (Table 5.1). The GC content was within the expected percentage for *S. aureus* strains. Of note, the genome size of W97 strain is slightly bigger than expected for *S. aureus* strains, but this could be due to the presence of plasmids and phages. Therefore a BLAST search of the W97 genome sequence for positive hits of different *S. aureus* plasmids and phages was conducted to identify the reason of the big genome size (Ekblom and Wolf, 2014). Several *S. aureus* mobile genetic elements (plasmids, pathogenicity islands and phages) were found on the W97 genome which could explain why this genome is bigger than the usual *S. aureus* genome. So, the W97 genome sequence continued to be used in this study for further analysis.

After that, a gene BLAST library was compiled in CLC Genomic Workbench containing known resistance determinants for nitrofurantoin (mentioned in the introduction of this chapter), identified both from the published literature and from the NCBI database (*ribE* [NP_687761.1], *oqxA* [YP_001693237.1] and *oqxb* [YP_001693238.1]) (Tettelin et al., 2002, Hansen et al., 2004). Then the sequences of these resistance determinants were used as the search terms to check for the presence of any of these genes in the DNA sequences of the three isolates using CLC Genomic Workbench, but none were found.

Table 5.1 Summary of the quality measurements for the three sequenced strains

Measurements Criteria	W80	W97	W99
Size (bp)	3,877,140	4,640,136	3,918,849
GC Content (%)	36.8	39.2	37.2
N75	31476	24658	48576
N50	68625	61944	85147
N25	406218	642923	545765
Minimum Contig Length	829	1007	919
Maximum Contig Length	631646	424309	860975
Average Contig Length	5649	9239	6526
L50	14	16	13
Number of Contigs (with PEGs)	699	1059	715
Number of Reads	9000000	9000000	9000000
Matched Reads	8896391	8434988	8535377
Not matched Reads	103609	565,012	464,623

W80, W97 and W99 are the strains with reduced nitrofurantoin susceptibility. N75 is the minimum contig length needed to cover 75% of the genome. N50 is the minimum contig length needed to cover 50% of the genome. N25 is the minimum contig length needed to cover 25% of the genome. L50 is the number of contigs whose summed length is N50. PEGs stand for "protein-encoding genes".

In a similar manner as the bacitracin chapter, RAST was used to annotate the genomic sequences of the three strains with reduced nitrofurantoin susceptibility described above. Similarly, during the analysis, RAST provided a read-out of the closest neighbouring strains (as mentioned before in Chapter 4, Section 4.2.4) for each of these sequenced strains (Aziz et al., 2008,

Overbeek et al., 2014, Brettin et al., 2015). These closest neighbouring strains are the most closely related strains for which fully sequenced genomes exist in the public database. All three strains with reduced nitrofurantoin susceptibility have similar closest neighbouring strains. Consequently, three of these closest neighbouring strains (same as the bacitracin reference strains), which were available in the laboratory, were picked and tested for their susceptibility to nitrofurantoin. The nitrofurantoin MICs were 16 µg/mL for all the closest neighbouring strains, which means they could be considered as nitrofurantoin susceptible and they can be used as reference strains for the three strains with reduced nitrofurantoin susceptibility.

After that, the RAST output was used to perform further analysis to check if these three closest neighbouring strains are closely related to the three strains with reduced nitrofurantoin susceptibility. Therefore, an MLST approach was conducted to identify the sequence type and the clonal complex for each strain and how closely related they were. Among the three strains with reduced nitrofurantoin susceptibility and three of their closest neighbouring strains, the MLST analysis identified four different sequence types (ST5, ST22, ST36 and ST371) that belonged to three clonal complexes (CC5, CC22 and CC30) (Table 5.2). These clonal complexes are among the most frequent *S. aureus* MRSA clonal complexes associated with hospital acquired infections, and they are closely related, which supports the assumption that the six strains tested here are related (Figure 5.5) (Rijnders et al., 2009, Magro et al., 2018, Rasigade et al., 2018).

Table 5.2 Summary of the results of MLST analysis for the three reduced nitrofurantoin susceptibility strains and three of their closest neighbouring strains

Strain ID	Sequence Type (ST)	Clonal Complex (CC)
W80	22	CC22
W97	22	CC22
W99	36	CC30
VRSA1	5	CC5
VRSA2	5	CC5
VRSA3a	371	CC5

W80, W97 and W99 are the strains with reduced nitrofurantoin susceptibility. VRSA1 VRSA2 and VRSA3a are their closest neighbouring strains

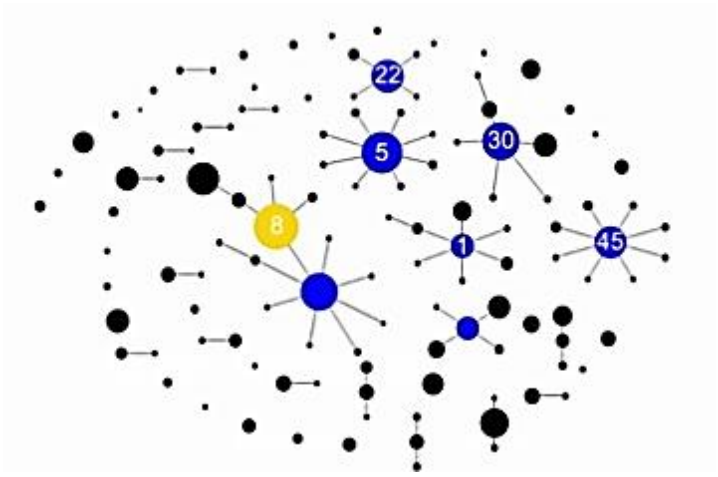

Figure 5.5 Schematic representation of the phylogenetic relationship between *S. aureus* clonal complexes. CC5, CC22 and CC30 are clustered next to each other on the top right side of the diagram, which indicates that these clonal complexes are closely related. Adapted from (Feil et al., 2003, Chambers and DeLeo, 2009, Rasigade et al., 2018).

Then, the results of the MLST analysis were used to provide further confirmation that these six strains were indeed related and to rule out the

possibility of contaminating/ incorrect strains. Therefore, it was important to obtain a phylogenetic relationship tree based on the sequence identity of 16S RNA, the 16S RNA was identified for each of the six strains by using the primers in (Edwards et al., 2012) to identify the 16S RNA sequence on each genome on CLC Genomic Workbench. Similarly, the 16S RNA sequences were identified in the same manner for several other closest neighbouring strains (*n*=7) that were obtained from RAST. 16S RNA sequence amplification and sequencing has been widely employed as a diagnostic tool for the identification of different bacterial species and also in detecting the diversity among microbial populations (Kommedal et al., 2009, Edwards et al., 2012). Here, universal PCR primers that specifically target short variable regions (V1-V9) within the 16S RNA gene were employed as the search term on the CLC Genomic Workbench to find the 16S RNA gene on each genome. After that, all the identified sequences of 16S RNA were uploaded to MEGA software to obtain a phylogenetic tree based on the sequence identity of 16S RNA. A neighbour-joining tree was constructed (Figure 5.6), and it showed that there is a relationship (i.e. they are related) between the strains with reduced nitrofurantoin susceptibility and their chosen closest neighbouring strains. Generally, in a phylogenetic tree, the tips of the trees are called the taxa, and the hypothetical ancestors are represented by the internal nodes. Hence, branches usually connect nodes and taxa, and several taxa that have the same ancestor are considered a clade (Takahashi et al., 2009, Hall, 2013). Usually the branching pattern of a phylogenetic tree indicates relatedness between different strains, and it is obvious from (Figure 5.6) that there are other strains that share most recent hypothetical ancestor (i.e. they are more

closely related) with the three strains with reduced nitrofurantoin susceptibility (W80, W97 and W99) rather than the three previously chosen closest neighbouring strains (VRSA1, VRSA2, VRSA3a). That can be explained by looking at the evolutionary distance between different taxa by adding up all the horizontal branch lengths that join them. The distance between W80, W97 and W99 and their chosen three closest neighbouring strains (VRSA1, VRSA2, VRSA3a) is longer than the distance between them (W80, W97 and W99) and other strains like S33R and VC40. Hence, taxa that share most recent common ancestors are more closely related, which means the choice of the closest neighbouring strains is rather speculative. However, they were used in this study because there is still a relationship between them and the three strains with reduced nitrofurantoin susceptibility and because these strains were considered as nitrofurantoin susceptible and were available in the O'Neill laboratory where the author worked. So those chosen closest neighbouring strains continued to be used in further analysis (genome comparison) as reference strains.

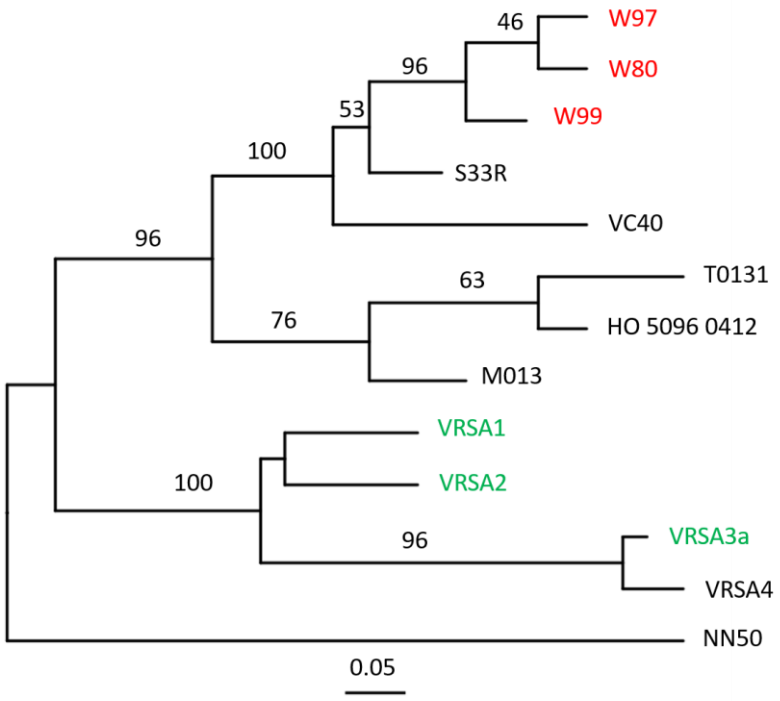

Figure 5.6 A phylogenetic tree obtained from MEGA for strains with reduced nitrofurantoin susceptibility (shown in red) and their closest neighbouring strains. Two of the strains with reduced nitrofurantoin susceptibility W97 and W80 are closely related, and both strains shared a common ancestor with W99 (the third strain with reduced nitrofurantoin susceptibility). The diagram also shows that there is a relationship between the W80, W97 and W99 and their chosen closest neighbouring (reference) strains (VRSA1, VRSA2, VRSA3a) (shown in green). But the distance between W80, W97 and W99 and their chosen three closest neighbouring (reference) strains (VRSA1, VRSA2, VRSA3a) is longer than the distance between them (W80, W97 and W99) and other strains like S33R and VC40 (they share most recent hypothetical ancestor). Bootstrap values with more than 50 % are shown on the nodes as percentages of 1000 replicates. The scale bar equals 0.05 change per nucleotide position.

Based on the literature, nitrofurantoin resistance in bacteria is usually the result of result of mutations in the nitroreductase genes (Breeze and Obaseiki-Ebor, 1983, Whiteway et al., 1998, Sandegren et al., 2008, Giske, 2015). Therefore, the next step was to determine if the reduced susceptibility towards nitrofurantoin identified here is caused by the same mechanism. So, a nucleotide-nucleotide search using the CLC Genomic Workbench 8.0 was

conducted for the sequences of the three strains with reduced nitrofurantoin susceptibility to identify the sequence and the location of all the nitroreductase genes which are available on the NCBI database and also commonly found in *S. aureus* genomes. Then, any BLAST hits in the sequences of the strains with reduced nitrofurantoin susceptibility showing 90-70% identity to those genes from the *S. aureus* genome were taken for further analysis.

Furthermore, pair wise alignment was performed using the online tool Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) for those nitroreductase genes which were identified by the BLAST search, by comparing these gene sequences of the three strains with reduced nitrofurantoin susceptibility and the three susceptible strains (closest neighbouring strains). They were examined for single-point mutations using Clustal Omega, but no such mutations were observed in those isolates when comparing all sequences on different sets. This suggests that the reduced susceptibility towards nitrofurantoin identified here was not caused by mutations in the nitroreductase genes.

Failure to identify likely resistance mutations in the nitroreductase genes and given the level of sequence similarity between the strains with reduced nitrofurantoin susceptibility and their reference strains (nitrofurantoin susceptible) led the author to conduct a comparative genome analysis to look for genes or regions (i.e. it might be possible to locate a mobile genetic element) which are unique to the strains with reduced nitrofurantoin susceptibility but are not their closest neighbouring strains. Therefore, MAUVE software was used to perform multiple alignments for the obtained DNA sequences to identify the differences and similarities between different

genomes of the strains with reduced nitrofurantoin susceptibility and the susceptible strains (closest neighbouring strains) (Darling et al., 2004, Darling et al., 2010).

First, the sequences of the three isolates with reduced nitrofurantoin susceptibility and three of their closest neighbouring strains were downloaded on MAUVE. Second, multiple alignments were performed for all six strains using MAUVE. Third, the alignment results were analysed visually by looking for unique regions or genes, which were only present in the three strains with reduced nitrofurantoin susceptibility (represented by the same coloured blocks on MAUVE) and could be considered candidate genes for conferring resistance (Figure 5.7 and Table 5.3). A 5 kb and 15 kb region were found to be unique to the three strains with reduced nitrofurantoin susceptibility, suggesting that the genes conferring nitrofurantoin reduced susceptibility could be in one of these regions.

Bioinformatic analysis did not identify any obvious candidates for nitrofurantoin resistance genes in these regions; the majority of the genes encoded hypothetical proteins, while the proteins of known or putative function are not obviously relevant to nitrofurantoin resistance (e.g. heavy metal homeostasis proteins) (Table 5.3, Appendix 5). Molecular biology experiments were therefore required to investigate whether these two regions participate in reduced nitrofurantoin susceptibility in those strains.

Table 5.3 Part of the Mauve alignment summary for the 5 kb region which was only found in the isolates with reduced susceptibility toward nitrofurantoin when comparing them with their closest neighbouring strains

Gene/ Protein name and position	W80	W97	W99	VRSA1	VRSA2	VRSA3a
Mobile Element Protein 441701 (Integrase)	√	√	√	X	X	X
Hydroxymethyl glutaryl-CoA synthase N-domain 442633	√	√	√	X	X	X
Phage Protein 459000	√	√	√	√	X	X
Hypothetical Protein 858045	√	√	√	X	X	X
Hypothetical Protein 875539	√	√	√	X	X	X
SA Bacteriophages 11, Mu50B 1258270	√	√	√	√	X	X
Hypothetical Protein Within Prophage 1289018	√	√	√	X	X	X
Type I Restriction Modification System 1712710	√	√	√	X	X	X
SA Bacteriophages 11, Mu50B 1826510	√	√	√	X	X	X
Hypothetical Protein Within Prophage 1840122	√	√	√	X	X	√

W80, W97 and W99 are the isolates with reduced susceptibility toward nitrofurantoin. VRSA1, VRSA2 and VRSA3a are the nitrofurantoin susceptible isolates (closest neighbouring strains/ reference strains).

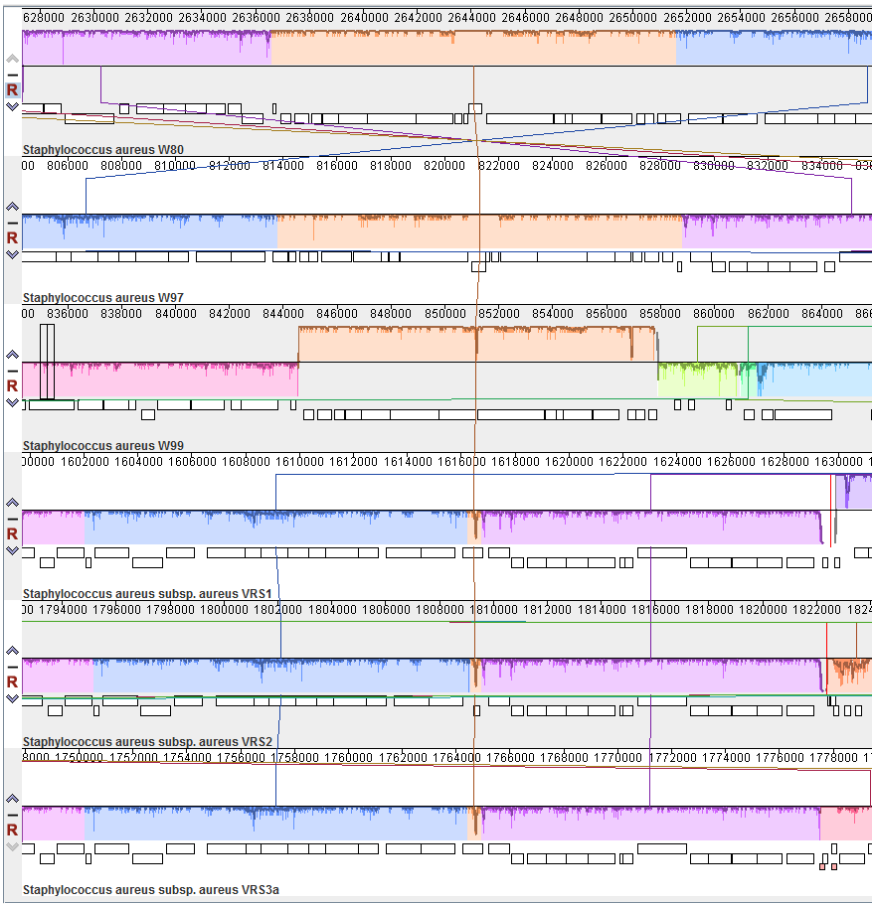

Figure 5.7 A screenshot from MAUVE for the alignment of the three nitrofurantoin reduced susceptibility strains with their closest neighbouring strains (nitrofurantoin-susceptible reference strains). The six horizontal sequences of blocks show ~30 kb regions of the genome sequences of W80, W97, W99, VRSA1, VRSA2 and VRSA3a. W80, W97 and W99 are the isolates with reduced susceptibility toward nitrofurantoin, whilst VRSA1, VRSA2 and VRSA3a are the nitrofurantoin susceptible reference strains. The large orange blocks in the sequences of the three strains with nitrofurantoin reduced susceptibility represent 15 kb fragments that are not present in the reference strains, suggesting that the genes conferring nitrofurantoin reduced susceptibility could be located in this region. The blue blocks represent a group of genes required for cell wall metabolism and coordination of cell division with DNA replication. The purple blocks represent a group of genes that are resistance or virulence-associated with a mobile element gene. The green block on W99 represents a couple of phage related genes (part of the 15 kb region) which are like those found in W80 and W97, but Mauve software annotated them differently. The genes organisation of W80 and W97 are arranged in the opposite order when compared to each other.

5.3.4 Determining if the two identified regions are responsible for reduced nitrofurantoin susceptibility

Regions of the chromosome uniquely found in the three nitrofurantoin reduced susceptibility strains isolates represent potential sites harbouring nitrofurantoin-resistance genes. To assess this, these regions were cloned and introduced into a susceptible strain to observe whether that resulted in any changes in nitrofurantoin susceptibility. Multiple sets of primers were designed for the amplification of portions of these two unique regions. One pair of primers was designed to amplify the 5 kb region while five sets of primers were generated to amplify the 15 kb region as a series of overlapping fragments to make sure that the possibility of multiple genes involved in nitrofurantoin resistance was assessed and also because it is hard to clone 15kb fragment at one time (5kb upper and lower, and 15kb 1-5 U and L, Appendix 3). The resulting amplicons were ligated into the *BstBI* site on the cloning vector pSK5487 and propagated in *E. coli* XL10-Gold. Once the colonies with the right insert were detected on the transformation plate by PCR amplification of the MCS of pSK5487 plasmid and confirmatory DNA sequencing, they were picked for plasmid extraction followed by transformation into *S. aureus* RN4220 (nitrofurantoin susceptible strain) competent cells by electroporation.

Transformation of these DNA fragments into RN4220 was successful, confirmed by detecting the required insert (one for the 5kb region and five for the 15 kb region) in 16 independent colonies each time by PCR using the correspondence cloning primers for each insert. However, no transformants showed any reduction in nitrofurantoin susceptibility, which stayed the same

as the WT RN4220 (MIC of 16 µg/mL). Similarly, RN4220 transformed with empty pSK5487 plasmid showed no change in nitrofurantoin susceptibility.

5.3.5 Transduction of putative nitrofurantoin resistance determinants into *S. aureus* RN4220

Since the above cloning experiment did not identify the genes responsible for nitrofurantoin resistance, an alternative approach was adopted that involved attempting to transfer nitrofurantoin resistance to a susceptible host by transduction. The transduction experiment was attempted multiple times using different bacteriophages (Φ80 and Φ11) to infect a variety of nitrofurantoin reduced susceptibility strains (donor strains) (*n*=8, MIC ≥32 µg/mL), and using *S. aureus* RN4220 (nitrofurantoin MIC of 16 µg/mL) as a recipient strain. As in Chapter 4, different selecting drug concentrations and exposure conditions were employed to induce the expression of nitrofurantoin resistance determinants and maximise the possibility of detecting resistance.

Briefly, after several experiments, W99 (one of nitrofurantoin reduced susceptibility strains) was successfully infected with Φ80 bacteriophage. Purified Φ80 bacteriophage particles recovered from W99 were used to infect RN4220, but no growth was observed on nitrofurantoin selection plates at 32 µg/mL. To confirm the efficiency of transduction, the experiment was repeated and the transfer of an unrelated marker (erythromycin resistance) was monitored to verify successful transduction. Erythromycin transductants were constructed in the same manner as the nitrofurantoin transductants because it was shown before that chromosomally located erythromycin resistance was able to be transferred by transduction.

The transduction experiment was conducted as previously mentioned in Section 2.5.9/ Chapter 2 but with the modifications in the antibiotic exposure conditions (as per Chapter 4) to ensure induction of the expression of any inducible nitrofurantoin resistance determinants. Although transduction of the erythromycin resistance determinants was successful at a frequency of $2.4X10^{-6}$ transductants per plaque forming unit, unfortunately no transductants that were able to confer nitrofurantoin resistance were detected. Only erythromycin-resistant transductants were able to be recovered on erythromycin plates (8 µg/mL), and no nitrofurantoin resistant transductants were detected on nitrofurantoin plates (32 µg/mL).

5.4 Discussion

This chapter focused on studying the prevalence and the basis of nitrofurantoin resistance in *S. aureus.* There was universal susceptibility of staphylococci to nitrofurantoin, and nitrofurantoin resistant mutants could not be selected (Figure 5.3 and Figure 5.4). Strains exhibiting reduced susceptibility to nitrofurantoin did not appear to possess a discrete resistance determinant that could be transferred to a susceptible host by transduction. Furthermore, whole genome sequencing and genetic comparison identified two regions (5 and 15 kb in size) only present in the strains with reduced nitrofurantoin susceptibility (Figure 5.7 and Table 5.3); however, transfer of these regions into a susceptible host did not result in co-transfer of resistance. Failure to select nitrofurantoin resistance in *S. aureus* after prolonged exposure to the drug contrasts with literature reports describing selection of nitrofurantoin resistance in other Gram-positive pathogens such as clostridia, for which nitrofurantoin-resistant mutants could be recovered at a frequency of 10^{-5} to 10^{-7} (Rafii and Hansen, 1998).

Determination of the susceptibility profile of nitrofurantoin in *S. aureus* could provide guidance for setting up a clinical breakpoint (a chosen concentration of an antibiotic which defines whether a species of bacteria is clinically susceptible or resistant to the antibiotic) (Kahlmeter et al., 2003). That would be of significance especially because no ECOFF value has been defined for nitrofurantoin in *S. aureus* (Turnidge and Paterson, 2007, EUCAST, 2017). The MIC breakpoint for nitrofurantoin in Gram-negative bacteria is well defined to be 32 µg/mL based on a large-scale study (Kahlmeter, 2000). Also, FDA,

EUCAST and CLSI guidelines suggest that the MIC breakpoint for nitrofurantoin in uropathogens, in general, is between 32 and 64 µg/mL (Shakti and Veeraraghavan, 2015, EUCAST, 2017). Thus, a bacterial strain with nitrofurantoin MIC equal or greater than 64 µg/mL is generally considered to be resistant, but this is a breakpoint that is not necessarily appropriate to *S. aureus*. Also, because of the large sample size and the geographical distribution of the isolates tested here, the results obtained in this study imply that nitrofurantoin susceptibility in *S. aureus* naturally spans a broad range. Furthermore, the ECOFF point for nitrofurantoin in *S. aure*us is 64 µg/mL, but further studies are required to investigate this.

Since no strain in this study grew above the MIC breakpoint in this panel of isolates, no instances of nitrofurantoin resistance were detected in this collection of *S. aureus*. Accordingly, the prevalence of nitrofurantoin resistance in *S. aureus* has generally been reported in the literature to be low, with figures ranging from 1.6 % to 3.8 %. Still, the information about nitrofurantoin resistance was limited because no further investigation on how this mechanism could emerge had been done and sometimes the sample size was small (Brown and Ngeno, 2007, Eksi et al., 2008, Babakir-Mina et al., 2012).

In terms of further confirmation of the nitrofurantoin susceptibility profile and to set up a break point in *S. aureus*, it will be essential to conduct large scale studies which link MIC values with treatment success/failure when using nitrofurantoin, with isolates obtained from a wide range of geographical places (to better represent the wider *S. aureus* population).

Of note, the genome size of the W97 strain is slightly bigger than expected for *S. aureus* strains, but the GC content was within the expected range for *S.*

aureus strains (Table 5.1). Likewise, the contig and read measurement criteria for the W97 genome and the genomes of the other two sequenced strains (W80 and W99) were similar to each other, which might indicate that there is no issue with the W97 sequencing data. Interestingly, a BLAST search of the W97 genome sequence identified several *S. aureus* plasmids, phages, and pathogenicity islands which could explain why the W97 genome is bigger than the usual *S. aureus* genome (Ekblom and Wolf, 2014). Moreover, the results of the MLST analysis further confirms that the data for the W97 genome is suitable to work with. Therefore, the W97 genome sequence continued to be used in this study for further analysis.

Overall, based on the whole genome sequencing and data analysis, it was not possible to detect any single-point mutation in the nitroreductase genes in the nitrofurantoin reduced susceptibility strains. Nitrofurantoin resistance without the loss of nitroreductase activity had been previously reported more than once (Breeze and Obaseiki-Ebor, 1983, Sastry and Jayaraman, 1984, Rafii and Hansen, 1998). Conversely, nitrofurantoin resistance has been observed to be conferred by mutations in the oxygen-insensitive nitroreductases (*nfsA* and *nfsB)* as seen with prior work in *E. coli* (McCalla et al., 1978, Sandegren et al., 2008). Therefore, the basis of reduced nitrofurantoin susceptibility in *S. aureus* observed in the clinical isolates of this study could not be attributed to nitroreductase genes at present.

To investigate this further, an experiment involving the reduction of the production of different nitroreductase enzymes can be conducted to determine if any of those enzymes are responsible for this reduced susceptibility. Also, there is a possibility that mutations have been acquired by any other

uncharacterised genes (but could not be characterised here in this study) lead to reduced susceptibility towards nitrofurantoin.

Whole genome sequencing and genetic comparison identified two genetic regions only present in the strains with reduced nitrofurantoin susceptibility (Figure 5.7 and Table 5.3). However, the transfer of these regions into a susceptible host did not transfer resistance. This implies that the recipient strain may be unable to express the proteins of interest properly, leading to the presence of the insert with no function being deployed. Sometimes, protein misfolding or aggregation can lead to protein degradation and insufficient expression. It is possible to solve this by expressing additional chaperones (proteins) at the same time as the protein of interest to help a proper folding of proteins, but this can promote a decline in the expression of proteins (Glick and Whitney, 1987, Schumann and Ferreira, 2004, Fakruddin et al., 2013). Another explanation is that these two regions might not be responsible for reduced nitrofurantoin susceptibility in those strains and that there might be other regions or genes responsible for nitrofurantoin resistance.

Of note, strains exhibiting reduced susceptibility to nitrofurantoin did not appear to possess a discrete resistance determinant that could be transferred to a susceptible host by transduction. The success of a transduction experiment is usually recognised by the transfer of a specific marker (usually a resistance or virulence gene) from one strain to another. Therefore, transduction would create a strain that had never existed before like the case here with erythromycin resistant transductants.

Additionally, there are several factors that could cause the problem encountered here during the recovery of transductants. For instance, reduced

susceptibility could be caused by multiple polymorphisms at different sites on the chromosome so that it could not be transduced. This idea is perhaps corroborated by the fact that nitrofurantoin resistance is fairly low-level in *S. aureus* as the strains with reduced nitrofurantoin susceptibility only have 4 times the MIC (4X MIC) of some of the susceptible strains in this study. In addition, another explanation is that nitrofurantoin resistance determinants may be located on a chromosome or large (over 40-45 kb) operonic structure which could make the transfer of those genes by bacteriophage critical or maybe there is an expression problem for those genes in the recipient strain. Also, one of the causes of transduction failure is that the bacteriophage particles released from the infected cells can kill the transductants, but this was controlled by the transfer of erythromycin resistance (Balcazar, 2014, Sackman et al., 2015).

Chapter 6 General Conclusions

The process of bacterial evolution, the widespread of antibiotics, and misguided treatment strategies contribute substantially to the problem of antibiotic resistance (Palmer and Kishony, 2013). Nonetheless, resistance to certain antimicrobial agents is poorly understood, with knowledge gaps regarding both the prevalence and genetic basis of resistance. Hence, understanding different mechanisms of resistance and the availability of updated epidemiological data on the resistance of antimicrobial agents in commonly encountered bacterial pathogens will help collectively on choosing the right treatment strategy, deploying effective antimicrobial stewardship programs and in designing new antimicrobial agents (Ventola, 2015b, Frieri et al., 2017).

Therefore, in this research, the epidemiological susceptibility, the genetic basis, and the propensity of certain bacterial species to develop resistance to three antimicrobial agents (silver, bacitracin, and nitrofurantoin) in clinically significant pathogens were investigated. This research could provide insights into the susceptibility and mechanism of resistance to these agents, and thus by deploying these data in clinical settings, it would guide clinicians to select genotype-based treatments against pathogens that are most resilient to developing resistance (Morar and Wright, 2010, Novais et al., 2010).

In Chapter 3, overt silver resistance was not found to be prevalent in a cross-section of clinical Gram-negative isolates. Nevertheless, a large proportion of *Klebsiella* spp. and *Enterobacter* spp. isolates have the potential to readily acquire high-level (MIC >128 μg/mL) silver resistance with resistance arising apparently as a consequence of single point mutations in *silS* that led to

activation of the Sil system. For these genera, the development of silver-resistant strains is likely in clinical settings, especially with the high frequencies ($\sim 10^{-8}$) with which silver resistance emerged in these isolates, which are comparable to those seen for antimicrobial agents that act upon a single cellular target (O'Neill and Chopra, 2004). In these strains, spontaneous mutation in *silS* leads to changes in the encoded regulator that likely causes a sensory 'false positive' or confers a phosphatase-minus phenotype. It would be of value to confirm the role of these SilS mutations in silver resistance by generating a reporter construct in *E. coli* using the first part of *sil* operon then cloning the *silS* which have a mutation (Gerlach et al., 2007, Clark et al., 2019). Also, it appears that the presence of *silS* is synonymous with the potential for spontaneous resistance to arise, and therefore PCR-based detection – or alternatively, mutation frequency determinations to silver resistance - could in principle be employed by clinicians to detect strains that carry the *sil* genes and are therefore associated with a risk of evolving overt silver resistance. According to the *in vitro* results presented here, cryptic silver resistance has the potential to compromise the efficacy of silver treatment.

In Chapter 4 of this study, it was identified that bacitracin resistance was not prevalent in a collection of *S. aureus* strains, and it could not be readily achieved by mutation. These findings suggest that in *S. aureus,* bacitracin resistant mutants are unlikely to arise during treatment, and that acquired resistance is more likely to arise by the exogenous route (horizontal gene transfer). This study established an ECOFF for *S. aureus*, which indicates that strains can be considered resistant if their MICs to bacitracin are ≥512 μg/mL. Transformation and transduction experiments showed that bacitracin

resistance is transferable, and that the *bcrABRS/bacA* locus was responsible for resistance in the isolates under study.

These findings represent the first-time bacitracin resistance in *S. aureus* has been characterised and studied at the genetic level. It would be beneficial to perform an experiment that involves generating constructs (e.g. transposon mutagenesis) to select for *S. aureus* mutants with different *bcr* genes being deleted and also test different levels of protein expression to identify which *bcr* genes are responsible for this resistance (Buhlmann et al., 2003, Manson et al., 2004, Godbole et al., 2015). Also, to determine if the pNRS384 plasmid is conjugative or mobilizable a conjugation experiment could be performed in the presence and absence of a conjugative plasmid (Hobot, 2015, Møller et al., 2017). Although the clinical use of bacitracin is limited to topical application in humans, these findings might have significant implications for the human and veterinary use of bacitracin, since they reveal that bacitracin resistance is transferrable among *S. aureus* strains and can be carried alongside multiple other antibiotic resistance genes on plasmids.

In Chapter 5, after examining the nitrofurantoin susceptibility profile of a group of *S. aureus* isolates, it was apparent that MICs for nitrofurantoin naturally span a broad range. No nitrofurantoin resistance was detected, and a suggested ECOFF point for nitrofurantoin resistance in *S. aureus* is therefore 64 µg/mL. Whole genome sequencing and genetic comparison with fully nitrofurantoin-susceptible isolates identified two genetic regions (5 and 15 kb regions) only present in strains that exhibit reduced susceptibility to nitrofurantoin. However, transfer of these regions into a susceptible host did not result in transfer of reduced nitrofurantoin susceptibility. Strains exhibiting

reduced susceptibility to nitrofurantoin did not, therefore, appear to possess a discrete resistance determinant that could be transferred to a susceptible host by transduction. Thus, it seems that the activity of nitrofurantoin against staphylococci is not under imminent risk from resistance.

Further studies are required to investigate nitrofurantoin susceptibility in *S. aureus* by conducting large scale studies which link MIC values with treatment success/failure when using nitrofurantoin, with isolates obtained from a wide range of geographical sources (Falagas et al., 2012). To further investigate if the nitroreductase enzymes are responsible for nitrofurantoin reduced susceptibility, an experiment could be conducted which involves reducing the production of different nitroreductase enzymes either by bioinformatic approach or artificially to examine if these genes or their upstream expression signals vary depending on their nitrofurantoin susceptibility (Gräslund et al., 2008, Lipońska et al., 2019).

Based on these findings, nitrofurantoin could therefore have a wider application for treating infections caused by *S. aureus*, since the *in vitro* studies described here suggest that the likelihood of resistance developing are low. Nevertheless, the fact that nitrofurantoin resistance has already been reported in several bacterial species to date means that the potential for a future nitrofurantoin resistance problem in *S. aureus* cannot be ruled out.

Together, this research provides insights into the susceptibility and the characterisation of the genetic elements involved in resistance against silver, bacitracin and nitrofurantoin, which would be potentially employed to guide their future applications as antimicrobial agents and to underpin future work to dissect the biochemical and structural basis for resistance.

Appendix 1 Antimicrobial agents and chemical reagents used in this study

Antimicrobial Agent/ Chemical Reagent	Manufacturer
Silver nitrate	Sigma-Aldrich, St. Louis, USA
Bacitracin	Sigma-Aldrich / St. Louis/ USA
Nitrofurantoin	Sigma-Aldrich / St. Louis/ USA
Erythromycin	Sigma-Aldrich / St. Louis/ USA
Ampicillin	Sigma-Aldrich / St. Louis/ USA
Tetracycline	Sigma-Aldrich / St. Louis/ USA
Cefoxitin	Sigma-Aldrich / St. Louis/ USA
Penicillin G	Sigma-Aldrich / St. Louis/ USA
Oxacillin	Sigma-Aldrich / St. Louis/ USA
Kanamycin	Sigma-Aldrich / St. Louis/ USA
Daptomycin	Cubist Pharmaceuticals
Chloramphenicol	Sigma-Aldrich / St. Louis/ USA
Calcium chloride	Sigma-Aldrich / St. Louis/ USA
Sodium citrate	BDH Laboratory, Poole, UK
Yeast extract	Milford Laboratories, Ipswich, UK
Sucrose solution	Sigma-Aldrich / St. Louis/ USA

Appendix 2 List of NARSA strains used in this study

NARSA Strain	Phenotypic Description
NRS3	*S. aureus*, strain HIP5827 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP5827 was deposited as positive for *mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, and *vanE*; MLST sequencing type (ST) 5; eGenomic *spa* type 12, eGenomic *spa* repeats TJMGMK; Ridom *spa* type t062.
NRS4	*S. aureus*, strain HIP5836 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP5836 was deposited as positive for *mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD* and *vanE*; MLST sequencing type (ST) 5; eGenomic *spa* type 230, eGenomic *spa* repeats TMBMDMGMK; Ridom *spa* type t010.
NRS11	*S. aureus*, strain SA MER was deposited as a heterogeneous vancomycin-intermediate *S. aureus* (hVISA) strain, but unlike most hVISA strains, it is susceptible to methicillin. *S. aureus*, strain SA MER was deposited as resistant to benzylpenicillin; negative for *mecA*, *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD* and *vanE*; MLST sequencing type (ST) 5; eGenomic *spa* type 2, eGenomic *spa* repeats TJMBMDMGMK; Ridom *spa* type t002. SA MER was the parental strain used to derive strains SA MER-S6 (NRS-12), SA MER-S12 (NRS13) and SA MER-S20 (NRS14) by exposing this strain to increasing levels of vancomycin resulting in the strains growing in the presence of 6 µg/mL, 12 µg/mL and 20 µg/mL vancomycin, respectively.
NRS17	*S. aureus*, strain HIP06297 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP06297 was deposited as positive for *mec* (subtype II); negative for the vancomycin resistance genes; MLST sequencing type (ST) 5; eGenomic *spa* type 2, eGenomic *spa* repeats TJMBMDMGMK; Ridom *spa* type t002. *S. aureus*, strain HIP06297 is reported to be resistant to erythromycin, ciprofloxacin and rifampin and susceptible to trimethoprim/sulfamethoxazole, chloramphenicol, and tetracycline. It is hypothesized that this VISA strain emerged from a methicillin-resistant *S. aureus* (MRSA) strain isolated from the patient during an infection 3 months prior. Based on pulsed-field electrophoresis gel patterns, *S. aureus*, strain HIP06297 is closely related to the multi-drug resistant New York clone.
NRS19	*S. aureus*, strain HIP07256 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP07256 was deposited as positive for *mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, and *vanE*; MLST sequencing type (ST) 5; eGenomic *spa* type 2, eGenomic *spa* repeats TJMBMDMGMK; Ridom *spa* type t002.
NRS21	*S. aureus*, strain HIP07920 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP07920 was deposited as positive for *mec* (subtype IV); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, and *vanE*; MLST sequencing type (ST) 8; eGenomic *spa* type 7, eGenomic *spa* repeats YHGCMBQBLO; Ridom *spa* type t064.
NRS22	*S. aureus*, strain HIP07930 is a hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) strain. Strain HIP07930 was deposited as resistant to erythromycin, clindamycin, trimethoprim/sulfamethoxazole, gentamicin and levofloxacin; positive for *mec* (subtype II); negative for PVL, *tsst*, *sea*, *seb*, *sec*, *sed* and *see*; MLST sequence type (ST) 45; pulsed-field type USA600; eGenomic *spa* type 10, eGenomic *spa* repeats A2AKEEMBKM; Ridom *spa* type t266; *agr* group I.S. aureus, strain HIP 07930 is a USA600 isolate. USA600 isolates have the same MLST profile (ST 45), SCC*mec* (subtype II or IV), *agr* group (I) and *spa* repeats (A2AKEEMBKB). They are PVL negative and resistant to erythromycin and

	clindamycin. Isolates are predominantly found in nares of healthy individuals and bloodstream infections. These isolates are associated with a higher rate of clinical failure and mortality, particularly those with a heterogeneous vancomycin-intermediate *S. aureus* (hVISA) phenotype. While USA600 isolates are uncommon the United States, the clonally-related Berlin strain is widespread throughout Germany, the Netherlands, and Ontario, Canada.
NRS24	*S. aureus*, strain HIP09143 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP09143 was deposited as positive for *mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, and *vanE*; MLST sequencing type (ST) 5; eGenomic *spa* type 230, eGenomic *spa* repeats TMBMDMGMK; Ridom *spa* type t010.
NRS27	*S. aureus*, strain HIP09433 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP09433 was deposited as positive for *mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, and *vanE*; MLST sequencing type (ST) 45; eGenomic *spa* type 15, eGenomic *spa* repeats A2AKEEMBKB; Ridom *spa* type t004.
NRS35	*S. aureus*, strain LIM 1 is a methicillin-resistant *S. aureus* (MRSA) and a vancomycin-sensitive *S. aureus* (VSSA) strain. *S. aureus*, strain LIM 1 was deposited as positive for *mec* (subtype I); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, and *vanE*; MLST sequencing type (ST) 572; eGenomic *spa* type 4, eGenomic *spa* repeats YHFGFMBQBLO; Ridom *spa* type t051. Strain LIM 1 was the first MRSA isolate recovered from this patient. During the course of treatment, which included the use of glycopeptides, three additional MRSA strains, LIM 2 (NRS36), LIM 3 (NRS37) and LIM 4, were isolated and found to have a vancomycin-intermediate *S. aureus* (VISA) phenotype. Results from pulsed-field gel electrophoresis suggest that these three VISA strains were selected from LIM 1.
NRS51	*S. aureus*, strain HIP09740 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP09740 was deposited as positive for *mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, and *vanE*; MLST sequencing type (ST) 5; eGenomic *spa* type 24, eGenomic *spa* repeats TJMEMDMGMK; Ridom *spa* type t242. Two VISA strains were isolated from this patient, strain HIP09740 and strain HIP09737 (NRS52), which have identical antibiotic susceptibility profiles except for oxacillin and have closely related pulsed-field gel electrophoresis (PFGE) types.
NRS54	*S. aureus*, strain BR 15 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain BR 15 was deposited as positive for *mec* (subtype III); negative for the vancomycin resistance genes; MLST sequencing type (ST) 239; eGenomic *spa* type 3, eGenomic *spa* repeats WGKAOMQ; Ridom *spa* type t037. Based on MLST sequencing type and the SCC*mec* type, *S. aureus*, strain BR 15 is most likely an isolate of the Brazilian Endemic Clone (BEC) which is reported to represent almost 80% of MRSA isolates in Brazil.
NRS56	*S. aureus*, strain BR 5 is a methicillin-resistant *S. aureus* (MRSA) strain and a vancomycin-intermediate *S. aureus* (VISA) strain. It is also reported to be resistant to a number of other antibiotics. *S. aureus*, strain BR 5 was deposited as positive for *mec* (subtype III); negative for the vancomycin resistance genes; MLST sequencing type (ST) 239; eGenomic *spa* type 3, eGenomic *spa* repeats WGKAOMQ; Ridom *spa* type t037. Based on MLST sequencing and the SCC*mec* type, *S. aureus*, strain BR 5 is most likely an isolate of the Brazilian Endemic Clone (BEC) which is reported to represent almost 80% of MRSA isolates in Brazil.
NRS65	*S. aureus*, strain LY-1999 0620-03 is a glycopeptide-intermediate *S. aureus* (GISA) strain. *S. aureus*, strain LY-1999 0620-03 was deposited as positive for *mec* (subtype III); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD* and *vanE*; MLST sequencing type (ST) 372; eGenomic *spa* type 3, eGenomic *spa* repeats WGKAOMQ;

	Ridom *spa* type t037. Strain LY-1999 0620-03 and strain LY-1999 0620-02 (NRS64) were isolated on the same day from the patient and are related by pulsed-field gel electrophoresis (PFGE).
NRS103	*S. aureus*, strain Becker is a methicillin-sensitive *S. aureus* (MSSA) strain. Strain Becker was deposited as displaying spontaneous rifampicin resistance; negative for *mecA*; MLST sequence type (ST) 508; eGenomic *spa* type 715, eGenomic *spa* repeats XKAKBMBKB; Ridom *spa* type t630. Strain Becker is a prototype strain for capsular polysaccharide type 8, which is the most commonly recovered capsular serotype in human isolates. It is believed that capsules produced by these polysaccharides enhance *S. aureus* virulence by impeding phagocytosis as well as promoting abscess formation.
NRS883	*S. aureus*, strain TN-305 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain TN-305 was deposited as positive for *mec* (subtype IV) and PVL; negative for *tsst*; pulsed-field type USA100.
NRS18	*S. aureus*, strain HIP06854 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP06854 was deposited as positive for *mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, and *vanE*; MLST sequencing type (ST) 5; eGenomic *spa* type 2, eGenomic *spa* repeats TJMBMDMGMK; Ridom *spa* type t002.
NRS170	*S. aureus* strain of unknown origin
NRS192	*S. aureus*, strain C1999000193 is a community-associated methicillin-resistant *S. aureus* (CA-MRSA) strain. *S. aureus*, strain C1999000193 was deposited as positive for *mec* (subtype IV), PVL and the staphylococcal enterotoxin gene *seb*; negative for *tst*; MLST sequence type (ST) 1; eGenomic *spa* type 194, eGenomic *spa* repeats UJFKKPFKPE; Ridom *spa* type t175. Based on pulsed field gel electrophoresis, *S. aureus*, strain C1999000193 is closely related to *S. aureus* strains MW2 (NRS123), and C1999000529 (NRS194).
NRS218	*S. aureus* strain of unknown origin
NRS248	*S. aureus* strain of unknown origin
NRS263	*S. aureus* strain of unknown origin
NRS644	*S. aureus* strain of unknown origin
NRS654	*S. aureus* strain of unknown origin
NRS658	*S. aureus* strain of unknown origin
NRS663	*S. aureus* strain of unknown origin
NRS108	*S. aureus*, strain A960649 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain A960649 was deposited as positive for *mec* (subtype I), 4'-4"-aminoglycoside nucleotidyltransferase (*ant4'*; responsible for tobramycin resistance) and 2"-aminoglycoside phosphotransferase-6'-aminoglycoside acetyltransferase (*aac6'-aph2"*; responsible for amikacin, tobramycin and gentamicin resistance); MLST sequence type (ST) 8; eGenomic *spa* type 1, eGenomic *spa* repeats YHGFMBQBLO; Ridom *spa* type t008.
NRS114	*S. aureus*, strain MNHOCH is a methicillin-sensitive *S. aureus* (MSSA) strain. Strain MNHOCH was deposited as positive for *seb*; negative for *mec*; MLST sequence type (ST) 8; eGenomic *spa* type 363, eGenomic *spa* repeats YGFMBQBLO; Ridom *spa* type t024. *S. aureus*, strain MNHOCH is a staphylococcal enterotoxin B (SEB) producing strain. SEB, like other staphylococcal enterotoxins, is a highly stable, heat and proteolytic resistant, secreted protein that is a cause of TSS and staphylococcal food poisoning.
NRS119	*S. aureus*, strain SA LinR #12 is a linezolid-resistant *S. aureus* (LRSA), methicillin-resistant *S. aureus* (MRSA) strain. It was deposited as resistant to linezolid and tedizolid; positive for *mec* (subtype IV); MLST sequence

	type (ST) 507; eGenomic *spa* type 7, eGenomic *spa* repeats YHGCMBQBLO; Ridom *spa* type t064. *S. aureus*, strain SA LinR #12 was co-isolated with SA LinR #13 (NRS120) and SA LinR #14 (NRS121) from the first clinically reported case of a MRSA infection that demonstrated resistance to linezolid. Based on pulsed-field gel electrophoresis, SA LinR #12 and SA LinR #13 are identical and SA LinR #14 is closely related to both. While each strain has a different antibiogram, all three are resistant to linezolid due to a G2576T mutation in domain V in one or more 23S rRNA genes (*Escherichia coli* numbering).
NRS121	*S. aureus*, strain SA LinR #14 is a methicillin-resistant *S. aureus* (MRSA) strain. It was deposited as resistant to linezolid; positive for *mec* (subtype IV); MLST sequence type (ST) 507; eGenomic *spa* type 7, eGenomic *spa* repeats YHGCMBQBLO; Ridom *spa* type t064. *S. aureus*, strain SA LinR #14 was co-isolated with SA LinR #12 (NRS119) and SA LinR #13 (NRS120) from the first clinically reported case of a MRSA infection that demonstrated resistance to linezolid. Based on pulsed-field gel electrophoresis, SA LinR #12 and SA LinR #13 are identical and SA LinR #14 is closely related to both. While each strain has a different antibiogram, all three are resistant to linezolid due to a G2576T mutation in domain V in one or more 23S rRNA genes (*Escherichia coli* numbering).
NRS127	*S. aureus*, strain M1712 is a linezolid-resistant *S. aureus* (LRSA), methicillin-resistant *S. aureus* (MRSA) strain. It was deposited as resistant to linezolid and intermediately susceptible to tedizolid; positive for *mec* (subtype II); MLST sequence type (ST) 5; eGenomic *spa* type 2, eGenomic *spa* repeats TJMBMDMGMK; Ridom *spa* type t002. *S. aureus*, strain M1712 is reported to have a ΔS145 mutation in the L3 ribosomal protein.
NRS167	*S. aureus*, strain A970675 is a methicillin-sensitive *S. aureus* (MSSA) strain. *S. aureus*, strain A970675 was deposited as negative for *mec*; positive for the enterotoxin gene cluster (*egc*) operon, the exfoliatin A gene (*eta*) and the hemolysin genes *hla*, *hld* and *hlg*; MLST sequencing type (ST) 1446; eGenomic *spa* type 886, eGenomic *spa* repeats TKR; Ridom *spa* type t1977; *agr* group I.
NRS172	*S. aureus*, strain A970230 is an exotoxin-producing, methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain A970230 was deposited as positive for *mec* (subtype IV), staphylococcal enterotoxins genes *sea*, *sed* and *sej*, hemolysin genes *hla*, *hld* and *hlgv* and leukocidin genes *lukE* and *lukD*; MLST sequencing type (ST) 8; eGenomic *spa* type 1, eGenomic *spa* repeats YHGFMBQBLO; Ridom *spa* type t008; *agr* group I.
NRS188	*S. aureus*, strain A950206 is an exotoxin-producing, methicillin-sensitive *S. aureus* (MSSA) strain. *S. aureus*, strain A950206 was deposited as negative for *mec*; positive for the enterotoxin gene cluster (*egc*) operon, staphylococcal enterotoxins genes *sed* and *sej*, the hemolysin genes *hla*, *hlb*, *hld* and *hlgv* and the leukocidin genes *lukE* and *lukD*; MLST sequencing type (ST) 1447; eGenomic *spa* type 903, eGenomic *spa* repeats UMDMGMK; Ridom *spa* type t6709; *agr* group II.
NRS209	*S. aureus*, strain No. 315 is a methicillin-resistant *S. aureus* (MRSA) strain. It was deposited as resistant to methicillin, ampicillin, and penicillin; positive for *mec* (subtype IV); MLST sequence type (ST) 247; eGenomic *spa* type 4, eGenomic *spa* repeats YHFGFMBQBLO; Ridom *spa* type t051.
NRS232	*S. aureus*, strain HT 20020065 is a clinically associated methicillin-sensitive *S. aureus* (MSSA) strain. *S. aureus*, strain HT 20020065 was deposited as negative for *mec*; positive for the enterotoxin gene cluster (*egc*) operon, a γ-hemolysin gene (*hlg*) and the Panton Valentine leukocidin (PVL) components *lukS* and *lukF*; MLST sequencing type (ST) 22; eGenomic *spa* type 113, eGenomic *spa* repeats TJEJNCMOMOKR; Ridom *spa* type t005; *agr* group I.

NRS241	*S. aureus*, strain HT 20020233 is a clinically associated methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain HT 20020233 was deposited as positive for *mec* (subtype IV), the staphylococcal enterotoxin genes *seb* and *sel* and the gamma-hemolysin gene *hlgv*; MLST sequencing type (ST) 59; eGenomic *spa* type 17, eGenomic *spa* repeats ZDMDMNKB; Ridom *spa* type t216; *agr* group I.
NRS245	*S. aureus*, strain HT 20020320 is a clinically associated methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain HT 20020320 was deposited as positive for *mec* (subtype IV), the staphylococcal enterotoxin genes *sea* and *sed*, the gamma-hemolysin gene *hlgv* and the leukocidin genes *lukE* and *lukD*; MLST sequencing type (ST) 8-SLV; eGenomic *spa* type 914, eGenomic *spa* repeats YHGFHGFMBLO; *agr* group I.
NRS249	*S. aureus*, strain HT 20020341 is a clinically associated methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain HT 20020341 was deposited as positive for *mec* (subtype IV), the staphylococcal enterotoxin gene *sea*, the gamma-hemolysin gene *hlgv* and the leukocidin genes *lukE* and *lukD*; MLST sequencing type (ST) 247; eGenomic *spa* type 40, eGenomic *spa* repeats YFGFMBQBLO; Ridom *spa* type t052; *agr* group I.
NRS254	*S. aureus*, strain HT 20020365 is a methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain HT 20020365 was deposited as positive for *mec* (subtype IV); the staphylococcal enterotoxin gene *sed*, the hemolysin genes *hlb* and *hlgv* and the leukocidin genes *lukE* and *lukD*; MLST sequencing type (ST) 8; eGenomic *spa* type 363, eGenomic *spa* repeats YGFMBQBLO; Ridom *spa* type t024; *agr* group I.
NRS258	*S. aureus*, strain HT 20020376 is a methicillin-sensitive *S. aureus* (MSSA) strain. *S. aureus*, strain HT 20020376 was deposited as negative for *mec*; positive for the enterotoxin gene cluster (*egc*) operon, the exfoliatin A (*eta*) and B (*etb*) genes, the hemolysin gene *hlgv* and the leukocidin genes *lukE* and *lukD*; MLST sequencing type (ST) 121; eGenomic *spa* type 312, eGenomic *spa* repeats I2Z2EGMMJH2M; Ridom *spa* type t159; *agr* group IV.
NRS49	*S. aureus*, strain NRS49 is a glycopeptide-intermediate *S. aureus* (GISA) strain. Strain NRS49 was deposited as positive for SCC*mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD* and *vanE;* MLST sequence type (ST) 5; eGenomic *spa* type 232, eGenomic *spa* repeats TJMBBMDMGMK*;* Ridom *spa* type t601. Strain NRS49 is reported to be resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, imipenem, oxacillin, and tetracycline, intermediately susceptible to vancomycin and teicoplanin, susceptible to rifampin and cotrimoxazole and to have a VraR A113V mutation.
NRS76	S. aureus, strain C2000001227 was reported as a vancomycin-intermediate S. aureus (VISA) strain. S. aureus, strain C2000001227 was deposited as positive for SCCmec (subtype II); negative for vanA, vanB, vanC1, vanC2, vanD and vanE; MLST sequencing type (ST) 5; eGenomic spa type 2, eGenomic spa repeats TJMBMDMGMK; Ridom spa type t002.
NRS171	*S. aureus* strain of unknown origin
NRS193	*S. aureus*, strain C1999000193 is a community-associated methicillin-resistant *S. aureus* (CA-MRSA) strain. *S. aureus*, strain C1999000193 was deposited as positive for *mec* (subtype IV), PVL and the staphylococcal enterotoxin gene *seb*; negative for *tst*; MLST sequence type (ST) 1; eGenomic *spa* type 194, eGenomic *spa* repeats UJFKKPFKPE; Ridom *spa* type t175. Based on pulsed field gel electrophoresis, *S. aureus*, strain C1999000193 is closely related to *S. aureus* strains MW2 (NRS123), C1998000370 (NRS192) and C1999000529 (NRS194).
NRS223	*S. aureus* strain of unknown origin

NRS251	*S. aureus* strain of unknown origin
NRS265	*S. aureus* strain of unknown origin
NRS645	*S. aureus*, strain CA-224 is a clinically-associated methicillin-resistant *S. aureus* (MRSA) strain. Strain CA-224 was deposited as positive for *mec* (subtype IV); negative for PVL and *tst*. *S. aureus*, strain CA-224 is an isolate of the Iberian clonal lineage. Iberian isolates typically have the same MLST profile (ST247), SCC*mec* (subtype I) and *spa* repeats (YHFGFMBQBLO), negative for the PVL and arginine catabolic mobile element (ACME) genes, and are resistant to β-lactams and other commonly used antimicrobials. The Iberian clone (also referred to as PFGE A and EMRSA5) is a contemporary successor of the Archaic MRSA clone (ST 250). Both cluster closely with USA300 and USA500 which all originated from the clonal complex (CC) 8 lineage.
NRS655	*S. aureus* strain of unknown origin
NRS659	*S. aureus*, strain CA-78 is a clinically-associated methicillin-resistant *S. aureus* (MRSA) strain. Strain CA-78 was deposited as positive for *mec* (subtype IV); positive for PVL; negative for *tsst*; pulsed-field type USA300. *S. aureus*, strain CA-78 is a USA300 isolate. USA300 isolates have the same MLST profile (ST 8), SCC*mec* (subtype IV), *spa* repeats (YHGFMBQBLO), Ridom *spa* type (t008), contain the PVL and arginine catabolic mobile element (ACME) genes and are usually resistant to both erythromycin and β-lactams. USA300 is the most common cause of community-associated MRSA infection and an increasing cause of hospital-acquired infections.
NRS665	*S. aureus* strain of unknown origin
NRS271	*S. aureus*, strain H2138 (Isolate 10) is a linezolid-resistant *S. aureus* (LRSA), methicillin-resistant *S. aureus* (MRSA) strain and is the second clinically isolated linezolid-resistant MRSA strain. It was deposited as resistant to linezolid, tedizolid, ciprofloxacin, oxacillin and penicillin; intermediate resistance to erythromycin; positive for *mec* (subtype IV); MLST sequence type (ST) 22; eGenomic *spa* type 382, eGenomic *spa* repeats TJJEJNF2MNF2MOMOKR; Ridom *spa* type t032; phage type E-MRSA 15. *S. aureus*, strain H2138 (Isolate 10) developed from a linezolid susceptible strain during treatment. It is reported to have a G2576T mutation in domain V of the 23s rRNA gene (*Escherichia coli* numbering) and a Q3K mutation in the L3 ribosomal protein.
NRS283	*S. aureus*, strain 160013 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain 160013 was deposited as positive for *mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, and *vanE*; MLST sequencing type (ST) 36; eGenomic *spa* type 16, eGenomic *spa* repeats WGKAKAOMQQQ; Ridom *spa* type t018.
NRS382	*S. aureus*, strain 626 is a methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain 626 was deposited as resistant to erythromycin, clindamycin and levofloxacin; positive for *mec* (subtype II); *sed*$^{+}$; pulsed-field type USA100; MLST sequence type (ST) 5; eGenomic *spa* type 2, eGenomic *spa* repeats TJMBMDMGMK; Ridom *spa* type t002; *agr* grp II.*S. aureus*, strain 626 is a USA100 isolate.USA100 isolates have the same MLST profile (ST 5) and SCC*mec* (subtype II) and are usually resistant to β-lactams, erythromycin and spectinomycin as well as being multiresistant to other commonly used therapeutic agents. USA100 is the most prevalent U.S health care-associated pulse-field type and is endemic in many U.S. hospitals.
NRS383	*S. aureus*, strain 96758 is a methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain 96758 was deposited as resistant to erythromycin, clindamycin and gentamicin; positive for *mec* (subtype II), *tsst* and *sea*; pulsed-field type (PFT) USA200; MLST sequence type (ST) 36; eGenomic *spa* type 16, eGenomic *spa* repeats WGKAKAOMQQQ;

	Ridom *spa* type t018; *agr* grp III. *S. aureus*, strain 96758 is a USA200 isolate. USA200 isolates have the same MLST profile (ST 36), SCC*mec* (subtype II), *spa* repeats (WGKAKAOMQQQ) and are resistant to erythromycin, β-lactams and in some cases, spectinomycin. USA200 is the second most common health care-associated pulsed-field type in U.S.
NRS384	*S. aureus*, strain USA300-0114 is a community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strain. Outbreaks of this strain have been reported in correctional facilities (located in MS, GA, TN, TX, and CA); athletic teams (located in PA and CA); and male homosexual behaviour (observed in CA). Strain USA300-0114 was deposited as resistant to erythromycin and tetracycline; positive for *mec* (subtype IV); *pvl*$^{+}$; MLST sequence type (ST) 8; eGenomic *spa* type 1, eGenomic *spa* repeats YHGFMBQBLO; Ridom *spa* type t008; *agr* grp I.
NRS385	*S. aureus*, strain 95938 is a hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) strain. *S. aureus*, strain 95938 was deposited as resistant to erythromycin, clindamycin, trimethoprim/sulfamethoxazole, levofloxacin, gentamicin and tetracycline; positive for *mec* (subtype IV), *sea* and *seb*; MLST sequence type (ST) 8; pulsed-field type USA500; eGenomic *spa* type 7, eGenomic *spa* repeats YHGCMBQBLO; Ridom *spa* type t064; *agr* grp I. *S. aureus*, strain 95938 is a USA500 isolate. USA500 isolates have a common MLST sequence type (ST 8), *spa* motif (MBQBLO) and *agr* group (I) and are PVL-negative. USA500 isolates are multi-drug resistant healthcare-associated MRSA strains, but have been also been associated with sporadic community-acquired infections. USA500 is believed to be the predecessor of the most common community-associated pulsed-field type, USA300.
NRS386	*S. aureus*, strain 1078 is a methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain 1078 was deposited as resistant to erythromycin; positive for *mec* (subtype IV); MLST sequence type (ST) 72; pulsed-field type USA700; eGenomic *spa* type 49, eGenomic *spa* repeats UJGFMGGM; Ridom *spa* type t126; *agr* grp I. *S. aureus*, strain 1078 is a USA700 isolate. USA700 isolates have the same MLST profile (ST 72), SCC*mec* (subtype IV), *spa* repeats (UJGFMGGM) and negative for PVL, *tsst* and enterotoxins. USA700 is associated with infections in both community and healthcare settings.
NRS387	*S. aureus*, strain 1045 is a methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain 1045 was deposited as positive for *mec* (subtype IV) and *seb*; pulsed-field type (PFT) USA800; MLST sequence type (ST) 5; eGenomic *spa* type 29, eGenomic *spa* repeats TJMBMDMGGMK; Ridom *spa* type t088; *agr* grp II. *S. aureus*, strain 1045 is a USA800/Paediatric isolate. USA800 isolates have the same MLST profile (ST 5), *agr* group (II), SCC*mec* subtype (IV), *spa* motif (MDMGMK), and Ridom *spa* types (t002 and related) and are positive for *sem* and *seo* toxin genes. USA 800 isolates are resistant to β-lactams and fluoroquinolones with some isolates being resistant to additional antibiotics. While first isolated in paediatric patients, USA800 strains recently have been isolated in adults.
NRS403	*S. aureus*, strain HIP13057 is a vancomycin-intermediate *S. aureus* (VISA) strain. *S. aureus*, strain HIP13057 was deposited as positive for *mec* (subtype II) and *vanC2*; negative for *vanA*, *vanB*, *vanC1*, *vanD* and *vanE*; sequencing type (ST) 5; eGenomic *spa* type 2, eGenomic *spa* repeats TJMBMDMGMK; Ridom *spa* type t002. *S. aureus*, strain HIP13057 tested positive for the vancomycin resistance gene *vanC2*, which is responsible for intrinsic resistance to low levels of vancomycin in *Enterococcus casseliflavus*.
NRS642	*S. aureus*, strain CA-126 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain CA-126 was deposited as positive for *mec* (subtype II); negative for PVL and *tsst*; pulsed-field type USA100. *S. aureus*, strain CA-126 is a USA100 isolate. USA100 isolates have the same MLST profile (ST 5), SCC*mec* (subtype II) and *spa* motif (MDMGMK) and are usually resistant

	to erythromycin and spectinomycin as well as being multiresistant to other commonly used therapeutic agents. USA100 is the most prevalent U.S health care-associated pulsed-field type and is endemic in many U.S. hospitals.
NRS643	*S. aureus*, strain CA-127 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain CA-127 was deposited as positive for *mec* (subtype IV) and PVL; negative for *tsst*; pulsed-field type USA300. *S. aureus*, strain CA-127 is an isolate of the USA300 clone. USA300 isolates have a common MLST profile (ST 8), SCC*mec* type (subtype IV), *spa* motif (MBQBLO) and *agr* group (I), carry the PVL and arginine catabolic mobile element (ACME) genes and are usually resistant to both erythromycin and β-lactams. USA300 is the most common cause of community-associated MRSA infection and an increasing cause of hospital-acquired infections.
NRS651	*S. aureus*, strain CA-409 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain CA-409 was deposited as positive for *mec* (subtype II) and *tsst*; negative for PVL; pulsed-field type USA200. *S. aureus*, strain CA-409 is a USA200 isolate. USA200 isolates have the same MLST profile (ST 36), SCC*mec* (subtype II), *spa* repeats (WGKAKAOMQQQ) and are resistant to erythromycin and β-lactams and, in some cases, spectinomycin. USA200 is the second most common health care-associated pulsed-field type in U.S.
NRS653	*S. aureus*, strain CA-513 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain CA-513 was deposited as positive for *mec* (subtype IV); negative for PVL and *tsst*; pulsed-field type USA800. *S. aureus*, strain CA-513 is a USA800/Paediatric isolate. USA800 isolates have the same MLST profile (ST 5), *agr* group (II), SCC*mec* subtype (IV), *spa* motif (MDMGMK) and Ridom *spa* types (t002 and related) and are positive for *sem* and *seo* toxin genes. USA 800 isolates are resistant to β-lactams with some isolates being resistant to additional antibiotics. While first isolated in paediatric patients, USA800 strains recently have been isolated in adults.
NRS662	*S. aureus*, strain CO-34 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain CO-34 was deposited as positive for *mec* (subtype IV); positive for PVL; negative for *tsst*; pulsed-field type USA300. *S. aureus*, strain CO-34 is a USA300 isolate. USA300 isolates have the same MLST profile (ST 8), SCC*mec* (subtype IV), *spa* repeats (YHGFMBQBLO), Ridom *spa* type (t008), contain the PVL and arginine catabolic mobile element (ACME) genes and are usually resistant to both erythromycin and β-lactams. USA300 is the most common cause of community-associated MRSA infection and an increasing cause of hospital-acquired infections.
NRS668	*S. aureus*, strain CO-72 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain CO-72 was deposited as positive for *mec* (subtype IV); negative for PVL and *tsst*; pulsed-field type USA800. *S. aureus*, strain CO-72 is a USA800/Paediatric isolate. USA800 isolates have the same MLST profile (ST 5), *agr* group (II), SCC*mec* subtype (IV), *spa* motif (MDMGMK), and Ridom *spa* types (t002 and related) and are positive for *sem* and *seo* toxin genes. USA 800 isolates are resistant to β-lactams with some isolates being resistant to additional antibiotics. While first isolated in paediatric patients, USA800 strains recently have been isolated in adults.
NRS68	*S. aureus*, strain NRS68 was deposited as a vancomycin intermediate *S. aureus* (VISA) strain. *S. aureus,* strain NRS68 was deposited as positive for SCC*mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD* and *vanE*; MLST sequencing type (ST) 5; eGenomic *spa* type 2, eGenomic *spa* repeats TJMBMDMGMK; Ridom *spa* type t002.
NRS79	*S. aureus*, strain IL (isolate F) is a vancomycin-intermediate *S. aureus* (VISA) strain and 1 of 6 isolates collected from the patient during her hospitalization. Strain IL (isolate F) was deposited as positive for *mec* (subtype II); negative for *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD* and *vanE;* MLST sequence type (ST) 231; eGenomic *spa* type 2, eGenomic *spa* repeats TJMBMDMGMK*;* Ridom *spa* type t002.

NRS173	*S. aureus* strain of unknown origin
NRS194	*S. aureus*, strain C1999000193 is a community-associated methicillin-resistant *S. aureus* (CA-MRSA) strain. *S. aureus*, strain C1999000193 was deposited as positive for *mec* (subtype IV), PVL and the staphylococcal enterotoxin gene *seb*; negative for *tst*; MLST sequence type (ST) 1; eGenomic *spa* type 194, eGenomic *spa* repeats UJFKKPFKPE; Ridom *spa* type t175. Based on pulsed field gel electrophoresis, *S. aureus*, strain C1999000193 is closely related to *S. aureus* strains MW2 (NRS123), and C1998000370 (NRS192).
NRS230	*S. aureus* strain of unknown origin
NRS255	*S. aureus* strain of unknown origin
NRS266	*S. aureus*, strain HT 20020455 is a methicillin- sensitive *S. aureus* (MSSA) strain. *S. aureus*, strain HT 20020455 was deposited as negative for *mec*; positive for *seb*, *egc*, *eta*, *etb*, *lukE-lukD*, *edinA-C* and *hlgv*; MLST sequence type (ST) 121; eGenomic *spa* type 683, eGenomic *spa* repeats I2Z2EGJH2M; Ridom *spa* type t2155; *agr* group IV.
NRS649	*S. aureus* strain of unknown origin
NRS656	*S. aureus* strain of unknown origin
NRS660	*S. aureus* strain of unknown origin
NRS670	*S. aureus*, strain CT-110 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain CT-110 was deposited as positive for *mec* (subtype II); negative for PVL and *tsst*; pulsed-field type USA100. *S. aureus*, strain CT-110 is a USA100 isolate. USA100 isolates have the same MLST profile (ST 5), SCC*mec* (subtype II) and *spa* motif (MDMGMK) and are usually resistant to erythromycin and spectinomycin as well as being multiresistant to other commonly used therapeutic agents. USA100 is the most prevalent U.S health care-associated pulsed-field type and is endemic in many U.S. hospitals.
NRS678	*S. aureus*, strain CT-58 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain CT-58 was deposited as positive for *mec* (subtype IV); negative for PVL and *tsst*, and pulsed-field type USA500. *S. aureus*, strain CT-58 is a USA500 isolate. USA500 isolates have a common *spa* motif (MBQBLO), MLST sequence type (ST 8) and *agr* group (I) and are PVL-negative. USA500 isolates are multi-drug resistant healthcare-associated MRSA strains but have been also been associated with sporadic community-acquired infections. USA500 is believed to be the predecessor of the most common community-associated pulsed-field type, USA300.
NRS683	*S. aureus*, strain GA-298 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain GA-298 was deposited as positive for *mec* (subtype IV) and PVL; negative for *tsst*, and pulsed-field type 300. *S. aureus*, strain GA-298 is a USA300 isolate. USA300 isolates have a common MLST profile (ST 8), SCC*mec* type (subtype IV), *spa* motif (MBQBLO) and *agr* group (I), carry the PVL and arginine catabolic mobile element (ACME) genes and are usually resistant to both erythromycin and β-lactams. USA300 is the most common cause of community-associated MRSA infection and an increasing cause of hospital-acquired infections.
NRS686	*S. aureus*, strain GA-356 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain GA-356 was deposited as positive for *mec* (subtype IV); negative for PVL and *tsst*, and pulsed-field type Iberian. *S. aureus*, strain GA-356 is an isolate of the Iberian clonal lineage. Iberian isolates have the same MLST profile (ST247), SCC*mec* (subtype I) and *spa* repeats (YHFGFMBQBLO), negative for the PVL and arginine catabolic mobile element (ACME) genes and are resistant to β-lactams and other commonly used antimicrobials. The Iberian clone (also referred to as PFGE A and EMRSA5) is a contemporary successor of the Archaic MRSA clone (ST 250). Both

	clusters closely with USA300 and USA500 which all originated from the clonal complex (CC) 8 lineages.
NRS689	*S. aureus*, strain GA-442 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain GA-442 was deposited as positive for *mec* (subtype IV); negative for PVL and *tsst*; pulsed-field type USA700. *S. aureus*, strain GA-442 is a USA700 isolate. USA700 isolates have the same MLST profile (ST 72), SCC*mec* (subtype IV), *spa* repeats (UJGFMGGM) and negative for PVL, *tsst* and enterotoxins. USA700 is associated with infections in both community and healthcare settings.
NRS692	*S. aureus*, strain GA-656 is a methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain GA-656 was deposited as positive for *mec* (subtype IV); negative for PVL and *tsst*; pulsed-field type (PFT) USA800. *S. aureus*, strain GA-656 is a USA800/Paediatric isolate. USA800 isolates have the same MLST profile (ST 5), *agr* group (II), SCC*mec* subtype (IV), *spa* motif (MDMGMK), and Ridom *spa* types (t002 and related) and are positive for *sem* and *seo* toxin genes. USA 800 isolates are resistant to β-lactams with some isolates being resistant to additional antibiotics. While first isolated in paediatric patients, USA800 strains recently have been isolated in adults.
NRS694	*S. aureus*, strain GA-92 is a clinically associated methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain GA-92 was deposited as positive for *mec* (subtype IV) and PVL; negative for *tsst*; pulsed-field type USA300. *S. aureus*, strain GA-92 is a USA300 isolate. USA300 isolates have a common MLST profile (ST 8), SCC*mec* type (subtype IV), *spa* motif (MBQBLO) and *agr* group (I), carry the PVL and arginine catabolic mobile element (ACME) genes and are usually resistant to both erythromycin and β-lactams. USA300 is the most common cause of community-associated MRSA infection and an increasing cause of hospital-acquired infections.
NRS703	*S. aureus*, strain MN-095 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain MN-095 was deposited as positive for *mec* (subtype IV) and PVL; negative for *tst*; pulsed-field type 300. *S. aureus*, strain MN-095 is a USA300 isolate. USA300 isolates have a common MLST profile (ST 8), SCC*mec* type (subtype IV), *spa* motif (MBQBLO) and *agr* group (I), typically carry the PVL and arginine catabolic mobile element (ACME) genes and are usually resistant to both erythromycin and β-lactams. USA300 is the most common cause of community-associated MRSA infection and an increasing cause of hospital-acquired infections.
NRS715	*S. aureus*, strain NY-315 is a hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) strain. Strain NY-315 was deposited as positive for *mec* (subtype II); negative for PVL and *tsst*; pulsed-field type USA600. *S. aureus*, strain NY-315 is a USA600 isolate. USA600 isolates have the same MLST profile (ST 45), SCC*mec* subtypes (II or IV), *agr* group (I), and *spa* repeats (A2AKEEMBKB). They are PVL negative and resistant to erythromycin and clindamycin. Isolates are predominantly found in nares of healthy individuals and bloodstream infections. These isolates are associated with a higher rate of clinical failure and mortality, particularly those with a heterogeneous vancomycin-intermediate *S. aureus* (hVISA) phenotype. While USA600 isolates are uncommon the United States, the clonally related Berlin strain is widespread throughout Germany, the Netherlands, and Ontario, Canada.
NRS716	*S. aureus*, strain NY-336 is a methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain NY-336 was deposited as positive for *mec* (subtype IV) and PVL; negative for *tsst*; pulsed-field type USA300. Strain NY-336 is a USA300 isolate. USA300 isolates have a common MLST profile (ST 8), SCC*mec* type (subtype IV), *spa* motif (MBQBLO) and *agr* group (I), carry the PVL and arginine catabolic mobile element (ACME) genes and are usually resistant to both erythromycin and β-lactams. USA300 is the most common cause of community-associated MRSA infection and an increasing cause of hospital-acquired infections.

NRS721	*S. aureus*, strain OR-130 is a clinically associated methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain OR-130 was deposited as positive for *mec* (subtype II); negative for PVL and *tsst*; pulsed-field type USA100. *S. aureus*, strain OR-130 is a USA100 isolate. USA100 isolates have the same MLST profile (ST 5), SCC*mec* (subtype II) and *spa* motif (MDMGMK) and are usually resistant to erythromycin and spectinomycin as well as being multiresistant to other commonly used therapeutic agents. USA100 is the most prevalent U.S health care-associated pulsed-field type and is endemic in many U.S. hospitals.
NRS722	*S. aureus*, strain OR-131 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain OR-131 was deposited as positive for *mec* (subtype II); negative for PVL and *tsst*; pulsed-field type USA200. *S. aureus*, strain OR-131 is a USA200 isolate. USA200 isolates have the same MLST profile (ST36), SCC*mec* (subtype II) and *spa* repeats (WGKAKAOMQQQ) and are resistant to erythromycin, β-lactams and, in some cases, spectinomycin. USA200 is the second most common health care-associated pulsed-field type in the U.S.
NRS740	*S. aureus*, strain TN-82 is a clinically associated methicillin-resistant *S. aureus* (MRSA) strain. *S. aureus*, strain TN-82 was deposited as positive for *mec* (subtype II) and *tsst*; negative for PVL; pulsed-field type USA200. *S. aureus*, strain TN-82 is a USA200 isolate. USA200 isolates have the same MLST profile (ST 36), SCC*mec* (subtype II), *spa* repeats (WGKAKAOMQQQ) and are resistant to erythromycin, β-lactams and in some cases, spectinomycin. USA200 is the second most common health care-associated pulsed-field type in U.S.
NRS860	*S. aureus*, strain VCU089 (also referred to as NRS860) is of unknown origin.
NRS882	*S. aureus*, strain TN-296 is a methicillin-resistant *S. aureus* (MRSA) strain. Strain TN-296 was deposited as positive for *mec* (subtype IV); negative for *tsst*; pulsed-field type USA100.
NRS73	*S. aureus* strain of unknown origin
NRS110	*S. aureus* strain of unknown origin
NRS179	*S. aureus* strain of unknown origin
NRS214	*S. aureus* strain of unknown origin
NRS243	*S. aureus* strain of unknown origin
NRS256	*S. aureus* strain of unknown origin
NRS482	*S. aureus* strain of unknown origin
NRS650	*S. aureus* strain of unknown origin
NRS657	*S. aureus* strain of unknown origin
NRS661	*S. aureus*, strain CO-23 is a clinically associated methicillin-resistant *S. aureus* (MRSA) strain. Strain CO-23 was deposited as positive for *mec* (subtype II); negative for *tst* and PVL; and pulsed-field type 100. *S. aureus*, strain CO-23 is a USA100 isolate. USA100 isolates have the same MLST profile (ST 5) and SCC*mec* (subtype II) and are usually resistant to β-lactams, erythromycin and spectinomycin as well as being multiresistant to other commonly used therapeutic agents. USA100 is the most prevalent U.S health care-associated pulsed-field type and is endemic in many U.S. hospitals.

Appendix 3 List of oligonucleotides primers used in this study

Primer Name	Sequence	Description
***silS* upper**	AGCGACTCCGCGCTAAAATA	Amplification and detection of *silS*
***silS* lower**	GGCTTCTGTTTGCTGCATGA	Amplification and detection of *silS*
***silS* seq 1**	CTCCATTACCTCGATGCGCT	Sequencing of *silS*
***silS* seq 2**	GTTCCTGGCACAGGCAGATA	Sequencing of *silS*
***silE* upper**	GCTGGGCTTTGGCTTAATTTCTTC	Detection of *silE*
***silE* lower**	GCACTGAGCATGCGACGATGCGC	Detection of *silE*
***silR* upper**	CAAACCTTCCAGACGCCCTTT	Detection of *silR*
***silR* lower**	GTTTGCTGCATGACAGGCTAA	Detection of *silR*
***bacA* upper**	CTGATATAAGTTAAAAGGAGG	Detection of *bacA*
***bacA* lower**	GAACCTCCTAACTGTATTACC	Detection of *bacA*
***bcrA* upper**	CAAAGCAATATGGAAGTCAGA	Detection of *bcrA*
***bcrA* lower**	CTCTCATCTGGAAAACATCAA	Detection of *bcrA*
***bcrB* upper**	AATCTACTCCGTACAGAATTT	Detection of *bcrB*
***bcrB* lower**	GGTAATGTTCCTCTAGATATG	Detection of *bcrB*
***Bcr* operon U**	TATATAAATTCGAACATACAGAAG ACTCCTTTTTG	Amplification of the *bcr* cluster
Bcr operon L	ATATATATTTCGAAATTAAGAGAAC GTGGTGTAAA	Amplification of the *bcr* cluster
NRS384 plasmid U	CAACTACTTTTAAGGTGGTAATAC AGTTAG	Amplification of pNRS384 plasmid
NRS384 plasmid L	TATGAACAGAACTGGTACTTAATC GGTTTT	Amplification of pNRS384 plasmid
NRS384 plasmid extension U	AAGTGGGTGCATTGATTACAG	Amplification of pNRS384 plasmid

NRS384 plasmid extension L	GCGGAGTGTTATTATGGATAT	Amplification of pNRS384 plasmid
5kb upper	TTAACAATGATTCAAAATACC	Amplification of the 5kb region (nitrofurantoin)
5kb lower	AGCTTAAACGTATGATTAGTG	Amplification of the 5kb region (nitrofurantoin)
15kb 1 U	AGATTGAGTTTGATATATGAC	Amplification of the 15kb region (nitrofurantoin)
15kb 1 L	ACTTGGACAATATAATGAACC	Amplification of the 15kb region (nitrofurantoin)
15kb 2 U	CACACATAAAATGATGATGAC	Amplification of the 15kb region (nitrofurantoin)
15kb 2 L	CTTTATTGAAGTGTATCTTTT	Amplification of the 15kb region (nitrofurantoin)
15kb 3 U	GATAAGGCTGTATACGTATAC	Amplification of the 15kb region (nitrofurantoin)
15kb 3 L	GAGTTGGTGACTGGATAAGTA	Amplification of the 15kb region (nitrofurantoin)
15kb 4 U	CGTTAAATACATACCATAGGG	Amplification of the 15kb region (nitrofurantoin)
15kb 4 L	TGGTTATGTAAACAAGTTGCT	Amplification of the 15kb region (nitrofurantoin)
15kb 5 U	ATACGCTTTTTTGATTCTTCT	Amplification of the 15kb region (nitrofurantoin)
15kb 5 L	ACACTTTTATTATTCTGATGC	Amplification of the 15kb region (nitrofurantoin)

Appendix 4 Summary of the genes identified in pNRS384

Gene/ Protein Name	Length (bp)	Function
WYL	136	Conserved hypothetical protein, possible transcriptional regulator
Tn1545	366	Mobile element protein
aphA	795	Aminoglycoside phosphotransferase
aadE	531	Aminoglycoside acetyltransferase, Streptomyces lavendulae type
Sat	490	Aminoglycoside resistance
IS6	675	Mobile element protein
HP	135	Hypothetical protein
HP	155	Hypothetical protein
binL	579	DNA-invertase
BlaI	381	β- lactamase repressor
BlaR1	1758	β- lactamase regulatory sensor-transducer
blaZ	846	β- lactamase protein
MarR	210	Transcriptional regulator, MarR family
NADPH	972	Alcohol dehydrogenase, 5-FCL-like protein, butanol biosynthesis, fermentations: mixed acid, glycerolipid and glycerophospholipid metabolism in bacteria
cadX	348	Cadmium efflux system accessory protein
cadD	618	Cadmium resistance protein
HP	123	Hypothetical protein
HP	189	Hypothetical protein
Rep	996	Replication protein
HP	174	Hypothetical protein
HP	582	Hypothetical protein
HP	195	Hypothetical protein

HP	230	Hypothetical protein
Gpos_C8	693	Putative immunity protein/bacteriocin
HP	150	Hypothetical protein
Gpos_C8	255	Putative immunity protein/bacteriocin
HP	150	Hypothetical protein
Rep	171	Replication protein
Rep	984	Replication protein
DUF536	744	Replication-associated protein
PBSX	186	Transcriptional regulator, PBSX family
HP	339	Hypothetical protein
mphC	609	ATP-binding protein p271, Macrolide resistance
msrA	900	Macrolide 2'-phosphotransferase
msrA	1467	Macrolide 2'-phosphotransferase
IS6	675	Mobile element protein
bcrR	366	Two-component response regulator, bacitracin stress response
bcrS	603	Putative two-component bacitracin sensor histidine kinase
bcrA	930	Bacitracin ABC transporter, ATP-binding protein
bcrB	738	Bacitracin ABC transporter, permease protein, putative
bacA	204	Bacitracin undecaprenyl-diphosphatase

Appendix 5 List of the genes (5 and 15 kb regions) that have been found only in the isolates with reduced nitrofurantoin susceptibility and their proposed function

Gene/ Protein Name	Notes
Hypothetical protein 117bp (2636616)	Hypothetical protein [*S. aureus*]
Transposase 354bp (2636937)	IS30 family transposase [*S. aureus]*
FIG01108533: hypothetical protein 504bp (2637454)	HTH superfamily, helix-turn-helix domain, integrase.
FIG01107943: hypothetical protein 357bp (2638066)	Lipoprotein (DUF4467)
FIG01107943: hypothetical protein 591bp (2638478)	Uncharacterised N-terminal domain of peptidoglycan hydrolase CwlO [Function unknown]; Putative exported protein [*S. aureus*].
Secretory antigen SsaA-like protein transposon-related 1047bp (2639075)	MULTISPECIES: mannosyl-glycoprotein endo-beta-N-acetylglucosamidase [*S. aureus*] NlpC/P60 family. The function of this domain is unknown. It is found in several lipoproteins.
Membrane protein, putative 1848bp (2640111)	MAP7 (E-MAP-115) family. The organisation of microtubules varies with the cell type and is presumably controlled by tissue-specific microtubule-associated proteins (MAPs). The 115-kDa epithelial MAP (E-MAP-115/MAP7) has been identified as a microtubule-stabilising protein predominantly expressed in cell lines of epithelial origin. The binding of

	this microtubule-associated protein is nucleotide independent.
FIG01108595: hypothetical protein 1359bp (2641963)	Cell division protein FtsK
Hypothetical protein 267bp (2643384)	No putative conserved domains have been detected
Hypothetical protein 144bp (2643725)	No putative conserved domains have been detected
Hypothetical protein 492bp (2643888)	No putative conserved domains have been detected
Conjugal transfer protein transposon-related 2496 bp (2644552)	AAA family ATPase [*S. aureus*]
FIG01107943: hypothetical protein 384bp (2647082)	Conjugal transfer protein [*S. aureus*] putative TcpE family protein
FIG01107943: hypothetical protein 261bp (2647477)	MULTISPECIES: hypothetical protein [*S. aureus*]
FIG01107943: hypothetical protein 1056bp (2647742)	MULTISPECIES: conjugal transfer protein [*S. aureus*]
Transcriptional regulator, Cro/CI family protein transposon-related 1092bp (2648858)	Replication initiation factor [*S. aureus*]
FIG01107943: hypothetical protein 303bp (2650124)	Transposon-related protein [*S. aureus*]
FIG01107943: hypothetical protein 321bp (2650440)	MULTISPECIES: hypothetical protein
FIG01107943: hypothetical protein 285bp (2650911)	MULTISPECIES: hypothetical protein

FIG01107980: hypothetical protein 540bp (2651227)	Membrane protein [*S. aureus*]
Mobile element protein 684bp (2760449)	IS5/IS1182 family transposase
Multicopper oxidase 1434bp (2761199)	Multicopper oxidase with three cupredoxin domains (includes cell division protein FtsP and spore coat protein CotA) [Cell cycle control, cell division, chromosome partitioning, Inorganic ion transport and metabolism, Cell wall/membrane/envelope biogenes].
Lead, cadmium, zinc, and mercury transporting ATPase; Copper translocating P-type ATPase 2046bp (2762647)	Subsystem Copper Transport System, Copper homeostasis cation exporting ATPase.
DNA-invertase 576bp (2764959)	MULTISPECIES: transposase

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