School of Pharmacy and Biomedical Sciences

Antimicrobial Resistance at the Human-Animal Interface

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Abstract

Antimicrobial drugs have had a profound effect on human and animal health. By mass, animals are the largest consumers of antimicrobials in Australia. The use of antimicrobials promotes the development of antimicrobial resistance (AMR), which may occur rapidly by the transmission of antimicrobial resistance genes between bacteria within mobile genetic elements. AMR reduces the effectiveness of antimicrobials to treat infections. While many antimicrobials used for human healthcare are restricted to human-use only to limit the development of AMR, some antimicrobials are used in the treatment of both human and animal infections. Additionally, many AMR genes confer resistance to multiple antimicrobials of the same class and may also confer resistance to antimicrobials of different classes. Resistance to antimicrobials which are restricted for human use can therefore provide resistance to antimicrobials allowed for animal use and vice versa. The use of antimicrobials in animals, despite restrictions on their use, may therefore still pose a risk to human health by the dissemination of cross-resistant bacterial pathogens from animals to humans and by the transmission of resistance genes between human- and animal-associated bacterial pathogens. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an opportunistic bacterial pathogen which is known for its ability to rapidly acquire AMR and is a major cause of infection in hospitals and the community, worldwide. *S. aureus* naturally colonise animal nasopharynx and is capable of transmitting between humans and animals via aerosols and direct contact. As such MRSA have increasingly become a cause of infection in people who work with animals and livestock, including veterinarians, livestock workers and meat processing workers. Livestock-associated MRSA (LA-MRSA) are emerging as a significant public health issue in many European nations, with infections increasing in frequency and severity. LA-MRSA were only detected for the first time in Australia in 2009-2010, but have been detected at higher frequency since then. In this thesis I examined MRSA, isolated from humans and animals in Australia, to determine whether transmission of MRSA has occurred between humans and animals, and examined the impact of antimicrobial use in Australia on the dynamics of MRSA transmission. Additionally, I examined the mobile genetic elements shared between MRSA of human and animal origin identifying instances of potential AMR transmission between these lineages of MRSA.

Overview

Chapter 3 examined sequence type (ST) 612 MRSA, following a bloodstream infection of a Western Australian patient. ST612 MRSA have been reported only sporadically in Australia, isolated from veterinarians and from racehorses during an MRSA outbreak, but had not previously caused disease in Australian patients. In South Africa, ST612 MRSA is a dominant community-associated MRSA and is a frequent cause of bacteraemia. The study attempted to determine if Australian equine-associated ST612 MRSA and South African community-associated MRSA are distinct and if so, identify which lineage of MRSA was responsible for the bloodstream infection of a Western Australian patient. ST612 MRSA were collected from the aforementioned outbreak in horses, from Australian veterinarians, from Western Australian healthcare screening collections and from Tygerberg Hospital, South Africa. Whole-genome sequencing (WGS) was performed on all isolates and the core genome phylogeny was produced. Additional long-read sequencing was performed on a single Australian ST612 MRSA to produce a high-quality reference genome. Phylogenetic analysis indicated the Australian and South African ST612 MRSA were genetically distinct. Although several isolates detected in Western Australian healthcare screening were closely related to those of South African origin, the isolate responsible for the Western Australian bacteraemia was most likely of Australian equine-associated origin. The patterns of AMR in all ST612 complements the pattern of antimicrobial use in South African HIV prophylactic antimicrobial treatment, and in Australian equine veterinary medicine, and may provide an environment suitable for ST612 MRSA to persist in humans and in horses.

Chapters 4 and 5 examined MRSA isolates from Australian pigs, pig farm workers and the pig farm environment. Employees of an Australian pig farm experienced MRSA infections over a three year period leading up to, and prompting, an MRSA surveillance study in 2015. The study identified community-associated ST93 MRSA and livestock-associated ST398 MRSA in Australian pig farms and also identified the first Australian linezolid-resistant MRSA. The presence of ST93 MRSA alongside the multi-resistant ST398 LA-MRSA raised the possibility that ST93 MRSA could gain AMR genes from ST398 and be retransmitted to humans, well adapted to human infection but armed with new defence against antimicrobial therapy. Chapter 4 analysed sequence data from the 2015 study. In addition, one LA-MRSA isolate was selected for long-read sequencing to produce a reference genome for Australian LA-MRSA. To determine if ST93 MRSA had acquired AMR genes from ST398 LA-MRSA, the mobile genetic elements of each lineage were identified, tabulated and compared. A custom bioinformatic workflow was developed to identify known and novel mobile genetic elements, which

were then compared phylogenetically to determine transmission between lineages. Phylogeny and comparison with ST93 MRSA in previous studies concluded several mobile genetic elements were likely to have been transmitted from ST398 LA-MRSA to ST93 MRSA, including a plasmid which encoded macrolide resistance and a transposon which encoded phenicol resistance. In addition, cryptic plasmids, a large composite SCC*mec* methicillin-resistance element and a potentially novel AMR gene were identified. The transmission of resistance between ST398 and ST93 in these instances may not represent an immediate threat to human health, but demonstrate a pathway for the development of a reservoir of multi-resistant, human-infectious MRSA and the development of a new LA-MRSA.

Chapter 5 examined LA-MRSA from the same study as Chapter 4, isolated from pigs and pig farm workers, which carried the linezolid resistance gene, *cfr*. While examining the genomic environment of *cfr* for evidence that the gene may be mobile, it was observed that only one ST398 isolate was phenotypically linezolid resistant, despite three other isolates carrying *cfr*. The three isolates carried a novel variant of the *cfr* gene, which I called *cfrAB,* containing a single base deletion which produced a premature stop codon and divided the open reading frame of the gene into two inactive portions. Although the gene appeared to be functionally truncated, residual linezolid resistance (3 µg/mL) was detected indicating the variant may still be functional. Additionally, the site of the deletion in *cfrAB* occurred in a poly-A sequence, which are known sites of ribosomal frameshifting. Ribosomal frameshifting is a mechanism known to regulate transcription of some genes. To determine whether *cfrAB* was being expressed by a ribosomal frameshift, a tandem affinity purification tagged *cfrAB* fusion-protein was constructed and expressed in *E. coli.* Frameshifted fusion-protein was purified, visualised and sequenced, confirming that frameshifted *cfr* was expressed from the *cfrAB* allele. The rate of *cfrAB* frameshifting was determined by the construction of *cfrAB*-β-galactosidase fusion protein which was expressed in *E. coli* and measured in a fluorescence reporter assay. The rate of frameshifting was 2.3% of *cfr* expression, which was consistent with the expression of the fusion protein observed in the previous assay, and with the reduced linezolid resistance identified in the *cfrAB* isolates relative to the *cfr* isolate. These data suggest *cfrAB* may have evolved to reduce expression of the Cfr protein. Additionally, *cfrAB* isolates were found to spontaneously mutate to *cfr* and demonstrated increased resistance to florfenicol after growth on media containing high concentrations of florfenicol. The 'silent' phenotype of *cfrAB* could go undetected by phenotypic screening, mutating to *cfr* and achieving clinical linezolid resistance upon treatment. The *cfrAB* variant may represent an example of AMR evolution as a consequence of antimicrobial use in animals.

Together, this work highlights the effect antimicrobial use in animals has on the development of persistent reservoirs of MRSA, and presents the dangers the resulting antimicrobial-resistant reservoirs pose to those who work closely with animals, and to the wider community. A One Health approach, which recognises the connection between the health of humans, animals and the environment, combining surveillance of AMR and MRSA from humans, animals and livestock environments, and cooperation between governments, health departments and private industry should be pursued to tackle the development of AMR and maintain the effectiveness of antimicrobials into the future. The work provides insight into the movement of AMR at the human and animal interface and indicates a need for improved hygiene and infection control measures amongst people who live and work with animals to limit the evolution and spread of AMR. Ideally, further restrictions on antimicrobial use in animals should be considered in light of evolving linezolid resistance in Australian LA-MRSA.

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List of publications included in thesis

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The author of this thesis was the first author and main contributor of the work presented in this publication. The nature and extent of my contributions to this publication are described below.

• I contributed to the generation of data, interpretation of results, and development of the concepts described in the publication with JR.

• I generated genome sequence data presented in this publication, with assistance from YTL and SP. The bacterial isolate was provided by GWC and MOD.

• I performed all bioinformatics analyses with the guidance of JR and SP.

• I was responsible for the planning, drafting, and editing of the publication with TB, JC and AD.

• All co-authors reviewed and edited the manuscript.

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Murphy, RJ, Ramsay J.P, Lee Y.T., Pang, S., O'Dea, M.A., Pearson, J.C., Axon, J.E, Raby,E., Abdulgader, S.M., Whitelaw, A., Coombs, G.W. (2019) Multiple introductions of methicillin-resistant *Staphylococcus aureus* ST612 into Western Australia associated with both human and equine reservoirs. International Journal of Antimicrobial Agents, 19, 30240-7

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• I contributed to the generation of data, interpretation of results, and development of the concepts described in the publication with GC and JR.

• I generated bacterial genome sequence data presented in this publication with technical assistance from SP and YTL. Several bacterial isolates and relevant metadata were kindly provided by JA, ER, SMA and AW.

• I performed all bioinformatics analyse, guided by JR and SP.

• I was responsible for the planning, drafting, and editing of the publication with GC, AW and JR.

• All co-authors reviewed and edited the manuscript.

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Relevant publications produced during candidature

Perry B.J., Sullivan J.T., Colombi E, **Murphy R.J.T.**, Ramsay J.P., Ronson C.W. Symbiosis islands of Loteae-nodulating *Mesorhizobium* comprise three radiating lineages with concordant *nod* gene complements and nodulation host-range groupings. Microb Genom. 2020 Sep;6(9). doi: 10.1099/mgen.0.000426. Epub 2020 Aug 26. PMID: 32845829.

Glady-Croue, J., Niu, X.-Z., Ramsay, J.P., Watkin, E., **Murphy, R.J.T.** and Croue, J.-P. (2018) Survival of antibiotic resistant bacteria following artificial solar radiation of secondary wastewater effluent. Science of the Total Environment, 626, 1005-1011.

Khaleque, H.N., Ramsay, J.P., **Murphy, R.J.T.**, Kaksonen, A.H., Boxall, N.J. and Watkin, E.L. (2017) Draft genome sequence of the acidophilic, halotolerant, and iron/sulfur-oxidizing *Acidihalobacter prosperus* DSM 14174 (strain V6). Genome announcements, 5, e01469-01416.

Khaleque, H.N., Ramsay, J.P., **Murphy, R.J.T.**, Kaksonen, A.H., Boxall, N.J. and Watkin, E.L. (2017) Draft genome sequence of *Acidihalobacter ferrooxidans* DSM 14175 (strain V8), a new iron-and sulfur-oxidizing, halotolerant, acidophilic species. Genome announcements, 5, e00413-00417.

Ramsay, J.P., Kwong, S.M., **Murphy, R.J.T.**, Yui Eto, K., Price, K.J., Nguyen, Q.T., O'Brien, F.G., Grubb, W.B., Coombs, G.W. and Firth, N. (2016) An updated view of plasmid conjugation and mobilization in Staphylococcus. Mobile genetic elements, 6, e1208317.

O'Brien, F.G., Yui Eto, K., **Murphy, R.J.T.**, Fairhurst, H.M., Coombs, G.W., Grubb, W.B. and Ramsay, J.P. (2015) Origin-of-transfer sequences facilitate mobilisation of non-conjugative antimicrobial-resistance plasmids in *Staphylococcus aureus*. Nucleic acids research, 43, 7971-7983.

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Contents

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Abbreviations

Abbreviation	Definition
ABC	Adenosine triphosphate-binding cassette
AMR	Antimicrobial resistance
AURA	Antimicrobial Use and Resistance in Australia
BLAST	Basic local alignment search tool
CA-MRSA	Community-associated MRSA
CRISPR	Clustered Regularly Interspersed Palindromic Repeat –
crRNA	Ribonucleic Acid
DUF	Domain of unknown function
GFP	Green fluorescent protein
HA-MRSA	Healthcare-associated MRSA
HGT	Horizontal gene transfer
IS	Insertion sequence
LA-MRSA	Livestock-associated MRSA
LB	Lysogeny broth
MBP	Maltose-binding protein
MDR	Multidrug-resistant
MGE	Mobile genetic element
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*

MSSA	Methicillin-sensitive *Staphylococcus aureus*
NSW	New South Wales
ORF	Open reading frame
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PRF	Programmed ribosomal frameshift
PVL	Panton-Valentine leucocidin
RCR	Rolling-circle replication
rpm	Revolutions per minute
RM	Restriction-modification
SaPI	*Staphylococcus aureus* pathogenicity island
SCC*mec*	Staphylococcal cassette chromosome *mec*
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
SSTI	Skin and soft-tissue infection
ST	Sequence type
Tn	Transposon
WA	Western Australia
WGS	Whole-genome sequencing
WHO	World Health Organization

Chapter 1

Introduction

1.1 Chapter outline

This chapter presents an introduction to the thesis and is broadly divided into two parts. The first part of the chapter provides a comprehensive review of antimicrobials and AMR. The second part of the chapter introduces the subject microbe Methicillin-Resistant *Staphylococcus aureus* and outlines the key knowledge gaps and aims of the study.

1.2 Life before antibiotics

Throughout history, bacterial infections have affected the quality and longevity of human life. Historical records of bacterial disease outbreaks existed prior to the scientific understanding of infectious diseases and illustrate life in a world completely vulnerable to bacterial pathogens. With a lack of understanding of infections and their causes, military conflicts, famine, poor hygiene and sanitation and no reliable treatments, outbreaks of infectious disease progressed unhindered.

Amongst the most prolific of outbreak pathogens is *Yersinia pestis*; the likely cause of the Justinian Plague (6th century Mediterranean), the infamous Black Death pandemic of 14th century Europe, and the later Third Pandemic of 19th century Asia. Transmitted from rodents to humans via fleas and lice, it is estimated that up to a third of the European population perished as a result of the Black Death pandemic (6). In its bubonic stage the disease is highly contagious and has a mortality rate of 40-70%. Without prompt antibiotic treatment, the pneumonic and septicaemic forms of plague are almost always fatal (7). Although still encountered today, the use of antimicrobials has limited the large scale of outbreaks of *Y. pestis.*

Many bacterial infections, which are easily preventable and treatable today, plagued much of the world and were a significant cause of disability. For example, syphilis, a sexually transmitted disease caused by the spirochete bacterium *Treponema pallidum* caused a pandemic in medieval Europe (8). Syphilis has many superficial manifestations, progressing from chancre sores and disfiguring rashes to the destruction of the nose and palate (9). Late stage syphilis can involve the eyes, central nervous system and cardiovascular system (10) while maternal syphilis increases the risk of stillbirths (11).

For much of human history treatment options have consisted of various herbal remedies. Although most offered little more than placebo effects, some remedies have been shown to have had truly beneficial antibiotic properties. Examples include, although are not limited to, the use of quinine-containing *Cinchona* bark by Peruvian Indians to treat malaria (12); the topical uses of fungi to treat infected wounds (13); and the European use of mercury to treat leprosy and syphilis (14). However, the mechanisms of disease and their treatments were not understood, and the most effective remedies were not readily accessible throughout the world.

1.3 The early years of microbiology

Prior to the 19th century, understanding the cause of infectious diseases was limited or at least sporadic. However, there has long been understanding of the spread of disease by direct contact. An early example of this understanding can be seen in the writing of Italian physician Girolamo Fracastoro, who recognised in 1546 that syphilis was a disease spread by intimate contact (12).

Microorganisms had been directly observed as early as 1665 by Robert Hooke and 1676 by Antonie van Leeuwenhoek, with the development of the microscope. However, the causal relationship between microbes and infectious disease took much longer to develop. The prevailing theory of disease in the western world, originally posed by Hippocrates in Classical Greece and was accepted

well into the 17th century, was humourism (15), a theory based on the balance of four elements consisting of blood, phlegm, yellow bile and black bile (16).

Towards the end of the 19th century, understanding of microorganisms and infection increased rapidly. Ignaz Semmelweis, while seeking a cause for 'childbed fever' (puerperal fever), performed many autopsies on women who had died during childbirth. In 1847, his friend and forensic pathologist, Professor Jakob Kolletschka, died after accidentally cutting his own finger while performing an autopsy. Upon examining Kolletschka's autopsy, Semmelweis noticed that his death was very similar to the puerperal fevers he had been studying. He hypothesised that "cadaveric particles" infected Kolletschka through the cut in his finger and similarly, these particles could be spread to pregnant women by medical staff who performed autopsies and assisted in delivering babies. While staff washed hands between the two operations, Semmelweis noted the cadaveric particles may have still remain on the hands, made evident by the smell of the hands which remained after autopsy and handwashing. Semmelweis experimented with alternatives to soap and water, determining a chlorine solution to be most effective at removing hand smell after performing autopsies. A trial was performed from June to August using the chlorine hand washing solution which reduced maternal puerperal fever mortality from 7.8% to 1.8%. It was a further 10 years before his work was formally published and longer still before it was generally accepted by the scientific community (17).

Through the mid-1800s, John Snow, Louis Pasteur, Robert Koch and Ferdinand Cohn developed microbiological techniques, understanding of microbial biology and contributed to the Germ Theory of disease, wherein infectious disease was attributed to microbes. With an understanding that microbes could cause disease and be spread, but also that they could be killed, the foundations for chemotherapeutics had been laid. Joseph Lister first described the use of carbolic acid to dress wounds before surgery in 1867, noting it prevented the suppuration of wounds after surgery (18). The complexity and scope of diseases that could be addressed by surgery was still limited by infection, but the successful demonstration of chemical antisepsis helped to solidify the germ theory of disease.

Even as sanitation, hygiene and medical understanding improved, the accounts of military medics from as recently as World War I show us that death and loss of limbs in war were often the effect of bacterial infection, rather than from direct trauma. Major General Escritt, aboard the Hospital Ship *Letitia* described the injuries of the soldiers aboard quickly becoming uncontrollably infected: "after a couple of days, nearly all the cases became septic and the odour of pus was overwhelming" (19).

1.4 The antibiotic age

The growing acceptance of germ theory, the demonstrated beneficial effect of antiseptics on wounds and in surgery, and the rapidly expanding field of chemistry in the late 1800s raised the possibility of internal antisepsis (20). The effects of internally administering antiseptics, typically orally or as a vapour, predictably presented issues of toxicity ranging from abdominal discomfort (from the use of phenol and various coal-tar derived products) (20) to burning of the bladder and urethra, and nephrotoxicity (urotropine) (21). The therapeutic benefit of such attempts at internal antisepsis barely outweighed the adverse effects.

Truly selective antibiotic chemotherapy, developed with the idea of selective toxicity, is often attributed to Paul Ehrlich. He noted chemicals which have an affinity for the agents of infectious disease were also likely to share an affinity with human cells and, "therefore one must search for therapeutic agents that are selective and that possess a relatively high affinity, and toxicity, for the parasite in relation to the animal body, so that it is possible to kill the parasites without serious damage to the body". The use of dyes to selectively stain tissues gave a visual representation of their effect on cells – a visual demonstration of his concept of selective toxicity. Through the study of chemically modified arsenical dyes, Ehrlich's lab identified a compound in 1907 which could kill the spirochete responsible for syphilis, *Treponema pallidum,* without harming the infected ape host. This compound,

the 606th tested, was patented and called arsphenamine/Salvarsan (22), and was the first effective medicinal treatment for syphilis.

Many other companies emerged in the search for chemotherapeutics, following the medical and commercial success of salvarsan, employing the same industrial approach of Ehrlich's lab. Gerhard Domagk, working at IG Farben, continued Ehrlich's work on azo dyes and discovered that a sulphonamide-dye compound was effective against *Streptococcus pyogenes.* Domagk used the new drug to cure his own daughter, leading to a patent for the drug Prontosil in 1935 (23).
Failing to realise that the active constituent of prontosil was the sulphonamide group, the active metabolite sulphanilamide was unpatented and lead to an explosion of companies producing the compound and derivatives, and was widely used during World War II. The benefit of these first antibiotics were immediately evident and between 1937 and 1943; sulphonamides alone were estimated to have caused an overall decrease of mortality of 2-3% with a specific decline in maternal mortality (24-36%), pneumonia (17-32%) and scarlet fever (52-56%) (24).

The outbreak of World War I highlighted the Allies' dependence on Germany for the manufacture of synthetic antibiotics (25) and helped to spur interest in the discovery of new antibiotics. The discovery of penicillin by Fleming, published in 1929, was based on the observation of a contaminant *Penicillium* mould on a staphylococcal agar culture, which had produced a substance able to diffuse into the agar which lysed the surrounding bacteria (26). Fleming's work was progressed by Howard Florey, who saw the need for better therapeutic agents to combat the infections of World War II. He assembled a research group to scale-up the production of penicillin, from culture and extraction to biochemical analysis, and helped to demonstrate its safe and rapid effect in treating bacterial infections (13).

While it is disputed whether Fleming was the first to discover or even publish on the antibacterial potential of fungi, his discovery drew attention to the vast, untapped potential for bioactive compounds in nature and provided a simple method to screen for them (27). Shortly after the success

of penicillin, Selman Waksman, who had earlier noted the antibiotic properties of *Actinomycetes* but had not considered their clinical potential, began earnestly screening the products of *Actinomycetes* and discovered streptomycin in 1944 (28), which was found to be an effective treatment for tuberculosis (29).

Many natural microbially-produced antibiotics have issues with chemical stability and bioavailability, which can be overcome by the addition of sidechains to produce semi-synthetic antibiotics. The concept of modifying naturally-occurring antibiotics with side-chains became popular after 6-amino penicillanic acid (6-APA) was isolated from fermentation broths and, as a penicillin core with no sidechains, was found to have no antibacterial activity (30). Many semi-synthetic penicillins were then described in the 1960s including cloxacillin, ampicillin, amoxicillin, piperacillin and methicillin. Aminoglycosides derivatives followed with the discovery of amikacin in the 1971 (31).

The discovery of antibiotics represents one of the greatest benefits of science to humanity in the modern age and while it is impossible to know the exact impact of the introduction of antibiotic chemotherapy into medicine, the use of antibiotics has undoubtedly decreased the mortality rates of many bacterial diseases. In 1900, 30% of all deaths in the United Stated of America (USA) occurred in children under five years of age, reducing the nation's average life expectancy. The three leading causes of death in the USA in 1900 were pneumonia (all forms) and influenza, tuberculosis, and diarrhoea, enteritis, and ulceration of the intestines (https://www.cdc.gov/nchs/nvss/mortality_historical_data.htm), all of which are bacterial or viral diseases. In 2016, the top three causes of death globally were ischaemic heart disease, stroke and chronic obstructive pulmonary disease, all of which are non-communicable diseases (https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death). From 1900 to 1980, infectious disease mortality declined from 797 deaths per 100000 to just 36 deaths per 100000 (32). While the decline of many infectious diseases, such as tuberculosis, had already begun before the advent of antibiotics possibly as a result of improved standards of living (33) and therefore cannot be attributed to antibiotics alone, the benefit of antibiotics is definitely seen in the reduction of

mortality for specific diseases. The fatality risk of typhoid fever (caused by *Salmonella enterica*) was approximately 10-30% in the pre-antibiotic era, while current mortality rates with antibiotic treatment are below 1% (34). Similarly, fatality from the tropical mite-borne disease Scrub Typhus (caused by *Orientia tsutsugamushi*) has decreased from an average of 6% without antibiotic treatment to 1% with treatment; however, rates can reach 70% fatality in the absence of any appropriate treatment (35). The introduction of multidrug antibiotic therapy for leprosy has reduced the global prevalence by 45% (36).

Following the therapeutic success of antibiotics, their use expanded to infection prevention. The value of antibiotic prophylaxis in surgery was recognised in the 1960s (37) and has since become the standard of care. Surgical prophylaxis and medical prophylaxis are the first and third most common indications for antibiotic prescribing in Australian hospitals (as of 2017) and represents 13% and 7.3% of all Australian hospital antibiotic prescribing, respectively (38). Antibiotics have provided great benefits to human health and have become an essential tool in modern healthcare.

The term ‘antibiotic’, while commonly used in reference to pharmaceutical antibacterial drugs, is formed of the Latin words ‘anti’ meaning against and ‘bio’ meaning life, and as such is not specific to bacteria. Therefore, the term ‘antimicrobial’ will be used hereafter to describe organic compounds, of natural or synthetic origin, which inhibit or kill bacteria and not mammalian cells.

1.5 Antimicrobial resistance

AMR is defined as a decrease in the ability of antimicrobials to kill or inhibit bacteria as a result of genetic changes that have occurred in those bacteria. The genetic changes can be the result of natural selection acting on random DNA mutations, or result from resistance gene acquisition. AMR has been present in bacterial populations long before the chemotherapeutic use of antimicrobials by humans.

The first case of resistance to penicillin occurred in 1940 (39), several years before penicillin was mass produced and used as a therapeutic. AMR is hypothesised to be a natural defence for microbes who share biological niches with antimicrobial producers, or as a self-defence against those compounds by producers (40). Examples of antimicrobial producers carrying resistance genes to the antimicrobials they produce are seen in the soil bacteria of the *Streptomyces* family, a family of bacteria responsible for producing the majority of discovered antimicrobial compounds. *Streptomyces kanamyceticus* and *S. specabilis* produce acetyltransferases which inactivate kanamycin, gentamicin and neomycin. The similarity of these acetyltransferases to those found in clinically-resistant gram-negative bacteria supports the idea that clinical AMR may be derived originally from antimicrobial producers (41).

AMR has serious medical consequences. AMR causes increased mortality and morbidity (length of hospital stays and rate of complications), and places increasing financial burdens on patients and healthcare systems. A meta-analysis of *S. aureus* bacteraemia studies published between 1980 and 2000 found a significant increase in mortality associated with methicillin-resistant *S. aureus* (MRSA) bacteraemia (42). In a study of patients admitted to a USA hospital between 1997 and 2000, surviving either methicillin-susceptible *S. aureus* (MSSA) or MRSA bacteraemia, patients with MRSA had a greater median hospital stay (9 days vs 7 days, P = 0.045), as well as significantly greater median costs associated with their hospital stays ($19,212 vs $26,424, P = 0.008) (43). Resistance to first line antimicrobials requires the use of alternative, often less-effective, more toxic or more expensive antimicrobials. MRSA, unable to be treated with semi-synthetic penicillinase-resistant penicillins such as methicillin and flucloxacillin, is treated with rifampicin, fusidic acid and in serious MRSA infections, vancomycin, daptomycin or linezolid. Systemic fusidic acid formulations cannot be prescribed alongside statin medications, as the combination may cause severe and occasionally fatal rhabdomyolysis (44). Given the widespread use of statins in Australia (up to 46% of geriatric patients in a large subacute care unit of an Australian hospital (45)), the opportunity for appropriate fusidic acid prescribing is limited. While safe and effective for severe MRSA infection, staphylococci rapidly develop resistance to rifampicin and so it must be used in combination with at least one other antimicrobial agent (46). Vancomycin use is associated with significant nephrotoxicity (47), while the extended use of linezolid has resulted in fatal lactic acidosis (48). In addition to adverse effects,

vancomycin can cost 30% - 40% more than linezolid over the duration of treatment for MRSA-pneumonia (49). In gram negative bacteria, resistance to cephalosporins has caused colistin to be brought back into therapeutic use, despite colistin being abandoned in the 1980s due to toxicity and the development of second and third-generation cephalosporins (50).

A significant concern in the development of AMR is the rise of 'super bugs'; bacterial pathogens which are pan-resistant (effectively resistant to all available antimicrobials). Superbugs pose a serious threat, as they can be resistant to all clinically available antimicrobials, leaving no reliable therapies for their treatment. Infections by *Klebsiella pneumoniae* (51), *Pseudomonas aeruginosa* (52) and *Acinetobacter baumanii* (53) superbugs can be fatal. Extensively drug-resistant *Mycobacterium tuberculosis* have been an ongoing issue in India for at least 10 years (54) and several pan-resistant tuberculosis isolates have been collected across the developing world (55).

It is important to prevent, monitor and control the rise of AMR. Given the medical and financial implications of AMR, there is an urgent need to conserve the effectiveness of our current antimicrobial arsenal and halt the spread of AMR. Aside from concerns for current antimicrobial efficacy, there is concern new antimicrobials may not be developed fast enough to replace the old antimicrobials to which resistance has become commonplace. The development of new antimicrobial drugs has slowed, with USA Federal Drug Administration approvals for new antibacterial agents decreasing 56% when comparing approvals from 1998-2002 and 1983-1987 (56). The time and costs associated with developing new antimicrobials are large. Antimicrobials have short durations of use relative to drugs used to treat chronic illnesses and thus are less attractive to develop from a financial perspective. Research to identify totally novel antimicrobials through target-based discovery approaches is expensive and has a high failure rate. These factors together with increasing regulatory requirements for new therapeutic drug registrations make antimicrobials a poor financial choice for pharmaceutical companies to pursue (57). The cost to develop new drugs is estimated to be greater than US $1B (58) and requires greater than 10 years to bring a new drug to market, with investors and companies carrying the financial risks a drug will fail to meet safety standards in clinical trials. Additionally, drugs

may pass clinical trials and receive USA Federal Drug Administration approval, yet still face issues upon market release (such as unpredictable adverse drug events), causing the drugs to be less widely used or be completely withdrawn from market. For example, severe adverse reactions to the fluoroquinolone trovafloxacin resulted in its withdrawal in 1999 (59). New antimicrobials which are demonstrated to be safe and effective become reserved for severe infections, to prevent the development of resistance, which limits their use initially and further reduces the incentive to develop new antimicrobials. Given the poor outlook for the development of new antimicrobial drugs, it is essential that we conserve the efficacy of antimicrobials we have currently available.

The frequency of AMR correlate with prevalence of antimicrobial use. Antimicrobials directly select for AMR, for example this can be seen *in vitro* by the selection of antimicrobial-resistant mutants on antimicrobial-supplemented agar culture. Even in 1904, the earliest days of antimicrobial chemotherapy, Ehrlich noted how resistance developed to distinct classes of drugs in strains of trypanosomes after repeated passaging in the presence of low concentrations of drugs (60). The phenomenon of mutational resistance has been demonstrated for many antimicrobial drugs (61) and is monitored during the clinical trial of new antimicrobial compounds (62). The introduction of new antimicrobials throughout the past 100 years has been met with new resistant pathogenic bacteria, often within very short timeframes. The first clinically significant cases of AMR were noted after treatment failures of gonorrhoea with sulphonamides in 1942 (63) and penicillin in 1948 (64). Resistance to sulphanilamide emerged within five years of its clinical introduction, while penicillin resistance was observed even before mass production had developed**.** Clinical resistance to new antimicrobials has closely followed the discovery of antimicrobials (

Table 1-1), which does not take into account the time taken for the introduction of antimicrobials to therapeutic use.

Table 1-1 - Timeline of antimicrobial discovery and resistance

Drug	**Class**	**Discovery Date (reference)**	**Clinical Resistance (reference)**

Salvarsan	Arsenical	1907 (65)	1932 (66)
Prontosil	Sulphonamide	1935 (67)	1944 (23)
Penicillin	β-lactam	1929 (26)	1942 (68)
Streptomycin	Aminoglycoside	1944 (28)	1946 (69)
Cephalosporin	β-lactam	1948 (70)	1963 (71)
Chloramphenicol	Phenicol	1948 (72)	1954 (73)
Aureomycin (Chlortetracycline)	Tetracycline	1948 (74)	1953 (75)
Neomycin	Aminoglycoside	1949 (76)	1954 (77)
Erythromycin	Macrolide	1952 (78)	1954 (79)
Vancomycin	Glycopeptide	1955 (80)	1988 (81)
Methicillin	β-lactam	1959 (82)	1960 (83)
Fusidic acid	Tetracyclic triterpenoid	1962 (84)	1965 (85)
Gentamicin	Aminoglycoside	1963 (86)	1971 (87)
Co-trimoxazole	Sulphonamide/trimethoprim	1962 (88)	1972 (89)
Rifampin	Rifamycin	1966 (90)	1968 (91)
Ciprofloxacin	Quinolone	1983 (92)	1984 (93)
Azothromycin	Macrolide	1987 (94)	1993 (95)
Quinupristin/dalfopristin	Streptogramin	1999 (96)	2000 (97)
Tigecycline	Glycylcycline	1999 (98)	2005 (99)
Linezolid	Oxazolidinone	1996 (100)	1999 (101)
Telithromycin	Ketolide	1997 (102)	2000 (103)
Teixobactin	Macrocyclic depsipeptide	2015 (104)	N/A

The link between the widespread use of antimicrobials and rising AMR in common hospital pathogens became apparent by the 1950s. A significant increase in the frequency of penicillin resistance in

S. aureus occurred in the 1940s, from 10% penicillin resistance in 1943 to 50-90% resistance in 1954. Increases in penicillin minimum inhibitory concentration (MIC) were also observed in meningococci following the adoption of penicillin treatment in the late 1980s in Spain (Table 1-2). These observations

led several groups to the conclusion that AMR was the direct consequence of antimicrobial use (105-108).

Table 1-2 – Increasing penicillin resistance in meningococcal isolates following the adoption of penicillin treatment (109)

	N. meningitidis isolates by year					
Penicillin MIC (μg/mL)	1985	1986	1987	1988	1989	1990
≤ 0.05	266	241	366	236	191	67
0.1	0	0	4	4	8	13
0.2	1	12	18	33	31	33
0.4	0	2	7	7	9	18
0.8	0	0	0	2	0	0
Total isolates	267	255	385	282	239	131
% of isolates with MIC ≥ 0.1	0.4	5.3	7.3	16.3	20.1	46

1.5.1 Coordinated government antimicrobial-resistance strategies

Government bodies have recognised AMR as a major threat, with significant mortality and healthcare costs associated. The recognition of AMR as a significant threat has resulted in the development of several interconnected action plans to combat the development and spread of AMR. Each of these actions plans are united under a shared One Health philosophy.

A large study published in *The Lancet* estimated the deaths and disability-adjusted life-years, which could be attributed to or associated with the bacterial AMR, of 23 pathogens in 204 countries and territories in 2019. Using predictive statistical modelling, they estimated that there were 4.95 million deaths associated with bacterial AMR in 2019, of which 1.27 million deaths were attributable to bacterial AMR. The all-age death rate attributable to AMR was highest in western sub-Saharan Africa, and lowest in the Australasia region. The six leading pathogens were *Escherichia coli, Staphylococcus*

aureus, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, in descending order of deaths associated with AMR. These six pathogens alone were estimated to be responsible for 72% of deaths associated with AMR and 73% of deaths attributable to AMR (110).

The World Health Organisation (WHO), founded in 1948, is a United Nations agency formed to promote and improve global health, working with governments of countries around the world, and non-government organisations to improve global health. The WHO declared bacterial AMR as one of the top 10 public health threats, endorsing a global AMR action plan in 2015. The plan includes measures to improve awareness and understanding of antimicrobial resistance, to strengthen surveillance and research, reduce the incidence of infection, optimise the use of antimicrobial medicines and to ensure sustainable investment in countering antimicrobial resistance (111). One of the initiatives of this plan is the formation of a Global Antimicrobial Resistance and Use Surveillance System, which publishes annual surveillance reports of AMR across 109 countries (112). In addition to monitoring AMR associated with urinary tract infections, gastrointestinal and genital infections, in 2019 the WHO began reporting specifically on MRSA bloodstream infections. In 2021, the WHO reported that the median rate for methicillin-resistance in *S. aureus* bloodstream infection was 24.9% and found that low-middle-income countries had higher incidence of MRSA bloodstream infection than high income countries (113).

The US Centre for Disease Control (CDC), a branch of the US Health Department, has put forward a National Action Plan for Combating Antibiotic-Resistant Bacteria, 2020-2025 (114), which builds on their previous National Action Plan (2015). The current National Action Plan sets similar goals to the WHO, with five goals, focused on surveillance, rapid testing, identification and characterisation of AMR bacteria, accelerated research and increased international collaboration. MRSA is categorised as a serious threat by the US CDC, the second highest threat level. The CDC estimated that MRSA infections have caused 10,600 deaths, 323,700 hospitalisations and resulted in $1.7B in healthcare costs in 2017. Rates of CA-MRSA bloodstream infections have decreased year-on-year in the US, while the rate of

HA-MRSA bloodstream infection have stalled since 2013. These decreases of MRSA bloodstream infection have been attributed to targeted control measures implemented across Veterans Affairs hospitals and include screening of patients upon hospital admission, tracking of MRSA cases and an emphasis on preventative hygiene practices (115).

The Australian government Department of Health has a strategy to combat AMR called Australia's National Antimicrobial Resistance Strategy – 2020 and Beyond, which also builds on a 2015 action plan (116). The strategy has seven objectives: clear governance for AMR initiatives, prevention and control infection and AMR spread; greater engagement in AMR initiatives; appropriate antimicrobial usage and stewardship; integrated surveillance of AMR and antimicrobial usage; collaboration with research; and strengthened global collaboration. The strategy aligns and collaborates with the WHO Global Action Plan. Several public and private governing bodies will join forces under the plan to implement the strategy, with AMR surveillance provided by the Antimicrobial Use and Resistance in Australia (AURA) Surveillance System (117). AURA is tasked with reporting *S. aureus* bacteraemia, detecting methicillin-resistance in bacteraemia isolates and analysing their molecular epidemiology. In Australia in 2019, AURA reported 3,157 *S. aureus* bacteraemia cases reported, of which 79.8% were community-associated and 18.5% were MRSA (118).

Each of these strategies to combat AMR are based in One Health principles. These principles recognise that the health of humans, animals and the environment are interconnected, and therefore, attempts to improve overall health requires cooperation between governments and organisations involved in each area. The strategies described above aim to implement surveillance and antimicrobial usage guidelines within human, animal and environmental health to detect and control AMR infection and AMR gene spread, while promoting research and funding for One Health initiatives.

1.6 Antimicrobial targets and mechanisms

To be effective, antimicrobials must exhibit selective toxicity towards bacteria. That is, they must cause more damage to the bacteria than to the host organism (human or animals). To achieve selective toxicity, antimicrobials must therefore target processes and/or structures which are unique to bacteria, are non-essential in mammals, or which may exist in mammalian cells but which are different enough to bacterial cells that they cause more damage to bacteria than to the host. Such structures and processes include bacterial cell walls, which mammals lack, and bacterial ribosomes, which differ from eukaryotic ribosomes. Most antimicrobials exert their inhibitory effects on bacteria by the competitive inhibition of target enzymes or by irreversible covalent attachment to target enzymes.

There are multiple classes of antimicrobials, grouped together by structure and therefore, by function. The main antimicrobial drug classes, in order of discovery, are the sulphonamides, penicillins, tetracyclines, amphenicols, macrolides, glycopeptides, quinolones, lincosamides and oxazolidinones. Drugs within these classes typically share mechanistic targets, for example chlortetracycline and oxytetracycline are members of the tetracycline class, and inhibit bacterial protein synthesis by binding to the 50S bacterial ribosome. Additionally, some antimicrobials from different classes share mechanistic activity, for example macrolides and tetracyclines target bacterial ribosomes, despite their structural dissimilarity. Antimicrobials kill or inhibit bacterial growth by targeting cell wall synthesis, protein synthesis, nucleic acid synthesis, cell membrane function, ATP synthesis and various other metabolic processes. The processes most relevant to this thesis are cell wall synthesis and protein synthesis, and so these processes are explained below. For reviews on antimicrobial inhibition of nucleic acid synthesis (119), cell membrane function (120) and various other metabolic processes (e.g. folate production (121)) can be found in the citations corresponding to each process. More recently, antimicrobials have been developed to target ATP synthesis (122).

1.1.1 Cell wall-targeting antimicrobials

For most bacteria, cell walls are an essential component for microbial survival under normal environmental conditions and provide structure to bacterial cells which allow regulation of the intracellular environment. Bacterial cell walls are comprised of alternating units of *N*-acetylglucosamine linked with *N*-acetylmuramic acid by β 1 → 4 glycosidic linkages. *N*-acetylmuramic acid units are cross-linked by pentapeptide bridges (L-Ala–D-Glu–L-Lys–D-Ala–D-Ala). The structure provides great strength to the microbial envelope to withstand turgor pressure, which is the intracellular hydrostatic pressure caused by the osmotic differential across the membrane of the cell.

The synthesis of cell walls occurs in three broad stages – the cytoplasmic, the membrane-associated and the extracytoplasmic stages which, in short, comprise the production of cell wall components (*N*-acetylmuramic acid, *N*-acetylglucosamine and peptide bridges), translocation of cell wall components across the cell membrane, and the joining of peptidoglycan chains into a mesh-like structure. Whilst an in-depth discussion of cell wall synthesis is beyond the scope of this study, a detailed review of cell wall synthesis has been written by Liu and Breukink (123). β-lactam antimicrobials target the third stage of cell-wall synthesis and exert their antimicrobial effects by mimicking the natural D-Ala-D-Ala substrate of the family of enzymes known as penicillin-binding proteins (PBP), which are responsible for cross-linking the peptidoglycan component of the bacterial cell wall (124). β-lactam antimicrobials form a penicilloyl-enzyme complex with PBP which inhibits their transpeptidase activity (125), disrupting the integrity of the cell wall and ultimately resulting in cell lysis.

Glycopeptide antimicrobials such as vancomycin also inhibit the transpeptidation stage of cell wall synthesis, interfering with the activity of the PBP, however, unlike β-lactams they do not directly attack the PBP enzyme. Vancomycin forms hydrogen bonds with the D-Ala-D-Ala moieties of the *N*-acetylmuramic acid peptide bridges of forming cell walls, preventing PBP from binding to the D-Ala-D-Ala substrate and thus preventing the transpeptidation reaction which provides the strength to the cell wall (126, 127).

While the majority of cell-wall targeting antimicrobials are involved in inhibiting transpeptidation, a number of other antimicrobials target different stages of cell wall synthesis, or disrupt the cell membrane. An overview of cell-wall synthesis reactions and the antimicrobials which target those processes is provided in Figure 1-1.

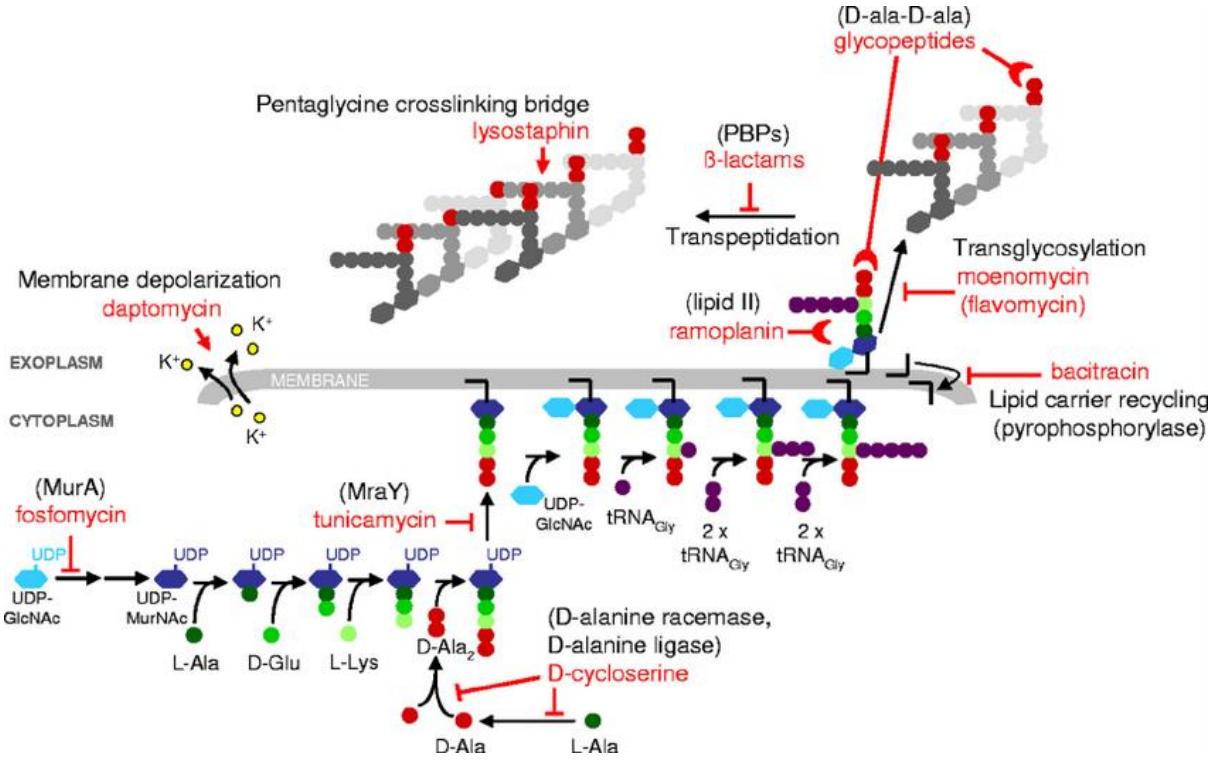

Figure 1-1 - Molecular targets of cell-wall targeting antimicrobials.

Antimicrobials are labelled in red. Reproduced from McCallum et al, 2011 (1).

1.1.2 Protein translation and ribosome-targeting antimicrobials

Ribosomes are the cellular macromolecules responsible for protein synthesis, a fundamental process of cellular biology and a common antimicrobial target. The bacterial ribosome is composed of RNA subunits including the 16S, 23S and 5S rRNA, and a varying number of ribosomal proteins. The *E. coli* genome encodes 54 ribosomal proteins and the majority are essential for survival (128). In *E. coli* the

small ribosomal subunit (or 30S subunit) is composed of the 16S rRNA and 21 ribosomal proteins, while the large ribosomal subunit (or 70S subunit) is composed of the 23S and 5S rRNA and 33 ribosomal proteins. During protein synthesis, the ribosome polymerises amino acids into polypeptide chains in a process which can be broadly categorised into four steps: initiation, elongation, termination and recycling. Initiation requires the formation of a 70S ribosome from the smaller 30S and 50S rRNA subunits and the coordination of the initiator tRNA with the start codon of the messenger RNA (mRNA) at the ribosomal P-site (peptidyl-tRNA binding site). Elongation involves the introduction of an aminoacylated tRNA to the A-site of the ribosome, monitored interaction of the tRNA with the mRNA codon, peptide bond formation of the A- and P-site tRNAs, and the translocation of the tRNAs from the A- and P-sites to the P- and E-sites (exit site), elongating the chain out through the exit tunnel of the 50S subunit. Termination occurs upon recognition of a stop codon along the mRNA by termination release factors, which release the polypeptide chain from the ribosome. Recycling involves the disassembly of the post-termination complex, allowing the components to be reused in the next round of translation initiation. Ribosome-targeting antimicrobials inhibit one or more of these steps, preventing the production of biomolecules essential to the growth and function of the bacteria. Additionally, prokaryotic RNA polymerases bind to ribosomes and allow protein translation to proceed while transcription is occurring. The inhibition of translation can therefore result in genome-wide stalling of transcription and can slow DNA replication by obstructing the DNA replication machinery (129, 130). The bacterial ribosome is therefore a very effective target for antimicrobial chemotherapy.

Molecules from a variety of antimicrobial classes target the ribosomal elongation cycle, including the aminoglycosides, tetracyclines, phenicols, lincosamides, macrolides, oxazolidinones, streptogramins and fusidic acid (Figure 1-2). Some antimicrobials bind the 30S subunit, inhibiting translation initiation or elongation by interfering with the interactions of tRNAs with the P-site or delivery/translocation of tRNAs at the A-site. Other antimicrobials bind the 50S subunit at or near the peptidyl-transferase centre, responsible for peptide formation between amino acids during elongation (2). Tetracyclines are a diverse, clinically and agriculturally important antimicrobial class whose members target various stages of protein synthesis. Tetracyclines bind to the ribosomal 30S subunit at ribosomal protein S7

and at bases G693, A892, U1052, C1054, G1300 and G1338 of the 16S rRNA, ultimately disrupting tRNA binding at the A-site (131).

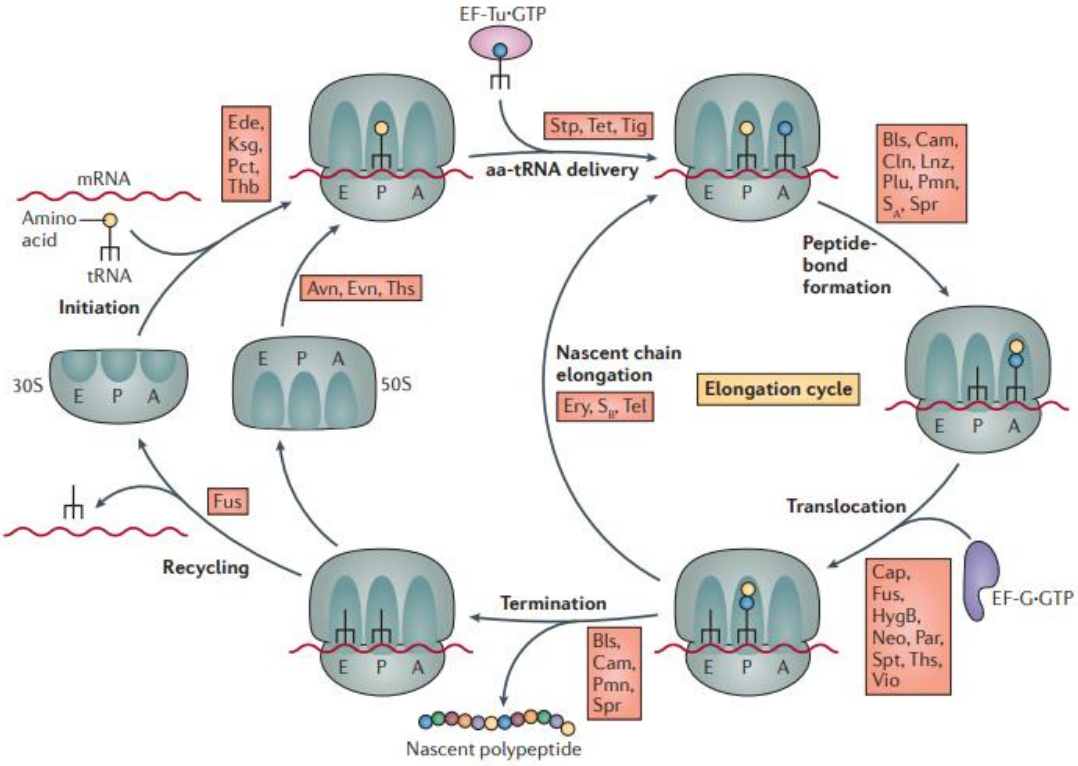

Figure 1-2 - Molecular targets of protein-synthesis inhibiting antimicrobials.

Components of the protein synthesis cycle are labelled (upper left), while the stages of the cycle are indicated in bold text. Red boxes indicate antimicrobials which inhibit protein-synthesis at the step they are located. Antimicrobials: edeine (Ede), kasugamycin (Ksg), pactamycin (Pct) thermorubin (Thb), avilamycin (Avn), evernimicin (Evn), thiostrepton (Ths), streptomycin (Stp), tetracyclines (Tet), tigecycline (Tig), blasticidin S (Bls), chloramphenicol (Cam), clindamycin (Cln),linezolid (Lnz), pleuromutilins (Plu), puromycin (Pmn), streptogramin A (SA), sparsomycin (Spr), capreomycin (Cap), viomycin (Vio), hygromycin B (HygB), neomycin (Neo) and paromomycin (Par), fusidic acid (Fus),

spectinomycin (Spt), erythromycin (Ery), streptogramin B (SB) and telithromycin (Tel). Reproduced from Wilson, 2014 (2).

1.6.1 Linezolid

Linezolid is classified as a "last line" antimicrobial in the treatment of infectious disease in human health and its use is restricted. Linezolid is the first member of the fully-synthetic family of oxazolidinone antimicrobials to be approved for clinical use. Linezolid is a bacteriostatic drug, effective against many common gram positive pathogens (132), and is used in the treatment of a variety of serious human infections including multidrug-resistant tuberculosis (133), streptococcal and MRSA pneumonia (134), and complicated skin and soft tissue infections (135). The oxazolidinone family of antimicrobials are effective against methicillin and vancomycin-resistant pathogens, arriving on the market when fears of vancomycin-resistant *Staphylococcus aureus* were increasing (136). Oxazolidinones are classed as high priority, critically important antimicrobials by the World Health Organisation (137). Linezolid has 100% oral bioavailability and reaches high concentrations in most tissues (138), enabling intravenous to oral switching for outpatient treatment, which reduces hospital stay duration and cost when compared to vancomycin (139). Linezolid is well tolerated, although serious side-effects may arise following long-term use including hyperlactataemia (140), metabolic acidosis, myelosuppression and some optic and digestive disturbances. However, short-term side effects may be dose-dependent and are often reversible (141). The effectiveness of linezolid against a broad range of common and otherwise resistant pathogens, its ease of administration and acceptable safety profile place linezolid as an important therapeutic antimicrobial and its use should be carefully guarded.

The bacteriostatic mechanism of linezolid is similar to other ribosome-targeting antimicrobials and resistance, both mutational and enzymatic, have arisen. Linezolid inhibits protein synthesis by binding to 50S ribosomal subunit which interrupts the formation of the ribosomal initiation complex and distorts the initiator-fMet-tRNA binding site (142, 143). Crystal structures of linezolid-50S ribosome

binding indicates linezolid binds to the 50S A-site, near the catalytic centre and interacts extensively with nucleotides of the 23S rRNA (144). Other ribosome-targeting antimicrobials including macrolide, lincosamide and streptogramin antimicrobial classes also bind the 50S ribosomal subunit but inhibit peptide elongation, rather than the initiation of protein synthesis (145).

1.7 Antimicrobial resistance mechanisms

While the majority of antimicrobials in clinical use target relatively few unique bacterial molecular structures, there are a wide variety of mechanisms through which bacteria can evade antimicrobial activity. These include genetic mutation, efflux, inactivation or modification of the antimicrobial, modification of the drug target, and production of resistant functional molecular homologues of drug targets. AMR can be achieved by mutation. Natural genetic variation between bacteria arises from genetic mutations which occur as the result of DNA damage and/or DNA replication errors during cell division. Mutations which allow survival under antimicrobial selection are able to continue growing and the mutation becomes fixed in the population. Single Nucleotide Polymorphism (SNP) mutations occur at a rate of 1×10^{-3} per genome, per generation in wild-type *E. coli,* exceeding DNA mismatch repair capacity (146). The rate and efficiency of mismatch-repair mechanisms can decrease relative to the background mutation rate according to environmental stresses (such as nutrient deficiency, heat and osmotic stress), following mismatch-repair system downregulation by global stress regulators RPoS and Hfq (147) in a process called stationary-phase mutation (148). Several mutational resistance phenotypes are predictable. For instance, mutations in *rpoB* confer rifampicin resistance by reducing the affinity of RNA polymerase for rifampin in *S. aureus* (149, 150). Mutations in ribosomal rRNA genes confer resistance to ribosome-targeting antimicrobials and the mutation of some nucleotides provide reduced susceptibility and/or resistance to multiple different classes of antimicrobials (151), for example the G2057A mutation provides resistance to erythromycin and chloramphenicol (152). A thorough review of mutational resistance is provided by Woodford and Ellington (61). Mutational resistance tends to be lost from populations in the absence of antimicrobial use. The phenomenon has been demonstrated *in vitro* for mutational rifampicin resistance in *Rhodococcus equi*, where rifampicin

resistance was lost in 2/3 isolates following 100 sequential subcultures in antimicrobial-free media (153).

AMR can be achieved by antimicrobial target overexpression. In *E. coli,* resistance to trimethoprim results from upregulation of dihydrofolate reductase. Trimethoprim is an inhibitor of bacterial dihydrofolate reductase, an enzyme essential to the biosynthesis of purines, pyrimidines and some amino acids (154). Mutations in the promoter region of the *E. coli* dihydrofolate reductase increase transcriptional efficiency and result in a 200-fold over-production of dihydrofolate reductase. The increased production of dihydrofolate reductase results in a trimethoprim minimum inhibitory concentration (MIC) of more than 1000 μg/mL (155), which is 250 fold greater than the 4 μg/mL clinical breakpoint MIC for Enterobacterales (https://eucast.org/clinical_breakpoints/).

Cells can gain protection from antimicrobials by the action of efflux pumps, which expel antimicrobials out of the cell and lower the intracellular concentration of antimicrobials to tolerable levels. Efflux pumps are protein complexes, categorised into five families: the Major Facilitator Superfamily, ATP-Binding Cassette (ABC) transporter family, the small multidrug resistance family, and the resistance-nodulation-division superfamily and the multidrug and toxic compound extrusion (MATE) family. The Major Facilitator Superfamily is the largest family of membrane transporters and is present in prokaryotes and eukaryotes alike (156). Several Major Facilitator Superfamily efflux pumps provide resistance to an array of antiseptics (e.g quaternary ammonia) and antimicrobials (fluoroquinolones) in *S. aureus*. Examples of Major Facilitator Superfamily efflux pumps include the antiporter efflux pumps NorA, QacA and QacC, which offer only limited fluoroquinolone and quaternary ammonium resistance respectively, but which aid survival and allow time for the acquisition of other, more effective resistance genes (157). A variety of less common efflux pumps may provide resistance to tetracyclines (*tet38* (75)), phenicols (*fexA*), streptogramins, lincosamides and pleuromutilins (*vgaA*).

Enzymatic modification of an antimicrobial reduces its antimicrobial activity, often by affecting its ligand binding affinity. The most common enzymatic activities encoded by AMR genes include acetylation, phosphorylation and adenylation. Antimicrobial-modifying enzymes differ in their substrate specificity, differing in the antimicrobial they can modify (for example spectinomycin but not kanamycin) and also in sites of a given antimicrobial that they can modify, while some even have dual biochemical activity (for example adenylation and phosphorylation activity) as seen in the *aac-aph* aminoglycoside resistance gene of *S. aureus* (158). Some examples include aminoglycoside and chloramphenicol resistance genes *aadD* and *cat*, respectively. *aadD(9)* is an aminoglycoside adenylyltransferase gene, which inactivates spectinomycin in staphylococci by catalysing the addition of an adenyl group (159). *cat* is a chloramphenicol acetyltransferase, which acetylates chloramphenicol and is found on small staphylococcal plasmids (160, 161). A different type of enzymatic antimicrobial modification is achieved by the destruction of the active moiety of the antimicrobial. Resistance to β-lactams is conferred by β-lactamase enzymes. β-lactam antimicrobials covalently (and irreversibly) bind to the PBP by their beta-lactam moiety, thus by cleaving the moiety, β-lactam antimicrobials are inactivated. Notably, antimicrobial-modifying enzymes such as β-lactamase, can be secreted into the extracellular environment and provide protection for neighbouring cells which may not be carrying resistance (162, 163).

Resistance can be achieved by modifying the molecular targets of antimicrobials. Many antimicrobials exert their effects by binding to enzymes that are critical to biological pathways or by inhibiting the binding of enzymes with their native substrates and thus disrupting the pathway. Modification of enzyme targets can protect the binding site of the enzyme from antimicrobials while still allowing the native substrate for the enzyme to bind. Many examples of target modification-type resistance exist, particularly against ribosome-targeting antimicrobials. For example, resistance to tetracycline can be mediated by either *tetM* or *tetO* genes, the products of which directly bind the 70S ribosome and inhibit binding of tetracycline to the ribosome by creating conformational change of the ribosome and steric clashes, enabling translation to proceed normally (164, 165). Macrolide resistance is frequently mediated by *erm* genes (***e***rythromycin ***r***ibosomal ***m***ethylation), which methylate the rRNA. The *erm* genes encode enzymes which mono- or dimethylate the adenine residue A2058 of the 23rRNA of the

50S ribosomal subunit (166). Methylation, within the V-domain of the 50S ribosomal subunit, interferes with the binding of macrolides at this site, while allowing translation to proceed. A single A2058T mutation can confer high-level macrolide resistance in *Moraxella catarrhalis*, indicating the importance of this single residue to macrolide binding (167). Several ribosome-targeting antimicrobial (macrolides, lincosamides, and streptogramin B) act on overlapping sites in the 23S rRNA, therefore methylation of the ribosome by *erm* genes can confer cross-resistance to several ribosome-targeting antimicrobials (168).

AMR can also be achieved by the production of an alternative molecule which replaces the function of the drug target. β-lactams antimicrobials exert their bactericidal effect by inhibiting the PBP responsible for cross-linking peptidoglycan chains, which disrupts the cell wall (section 1.1.1). β-lactamase-resistant penicillin-derivatives, such as methicillin, whose lactam ring is protected from β-lactamase cleavage by the addition of a *ortho*-dimethoxybenzoyl group to the sidechain of the lactam group, are able to inhibit PBP despite the presence of penicillin resistance-conferring β-lactamases (169). Resistance to β-lactamase-resistant antimicrobials, such as methicillin, is achieved by the *mecA* gene which encodes an alternative PBP (PBP2a) which has a lower affinity for β-lactams (170). PBP2 and PBP2a function together to provide the transpeptidation and transglycosylation activities necessary to crosslink peptidoglycan and construct a functional cell wall even in the presence of β-lactam antimicrobials (171). Acquisition of *mecA* provides resistance to all β-lactams and most β-lactam-derivatives (172).

1.7.1 Linezolid resistance

As linezolid is fully synthetic, resistance to linezolid was initially thought to be unlikely or to develop more slowly than to naturally-occurring antimicrobials. However, mutational and acquired linezolid resistance has developed clinically. Linezolid resistance can occur through spontaneous mutations in the 23S rRNA nucleotides with which it interacts. Nucleotide mutations within the binding pocket of

the peptidyl transferase centre can confer linezolid resistance, including the nucleotides G2061, C2452, A2503, U2504 and G2505. Several other mutations distal to these typically-conserved nucleotides can also confer resistance (173). Some of the distal mutations are sufficient to provide linezolid resistance alone, such as G2032A in *E. coli* (174). Examination of the linezolid-bound 50S subunit of the resistance-conferring H50S G2032A-C2499A double mutant ribosomal subunit protein of *Mycobacterium smegmatis* indicates peptidyl transferase centre-distal mutations can affect the binding affinity of linezolid (175). Additionally, a variety of mutations in the 50S ribosomal protein L3 and L4 proteins are able to provide linezolid resistance (173). Linezolid resistance however, is not limited to spontaneous mutation and can be acquired by the *cfr* methyltransferase gene (176). Cfr methylates the A2503 residue of the 23S rRNA at position C-8 (177). Linezolid binds to a site within the peptidyl transferase centre of the ribosome which overlaps with several other ribosomal protein synthesis inhibitors and therefore, methylation within the peptidyl transferase centre by Cfr confers a PHLOPSa (phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A) phenotype (178). Molecular modelling and the examination of crystal structure data suggests methylation causes a steric clash within the peptidyl transferase centre of the ribosome, rejecting several structurally diverse antimicrobials and thus providing resistance, while maintaining the function of the ribosome. Clinically, reduced susceptibility to linezolid is rarely the result of *cfr* carriage alone and increasing linezolid MIC values can occur where *cfr* carriage is combined with 23S rRNA mutations, as seen in the isolates of the 2014 USA linezolid LEADER surveillance program (179).The combinatorial effect of *cfr* and ribosomal mutations however, are not always predictable or synergistic (180).

Linezolid resistance due to *cfr* is mobile and is at risk of further dissemination. The *cfr* gene was first identified in the year 2000 from a *S. sciuri* isolate, obtained from the nasal swab of a calf suffering a respiratory infection. The isolate was resistant to multiple antimicrobials including chloramphenicol and florfenicol, hence the gene was named *cfr* (176). The authors note chloramphenicol resistance is typically mediated by the chloramphenicol acetyltransferase *cat* gene in staphylococci which cannot provide resistance to florfenicol, a fluorinated derivative of chloramphenicol. *cfr* has since been discovered in *Enterococcus* species (181), *E. coli* (182, 183) and several staphylococci including *S. aureus, S. capitis* (184), *S. epidermidis*, *S. hominis, S. lugdunensis* and *S. arelettae* (185, 186). *cfr* is

typically carried by plasmids and several *cfr*-plasmids are capable of conjugative transmission. *cfr*-plasmid conjugation has been demonstrated in *E. faecalis* (187) and in *S. aureus* (188, 189), confirming *cfr* is capable of interspecies horizontal gene transfer (HGT).

cfr has a strong association with meat and meat production, and is only sporadically detected in human clinical isolates. *cfr*-positive staphylococci have been identified in 18.4% of samples from retail meats in China (190). *cfr* was also detected in 15.7% of staphylococci from a total of 765 samples, taken from six pig farms in four geographically distinct provinces in China (191). Clinical isolates and small outbreaks of *cfr*-positive linezolid resistant staphylococci have since occurred at hospitals in Mexico (192), Spain (193), the USA (194) and Ireland (195). The first *cfr*-mediated linezolid resistant clinical isolates were MRSA emerging in Columbia and the USA in 2005 (196, 197). *cfr* has since been identified in the epidemic ST8 IV (USA300) and ST22-IV (EMRSA-15) MRSA clones (5, 188).

1.8 Mobile genetic elements and horizontal gene transfer

When resistance is provided by discrete genes or cassettes of genes, the genes (and therefore the AMR they confer), can be transferred by mobile genetic elements (MGE). MGEs can be transmitted vertically and horizontally. Vertical transmission occurs when cells divide and the MGE is replicated and segregated with genomic DNA. HGT is the dissemination of MGEs between distinct cells, strains and even different species and genera. Different physical mechanisms of HGT include conjugation, transformation and transduction (Figure 1-3, (3)). HGT occurs almost universally in prokaryotes and is the primary mechanism of the spread of AMR between bacteria. The transmission of MGEs between bacteria contribute to the success of their host by encoding accessory genes, such as AMR and virulence genes, facilitating rapid adaptation of bacteria to their environment (3).

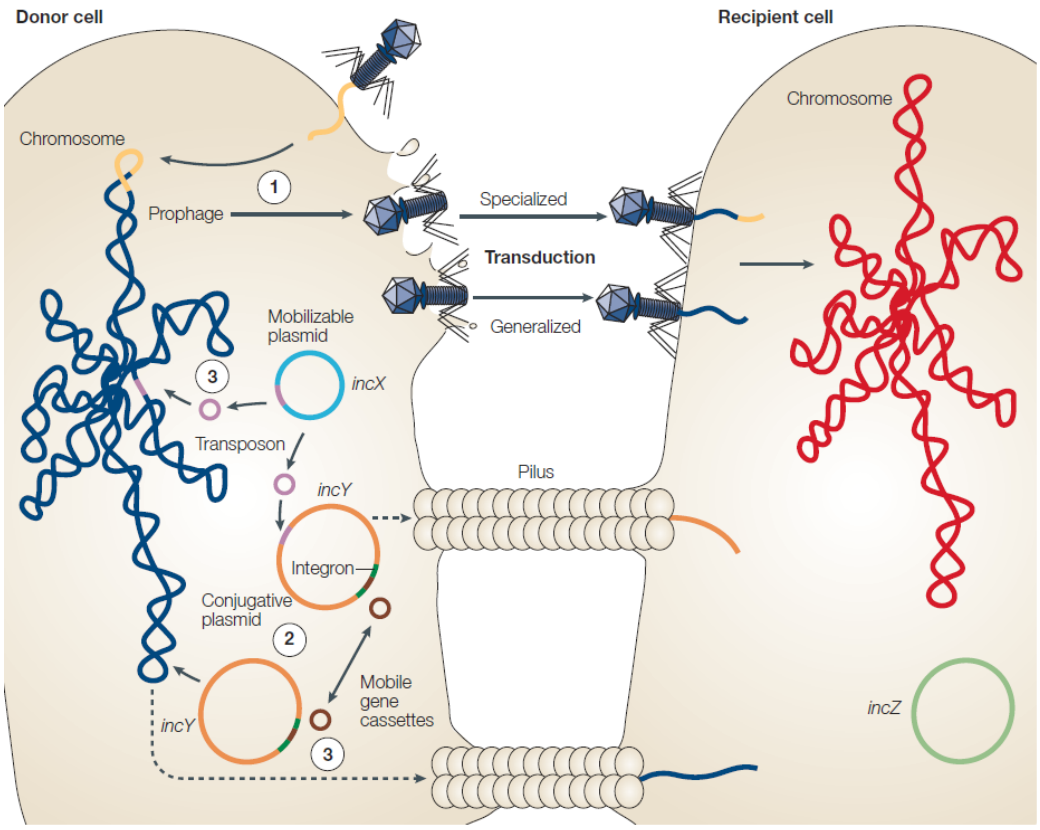

Figure 1-3 - Mechanisms of horizontal gene transfer.

Transduction (1). A temperate phage inserts its DNA (yellow) into the chromosomal DNA (dark blue) of a bacterium. The phage may later replicate, packaging host DNA (generalized transduction) or a combination of host DNA and phage DNA (specialized transduction). The phage lyses the donor cell and infects a recipient cell, transferring the DNA to the recipient chromosome (red). Conjugation (2). Conjugative plasmids (orange) encode proteins to form a mating pore between donor and recipient cells, as well as proteins to nick, unwind and guide the plasmid DNA to and through the pore to the recipient where the plasmid recircularises. Mobilisable plasmids and parts of the chromosome may also occasionally be transferred during plasmid conjugation through the mating pore. Recipient cells may already contain resident plasmids (light green), and maintenance of multiple plasmids can occur where different *rep* types are present (labelled *incX, incY, inZ*). Transposition (3). Transposons (pink) and integrons (dark green) transfer between plasmid and chromosomal DNA through recombination and can thus be transferred by conjugative elements. Reproduced from Frost *et al,* 2005 (3).

1.8.1 Conjugation

Conjugation is the transfer of genetic material by two cells in contact and is a process that allows some MGEs to be self-transmissible. Conjugation requires a large cassette of genes, which encode the proteins required for the formation of a type IV secretion-system pore and coupling proteins which guide the plasmid DNA the cell wall and membranes into the cytoplasm of a neighbouring HGT recipient (198). DNA transferred by conjugation is recognised by a relaxase protein, which recognises, nicks and covalently joins to a specific DNA site called the origin of transfer (*oriT*). The resulting DNA-nucleoprotein complex, termed the relaxasome, with the help of a type IV secretion-system coupling protein, guides the DNA to the type IV secretion-system. The relaxase assists the re-circularisation and replication of the ssDNA copy in donor cell and the new host (199-203). Conjugation genes are found on extrachromosomal circular DNA plasmids and chromosomally-integrated 'Integrative and Conjugative Elements' (ICEs). Transposon (Tn) Tn*5801* is a widespread integrative and conjugative element found in many pathogenic gram positive bacteria and typically carries the tetracycline resistance *tetM* or *tetL* genes (204).

Not all mobile plasmids or chromosomally-integrated MGEs encode their own genes for conjugation. Conjugative mobilisation is a mechanism of transfer where a MGE exploits the conjugative machinery encoded by another conjugative element resident in the same cell. Mobilisation requires only part of the components of the relaxasome to be encoded, often a distinct relaxase (*mob*) protein and an *oriT* sequence (203). During mobilisation, a relaxase nicks and unwinds the plasmid at the *oriT*, binds and guides the ssDNA to the coupling protein of the type IV secretion-system in the same manner as conjugative plasmids, allowing the plasmid to be transferred alongside a conjugative plasmid. Non-conjugative staphylococcal plasmids are able to be mobilised by a relaxase-*in trans* mechanism, where the plasmid carries an *oriT* mimic of a conjugative plasmid and is able to exploit the relaxase of the conjugative plasmid to transfer during conjugation (205). Mobilisation frequencies are typically lower than conjugation frequencies, however, the vast distribution of plasmids carrying relaxase/*oriT* and/or

oriT mimics suggests mobilisation is a widely employed method of plasmid (and thus AMR) dissemination.

1.8.2 Transformation

Transformation involves the uptake of exogenous DNA from the immediate environment across the cell wall and cell membrane. Bacteria able to perform transformation are called naturally competent. Many species of gram-positive and negative bacteria are naturally competent. The process of transformation requires the expression of genes for DNA-uptake genes, for example pili, ssDNA-protective DNA-binding proteins and recombinases. Natural competence is a regulated process which occurs within subsets of bacterial populations, triggered by environmental conditions (e.g. high cell density or low nutrient availability) and intrinsic factors (e.g. cell cycle stage). Transformation can allow the use of exogenous DNA as a nutrient, but can also allow the acquisition of new genetic material through the capture of plasmids, or the integration of foreign DNA into the chromosome. A review of transformation is provided by Blokesch (206).

1.8.3 Transducing MGE

Transduction is the process of DNA transfer between bacterial cells mediated by a bacteriophage. Bacteriophages (phage), are bacterial viruses and are thought to be the most abundant biological entity on the planet, rivalling bacterial counts in natural aquatic environments (207). Phage can reside integrated to the chromosome or replicate as a plasmid in a latent, temperate state called prophage, which replicates and is inherited vertically with the host DNA. Upon induction of the lytic cycle, prophage excise, produce viral particles, package their DNA into the viral particles and lyse the host bacterium. Genomic sequences called *pac* (packaging site, (208)) and *cos* (cohesive end site, (209)) sites determine where phage terminases cut the DNA to be packaged into the phage head. There are three main forms of transduction; generalised, specialised and lateral transduction, each differing at

the phage head packaging stage. Generalised transduction involves the packaging of host DNA into phage heads instead of viral DNA, based on the mistaken identification of pseudo-*pac* sites in genomic DNA. Specialised transduction involves the aberrant prophage excision events which result in the phage DNA acquiring prophage-adjacent genes. In lateral transduction, packaging of the phage head occurs from the true *pac* site, packaging the viral DNA while still attached to the host chromosome, continuing to package the adjacent chromosomal DNA for up to seven phage heads full (210), resulting in the transfer of large spans of contiguous genomic DNA at relatively high frequencies. Each transduction type is capable of transferring AMR genes (211) and, by transferring pathogenicity islands, virulence genes (212). The Panton-Valentine Leucocidin (PVL)-associated genes are an example of virulence gene transmitted by phage. PVL is a two-component, secreted, pore-forming toxin which destroys neutrophils (213). Pathogenicity islands are chromosomally integrated MGEs which, like mobilisable MGEs, lack their own HGT genes but can exploit co-resident infecting phage to replicate, excise and transfer to another host via a modified phage particle. *S. aureus* Pathogenicity Islands (SaPI) typically carry virulence factors such as toxic shock proteins. SaPI-encoded terminases form oligomers with phage terminases, altering their specificity to recognise *pac* sites specific to SaPI, instead of their intended phage *pac* sites. Recognition of the SaPI pac sites results in the encapsidation of the SaPI genome into the phage-derived particles in place of the phage genome, while using the phage packaging machinery. This allows SaPI to transfer to other staphylococci at high frequencies (214), facilitating the transmission of staphylococcal enterotoxin genes (215).

1.8.4 Transposable elements

While horizontally transferring MGEs provide the major mechanisms of DNA transfer between bacteria, intragenomic movement of DNA between the chromosome, plasmids and other MGEs is also critical for the spread of AMR. Insertion sequences (IS) are ubiquitous and abundant MGE, consisting of one or two transposase genes flanked by inverted terminal repeats, which essentially cut/copy and paste themselves (although the actual mechanisms are more complex) between distinct sites in the genome.

IS elements are composed of insertion sequences and their recombinases, however, more complex configurations of IS elements exist. Composite transposons comprise two IS elements flanking genes which may be transposed together. Unit transposons carry genes for transposition, a site-specific recombinase or resolvase and accessory genes such as AMR, flanked by terminal repeats (Figure 1-4). Unit transposons are distinct from composite transposons as unit transposons always transpose as a single unit, while composite transposons can transfer independent of accessory genes (216).

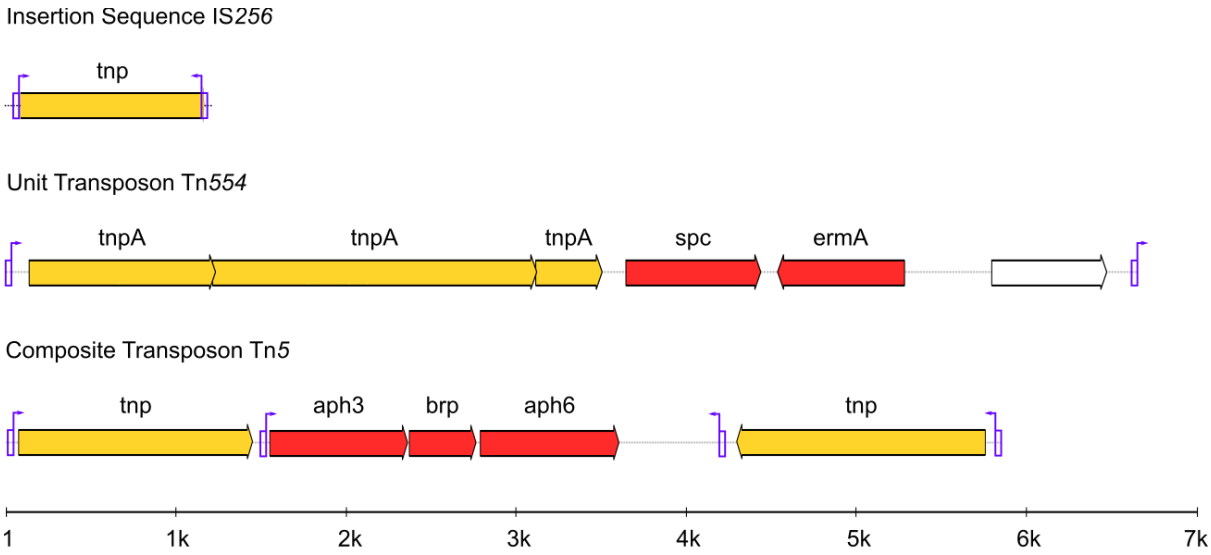

Figure 1-4 - Classes of transposable elements.

Gene names are indicated in text above arrows, which represent coding sequences. Arrow colour indicates gene function, either transposition (yellow), AMR (red) or unknown function (white). Blue box-arrows indicate identical repeat sequences and their respective orientation. Ruler indicates DNA sequence length in basepairs.

Transmission of IS and transposons from the chromosome to conjugative plasmids or other horizontally-transferring MGEs facilitates their movement to new hosts via HGT. IS sequences mediate

the transposition of AMR genes between chromosome and plasmid, and can mediate the reorganisation and chromosomal integration of plasmids. IS*257* is abundant in gram positive bacteria and is associated with the integration of tetracycline resistance into the staphylococcal cassette chromosome *mec* (SCC*mec*) region of *S. aureus* (217). Additionally, IS*257* affects the evolution of plasmids, exemplified by the capture of aminoglycoside resistance plasmid pUB110 by *S. aureus* conjugative plasmids (218) and the formation of the largest recorded *S. aureus* conjugative plasmid (pWBG731) by several stepwise IS*257*-mediated recombination events(219). The unit transposon Tn*552* carries inducible β-lactam resistance (220) and is widely disseminated, present on nearly all *S. aureus* plasmids (221). Partridge *et al.* have reviewed the IS in the context of AMR transmission extensively (222). Transmission of AMR from plasmids to chromosome can secure the AMR in the genome, preventing its accidental loss as plasmids fail to segregate into daughter cells (223).

Transposable elements also play a role in shaping bacterial evolution and can enable rapid phenotype switching. Depending on where these elements reintegrate, they can disrupt various functional genetic regions (e.g. promoter regions, open reading frames), which can potentially be lethal or result in new phenotypes e.g. upregulated expression of genes through the disruption of repressor genes, the reduced expression of genes by the disruption of promoter sequences, or the abrogation of coding sequences by disrupting the open reading frame. IS element transposition activity is controlled by the translation initiation inhibition (e.g. transcriptionally inhibitory secondary structures in IS mRNA), programmed ribosomal frameshift motif (PRF), transposase stability and/or a number of host factors (224). PRF is the controlled slipping of a ribosome into an alternative reading frame, allowing two proteins to be expressed from the same mRNA and is a process which occurs at 'slippery' sequences (for example X XXY YYZ) downstream of a structure that stalls the ribosome, such as a mRNA stem-loop or pseudoknot (225). It is a process employed by prokaryotes, eukaroytes and viruses, often as a regulator of gene expression, for example, in downregulating the expression of copper transporter *copA* in *E. coli* in elevated concentrations of extracellular copper ions (226).

1.9 Barriers to MGE transfer

While MGE can provide benefits to their host by introducing new antimicrobial resistance genes and virulence factors, MGE are not always beneficial and are often detrimental to the host. Lytic phage, for example, parasitise the host to propagate copies of their own DNA and structural components before lysing the host (as described in 1.8). To prevent parasitic infections of host bacterium by MGE, bacteria have evolved several barriers to MGE transfer including restriction-modification systems and clustered regularly interspaced short palindromic repeats (CRISPR) systems. Additional defences against MGE are found in bacteria, including bacteriophage abortive infection systems (227), prokaryotic argonaute proteins (228) and the RecBCD complex (229). These systems are primarily involved in defence against bacteriophage and so will not be further explored here.

1.9.1 Restriction-modification systems

Restriction-modification (RM) systems are barriers to bacterial HGT, which recognise specific, short, often palindromic DNA sequences and cleave DNA based on the recognition of these sequences.

As these short sequences are likely to appear within the host genome, RM systems protect the host genome by methylating bases within recognition sequences, preventing cleavage by the RM endonucleases (230). When foreign DNA, which lacks the methylation pattern of the host RM system, enters the cytoplasm of the host, it is acted on by RM restriction endonucleases, before being further degraded by host nucleases (231). This cleavage and eventual destruction of the MGE, preventing replication and expression of genes encoded by the MGE.

There are four types of RM systems, classified according to sequence recognition, cleavage position relative to recognition sequences, subunit composition and substrate specificity. Type I RM systems comprise discrete protein subunits with restriction endonuclease, methyltransferase and specificity

functions, which form complexes (231). These systems typically cleave DNA 100 bp – 1000 bp away from their symmetrical recognition sequences (232). The protein-coding genes of type I RM systems are typically labelled host specificity of dna (*hsd*) and a letter denoting their function i.e specificity (S) protein, DNA modification (M) protein and a restriction (R) endonuclease (231). Type II RM systems are some of the best characterised and comprise independent restriction endonuclease and methyltransferase proteins, lacking a discrete specificity protein. Type II RM systems cleave within or near the recognition sequence at a fixed distance from that sequence (232). Type III RM systems comprise restriction and modification subunit complexes of multiple different restriction endonuclease and methyltransferases and DNA-dependent nucleoside triphosphatases, which recognize short five or six base-pair asymmetric sequences and cleave ~25 bp away from the recognition site (233). Type IV RM systems comprise only restriction endonucleases and only cleave modified DNA substrates (i.e. methylated or hydroxymethylated DNA) at recognition sequences (234). Additionally, orphan methyltransferases have been recorded, which are not associated with restriction endonucleases, and which affect important cellular processes like the regulation of DNA repair , initiation of DNA replication and control of gene expression (235).

Staphylococci have been found to encode three of the four RM types - only type III is absent. Most *S. aureus* strains carry two separate Type I systems (*saul,* (236)). Specificity of the *S. aureus* Type I RM system is varied and the 10 dominant lineages of *S. aureus* carry unique combinations of *sau1hsdS* genes that control the sequence specificity of the system, with implications for HGT between lineages. For example, Sau1 system prevents transmission of plasmids between CC5 and CC8 lineages (236). Some *S. aureus* contain the Sau3A type II RM system, which targets GATC sites (237)). The clinical *S. aureus* lineage USA300 encodes active type IV systems (238). The Type IV system in *S. aureus* comprise a single gene called *sauUSI* which recognises methylated cytosine bases in the motif C/G^{m}CNGC/G (238). Knockout of Type IV RM systems has been demonstrated to allow transfer of *E. coli* plasmids into *S. aureus* (239). These RM systems represent significant barriers to HGT in *S. aureus* and will be further examined throughout this study. A more comprehensive review of staphylococcal RM systems is published by Monk and Foster, 2012 (240).

1.9.2 CRISPR-Cas

Clustered regularly interspersed short palindromic repeats (CRISPR) systems, named for their genetic structure (241), provide host bacteria with resistance against bacteriophages (242) and limit HGT of other MGE (243). CRISPR systems comprise a cassette of repeat sequences (244), found adjacent to an operon of CRISPRI-associated (*cas*) protein-coding genes (242). The Cas proteins allow the CRISPR cassette to be transcribed and processed into short CRISPR RNAs (crRNAs) which individually bind to, and guide, Cas endonucleases to recognise and degrade DNA which shares homology with the crRNA (245). Cas proteins also control the addition fragments of unrecognised DNA to be added to the CRISPR cassette, to protect against future exposure (246). Short, often 33 bp sequences of foreign DNA ('spacers') are taken from the MGE and are integrated between the repeats of the CRISPR cassette (247) and in this way, acts like primitive a bacterial homologue of the human adaptive immune system. Similar to RM systems, CRISPR systems are categorised into two broad classes and six types. Systems are typed according to their genetic architecture, and are classed according to the multifunctional or discrete functions of their Cas effector proteins (248). Although CRISPR-Cas systems have been found in Staphylococci (249), including the type II SaCas9 which has found use as a highly site-specific tool for biotechnological applications (250), they are predominantly found in gram negative bacteria, are not commonly found in *S. aureus* (251) and are not considered a significant barrier to HGT in *S. aureus.*

1.10 Methicillin Resistant *Staphylococcus aureus*

MRSA is the primary focus of this thesis. The following section describes the evolution, medical relevance, molecular methods of typing and current state of MRSA infection in Australia and elsewhere in the world.

S. aureus is a bacteria from the genus *Staphylococcus* and the family Staphylococcaceae. The Staphylococcaceae family are gram-positive cocci that grow in irregular clusters, are non-motile, non-

spore-forming, catalase-positive, mannitol-fermenting bacteria (252). According to the List of Prokaryotic names with Standing in Nomenclature, there are currently 71 validly published species of *Staphylococcus*, including *S. aureus* (253). *S. aureus* is a gram-positive bacterium, carried in the nasopharynx of 20-30% (254) of the population and is an opportunistic pathogen of humans and animals. *S. aureus* predominantly causes skin and soft tissue infections but is also a significant cause of serious infections of the heart, bones and blood. *S. aureus* carries many virulence factors and is capable of rapidly acquiring AMR. The first penicillinase-producing *S. aureus* isolates were detected in 1944 (255), just years after the introduction of penicillin. Penicillin-resistance rapidly emerged in *S. aureus*, resulting in difficulties to treat *S. aureus* infections until the development of the penicillinase-resistant semi-synthetic penicillins. Modified penicillins such as methicillin, oxacillin, and the structurally-related cephalosporins were developed (

Table 1-1) in the late 1940s and 1950s, however, MRSA was detected soon after the introduction of methicillin in the UK in 1960 (83), and in many European and Asian nations, the USA and in Australia in 1968 (256). Methicillin resistance results in less favourable patient outcomes (as discussed in section 1.5). MRSA is more difficult to treat than MSSA as methicillin resistance confers resistance to all β-lactams, requiring treatment with pharmacologically less favourable antimicrobials (e.g. vancomycin), causes extended hospital stays, and results in higher mortality and morbidity. Initially, MRSA was largely limited to transmissions and infection within and between hospitals and was referred to as Healthcare-Associated MRSA (HA-MRSA), but infections of patients without previous exposure to healthcare environments soon followed. The first cases of MRSA infections arising from the community, now referred to as Community Associated MRSA (CA-MRSA) were reported in Detroit, USA in the early 1980s (257) and in Perth, Western Australia (WA) in 1993.

Although WA is geographically isolated and has a dispersed population, the Health Department has maintained a high level of MRSA surveillance and infection control. The first major MRSA outbreak in WA occurred in a large metropolitan teaching hospital in 1982 (258). The outbreak was eliminated from the hospital and an MRSA screening policy was implemented for all staff and patients who had been hospitalised outside WA in the previous 12 months. A study was conducted in 1993, following an

increasing number of MRSA-positive patients in Perth hospitals who did not have a history of hospitalisation in the previous 12 months. Typing of the MRSA isolates using pulsed-field gel electrophoresis revealed the isolates were distinct from those causing the 1982 hospital outbreak and notably, the patients were from small, remote communities in the far north Kimberley region of WA, over 2,000 km from the city of Perth. The patients were from nine distinct Kimberley communities, separated by no closer than 200 km and no further than 400 km (259). While other examples at the time suggested MRSA had been acquired in patients from the community, the study provided solid evidence MRSA could now emerge and cause infection outside of healthcare environments. Thus CA-MRSA were recognised as a clinical subtype of MRSA by the late 90s, based on their propensity to infect typically young and healthy individuals lacking traditional healthcare risk factors (260). CA-MRSA infections were soon recorded globally, with ST1, ST5, ST8, ST22, ST30, ST59 and ST80 being some of the most widely distributed lineages (reviewed by Mediavilla *et al*, 2012 (261)). In Australia, the dominant CA-MRSA lineage is ST93, a PVL-positive MRSA first reported in 2000 in Queensland, Australia (262). Subsequently, ST93 has been reported in the UK and New Zealand and typically causes skin infections (263). ST93-MRSA is highly virulent, being responsible for the majority (71.4%) of CA-MRSA bacteraemias in Australia (118) and may cause necrotising pneumonia in young patients (264)**.** In Australia, the majority of MRSA sepsis cases are now caused by CA-MRSA (76.4% as of 2018, (265)).

1.10.1 Risk factors and treatment of MRSA acquisition and infection

Risk factors differ for the acquisition of CA-MRSA and HA-MRSA. Risk factors for HA-MRSA acquisition include chronic disease, use of medical devices (eg catheters), long hospital stays, invasive procedures and patients having burns (266). Those who acquire CA-MRSA often don't have traditional risk factors for infection, such as chronic disease. Those with a higher risk of CA-MRSA acquisition include military personnel (267), contact-sports athletes (268), intravenous drug users (269), the incarcerated (270) and recent hospitalization, admission to nursing homes (270). Antibiotic exposure increases the risk of CA-MRSA and HA-MRSA colonisation by 1.8 fold (271).

Treatment options for MRSA consist of decolonisation, drainage of infections and antimicrobial therapy for invasive infections. Decolonisation may be offered where recurrent CA-MRSA infections occur in individuals or households, for healthcare workers or carers or where an increased risk of infection occurs due to other existing medical conditions. Decolonisation includes the use of triclosan or chlorhexidine gluconate body wash for 5 days, nasal mupirocin ointment treatment for 5 days, soaking of dentures overnight and for throat carriage, twice-daily use of chlorhexidine-based mouthwash (272). Western Australian CA-MRSA treatment for skin and soft-tissue infections (SSTIs) include incision and drainage as a priority and the prescription of antibiotics if incision and drainage isn't possible or if the patient has significant co-morbidities. Antibiotic therapy is decided by an assessment of infection severity, while the choice of antimicrobial prescribed is dependent on patient age, allergy history and presence of patient co-morbidities. First line antimicrobial for treatment of adult CA-MRSA is 450mg oral clindamycin, three times per day. Second and third-line treatments are trimethoprim-sulphamethoxazole and doxycycline respectively (273). Treatment of upper respiratory tract infection, necrotizing pneumonia and bacteraemia caused by MRSA, regardless of whether it is CA-MRSA or HA-MRSA, includes intravenous vancomycin or linezolid as first-line and alternative first-line treatments respectively (274). A range of antimicrobials are available or are undergoing development for the treatment of MRSA. A comprehensive list of these antimicrobials can be found in a 2019 review by Fowler Jr et al (4).

1.10.2 SCCmec

As described in section 1.7, Methicillin resistance is provided by the *mecA* gene, which encodes an alternative PBP with lower affinity for beta-lactams and is carried by the chromosomally-integrated MGE SCC*mec* (275). SCC*mec* is a complex and highly variable MGE, typically between 20 and 60 kb in length, consisting of a *mec* gene complex, ccr-gene complex which encodes the recombinases *ccrA* and *ccrB* or *ccrC,* and J-regions (Figure 1-5). SCC*mec* may also contain additional AMR genes and integrated MGEs (such as transposon Tn*554* and plasmids pT181 and pUB110), which are found within J-regions. The element integrates into a chromosomal open reading frame called *rlmH* (originally denoted *orfX*).

The *mec* gene complex comprises a *mec* gene (or *mec* variant) and regulatory elements *mecR1* and *mecI* (sensor/transducer and repressor respectively). The J regions exist outside the *ccr* and *mec* gene complexes and contain variations which form the basis for SCC*mec* subtyping (276). SCC*mec* is found in diverse lineages of MRSA, indicating it has been independently acquired by HGT (277). SCC*mec* does not encode components of a type IV secretion-system and is therefore non-conjugative, nor does it encode genes required for replication after circularisation, however, SCC*mec* is able to form cointegrates with conjugative plasmids via IS recombination and transfer with conjugative plasmids (278), although it is uncertain whether this a common route for SCC*mec* dissemination. Transfer of SCC*mec* has also been demonstrated by transformation (279) and transduction (280), however, it is unclear if any of these transfer mechanisms are utilised by SCC*mec* outside the laboratory.

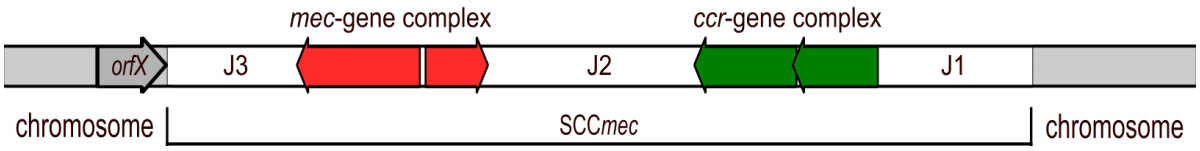

Figure 1-5 - Archetypal SCC*mec*

The archetypal SCC*mec* element is divided into the *mec*-gene complex (red), *ccr*-gene complex (green) and J-regions (white). J-regions are variable and often contain insertion sequences and AMR genes. The element is integrated into the chromosome (grey), disrupting the *rlmH* gene (*orfX*). The borders of the element are flanked by direct and indirect repeats.

1.10.3 Epidemiology of MRSA

Several methods are used to distinguish closely related MRSA to monitor transmission, dissemination and the progression of MRSA epidemics, including multi-locus sequence typing (MLST), staphylococcal protein A (spa) typing, SCCmec typing and the presence/absence of the PVL-associated toxin genes. MLST categorises bacteria into a given sequence type according to the combination of alleles of house-keeping genes, which themselves are differentiated and assigned allele numbers according to

sequence variation. S. aureus MLST is based on variation within seven house-keeping genes: arcC, aroE, glpF, gmk, pta, tpi, and yqiL. The S. aureus MLST database is available at https://pubmlst.org/saureus/ and is based on the Bacterial Isolate Genome Sequence database (281, 282). STs can be grouped into a Clonal Complex (CC) by the eBURST method (283). The spa gene (encoding the antiphagocytic S. aureus Protein A (284)) contains a variable region of short (24bp) repeats, which vary in sequence and copy number. Sequencing of the polymorphic region of spa and the classification of repeat type and count produces a numeric spa type (e.g. t008) (285). SCCmec-typing is based on the combination of mec and ccr complexes. SCCmec elements can be further subtyped according to the contents of the J region (276). The SCCmec type is represented as a roman numeral and, where applicable, a subtype is given by a lower case letter. For example, ST8-IVa is an MLST 8 and has a type 4, subtype 'a' SCCmec element. As of 2020, 14 SCCmec types have been described (286, 287), although novel types and composite elements have arisen through recombination which have proven difficult to type with the current schema. Epidemic CA-MRSA strains are typically associated with the carriage of the genes encoding the PVL toxin (288). Although the role of PVL in the virulence of CA-MRSA is controversial (289), it remains a useful molecular marker for the differentiation of closely-related MRSA strains. PVL is detected by the polymerase chain reaction (PCR) amplification of a 433-bp fragment of the *lukS-PV* and *lukF-PV* genes, which encode the two components of the PVL toxin (290). PVL can be further typed according to the bacteriophage on which PVL is carried by PCR-detection of phage-specific integrase genes (291).

An effective way to differentiate and examine the relationships between closely bacterial genomes is by the construction of a phylogenetic tree, based on the alignment of genomes. However, many genomes contain large regions of variation due to integrated MGE. Instead of trying to completely align whole genomes, multiple genome alignment strategies often first produce a core genome. Core-genome alignment focuses on identifying orthologous sequences conserved across all of genomes (generally containing essential genetic information related to cellular metabolism and replication) in an alignment. This strategy simplifies the process of otherwise aligning very large stretches of DNA. The core genome consists of conserved, essential genes which are typically inherited vertically, and which therefore will be present and comparable with closely related bacteria. Using core genes to

produce an alignment allows more robust inferences of phylogeny than one which attempts to align highly variable and rapidly moving MGE. Reducing the physical size of a whole genome alignment further can be done by simply identifying single-nucleotide polymorphisms in these core genes and concatenating them into short, but highly informative sequences for alignment. Core-genome SNP alignments have become the standard method for producing large phylogenies of closely related bacterial genomes (292).

Different lineages of MRSA are predominant in invasive infection across different regions of the world. These lineages are typically described by the sequence type and/or clonal complex to which they belong. ST239 (CC8), is a widely distributed HA-MRSA which has been reported in the Middle East, Asia-Pacific and across Europe (293, 294). CA-MRSA ST80 has been well reported in western Europe, the Middle East and North Africa (293-295). ST772 (the Bengal Bay clone) is dominant in India (296) and has spread globally from India and Bangladesh (297). Other Asian MRSA are diverse, with ST72 (CC8) reported in Korea, ST59 in Taiwan, ST30 or ST8 in Japan, and vast diversity of reported MRSA in China (298) (299). ST30 and ST22 are dominant HA-MRSA in continental Europe (300), while ST30 (CC30) has also spread through Asia–Pacific and parts of the Americas (298, 301, 302). CC30 is associated with higher invasive infection rates and mortality (303). USA300 (ST8, CC8) is the dominant in the United States, while ST30, ST22 and ST80, are uncommon in the United States (304). ST93 is the dominant CA-MRSA across Australia while the dominant HA-MRSA (in Australian sepsis cases) is ST22 (118). A map of the distribution and diversity of global MRSA lineages is provided in Figure 1-6. It is important to note that the emergence and regional dominance of distinct MRSA clones continues to change over time (295, 305). An example is the recent dominance of ST59 (CA-MRSA) over ST239 (HA-MRSA) as the dominant MRSA in China (306). Also, as global MRSA surveillance expands under One Health national strategies, greater emphasis on reporting MRSA associated with food-production systems may become more common.

Multidrug-resistance (MDR), defined as resistance to one or more classes of antimicrobial agents, is common in MRSA, as the SCC*mec* element which provides methicillin resistance also provides

resistance to virtually all β-lactam antimicrobials. In Australia, the MDR MRSA clone ST45-VT [5C2&5] accounted for 12.7% of CA-MRSA sepsis cases in 2019 (307, 308).

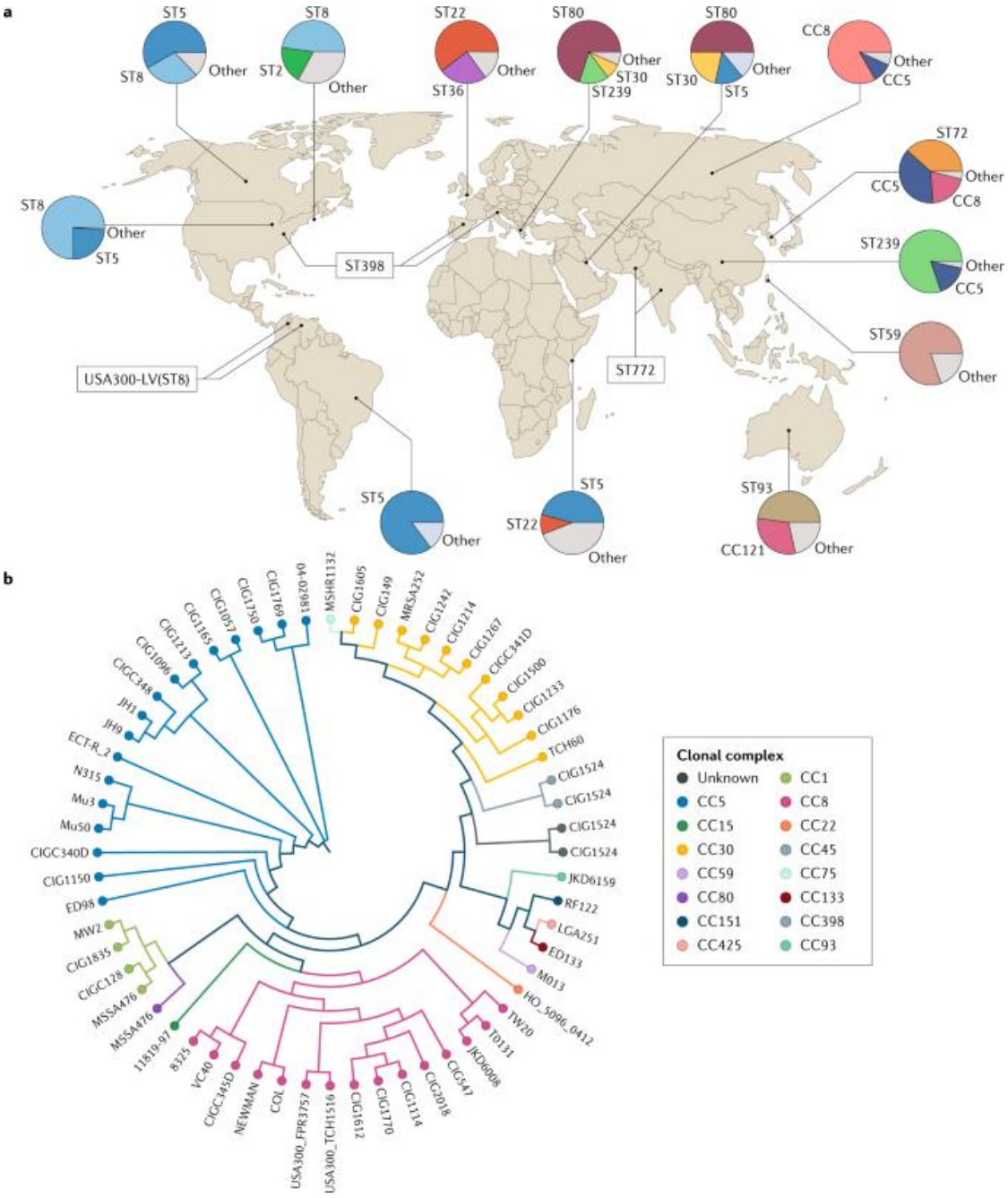

Figure 1-6 - Global distribution and diversity of MRSA

(a) A map of the major strains across the world with regional strain prevalence. (b) A maximum-likelihood SNP dendrogram produced from 60 *Staphylococcus aureus* isolates, representing relationships between major clonal complexes. Reproduced with permission from Fowler JR *et al,* 2019 (4).

1.10.4 Clonal complex 8 MRSA: prolific pathogens

MRSA was first identified in a member of *S. aureus* CC8 and many historic epidemic MRSA strains are members of CC8, including ST239, ST250 (the Archaic Strain), ST247 (the Iberian Clone), ST8 and several sporadic epidemic clones (e.g. the Hannover clone, ST254). CC8 MRSA form two subgroups, whose ancestral strain is likely an SCC*mec*-I ST250 MRSA evolved from a successful ST8 MSSA (277) which horizontally acquired SCC*mec*-I. ST250-I, the first epidemic MRSA clone, emerged in the UK and spread through Europe in the 1960s and 1970s, becoming less prevalent before being supplanted by ST247 (309). ST247-I (the Iberian clone) was first detected in Spain in 1989, and spread globally, while causing persistent outbreaks in Europe into the late 1990s (310).

MRSA also spread to the American continents. CC8 ST239 MRSA (the Brazilian clone) is a HA-MRSA first isolated in 1992 in Brazil (311). The clone is prevalent in South America, Eastern Europe and Asia, is typically resistant to multiple antimicrobials and carries a higher risk of bacteraemia than other MRSA strains (312). Some of the first CA-MRSA isolated in Australia (259) were also ST8 and an ST8-derived CA-MRSA became the dominant MRSA in the USA (as pulsed-field gel electrophoresis-type USA300), accounting for 48% of the MRSA collected in the USA in 2011 and was responsible for 37% of bloodstream infections (313). USA300 is hyper-virulent and is frequently isolated from athletes, children, military recruits and prisoners. USA300 is distinguished from other MRSA by its lack of capsular polysaccharide, a t008 *spa*-type, type-IV SCC*mec,* carriage of the PVL-associated toxin genes and variably, the carriage of the arginine catabolic mobile element (314).

USA500 is another member of CC8 which is closely related to USA300. USA500 is a pulsed-field gel electrophoresis-subtype of ST8 first described in the USA in 2003 and is variably a multi-resistant HA-(315) or CA-MRSA (316). USA500, although less common than USA300, was reported as the third most common MRSA detected in the USA (from 2005 – 2013), representing 13% of all HA-MRSA infections in 2013 (316). USA500 is also prevalent in bacteraemia, representing the cause of 99% of bacteraemias in one American surveillance study (317). USA300 and USA500 are genetically very similar, however, USA500 does not typically carry the PVL-associated toxin genes and frequently contains multiple copies of IS*256* elements, whereas IS*256* is less commonly carried in USA300 (316, 318). USA500 often carries *spa*-type t064 (319), while USA300 is more frequently t008. USA500, like USA300, is unable to produce capsule (319) and additionally contains IS*256*-mediated disruption of the repressor of toxin gene (*rot*), which contributes to a 'hyper-virulent' phenotype in animal models (318).

Members of CC8 appear to have zoonotic potential. CC8 MRSA ST8, ST8-USA500, ST254 and ST259 have been reported frequently isolated from horses and/or veterinarians that have had contact with horses in France, Germany, Denmark, Austria, the UK, and Ireland (320-322). CC8 are also occasionally isolated from dogs and cats (323). ST8 are sporadically isolated from cows (324) and retail meat (including beef, chicken, turkey and pork) (325).

ST612 is a member of CC8 and shares significant genomic relatedness to USA500 2953 (GenBank: CP007499) (326). ST612 MRSA is frequently identified in South Africa (SA) (327) where it is over-represented in bacteraemia cases (328), however, it is not a frequently reported cause of human infections elsewhere. In Australia, ST612-MRSA has been isolated from veterinarians and from New South Wales (NSW) horses (329). Furthermore ST612-MRSA are increasingly detected in human patients living in WA, with at least one case leading to serious bacteraemia (330).

1.10.5 Livestock-associated MRSA

Livestock-Associated MRSA (LA-MRSA) are MRSA, mostly from CC5, CC398 or CC9, associated predominantly with the colonisation and infection of poultry, pigs and cattle. *S. aureus* naturally colonise and are opportunistic pathogens of a wide variety of domesticated and wild mammals (331). MRSA have been isolated from livestock as early as 1975 (332). LA-MRSA have direct impacts on animal health and MRSA afflicts animals with similar infections to humans, largely comprising skin and soft tissue infections, respiratory infections and less frequently, invasive infections which typically follow invasive procedures. Horses are vulnerable to a range of MRSA disease, predominantly joint, incision and skin and soft tissue infections, although other infections are possible such as pneumonia and infections of the reproductive tract and bloodstream infections (333). In dogs and cats, cutaneous wound infections, surgical site infections, pyoderma, and UTI are common (333). MRSA infection in pigs is rare, despite high rates of MRSA colonization. MRSA infections in pigs can cause 'greasy pig disease', a type of exudative epidermatitis, metritis and UTI (333, 334). Specific lineages of MRSA are more likely to colonise and cause disease in animals. While MRSA can cause zoonotic infection, the opposite is also true. Anthroponosis is the transmission of infectious disease from humans to animals. Increased industrialisation and intensification of animal food production has likely increased the opportunities for anthroponotic transmission of MRSA, and may have increased the opportunities for adaptation of typically human-host MRSA to animal hosts; the theoretical origins of LA-MRSA (335). CC8 and CC5 MRSA are a major cause of bovine mastitis in cows while CC5 MRSA is a major cause of skeletal infections of poultry (336). CC398 MRSA have become synonymous with the LA-MRSA designation, despite being only sporadically isolated in direct animal infections. CC398 LA-MRSA originates from a human ST398 MSSA which acquired SCC*mec* after introduction into livestock (337). The LA-MRSA lineage of ST398 is typically SCC*mec*-V or IV in Europe, does not carry the PVL-associated toxin genes and is *spa* type t011 or t034 (337, 338). ST398 LA-MRSA lineage has been identified across Europe, Asia, Oceania and America. LA-MRSA colonisation of farmed pigs is increasing, which poses the threat of zoonotic transmission and infection of humans. CC398 LA-MRSA spread rapidly and in parts of the world has become endemic on pig farms. A 2016 Danish government screen for LA-MRSA

in pigs found 88% of farms were MRSA-positive, up from 3% only eight years prior (339).

Treatment of MRSA infection in animals consists of antimicrobial therapy in Australia. Veterinarians have prescribing powers and are expected to comply with the legislation of the state or territory they operate in and to adhere to the guidelines set out by the Australian Veterinary Prescribing Guidelines (340). The guidelines promote antimicrobial stewardship and best-practices, as well as recommending particular antimicrobials for infection types. While many antimicrobials are registered for veterinary use, only rifampicin and sodium fusidate are registered and indicated for veterinary treatment of MRSA (341).

LA-MRSA is an emerging pathogen. Human carriage of LA-MRSA ST398 was first identified in the Netherlands (342) and France (343) in early 2005, in pig farmers and their herds, and as early as 2003 in the Netherlands in patients with predicted animal contact (344). Danish incidence of human LA-MRSA CC398 infections increased 66% between 2004 and 2011 (338). Despite loss of the human immune evasion cluster genes, which would suggest diminished human infectious capability, ST398 LA-MRSA is capable of zoonotic transmission and causing serious disease in humans. A 2007 study of 15 European countries pathology lab submissions determined 1.4% of all clinical MRSA infection submissions (n=113/3435) were ST398, of which 67% were isolated from skin and wounds, 17.7% were respiratory isolates and 1.8% were isolated from blood (345). The European average presented downplays the issue of ST398 infection somewhat, as ST398 infection correlates with the total pig population of a country (345). For example, Denmark is a large pork producer and LA-MRSA CC398 accounted for 16% of all MRSA infections in Denmark in 2016 ((346)). LA-MRSA is an emerging issue in Asia, with increasing reports of various LA-MRSA lineages across several Asian nations (347).

Risk factors for LA-MRSA infection include colonisation, farm density and time spent with animals. A causal association between *S. aureus* nasal carriage and infection has been known since 1931, supported by the shared genotype/phage type of infection and carriage strains, and by a reduction in infection risk following decolonisation with nasal antistaphylococcal drug application (348, 349). LA-MRSA CC398 carriage in humans can persist for over a year (350). Farmers and farm workers often

carry the same ST398 as those found in the animals on the farms where they work (351). MRSA rates on farms increase with farm size, determined by the number of pigs per farm (351), and proximity of pig farms to other farms. The total time spent working with pigs, as well as how intense the contact is (high intensity contact includes birthing and castration, earmarking is considered lower intensity) correlates with MRSA acquisition (352, 353). Aerosolised MRSA has been identified as a source of transmission (354). The food production chain is potentially a significant point for LA-MRSA transmission to the community, with frequent reports of MRSA being isolated from retail meat (336). Meat products may not be a significant source of human MRSA infection or colonisation, and may instead be indicative of the amount of MRSA carriage by animals and meat-and-livestock workers at earlier stages of production (355).

LA-MRSA can be transmitted from animals to the community, placing animals as a source of indirect MRSA infection. Human infection with LA-MRSA without any prior animal contact has been documented, suggesting human-human transmission of LA-MRSA. A retrospective study of human infection isolates in Danish patients with no prior livestock contact from as early as 1999 match LA-MRSA CC398 (338). These infections were determined to be the result of 'repeated spill over' from farms (356). Spread of LA-MRSA CC398 into the community is a concern, as it can be introduced to the immunocompromised and the elderly, where it can cause severe disease and death (357, 358). The contamination of meat products with MRSA is a potential route of LA-MRSA transmission into the community. Agricultural run-off and the use of livestock manure in crop fields are another potential route for LA-MRSA infection. Proximity to pig manure fertilisation of crop fields correlates with MRSA skin and soft tissue infection (359).

LA-MRSA carry diverse AMR genes on a variety of MGE. Many MGEs associated with LA-MRSA are novel (360) and often encode AMR genes uncommon in other MRSA (361). The AMR genes provides resistance to macrolide-lincosamide-streptogramin B (MLS) and resistance genes such as *ermC* or *ermT*, lincosamide resistance genes *lnuA* and *lnuB*, and pleuromutilins-lincosamide-streptogramin A resistance genes *vgaA* or *vgaC.* Spectinomycin resistance (*spd*) and trimethoprim resistance (*dfrK*) are also commonly found on small plasmids (360). Some LA-MRSA plasmid-borne AMR genes are specific

to antimicrobials used in agriculture, such as florfenicol resistance gene *fexA* and apramycin resistance gene *apmA.* Dissemination of plasmids by LA-MRSA is likely, as many of the plasmids are found in diverse non-*aureus* staphylococci, suggesting ST398 LA-MRSA are either recipients or donors of plasmids (361).

A 2013 study investigating the potential presence of ST398 in *S. aureus* from Australian livestock in samples collected from 22 Australian veterinary diagnostic laboratories identified only one ST398 isolate from a pig veterinarian (362, 363). Between 2011 and 2013, neighbouring New Zealand (NZ) reported nine human infections with ST398 MRSA, three of which were in patients who worked with pigs (364). By 2015, the total number of human ST398 LA-MRSA detected had escalated to 36 (24 clinical isolates and 12 colonising isolates detected during screening). An epidemiological study determined the isolates had been introduced from Western Europe, possibly contributed to by an increase in the use of tetracyclines in NZ (365). ST398 LA-MRSA was first detected in Australian pigs and pig-farm workers in a 2017 surveillance study of five commercial pig farm sites alongside ST93 MRSA, a common Australian CA-MRSA (366). 60% of farm workers and 74% of pigs were MRSA positive, with the majority of isolates typed ST93. All ST398 MRSA were tetracycline resistant and carried a type V SCC*mec,* consistent with LA-MRSA and were likely introduced into Australia from Europe or North America. Consistent with other LA-MRSA studies, risk factors for MRSA colonisation of pig farm workers included direct pig contact, farm roles which involved direct contact (i.e. farrowing) and total time spent in contact with pigs (367).

LA-MRSA has the potential to become a significant problem in Australia. LA-MRSA is closely associated with pigs and Australia has around 2,700 pig-producing farms, ranging from small farms to large commercial operations, producing approximately 360,000 tonnes of meat per year. By comparison, Denmark is a country with significant CC398 LA-MRSA detection on pig farms and increasing community infection and had 2890 pig farms in 2019 (356, 368, 369). With a significant number of pig farms and a known population of LA-MRSA in Australian piggeries, Australian LA-MRSA require further monitoring. While recent studies have examined the prevalence of LA-MRSA in Australian pigs and pig farms, the MGEs carried by LA-MRSA and the potential mobility of these MGE currently lie unknown.

The 2017 MRSA surveillance study by Sahbizada et al reported that Australian LA-MRSA encode many AMR genes, however the MGE which carry these genes are unknown.

1.10.6 The MRSA genome

Bacterial genomes can be separated according to whether genes are present in all isolates, called core the core genome, and accessory. The core genome is highly conserved, contains genes required for replication and metabolism, and typically comprises ~75% of the 2.8 Mb *S. aureus* genome (370). The accessory genome contains genes involved in antimicrobial resistance, immune evasion and virulence, and typically accounts for the remaining 25% of the genome. The accessory genome comprises MGEs such as plasmids, integrated phage, pathogenicity islands, transposons and SCC*mec* elements (as described in sections 1.8 and 1.10.2, and depicted in Figure 1-7), in MRSA. As these elements are mobile, they account for much of the genomic variability between strains of MRSA. The structure of a typical ST8 genome is presented in Figure 1-7 below. Regions where MGE are integrated in the genome are hotspots for integration and are variable between MRSA.

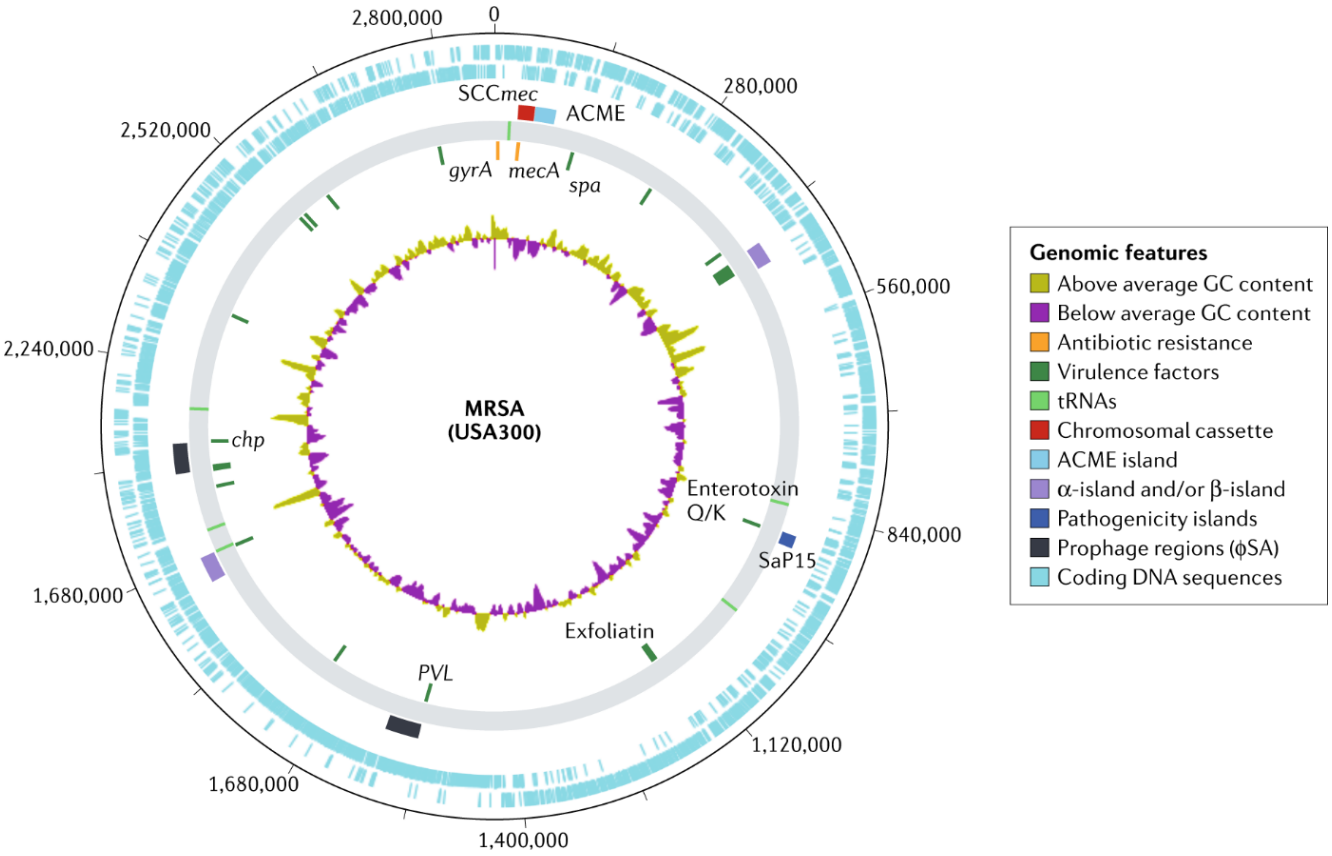

Figure 1-7 - Genomic features of ST8 MRSA USA300

The genome of ST8 MRSA USA300 is presented with coding sequences noted on the outer track, mobile genetic elements (the accessory genome) marked on the inner track and the GC content represented by the innermost circle. Variation between MRSA genomes largely occurs within the accessory genome; for example features such as the ACME element shown above, are found almost exclusively in ST8 MRSA. Reproduced with permission from Nature Publishing (4).

Recombination is a source of core-genome variation in bacteria, which provides adaptation to their environment and is largely mediated by the exchange of genomic DNA between related bacteria via conjugation, transduction and transformation (371). Because of the strong RM system barriers to transfer between lineages of MRSA and a lack of natural competence, MGE are predicted to be the

major drivers of core-genome recombination in *S. aureus*, based on the presence of core genome transfer hot spots adjacent to MGE such as conjugative transposons and the SCC*mec* region (372). Lysogenic phage and SaPI elements have been demonstrated to mobilise plasmids and large segments of genomic DNA between both laboratory and clinical isolates of MRSA, suggesting that these MGE are significant sources of genetic variation in *S. aureus* and that transduction is a viable mechanism for facilitating recombination (210, 214, 373, 374). In addition to the biological significance, genomic recombination in bacteria and can affect the accuracy of phylogenetic tree construction (topology and branch length), affecting the accuracy of the inferences drawn from those phylogenies (375). Rates of recombination can be detected and accounted for in the creation of phylogenies. ClonalFrame is a tool used for detecting recombination, which estimates relative probabilities that a nucleotide substitution has occurred as a result of mutation or recombination (376). A study using ClonalFrame to examine rates of recombination across bacteria and archaea determined that rates of homologous recombination are very low in *S. aureus* (377). Furthermore, a study by Hedge and Wilson (2014) found that methods of phylogenetic tree construction which did not remove recombining sites from alignments resulted in phylogenies of accurate topology, although branch lengths were inaccurate. The authors additionally found that corrections made for recombination could exacerbate branch length inaccuracy (378). Recombination will be ignored during the construction of phylogenies in this study. Additionally, the selection of evolutionary model on which to calculate a phylogeny is not crucial to the accuracy of the tree topology (379).

1.10.7 MRSA plasmids

S. aureus frequently carry plasmids, which are an abundant source of AMR genes. It's estimated that

90% of *S. aureus* carry plasmids (221). Plasmids in *S. aureus* range in size from ~1kb to greater than 60 kb. The plasmids of *S. aureus* are typically categorised according to their replication style. Small plasmids are often encode a single accessory gene (often an AMR gene) and replicate via a rolling-circle replication (RCR) mechanism (380). These plasmids can be further categorised by the type of replication protein they encode, represented by the prototypes pT181, pSN2, pE194, and pC194 ((381, 382)). These small plasmids encode chloramphenicol (pC221 and pC194), tetracycline (pT181 and pE194-family plasmid pMV158), erythromycin (pSN2-family plasmids) or aminoglycoside resistance (pUB110, a pC194-family plasmid) (380). Seven distinct replication initiation (*rep*) proteins have been identified in MRSA plasmids according to their conserved domains including Rep_1, Rep_2, Rep_trans and RepL (RCR plasmids), and RepA_N, Rep_3, and PriCT_1 (theta-replicating plasmids) (380). Staphylococcal plasmids larger than 8 kb use a theta (θ)-type replication mechanism and fall into one of two main classes according to their conjugative ability (382). Non-conjugative theta-replicating plasmids include pSK1-family plasmids (which encode heavy metal resistance and β-lactamase resistance) and pSK639-family plasmids. Two broad families of conjugative plasmids in *S. aureus* include the pSK41 and pWBG749 families. pSK41 was the first *S. aureus* conjugative plasmid family identified in the 1980s as the cause of rising gentamicin resistance (383). Plasmids from this family have been found to confer resistance to antiseptics and disinfectants, aminoglycosides, β-lactams, bleomycin, trimethoprim, tetracycline, mupirocin, macrolides, lincosamides and streptogramin B (203). The pWBG749 conjugative-plasmid family, first discovered in *S. aureus* from remote Australian Indigenous communities, typically carry less AMR genes than pSK41-family plasmids, but has been found to encode β-lactam, heavy-metal, gentamicin, trimethoprim, mupirocin, chlorhexidine, aminoglycoside and vancomycin resistance (205, 219, 221, 384). Small MRSA plasmids can be mobilised by larger conjugative plasmids via mobilisation (described previously in section 1.8.1), facilitating the spread of small AMR plasmids.

1.11 Aims of the study

Broadly, the study is divided into two major and complimentary projects. The first project (Chapter 3) examines the dual equine-associated and human-bacteraemic ST612-MRSA, which has been increasingly infecting humans in Australia, to determine the origin of Australian ST612 MRSA. The second project (Chapters 4 and 5) examines the CA-MRSA and LA-MRSA discovered in Australian pigs and pig farm workers with a particular focus on the transmission of MGEs and subsequent AMR between the lineages. Additionally, upon the discovery of a novel linezolid-resistance gene variant in Australian LA-MRSA, the project examined the genomic background and molecular function of the variant (Chapter 5).

The aims of the study were to:

- Characterise the genomes of Australian equine-associated MRSA.
- Determine the origin of Australian equine-associated MRSA by phylogenetic analysis.
- Characterise the genomes and MGEs of Australian LA-MRSA.
- Predict direction of transmission of MGEs between Australian CA-MRSA and LA-MRSA.
- Characterise novel linezolid-resistance gene variant, *cfrAB.*
- Determine changes in functional expression of *cfrAB*.

Chapter 2

Materials and methods

2.1 Media and growth conditions

Unless otherwise specified, all *E. coli* and *S. aureus* were grown in Lysogeny Broth (LB, Table 7-1 - S1 Media) (385) or LB Agar (Table 7-1 - S1 Media) overnight at 37 °C. *E. coli* carrying pUC19 and pUC19-derivatives were maintained in LB or LBA supplemented with 0.4% (w/v) glucose. All broth cultures, unless otherwise specified, were incubated at 37 °C, with shaking at 180 revolutions per minute (rpm).

2.1.1 Bacterial isolates and plasmids

The bacterial isolates and plasmids used in Chapter 4 and Chapter 5 are listed in Table 2-1. Additional isolates used in Chapter 3 are listed in Chapter 3. Isolates were collected and confirmed as MRSA using chromogenic agar as described in the references provided for each isolate in Table 2-1. Isolate collection, chromogenic agar culture and epidemiological typing of ST398 and ST93 MRSA was performed by the authors of the study, Sahibzada et al (366). All ST398 and ST93 isolates used in this study, and listed in the table below, were collected between May and August 2015 from two Australian pig farm sites, as described in Sahibzada et al (366). Additionally, the isolates from that study were named to reflect their source of isolation (H indicating human; P or W indicating pig; E indicating isolation from the physical farm environment). Isolates with the Reference label “Sahibzada” have been kindly provided by Shafi Sahibzada and are yet unpublished.

Isolates are cryopreserved in 0.5 mL overnight broth cultures in 16.7% (v/v) dimethyl sulfoxide at -80 °C.

Table 2-1 - Bacterial isolates and plasmids

Isolate/Plasmid	Description	Reference
S. aureus		
E-008	ST93-IV LA-MRSA isolated from a farm environment	(366)
E-017	ST93-IV LA-MRSA isolated from a farm environment	(366)
E-021	ST93-IV LA-MRSA isolated from a farm environment	(366)
E-026	ST398-V LA-MRSA isolated from a farm environment	(366)
ETDrA	ST93-IV LA-MRSA isolated from a farm environment	(366)
ETWeP	ST398-V LA-MRSA isolated from a farm environment	(366)
H-2	ST93-IV LA-MRSA isolated from a human	(366)
H-3	ST93-IV LA-MRSA isolated from a human	(366)
H-4	ST93-IV LA-MRSA isolated from a human	(366)
H-6	ST93-IV LA-MRSA isolated from a human	(366)
H-7	ST93-IV LA-MRSA isolated from a human	(366)
H-8	ST93-IV LA-MRSA isolated from a human	(366)
H-9	ST93-IV LA-MRSA isolated from a human	(366)
H-11	ST93-IV LA-MRSA isolated from a human	(366)
H-13	ST93-IV LA-MRSA isolated from a human	(366)
H-14	ST93-IV LA-MRSA isolated from a human	(366)
H-15	ST93-IV LA-MRSA isolated from a human	(366)
H-16	ST93-IV LA-MRSA isolated from a human	(366)
H-17	ST93-IV LA-MRSA isolated from a human	(366)
H-18	ST93-IV LA-MRSA isolated from a human	(366)

H-19	ST93-IV LA-MRSA isolated from a human	(366)
H-20	ST398-V LA-MRSA isolated from a human	(366)
H-21	ST398-V LA-MRSA isolated from a human	(366)
H-23	ST398-V LA-MRSA isolated from a human	(366)
H-24	ST93-IV LA-MRSA isolated from a human	(366)
H-25	ST93-IV LA-MRSA isolated from a human	(366)
H-26	ST93-IV LA-MRSA isolated from a human	(366)
H-27	ST93-IV LA-MRSA isolated from a human	(366)
H-29	ST93-IV LA-MRSA isolated from a human	(366)
H-31	ST93-IV LA-MRSA isolated from a human	(366)
H-33	ST398-V LA-MRSA isolated from a pig	(366)
HT3	ST93-IV LA-MRSA isolated from a human	(366)
HT13	ST93-IV LA-MRSA isolated from a human	(366)
HT19	ST398-V LA-MRSA isolated from a human	(366)
HW-2	ST93-IV LA-MRSA isolated from a human	(366)
HW-15	ST93-IV LA-MRSA isolated from a human	(366)
HW-24	ST93-IV LA-MRSA isolated from a human	(366)
HW-31	ST398-V LA-MRSA isolated from a human	(366)
P1N1	ST398-V LA-MRSA isolated from a pig	Sahibzada
P-216	ST398-V LA-MRSA isolated from a pig	(366)
P-221	ST398-V LA-MRSA isolated from a pig	(366)
P-223	ST398-V LA-MRSA isolated from a pig	(366)
P-236	ST398-V LA-MRSA isolated from a pig	(366)
P-244	ST398-V LA-MRSA isolated from a pig	(366)
P-257	ST398-V LA-MRSA isolated from a pig	(366)
P-271	ST93-IV LA-MRSA isolated from a pig	(366)
P-306	ST93-IV LA-MRSA isolated from a pig	(366)
P-325	ST93-IV LA-MRSA isolated from a pig	(366)
P-330	ST398-V LA-MRSA isolated from a pig	(366)

PTDrAP2	ST398-V LA-MRSA isolated from a pig	(366)
PTDrAP2 CM-700	ST398-V LA-MRSA chloramphenicol resistant mutant	This study
PTDrAP2-Em	ST398-V LA-MRSA with confirmed erythromycin resistance	(366)
PTDrAP2 FM-1	ST398-V LA-MRSA florfenicol resistant mutant	This study
PTDrAP2 FM-2	ST398-V LA-MRSA florfenicol resistant mutant	This study
PTDrAP2 FM-3	ST398-V LA-MRSA florfenicol resistant mutant	This study
PTDrAP2 FM-4	ST398-V LA-MRSA florfenicol resistant mutant	This study
PTDrAP4	ST93-IV LA-MRSA isolated from a pig	(366)
PTDrBP4	ST93-IV LA-MRSA isolated from a pig	(366)
PTDrBP5	ST398-V LA-MRSA isolated from a pig	Sahibzada
PTF1P3	ST93-IV LA-MRSA isolated from a pig	Sahibzada
PTGrAP4 MSSA	ST398 MSSA isolated from a pig	Sahibzada
PTGrAP5	ST93-IV LA-MRSA isolated from a pig	(366)
PTGrBP1	ST398-V LA-MRSA isolated from a pig	(366)
PTPgP1	ST93-IV LA-MRSA isolated from a pig	(366)
PTWeP5	ST398-V LA-MRSA isolated from a pig	(366)
RN4220	Restriction deficient and phage-cured *S. aureus* laboratory control strain Δ*sau1* Δ*hsdR*	(386)
W1Bb-4	ST93-IV LA-MRSA isolated from a pig	(366)
W1Dr-3	ST93-IV LA-MRSA isolated from a pig	(366)
W1Fi-5	ST93-IV LA-MRSA isolated from a pig	(366)
W1Fi-9	ST93-IV LA-MRSA isolated from a pig	(366)
W1FPa-4	ST398-V LA-MRSA isolated from a pig	(366)
W1FPB-20	ST398-V LA-MRSA isolated from a pig	(366)
W1FPb-22	ST93-IV LA-MRSA isolated from a pig	(366)
W1FSb-2	ST398-V LA-MRSA isolated from a pig	(366)

W1Gr-12	ST398-V LA-MRSA isolated from a pig	(366)
W1Gr-24	ST93-IV LA-MRSA isolated from a pig	(366)
WWeU-6	ST93-IV LA-MRSA isolated from a pig	(366)
WweU-9	ST398-V LA-MRSA isolated from a pig	(366)
Escherichia coli		
BL21(DE3)pLys	Competent T7 expression, protease deficient protein-expression strain F–, *omp*T, *hsd*S_B (r_B–, m_B–), *dcm*, *gal*, λ(DE3), pLysS, Cm^r	Novagen
DH5α	Competent transformation strain F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Invitrogen
NiCo21(DE3)	Competent T7 expression, protease deficient protein-expression strain can::CBD *fhuA2* [lon] *ompT* gal (λ DE3) [*dcm*] *arnA*::CBD *slyD*::CBD *glmS6Ala* Δ*hsdS* λ DE3 = λ sBamHIo ΔEcoRI-B int::(*lacI*::PlacUV5::T7 gene1) i21 Δnin5	NEB
DC10B	Dcm Methylase-deficient competent strain F– *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80lacZΔM15 ΔlacX74 *recA1 endA1 araD139* Δ (ara-leu)7697 *galU galK* λ– *rpsL*(Str^R) *nupG Δdcm*	(239, 387)
Plasmids		
pETM-11	Protein expression plasmid with TEV-cleavable 6-histidine tag (6-his)	(388)
pETM-11-*cfrAB*	pETM-11 containing fragment of *cfrAB* gene for expression of -1 frameshift protein	This study

pETM-41	Protein expression plasmid with T7 promoter, 6-his tag and TEV-cleavable maltose-binding protein (MBP)	European Molecular Biology Laboratory
pETM-41-*cfr*AB-sfGFP	*cfrAB-lacZα* gene from pUC19-*cfr*AB cloned in-frame with MBP and super-folded green fluorescent protein (GFP) for the expression and purification of frameshifted CfrAB protein	This study
pUC19	High copy number cloning plasmid with *lac* promoter	(389)
pUC19-*cfr*	pUC19 containing 128 bp of *cfr* gene cloned in-frame with lacZα to detect baseline expression	This study
pUC19-*cfr*AB	pUC19 containing 128 bp of *cfrAB* gene cloned in-frame with lacZα to detect -1 frameshift expression	This study
pUC19-*cfr*ABneg	pUC19 containing 128 bp of *cfrAB* gene cloned in-frame with lacZα, with an additional -1 reading frame stop codon to act as an expression negative control	This study

The primers and corresponding sequences used for the cloning of plasmids in Chapter 5 are listed in Table 2-2

Table 2-2 - Primer sequences

Primer name	Sequence 5' to 3'	Use
*cfr*_NcoI_fwd	AGGATGCCATGGTTATGAATTTTAATAATA AAACA	Amplifies *cfr* gene, adds NcoI site and stays in-frame for cloning into pETM-11
*cfr*_EcoRI_rev	GTTCAGAATTCTTGGCTATTTTGATAATTAC CAT	Amplifies *cfr* gene, adds NcoI site and stays in-frame for cloning into pETM-11
cfr_screening_fwd	GAG CAA ATT GTG AAA GGA TGA	Screening *cfrAB>cfr* mutants
cfr_screening_rev	ACC TTG TTC AAT ACT ATT GGC	Screening *cfrAB>cfr* mutants
LacZa_MBP_NotI_fwd	GCGCGGCCGCCTATGCGGCATCAGAG	Amplify pUC19-*cfr* LacZa-fusion for cloning into pETM-41
LacZa_MBP_NcoI_Rev	CAGGCCATGGCTATGACCATGATTACGCCA AGC	Amplify pUC19-*cfr*LacZa-fusion for cloning into pETM-41
M13fwd	CGCCAGGGTTTTCCCAGTCACGAC	M13 sequencing primer
M13rev	TCACACAGGAAACAGCTATGAC	M13 sequencing primer

GBlocks used for the cloning of pUC19 expression vectors in Chapter 5 are listed in Table 2-3.

Table 2-3 – GBlock dsDNA sequences

GBlocks	Sequences 5’ to 3’
lacZ_*cfr*	CCGCGCCAAGCTTGGAGAGAGTAGAAACGGTAAACATGAAGTATAAAGCAGGTTGG GAGTCATTTTGTATATCATCACAATGCGGATGTAATTTTGGGTGTAAATTTTGTGCTA CAGGCGACATTGGATTGAAAAAAAACCTAACTGTAGATGAGATAACAGATCAAGTTT TATACTTCCATTTATTAGGTCATCAAATTGATAGCATTTCTTTTATGGGAATGGGTGAA GCTCTAGCCAACCGTCAAGTATTTGATGCTCTTGATTCGTTTACGGATCCTAATTTATT TGCATTAAGTCCTCGTAGACTTTCTATATCAACGATTGGTATTATACCTAGTATCAAAA AAATAACCCAGGAATATCCTCAAGTAAATCTTACATTTTCATTACACTCACCTTATAGT GAGGAACGCAGCAAATTGATGCCAATAAATGATAGATACCCAATATCGAATTCACCG CG
lacZ_*cfr*AB	CCGCGCCAAGCTTGGAGAGAGTAGAAACGGTAAACATGAAGTATAAAGCAGGTTGG GAGTCATTTTGTATATCATCACAATGCGGATGTAATTTTGGGTGTAAATTTTGTGCTA CAGGCGACATTGGATTGAAAAAAACCTAACTGTAGATGAGATAACAGATCAAGTTTT ATACTTCCATTTATTAGGTCATCAAATTGATAGCATTTCTTTTATGGGAATGGGTGAA GCTCTAGCCAACCGTCAAGTATTTGATGCTCTTGATTCGTTTACGGATCCTAATTTATT TGCATTAAGTCCTCGTAGACTTTCTATATCAACGATTGGTATTATACCTAGTATCAAAA AAATAACCCAGGAATATCCTCAAGTAAATCTTACATTTTCATTACACTCACCTTATAGT GAGGAACGCAGCAAATTGATGCCAATAAATGATAGATACCCAATATCGAATTCACCC GCG
LacZ_*cfrAB*neg	CCGCGCCAAGCTTGGAGAGAGTAGAAACGGTAAACATGAAGTATAAAGCAGGTTGG GAGTCATTTTGTATATCATCACAATGCGGATGTAATTTTGGGTGTAAATTTTGTGCTA CAGGCGACATTGGATTGAAAAAAACCTAACTGTAGATGAGATAACAGATCAAGTTTT ATACTAGCATTTATTAGGTCATCAAATTGATAGCATTTCTTTTATGGGAATGGGTGAA GCTCTAGCCAACCGTCAAGTATTTGATGCTCTTGATTCGTTTACGGATCCTAATTTATT TGCATTAAGTCCTCGTAGACTTTCTATATCAACGATTGGTATTATACCTAGTATCAAAA AAATAACCCAGGAATATCCTCAAGTAAATCTTACATTTTCATTACACTCACCTTATAGT GAGGAACGCAGCAAATTGATGCCAATAAATGATAGATACCCAATATCGAATTCACCC GCG

2.2 Antimicrobial susceptibility testing

AMR phenotyping of ST398 and ST93 MRSA in Chapter 4 was performed previously by Sahibzada et al (366).

2.2.1 ETEST® MIC assay

Detection of *cfr* and *cfrAB*-mediated linezolid resistance was performed by ETEST® MIC assay. Isolates were plated on Tryptic Soy Agar (Table 7-1 - S1 Media) and raised overnight at 37 °C. Inocula were prepared by taking up to three colonies were selected with a sterile cotton swab, which were resuspended in 4 mL of Mueller Hinton (MH) broth (Table 7-1 - S1 Media). Inocula were adjusted manually to match turbidity of a 0.5 McFarland standard. A sterile swab was dipped in the inoculum and excess was allowed to drain before swabbing on to a MH agar (Table 7-1 - S1 Media) plate in three directions. *S. aureus* RN4220 were used as negative control strains. ETEST® (Biomerieux) linezolid strips were applied to the surface of the MH agar with sterile forceps before plates were incubated for 24 hours at 37 °C. The MIC was interpreted as the point where the edge of bacterial growth intersects the strip, with the concentration of antimicrobial indicated on the strip. Clinically significant *S. aureus* linezolid resistance was based on the European Committee on Antimicrobial Susceptibility Testing disk diffusion assay breakpoint of ≥ 4 µg/mL (https://eucast.org/clinical_breakpoints/).

2.3 Preparation of *E. coli* competent cells and electroporation methods

E. coli electrocompetent cells were prepared using the protocol provided by the electroporation equipment manufacturer (Biorad). *E. coli* cells were cultured in super optimal broth (SOB) medium (Table 7-1 - S1 Media). Plasmids were introduced into *E. coli* cells by electroporation using the following protocol: keeping the DNA and cells on ice, 2 µL of purified plasmid or 20 µL of purified

plasmid ligation were added to 40 µL of electrocompetent cells, and transferred into a 0.2 mm gap cuvette (Fisher Biotechnology). Cuvettes were charged with a single 5 ms 1.8 kV pulse (at 25 µF resistance and 200 ohms) using a Gene Pulser II (Biorad). The cells were immediately resuspended in 1 mL of LB and transferred to a 30 mL tube before incubation at 37°C on a rotary shaker (180 rpm) for 30 minutes. Transformation reactions were spread in serial dilutions onto selective LB agar media and incubated overnight at 37°C.

2.4 Nucleic acid techniques

2.4.1 Whole-genomic, plasmid DNA extraction and purification

Cells were grown in overnight 5 mL broth culture, in 30 mL tubes. *E coli* was grown in LB (Table 7-1 - S1 Media), while *S. aureus* were grown in Tryptic Soy Broth (Table 7-1 - S1 Media). Plasmid extractions were performed using FavorPrep Plasmid DNA Extraction mini kits (Favorgen) and PCR-amplified DNA was purified using FavorPrep PCR Clean-Up kits (Favorgen, Taiwan), each according to the manufacturer's instruction.

S. aureus whole genomic DNA was extracted using FavorPrep Blood / Cultured Cell Genomic DNA Extraction Mini kits (Favorgen) according to the manufacturer's instruction ("General Protocol"), with the following modifications: Overnight 5 mL Tryptic Soy Broth (Table 7-1 - S1 Media) culture was centrifuged at 14000 x g to produce cell pellets, from which the supernatant was removed and which were resuspended in 200 µL of FAPD1 buffer (FavorPrep Plasmid DNA Extraction mini kits) containing RNAse A (50 mg/mL). As per the manufacturers' instruction, 200 µL of FabG lysis buffer (FavorPrep Blood / Cultured Cell Genomic DNA Extraction Mini Kit buffer) was added to the cell suspension with 20 µL of ProteinaseK (10 mg/mL), and the sample was incubated at 60 °C. During incubation, the sample was not vortexed but was instead gently inverted to reduce DNA shearing. Notably, lysostaphin was omitted from *S. aureus* extractions due to the lack of appreciable benefit to DNA yield or quality.

DNA extraction proceeded according to the manufacturers' instruction. DNA prepared for PacBio Single-Molecule Real-Time (SMRT) Long Read sequencing was concentrated using a Savant DNA Speed Vac concentrator (ThermoFischer), with vacuum, at ambient temperature to ensure DNA integrity. Concentrated DNA integrity was examined visually by gel electrophoresis (2.4.3) on 1.5% agarose gel or 1% agarose gel for high molecular weight DNA.

2.4.2 DNA Quantification and quality assessment

DNA quantity was determined by NanoDrop 2000 UV Visible Spectrophotometer (Thermo Scientific), with quality determined by 260/280 and 230/260 ratios. Where greater accuracy of dsDNA quantification was required, the Qubit 2.0 fluorometer (Invitrogen) was used, according to the manufacturer's instruction. DNA integrity was determined visually by gel electrophoresis. Average DNA fragment size of Illumina MiSeq sequencing libraries was determined with a LabChip GXII Touch nucleic acid analyser (Perkin Elmer).

2.4.3 Agarose gel electrophoresis

PCR products and plasmids were electrophoresed at 9 V cm-1 for 45 – 60 minutes in 1.5% (w/v) agarose (Fisher Biotechnology) prepared in 1x TAE Buffer (Table 7-2 - S2 Buffers) and 1x Gelred dye (Fisher Biotechnology). The 1 kb, 1 kb-plus or 2-log DNA ladders (NEB) were used as molecular weight markers as appropriate for the expected PCR product size. Gels were visualised with ultraviolet light on a Chemi-Doc transilluminator (Biorad).

2.4.4 Polymerase chain reaction (PCR)

Primers were designed in Ugene (v 34.0, (390)). Primer pair melting temperatures were estimated using the NEB Tm Calculator (v 1.12.0, https://tmcalculator.neb.com/#!/main)) and primer lengths were manually adjusted to ensure primer melting temperatures were within 5 °C of each other. Custom oligo DNA primers were ordered from Integrated DNA Technology (IDT) and were diluted to 10 µM with filtered ddH_20 before use. PCR amplifications for cloning were performed in 50 µL reactions using 25-200 ng of DNA template, 1x Phusion reaction buffer, 0.2 mM deoxynucleotide triphosphates, 500 nM forward and reverse primers (Table 2-2), 1 unit of Phusion high-fidelity DNA polymerase and 0.2 µM filtered sterile DdH20 water, and thermal cycle conditions used as per polymerase manufacturer's instructions. All PCR reactions were carried out on a Veriti 96 well Thermal Cycler (Applied Biosystems) and post-reaction, PCR products were purified using FavorprepTM GEL/PCR Purification Kit (Favorgen) as per manufacturer's recommendations and eluted using 50 µL of 0.2 µm filtered DdH20 water.

2.4.5 Preparation of plasmid DNA for cloning

PCR products and plasmids used for cloning were digested using restriction enzymes from NEB. Selection of buffers, incubation temperatures and durations were to the manufacturer's instructions. Restriction enzyme-digested cloning vectors were dephosphorylated in 1x Antarctic phosphatase buffer (NEB) with 3 U of Antarctic phosphatase and incubated at room temperature for 2 hours.

2.4.6 DNA Ligation

PCR products and digested gBlocks were purified using the Favorprep^{TM} PCR mini-prep kit. Dephosphorylated vectors were gel purified using the same kit (Favorgen) as per manufacturer's recommendations. Purified DNA was eluted in 50 µL of 0.2 µm filtered ddH_20 water. Ligation reactions consisting of 10 µL dephosphorylated plasmid, 16 µL or five molar equivalent of digested PCR product, 3 µL of 10x T4 ligase buffer (NEB), 1 µL T4 ligase, and 1mM ATP (NEB). Ligation reactions were

incubated at 4°C for 24 hours and were purified using the Favorprep^{TM} PCR mini-prep kit. Purified, ligated plasmid was elution in 40 µL of 0.2 µm filtered ddH_20 water.

2.4.7 Cloning of Plasmids

Enzymes were purchased from New England Biolabs (NEB), primers and gBlock oligonucleotides were purchased from Integrated DNA Technologies (IDT). Primers and oligonucleotides used to construct plasmids in this study are listed in Table 2-2. Digested plasmids were treated with alkaline phosphatase as described in section 2.4.5**.**, and purified by gel electrophoresis using the FavorprepTM GEL/PCR Purification Kit (Favorgen) as per manufacturer's instructions, prior to ligation (described above in 2.4.6). Ligated plasmids were purified and introduced into *E.coli* DH5α by electroporation (described in 2.3), then transformed cells were screened for correct plasmid by colony-polymerase chain reaction (PCR) (2.4.4) using primers listed in Table 2-2. Cloning insert sequence was confirmed by Sanger sequencing (2.5.1).

pUC19. Purified pUC19 plasmid was digested with EcoRI and HindIII, dephosphorylated and was gel-purified. Purified plasmid DNA was diluted and quantified.

pUC19-cfr, -*cfrAB* and –*cfrAB*neg. gBlocks (listed in Table 2-3) were digested with ecoRI and HindIII, purified and ligated into EcoRI/HindIII-digested pUC19.

pETM-11-*cfrAB*. pETM-11 was digested with EcoRI and NcoI, dephosphorylated and gel-purified. Primers *cfr*_NcoI_fwd and *cfr*_EcoRI_rev were used to PCR amplify purified HW31 genomic DNA. PCR products were purified and digested with NcoI/EcoRI. Digested PCR products were purified and ligated into NcoI/EcoRI digested pETM-11.

pETM-41. Purified pETM-41 plasmid was digested with NotI and NcoI, dephosphorylated and was gel-purified. Primers LacZa_MBP_NotI_fwd and LacZa_MBP_NcoI_Rev were used to amplify pUC19-*cfr*

plasmid DNA. The PCR products were purified and digested with NcoI/NotI. Digested PCR products were purified and ligated into NotI/NcoI digested pETM-41.

2.5 DNA sequencing

2.5.1 Sanger sequencing

Sanger sequencing was used to confirm cloned plasmids and *cfr* mutants. Plasmid DNA was extracted and PCR products were purified as described in 2.4.1. Samples were diluted to the concentrations recommended by the Australian Genome Research Facility (e.g. 30-75 ng of >800 bp PCR products, 600-1500 ng of plasmid dsDNA) with ddH_20 to a volume of 14 µL and mixed with 1 µL of appropriate sequencing primer (Table 2-2), diluted to 10 pmol with ddH_20. Sanger sequencing was performed by the Australian Genome Research Facility (https://www.agrf.org.au/sanger-sequencing). Sequence data was analysed using Ugene v34.0 software (390) or the Benchling server (https://www.benchling.com/).

2.5.2 Whole –genome sequencing: Illumina MiSeq and PacBio SMRT long-read sequencing

WGS was performed on bacterial isolates in Chapters 3, 4 and 5. *S. aureus* genomic DNA extraction was performed as described in section 2.4.1. Preparation of 5 nM sequencing libraries was performed with the Nextera XT library preparation kit according to the manufacturer's instructions with the following variations: A DNA High-Sensitivity assay kit was used to prepare genomic DNA samples, as per the manufacturers instruction, for analysis on a Labchip GXII Touch instrument (PerkinElmer) to determine the molarity and integrity/fragment size distribution of Nextera XT sequencing libraries. Degraded libraries, or those with average fragment sizes too large or too small were discarded and reprepared. Based on average library fragment size in bp, libraries were diluted with ddH20 according

to Illumina's Concentration Conversion guide to achieve final library concentrations of 4 nM. ST398 and ST612 MRSA libraries were sequenced on Illumina MiSeq sequencing platform at Murdoch University (Western Australia), and PTDrAP2 florfenicol *cfrAB>cfr* mutant isolates were sequenced on the Illumina MiSeq platform at Curtin University (WA).

Reference genomes for Chapters 3 and 4 were sequenced with Pacbio SMRT Long-Read sequencing. Whole genomic DNA was extracted and purified as described in 2.4.1. Purified DNA (16 µg per sequencing run) was sent to Pacific Bio (South Korea) for sequencing. The PacBio reads were assembled with Canu (v1.6, (391)) using default parameters. The assembled sequences were trimmed to remove overlaps, and the origin of the genome was set to *dnaA* using Circlator (v1.5.5 (392)). Plasmid DNA was extracted from Illumina MiSeq reads by mapping the Illumina MiSeq reads to the Canu assembly using BWA-index and BWA-mem (Burrows-Wheeler Aligner [BWA] v0.7.17-r1188 (393)). Samtools (394) was then used to extract reads which did not map to the Canu assembly (i.e. non-chromosomal DNA), which were then assembled with SPAdes v3.9.0 (395). Plasmid contigs were manually inspected trimmed to remove overlaps using Ugene v34.0 (390). The Illumina reads and draft PacBio assembly were used together as input to Pilon v1.22 (https://github.com/broadinstitute/pilon/releases, (396)) to polish the PacBio assembly. This served as a quality control step, in addition the high coverage provided already by PacBio sequencing, by comparing base-calls with the MiSeq reads, effectively boosting the overall depth of coverage of the assembly. Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline for ST612 SVH7513 and Prokka was used to annotate the ST398 PTDrAP2 genome.
Reads for SVH7513 are deposited in the Sequence Read Archive under accession SRP151517.

2.6 Protein Techniques

2.6.1 Bacterial protein expression

Expression plasmids (based on either pETM-11 or pETM-41) were electroporated into *E. coli* BL21(DE3) pLys or NiCo21(DE3) competent cells. Cells were recovered in 1 mL of LB broth at 37° C with shaking for one hour, before 100 µL was spread on LB agar plates containing appropriate antimicrobials (50 µg/mL kanamycin for pETM-11/41 plasmids and ampicillin 100 µg/mL for pUC19) and 0.4% glucose, to repress protein expression. After overnight incubation at 37 °C a single colony was inoculated into 5 mL LB broth, supplemented with the appropriate antimicrobial and 0.4% glucose and grown overnight at 37 °C with shaking at 180 rpm. A 250 - 1000 mL LB broth (maximum 500 mL in a 2 L flask) was inoculated with the 5 mL overnight culture and incubated at 25 °C with shaking at 200 rpm until an Optical Density at 600 nm (OD_{600}) of 0.6 was reached. Flasks were incubated on ice while isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The culture was incubated for a further 16 hours at 18 °C at 180 rpm. The culture was centrifuged at 8000 g at 4 °C and the supernatant discarded. Cells pellets were kept on ice or stored at -20 °C until ready for protein purification.

2.6.2 Protein purification

A cell disruptor (CF1 Cell Disruptor, Constant Systems) was pre-chilled to an operating temperature of 4 °C. The disruptor was washed with 250 mL of 70% ethanol and rinsed with 250-500 mL cold, deionised, autoclaved water. Cell pellets, thawed and resuspended in 50-150 mL of nickel-affinity binding buffer (Table 7-2 - S2 Buffers) supplemented with 2 µg/mL of DNase I nuclease (Astral) and 300 µg/mL of lysozyme (Sigma), were loaded into a cell disruptor. Cells were disrupted at 20k PSI and the supernatant maintained on ice to prevent proteolytic degradation of the sample. The lysate was centrifuged at 24,000 g for 45 minutes at 4 °C before syringe-filtration (Millex-GP 0.22 µm syringe filter, Merck Millipore) to sterilise and remove any remaining large particles from the lysate. The filtered lysate was loaded on to a 5 mL Ni-charged HisTrap HP column (GE Healthcare), which was washed with 3-5 column-volumes of wash buffer. The histidine-tagged fusion proteins were eluted using an imidazole gradient (25-500 mM) over eight column-volumes and the fraction containing the highest protein concentration bound to a HiLoad 16/60 Superdex 75 size exclusion column (GE

Healthcare) using size-exclusion buffer (Table 7-2 - S2 Buffers) at 1 mL/minute. Chromatography purification steps were performed using an AKTA purifier HPLC system (GE Healthcare) at 4 °C and absorbance traces at 280 nm only (AKTA Start), or 280, 260 and 230 nm (AKTA Purifier) were constantly monitored.

Additional amylose resin purification was performed. A 1 mL amylose resin column (NEB) was maintained at 4 °C and washed with five volumes of amylose column buffer (Table 7-2 - S2 Buffers) at a flowrate of 0.8 mL/min. The fraction with the highest concentration of eluted protein (as determined by the AKTA 280 nm absorbance trace) was selected and loaded directly into the amylose resin column. The column was washed with 10 column-volumes of column buffer, before elution with column buffer supplemented with 10 mM maltose.

2.6.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualise cfrAB frameshift products and assess protein integrity before Mass-spectrometry. Protein samples (9 µL) were mixed with 4x SDS loading buffer (1 µL, Table 7-2 - S2 Buffers) and were incubated at 95°C for 10 minutes. 10 µL samples were loaded onto an AnykD™ tris-glycine gel (Mini-PROTEAN TGX, Bio-Rad). Electrophoresis was performed in 1x Protein gel running buffer (Table 7-2 - S2 Buffers) at 110V. Gels were stained with Coomassie blue stain (Table 7-2 - S2 Buffers) for 20 minutes and destained with Coomassie blue destaining solution (Table 7-2 - S2 Buffers) until protein samples and protein ladder bands were clearly visible. Gels were imaged using a Gel-doc transiluminator (Bio-Rad). Gels were run with 5 µL of 10 – 200 kDa protein ladder (NEB). The molecular weights of protein products were predicted using the *ExPasy Protparam* webtool (https://web.expasy.org/protparam/, (397)). Gel image files were imported to a local computer and relative abundance of protein was determined using ImageJ software (https://imagej.nih.gov/ij/, (398).

2.6.4 Mass spectrometry peptide sequencing

Mass spectrometry was performed by Proteomics International (Perth, Australia). Protein samples were Glu-C digested and peptides extracted according to standard techniques (399). Peptides were analysed by electrospray ionisation mass spectrometry using the Shimadzu Prominence nano HPLC system (Shimadzu) coupled to a 5600 TripleTOF mass spectrometer (Sciex). Glu-C peptides were loaded on to an Agilent Zorbax 300SB-C18, 3.5 µm (Agilent Technologies) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with *Bos taurus* and a custom p41*cfr*ABsfGFP database containing the full amino acid sequence of the -1 frameshifted MBP-CfrAB-sfGFP protein. The possibility of alternative PRF sites and types in the MBP-*cfrAB*-sfGFP sequence were explored and were included in the peptide sequencing database (Figure 2-1), which included peptides which would be produced by a +2 frameshift in the poly-A tract and every combination of -1 and +1 frameshifted sequences. Additionally, four sequences which would be produced by frameshifts which terminate abruptly were included (sequences labelled 'dud').

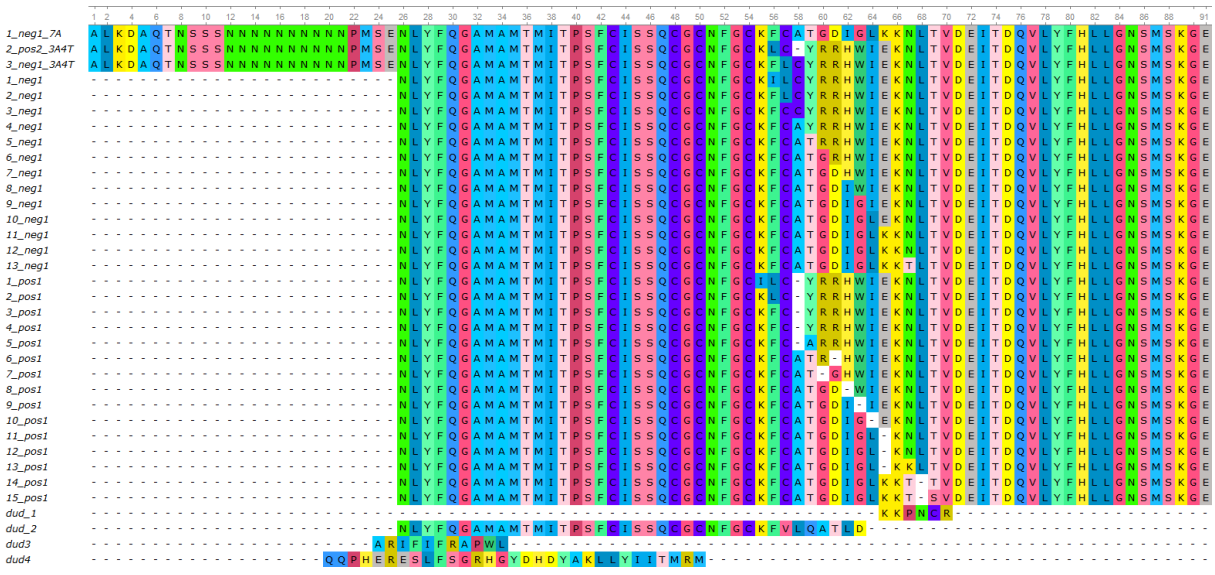

Figure 2-1 - p41*cfr*ABsfGFP peptide sequencing database.

Sequences were named according to the type of frameshift (neg1 = negative 1 base frameshift, pos1 = positive 1 base frameshift) and were assigned a number in order of frameshift site relative to the first A in the poly-A PRF sequence. 'Dud' denotes frameshifts which result in premature sequence terminations. The full-length MBP-CfrAB-sfGFP sequence is included in the database but is not included in the figure due to its length.

2.7 β-Galactosidase reporter assay

For expression assays described in Chapter 5, 5 mL LB (supplemented with 100 µg/mL ampicillin and 0.4% glucose) inoculated from single colonies of *E. coli* DH5α cells carrying pUC19-*cfr*, pUC19-*cfrAB* and pUC19-*cfrAB*neg (negative control) were grown for 24 h. Fresh broths containing 0.1 mM IPTG were inoculated from the 24 hour cultures (1/100 dilution) and grown for 24 h at 37 °C. Cell density was measured at OD_{600} and all samples were adjusted to OD_{600}=0.5. Cells were analysed for β-galactosidase expression by using the fluorescent substrate 4-methylumbelliferyl β-D-galactoside (MUG) and an Enspire Multimode plate reader (Perkin Elmer, as described (400)). Plate reader data was converted to Relative Fluorescence Units/s/Ab_{600}. Assays were performed in biological triplicate.

2.8 Isolation of *cfrAB>cfr* mutants and determination of mutation rate

To isolate PTDrAP2 *cfrAB>cfr* mutants, 5 mL LB cultures were incubated in 30 mL tubes overnight at 37 °C with shaking at 180 rpm. Undiluted 100 µL aliquots of the overnight culture were applied to LB agar (Table 7-1 - S1 Media) plates supplemented with 80 µg/mL florfenicol, and were spread evenly over the surface with a flame-sterilised glass hockey stick spreader until dry. Agar plates were incubated for 36-48 hours, until colonies appeared. Colonies were picked with a flame-sterilised loop and streaked for single colonies on LB agar plates supplemented with 80 µg/mL florfenicol and incubated overnight at 37 °C. Single colonies were raised in LB broth supplemented with 80 µg/mL florfenicol and incubated overnight at 37 °C with shaking at 180 rpm. Genomic DNA was extracted

(2.4.1) and *cfr* alleles were amplified for sequencing by PCR (described in 2.4.4) using *cfr* screening primers (Table 2-2). PCR products were purified using FavorprepTM GEL/PCR Purification Kit (Favorgen) as per manufacturer's recommendations and eluted using 50 µL of 0.2 µm filtered DdH20 water. Samples were sent to AGRF for Sanger sequencing (described in 2.5.1) to confirm mutation. To confirm that there were no additional chromosomal mutations, five additional isolates were whole-genome sequenced using Illumina MiSeq (2.5.2).

To determine the rate of *cfrAB>cfr* mutation, *cfrAB* isolates PTGrBP1, PTGrAP4 and PTDrAP2; *cfr* isolate HW-31; ST93 *cfr*-negative isolate PTF1P3, and control strain RN4220 were colony purified and raised in 5 mL overnight LB in triplicate, at 37 °C with shaking at 180 rpm. A 100 µL aliquot of the overnight culture, diluted to 1 x 10^{-6} in LB media, was spread plated on antimicrobial-free LB agar to determine total Colony-Forming Units/mL of the overnight cultures. 100 µL of broth culture was flood inoculated on to LB agar plates supplemented with 80 µg/mL florfenicol and spread with a sterile glass spreader. Inoculated LB agar plates were incubated 48 hr at 37 °C. The number of colonies emerging on LB agar plates containing 80 µg/mL florfenicol were counted and divided by the initial Colony-Forming Units/mL to determine the mutation rate per Colony-Forming Units/mL. Florfenicol was used instead of linezolid as the cost and availability of linezolid was prohibitive, and the linezolid-resistance determinant, *cfr*, provides resistance to both antimicrobials.

2.9 Microbial Genomics

2.9.1 Computing equipment

All computing was performed in the Ubuntu environment on a local PC**.** The PC comprised an AMD Threadripper 3.4Ghz processor (16 cores, 32 threads, AMD) with 32 Gb of RAM and Gigabyte GeForce GTX1050 OC 2GB GDDR5 graphics card (Nvidia) connected to a ASRock X399 Taichi TR4 ATX Desktop

Motherboard (Taichi). The hardware was cooled with a Noctua NH-U14S AMD Threadripper TR4 CPU Cooler (AMD).

2.9.2 Manual DNA sequence visualisation and analyses

DNA sequences were manipulated using Ugene v34.0 (390). Custom databases were created using formatdb (v 2.9.0+) and were used to search genomes with standalone nucleotide Basic Local Alignment Search Tool (BLAST) v2.9.0 (401), using default settings (expectation value = 10, culling limit = 20, word size = 11, gap cost = 2 2 and match score = 1 -3). Local BLAST+ was used as a plugin in Ugene (390).

2.9.3 Reference genomes

Reference genomes used in this study for the construction core-SNP genome phylogenies include Australian ST612 MRSA SVH7513 (GenBank: CP029166.1), ST398 MRSA S0385 (GenBank: AM990992.1), the first sequenced LA-MRSA (402), and CA-MRSA ST93 JKD6159 (GenBank: NC_017338.1). SVH7513 was used as a genomic reference for ST612 MRSA core-SNP genome phylogeny construction in section 3.4.2; S0385 was used as a reference for ST398 LA-MRSA in 4.3.1; and JKD6159 was used as a reference for ST93 CA-MRSA phylogeny in 4.5.

2.9.4 Genome assembly, annotation and core-SNP phylogeny

Genomes were sequenced on the Illumina MiSeq platform as described in 2.5.2. Sequence reads were cleaned, assembled and annotated using the powerful Nullarbor bioinformatic pipeline software

package (github.com/tseemann/nullarbor). The Nullarbor pipeline takes paired-end MiSeq reads and performs a series actions per isolate and per dataset to produce complete, annotated genomes and core-SNP genome phylogenies. For each individual genome, Nullarbor clean reads to remove adaptor sequences, and low quality bases and reads using Trimmomatic (v 0.22 (403); performs species identification by k-mer analysis against known genome database using Kraken (v 0.10.5-beta, https://github.com/DerrickWood/kraken); performs *De novo* genome assembly using Megahit (v 1.1.1, (404) [although SPAdes v 3.11.1, (395) was used for the assembly of ST93 and ST398 genomes]; annotates the genome using Prokka v 1.12, (405); performs MLST typing from the genome assembly using a search algorithm and MLST database using the mlst algorithm (v 2.8, https://github.com/tseemann/mlst) and *PubMLST* database (281); computes the resistome from the assembly using abricate (v 0.4, https://github.com/tseemann/abricate) with the *Resfinder* AMR gene database (406); and calls SNP variants from reads aligned to a reference sequence using snippy (v 3.1, https://github.com/tseemann/snippy). Per set of isolates, Nullarbor produces a core genome SNP alignment from the input read sequences using snippy-core (v 3.1, https://github.com/tseemann/snippy/blob/master/bin/snippy-core); infers core SNP phylogeny, by default selecting a Maximum-likelihood GTR+G4 model, computed using FastTree (v 2.1.8); calculates a SNP distance matrix using snp-dists; and determines the pan genome from annotated contigs using Roary. Nullarbor also produces a report of isolate information in HTML format.

MRSA *spa* types were determined with 'Get spa type' (v 0.10, https://github.com/mjsull/spa_typing) using the Ridom SpaServer database (https://spaserver.ridom.de/, (407)). Virulence factors were detected in ST612 MRSA using the abricate v0.4 (https://github.com/tseemann/abricate) to query the Virulence Factor Database (http://www.mgc.ac.cn/VFs/main.htm). CRISPR-Cas loci were detected using CrisprCasFinder ((408)).

2.9.5 Quality control of assemblies

Quality control of assemblies was performed within the Nullarbor pipeline (github.com/tseemann/nullarbor). Trimmomatic,was used the flags "LEADING:10 TRAILING:10 MINLEN:30" to filter low quality sequence reads and to clip Illumina MiSeq sequencing adaptor sequences. The Nullarbor pipeline was used to assess genome assembly quality, including total reads and yield (bp), minimum and average contig length (bp) and average read depth of coverage.

2.9.6 Construction of local BLAST databases

makeblastdb (409) was used to generate BLAST-formatted databases from concatenated multifasta files. Individual BLAST databases were made with the assembled ST612 MRSA genomes, ST398 LA-MRSA genomes and ST93 CA-MRSA genomes for analyses in Chapter 3 and 4. Protein domain searches were performed with BLASTx to query a local SWISSProt protein domain database (https://www.uniprot.org/downloads, downloaded July 2018). A staphylococcal Rep-typing database was made with makeblastdb using a multifasta file containing the sequences of representative plasmids for each rep-type, using the genbank accession listed in the supplementary materials Kwong et al (380)) to locate and download the plasmid sequences from Genbank (https://www.ncbi.nlm.nih.gov/nucleotide/). A SaPI-finder database was made containing the genomes of SaPI listed in section 3.6, downloaded from Genbank. A phage-finder database was made containing *att, cos* and *pac* sequences (410). A database of the RN0450 (NCTC 8325-4, GenBank NC_007795.1), which was cured of phage (411), was made using formatdb+ in Ugene v36.0.

2.9.7 Identification of SaPI and Phage

SaPI elements were identified in ST612 SVH7513 and ST398 PTDrAP2 annotated genome sequences by local BLAST against the SaPI-finder database (2.9.6). Search for novel SaPI and phage was performed by local BLAST of ST612 and ST398 genomes against the phage-finder database (2.9.6) with modified BLAST parameters for short, nearly exact sequences (Expectation value = 1000, word size = 16).

2.9.8 MGE identification from assembled genome contigs

Plasmid contigs were identified from ST93 and ST398 local databases (2.9.6) using PlasFlow (412). Additional MGE contigs were extracted from ST93 and ST398 local BLAST databases with Fastagrep (v 2.0, https://github.com/rec3141/rec-genome-tools/blob/master/bin/fastagrep.pl), using the output from Abricate as input for the sequence search tool. MGE fasta contigs were manually sorted by sequence length and assigned to 'plasmid/transposon' (<30 kb) or 'chromosomal' (>30 kb) categories, using file size as a proxy for sequence length. 'Plasmid/transposon' contigs and 'chromosomal' contigs multiple sequence alignments were performed ClustalX (v 1.2.4, http://www.clustal.org/clustal2/) or MUSCLE (413) in Ugene. Prokka (405) was used to annotate representative fasta contigs selected from each sub-alignment.

Chromosomally-integrated MGE (transposons, SCC*mec*, phage, SaPI and degraded remnants of MGE) were identified in closed genomes (not contigs) by local BLAST search against the RN0450 database (2.9.6). BLAST sequence-alignment gaps were extracted and searched with abricate to locate AMR genes and virulence genes (using the ResFinder and VFDB databases). RN0450 is cured of prophage (411) and does not encode any resistance genes, aside from NorA (confirmed with abricate). Gaps in an alignment indicated genes not found in RN0450 genome and are more likely to contain phage and AMR-containing MGE. Potential integrated MGE sequences were trimmed and copied to new files for further typing and analysis.

2.9.9 Plasmid Rep-typing

Prokka-annotated plasmid contigs (2.9.8) were visually inspected for genes annotated '*rep*', 'mob', 'pre' and 'transposase'. Genes annotated 'rep', 'mob' and 'pre' were used to query the Rep-typing database (2.9.6) using BLAST (2.9.2). Rep-family classification of genes which couldn't be confidently typed with the local rep-typing database were assigned a family according to rep PFAM domains

identified by protein BLAST query (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Plasmid contigs were confirmed circular by manually inspecting for the presence of overlapping (identical) sequence at the contig ends. These overlaps were manually removed and the plasmid sequences manually reoriented start at the *rep* gene.

2.9.10 Transposon characterisation

Prokka-annotated transposon contigs (2.9.8) were visually inspected for genes annotated 'transposase', 'recombinase' or 'resolvase' to find transposase genes. Transposase/resolvase genes were typed by domain Protein BLAST Conserved Domain search (414). Where a probable match to an established transposon or IS element occurred, the entire predicted transposon/IS sequence was aligned by nucleotide BLAST against the transposon. If >98% nucleotide identity occurred between the hypothetical transposon/IS and a characterised transposon/IS, the transposon/IS was confirmed (for example Tn*916*) or otherwise was given the tentative suffix '-like' for the closest matching characterised transposon e.g. Tn*554*-like.

2.9.11 Construction of plasmid phylogenetic trees

Alignment of plasmid sequences of ST93 and ST398 MRSA was performed in Ugene v34.0 using the ClustalW (v 2.1) algorithm (415) and were manually inspected/corrected in the Ugene alignment viewer. Plasmid Maximum-Likelihood phylogenetic trees were computed from Clustal multiple sequences alignments in MEGA-X v 10.2.4, (416) using the Tamura-Nei substitution model of evolution (417) and 100 bootstrap replicates.

2.9.12 Determining distribution of MGE in genomes

Characterised MGE were queried by local BLAST against ST612, ST93 and ST398 MRSA databases (2.9.6); full-length, high identity (>98% nt identity) alignments of MRSA contigs was considered positive confirmation of MGE carriage. Partial BLAST alignments were extracted from BLAST databases by contig ID and annotated using PROKKA and manually inspected in UGENE to determine if the contig represents an MGE variant or is caused by a short contig/low genome coverage region. Results of MGE carriage were manually tabulated.

2.9.13 Analysis of restriction-modification barrier integrity.

RM system genes were identified in JKD6159 ST93 MRSA genome by the locus tag information published in Chua et al (418). RM system genes in PTDrAP2 were identified by a text search of the GenBank file for gene annotations containing phrases “hsd” and “Restriction” revealed 3 candidate RM system loci. The RM functions within the loci were confirmed by PaperBLAST (419) of the translated amino acid sequence of the candidate RM system genes. Each gene was individually BLAST queried against ST93 and ST398 databases (2.9.6); full-length, identical (100% nt identity) alignments of MRSA contigs was considered positive confirmation of MGE carriage. No partial matches were detected. Gene presence/absence was manually tabulated.

JKD6159 Genbank Gene locus tags of RM system loci:

RM1 – *hsdR1* (SAA6159_01737), *hsdM2* (SAA6159_01738)
RM2 – *hsdM1* SAA6159_00386, *hsdS2* SAA6159_00387
RM3 – *sauUSI* SAA6159_00387
RM4 - *hsdR1* SAA6159_00176
RM5 – *hsdR1* SAA6159_00054, *hsdS* SAA6159_00055, *hsdR* SAA6159_00056

PTDrAP2 Genbank Gene locus tags of RM system loci*:*

398_1 – hsdM DD562_RS00700, *hsdR* DD562_RS00690

398_2 – *hsdM* DD562_02840, *hsdS* DD562_RS02845

398_3 – *hsdR* DD562_RS01375

Chapter 3

Genomics and origins of sequence type 612 Methicillin Resistant Staphylococcus aureus in Western Australia

3.1 Introduction

3.1.1 ST612 MRSA in Western Australia: first Australian ST612 bacteraemia

ST612 is a member of CC8 and is closely related to ST8, a prolific CA-MRSA in the USA (as described in section 1.10.4). In South Africa, ST612 is one of the most common MRSA in hospitals and is a frequent cause of skin and soft tissue infection and bacteraemia (328, 420-422). The number of ST612 MRSA notifications in WA has historically been low, with isolates identified by routine screening of staff and patients in WA hospitals. While the notifications of ST612 MRSA in WA are low, isolated sporadically across four Perth metropolitan hospitals and pathology centres (an 80 km area), the frequency of isolation increased between 2004 and 2015 (

Table 3-1). This data was provided by Dr Geoffrey W Coombs (Department of Microbiology, PathWest Laboratory Medicine WA, Fiona Stanley Hospital, Murdoch, Western Australia, Australia).

Table 3-1 - Western Australian ST612 MRSA notifications

Year	Hospital[1]	Isolate	Clinical/Screening[2]	Source
2004	WA Hospital 1	WA HCW 1	Screen	Nose
2009	WA Hospital 2	WA HCW 2	Screen	Throat
2012	WAC	WA Patient 2	Clinical	Left Ear
2012	WA Hospital 2	WA Patient 3	Clinical	Unknown
2013	WA Hospital 3	WA Patient 4	Screen	Nose
2014	WAC	WA Patient 5	Clinical	Elbow Wound
2014	WAC	WA Patient 6	Clinical	Eye
2014	WA Hospital 1	WA Patient 7	Screen	Nose
2014	WA Hospital 3	WA Patient 8	Screen	Nose
2014	WA Hospital 1	WA HCW 3	Screen	Unknown
2015	WA Hospital 3	WA Patient 9	Screen	Nose
2015	WA Hospital 4	WA Patient 10	Screen	Unknown

2016	WA Hospital 4	WA Patient 1	Clinical	Bloodstream

[1] WAC denotes a WA community pathology submission

[2]"Screen" isolates denote MRSA detected by routine nasal swab for all patients who were admitted to hospitals from different states or overseas and all new staff members who had worked outside WA in the previous 12 months under WA Health policy(423). These isolates represent colonization and are not part of an active infection. "Clinical" isolates are obtained from an infection.

In 2015, a 71-year-old male (WA Patient 9) presented to a WA hospital with multiple traumas following a motor-vehicle accident. ST612 MRSA was cultured from the patient's nasal swab and was resistant to co-trimoxazole, rifampicin, erythromycin and doxycycline. Within a year, the 71-year-old wife of WA Patient 9, (WA Patient 1) while receiving treatment for lymphoma, had an episode of febrile neutropaenia. ST612 MRSA was isolated from a peripheral blood culture, with no growth from central line insertion site and tip cultures. The couple operated a horse stud, south of Perth, WA. The sudden escalation of ST612 MRSA disease to bloodstream infection, the potential for virulence through its membership in the prolific CC8 group, and the reputation of ST612 MRSA as a dominant human pathogen in South Africa warranted an investigation into the origins of ST612 MRSA in WA.

3.2 Project aims

In this chapter we aimed to characterise and identify the potential origins of ST612 MRSA in WA by

- Producing a high-quality, closed ST612 genome using PacBio SMRT Long-Read sequencing;
- Performing short-read WGS on Western Australian, South African, Australian horse and veterinarian-isolated ST612 MRSA genomes and constructing a core genome phylogenetic tree to determine the evolutionary relationship between isolates and infer epidemiological origins;

- Identifying genetic context of AMR, virulence factor and the MGE composition of isolates to strengthen inferences drawn from core genome phylogeny and identify potential molecular factors associated with the host of isolation.

3.3 Chapter overview

The work presented in this chapter includes publications presented in sections 3.4.1 and 3.4.2, which addressed aims (i) and (ii-iv), respectively. Supplementary materials supporting the publications are provided in section 3.6. Additional conclusions from the combined work are presented in section 3.7.

3.4 Results

3.4.1 Publication: Complete Genome Sequence of a *Staphylococcus aureus* Sequence Type 612 Isolate from an Australian Horse

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GENOME SEQUENCES

Complete Genome Sequence of a *Staphylococcus aureus* Sequence Type 612 Isolate from an Australian Horse

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ABSTRACT *Staphylococcus aureus* is a serious pathogen of humans and animals. Multilocus sequence type 612 is dominant and highly virulent in South African hospitals but relatively uncommon elsewhere. We present the complete genome sequence of methicillin-resistant *Staphylococcus aureus* strain SVH7513, isolated from a horse at a veterinary clinic in New South Wales, Australia.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious pathogen of humans and animals. Multilocus sequence type 612 (ST612) is the most commonly isolated sequence type in South African hospital-isolated bacteremia (1, 2). ST612 is relatively uncommon elsewhere but has been sporadically isolated in association with horses and horse veterinarians in Australia (3, 4). ST612 is a close relative of American clones USA500, a health care-associated MRSA strain (5), and the epidemic community-associated USA300 (ST8) (6), both of which are members of clonal complex 8 (CC8). ST612 may pose a threat to public health as a proven human-adapted pathogen and horse-colonizing strain, where colonized horses may act as a reservoir. Here, we present the genome of MRSA strain SVH7513, isolated from a horse at a veterinary clinic in New South Wales, Australia. This genome may present clues to the genetic requirements of equine zoonosis; it provides a quality reference for the assembly of other ST612 genomes and is the first published complete sequence of an ST612 isolate.

SVH7513 was grown in tryptic soy broth (BD Biosciences, Sparks, MD), and cells were lysed in a FavorPrep plasmid extraction minikit (Favorgen, Taiwan) with buffer FAPD 1, containing RNase, with the addition of lysostaphin (Sigma-Aldrich; 2 mg/ml, 37°C, 20 min). Genomic DNA was extracted using a blood genomic DNA extraction kit (Favorgen). The genome of strain SVH7513 was sequenced using Pacific Biosciences (PacBio) RS II single-molecule real-time (SMRT) cell sequencing technology (Macrogen, South Korea). SMRT cell sequencing produced 152,425 subreads with an average subread length of 11,790 bp. Additionally, an Illumina genomic library was prepared using the Nextera XT Library prep, which was sequenced using the Illumina MiSeq platform and 600-cycle reagent kit (v3) (2 × 300-bp paired-end reads), producing 295,192 reads. PacBio reads were assembled with Canu (v1.6) using default parameters, which produced a circular 2,915,384-bp chromosome and a circular 27,887-bp plasmid, pSVH7513a.

The plasmid and chromosome had a 616-fold average depth of coverage. MiSeq reads mapped to these contigs with an average 29-fold depth of coverage. Mapping was used to identify likely sequence errors (Burrows-Wheeler Aligner [BWA] v0.7.17-r1188), but no differences between the Canu assembly and BWA-mapped reads were

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detected. Separate SPAdes (v3.11.1) assembly, using default parameters, of unmapped Illumina reads (extracted using SAMtools v1.6) identified a 2,495-bp *ermC* plasmid, pSVH7513b.

The assembled sequences were trimmed to remove overlaps, and the origin of the genome was set to *dnaA* using Circlator (v1.5.5). Plasmid origins were manually oriented to the start codon of predicted plasmid replication genes. Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline. A total of 3,122 chromosomal genes were identified, of which 3,040 were coding sequences (CDS), 109 were pseudogenes, 19 were rRNA genes, and 59 were tRNA genes.

Data availability. This whole-genome sequencing project has been deposited in GenBank under the accession numbers CP029166, CP029167, and CP029165 for the genome and plasmids pSVH7513a and pSVH7513b, respectively.

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REFERENCES

1. Oosthuysen WF, Orth H, Lombard CJ, Sinha B, Wasserman E. 2014. Population structure analyses of *Staphylococcus aureus* at Tygerberg Hospital, South Africa, reveals a diverse population, a high prevalence of Panton–Valentine leukocidin genes, and unique local methicillin-resistant *S. aureus* clones. Clin Microbiol Infect 20:652–659. https://doi.org/10.1111/1469-0691.12452.
2. Perovic O, Iyaloo S, Kularatne R, Lowman W, Bosman N, Wadula J, Seetharam S, Duse A, Mbelle N, Bamford C, Dawood H, Mahabeer Y, Bhola P, Abrahams S, Singh-Moodley A. 2015. Prevalence and trends of *Staphylococcus aureus* bacteraemia in hospitalized patients in South Africa, 2010 to 2012: laboratory-based surveillance mapping of antimicrobial resistance and molecular epidemiology. PLoS One 10:e0145429. https://doi.org/10.1371/journal.pone.0145429.
3. Smyth DS, Feil EJ, Meaney WJ, Hartigan PJ, Tollersrud T, Fitzgerald JR, Enright MC, Smyth CJ. 2009. Molecular genetic typing reveals further insights into the diversity of animal-associated *Staphylococcus aureus*. J Med Microbiol 58:1343–1353. https://doi.org/10.1099/jmm.0.009837-0.
4. Groves MD, Crouch B, Coombs GW, Jordan D, Pang S, Barton MD, Giffard P, Abraham S, Trott DJ. 2016. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* isolated from Australian veterinarians. PLoS One 11:e0146034. https://doi.org/10.1371/journal.pone.0146034.
5. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol 41:5113–5120. https://doi.org/10.1128/JCM.41.11.5113-5120.2003.
6. Planet PJ. 2017. Life after USA300: the rise and fall of a superbug. J Infect Dis 215:S71–S77. https://doi.org/10.1093/infdis/jiw444.

3.4.2 Publication: Multiple introductions of Methicillin-resistant *Staphylococcus aureus* ST612 into Western Australia associated with human and equine reservoirs

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Multiple introductions of methicillin-resistant *Staphylococcus aureus* ST612 into Western Australia associated both with human and equine reservoirs

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ABSTRACT

Staphylococcus aureus is a serious human and animal pathogen. Multilocus sequence type 612 (ST612) is the dominant methicillin-resistant *S. aureus* (MRSA) clone in certain South African hospitals and is sporadically isolated from horses and horse-associated veterinarians in Australia. Colonisation and infection by ST612-MRSA is increasing in Western Australia. Whole-genome sequencing was performed for 51 isolates of ST612-MRSA from Western Australian patients and healthcare workers, South African hospital patients, Australian veterinarians and New South Wales horses. Core genome phylogenies suggested that Australian equine and veterinarian-associated ST612-MRSA were monophyletic. Individual Western Australian isolates grouped either with this equine-associated lineage or more diverse lineages related to those in South African hospitals. Bioinformatic analyses of the complete ST612-MRSA reference genome SVH7513 confirmed that ST612-MRSA was closely related to ST8 USA500 MRSA. Common use of rifampicin in South Africa and equine veterinarian practice may favour ST612-MRSA in these settings. Humans and horses colonised with ST612-MRSA are potential reservoirs for MRSA in Australia.

1. Introduction

Staphylococcus aureus is a versatile and virulent opportunistic pathogen of humans and animals [1]. The organism is increasingly resistant to multiple antimicrobials, which has led to reduced therapeutic options and increased morbidity and mortality. The emergence of methicillin-resistant *S. aureus* (MRSA) within hospitals has prompted increased surveillance and infection control measures. Since the 1990s, MRSA has also been increasingly associated with infections acquired outside of the hospital environment, referred to as community-associated MRSA (CA-MRSA). Although MRSA is associated with human hosts, MRSA carriage, infection and transmission is also observed in domestic animals such as cats, dogs, horses and livestock, e.g. pigs and cows. As such, animals may also act as MRSA reservoirs and may account in part for the rise of CA-MRSA [2].

Expansion of the MRSA host range can be attributed to the acquisition of host-specific virulence and colonisation factors carried by a variety of mobile genetic elements [3]. Multilocus sequence type 612 (ST612) is a member of clonal complex 8 (CC8), which includes the dominant CA-MRSA USA300 and the closely related USA500 lineage [4]. Although ST612-MRSA is frequently identified in South Africa [5], where it is over-represented in bacteraemia cases [6], it is not a frequently reported cause of human infections elsewhere. In Australia, ST612-MRSA has been isolated from veterinarians and from New South Wales (NSW) horses [7]. Furthermore ST612-MRSA is also increasingly detected in human patients living in Western Australia (WA), with at least one case leading to seri-

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ous bacteraemia [8]. In brief, ST612-MRSA was cultured from the nasal swab of WA Patient 9. The isolate was resistant to trimethoprim/sulfamethoxazole (co-trimoxazole), rifampicin, erythromycin and doxycycline. Within a year, the partner of WA Patient 9, called WA Patient 1, had bacteraemia with ST612-MRSA. The couple had a close association with horses. Here we collected and analysed the genome sequences of human-associated ST612-MRSA from WA, NSW, South Australia and South Africa, along with horse-associated ST612-MRSA from NSW, in an attempt to identify potential origins of ST612-MRSA in WA.

2. Materials and methods

2.1. Isolates

The ST612-MRSA isolates ($n = 51$) used in this study included: isolates from patients ($n = 10$) and healthcare workers ($n = 3$) living in WA; patient isolates from Tygerberg Hospital (Cape Town, South Africa) ($n = 8$); isolates from the Scone Veterinary Hospital (Scone, NSW, Australia) isolated from horses ($n = 24$) and veterinarians ($n = 3$) [7]; and isolates from South Australian veterinarians attending a series of Australian veterinary conferences in 2009 ($n = 3$) (previously described [9]). Isolate details are provided in Supplementary Table S1.

2.2. Genome sequencing and assembly

All isolates were grown in overnight cultures of tryptic soy broth with shaking at 37 °C. Whole DNA extraction was performed as described previously [10]. Genomic DNA libraries were prepared using a Nextera XT Library Prep Kit (Illumina Inc., San Diego, CA, USA) and were sequenced on an Illumina MiSeq platform (Illumina Inc.). Reads were cleaned, assembled and annotated using the nullarbor bioinformatic pipeline software package (github.com/tseemann/nullarbor). Isolate SVH7513 from the Scone Veterinary Hospital collection was sequenced using long-read SMRT (single molecule, real-time) cell sequencing to produce a finished high-quality reference genome as previously described [10]. Genome sequence assemblies have been deposited in GenBank under BioProject accession **PRJNA558684**.

2.3. Typing and identification of mobile genetic elements

Multilocus sequence typing (MLST) was performed with MLST v.2.10 (github.com/tseemann/mlst). Staphylococcal protein A (*spa*) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing were performed in silico using spaTyper 1.0 (https://cge.cbs.dtu.dk/services/spatyper) and SCCmecFinder (https://cge.cbs.dtu.dk/services/SCCmecFinder/) software, respectively. Plasmids were detected using PlasFlow [11]. Antimicrobial resistance and virulence genes were detected using abricate v.0.8 (github.com/tseemann/abricate) to query the ResFinder database (https://bitbucket.org/genomicepidemiology/resfinder_db.git) and Virulence Factor Database (VFDB), respectively. *Staphylococcus aureus* pathogenicity island (SaPI) elements were identified manually with BLASTn by searching genomes for previously described *att* sites [12], whilst comparisons of SaPIs were made with a BLASTn library of SaPI elements retrieved from GenBank (Supplementary Table S2) and visualised in UGENE v.1.3.

2.4. Comparison of genomes and phylogenetics

The reference ST612-MRSA genome SVH7513 (**CP029166.1**) was aligned with USA500 (**CP007499**) using BRIG v.0.95 [13] as were their associated plasmids pSVH7513a (**CP029167.1**) and pUSA500 (**CP007500.1**), respectively. Core genome alignments were produced using Snippy v.3.2 (github.com/tseemann/snippy). Approximately maximum-likelihood phylogenetic trees were computed using FastTree v.2.1.10 [14] with the generalised-time reversible substitution model. Trees were visualised with Interactive Tree of Life (iTOL) v.3 (https://itol.embl.de/).

3. Results

3.1. Genome composition of the ST612-MRSA reference genome SVH7513

SVH7513, isolated from an Australian horse in 2008, was selected as a reference genome. Initial queries of the National Center for Biotechnology Information (NCBI) database confirmed that SVH7513 was closely related to the ST8 strain USA500. The closest related USA500 strains were from CC8-USA500 clade I, the same clade as the USA500 clinical isolate reference genome USA500 2395 [15,16]. Whole-genome comparisons revealed that SVH7513 and USA500 2395 (**CP007499**) shared 99% identity and >97% coverage of the genome. USA500 is a highly virulent hospital and CA-MRSA strain whose virulence has been attributed to the modulation of virulence gene expression through the acquisition of insertion sequence (IS) elements within virulence-associated gene-regulatory regions. Sixteen copies of IS*256* are found throughout the USA500 chromosome, whilst fourteen copies were found in the SVH7513 chromosome. Seven copies of IS*256* were located in identical positions to those in USA500 2395 (Supplementary Table S3), including a copy located upstream of the virulence-associated fibrinogen-binding gene *sdrD* and one copy interrupting the 'repressor of toxins' gene *rot*, to which to the enhanced virulence of USA500 is attributed [16]. Of the seven unique IS*256* disruptions in SVH7513, five were within intergenic regions, whilst two occurred within coding sequences for predicted genes with no homologues (Supplementary Table S3).

Sequence queries of SVH7513 and USA500 2395 genomes with the VFDB revealed both lineages share the same number of known virulence-associated genes ($n = 68$), including leukotoxin *lukEv–lukDv* and cytotoxin genes (*hla, hld* and *hlgABC*). Like USA500, SVH7513 carried a type IVd SCC*mec* element and a 27 887-bp multiresistance plasmid named pSVH7513a (**CP029167**), which was 99% identical (98% coverage) to pUSA500. pSVH7513a carried resistance genes for trimethoprim (*dfrC*), cadmium (*cadC*), aminoglycosides (*aac6–aph2*) and β-lactams (*blaZ*). In addition, SVH7513 carried a small 2496-bp plasmid, named pSVH7513b (**CP029165.1**), which encodes the inducible erythromycin resistance gene *ermC* and the *repL*-family rolling circle replication initiation gene. USA500 2395 and SVH7513 shared several prophage and pathogenicity islands, including *hlb*-converting prophage φSA3, which carries the human immune evasion genes *sak* and *scn* and enterotoxin *sea*, and the staphylococcal pathogenicity island SaPI3 encoding enterotoxin genes *seb, selk* and *selq*. The SVH7513 genome was 40.3 kb smaller than the USA500 2395 genome, primarily due to variations within SaPI elements and prophage φSA2 and the absence of prophage φSA7. SVH7513 contained a previously unidentified SaPI, named here SaPIsvh7513 (Fig. 1).

3.2. Sequencing and whole-genome comparisons of ST612-MRSA isolated from humans and horses in Australia and from humans in South Africa

To determine the origin of ST612-MRSA in WA, ST612-MRSA ($n = 51$) genomes were sequenced with an average 56-fold depth of coverage. The majority of isolates (42/51; 82%) were *spa* type t064. The remaining isolates were t1257 (6/51; 12%), t723 (2/51;

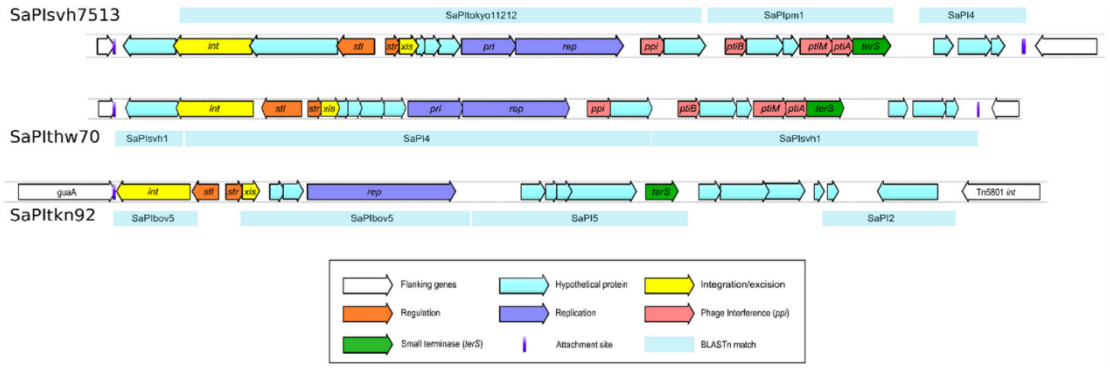

Fig. 1. Unique *Staphylococcus aureus* pathogenicity island (SaPI) elements found in ST612-MRSA genomes. BLASTn matches represent regions of >95% nucleotide identity to the indicated SaPI elements. SaPIsvh7513 and SaPIthw70 both integrate into the 30S ribosomal S18 gene, whilst SaPItkn92 has displaced Tn*5801* at the *guaA* GMP synthase gene site. Distribution of these SaPI among ST612-MRSA isolates is indicated in Fig. 2. ST, sequence type; MRSA, methicillin-resistant *S. aureus*.

4%) and a single t7571, all of which are closely related to t064, containing either insertions, rearrangements or deletions of *spa* repeat sequences. Overall, there was little variation in the core genome between ST612-MRSA genomes. All isolates harboured SCC*mec* IVd, *lukEv–lukDv* and carried a tetracycline resistance gene (*tetM*) on an integrative and conjugative element (ICE) related to Tn*5801*, integrated at the 3′ end of the GMP synthase gene *guaA*. The isolates shared identical RNA polymerase subunit B (*rpoB*) genes conferring rifampicin resistance (His481→Asn, Ile527→Met). All isolates carried pSVH7513a (described above), whilst the erythromycin resistance plasmid pSVH7513b was sporadically present in 59% of isolates (30/51). The β-lactamase gene *blaZ* was present in all isolates and was disrupted by an IS*256* insertion in all but three isolates (NSW Horse 18 and 21, and South African Patient 4). Some resistance genes were present only sporadically, such as the macrolide–streptogramin resistance gene *msrA* ($n = 3$), the quaternary ammonium disinfectant resistance gene *qacB* ($n = 1$), and the chloramphenicol (*catA7*) and streptomycin (*str*) resistance genes ($n = 1$). In two isolates, loss of prophage φSA3 resulted in restoration of the β-haemolysin gene *hlb*. Two unique SaPIs were identified among the South African isolates, named SaPItkn92 and SaPIthw70 (Fig. 1). Differences in virulence-associated and antimicrobial resistance genes are depicted in Fig. 2.

3.3. Equine-associated ST612-MRSA form a distinct clade and both human and equine-associated lineages are possible sources of ST612-MRSA in Western Australia

A maximum-likelihood phylogenetic tree was constructed using the core genomes of the 51 ST612-MRSA isolates and represented a total of 3988 single nucleotide polymorphisms (SNPs) across a core genome of 2 915 384-bp. All horse and veterinarian-isolated ST612-MRSA grouped within a single clade together with several WA patient isolates (Fig. 2). The greatest SNP difference was between WA Patient 2 and South African Patient 3 (134 bp), whilst the minimum number of SNP differences were between NSW Horses 16, 18 and 22 (0 bp) and NSW Horses 1 and 15 (0 bp). Five of the WA human isolates grouped with the equine-associated clade, whilst the remaining eight clustered with various more diverse South African ST612-MRSA isolates.

4. Discussion

This study aimed to identify the origins of ST612-MRSA in WA. Characterisation of ST612-MRSA was performed by comparison with a high-quality ST612-MRSA genome and a closely-related and well-characterised USA500 MRSA genome (**CP007499**) [16]. Except for variation in prophages and SaPIs, ST612-MRSA SVH7513 shared many mobile genetic elements and an identical suite of characterised virulence-associated genes.

'Hypervirulence' of USA500 has been attributed to the increased expression of virulence genes mediated by chromosomal insertions of IS*256*. Many IS*256* sites of USA500 were shared with SVH7513, including a copy inserted into the 'repressor of toxins' gene *rot*. Insertion of IS*256* in *rot* causes increased toxin production, resulting in enhanced spleen colonisation and survival in the presence of neutrophils [16]. Disruption of *rot* suggests that SVH7513 may share a similar virulence gene expression profile as the hypervirulent USA500. Variations in IS*256* distribution amongst ST612-MRSA genomes, such as the absence of a disruptive IS*256* insertion in the *blaZ* gene of three isolates, as well as the different distributions of seven IS*256* in SVH7513 relative to USA500, demonstrates the mobility of IS*256* in these isolates.

The ST612-MRSA core genome phylogeny was consistent with multiple introductions of ST612-MRSA into WA. The phylogenetic tree formed a single low-diversity clade containing all horse and veterinarian-associated ST612-MRSA along with several WA ST612-MRSA isolated from humans. The remainder of Western Australian and South African ST612-MRSA were relatively more diverse, but groupings suggested multiple exchanges of distinct ST612-MRSA. WA has a significant South African population (1.7% of the WA population [17]), so the potential for direct introductions of ST612-MRSA from South Africa by travel is not unreasonable.

The monophyletic grouping of equine and veterinarian-isolated ST612-MRSA in this study is unsurprising, as previous studies have identified an increased risk of carriage of ST612-MRSA for equine veterinarians [9]. It is possible that this lineage of ST612-MRSA has adapted to persistently colonise horses and may therefore present a reservoir of MRSA for human infections. Indeed, the most serious case of ST612-MRSA infection in WA (WA Patient 1) occurred in a patient who had direct contact with horses, suggesting transmission of virulent ST612-MRSA to and from horses. However, we additionally sampled 39 horses on the property of WA Patient 1 by nasal swab in March 2016 in an attempt to isolate ST612-MRSA, but no MRSA were detected. Thus, the source may have been a different stable, introduced for example by personnel working between stables or following interstate travel.

The equine and veterinarian-associated ST612-MRSA in this study were isolated in 2008–2009, whilst the most recent and

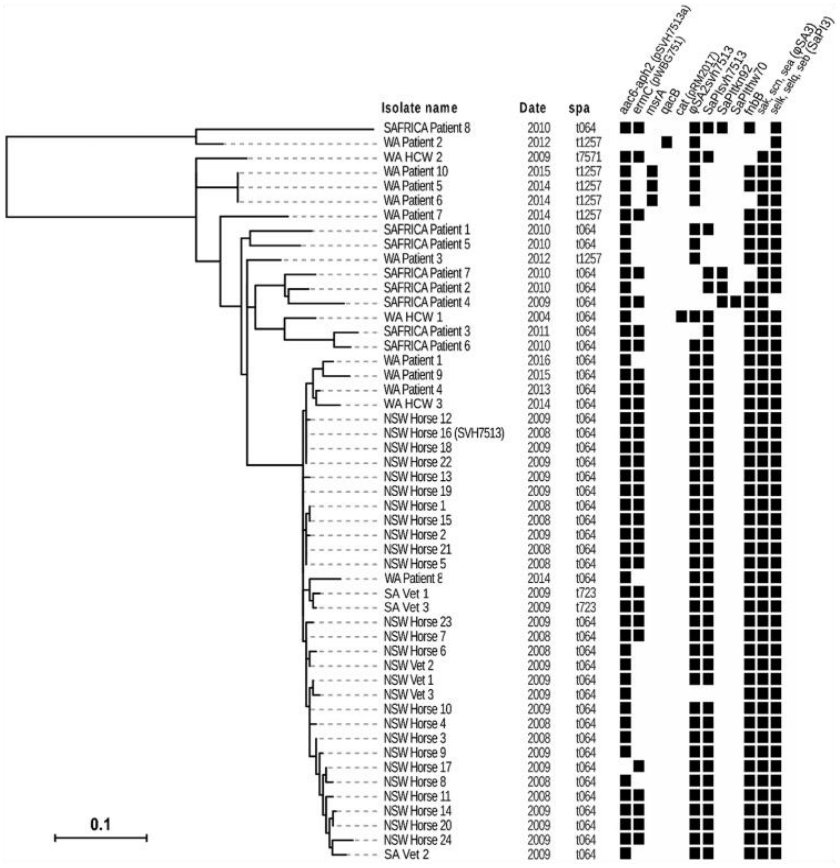

Fig. 2. Maximum-likelihood phylogenetic tree of core genomes from 43 Australian and 8 South African ST612-MRSA. Isolate names indicate origins of the represented genomes: SAFRICA, South Africa; WA, Western Australia; NSW, New South Wales; and SA, South Australia. Year of isolation (date) and staphylococcal protein A (*spa*) type are shown. The scale bar indicates average substitutions per site. Presence of resistance and virulence genes, prophage φSA3 and *S. aureus* pathogenicity island (SaPI) is indicated by filled squares. Names of associated mobile genetic elements are bracketed if known. ST, sequence type; MRSA, methicillin-resistant *S. aureus*; HCW, healthcare worker.

likely zoonotic infection from a member of this clade occurred in 2016, suggesting that ST612-MRSA has been circulating in Australian horse populations for at least 7 years with little genetic change occurring during that time. The presence of genetically close ST612-MRSA in horses and humans over a wide span of years and geographic range suggests that ST612-MRSA may have a combination of virulence factors and antimicrobial resistance determinants that allow it to persist in at least one of these hosts. The equine-adaptation phage ΦSaeq1 was not identified in any of the isolates here and therefore is not a requisite for colonisation or infection of horses. The presence of a φSA2-type prophage (φSA2svh7513) and SaPIsvh7513, all of which encode genes of unknown function within accessory gene regions, could contribute to equine host adaptation. The ability of ST612-MRSA to persist in South Africa and the Australian horse population, however, may be an unfortunate consequence of antimicrobial selection favouring ST612-MRSA. All ST612-MRSA in this study had an identical mutant *rpoB* gene providing constitutive rifampicin resistance and are consistent with those previously identified in ST612-MRSA from South Africa [5]. In addition, all ST612-MRSA carried the trimethoprim resistance plasmid pSVH7513a. Jansen van Rensburg et al. suggested that the prevalence of ST612-MRSA in South Africa could be the result of selection by the use of rifampicin and co-trimoxazole [18]. Rifampicin is used in South Africa for the treatment of tuberculosis [5], whilst co-trimoxazole is used prophylactically for the control of bacterial and *Pneumocystis* infections in human immunodeficiency virus (HIV)-infected patients. Rifampicin is uncommonly prescribed in Australia, with only 6020 prescriptions recorded in 2015 by the national Prescribing Benefit Scheme compared with 5.6 million prescriptions for the commonly prescribed antibiotic cefalexin [19]. However, Saputra et al. suggest that the prevalence of rifampicin-resistant ST612-MRSA in Australian horses is the result of the treatment of foals with rifampicin, in combination with macrolides, for the prevention of *Rhodococcus equi* infection [20]. Trimethoprim has been used orally in horses for the treatment of respiratory infections, which would further select for ST612-MRSA in Australian horses.

These findings support current local policy of MRSA screening at the time of hospital admission for patients recently treated in overseas health facilities, but a targeted campaign raising awareness among equine veterinarians may also be warranted. The zoonotic potential of ST612-MRSA also raises the question of whether the problem could be greater in nations with larger populations both of HIV infection and domesticated horses.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.08.022.

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Ethical approval: Not required.

References

[1] Walther B, Wieler LH, Friedrich AW, Hanssen AM, Kohn B, Brunnberg L, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from small and exotic animals at a university hospital during routine microbiological examinations. Vet Microbiol 2008;127:171–8.
[2] Cuny C, Wieler LH, Witte W. Livestock-associated MRSA: the impact on humans. Antibiotics (Basel) 2015;4:521–43.
[3] Richardson EJ, Bacigalupe R, Harrison EM, Weinert LA, Lycett S, Vrieling M, et al. Gene exchange drives the ecological success of a multi-host bacterial pathogen. Nat Ecol Evol 2018;2:1468–78.
[4] Li M, Diep BA, Villaruz AE, Braughton KR, Jiang X, DeLeo FR, et al. Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. Proc Natl Acad Sci U S A 2009;106:5883–8.
[5] Jansen van Rensburg MJ, Whitelaw AC, Elisha BG. Genetic basis of rifampicin resistance in methicillin-resistant *Staphylococcus aureus* suggests clonal expansion in hospitals in Cape Town, South Africa. BMC Microbiol 2012;12:46.
[6] Perovic O, Iyaloo S, Kularatne R, Lowman W, Bosman N, Wadula J, et al. Prevalence and trends of *Staphylococcus aureus* bacteraemia in hospitalized patients in South Africa, 2010 to 2012: laboratory-based surveillance mapping of antimicrobial resistance and molecular epidemiology. PLoS One 2015;10:e0145429.
[7] Axon JE, Carrick JB, Barton MD, Collins NM, Russell CM, Kiehne J, et al. Methicillin-resistant *Staphylococcus aureus* in a population of horses in Australia. Aust Vet J 2011;89:221–5.
[8] Raby E, Coombs GW, Pang S, Pearson J, Robinson OJ, Dyer J. Zoonotic transmission of the methicillin-resistant Staphylococcus aureus ST612-IV equine strain. Australian Society for Antimicrobials; 2016. Break Point Newsletter p. 4–6.
[9] Groves MD, Crouch B, Coombs GW, Jordan D, Pang S, Barton MD, et al. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* isolated from Australian veterinarians. PLoS One 2016;11:e0146034.
[10] Murphy RJT, Lee YT, Pang S, Bastholm TR, Crow JE, Davis AM, et al. Complete genome sequence of a *Staphylococcus aureus* sequence type 612 isolate from an Australian horse. Microbiol Resour Announc 2018;7 pii: e00869-18.
[11] Krawczyk PS, Lipinski L, Dziembowski A. PlasFlow: predicting plasmid sequences in metagenomic data using genome signatures. Nucleic Acids Res 2018;46:e35.
[12] Novick RP, Ram G. Staphylococcal pathogenicity islands—movers and shakers in the genomic firmament. Curr Opin Microbiol 2017;38:197–204.
[13] Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics 2011;12:402.
[14] Price MN, Dehal PS, Arkin AP. FastTree 2—approximately maximum-likelihood trees for large alignments. PLoS One 2010;5:e9490.
[15] Frisch MB, Castillo-Ramirez S, Petit RA 3rd, Farley MM, Ray SM, Albrecht VS, et al. Invasive methicillin-resistant *Staphylococcus aureus* USA500 strains from the U.S. Emerging Infections Program constitute three geographically distinct lineages. mSphere 2018;3 e00571-17.
[16] Benson MA, Ohneck EA, Ryan C, Alonzo F 3rd, Smith H, Narechania A, et al. Evolution of hypervirulence by a MRSA clone through acquisition of a transposable element. Mol Microbiol 2014;93:664–81.
[17] . *2016 Census of Population and Housing: Western Australia*. Australian Bureau of Statistics; 2016.
[18] Jansen van Rensburg MJ, Eliya Madikane V, Whitelaw A, Chachage M, Haffejee S, Gay Elisha B. The dominant methicillin-resistant *Staphylococcus aureus* clone from hospitals in Cape Town has an unusual genotype: ST612. Clin Microbiol Infect 2011;17:785–92.
[19] Mabbott V, Storey P. Australian statistics on medicines. Australian Government, Department of Health, Pharmaceutical Benefits Advisory Committee; 2015.
[20] Saputra S, Jordan D, Worthing KA, Norris JM, Wong HS, Abraham R, et al. Antimicrobial resistance in coagulase-positive staphylococci isolated from companion animals in Australia: a one year study. PLoS One 2017;12:e0176379.

3.5

3.6 Supplementary materials

Murphy, Riley; Ramsay, Josh; Lee, Y.; Pang, S.; O'Dea, M.; Pearson, J.; Axon, J.; Raby, E.; Abdulgader, S.; Whitelaw, A.; Coombs, G. (2019) Multiple introductions of methicillin-resistant *Staphylococcus aureus* ST612 into Western Australia associated with both human and equine reservoirs.

Technical appendix – Table of isolates and SVH7513 *IS256* genomic locations.

Table S1. Isolates analysed in this study

Study ID	Original Isolate ID	Year Isolated	Source[1]	Reference
NSW Horse 1		2008	Scone Vet Hospital	(329)
NSW Vet 1		2009	Scone Vet Hospital	(329)
NSW Horse 2		2009	Scone Vet Hospital	(329)
NSW Vet 2		2009	Scone Vet Hospital	(329)
NSW Horse 3		2008	Scone Vet Hospital	(329)
NSW Vet 3		2009	Scone Vet Hospital	(329)
NSW Horse 4		2008	Scone Vet Hospital	(329)
NSW Horse 5		2008	Scone Vet Hospital	(329)
NSW Horse 6		2008	Scone Vet Hospital	(329)
NSW Horse 7		2008	Scone Vet Hospital	(329)
NSW Horse 8		2008	Scone Vet Hospital	(329)
NSW Horse 9		2009	Scone Vet Hospital	(329)
NSW Horse 10		2009	Scone Vet Hospital	(329)
NSW Horse 11		2008	Scone Vet Hospital	(329)
NSW Horse 12		2009	Scone Vet Hospital	(329)
NSW Horse 13		2009	Scone Vet Hospital	(329)
NSW Horse 14		2009	Scone Vet Hospital	(329)
NSW Horse 15		2008	Scone Vet Hospital	(329)
NSW Horse 16	SVH7513	2008	Scone Vet Hospital	(329, 424)
NSW Horse 17		2009	Scone Vet Hospital	(329)
NSW Horse 18		2009	Scone Vet Hospital	(329)
NSW Horse 19		2009	Scone Vet Hospital	(329)
NSW Horse 20		2009	Scone Vet Hospital	(329)
NSW Horse 21		2008	Scone Vet Hospital	(329)
NSW Horse 22		2009	Scone Vet Hospital	(329)
NSW Horse 23		2009	Scone Vet Hospital	(329)
NSW Horse 24		2009	Scone Vet Hospital	(329)
WA HCW 1	17052	2004	WA Hospital 1	(425)

WA HCW 2	15534	2009	WA Hospital 2	This study
WA Patient 2	18541	2012	WAC	This study
WA Patient 3	18863	2012	WA Hospital 2	This study
WA Patient 4	21557	2013	WA Hospital 3	This study
WA Patient 5	15042	2014	WAC	This study
WA Patient 6	16184	2014	WAC	This study
WA Patient 7	16861	2014	WA Hospital 1	This study
WA Patient 8	17563	2014	WA Hospital 3	This study
WA HCW 3	21818	2014	WA Hospital 1	This study
WA Patient 9	16198	2015	WA Hospital 3	This study
WA Patient 10	20160	2015	WA Hospital 4	This study
SA Vet 1	778	2009	Equine Vet	(362)
SA Vet 2	570	2009	Mixed Animal Vet	(326, 362)
SA Vet 3	723	2009	Mixed Animal Vet	(362)
WA Patient 1	15-24259	2016	WA Hospital 4	This study
SAFRICA Patient 1	TKN-52	2010	Tygerberg Hospital	This study
SAFRICA Patient 2	TKN-92	2010	Tygerberg Hospital	This study
SAFRICA Patient 3	TKN-119	2011	Tygerberg Hospital	This study
SAFRICA Patient 4	THW-70	2009	Tygerberg Hospital	(426)
SAFRICA Patient 5	THW-93	2010	Tygerberg Hospital	(426)
SAFRICA Patient 6	THW-263	2010	Tygerberg Hospital	This study
SAFRICA Patient 7	THW-371	2010	Tygerberg Hospital	This study
SAFRICA Patient 8	THW-380	2010	Tygerberg Hospital	This study
USA500	2395	1996	New York-Presbyterian Hospital	(318)

[1] Source abbreviations: WAC = Western Australian community pathology submission, NSW = New South Wales, Vet = Veterinarian, WA = Western Australian, HCW = Healthcare Worker, SA = South Australia, SAFRICA = South Africa

Table S2. IS*256* locations and genomic context within the *S. aureus* SVH7513 genome

IS256	Start	End	Length (bp)	Intergenic	Nearest gene	Protein ID	Distance† (bp)
1	35588*	36760	1173	Yes	DUF3578 Domain-containing protein	AWI92111.1	357
2	79406	80578	1173	Yes	LysR family transcriptional regulator	AWI92152.1	297
3	147224*	148396	1173	No	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	AWI92213.1	1735
4	483767*	484957	1191	Yes	ATP-dependent endonuclease	AWI92512.1	133
5	664281	665453	1173	Yes	SdrC	AWI92667.1	268
6	712133*	713305	1173	No	DUF443 domain-containing protein	AWI92715.1	0
7	1380910*	1382082	1173	Yes	Hypothetical protein	AWI93379.1	766
8	1925745*	1926917	1173	Yes	Rot (−317) and alpha/beta hydrolase	AWI93897.1	1321
9	1970035	1971207	1173	No	Hypothetical NTPase	AWI93941.1	48
10	2023782	2024954	1173	Yes	Hypothetical protein	AWI93995.1	179
11	2388545*	2389717	1173	No	ClpA (+399) ~ inverted orientation, slightly interrupting start of hypothetical protein DD555 12585		193
12	2626529	2627701	1173	Yes	Putative ABC transporter, ATP-binding protein	AWI94616.1	120
13	2704773	2705945	1173	No	Disrupts putative peptide ABC transporter permease	AWI94686.1	0
14	2859366	2860538	1173	Yes	Between Serine-rich glycoprotein adhesin and putative flavin reductase	AWI94821.1	388

* IS elements in identical genetic context as USA500

† Distance to stop or start codon of nearest gene

Table S3. List of Staphylococcal Pathogenicity Islands (SaPI) used to construct a nucleotide BLAST database for the identification and comparison of SaPI found in ST612 MRSA.

SaPI Name	Isolate	GenBank Accession
SaPIpm1	PM1	AB690438
SaPIivm10	IVM10	AB716349
SaPIj11	J11	AB704541
SaPIbov4	BA4	HM211303
SaPITokyo11212	Tokyo11212	AB860416
SaPIhhms2	HHMS2	AB704540
SaPIivm60	IVM60	AB704539
SaPI1	RN4282	U93688
SaPI2	RN3984	EF010993
SaPI2R	OC3	AB983199
SaPI3	COL	AF410775
SaPI4	MRSA252	BX571856
SaPI5	USA300_FPR3757	NC_007793
SaPIbov	RF122	AF217235
SaPIbov2	V329	AY220730
SaPIbov5	JP5338	HM228919
SaPIeq1	DL584	HM228920
SaPImw2	MW2	BA000033

3.7 Chapter summary and conclusions

This chapter examined the potential origins of ST612 MRSA in WA and resulted in the assembly of a high-quality reference genome sequence for the future study of ST612. While the history of ST612 MRSA in Australia has been relatively benign to date and the total number of ST612 MRSA infections in WA has remained consistently low, the first incidence of a systemic ST612 MRSA infection warranted an investigation. ST612 MRSA is endemic in South Africa, where it is responsible for a high rate of bacteraemia. WA has a relatively large population of expatriate South Africans and travel between these locations invites the opportunity for the importation of a potentially more virulent ST612 MRSA into WA. As no genomes of ST612 were publicly available at the time, all ST612 MRSA had to be sourced and sequenced for the study to determine if the ST612 present in Australia was an indigenous strain (introduced with, and adapted to, horses for example) or whether they arrived from South Africa.

Analyses of the ST612 MRSA concluded domestic and international ST612 MRSA were distinguishable using core-genome phylogeny and ST612 MRSA detected in WA likely arrived from a monophyletic horse-associated clade present in Australia and through multiple introductions of more diverse ST612 related to those isolated in South Africa. The bloodstream infection which prompted this investigation appeared to originate from the domestic, horse-associated clade of ST612 MRSA. This suggested the horse-associated Australian clade had retained virulence against humans. The human virulence of the domestic ST612 MRSA clade was supported by the presence of the human immune evasion cluster, leucocidins *lukEv-Dv* and a similar distribution of IS*256* sites to USA500 2395, an isolate identified as 'hyper virulent' in part because of these insertions. This raised the possibility that there was a human-virulent, horse-adapted strain of MRSA colonising Australian horses. Additionally, their phylogeny suggests that ST612 MRSA can be transmitted between humans, as demonstrated by WA Patients 10, 5 and 6 who share an identical core-SNP genome and are likely clonal isolates.

Three novel SaPI and a novel phage were identified in the study, none of which appeared to carry any characterised virulence factors, however, their prevalence in the equine ST612 isolates suggests some biological significance in horses. Further studies are required to determine whether genes present in these SaPI or phage encode toxins, adhesins or other host-specificity factors.

All ST612 MRSA shared a rifampicin resistance gene *rpoB*: $H_{481}N$, $I_{527}M$, and carried a plasmid encoding trimethoprim resistance. It seems possible that this particular resistance profile may be responsible for the persistence of ST612 MRSA both in South Africa and in Australian horses. Rifampicin and co-trimoxazole are used in South Africa for the treatment of tuberculosis and in the prophylactic control of infections in human immunodeficiency virus (HIV)-infected patients, respectively. In Australia these drugs are relatively uncommonly prescribed in humans, however, they are commonly used in horses. Foals are treated with rifampicin, in combination with macrolides, for the prevention of *Rhodococcus equi* infection while trimethoprim has been used orally in horses for the treatment of respiratory infections. The work highlights the need for the prudent use of antimicrobials in animals and the careful prevention of microbial transmission from animals to humans.

Chapter 4

Comparative genomics of livestock-associated MRSA isolated from Australian pigs

4.1 Introduction

As described in section 1.10.5, LA-MRSA is present in Australia and is frequently associated with pig production. A 2017 study by Sahibzada *et al* (366) identified ST398 and ST93, a LA-MRSA and a common Australian CA-MRSA, at two farm sites of a commercial pig farm at high frequency. In this chapter I performed a survey of the genomic features of ST398 (n=26) and ST93 MRSA (n=45) from the five sites to identify AMR genes and their potential transmission between human-associated and animal-associated MRSA. Additionally, there was concern that there had been both zoonotic and anthropozoonotic transmission of ST398 and ST93 between humans and pigs. I examined the potential exchange of AMR between the human-associated ST93 and pig-associated ST398 isolates, as a consequence of their proximity in the farm environment. The isolates studied in this section are described in section 2.1.1 and have been deposited in GenBank under BioProject accession PRJNA560124.

4.2 Project aims

The work aimed to identify instances of potential AMR transfer between ST93 and ST398 MRSA lineages by

- Producing a high-quality, closed Australian ST398 reference genome by PacBio SMRT Long-Read sequencing;
- Analysing whole-genome sequences of Australian pig and human-associated MRSA genomes;
- Identifying MGEs and their distribution among the study isolates to infer strain origin and support potential MGE transfer events that have occurred between human-associated and LA-MRSA.

4.3 Results

4.3.1 Whole-genome sequence of PTDrAP2

For characterisation of the isolate and for downstream phylogenetic analysis of other Australian ST398 MRSA, I prepared a high quality reference genome of a selected ST398 MRSA from the study. The selection of an appropriate ST398 MRSA for the production of a representative and informative reference genome was informed by phylogeny and by the carriage of an alternative *cfr* allele (discussed further in Chapter 6), as detected previously by Sahibzada *et al* (366) by microarray. The isolate, PTDrAP2 (Figure 4-1) was selected and the genome was sequenced with Pacific Biosciences (PacBio) RSII sequencing technology, using one single-molecule real-time cell, in addition to Illumina MiSeq sequencing. PacBio sequencing produced subreads of a total 1,709,465,808 bases and produced a genome of 2,902,681 bp, with an estimated average 588 fold genome read coverage. The completed, high quality reference genome and plasmids of Australian ST398 MRSA PTDrAP2 were deposited in GenBank under accession under Assembly number GCF_003111745.1.

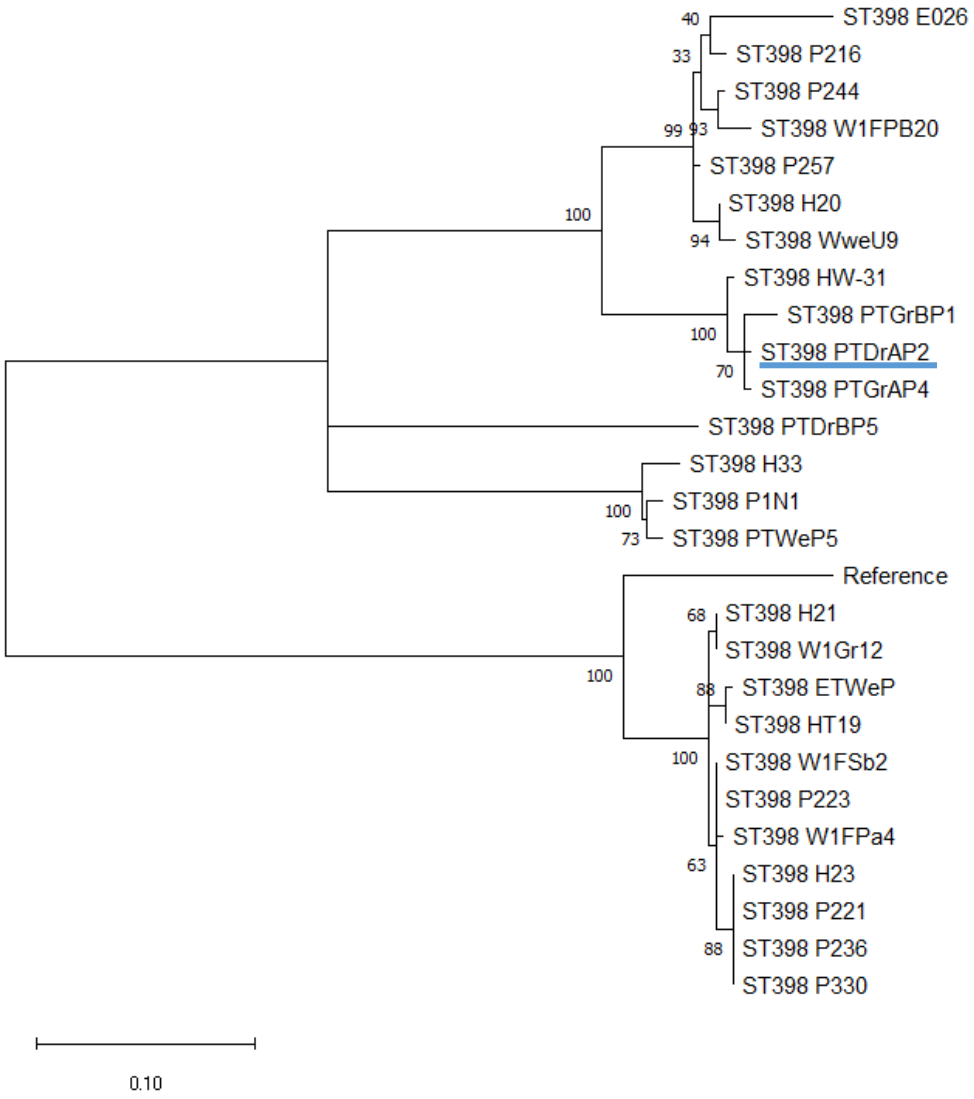


Figure 4-1 - Australian ST398 MRSA maximum-likelihood phylogenetic tree.

The unrooted maximum-likelihood 258 bp core-SNP genome tree was computed with 100 bootstrap replicates and the bootstrap values are overlaid on the tree nodes. The reference genome is ST398 MRSA (GenBank: AM990992.1). The ST398 strain selected for sequencing is underlined in blue. The scale bar represents SNP substitutions per site. The *cfrAB* strains PTDrAP2 and PTGrAP4 are closely related, with only 2 SNP differences in their core-SNP genomes across 258 sites.

4.3.2 Resistome of ST398 PTDrAP2

The resistance genes of PTDrAP2 were detected using abricate (v 0.4) together with the ResFinder database. Genes conferring resistance to the beta-lactams and daptomycin (*blaZ*, *sav1866*), lincosamide (*lnuB*), streptogramin A/virginiamycin (used in treatment of VRSA - v*gaA*), tetracycline (*tet38*, *tetK* and *tetM*), aminoglycosides (*ant6-Ia*), macrolides (*ermC*), trimethoprim (*dfrG*), phenicols (*fexA*) and fluoroquinolones (*norA*), oxazolidinones (*cfr*) were identified (Table 4-1). AMR phenotyping was performed by Sahibzada et al (366). Some AMR genes which were detected did not produce the expected AMR phenotype – for example, *ant6-Ia* did not produce an aminoglycoside resistance phenotype. The chromosome of ST398 MRSA genome S0385 (GenBank: AM990992.1) was used as a reference sequence for WGS alignments, as it was the first sequenced LA-MRSA (402). The S0385 genome encodes genes conferring resistance to methicillin, penicillins, tetracyclines and streptomycin and overall the PTDrAP2 genome encodes eleven additional resistance genes compared with S0385. The presence of so many distinct AMR determinants in PTDrAP2 suggested potential gene acquisition via HGT. Figure 4-2 provides a visual overview of the chromosomally integrated MGE of ST398 MRSA PTDrAP2.

Table 4-1 - Molecular characteristics of LA-MRSA PTDrAP2

Isolate	Type	Origin	AMR genes[a]	Phenotypic AMR[b]
PTDrAP2	ST398-V	Australian pig	blaZ, sav1866, lsaE, norA, vgaA, ermC, lnuB, cfr, fexA, tet38, tetK, tetM, dfrG, aadE, ant6-la, cfr, mecA	BLA, TET, ERY, CHL, CLI, QDA

[a]Several of these genes correspond to efflux pumps which are not may not reliably confer a biologically significant AMR phenotype, but which contribute to increasing antimicrobial MIC

[B]BLA – β-lactams, TET – tetracycline, ERY – erythromycin, CHL – chloramphenicol, QDA – quinupristin-dalfopristin.

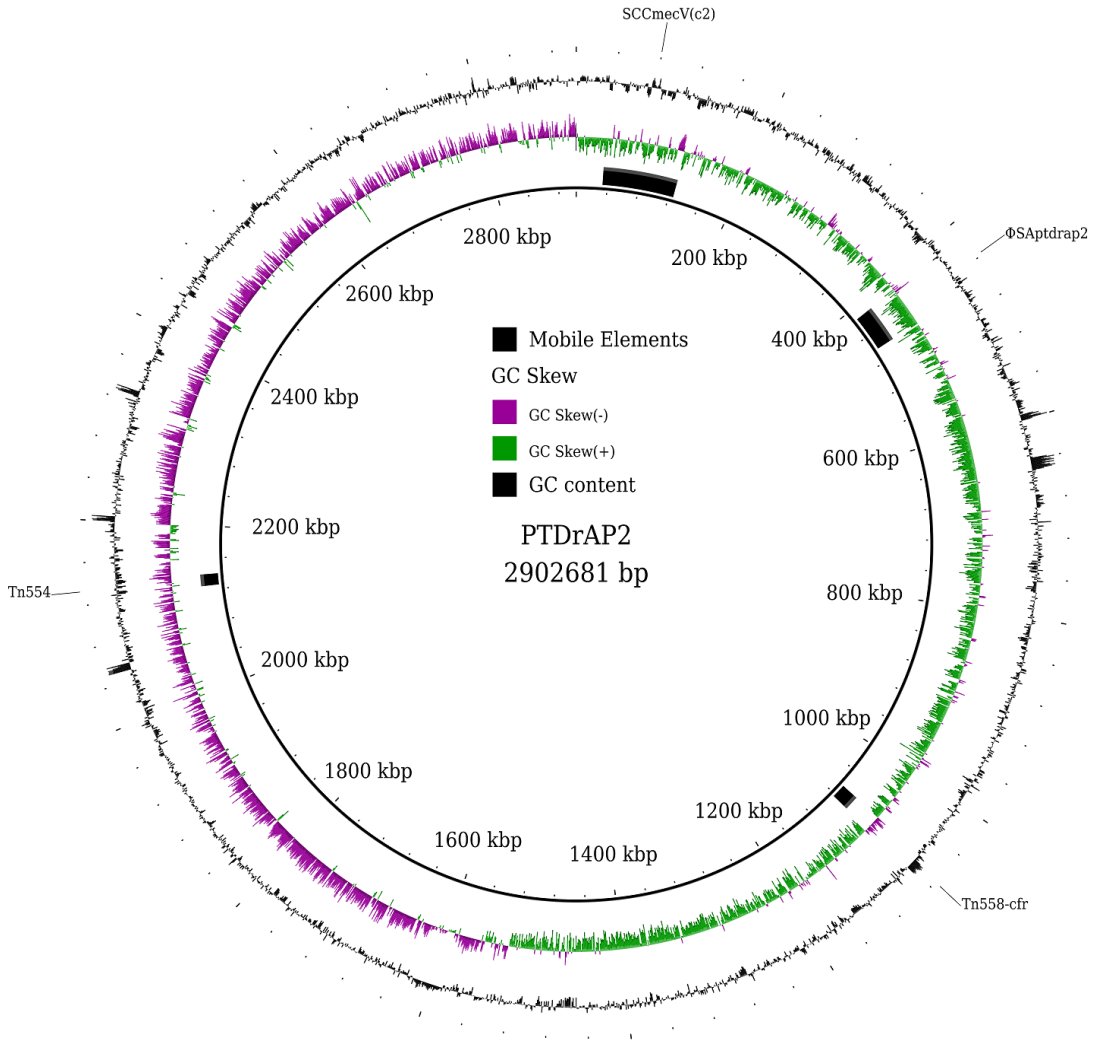


Figure 4-2 - Genomic features of ST398 MRSA PTDrAP2

The 2.9 mb genome of PTDrAP2 features several integrated MGE, including a very large type V SCC*mec* element, prophage and transposons. The outer and middle ring represent GC content and GC skew respectively, while the inner ring represents the backbone. Image produced with BRIG.

4.3.3 SaPI and phage of ST398 PTDrAP2

A search was performed for chromosomally-integrated phage and SaPI elements, as they often carry virulence factors and surface-adhesion molecules involved in host adhesion and host adaptation (427). To identify phage and SaPI, the PTDrAP2 genome was queried with PHAST (428). PHAST identified three prophage or phage-like elements. The first phage, phiSAptdrap2 (ΦSAptdrap2), was located at position 414,095 – 460,313 bp. The 46,218-bp phage was integrated within and disrupted the *geh* lipase gene and did not appear to contain any known virulence genes. phiSAptdrap2 was most similar to a phi3-type phage (GenBank: LT992460.1) described in a study of phage isolated from German LA-MRSA (429). No SaPI elements were identified and the examination of sites where SaPI typically integrate found them to be unoccupied.

4.3.4 Plasmids of ST398 PTDrAP2

Plasmids were identified using the MGE identification method described in Chapter 2. All plasmid contigs were found to be circular. PTDrAP2 carries four plasmids which include the 4.2 kb pE194-like plasmid carrying the *ermC* macrolide resistance gene; a 4.2 kb *repE E. faecium* plasmid, containing carbohydrate metabolism and rRNA L4 protein genes; a 3 kb *rep52*-type plasmid and 23s rRNA methyltransferase *rlmA*; a 2.6 kb rep52 plasmid, carrying *qacC* quaternary ammonia resistance. The plasmids were named pPTDrAP2a (GenBank: CP029168.1), pPTDrAP2b (GenBank: CP029169.1), pPTDrAP2c (GenBank: CP029170.1) and pPTDrAP2d (GenBank: CP029171.1) respectively. pPTDRaP2b may be a sequencing contaminant, as the sequence depth of coverage was low (6-fold) compared to the genome average (123-fold), and is identical to several *E. faecium* plasmids, for example pJS42 (Genbank: EU370688.1). Although the plasmid was not found in subsequent Illumina MiSeq resequencing, the data has been retained as it may have some biological significance.

4.3.5 SCCmec of ST398 PTDrAP2

The SCCmecFinder web tool was used for initial identification and characterisation of the SCC*mec* region in PTDrAP2, using the default search parameters, which identified a single *mecA* gene and a single SCC*mec* element with an 88% match to SCC*mec* Type Vc(5C2&5), [GenBank accession AB505629.1]. The *mecA* gene was identified as a class C2. Comparison of the SCC*mec* sequence with known subtypes revealed it to be closely related to SCC*mec* Vc(5c2&5) (Table 4-2). A comparison of the SCC*mec* Vc(5C2&5) reference sequence with the PTDrAP2 SCC*mec* revealed it contained an additional *ccrC1* allele.

Table 4-2 - SCCmecFinder output for PTDrAP2

SCC*mec* classification reference sequence [GenBank accession]	**Identity (%)**	**Length (bp)**	**Position in Contig**
mec-class-C2:5: [AB505629]	100.00	4408	44651..49058
subtype-Vc(5C2&5):10: [AB505629]	99.64	1935	69339..71273
ccrC1	92.90	1677	104512..106188
ccrC1-allele-8:1: [AB462393]	100.00	1677	40521..42197
ccrC1-allele-2:1: [AB512767]	98.45	1680	57131..58810

The SCC*mec* CcrC1 recombinase attachment (*att*) core sites (TATCATAA, (430)) were identified within a repeated sequence AGGCTTATCATAAAT flanking the SCC*mec* element. One *att* site was embedded in the 3' end of the *rlmH* gene, while the other *att* site was found adjacent to a hypothetical protein. Several additional mobile plasmids and transposon sequences were found integrated within the SCC*mec* element and were identified by a series of searches using the NCBI nucleotide and protein BLAST tool (431). The delineated SCC*mec* element of PTDrAP2 is a 92.2 kb composite element with highest similarity to a Type Vc element. The remainder of the element contains integrated plasmids and transposons carrying several resistance genes. An *hsdR* restriction-modification system was found

near *attR* of the SCC*mec*, a feature which has been identified in the type V SCC*mec* of an Australian ST45 MRSA (432). SCC*mec*$_{PTDrAP2}$ contains hypothetical proteins with predicted heavy metal resistance domains (Figure 4-3). An integrated pT181-like plasmid was identified downstream of the second *ccrC1* gene, harbouring *tetK* tetracycline resistance gene. Integrated copies of pT181 have been found in SCC*mec* types III and V previously (433), however, uniquely to SCC*mec*$_{PTDrAP2}$, a second integrated plasmid related to pE1774 (GenBank: LR135183.1), carrying *ant6-Ia* and *lnuH*-like genes, was located adjacent to pT181. A third *ccrC1*-like gene was found within a transposon related to Tn*54096* (GenBank: (AF186237.2, (434)) which carried the *vgaA* streptogramin A resistance gene. These integrated elements (Figure 4-3) extended the length of SCC*mec*$_{PTDrAP2}$ considerably and suggest a strong selective pressure to acquire AMR.

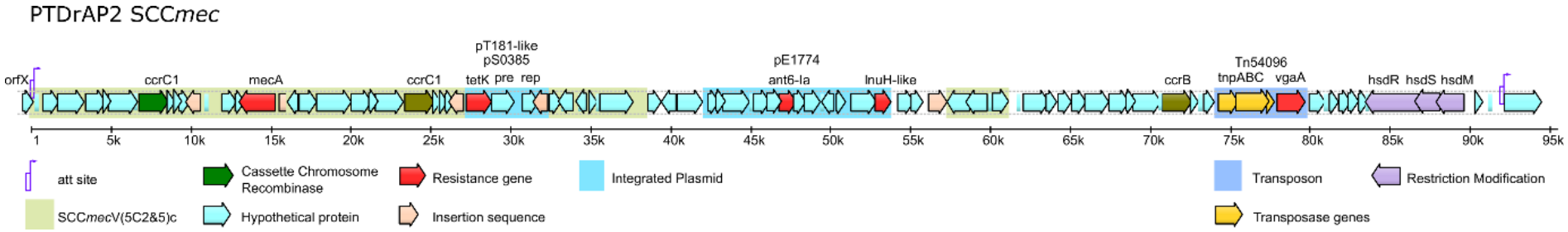


Figure 4-3 - SCC*mec* of ST398 MRSA PTDrAP2

Arrows represent predicted coding sequences. The key below indicates the predicted function of genes and regions of high nucleotide identity to SCCmec V (5C2&5)c (tan) and other MGE, with names of matching MGEs listed above the highlighted regions. The scale bar indicates the sequence length in base pairs. SCC*mec*$_{PTDrAP2}$ matches SCC*mec* V (5C2&5)c, but contains many additional integrated MGEs including tetracycline-, aminoglycoside- and lincomycin-resistance plasmids and a streptogramin-resistance transposon.

4.3.6 Transposons of ST398 PTDrAP2

The *tetM* tetracycline resistance gene of PTDrAP2 was found on a copy of the 18 kb Tn*916* transposon located 1,064 kb from the PTDrAP2 replication origin and was 99% identical to Tn*916* of *Bacillus subtilis* (GenBank: AF186237.2). The presence of the element was unsurprising, as *tetM* is a common resistance determinant in livestock-associated ST398, and because Tn*916* is extremely widespread in *S. aureus* and other gram-positive bacteria (435).

The *blaZ* beta-lactamase gene of PTDrAP2 was found on a 14-kb Tn*552*-like transposon, disruptively integrated into a YolD-like hypothetical protein (locus: SACOL1986). The element backbone sequence was almost identical to Tn*552* (99% identity, 63% coverage), except for the addition of four copies of IS*256*, which disrupted the *tnpC* gene. The *tnpC* gene is not necessary for the transposition of the element (436), suggesting the element may still be mobile. The presence of these four copies of IS*256* introduced several repeat sequences into the transposon, some of which may interfere with the function of the genes present due to their position, for example *tnpB* or *blaI* (Figure 4-4). The accumulation of IS*256* copies within the element suggested the IS elements may provide some advantage, perhaps affecting the regulation of gene expression, though it is uncertain if the IS*256* insertions have actually affected the function of the element in any way.

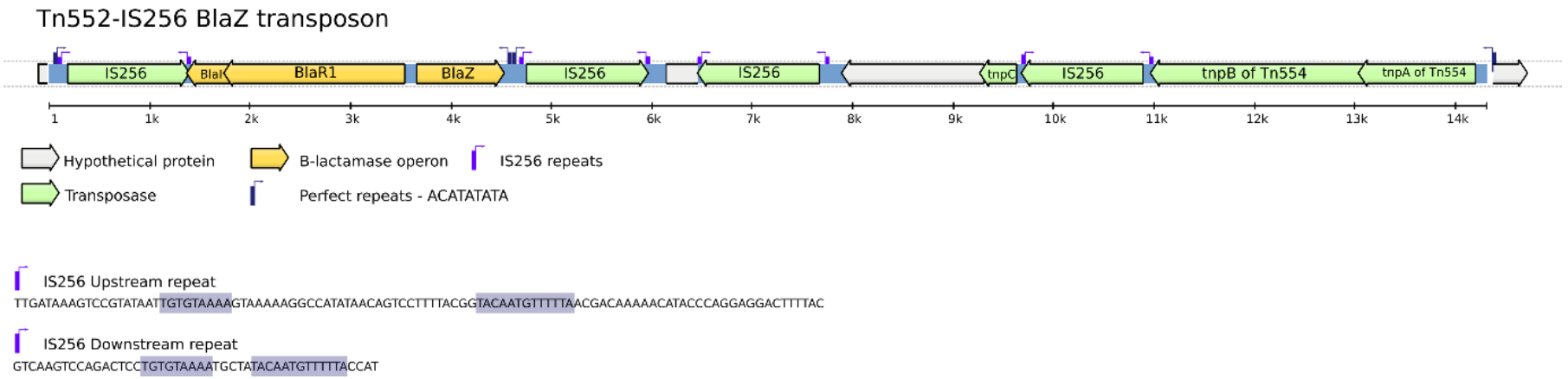


Figure 4-4 - IS*256*-interrupted Tn*552*-like blaZ element of PTDrAP2.

Arrows indicate coding sequences, coloured according to function as indicated in the legend (centre). Nucleotide sequence of repeats are indicated (bottom). Scale bar indicates sequence length in base pairs. Repeat sequences were identified using the Repeat Finder plugin in Ugene v34.0. Four copies of IS*256* interrupt the Tn*552* transposon.

4.3.7 Identification of novel, chromosomally-integrated *cfr* transposon

The linezolid resistance gene *cfr* was previously identified in Australian MRSA (366). Examination of the *cfr* gene in Australian ST398 MRSA isolate PTDrAP2 revealed it was carried by a chromosomally-integrated transposon. The element, Tn*558*-IS*256*-*cfrAB*, is 9,763 bp long and contains eight ORFs which include a hypothetical protein gene, *fexA* florfenicol resistance efflux pump, *orf138* putative oxidoreductase, *tnpC*, *cfr* chloramphenicol-florfenicol resistance gene, the transposase for IS*256, tnpB* (disrupted by IS*256*) and *tnpA* (Figure 4-5). The element is integrated adjacent to a conserved hypothetical protein (GenBank: WP_000171859.1) and into the end of the putative DNA repair protein gene, *radC*. The transposon *attL* site (GAGGGCGT TACATC TTATCAAG) and *attR* (ATGTTTAA TACATC ACCGGATG), each contained a Tn*554* att554 core sequence, TACATC. A BLAST search suggested the transposon is novel, as it has no identical matches on GenBank. Several MRSA plasmids have been isolated which carry *cfr* and *fexA*, contain IS elements and share a similar genetic organisational structure to Tn*558*-IS*256*-*cfrAB*, however the plasmids encode different IS elements (Figure 4-6). Several Tn*558*-like elements contain a *cfr* gene integrated via an IS element (184), while few contain IS*256* and those which do (for example plasmid pSAM12-0145, GenBank: KU521355.1) contain additional integrase/resolvases *istA* and *istB* which were not present in Tn*558*-IS*256*-*cfrAB.* The uniqueness of the transposon raises the possibility that *cfr* was independently acquired by Tn*558* in Australian ST398 MRSA by insertion-sequence-mediated integration.

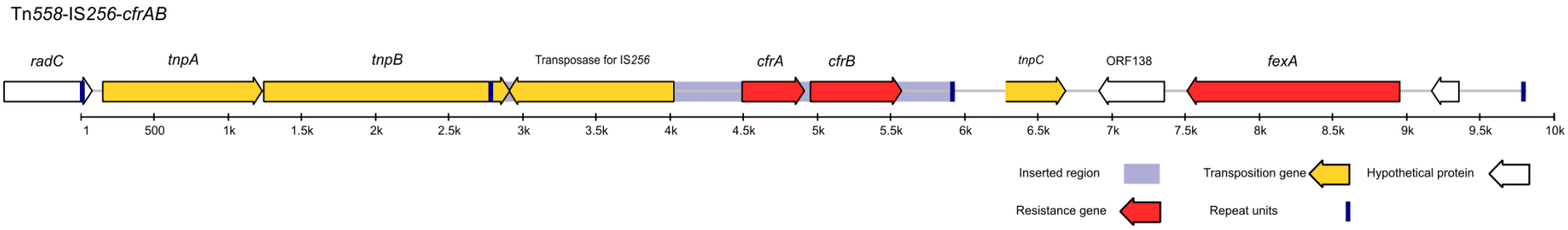


Figure 4-5 Schematic of Tn*558*-IS*256*-*cfr*AB element

Arrows indicate coding sequences. Colours indicate gene function, as indicated in the key. Scale bar indicates sequence length in base pairs.

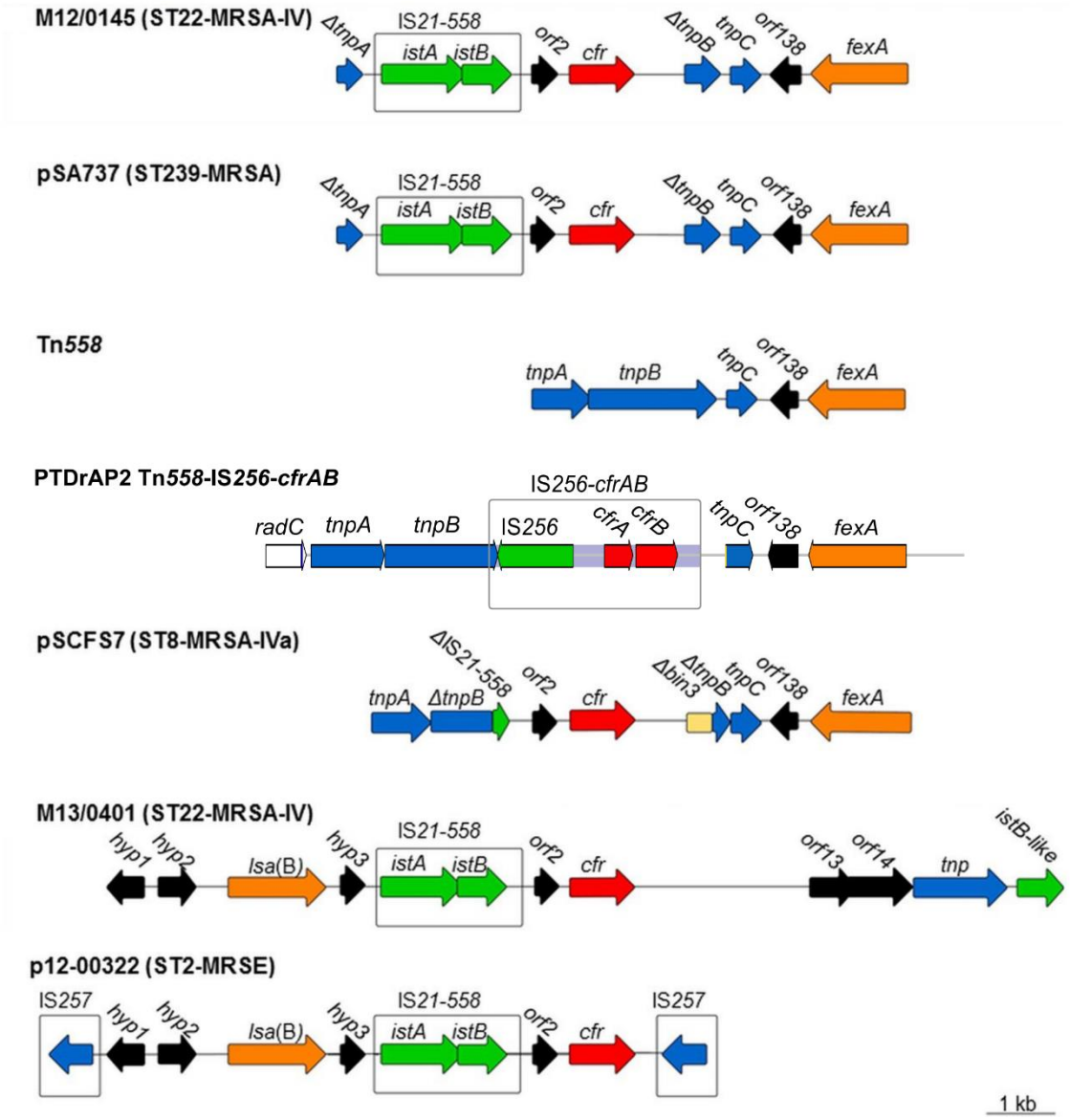


Figure 4-6 - Genomic context of *cfr* within other mobile genetic elements of MRSA.

Tn*558-cfr* elements in other studies share a similar architecture to the Tn*558*-IS*256-cfrAB* (centre), however they differ by the type of IS element present, namely IS*21-558* or IS*257* instead of IS*256*. Tn*558*-IS*256-cfrAB* is unique and not directly related to Tn*558-cfr* elements identified in previous studies. Modified from Shore et al. 2016 (5).

4.3.8 Insertion of IS*256-cfr* may have immobilised Tn*558*

The insertion of IS*256-cfr* into Tn*558* may have immobilised the Tn*558* transposon, making it more stable in the chromosome. Tn*558* does not carry conjugation genes, but can form circular intermediates which are required for integration into other mobile elements such as plasmids (437). The integration of IS*256-cfr* into Tn*558* disrupts the *tnpB* gene, one of three transposases required for the excision of the element from the chromosome (436). The *tnpB* genes of Tn*558*-IS*256-cfrAB* contained a number of missense mutations (residues 427 E>G and 429 I>V) within the coding sequence and at the peptide C-terminus (residues 525-547) before terminating 92 residues prematurely. I predict therefore, that the insertion of IS*256-cfr* has disrupted the function of *tnpB* and has immobilised the element. Additionally, the presence of the IS*256-cfr* insertion may have affected the function of the neighbouring gene, previously dubbed *orf138 (437)*. The gene *ofr138* of Tn*558* is found intact in all ST398 but is disrupted by a frameshift mutation in the three ST398 which carry the Tn*558*-IS*256-cfrAB* element. *orf138* has not been characterised but is predicted to be an NAD(P)H-dependent oxidoreductase. Given the location of the gene, adjacent to the *fexA* florfenicol efflux pump, the gene may assist *fexA* in the efflux or detoxification of florfenicol by helping to maintain an ion gradient, or by directly reducing florfenicol. The immobilisation of Tn*558*-IS*256-cfrAB* and apparent loss-of-function of *orf138* suggest the element may be evolving towards genetic stability, being less likely to be lost during formation of the Tn*558* circular intermediate and thus becoming fixed in the ST398 genome.

4.4 Mobile genetic elements present in ST398 and ST93 associated with piggeries in Australia

To identify potential transfer of elements between ST398 LA-MRSA and the typically human-associated ST93 epidemic "Queensland Clone" MRSA identified in the 2017 farm surveillance study, the types and quantities of MGEs was determined for each group. MGEs were discovered using the methods described in sections 2.9.7 to 2.9.10.

4.4.1 Mobile genetic elements of human- and livestock-associated MRSA

A large variety of MGEs were identified in the human-associated ST93 and ST398 LA-MRSA. Transposons identified in ST398 MRSA include a 13 kb Tn*552*-like *blaZ* transposon which confers beta-lactam resistance, the 17.8 kb Tn*916 tetM* tetracycline-resistance transposon, and a 12 kb Tn*554*-like *fexA* phenicol-resistance transposon. The Tn*916* transposon shares a consistent integration site among the ST398 isolates in this study. Notably, a large variety of plasmids were identified in ST398 and ST93 MRSA. The majority of the plasmids encode genes for AMR but many others lack any identifiable accessory genes. The plasmids identified are described and listed in Table 7-3. Except in plasmid pE026, no *oriT* sequences were identified.

Plasmids pPTF1P3 and pPTDRaP2a encoded macrolide resistance gene, *ermC*. pPTF1P3 shared 99% nucleotide identity with USA300 plasmid pUSA03 (GenBank: CP000258.1). The plasmid was identified throughout ST93 and ST398 study isolates and was the most common *ermC*-carrying plasmid found amongst the isolates. pPTF1P3 lacked any detectable mobilisation determinants. Alignment of the pPTF1P3 plasmids revealed those carried by ST93 isolates were identical but those carried by ST398 isolates had minor sequence variation. A 2,415-bp variant of pPTF1P3 was identified which was only found in ST398 MRSA. The variant encoded additional sequence, ATAAGTGGTTATAATGAATCGTTAATAAGCAAAATTCATTATAACCAAATTA, inserted upstream of the

ermC gene. The sequence may serve to regulate the *ermC* gene in a similar manner to the *ermCL* leader peptide of some macrolide resistance plasmids (438), however the sequence did not form an ORF and bore only 59% nucleotide sequence identity to the *ermCL* ORF. Plasmid pPTDrAP2a carried *ermC* and was larger than pPTF1P3. pPTDrAP2a was most similar (99% identity, 94% coverage) to *S. aureus* MRSA37 plasmid pSWS372 (GenBank: HG380318.1). pPTDrAP2a was found in three isolates in the study, with a variant carried by ST93 isolate H4 containing two copies of the *fomD* and *ermC* genes each. *Streptomyces fomD* fosfomycin biosynthesis pathway gene performs nucleophilic attack on phosphate moieties (439, 440). The significance of *fomD* carriage in pPTDrAP2a plasmids was unclear.

Two plasmids, pRGMX-2993 and pRGMX-3050, encoded a predicted 23S rRNA (guanine (745)-N(1))-methyltransferase, *rgmX*. Plasmid pRGMX-2993 had a 99% nucleotide BLAST identity to *S. aureus* RIVM4296 plasmid pRIVM4296 (GenBank: CP013626.1) and was found in ST93 and ST398 isolates. In isolate E008, the plasmid appeared to be chromosomally integrated into a bacteriocin gene (RefSeq: YP_005739044.1). Among ST398 isolates, pRGMX-2993 plasmids displayed backbone sequence variations occurring between *rep* and *rgmX* (Figure 4-7). pRGMX-3050 was identical to plasmid pPTDRaP2c (GenBank: CP029170.1) and was a 98% nucleotide match to *S*. *aureus* RIVM1295 plasmid pRIVM1295-2 (GenBank: CP013618.1), differing by the presence of an intergenic sequence: CCCTTACGGG. The *rgmX* gene of pRGMX-3050 was identical to the *rgmX* of pRGMX-2993. The plasmids differ in the type of *rep* gene they carry. The wide distribution of pRGMX plasmids among ST93 and ST398 isolates in the study suggests the *rgmX* gene confers some advantage to the host, however, no studies have addressed the function of *rgmX*.

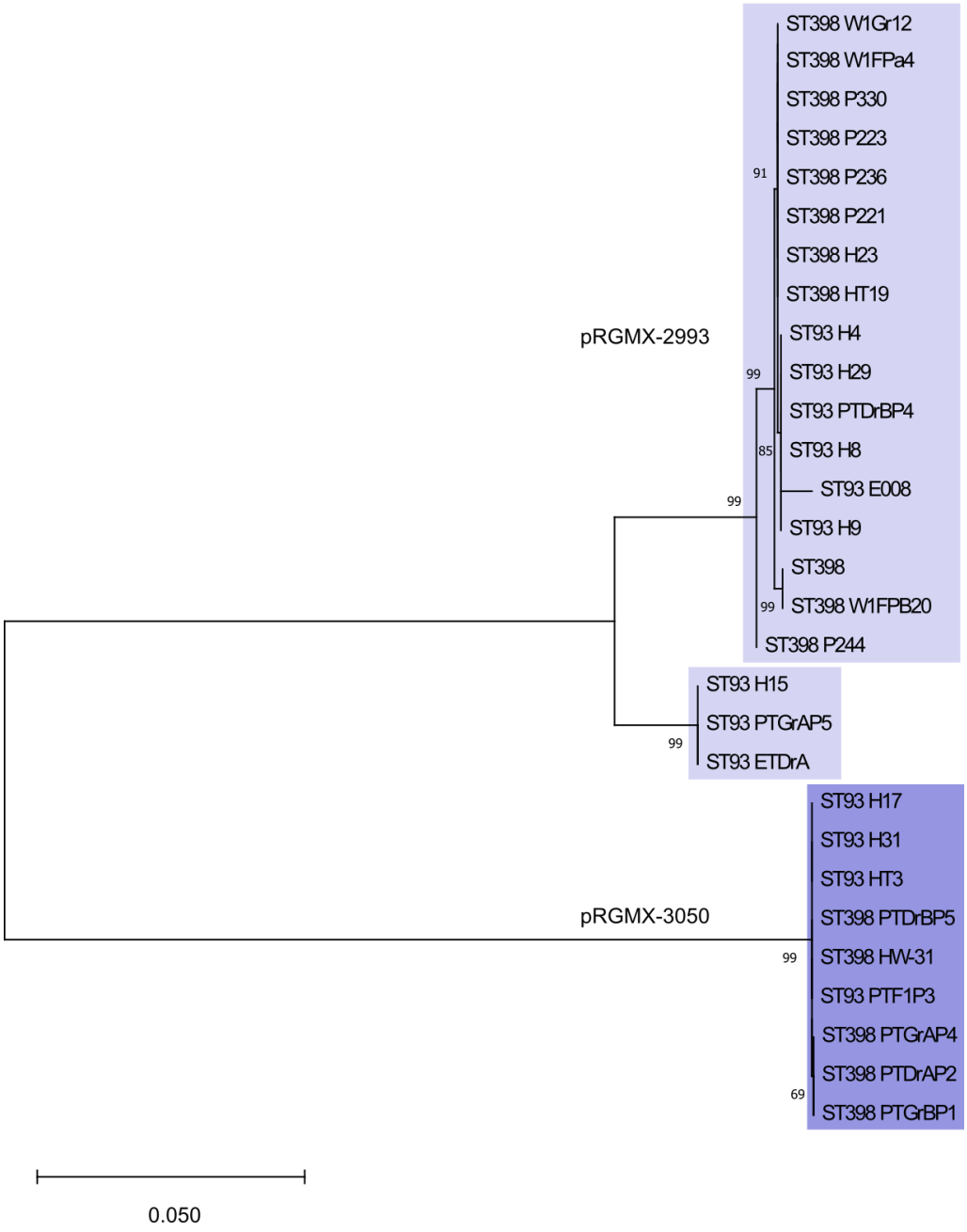


Figure 4-7 - Maximum-Likelihood phylogenetic tree of pRGMX plasmids

Maximum-likelihood phylogenetic tree generated from 100 bootstrap replicates. Bootstrap values are presented at each node. Plasmid designation is indicated by shaded sections for pRGMX-2993 (light

colour) and pRGMX-3050 (dark colour). Scale bar indicates nucleotide substitutions per site. pRGMX-2993 and pRGMX-3050 were genetically distinct. pRGMX-2993 plasmids contained more sequence variation than pRGMX-3050, which was carried by isolates of LA- and CA-MRSA.

Plasmids pH15 and pH4 encoded tetracycline resistance gene *tetL.* Plasmid pH15, which was 99% identical to *Bacillus cereus* plasmid pBCS16 (GenBank: U32369.1), also had high identity to many much larger *S. aureus* and *E. faecium* plasmids and chromosomal DNA sequences, where the plasmid typically appears chromosomally integrated. Plasmid pH4 is identical to plasmid pH15, except for the addition of kanamycin resistance gene *aadD,* which is integrated between *rep_1* and *tetL*. pH4 matches *S. sciuri* P723 plasmid pSSC723 (GenBank: KY389065.1), a plasmid which has been shown to be transduced by bacteriophage (441). An identical plasmid was also found integrated in the larger (50 kb) conjugative plasmid pERGB (JN970906.1), a plasmid which also carries *cfr* (189).

Plasmids pPTGrAP5, pP330 and pH14 encode *mobCA* mobilisation genes. Plasmid pPTGrAP5 has poor identity to other publicly available plasmids. Sequence matches align either to the *rep* gene (most common hits) or to the *mob* gene of the plasmid (e.g. a 93% nucleotide match *S. aureus* RIVM6519 plasmid pRIVM6519-1, GenBank: CP015174.1), not the combination of *rep* and *mob*, nor to the plasmid backbone. There is no significant variation between the pPTGrAP5 plasmids found in ST398 isolates and the ST93 PTGrAP5 plasmid. Plasmid pP330 has 94% identity (99% coverage) to *S. simulans* plasmid pACK4. pACK4 was shown to be mobilised by pG01 (442). pH14 encodes *mobCA* genes and tetracycline resistance gene *tetK*. The plasmid has no complete GenBank BLAST matches, but the *mobCAB* operon matches *S. epidermidis* plasmid pSE-12228-02, (GenBank: AE015931.1, >95% nucleotide identity). The plasmid was found only in isolate H14.

Plasmid pE008 shared an identical backbone with pRGMX-3050, but encodes *qacC* in place of *rgmX.* The *qacC* gene provides resistance to quaternary ammonium disinfectants (biocides), which are often used in hospitals and in pig farms (443). It was therefore unsurprising that *qacC* plasmids were found

in ST93 isolates in a pig farm-environment (E008), a human (H8), and a pig (PTDrBP4 and PTpGP1) (366). *qacC* carriage may enhance the survival of ST93 in livestock and healthcare environments.

pPTGrBP5 encoded tetracycline-resistance gene *tetK*. BLAST matches of the plasmid were almost exclusively to chromosomal DNA sequences, including in PTDrAP2 where the plasmid had an 84% nucleotide match to the SCC*mec* region, suggesting pPTGrBP5 is the circularised form of a typically chromosomally-integrated plasmid. *tetK* was found in the majority of ST398; however, in all isolates except PTGrBP5, *tetK* was encoded by plasmids which were integrated into the SCC*mec* element.

pE026 encoded an *hsdS* restriction-modification methylation-specificity gene. An *oriT* with 80% nucleotide identity to the pWBG749-family plasmid OT408 was identified (205). BLAST matches to the plasmid were incomplete, and matched either plasmid backbone or *hsdS* gene, which suggested the plasmid had recombined. The plasmid backbone was an 87% nucleotide match to *S. aureus* WBG4364 plasmid pWBG1773 (GenBank: EF537646.1). pE026 was present in ST398 isolates E026 and W1FPB20 only. The significance of carrying an additional restriction methyltransferase specificity subunit gene is uncertain.

pP221 encoded many *rep*-like proteins (ORFs 1, 3, 5 and 24), copper (ORFs 17 and 18) and cadmium resistance operons (ORFs 26 and 27), and a tetronasin resistance operon (ORFs 10-13, Table 7-4). pP221 was identical to *S. aureus* NZ15MR0322 genome assembly, plasmid: 2 (GenBank: LT699705.1). pP221 potentially confers resistance to ionophore antimicrobials, via the tetronasin resistance operon. Ionophores are used in livestock as coccidiostats (444), however, some ionophores such as salinomycin are used as growth promoters in pigs (445). Tetronasin is a polyether ionophore antimicrobial which can inhibit the growth of gram-positive bacteria (446), however, it is not registered for use in Australia (https://apvma.gov.au/). Tetronasin resistance ABC-transporter genes have been demonstrated to produce cross-resistance to the ionophore narasin (447), so although tetronasin is not registered for use in Australia, tetronasin resistance may still offer some protection from other ionophores and

therefore offer an advantage to LA-MRSA. Ionophore use in Australian pigs was low when last reported in 2010 (448), however use may have increased.

Additional elements of ST398 and ST93 include beta-lactamase resistance (*blaZ*) resistance plasmid pE017, and *fexA* florfenicol-resistance transposon Tn*558* (Figure 4-8). The 20.73-kb *blaZ* plasmid, pE017, is present in all ST93 except WweU6 and is absent in ST398. It shares 98% nucleotide identity with the well-characterised and widespread plasmid pMW2. These plasmids carry bacteriocin production and bacteriocin immunity genes, and beta-lactam and cadmium resistance genes (221). They also carried pWBG749-family *oriT* sequences of the OTUNA-subtype (81% nucleotide identity). *fexA* transposons (Tn*558* and Tn*558*-IS*256-cfr/AB*) were found in ST93 and ST398 isolates. Tn*558* and Tn*558*-IS*256-cfrAB* were described previously (4.3.7). Tn*558* transposon was found in all ST93 and in eight ST398 isolates, while Tn*558*-IS256-*cfr* was found in one ST398 and Tn*558*-IS256-*cfr*AB was found in three ST398 (Figure 4-9). Additional resistance genes were found amongst ST93 and ST398 isolates and were not plasmid bound. The additional genes were present within the SCC*mec* element in each isolate and included *ant-6-Ia* (aminoglycoside resistance), *lnuB* (lincomycin resistance), *vgaA* (quinupristin-dalfopristin resistance), *dfrG* (trimethoprim resistance), and *spc* (spectinomycin resistance). The complement of additional resistance genes was found only in 10 ST398 isolates (as shown in the PTDrAP2 SCC*mec* element, Figure 4-3) but was otherwise absent from ST398 and from all ST93 isolates. The SCC*mec* of the isolates appeared to be sites of intensive MGE integration.

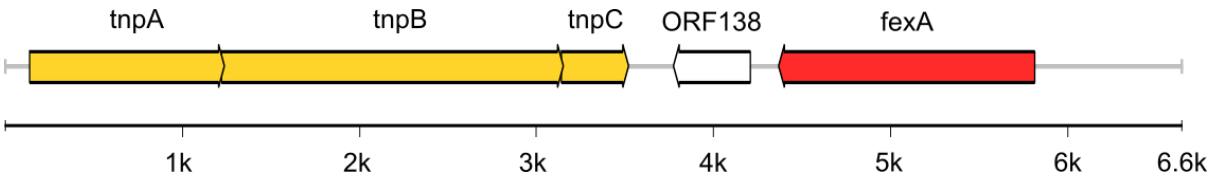


Figure 4-8 - Tn*558* florfenicol resistance transposon.

Arrows represent coding sequences and are coded according to function. Genes are involved in transposition (yellow), AMR (red), or are of unknown function (white). Gene names are indicated above arrows. Scale bar indicates sequence length in kilobase pairs.

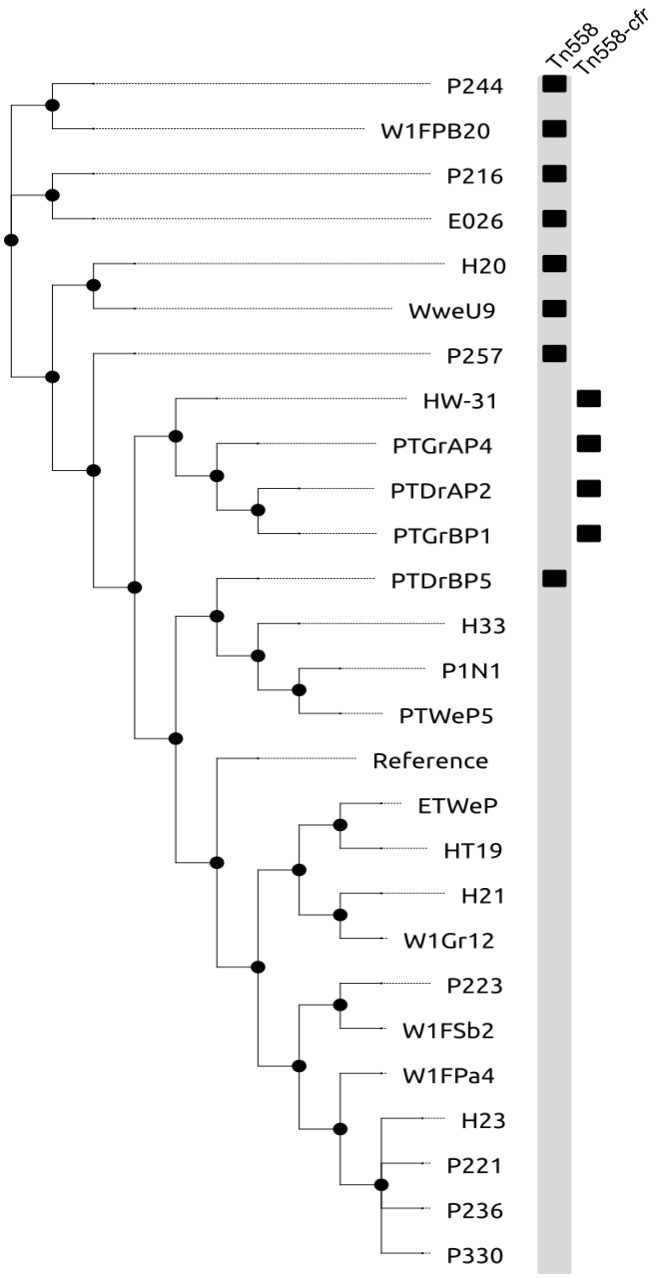


Figure 4-9 - Carriage of Tn*558* and Tn*558*-IS*256-cfr* by Australian ST398 MRSA

Maximum-likelihood core genome SNP cladogram of Australian ST398 MRSA. Transposon type is indicated (top) and carriage is indicated by black squares. A small subgroup carries Tn*558-cfr* indicating that a common ancestor of the group carried Tn*558* which then acquired IS*256-cfr.*

4.5 Evidence for transfer of MGEs between human and pig-associated MRSA

4.5.1 Integrity of barriers to HGT in study isolates.

ST93 is particularly difficult to manipulate genetically due to the carriage of three type I RM systems and a type IV RM system sauUSI (449) which targets cytosine-methylated DNA (238). Attempts to transfer DNA into ST93 isolates in ideal laboratory conditions has been met with limited success. For there to be any chance of successful HGT of MGE from ST398 to ST93, the RM system barriers would have to be deficient or missing. A local BLAST alignment of the ST93 MRSA database (2.9.6), comprising the contigs of all the ST93 MRSA from this study, against the RM system loci listed by Stinear *et al* 2011 (418) (2.9.13) revealed that several ST93 MRSA are missing all of the RM systems present in JKD6159 (Table 4-3). Nine ST93 lacked any of the RM systems found in JKD6159, all isolated from pigs (Table 7-5). Against expectations, the ST93 in this study may have better than average odds of HGT.

Table 4-3 - RM System integrity of ST93

Origin of isolation	**RM locus 1** (*hsdM* and *hsdR*)	**RM locus 2** (*hsdM* and *hsdS*)	**RM locus 3** (*sauUSI*)	**RM locus 4** (*hsdR*)	**RM locus 5** (*hsdM hsdS* and *hsdR*)
Human	14/26	19/26	17/26	16/26	13/26
Environment	0/4	2/4	0/4	3/4	1/4
Pig	0/15	0/15	0/15	0/15	6/15
Totals	14/45	21/45	17/45	19/45	20/45

The wide range of MGE identified in ST398 MRSA in this study suggests that the RM barriers of ST398 may be similarly compromised, however this is not the case. Performing a local BLAST query of RM system loci (2.9.13) against the ST398 MRSA database (2.9.6) revealed that all ST398 encoded at least one of the RM loci identified in PTDrAP2. Two isolates, W1Fsb2 and W1Gr12, encoded only an *hsdR*

gene so their genomes are potentially un-methylated, suggesting they could be broad HGT donors, as unmethylated DNA has been transferred to RM proficient *S. aureus* successfully (239).

Table 4-4 - RM system integrity of ST398

Origin of isolation	**RM locus 1** (*hsdM* and *hsdR*)	**RM locus 2** (*hsdM* and *hsdS*)	**RM locus 3** (*hsdR*)
Human	1/6	6/6	5/6
Environment	½	1/2	1/2
Pig	7/18	12/18	14/18
Totals	9/26	19/26	20/26

In addition to RM barriers, genomes of PTDrAP2 and JKD6159 were examined for CRISPRs (2.9.4), but did not find any CRISPR arrays or *cas* operons. While these two genomes do not account for all of the genetic variability of the other ST93 and ST398 MRSA datasets, CRISPR-Cas has not previously been published in ST93 MSRA and has only once been detected in ST398 (450).

4.5.2 Transmission of plasmids between LA- and CA-MRSA lineages

I examined the distribution of plasmids in both ST93 and ST398 lineages to assess if plasmid transfer may have occurred between lineages. Table 4-5 and Figure 4-10 summarise the distribution of plasmids in ST93 and ST398 isolates. Seven plasmids were found in ST398 and ST93 isolates and therefore may have been shared through HGT.

Table 4-5 – Percentage of shared plasmid carriage in ST93 and ST398 isolates

Resistance gene carried	ermC	ermC	rgmX	rgmX	aadD	None (*mobCA*)	qacC
Identifier	pPTF1P3	pPTDrAP2a	pRGMX-2993	pRGMX-3050	pH4	pPTGrAP5	pE008
Total ST93	34	2	8	4	2	4	4
Percentage	76%	4%	18%	9%	4%	9%	9%
Total ST398	12	1	12	4	1	1	1
Percentage	46%	4%	46%	15%	4%	4%	4%
Totals (all)	46	3	20	8	3	5	5
Percentage	65%	4%	28%	11%	4%	7%	7%

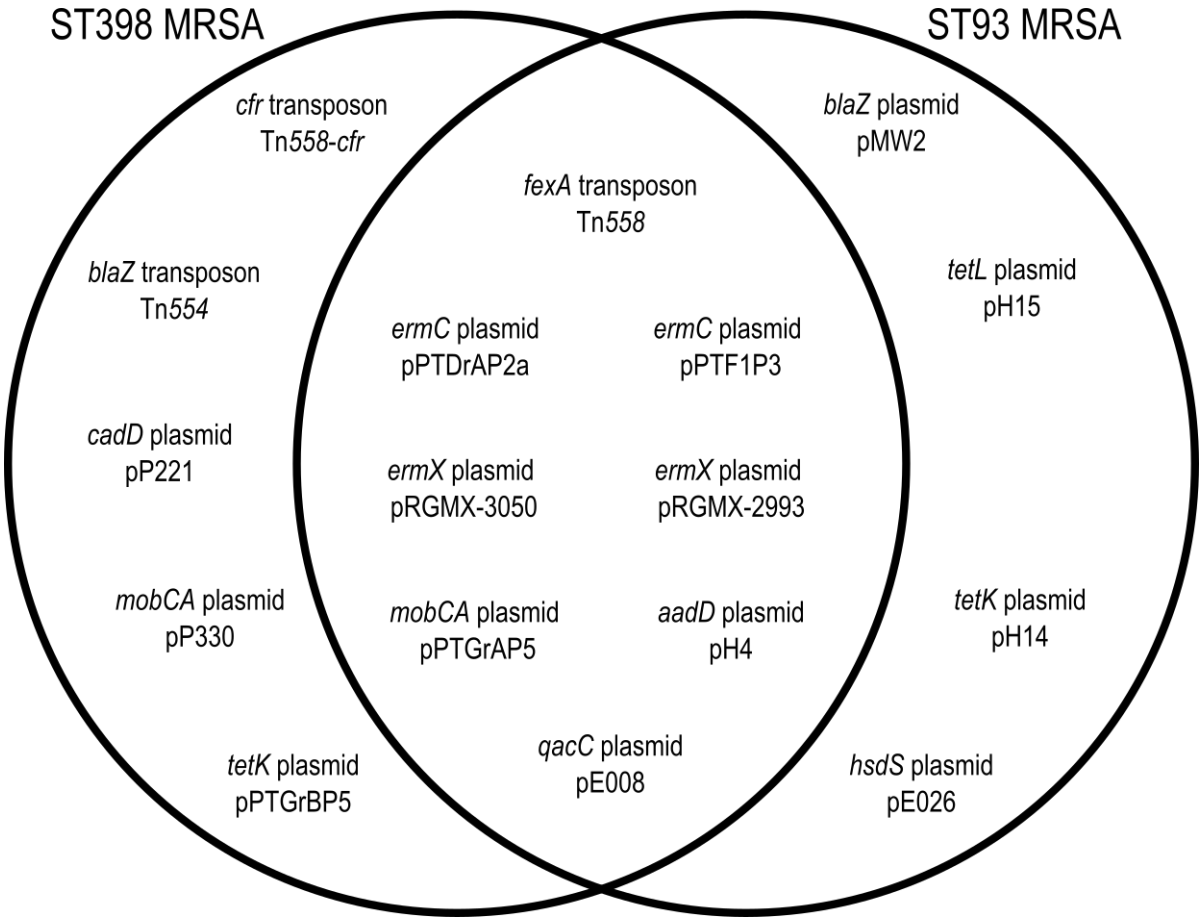


Figure 4-10 - Venn diagram of MGE distribution across ST398 and ST93 MRSA.

The most frequently carried plasmid was the small *ermC* plasmid pPTF1P3, found in 65% of all isolates, which was found in a higher proportion of ST93 isolates than ST398 isolates (76%, vs 46% respectively). Alignment and phylogenetic analysis of the *ermC* plasmids indicate that Plasmid A group forms three sub-groups (Figure 4-11 and Figure 4-12). One subgroup was only found in ST93, while the other two sub-groups were found in ST398. Variance between the pPTF1P3 subgroups occur as insertion upstream of the *ermC* gene, as 1-3 bp deletions/insertions upstream of *ermC,* and a five bp variant sequence (TAAAC > GTTTA) downstream of *ermC.* Inference of plasmid transfer events between ST93 and ST398 cannot be made on plasmid phylogeny and plasmid distribution alone.

Figure 4-11 - Phylogeny of *ermC* macrolide resistance plasmids in ST93 and ST398 MRSA.

Maximum-likelihood phylogenetic tree generated from 100 bootstrap replicates. Bootstrap values are presented at each node. Plasmid types are indicated by the branch labels, coloured blocks indicate host origin – pig (pink), human (blue) and environmental (yellow). Scale bar represents nucleotide substitutions per site. pPTDrAP2a is significantly divergent from pPTF1P3 and is shared by LA- and CA-MRSA.

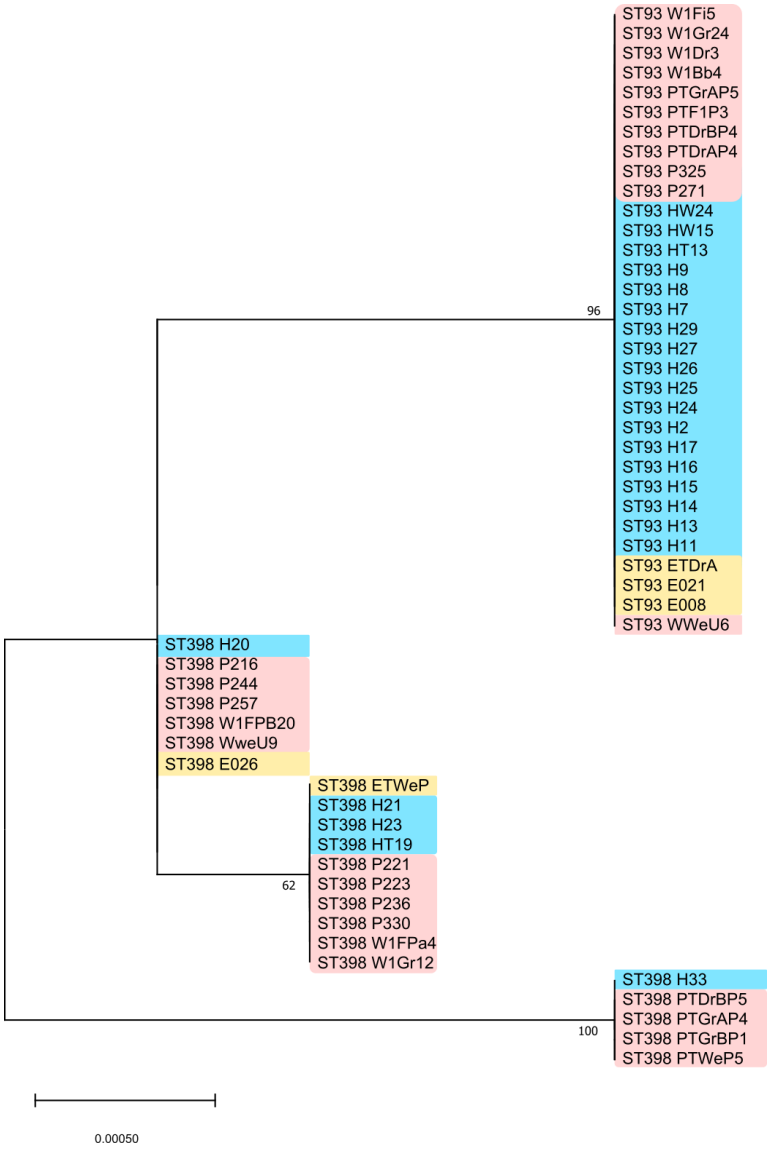


Figure 4-12 - Phylogeny of pPTF1P3 *ermC* macrolide-resistance plasmids in ST93 and ST398 MRSA.

Maximum-likelihood phylogenetic tree generated from 100 bootstrap replicates. Bootstrap values are presented at each node. Scale bar represents nucleotide substitutions per site. Coloured blocks indicate host origin – pig (pink), human (blue) and environmental (yellow). The tree indicates that variation across pPTF1P3 plasmids, which vary according to their host lineage. pPTF1P3 plasmids are highly conserved in ST93 MRSA and do not appear to have been recently transferred between LA- and CA-MRSA lineages.

The *ermC* gene is encoded by plasmids similar to pPTF1P3, and is carried by ST93 and ST398 at varied frequencies in other studies. The largest genomic study of ST93 MRSA and MSSA isolated globally, although largely comprising Australian ST93, between 1991 and 2012 showed a 20% (90/459) carriage rate of *ermC* (263). 56 of the 459 isolates were examined in a previous study and 18% carried *ermC* on pNE131-like plasmids (451) which were almost identical (100% identity, 95% coverage) to pPTF1P3, except for a single 108-bp insertion in the pNE131-like plasmid upstream of the *ermC* gene. Carriage of *ermC* in ST398 is highly variable, with studies observing *ermC* carriage ranging from 13% to 56% (452, 453). ST398 has a far greater global distribution than ST93 and the carriage of *ermC* is likely related to macrolide use in the specific study regions. Few studies in ST398 describe the plasmids which carry *ermC*, however, pSW371 (GenBank accession: HG380317.1) isolated from LA-MRSA ST398 had 99.92% identity (98% coverage) to pPTF1P3 and only varied by a 92-bp insertion upstream of pSW371 *ermC* and an intergenic SNP deletion in pPTF1P3. Once again, the insertion was not present in pPTF1P3 and occurred in the region immediately upstream of *ermC.* The region appeared to be hypervariable. The distribution of pPTF1P3-like *ermC* plasmids in ST93 and ST398 outside of my study was similar to the carriage of plasmids in my study, and in neither case was a non-study MRSA found to carry an *ermC* plasmid identical to pPTF1P3. Once again, direction of transmission or whether transmission of *ermC* plasmids had occurred between the ST93 and ST398 lineages at all, could not be inferred.

The *ermC* pPTDrAP2a was shared by ST93 and ST398, which may indicate a MGE transfer event. The plasmid was unique to the study, with a single match (99% identity, 94% coverage) on GenBank to plasmid pSWS372 (GenBank: HG380318.1). Plasmid pSWS372 was carried by an ST398 MRSA isolated from the dust of the broiler house of a chicken farm (454). pSWS372 differed from pPTDrAP2a in the length of the hypothetical protein, DUF402, which was 161 bp longer in pPTDrAP2a. Given the agricultural origins of pSWS272, being isolated from an ST398 MRSA, and sharing high overall identity to pPTDrAP2a of the study, which is otherwise unique among publicly available plasmids on GenBank, it seems possible pPTDrAP2a may have originated in ST398 and transferred to ST93.

The *rgmX*-encoding pRGMX-2993 plasmid was found in ST398 (46% carriage) and ST93 (18% carriage) lineages. pRGMX-2993 was present in each subclade of the ST398 isolates (Figure 4-13) and was largely concentrated in a single subclade of ST93 isolates (Figure 4-14), which may indicate transfer from any of multiple ST398 isolates to an ancestor of the ST93 subclade. pRGMX-2993 and pRGMX-3050 shared a similar organisational structure (Figure 4-14) but shared low nucleotide identity in intergenic regions and carried different *rep* gene types, which was reflected in their phylogenetic comparison (Figure 4-7). pRGMX-2993 carried consistent variations between ST398 and ST93, while pRGMX-3050 was identical and carried by each lineage, which suggests recent transmission of pRGMX-3050 between lineages and transmission, with lineage-specific evolution of pRGMX-2993. The origin of pRGMX plasmids in the MRSA can be inferred from the carriage of similar plasmids in MRSA outside of the study. While pRGMX-3050 had no full-length matches on GenBank, pRGMX-2993 was >97% identical to five plasmids on GenBank which listed animal sources of isolation; pSA01-04 isolated from "chicken" (GenBank: CP053078.1); GDC6P096P plasmid three isolated from "pig farms" (GenBank: CP065197.1); and plasmids pRIVM1295-2 (GenBank: MH785226.1), pRIVM4294 (GenBank: CP013625.1) and pRIVM4296 (GenBank: CP013626.1) isolated from ST398 LA-MRSA in human infections (455). Plasmids carrying *rgmX* were found in 19.4% of the 206 LA-MRSA sequenced in a Dutch LA-MRSA surveillance study (455). The connections, not only to *S. aureus* isolated from animals, but to ST398 LA-MRSA strongly suggested the *rgmX* plasmids identified in the study originated in ST398 and transferred to ST93.

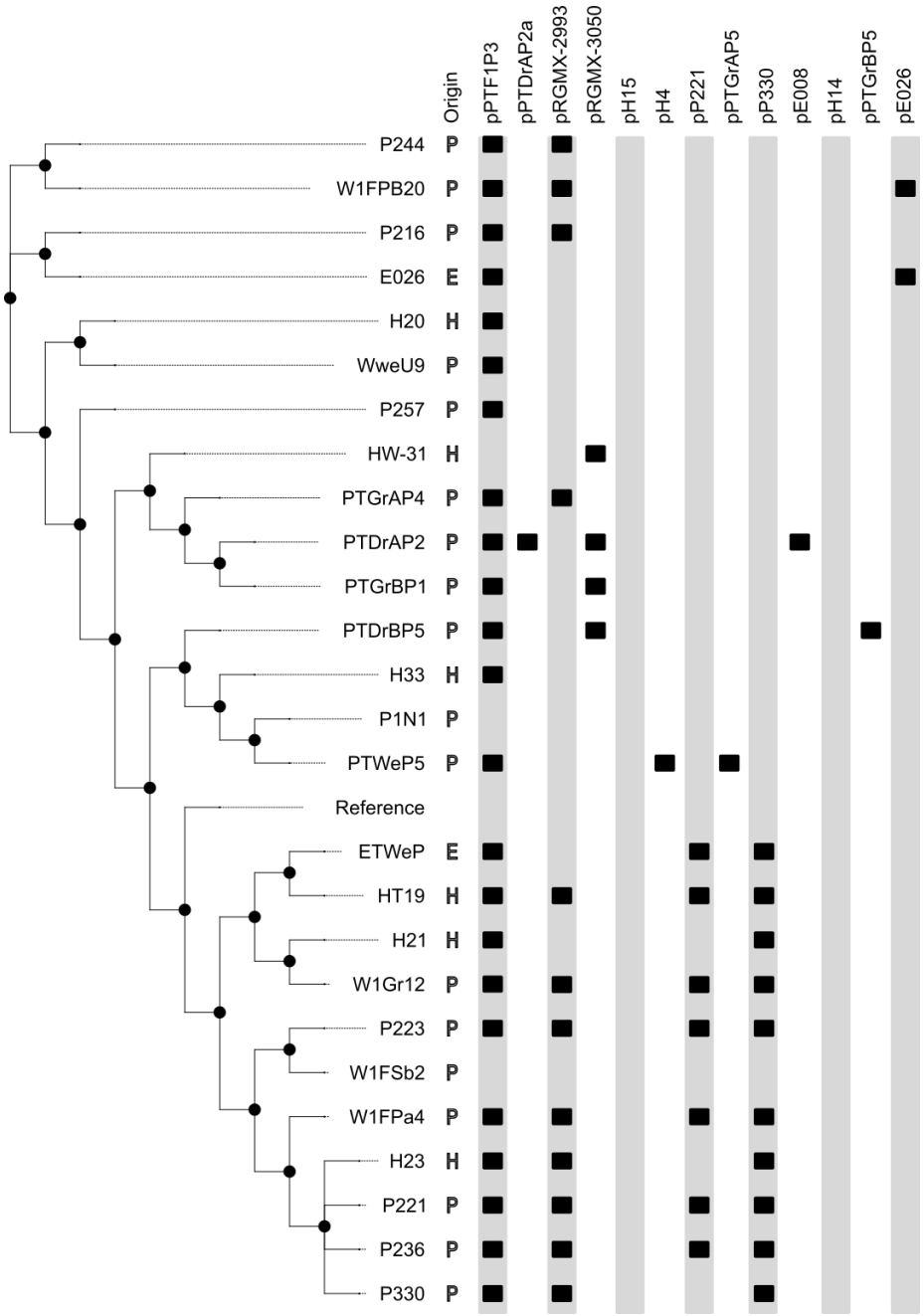


Figure 4-13 - Core phylogeny cladogram of ST398 isolates and presence of plasmids.

Maximum-likelihood core genome SNP cladogram of Australian ST398 MRSA. Black squares indicate carriage of plasmid, labelled at the top of each column. Origin column indicates isolates of human (H), pig (P) or environmental (E) origin.

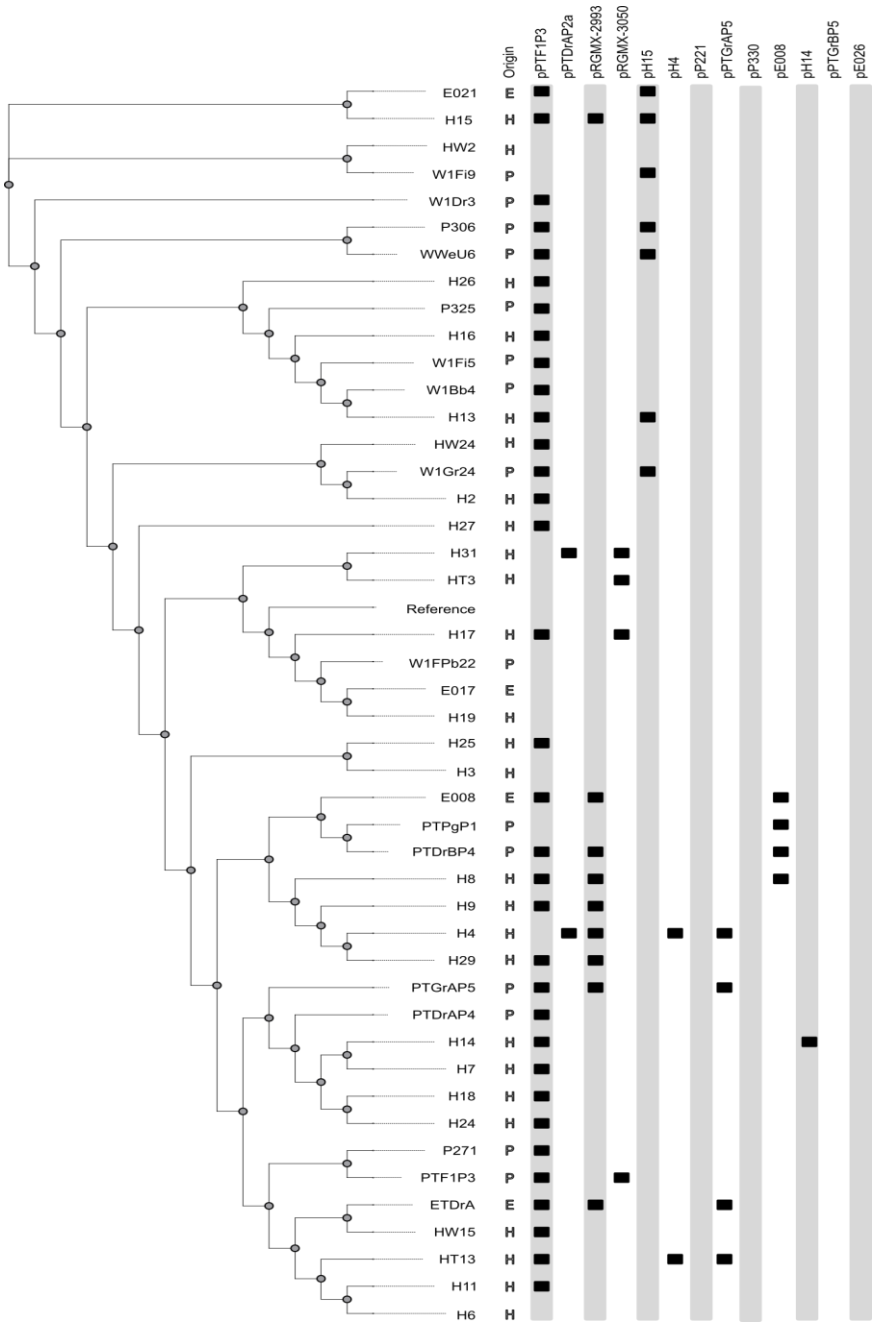


Figure 4-14 - Core phylogeny cladogram of ST93 isolates and presence of plasmids.

Maximum-likelihood core genome SNP cladogram of Australian ST393 MRSA. Black squares indicate carriage of plasmid, labelled at the top of each column. Origin column indicates isolates of human (H), pig (P) or environmental (E) origin.

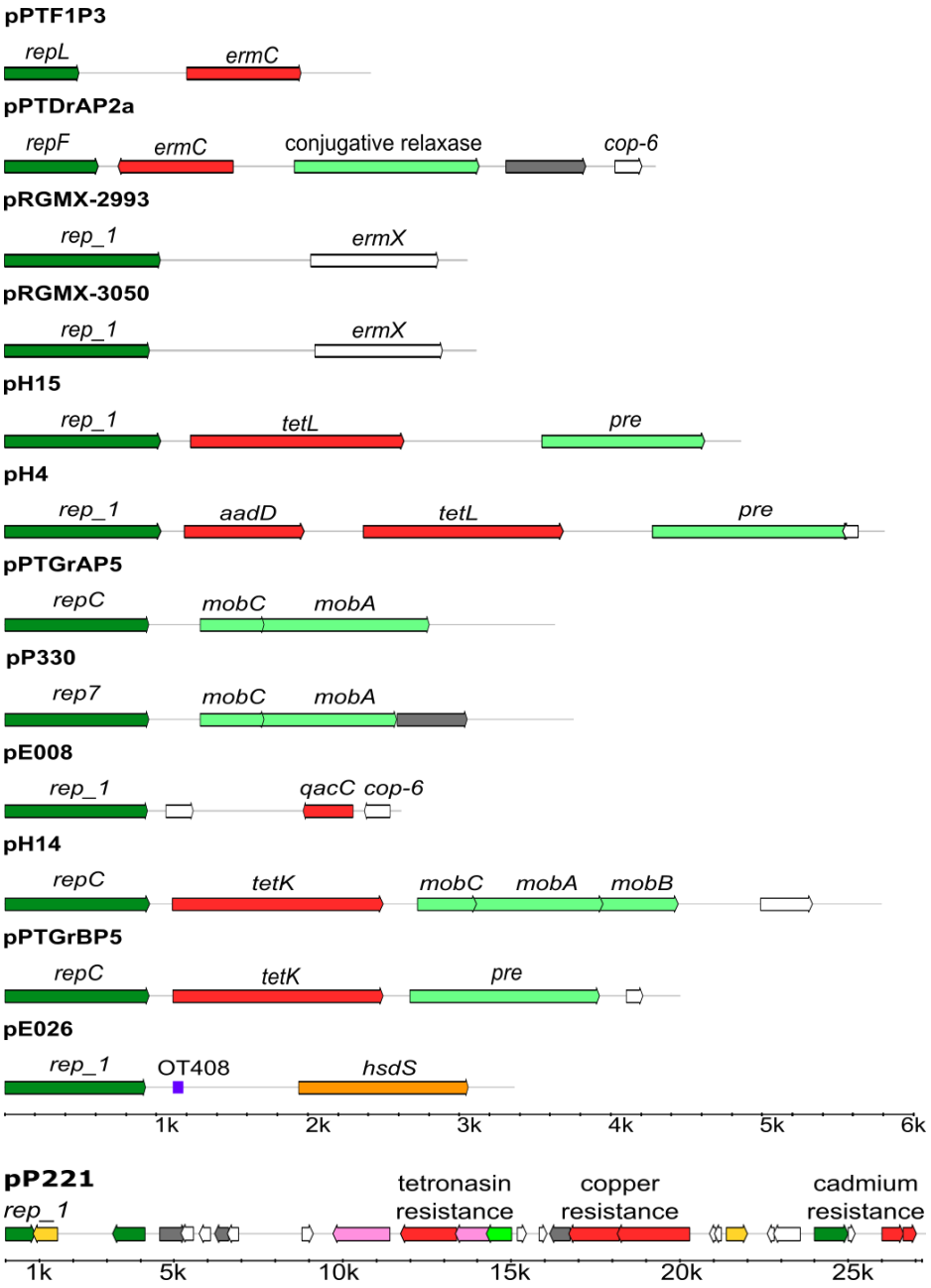


Figure 4-15 - Plasmids identified in Australian ST398 and ST93

Open Reading Frames (ORF) are represented by arrows and are colour-coded as follows: replication genes (green); resistance genes (red); mobilisation genes (light green); ABC-transporter genes (pink); transposition genes (yellow); methylation specificity genes (orange). Scale bar indicates plasmid length in base pairs.

4.5.3 Transmission of transposons between LA- and CA-MRSA lineages

The Tn*558* transposon (Figure 4-8) was found in all ST93 and in 12/26 ST398 MRSA. Nucleotide alignment and phylogenetic analysis of Tn*558* (data not shown) provide no further insight, as 45/54 Tn*558* were identical across 6,643 bp and the remaining nine Tn*558* vary by no more than one SNP each, with no polymorphisms shared between individual elements. The florfenicol resistance gene carried by Tn*558*, *fexA,* has not been identified previously in ST93, except in the publication from which the isolates in the present study were derived, and has only once been reported in Australian MRSA. *fexA* was discovered in a single WA CA-MRSA in 2005 (456). A 2016 study of 44 MRSA isolates from 771 Australian veterinarians found a variety of MRSA (including a single ST398 MRSA) but failed to detect *fexA* (362). No further publications list Tn*558* in Australian MRSA. It is therefore unlikely Tn*558* was introduced to the study isolates by a ST93 MRSA.

fexA has been identified in MRSA globally, although Tn*558* is less frequently reported. *fexA* has been reported in ST9 LA-MRSA from pigs in Thailand (457) and in several ST398 LA-MRSA including a clinical isolate from a Spanish pig farmer (458), from German pigs (459, 460) and cows (461, 462), a Brazilian pig (463), Belgian humans and animals (452), and three clinical isolates recovered from mongoose wounds in a UK zoo (464). A survey of commercial pig farms, slaughterhouses and markets in Beijing, China found *fexA* in 83% of ST9 LA-MRSA, of which 84% were Tn*558* positive (465). Tn*558*-like elements have been identified in clinical ST125 MRSA and ST398 LA-MRSA from a pig in Spain (466, 467). Despite Tn*558* being infrequently described in ST398, the many reports of *fexA* in ST398 LA-MRSA rarely describe the genomic context of the gene, and so it is probable some of the *fexA* genes are bound by Tn*558* elements. Based on the lack of reports of *fexA*, and thus Tn*558*, in ST93 outside of this study, it seems possible Tn*558* already present in the LA-MRSA lineage may have subsequently transferred to ST93, despite the higher prevalence of Tn*558* in ST93.

The conjugative tetracycline transposon Tn*916 tetM* was present in all ST398 in and was absent in ST93. This was surprising as Tn*916* appeared to be intact in these isolates and Tn*916* is a known conjugative element (468). Attempts to transfer tetracycline resistance by broth culture mating experiments were unsuccessful (data not shown).

The low number of *cfr*-positive ST398 MRSA suggests the *cfr-IS256* region may have been acquired more recently, independent of the acquisition of Tn*558*. IS*256* may have mediated the excision of *cfr* from a transient plasmid and its subsequent integration into Tn*558*. IS elements are able to circularise, and may mediate the co-integrant formation of, Tn*558*-like *fexA-cfr* transposons (186, 469). A transient *cfr*-carrying plasmid could have been acquired by an ancestor of the *cfr*-positive ST398 clade from non-*aureus* staphylococci within the farm environment. Various CoNS are found in pigs and are a known source of *cfr* plasmids (470-472). Tn*558-cfr* elements of similar genetic structure have been discovered in staphylococci from retail meats in China (190), and in ST9 MRSA from a pig and chicken in China (473). A Tn*558-cfr* element were discovered as the basis of linezolid-resistant MRSA and methicillin-resistant *S. haemolyticus* bacteraemias in Spain (466). The presence of Tn*558-cfr* elements in LA-MRSA suggests the element is beneficial to LA-MRSA.

4.5.4 Origins of tetracycline resistance plasmids in CA-MRSA and LA-MRSA

ST93 MRSA in the study carried tetracycline resistance plasmids. pH14 and pH15 carry tetracycline resistance determinants *tetL* and *tetK* (Figure 4-14). *tetL* pH15, found in 8 ST93 isolates, is almost identical (99% identity, 100% coverage) to *Bacillus cereus* plasmid pBC16. pBC16 has been mobilised between *B. thuringensis* and several other bacilli (474), indicating the plasmid has broad host range. It may be possible that ST93 acquired the plasmid from a soil bacillus, as predicted by early research comparing staphylococcal and bacillus plasmids (475). Tetracycline resistance provided by *tetK* has been recorded in ST93 (451) carried by a plasmid which was almost identical (98%) to *tetK* plasmid

pH14, which suggested that the pH14 identified in could have been present in ST93 H14 prior to exposure to the farm environment.

4.6 Chapter summary and conclusions

I have shown the genomes of human-associated and LA-MRSA study isolates carried abundant genes which confer resistance to antimicrobials, disinfectants and heavy metals, and were host to a wide array of chromosomal and extrachromosomal MGEs likely to be capable of disseminating these genes between strains. The diversity of AMR genes and MGEs was exemplified by the size and content of the large, composite SCC*mec* element of ST398 MRSA PTDrAP2. The variety and amount of resistance genes, and the array of MGEs identified in the LA-MRSA, suggests evolution to allow persistence in an environment of heavy antimicrobial selection pressures. MGEs identified were typical for their lineage (e.g. Tn*916* in ST398), although some were unexpected, such as pH15 which was identical to *B. cereus* tetracycline-resistance plasmid pCB16, and the *fexA*-carrying Tn*558* transposon which is infrequently reported in MRSA. The presence of such uncommon MGEs suggested that Australian pig farms provide unique selection pressures that favour florfenicol and tetracycline resistance.

The *cfr* gene appears to have been recently acquired by the Australian ST398 LA-MRSA and has become integrated within a Tn*558* transposon, which encodes phenicol resistance efflux gene *fexA.* Integration which appears to have been mediated by an IS*256* element, has disrupted the *tnpB* gene of Tn*558,* immobilising the transposon in the chromosome. The IS*256-cfr* element may have recombined from a transient plasmid. The capture of IS*256-cfr* by Australian LA-MRSA was likely a single event and via clonal expansion, resulted in several isolates carrying the gene, rather than transmission of Tn*558-cfr* among LA-MRSA. This is the first *cfr* gene identified in Australian MRSA and its origins remain unknown, as live import of pigs to Australia has been prohibited for many years (476).

Whilst evidence of MGE and AMR transfer between lineages is difficult to infer from sequence comparisons alone, it seems that MGE transfer between Australian CA-MRSA and LA-MRSA may have occurred. The discovery that many pig-isolated ST93 MRSA appear to lack all of the RM systems of JKD6159 suggests that these isolates would be good candidates to receive MGE from ST93 MRSA. The presence of two ST398 MRSA which carry only an *hsdR* gene and carry no immediately obvious RM *hsdM* methylases suggest that these isolates could be facilitators of inter-lineage MGE transfer. There is of course the possibility that other RM genes exist in the genomes and that my search could not find them, but there appears to be an overall sporadic carriage of RM systems. The fact that the RM systems were infrequently carried by most pig-isolated ST93 and completely absent in others implies a selection pressure against RM systems of MRSA when colonising porcine hosts.

The MGE identified in ST93 in this study may have come from another undetected lineage of *S. aureus,* or another commensal staphylococci altogether, however the presence of identical plasmids in the distantly-related ST93 and ST398 lineages, and the often sub-clade restricted nature of the plasmids within the lineages add weight to the probability of HGT having occurred between ST93 and ST398 MRSA. Of the MGEs studied, the elements which may have transferred between lineages were the *ermC* plasmid pPTDrAP2a, the cryptic plasmid pRGMX-3050 and *fexA* transposon Tn*558.* For each MGE, carriage has been reported in LA-MRSA and has never before been reported in CA-MRSA. The high numbers of LA-MRSA and CA-MRSA together in close proximity provides an opportunity for HGT to occur. Although HGT could not be demonstrated under laboratory conditions, this may simply indicate that conjugation (the only method of HGT tested) is not the most likely avenue for HGT to have occurred. A lack of conjugative MGE in ST398 and ST93 support a poor likelihood of conjugative transfer being the predominant method of HGT. Phage transduction however, does seem like a possible route of transmission. Transfer of plasmids between *S. aureus* via phage transduction has been demonstrated and is suggested as a viable method for the HGT of non-conjugative plasmids in nature (374). Demonstrating that phage transduction of the small plasmids identified in this study could occur between ST93 and ST398 could form the basis for future work.

The possibility of AMR transfer between human and livestock-associated lineages highlights the problems caused by high-volume antimicrobial use in livestock environments where human contact is close and frequent. The probable transfers of AMR raise two possibilities; the potential of resistance transfer from ST398 into the highly pathogenic human-associated ST93 lineages, which may then become fixed in that population and spread into the community, and the potential for virulence to transfer from human-pathogenic ST93 CA-MRSA into ST398 LA-MRSA. Such an increase in virulence capability in ST398 LA-MRSA would present a serious health concern to livestock-workers and ultimately the greater community. Whether *fexA, tetK* and other pig LA-MRSA-associated AMR pose a threat to the efficacy of human antimicrobial chemotherapy remains to be seen. Aside from the impact on human health, resistance to livestock antimicrobials reduces the effectiveness of antimicrobials used to maintain animal health.

The high prevalence of multidrug-resistance observed in the Australian LA-MRSA sheds light on the effectiveness of current agricultural antimicrobial-use controls. Whether the failure of antimicrobial stewardship has occurred at the farm level, the veterinary/commercial supplier level or at the point of legislation, the current regulatory environment has failed in this instance to prevent the development and spread of AMR in Australian pig farming. A ban on in-feed antimicrobial additives should be considered in Australia to reduce the total AMR carriage by Australian LA-MRSA to prevent the spread of such MGEs and their accompanying AMR into CA-MRSA.

Chapter

Identification and characterisation of *cfr* linezolid resistance gene variant *cfrAB*

5.1 Introduction

As discussed in section 1.10.5, the use of antimicrobials in livestock is widespread and raises concerns for the evolution and spread of AMR on a large scale. The dissemination of antimicrobial-resistant pathogens and genes into the community is a burgeoning problem with potentially severe consequences for human health. Whilst Chapter 4 examined the distribution and potential spread of AMR among human and livestock-associated MRSA in Australian piggeries, Chapter 5 examines the evolution of linezolid resistance within isolates from the same study population.

cfr was first reported in Australian LA-MRSA by Sahibzada *et al* in 2017 from a surveillance study of Australian pigs, pig farm workers and the pig farm environment (366). No clinical *cfr*-mediated linezolid-resistant *S. aureus* have been reported in Australia. Transferable linezolid resistance gene *cfr* was discussed in detail previously (section 1.7.1). Given the rapid spread of *cfr* among different genera of pathogenic bacteria in other parts of the world, the multi-resistance potential of the *cfr* gene, the association of *cfr* with livestock and the relative novelty of *cfr* in Australia, the *cfr*-positive Australian ST398 MRSA warrant further investigation.

5.2 Project Aims

In chapter 4 I examined the genetic background of the *cfr* gene in ST398 MRSA isolate PTDrAP2. In this chapter I examined the function and expression of *cfr* gene variant, *cfrAB,* to determine

- If *cfrAB* is predicted to be functional despite a disruptive frameshift deletion;
- If *cfrAB* contains a PRF site which could allow translation of functional Cfr from the *cfrAB* allele;
- The frequency of *cfrAB* frameshifting events.

5.3 Results

5.3.1 A *cfr* variant, *cfrAB,* is present in LA-MRSA genomes

Microarray resistome characterisation of ST398 MRSA (performed by Shafi Sahibzada) confirmed four isolates encoded *cfr*, however, VITEK® ® and disc diffusion susceptibility assays (performed by Shafi Sahibzada) determined only one of these isolates (HW-31) was phenotypically linezolid resistant (32 mg/mL, breakpoints: Clinical and Laboratory Standards Institute ≥ 8 µg/mL; European Committee on Antimicrobial Susceptibility Testing ≥ 4 µg/mL). Although the remaining three *cfr*-positive isolates (PTGrBP1, PTGrAP4 and PTDrAP2) had non-zero linezolid MIC values (2-3 µg/mL), the MICs were below the clinical resistance threshold and therefore were reported linezolid sensitive. The core SNP genome phylogeny of ST398 study isolates (Figure 4-1) indicate that all four *cfr* and *cfr*-variant MRSA are very closely related, ranging from 2-7 SNP difference across a core genome of 258 bp.

WGS data was examined to confirm the presence of *cfr* genes in PTGrAP4, PTGrBP1 and PTDrAP2. The WGS and assembly of ST93 sequence data was performed, and kindly provided by, Stanley Pang (366). A BLAST of the WGS data for the *cfr* gene produced a 99% nucleotide match in the linezolid-intermediates, which revealed a variant, truncated *cfr* gene of 387 bp. The SNP deletion occurred in the first 1/3 of the *cfr* gene in a poly-A tract between nucleotide position 379 to 387, which produced a frameshift in the coding sequence and resulted in a stop codon (Figure 5-1). The SNP deletion in the linezolid-intermediate isolates occurred at the same location of the *cfr* gene, within a poly-A tract. A start codon occurs in the -1 reading frame, 77bp downstream of the stop codon, initiating a second 663 bp ORF, encoding the remaining residues of the Cfr peptide (Figure 5-2). These separate ORFs were named *cfrA* and *cfrB.* Collectively, the *cfr* variant allele was therefore named *cfrAB.*

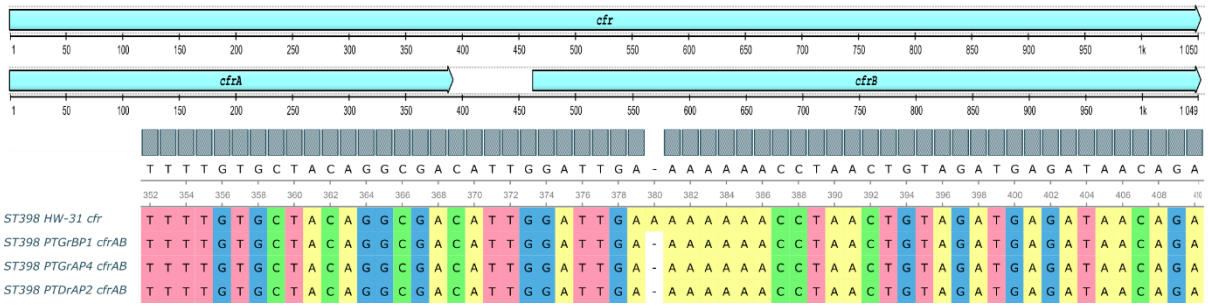


Figure 5-1 - Alignment of *cfr* and *cfr*AB nucleotide sequences

Arrows represent coding sequences of *cfr* (top) and *cfrAB* (middle, separate open reading frames of *cfrA* and *cfrB*). Partial alignment (bottom) shows the nucleotides 352-410 of the coding sequences, encompassing the SNP deletion within the poly-A site. Sequences are from isolates HW-31, PTGrBP1, PTGrAP4 and PTDrAP2 (accession: NZ_CP029172) from this study.

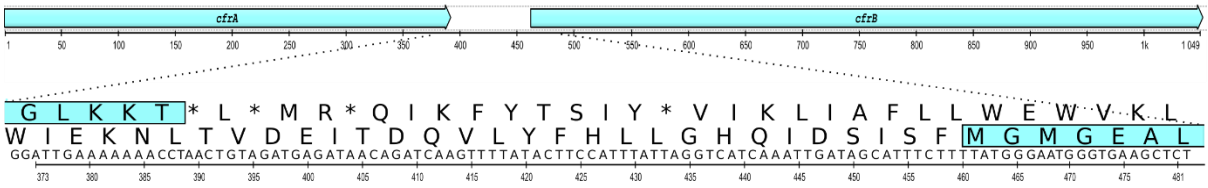


Figure 5-2 - Location of the *cfrA* stop codon and *cfrB* start codon

The separate *cfrA* and *cfrB* open reading frames of *cfrAB* are depicted by the arrows (top). The zoomed sequence shows the 0 and -1 reading frames and corresponding amino acid codons in addition to the nucleotide sequence. The absence of one A base in the poly-A sequence (bottom left) causes a stop codon to disrupt the 0 reading frame. A start codon in occurs in the -1 reading frame, continuing the *cfrB* peptide, however the lack of a nearby ribosomal binding site limits the likelihood that the start codon would be translated.

5.3.2 *cfrAB* increases linezolid MIC despite nonsense SNP mutation

ETEST® MIC assays were performed to confirm the initial MIC findings, using strain RN4220 as a *cfr*-negative control; PTDrAP2 to test the resistance conferred by *cfrAB;* HW-31 as a *cfr* positive control; and *cfrAB*>*cfr* mutant PTDrAP2-FM1 to observe the effect of restoring *cfrAB* to *cfr*. Isolation and validation of *cfrAB*>*cfr* mutant PTDrAP2-FM1 is described in section 2.8. Additionally, isolates HT19 (ST398) and PTF1P3 (ST93) were used to examine the effect of *fexA* carriage on linezolid resistance, as each isolate which carried *cfr* also carried *fexA*. ETEST® confirmed that *cfrAB* provides a clinically linezolid sensitive phenotype, but offered slightly elevated linezolid MIC when compared to *cfr*-negative controls after 48 hours (Table 5-1). *fexA* provided a very slight increase in linezolid MIC relative to the *fexA* negative RN4220, but could not provide resistance to levels matching *cfr* or *cfrAB*, after 48 hours incubation. None of the isolates displayed MIC > 8 µg/mL of linezolid resistance, however, the original MIC tests were performed with VITEK®, which is less accurate than ETEST® (477). Additionally, the 32 µg/mL MIC initially observed for *cfr* isolate HW-31 may have been the result of the isolates being 'primed' for linezolid exposure (i.e. with methylated ribosomes) following a shorter period between isolation and MIC determination. With an approximation for an isogenic strain, *cfrAB* provided 7.14% of the resistance to linezolid compared to *cfr*, where the effect of *cfrAB* alone is equal to:

$$= [(MIC_{cfrAB} - MIC_{fexA}) / (MIC_{cfr} - MIC_{fexA})] \times 100$$

$$= [(PTDrAP2 = 1.5 - PTF1P3 = 1)] / [(PTDrAP2\text{-}FM = 8 - PTFIP3 = 1)]$$

$$= [(1.5 - 1) / (8 - 1)] \times 100$$

$$= [0.5 / 7] \times 100$$

$$= 7.14\%$$

Table 5-1 – Linezolid ETEST® Minimum Inhibitory Concentration (MIC) assay of *cfr* and *cfrAB* isolates

Isolate	Genotype	[a]ETEST® MIC (24 hours)	[a]ETEST® MIC (48 hours)
RN4220	ST8. Cfr-, fexA-	0.25 µg/mL	0.75 µg/mL
HT19	ST398. Cfr-, fexA-	0.38 µg/mL	1 µg/mL
PTF1P3	ST93. Cfr-, fexA+	0.5 µg/mL	1 µg/mL
PTDrAP2	ST398. *cfr*AB+, fexA+	0.5 µg/mL	1.5 µg/mL
PTDrAP2-FM1	ST398. Cfr+, fexA+	3 µg/mL	8 µg/mL
HW-31	ST398. Cfr+, fexA+	3 µg/mL	8 µg/mL

[a] MIC results represent a single biological replicate

5.3.3 CfrA and CfrB were not predicted to be independently functional

Bioinformatic prediction of functional domains indicate that CfrA and CfrB protein should not be individually functional as a methyltransferase capable of producing linezolid resistance. The active sites of the Cfr protein were divided by the stop codon which separated *cfrA* and *cfrB*. Cfr is a Radical S-adenosyl-L-methionine enzyme which requires the coordination of an 4Fe-4S iron sulphide cluster and Cys338 of the Cfr protein to perform adenine methylation (478). Cys338, the predicted S-adenosyl-L-methionine binding sites and iron-sulphide-coordinating residues were all encoded by *cfrB* ORF of the *cfrAB* allele, occurring from residue 91 onwards (Uniprot: Q9FBG4, Figure 5-3) which suggested the C-terminus of Cfr, which is the larger portion of the protein, was essential to the function of *cfr* and that the *cfrA* portion alone should not be functional. CfrB was predicted to be insufficient for ribosomal methylation alone for the same reasons, and additionally, while there was a start codon present 74 bases after the *cfrAB* frameshift stop codon which could allow the *cfr*A-independent transcription of CfrB peptide, there was no preceding ribosomal binding site present within the coding sequence of the *cfr* gene which could have initiated translation of the peptide.

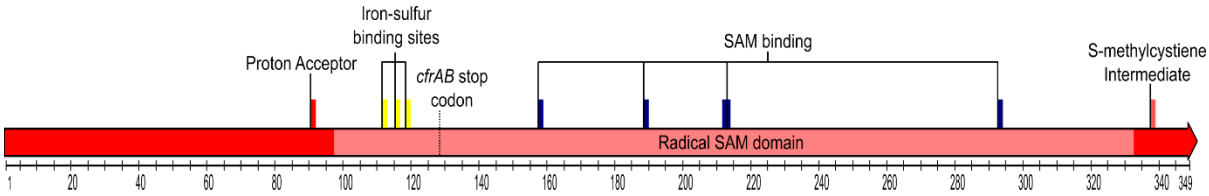
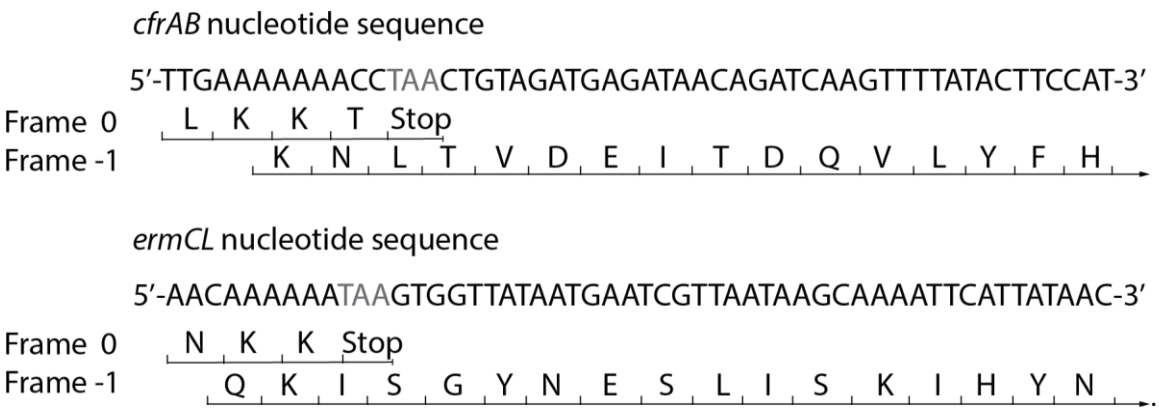


Figure 5-3 - Domains and catalytically active residues of the Cfr protein

The stop codon which divides the *cfrA* and *cfrB* portions also divides the Radical SAM domain, and separates the SAM-binding sites from the proton acceptor. The separation of the active sites strongly suggests that neither CfrA nor CfrB are independently functional as methyltransferases and therefore could not independently account for elevated linezolid resistance.

5.3.4 *cfrAB* contained a programmed ribosomal frameshift site

Functional Cfr protein was predicted to be expressed from the *cfrAB* allele, despite the presence of a disruptive frameshift deletion. *cfrAB* contains 7A poly-A tract as the result of a SNP deletion, where wild-type *cfr* contains an 8A poly-A tract. The SNP deletion caused the ORF to shift from AAC to ACC (N>T codon), and CTA to TAA (L>STOP codon), terminating the peptide (Figure 5-4)



Despite the introduction of a disruptive stop codon in the 0 coding frame, it was predicted that the slightly elevated MIC observed in *cfrAB*-carrying ST398 MRSA was the result of functional Cfr being expressed at low frequency from the *cfrAB* allele. The poly-A tract of the *cfrAB* allele SNP site bore resemblance to a characterised antimicrobial-dependent PRF (Figure 5-4). Poly-A sites can cause

ribosomes to slip into the -1 reading frame and are known PRF sites. The PRF motif occurs upstream of the *ermC* macrolide resistance gene and regulates its expression in a macrolide-dependent manner (479). The PRF-expression model-system *ermCL* contains a 6A poly-A site followed by a stop codon, which prevents the ribosome from reaching the transcriptional start codon of the *ermC* macrolide resistance gene. However, when a frameshift into the -1 reading frame occurs, the ribosome can continue along the mRNA to the start codon of the *ermC* gene, expressing the ErmC rRNA methyltransferase in full. Based on the sequence similarity between the *cfrAB* allele and *ermCL,* the SNP deletion of *cfrAB* was predicted to function as a PRF regulator of Cfr expression.

Figure 5-4 - Comparison of the *cfr*AB frameshift deletion site with the *ermCL* frameshift site

Sequences of PTDrAP2 *cfrAB* and the *ermCL* leader region, and corresponding amino acid codons of the 0 and -1 reading frames are shown. Similar poly-A sequences were present in each region adjacent to a stop codon in the 0 frame. The -1 reading frames of each sequence were uninterrupted downstream. Translation of a peptide beyond a stop codon of the 0 frame may occur when the ribosome slips into the -1 frame at a PRF site, such as the 8A seen in each sequence. The similarity of the sequences suggests regulated *cfrAB* expression may occur, despite the 0 frame stop codon, via a PRF in the same way *ermCL* PRF regulates expression of *ermC.*

5.3.5 Reporter assay detection of frameshifting and quantification of frameshift frequency

To further quantify the rate of *cfrAB* frameshifting, a reporter assay was devised where a fragment of the *cfrAB* allele, comprising 127 bp of the predicted frameshift site, was cloned in-frame into the *lacZα* gene of plasmid pUC19 to produce a *cfr-lacZα* fusion gene. Expression past the stop codon, when a frameshift occurs, results in the production of a Cfr-β-galactosidase fusion protein. The rate of frameshifting can be quantified by measuring the fluorescence generated by the degradation of a fluorogenic substrate, methylumbelliferyl green, by β-galactosidase. To determine the maximum and minimum levels of expression from the reporter assay for relative quantification of the *cfrAB* allele frameshifting rate (from construct pUC19-*cfr*AB), two additional constructs were cloned. A positive control (pUC19-*cfr*) was cloned containing the same region as the *cfr*AB construct, but with an 8A poly-A tract (the native *cfr* sequence) allowing constitutive expression. A negative control (pUC19-*cfr*Neg) was designed, comprising the *cfrAB* fragment with an additional codon-swap to create a secondary downstream stop codon in the -1 reading frame, to halt translation in the -1 reading frame after a ribosomal frameshift occurs (Figure 5-5). The reporter assay was performed in microplates (as per section 2.7) and the output was expressed as relative fluorescence units (RFU). β-galactosidase activity measured 251.53 RFU from pUC19-*cfr* positive control strains, 5.72 RFU from pUC19-*cfr*AB test strains, and 0.29 RFU from pUC19-*cfr*Neg negative control strains, which indicated the frameshift of *cfrAB* occured at 2.27% of the rate of the wild type *cfr.*

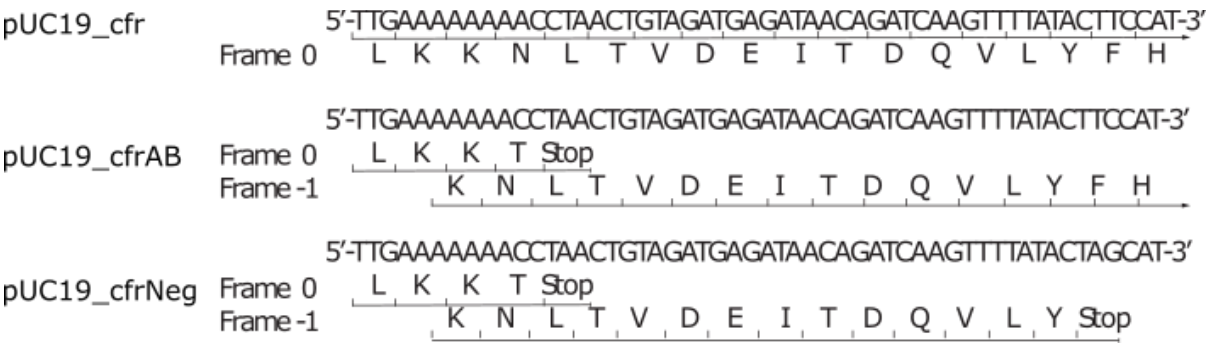


Figure 5-5 - Frameshift expression-assay construct frameshift-site sequences

Plasmid construct frameshift sequences are labelled (left). Partial nucleotide sequences are shown, comprising PRF site and downstream sequence, with corresponding 0 and -1 reading frame amino acid

codons labelled below. pUC19-*cfr*Neg contains an additional stop codon in the -1 reading frame to halt expression in the event of a -1 frameshift.

5.3.6 Proteomic confirmation of *cfr*AB -1 frameshift

To identify the potential site of *cfrAB* -1 ribosomal frameshifting, a histidine-tagged fusion protein was cloned into a protein expression vector, containing the poly-A tract of *cfrAB*, for expression and visualisation on gel, and subsequent peptide sequence confirmation. Based on the relative predicted physical mass of CfrA and CfrAB peptides, each peptide should be visible on SDS-PAGE gel and differentiated by size of the protein products expressed. A *cfr*AB-*lacZα* fusion protein was initially cloned using the pETM-11 plasmid and was used for protein expression, however, SDS-PAGE did not produce strong bands of predicted size. Following the failure of the pETM-11 *cfrAB-lacZα* constructs to produce significant protein, bioinformatic screening of the pETM-11 *cfrAB-lacZα* fusion protein was performed using the protein solubility evaluator web tool, PROSO II (480) which predicted the fusion protein was insoluble. Insolubility of the fusion protein may have caused the fusion protein to be lost in the insoluble fraction during protein purification, producing poor target protein yield and therefore low visibility of bands on SDS-PAGE gel.

To resolve the potential insolubility issue, a 127 bp region of *cfrAB* was cloned into protein expression vector pETM-41, comprising the 7-A frameshift site, 69 bp upstream and 51 bp downstream to produce pETM-41-*cfr*AB-sfGFP. The pETM-41 multiple cloning site contains maltose-binding protein and a 6-His tag for purification, improving the predicted solubility of the protein and providing a secondary means of purification by amylose resin affinity. Additionally, to aid in the differentiation of frameshifted and non-frameshifted protein products by size following the use of a short frameshift fragment of *cfrAB* rather than the full-length *cfrAB* allele, green fluorescent protein (GFP) was cloned in-frame after the frameshift fragment. The fusion protein is under control of a T7 promoter and T7 terminator. Two proteins products were expected to be expressed from the 6-His-MBP-*cfr*AB-sfGFP

gene; 6-His-MBP-CfrA (436 amino acids, with a predicted molecular weight of 48 kDa) and 6-His-MBP-Cfr-sfGFP (694 amino acids, predicted molecular weight of 77 kDa) (Figure 5-6).

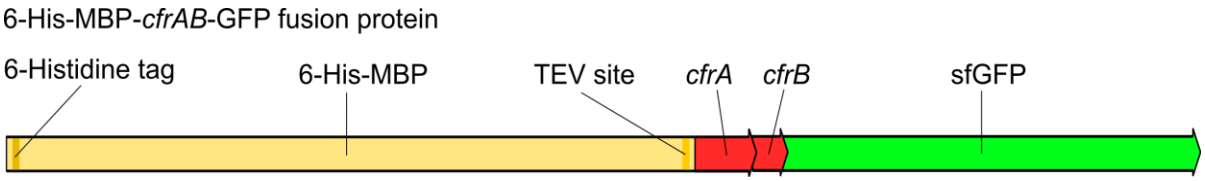


Figure 5-6 - MBP-*cfr*AB-sfGFP fusion protein

The fusion protein components are delineated by colour and are labelled.

As size-exclusion purification did not improve the purity of the protein and may have resulted in the loss or dilution of desired protein (Figure 5-7), amylose resin purification was used in an attempt to further purify the nickel-column eluted protein. 1 mL of each nickel-purification fraction was loaded on to a washed amylose resin column, washed with 10 mL PBS and eluted with 5 mL PBS containing maltose. Following amylose resin purification, fractions of the eluted protein were visualised on SDS-PAGE gel. The 77 kDa 6-His-MBP-CfrAB-sfGFP product became visible (Figure 5-8) and non-specific products were virtually eliminated. The presence of the 77 kDa product indicates that a frameshift had occurred.

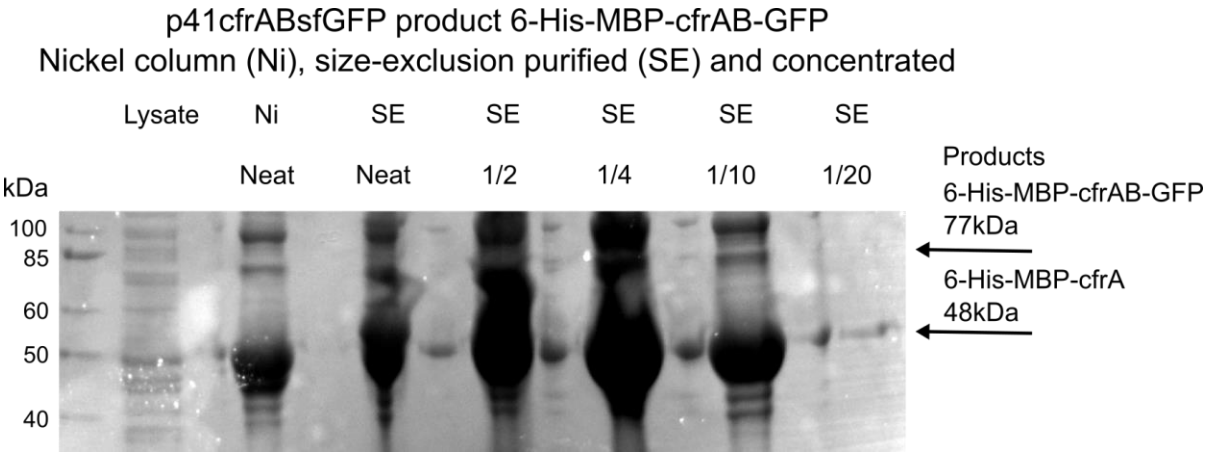


Figure 5-7 - SDS-PAGE visualisation of nickel-column and size-exclusion column purified protein fractions.

Top row labels indicate purification type; un-purified (lysate), nickel-column purified only (Ni), nickel-column and size-exclusion column purified (SE). Second row labels indicate dilution.

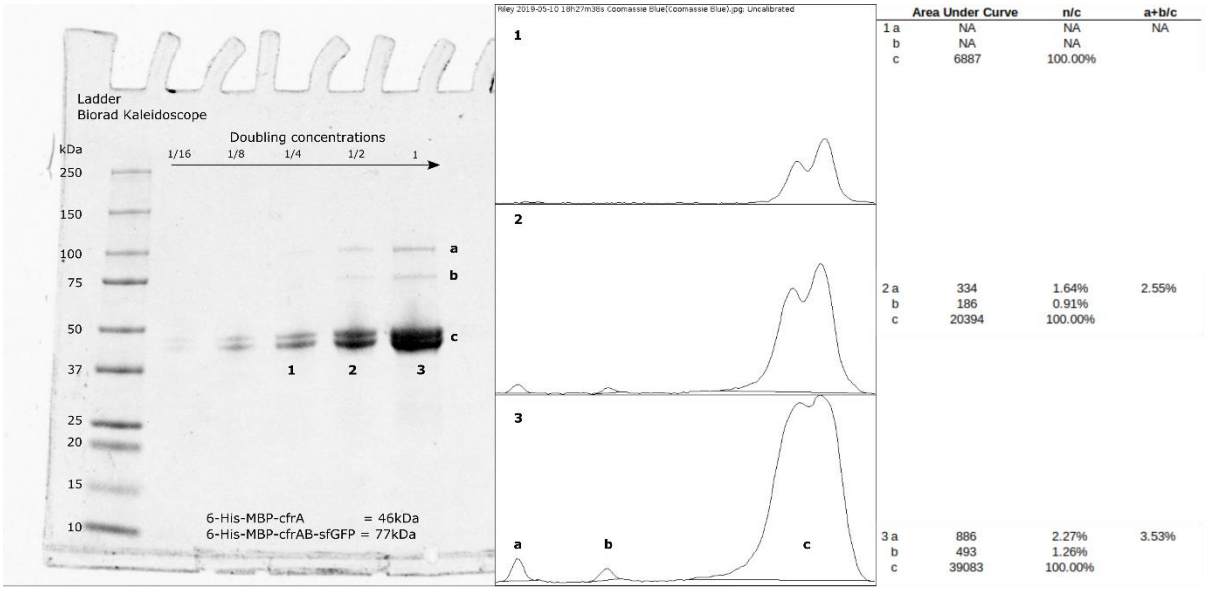


Figure 5-8 – Visual quantification of protein expression

To quantify the relative expression of frameshifted (6-His-MBP-CfrAB-sfGFP) and non-frameshifted (6-His-MBP-CfrA) fusion-protein products, gel image analysis was performed in ImageJ. The pixel intensity of protein bands was quantified, which indicated that expression of the 6-His-MBP-*cfr*AB-sfGFP product occurs at approximately 1.6% of the 6-His-MBP-CfrA product expression.

To confirm whether the band appearing at 77kDa was the expected 6-His-MBP-*cfrAB*-sfGFP product and had been produced as a result of a -1 frameshift within the 8A predicted PRF site, the ~40, 48, 77 and ~100 kDa bands were excised from the SDS-PAGE gel and sequenced by Proteomics International. Protein samples were analysed by electrospray ionisation mass spectrometry and the resulting spectra were analysed to identify proteins of interest against a custom database (p41*cfr*ABsfGFP, Figure 2-1) containing predicted frameshift peptides (described in section 2.6.4). Whilst complete coverage of the 6-His-MBP-CfrAB-sfGFP protein was not obtained, peptide sequencing confirmed peptides from up and downstream of the predicted frameshift site sequence. Importantly, a peptide which would only be produced if a -1 frameshift occurred within the 7-A predicted PRF sequence was identified (bold text, Figure 5-9). No matches were identified for any of the peptides produced by alternative (i.e. +1 or +2) frameshift events.

MKHHHHHHPMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGG
YAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNL
QEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWA
WSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEEL
AKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNNNNNPMSENLYFQ
GAMAMTMITPSFCISSQCGCNFGCKFCAT**GDIGLKKNLTVDE**ITDQVLYFHLLGNSMSKGEELFTGVVPILVELDGDVNG
HKFSVRGEGEGDATIGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDD
GKYKTRAVVKFEGDTLVNRIELKGTDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFTVRHNVEDGSVQLADHY
QQNTPIGDGPVLLPDNHYLSTQTVLSKDPNEKRDHMVLHEYVNAAGITLGMDEL

Figure 5-9 - Peptide sequencing coverage of 6-His-MBP-CfrAB-sfGFP fusion protein.

Red text indicates residues identified by peptide sequencing. Bold text indicates target peptides produced by a -1 ribosomal frameshift event.

The elution of fluorescent protein, presence of protein bands on SDS-page which corresponded to the predicted sizes of frameshifted and non-frameshifted peptides and the presence of key frameshift-product peptides indicated that a frameshift had occurred within the 7A predicted PRF sequence adjacent to the stop codon formed in *cfrAB*, and confirms that expression of a -1 frameshifted product was able to proceed beyond a stop codon. The relative abundance of CfrA and CfrAB products as visualised on SDS-page indicated that CfrA products comprised the vast majority of peptide products from the *cfrAB* allele. However, Cfr is expressed at a reduced rate of 1.6% of the CfrA products. The findings collectively suggest that the subclinical linezolid resistance provided by *cfrAB* is the result of intermittent Cfr expression, resulting from a -1 ribosomal frameshift occurring in the poly-A tract of the *cfrAB*.

5.3.7 *cfrAB* can spontaneously mutate to *cfr*

While initially trying to determine the MIC values of *cfrAB* isolates to phenicols, mutant colonies appeared on LB agar media containing 80 µg/mL of florfenicol, particularly after 36-48 hours of growth. When sub-cultured on to high concentrations of florfenicol media, growth of mutant colonies was confluent and comparable to the native *cfr*-carrying isolate, HW-31. I hypothesised the sudden increase in MIC was due to an insertion mutation in the poly-A tract, restoring *cfrAB* to *cfr,* restoring the function of *cfr* and thus providing florfenicol resistance. To confirm that the sudden increase of MIC values after extended incubation of *cfrAB*-carrying isolates was due to mutation of *cfrAB* to *cfr*, three colonies were picked from the experiments, whole-DNA extracted and sequenced to confirm the *cfrAB*>*cfr* mutation. Genomic DNA was amplified with *cfr*-screening primers (forward and reverse) and the resulting PCR products were sequenced in each direction using the same primers. Sanger sequencing was performed as per section 2.5.1. Results indicated that high-resistance mutants were *cfr* positive.

Additionally, four PTDrAP2 *cfrAB*>*cfr* florfenicol mutants and one chloramphenicol mutant were colony purified and WGS was performed on the MiSeq platform. These mutants were genetically identical to the parent isolate (PTDrAP2) except for the *cfrAB*>*cfr* insertion. Notably, the isolate grown on chloramphenicol-containing agar was able to tolerate 700 µg/mL of chloramphenicol. The isolate, dubbed PTDrAP2-Cm700, had an additional SNP mutation in a non-coding region (position 45205 C>A) in addition to a *cfrAB*>*cfr* mutation. The significance of the additional mutation is unknown, however, its location in a non-coding sequence may indicate it has no function and is merely random. Our data indicate the *cfr*AB allele readily mutates to *cfr* by an insertion in the poly-A tract and that the mutation increases the MIC of the isolates rapidly. Additionally, *cfr* can provide high levels of resistance to chloramphenicol.

5.3.8 Quantification of *cfrAB>cfr* mutation rate

Mutation rates were determined as described in section 2.8. Mutation ranged from 1.55 x 10^{-8} to 3.1 x 10^{-9} colony-forming units/mL. No resistance was observed in the genetically similar non-*cfr* (though *fexA*-positive) isolate PTF1P3 or laboratory strain RN4220 (Table 5-2), which suggested resistance was due to the presence of *cfrAB>cfr*.

Table 5-2 - Florfenicol *cfrAB>cfr* mutation rate

Isolate	[a]Average mutation rate (colony-forming units/mL)
PTGrBP1	2.1 x 10^{-8}
PTGrAP4	9.2 x 10^{-9}
PTDrAP2	1.2 x 10^{-9}
PTF1P3	0
RN4220	0

[a] Mutation rate represents the average of 3 biological and 3 technical replicates for a total of 9 replicates for each strain.

To confirm that the observed resistance was the result of mutation, one colony from each florfenicol mutation plate was colony purified twice and raised in 5mL broth culture. DNA was extracted and the *cfr* genes were PCR amplified. The PCR products were sequenced and four of six mutants carried *cfrAB>cfr* insertions in the poly-A tract. The two resistant non-mutants were PTGrAP4 isolates, which suggested either *cfr* reverted back to *cfrAB* during colony purification and broth culture, or that carriage of *cfrAB* alone was sufficient to provide resistance to florfenicol at 80 µg/mL.

5.4 Conclusions and discussion

I have identified a novel variant of the *cfr* 23S rRNA methyltransferase gene found in LA-MRSA isolated from Australian pigs and explored its function. Cfr provides resistance to antimicrobials frequently used in agriculture from multiple antimicrobial classes including lincosamides, phenicols and streptogramin (176). Among four *cfr*-positive ST398 MRSA, a *cfr* variant was detected, *cfrAB*. The variant contained a SNP deletion resulting in a premature stop codon, which interrupts the gene at 1/3 of the ORF. The *cfrAB* gene however, still provides higher phenicol and linezolid resistance than comparable, *fexA*-carrying *cfr*-negative isolates. I have demonstrated gene expression can occur beyond the stop codon as a result of a -1 frameshift through proteomic experiments and by a reporter assay, suggesting the production of functional Cfr from the *cfrAB* allele accounts for the slight phenicol/linezolid resistance observed. Proteomics and reporter assays indicate *cfrAB* expression occurs between 1-2.5% of *cfr* expression, while the linezolid MIC of *cfrAB* isolate PTDRAP2 is around 19% of PTDRAP2-FM *cfr* isolates (which are genetically identical to PTDrAP2 aside from the *cfr*>*cfrAB* mutation). This discrepancy may be due to the sensitivity and reliability of each assay used. For each lacZ reporter assay, there were at least three biological and three technical replicates producing a lot of data, which averages out the outliers. ETESTs were unfortunately only performed in a single biological replicate, so the sensitivity data more of an indication than a true measure of MIC. The ETEST may have been more informative with more paired isolates, for example *cfrAB*>*cfr* mutants of PTGrBP1 and PTGrAP4 to compare. Previous attempts to clone cfr and cfrAB onto a *S. aureus* shuttle vector and express the genes in RN4220 failed to demonstrate any appreciable change in MIC at all. Such a failure to demonstrate a linezolid resistance phenotype from a cloned AMR gene on a constitutively expressing plasmid does suggest that other features of the Tn*558*-cfr element may be important in the expression of *cfr*.

What benefit could LA-MRSA gain from the deprecation of an AMR gene? I initially hypothesised that the *cfr* variant *cfrAB* may have evolved as a result of the fitness cost associated with *cfr*-mediated ribosomal methylation. The fitness cost of *cfr* has previously been demonstrated to be negligible (481).

However, I envision that ribosomal methylation by *cfr* could impose a fitness cost on the host under certain conditions, for example the presence of multiple ribosomal methylating AMR genes being expressed in the same cell. Many of the ST398 examined in Chapter 4 carried *ermC* ribosome-methylating AMR genes, while the *cfr* strain HW31 was one of only two ST398 MRSA which did not encode *ermC.* It is possible that *ermC* is too deleterious to express alongside *cfr,* as both AMR genes methylate rRNA near the same site: *ermC* methylates the A2508, while *cfr* methylates the A2503 site (482, 483). Further work could easily determine if the two AMR genes compete and cause a fitness cost.

The SNP deletion of *cfrAB* may be a random event and may represent deprecation of the gene. Long poly-A sequences cause ribosomal slippages, resulting in erroneous, extended peptides and are thus selected against in bacterial coding sequences (484). Alternatively, the SNP may be a desirable feature, greatly reducing the expression of *cfr* whilst retaining the ability to rapidly revert to full-length wild-type *cfr* to increase expression when therapeutic concentrations of phenicols and linezolid are encountered. I found *cfrAB* isolates mutate readily to *cfr* and achieve AMR at concentrations matching the wild-type *cfr* isolate HW-31, and at mutation frequencies which may be biologically relevant. In mutation rate assays, resistant mutants typically emerged at 36-48 hours incubation. Linezolid is bacteriostatic and so this time period could represent the time it takes for frameshift correction to occur. Sequencing confirmed that *cfrAB*>*cfr* reversion mutation had occurred in each instance that a mutant colony emerged. Growth-based susceptibility methods for detecting phenotypic resistance typically do not extend to beyond 24 hours, raising the possibility that a *cfrAB* isolate may be falsely called linezolid sensitive. Linezolid treatment could fail as an infecting *cfrAB* isolate mutates to *cfr* and gains clinical resistance. The phenomenon of pathogenic bacteria harbouring 'silent' AMR determinants which are reactivated during treatment and cause treatment failure has been recently recognised and the genes designated as a SARM (silencing of antibiotic resistance by mutation) (485). These 'SARMs' were identified in up to 10% of isolates (152/1470), frequently arose through mutation within poly-A tracts within AMR genes and reverted to AMR phenotypes at frequencies of $>10^{-9}$. Notably, the phenomenon was observed for several resistance determinants including macrolide, lincosamide and tetracycline resistance, but did not identify a *cfr* SARM.

The use of macrolides and phenicols, particularly florfenicol, in pigs may be a key factor in the development of linezolid resistance development and dissemination. *Cfr* was discovered 5 years after the market release of florfenicol in Germany (176). Linezolid was not approved for clinical use until 2000 (486) and has maintained legal protection in Australia from use in food-producing animals. Florfenicol is widely used in the preventative treatment of pigs in China however, and the contamination of soils with florfenicol residue has resulted in the enrichment of florfenicol resistance genes of soil bacteria, including the *cfr* gene (487).

Linezolid is extremely valuable for the treatment of multidrug-resistant tuberculosis, however, the prolonged use of linezolid in tuberculosis treatment may also be a source of linezolid resistance development in commensal species. The use of linezolid in the treatment of multidrug-resistant tuberculosis can be prolonged (up to 32 months) (488) and may necessitate reduced dosing frequency to reduce mitochondrial toxicity (489). Prolonged exposure to linezolid at reduced selection pressure may provide an ideal environment for the formation of an AMR reservoir and subsequent evolution of a genetically stable and regulated *cfr* MGE in human commensal species (both staphylococcal and coliform). Carriage of *cfr* in tandem with methicillin and/or vancomycin resistance in gram-positive bacteria greatly reduces the available treatment options for common, but serious, infectious diseases.

cfr also threatens the effectiveness of agriculturally important antimicrobials such as phenicols and lincosamides. Agricultural use of antimicrobials conflicts with human use because of cross-resistance. Whilst the use of multiple classes of antimicrobials in pig production ensures economic productivity, food security and animal welfare, it drastically increases the potential for *cfr* to become established in Australian LA-MRSA. Continued use of drugs which select for *cfr* will allow the continued dissemination and evolution of the *cfr* gene in staphylococci and other human-infectious bacteria. Ultimately, the *cfr* gene has the potential to become as genetically complex, mobile, disseminated and problematic as *mecA* of SCC*mec* or *blaZ* of Tn*552*. Close monitoring of antimicrobial use and surveillance of *cfr* should

continue to prevent the development of a highly-regulated *cfr* MGE and subsequent evolution of untreatable multidrug-resistant pathogens.

Chapter 6

Conclusions and discussion

6.1 Introduction

MRSA is a cause of infections in hospitals, the community and in animals including livestock. MRSA rapidly adapts to antimicrobial use by acquiring AMR genes. Although LA-MRSA has received relatively little scientific attention in Australia, compared to HA and CA-MRSA, recent research suggests infections of humans by MRSA from animals is an increasing problem with the potential for severe and fatal infections. It is therefore important to monitor Australian LA-MRSA and to determine the extent of LA-MRSA carriage and transmission. Livestock are not the only animal reservoir of MRSA. Domestic pets and companion animals such as horses are known carriers of MRSA and may also represent a significant source of zoonotic MRSA transmission. Human and animal interactions are the key to zoonotic transmission of MRSA and may also come with indirect consequences, such as the sharing of MGEs, which assist the adaptation of MRSA to new biological niches.

My thesis examines two separate instances of zoonoses in Australia. The first study (Chapter 3) examined equine-associated ST612 MRSA, following the bloodstream infection of a patient with close association with horses. Australian ST612 MRSA was genetically compared with South African human clinical ST612 MRSA isolates to determine the origins and characteristics of Australian ST612 MRSA, and identify common molecular factors which would allow ST612 to thrive in each environment. The second study (Chapters 4 and 5) examined Australian ST398 LA-MRSA and ST93 CA-MRSA discovered in Australian pig farms during a previous MRSA surveillance study, following recurrent infections in the farm workers. This current work aimed to identify evidence of molecular adaptation in the different lineages, examine the exchange of MGEs, which may have supported host switching of CA- and LA-MRSA. Chapter 5 characterised a novel linezolid resistance gene variant (*cfrAB*) and determined a mechanism of expression of the gene which was not found in wild-type *cfr.*

6.2 Findings and significance

6.2.1 MRSA may become established in Australian animals

Phylogenetic analysis of Australian and South African ST612 MRSA suggested there are distinct human and equine-associated ST612 MRSA lineages. The equine lineage was genetically stable, as evidenced by only minor core genome variation and minor variation in the carriage of MGEs, despite isolates being gathered from across the Australian continent and across a period of eight years (2008-2016). Additionally, a genomic analysis of Australian equine ST612 MRSA revealed a close genetic relation to the highly virulent ST8 USA500 2395 strain, sharing features attributed to its 'hyper-virulence'. Chapter 4 examined isolates from a 2017 MRSA surveillance study of Australian pig farms and farm workers, which identified ST398 MRSA and ST93 MRSA. The ST398 MRSA carried molecular features which are shared by LA-MRSA in Europe (e.g. Germany, Denmark, the Netherlands, Italy etc.) and North America, including spa type t011, SCC*mec* type V (5c) and tetracycline resistance *tetM*. ST398 LA-MRSA has previously been identified in Australia from 2009-2010 (490), carried by an Australian veterinarian and at low frequency in a pig herd (0.9%) in NSW. Sampling of pigs in 2015 found 75% MRSA carriage, of which 29% were ST398 ((491), the isolates examined in this thesis). The increase of prevalence of LA-MRSA in the study of Australian pig farms and farm workers relative to the study five years prior suggests ST398 LA-MRSA is gaining a foothold in Australian pig farms. Chapter six of the 2018 PhD thesis of Shafi Sahibzada details another Australian LA-MRSA surveillance study which found lower rates of MRSA carriage on Australian pig farms. In this 2018 study, isolates were collected between January – October 2017 from 26 commercial pig herds and saw a decrease in MRSA carriage in pigs (40.28%) and overall decreased MRSA detection (53.9%) with detection of MRSA from 14 of 26 commercial herds (492). This is a significant decrease over the span of two years (from the date of isolate collection of the 2017 study), however it may indicate that the first study was simply too restricted in size (two farm sites) to infer the status of LA-MRSA across Australia. While the later study observed lower carriage, more than half of pig herds carried LA-MRSA. Notably, ST93 CA-MRSA was absent from any of the 26 pig herds (492), which suggests that the observation of ST93 CA-MRSA may have been an anomaly, or more hopefully, that infection control measures taken on the farm were successful in decolonizing the pigs and farm workers.

While the prevalence of LA-MRSA on Australian pig farms has appeared to decrease, a more recent study examining ST398 in Australia-wide *S. aureus* bacteraemia identified ST398-V (5C2&5) as the cause of invasive infection (and two deaths) in 4 Australian states. The authors suggested that ST398 is now likely widespread in Australian piggeries and their data suggest that ST398 MRSA does indeed have the potential for significant zoonotic disease (493).

The potential for these AMR genes to propagate in an antimicrobial-rich environment like a pig farm and be mobilised into CA-MRSA carried by farm workers, with close contact to pigs, to then be transmitted through the community is cause for concern. Greater regulation of livestock antimicrobial use and reporting appears necessary to prevent such a scenario. Additionally, continued microbial surveillance of pigs and farm workers is essential for a successful One Health approach, to identify reservoirs of AMR and infectious diseases in animals and implement measures to control them.

6.2.2 Use of phenicols has likely changed over time and may promote linezolid resistance

Florfenicol use in Australia has been insignificant, accounting for only 0.3% of antimicrobials sold for veterinary use in 2009 (494). Rates of florfenicol use in Australia after 2009 could not be found, however, the Australian antimicrobial prescribing guidelines for pigs 2020 (495) recommends florfenicol as a first-line treatment for pig *Actinobacillus pleuropneumoniae* respiratory disease for use in water, feed or intramuscular injection and it is therefore likely that florfenicol use has increased. A single *fexA*-positive MRSA had been reported in Australia in 2005, while *cfr* had not been detected in Australia before the 2017 study by Sahibzada et al (366). As AMR correlates strongly with antimicrobial use, the sudden spike in florfenicol resistance suggests florfenicol use may have risen significantly in Australia. Florfenicol has low human medical importance (496), so why is this an issue? *fexA* provides resistance to chloramphenicol, which is an antimicrobial of modest human importance due to toxicity and is not permitted for use in animals (497). More importantly though, the mobile genetic element which carries *fexA,* Tn*558*, also carried *cfr* (Chapter 5). A genetic linkage between *fexA* and *cfr* is apparent in other studies, where *cfr* is often integrated into Tn*558* by means of an IS element (Figure 4-6(498). The *cfr* gene also confers resistance to florfenicol and chloramphenicol, but more importantly, provides resistance to linezolid, an antimicrobial used to treat gram-positive bacteria which are resistant to multiple classes of antimicrobials including MRSA and vancomycin-resistant *Enterococcus* species. Tn*558* was found in 100% of ST93 MRSA and 45% of ST398 MRSA, of which four ST398 carried Tn*558* with integrated IS*256-cfr or* IS*256-cfrAB.* The novel configuration of IS*256-cfr/cfrAB* integrated into Tn*558* suggests either Tn*558*-IS*256-cfr* was widespread before losing IS*256-cfr*, or Tn*558* gained IS*256-cfr*/*cfrAB*. If the latter is the case, the ST93 carrying Tn*558* have the potential to acquire *cfr* via IS*256* recombination. The ability of *cfrAB,* discovered in this study, to spontaneously revert to *cfr* has serious clinical implications. Phenotypic AMR screening may fail to detect *cfrAB,* leading to mutation reversion throughout the course of antimicrobial treatment, which would extend hospital stays for patients and could lead to treatment failure. Data on the use of florfenicol in Australian pigs is outdated and may have increased significantly since the last survey in 2010 (494), and an increase in the use of florfenicol may explain the widespread carriage of florfenicol resistance.

Regardless, the use of florfenicol indirectly promotes linezolid resistance through the genetic linkage of *fexA* with *cfr* and the cross-resistance provided by *cfr* to florfenicol and linezolid.

6.2.3 Human-animal interactions provide an opportunity for AMR transfer between MRSA lineages

In the 2017 LA-MRSA surveillance study by Sahibzada *et al* (366), the authors concluded ST93 MRSA isolated from pigs were not indigenous to pigs, but were more likely transmitted from humans to animals. The authors also noted that the ST93 MRSA was showing signs of adaptation to pig hosts and loss of human virulence factors such as bacteriophage-encoded PVL-associated *luk-F PV* and *lukS-PV* genes. Within the pig farm environment, and in close proximity with ST398 LA-MRSA, it appears that ST93 MRSA acquired AMR through MGE transfer. ST93 MRSA are typically only resistant to β-lactam antimicrobials, and are resistant to ciprofloxacin and macrolides at a rate of < 2%. In this study, 82% of isolates carried a macrolide-resistance plasmid, 100% of isolates carried the Tn*558* florfenicol/chloramphenicol resistance transposon, and 26.7% carried a tetracycline resistance plasmid. While not all of these elements were shared with ST398, Tn*558* and the macrolide resistance plasmids have been linked to ST398 and were likely transmitted from ST398 to ST93, despite the known multiple RM system barriers of ST93. An examination of the RM systems of ST93 in this study found several pig-isolated ST93 which had lost all of their RM system genes (Table 4-3), increasing the probability that ST398 had shared some MGEs with ST93. The apparent loss of RM systems, barriers to HGT, upon taking residence in a new pig host may have been a survival necessity, to allow the uptake of AMR genes for rapid adaptation to an antimicrobial-rich environment. If antimicrobial selection pressures did facilitate the loss of the ST93 RM barriers to HGT, then antimicrobial selection pressures from the heavy use of antimicrobials on pig farms, in a broader sense, promotes AMR dissemination by decreasing the barriers to AMR MGE HGT.

The ST398 and ST93 MRSA examined in the present study carried several MGEs which are associated with LA-MRSA (section 4.4.1). The MGE conferred resistance to antimicrobials including β-lactams, tetracyclines, phenicols and macrolides, suggesting the isolates have had significant exposure to the antimicrobials used in a pig farm environment. The resistance genes observed in the present study align with the 'frequently used' antimicrobials reported by Australian pig farmers Sahibzada's thesis (492), which included penicillin (β-lactams), amoxicillin (β-lactams), oxytetracycline (tetracycline), tylosin (macrolide), tulathromycin (macrolide), lincomycin (macrolide) and florfenicol (phenicol). The presence of these AMR genes in ST93 MRSA suggests that ST93 MRSA has received MGE by HGT and that it may have be adapting to pig colonisation. The major concern is that although many of these antimicrobials are only used in animals, such as tylosin and florfenicol, they can select for AMR genes such as *ermC* and *cfr,* which confer resistance to critically important human antimicrobials, such as azithromycin, erythromycin (145, 499) and linezolid (193).

The pRGMX plasmids identified in this study carry an uncharacterised, putative resistance gene *rgmX.* Although the function of *rgmX* is unknown, many *rgmX*-bearing plasmids can be found on GenBank (4.4.1), isolated from livestock-associated staphylococci, suggesting *rgmX* provides some survival benefit in a livestock environment. These pRGMX plasmids were detected in both ST398 and ST93. For ST93, an MRSA which typically carries very few AMR genes and has few MGE, to have acquired AMR genes never before documented in ST93 in addition to plasmids with genes of unknown significance is concerning. The implications of ST93 acquiring AMR through the transfer of MGEs are two-fold. Firstly, it suggests ST93 may be in the early stages of adaptation to pig colonisation where it may become a veterinary and zoonotic threat; and secondly it suggests ST93 can readily acquire multiple AMR genes, making ST93 infection harder to treat. ST93 is currently the dominant CA-MRSA in Australia and is associated with severe illness. If ST93 became adapted to pig colonisation, while retaining human virulence capability, then pigs could become a significant reservoir for human multi-resistant zoonotic MRSA infection.

6.3 Strengths, weaknesses and future directions

The work published in this study gave some insight into the nature of Australian equine and LA-MRSA, detecting possible instances of potential equine MRSA zoonosis, potential transfer of MGE between Australian CA-MRSA ST93 and LA-MRSA ST398 lineages, and identified a novel linezolid-resistance gene variant with clinical implications. There were, however several limitations to these studies.

Each of the major findings presented in this thesis may simply be isolated events.

Although ST612 MRSA notifications in Western Australia were rising up until 2016 (section 3.1.1), ST612 has not become a significant source of disease and has not been reported in Western Australia. Globally, ST612 MRSA reports since our 2019 study (500) have been sporadic, causing disease in Canadian horses (501), as the predominant MRSA detected in the survey of a South African poultry farm (502) and in screening of Tanzanian healthcare workers (503). The adaptation of ST93 MRSA to pigs and the transmission of MGE between these and ST398 MRSA may not represent an ongoing problem. A larger follow-up MRSA-surveillance study of Australian pig farms was described in the thesis of Sahizada (492) which failed to find ST93. Similarly, there have been no reports of clinical linezolid resistance in Australia (308), nor any *cfr*-carrying MRSA in Australia since the 2017 parent study (366). While it is fortunate reports of zoonotic ST612 infection have stopped, ST93 MRSA in pig and pig farms have become undetectable and linezolid resistance has not been reported in Australia, it reduces the real world significance of the work presented in this thesis. However, the COVID-19 pandemic has greatly reduced global and domestic travel, reducing opportunities for MRSA transmission and this may change as travel resumes. Additionally, there is no ongoing surveillance of livestock and equine MRSA. ST612 MRSA may still persist in Australian horses, ST93 may have re-entered and become prevalent in Australian pig farms and *cfr* may still be circulating among Australian LA-MRSA. Without ongoing surveillance, these and other potential public health challenges may be developing undetected.

Technical/methodological limitations

Many of the hypotheses formed and tested throughout this thesis could not be definitely proven by the techniques employed. In trying to determine transmission of MRSA between humans and horses, and of AMR between lineages of MRSA, the phylogenies constructed did not allow me to draw strong conclusions. For the ST612 study, the geographic separation of MRSA isolation (NSW, South Africa and WA) limited the ability to make inferences of direct MRSA transmission. Phylogenies constructed for the inference of transmission of AMR between CA-MRSA and LA-MRSA did not take into account recombination and no test was performed to determine the optimal evolutionary model for my datasets. Although skipping these steps produces phylogenetic trees with inaccurate branch lengths, tree topology is still reliable (375, 379). Producing phylogenies based on the most appropriate evolutionary model, and accounting for recombination may have allowed me to make stronger inferences from my phylogenies.

Failure to demonstrate MGE transfer between ST93 and ST398 under laboratory conditions undermines the hypothesis that ST398 transferred MGE (*ermC* plasmids and Tn*558*) to ST93. Attempts were made, performing broth conjugations, to effect conjugative transfer of MGE between ST93 and ST398 MRSA. These attempts were unsuccessful and were therefore not included in this study. The inability to demonstrate MGE transfer under controlled conditions reduces the probability of HGT occurring between the lineages in the wild. Attempts were not made to transduce the MGE across lineages, however and so this still remains a possible mechanism of HGT for the MGE identified in the study which do not encode conjugation genes, and do not encode *oriT* sequences which would facilitate conjugative mobilisation. Additionally, as nine ST93 MRSA were found to lack detectable RM systems (Table 4-3 - RM System integrity of ST93) and two ST398 were found to carry no *hsdM* methylase, these isolates should have been selected for HGT studies, as they encode the least barriers to HGT.

Attempts to characterise the functional capability and biological significance of *cfrAB* did not employ robust MIC detection. The prohibitive cost of linezolid led to attempts to use microplates and a microplate reader to perform broth microdilution MIC assays failed to produce reliable data, due to

technical complications (contamination of control wells, uncontrolled evaporation of sample, followed by the use of non-breathable plate seals). The data generated from these assays was therefore discarded. ETEST assays were performed, however reproducibility, reliability and sensitivity of ETEST is lower than broth MIC assays (504). Regular broth MIC assays should have been performed instead. Additionally, the biological significance of *cfrAB* in ST398 could not be precisely determined conclusively with the isolates used in this study. Comparison of ETEST MICs of PTDrAP2 and *cfrAB>cfr* mutant PTDrAP2-FM provided an indication of *cfrAB* biological effect, however mutants should have been developed and MIC tested for each of the *cfrAB*-carrying ST398.

Future directions

The present study has left open many avenues for future study. Several novel SaPI were identified in ST612 MRSA. These SaPI contained uncharacterised genes within the accessory gene region of the SaPI, where virulence genes are typically located in characterised *S. aureus* SaPI. Study of SaPI isolated from ruminants detected an alternative von Willebrand factor-binding protein, encoded in the accessory region, which was specific to ruminant and equine prothrombin, potentially facilitating host adaptation (427). The uncharacterised genes in the ST612 SaPI may also allow host adaptation, which is a very interesting theory when considering SaPI detected in South African patients – who carried both equine-associated SaPIsvh7513 and SaPItkn92 or SaPIthw70, the latter two which were only found in human-isolated ST612. These SaPI could provide host-adaptive virulence factors for both human and animal colonisation, which has significant implications for zoonosis and anthroponosis, and warrants further study.

Plasmids encoding rgmX were widespread in ST398 and ST93, and are apparently widespread in the publicly avaible genomes of LA-MRSA and livestock commensal staphylococci (4.4.1). The function of *rgmX* has not been studied or published, despite the apparent ubiquity of this gene in staphylococci and its association with livestock. The gene has a predicted 23S rRNA (guanine (745)-N(1))-methyltransferase function, and as several commonly used agricultural antimicrobials target the

ribosome (tylosin, lincomycin, chlortetracycline, oxytetracycline, florfenicol), it is not unreasonable to assume that it could provide resistance to the antimicrobials, or boost the effect of other ribosome-methylating and ribosome-protecting AMR genes (e.g. *cfr,* the *erm* genes*,* the non-efflux *tet genes*). It seems worthwhile to determine the function of this gene, given its distribution and predicted rRNA-methylating features.

The apparent loss of all RM systems in pig-origin ST93 (Table 4-3) requires further study. HGT experiments should be performed with these isolates as recipients, for their lack of RM barriers could allow HGT across clonal complexes of MRSA (as hypothesised in this study) and could allow HGT across species barriers. The lack of barriers also presents the possibility of these strains being transformable and having use in genetic manipulation studies of ST93 MRSA. First however, a more comprehensive search of the genomes should be performed for *hsdS* and *hsdR* genes.

The *cfrAB* was shown to express full-length protein at around 2.3% of the rate of the uninterrupted *cfr* gene. The *cfrAB* gene could act as a sort of molecular chassis for the creation of fusion proteins for which a low level of expression is desired. Further work is needed to determine if the gene is regulated and at what rate Cfr is expressed from *cfrAB* in the wild-type.

Lastly, further MRSA surveillance experiments should be conducted on Australian farms to confirm the status of LA-MRSA in Australian piggeries. The recent invasive ST398-V MRSA infections, of which two were fatal (493), indicates that there is a reservoir of ST398 somewhere in Australia, even if it is not in piggeries. It is important to know if LA-MRSA is running rampant, or is declining to determine if further controls are necessary to prevent the spread of MRSA from the farms to the community.

6.4 Conclusions

The human-animal interface is the frontline of the battle with AMR. Animals are the largest consumers of antimicrobials by mass in Australia and antimicrobial use is directly correlated with AMR. ST398 LA-MRSA colonisation of Australia livestock has only fairly recently been detected for the first time and in the years since then, there have been fatal LA-MRSA infections in Australia. More MRSA in Australian animals will mean more MRSA infections in humans, as this study has demonstrated, MRSA can persist in animal populations for years, re-emerging to cause serious disease in humans. The health of humans, animals and the environment are all interconnected and so to address human health, we need to adapt a holistic approach.

On-going surveillance of meat and livestock production systems are essential to limiting the spread of zoonotic pathogens. Although ST93 was not found in the last MRSA surveillance study of Australian pig farms, ST93 may still be acquiring AMR and evolving its zoonotic and anthroponotic abilities undetected in Australian pig farms. Just as ST398 had human origins prior to being introduced into livestock, adaptation to pig colonisation, proliferation in pig herds and eventual zoonosis back into humans, ST93 could follow a similar trajectory. Without ongoing surveillance of LA-MRSA, we just don't know what the situation is.

The current state of large-scale industrial meat production in Australia appears to have insufficient controls in place over the use of antimicrobials in livestock to prevent the evolution and spread of AMR. With several international examples of 'worst-case' scenarios for LA-MRSA zoonosis, despite careful regulation and monitoring, the future of livestock agriculture will likely become a greater source of infections as demand for meat increases alongside the growth of the human population. Increased demand will likely require further intensification of food-animal production systems, which will increase infection risks for animals, which increases the need for antimicrobials, which promotes AMR. However, with strong scientific evidence to support the benefits of reduced antimicrobial use in combination with increased biosecurity and hygiene controls, a sustainable and safe livestock industry is possible. With continued vigilance and the prudent use of antimicrobials, the threat of zoonotic

MRSA infection in Australia may be controlled, and our antimicrobials will remain effective into the distant future.

Chapter 7

References

1. McCallum N, Meier PS, Heusser R, Berger-Bachi B. Mutational analyses of open reading frames within the *vraSR* operon and their roles in the cell wall stress response of *Staphylococcus aureus*. Antimicrob Agents Chemother. 2011;55(4):1391-402.
2. Wilson DN. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. Nat Rev Microbiol. 2014;12(1):35-48.
3. Frost LS, Leplae R, Summers AO, Toussaint A. Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol. 2005;3(9):722-32.
4. Turner NA, Sharma-Kuinkel BK, Maskarinec SA, Eichenberger EM, Shah PP, Carugati M, et al. Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. Nat Rev Microbiol. 2019;17(4):203-18.
5. Shore AC, Lazaris A, Kinnevey PM, Brennan OM, Brennan GI, O'Connell B, et al. First Report of *cfr*-Carrying Plasmids in the Pandemic Sequence Type 22 Methicillin-Resistant *Staphylococcus aureus* Staphylococcal Cassette Chromosome mec Type IV Clone. Antimicrob Agents Chemother. 2016;60(5):3007-15.
6. Dean KR, Krauer F, Walloe L, Lingjaerde OC, Bramanti B, Stenseth NC, et al. Human ectoparasites and the spread of plague in Europe during the Second Pandemic. Proc Natl Acad Sci U S A. 2018;115(6):1304-9.
7. Stenseth NC, Atshabar BB, Begon M, Belmain SR, Bertherat E, Carniel E, et al. Plague: past, present, and future. PLoS Med. 2008;5(1):e3.
8. Fornaciari A, Gaeta R, Minozzi S, Giuffra V. Syphilis in Maria Salviati (1499–1543), Wife of Giovanni de' Medici of the Black Bands. Emerg Infect Dis. 2020;26(6):1274-82.
9. Lopes C, Powell ML, Santos AL. Syphilis and cirrhosis: a lethal combination in a XIX century individual identified from the medical schools collection at the university of Coimbra (Portugal). Mem Inst Oswaldo Cruz. 2010;105(8):1050-3.
10. Peeling RW, Mabey D, Kamb ML, Chen XS, Radolf JD, Benzaken AS. Syphilis. Nat Rev Dis Primers. 2017;3:17073.
11. Gomez GB, Kamb ML, Newman LM, Mark J, Broutet N, Hawkes SJ. Untreated maternal syphilis and adverse outcomes of pregnancy: a systematic review and meta-analysis. Bull World Health Organ. 2013;91(3):217-26.
12. Lederberg J. Infectious history. Science. 2000;288(5464):287-93.
13. Arseculeratne SN, Arseculeratne G. A re-appraisal of the conventional history of antibiosis and Penicillin. Mycoses. 2017;60(5):343-7.
14. Rasmussen KL, Boldsen JL, Kristensen HK, Skytte L, Hansen KL, Mølholm L, et al. Mercury levels in Danish Medieval human bones. J Archaeol Sci. 2008;35(8):2295-306.
15. Richet C. An address on ancient humorism and modern humorism: Delivered at the International Congress of Physiology held in Vienna, September 27th to 30th. Br Med J. 1910;2(2596):921-6.
16. Jackson WA. A short guide to humoral medicine. Trends Pharmacol Sci. 2001;22(9):487-9.
17. Kadar N, Romero R, Papp Z. Ignaz Semmelweis: the "Savior of Mothers": On the 200(th) anniversary of his birth. Am J Obstet Gynecol. 2018;219(6):519-22.
18. Lister J. On the Antiseptic Principle in the Practice of Surgery. Br Med J. 1867;2(351):246-8.
19. Runcie H. Infection in a Pre-Antibiotic Era. J Anc Dis Prev Rem. 2015;03(02).
20. Crellin JK. Internal antisepsis or the dawn of chemotherapy? J Hist Med Allied Sci. 1981;36(1):9-18.

21. Thompson HP. The Prophylaxis of Scarlatinal Nephritis: Observations on 300 Cases of Scarlatina Treated with Urotropine, Hexamethylene-Tetramine and Metramine. Edinb Med J. 1907;21(2):103-13.
22. Parascandola J. The theoretical basis of Paul Ehrlich's chemotherapy. J Hist Med Allied Sci. 1981;36(1):19-43.
23. Landecker H. Antimicrobials before antibiotics: war, peace, and disinfectants. Palgrave Communications. 2019;5(1):45.
24. Jayachandran S, Lleras-Muney A, Smith KV. Modern Medicine and the Twentieth Century Decline in Mortality: Evidence on the Impact of Sulfa Drugs. Am Econ J Appl Econ. 2010;2(2):118-46.
25. Williams KJ. The introduction of 'chemotherapy' using arsphenamine - the first magic bullet. J R Soc Med. 2009;102(8):343-8.
26. Fleming A. On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of *B. influenzæ*. Br J Exp Pathol. 1929;10(3):226-36.
27. Mohr KI. History of Antibiotics Research. Curr Top Microbiol Immunol. 2016;398:237-72.
28. Schatz A, Bugie E, Waksman SA. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. 1944. Clin Orthop Relat Res. 2005(437):3-6.
29. Woodruff HB. Selman A. Waksman, winner of the 1952 Nobel Prize for physiology or medicine. Appl Environ Microbiol. 2014;80(1):2-8.
30. Batchelor FR, Doyle FP, Nayler JH, Rolinson GN. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature. 1959;183(4656):257-8.
31. Kawaguchi H. Discovery, chemistry, and activity of amikacin. J Infect Dis. 1976;134:S242-8.
32. Armstrong GL, Conn LA, Pinner RW. Trends in infectious disease mortality in the United States during the 20th century. JAMA. 1999;281(1):61-6.
33. Jones DS, Podolsky SH, Greene JA. The burden of disease and the changing task of medicine. N Engl J Med. 2012;366(25):2333-8.
34. Crump JA. Progress in Typhoid Fever Epidemiology. Clin Infect Dis. 2019;68:S4-S9.
35. Xu G, Walker DH, Jupiter D, Melby PC, Arcari CM. A review of the global epidemiology of scrub typhus. PLoS Negl Trop Dis. 2017;11(11):60-2.
36. Chaptini C, Marshman G. Leprosy: a review on elimination, reducing the disease burden, and future research. Lepr Rev. 2015;86(4):307-15.
37. Burke JF. The effective period of preventive antibiotic action in experimental incisions and dermal lesions. Surgery. 1961;50:161-8.
38. Australian Commission on Safety and Quality in Health Care. AURA 2017: Second Australian report on antimicrobial use and resistance in human health. Sydney: ACSWIH; 2017.
39. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. 1940. Rev Infect Dis. 1988;10(4):677-8.
40. Peterson E, Kaur P. Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. Front Microbiol. 2018;9(2928):2928.
41. Benveniste R, Davies J. Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc Natl Acad Sci U S A. 1973;70(8):2276-80.

42. Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y. Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. Clin Infect Dis. 2003;36(1):53-9.
43. Cosgrove SE, Qi Y, Kaye KS, Harbarth S, Karchmer AW, Carmeli Y. The impact of methicillin resistance in *Staphylococcus aureus* bacteremia on patient outcomes: mortality, length of stay, and hospital charges. Infect Control Hosp Epidemiol. 2005;26(2):166-74.
44. Deljehier T, Pariente A, Miremont-Salame G, Haramburu F, Nguyen L, Rubin S, et al. Rhabdomyolysis after co-administration of a statin and fusidic acid: an analysis of the literature and of the WHO database of adverse drug reactions. Br J Clin Pharmacol. 2018;84(5):1057-63.
45. Noaman S, Al-Mukhtar O, Abramovic S, Mohammed H, Goh CY, Long C, et al. Changes in Statin Prescription Patterns in Patients Admitted to an Australian Geriatric Subacute Unit. Heart Lung Circ. 2019;28(3):423-9.
46. Forrest GN, Tamura K. Rifampin combination therapy for nonmycobacterial infections. Clin Microbiol Rev. 2010;23(1):14-34.
47. Filippone EJ, Kraft WK, Farber JL. The Nephrotoxicity of Vancomycin. Clin Pharmacol Ther. 2017;102(3):459-69.
48. Mao Y, Dai D, Jin H, Wang Y. The risk factors of linezolid-induced lactic acidosis: A case report and review. Medicine (Baltimore). 2018;97(36):e12114.
49. Machado AR, Arns Cda C, Follador W, Guerra A. Cost-effectiveness of linezolid versus vancomycin in mechanical ventilation-associated nosocomial pneumonia caused by methicillin-resistant *Staphylococcus aureus*. Braz J Infect Dis. 2005;9(3):191-200.
50. Stein A, Raoult D. Colistin: an antimicrobial for the 21st century? Clin Infect Dis. 2002;35(7):901-2.
51. de Man TJB, Lutgring JD, Lonsway DR, Anderson KF, Kiehlbauch JA, Chen L, et al. Genomic Analysis of a Pan-Resistant Isolate of *Klebsiella pneumoniae*, United States 2016. mBio. 2018;9(2):e00440-18.
52. Xiong J, Deraspe M, Iqbal N, Krajden S, Chapman W, Dewar K, et al. Complete Genome of a Panresistant *Pseudomonas aeruginosa* Strain, Isolated from a Patient with Respiratory Failure in a Canadian Community Hospital. Genome Announc. 2017;5(22):e00458-17.
53. Nichols L. Death from pan-resistant superbug. Autops Case Rep. 2019;9(3):e2019106.
54. Udwadia ZF, Jain S, Rodrigues C, Mehta A. XDR tuberculosis in India: what's in a name? Lancet Infect Dis. 2007;7(7):441-2.
55. Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, ZiaZarifi AH, et al. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in iran. Chest. 2009;136(2):420-5.
56. Spellberg B, Powers JH, Brass EP, Miller LG, Edwards JE, Jr. Trends in antimicrobial drug development: implications for the future. Clin Infect Dis. 2004;38(9):1279-86.
57. Projan SJ. Why is big Pharma getting out of antibacterial drug discovery? Curr Opin Microbiol. 2003;6(5):427-30.
58. Towse A, Hoyle CK, Goodall J, Hirsch M, Mestre-Ferrandiz J, Rex JH. Time for a change in how new antibiotics are reimbursed: Development of an insurance framework for funding new antibiotics based on a policy of risk mitigation. Health Policy. 2017;121(10):1025-30.
59. Marchant J. When antibiotics turn toxic. Nature. 2018;555(7697):431-3.
60. Goldstein A. Antibacterial chemotherapy. N Engl J Med. 1949;240(4):137; passim.

61. Woodford N, Ellington MJ. The emergence of antibiotic resistance by mutation. Clin Microbiol Infect. 2007;13(1):5-18.
62. O'Dwyer K, Spivak AT, Ingraham K, Min S, Holmes DJ, Jakielaszek C, et al. Bacterial resistance to leucyl-tRNA synthetase inhibitor GSK2251052 develops during treatment of complicated urinary tract infections. Antimicrob Agents Chemother. 2015;59(1):289-98.
63. Cox OF. Chemotherapy in Gonococcal Infections. N Engl J Med. 1942;226(5):184-6.
64. Hughes RP, Carpenter CM. Alleged penicillin-resistant gonorrhea. Am J Syph Gonorrhea Vener Dis. 1948;32(3):265-71.
65. Dioxydiamidoarsenobenzol. Hospital (Lond 1886). 1911;49(1277):437-8.
66. Beerman H, Severac M. The Problem of Treatment-Resistant Syphilis. J Invest Dermatol. 1942;5(5):269-82.
67. Epitome of Current Medical Literature. Br Med J. 1935;2(3892):E21-E4.
68. Rammelkamp CH, Maxon T. Resistance of *Staphylococcus aureus* to the Action of Penicillin. Exp Biol Med. 1942;51(3):386-9.
69. Petroff BP, Lucas FV. Streptomycin in Urinary Infections. Ann Surg. 1946;123(5):808-18.
70. Abraham EP, Newton GG, Olson BH, Schuurmans DM, Schenck JR, Hargie MP, et al. Identity of cephalosporin N and synnematin B. Nature. 1955;176(4481):551.
71. Fleming PC, Goldner M, Glass DG. Observations on the nature, distribution, and significance of cephalosporinase. Lancet. 1963;1(7296):1399-401.
72. Ehrlich J, Gottlieb D, Burkholder PR, Anderson LE, Pridham TG. *Streptomyces venezuelae*, n. sp., the source of chloromycetin. J Bacteriol. 1948;56(4):467-77.
73. Anderson K. A strain of staphylococcus resistant to five antibiotics. J Clin Pathol. 1954;7(2):148-51.
74. Duggar BM. Aureomycin; a product of the continuing search for new antibiotics. Ann N Y Acad Sci. 1948;51(Art. 2):177-81.
75. Thaker M, Spanogiannopoulos P, Wright GD. The tetracycline resistome. Cellular and molecular life sciences : CMLS. 2010;67(3):419-31.
76. Waksman SA, Lechevalier HA. Neomycin, a New Antibiotic Active against Streptomycin-Resistant Bacteria, including Tuberculosis Organisms. Science. 1949;109(2830):305-7.
77. Roy TE, Collins AM, Craig G, Duncan IB. A survey of the incidence of resistance to antibiotics in bacteria isolated in a children's hospital. Can Med Assoc J. 1957;77(9):844-50.
78. Erythromycin. Br Med J. 1952;2(4793):1085-6.
79. Giles C, Shuttleworth EM. The sensitivity of various bacteria to antibiotics during the years 1951 to 1956. J Clin Pathol. 1958;11(2):185-9.
80. Welch H. Certification of antibiotics. Public Health Rep. 1956;71(6):594-9.
81. Leclercq R, Derlot E, Duval J, Courvalin P. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N Engl J Med. 1988;319(3):157-61.
82. Knox R. A new penicillin (BRL 1241) active against penicillin-resistant staphylococci. Br Med J. 1960;2(5200):690-3.
83. Jevons PM. “Celbenin” - resistant Staphylococci. British Medical Journal. 1961;1(5219):124-5.
84. Fucidin, A New Antibiotic. Can Med Assoc J. 1962;86(24):1122-3.
85. Evans RJ, Waterworth PM. Naturally-occurring fusidic acid resistance in staphylococci and its linkage to other resistances. J Clin Pathol. 1966;19(6):555-60.

86. Weinstein MJ, Luedemann GM, Oden EM, Wagman GH, Rosselet JP, Marquez JA, et al. Gentamicin, a New Antibiotic Complex from Micromonospora. J Med Chem. 1963;6(4):463-4.
87. Greene WH, Moody M, Schimpff S, Young VM, Wiernik PH. *Pseudomonas aeruginosa* resistant to carbenicillin and gentamicin. Epidemiologic and clinical aspects in a cancer center. Ann Intern Med. 1973;79(5):684-9.
88. Noall EW, Sewards HF, Waterworth PM. Successful Treatment of a Case of *Proteus* Septicaemia. Br Med J. 1962;2(5312):1101-2.
89. Fleming MP, Datta N, Gruneberg RN. Trimethoprim resistance determined by R factors. Br Med J. 1972;1(5802):726-8.
90. Maggi N, Pasqualucci CR, Ballotta R, Sensi P. Rifampicin: a new orally active rifamycin. Chemotherapy. 1966;11(5):285-92.
91. Cobbold RJ, Morrison GD, Willcox RR. Treatment of gonorrhoea with single oral doses of rifampicin. Br Med J. 1968;4(5632):681-2.
92. Wise R, Andrews JM, Edwards LJ. In vitro activity of Bay 09867, a new quinoline derivative, compared with those of other antimicrobial agents. Antimicrob Agents Chemother. 1983;23(4):559-64.
93. Barry AL, Jones RN. Cross-resistance among cinoxacin, ciprofloxacin, DJ-6783, enoxacin, nalidixic acid, norfloxacin, and oxolinic acid after in vitro selection of resistant populations. Antimicrob Agents Chemother. 1984;25(6):775-7.
94. Walsh M, Kappus EW, Quinn TC. In vitro evaluation of CP-62,993, erythromycin, clindamycin, and tetracycline against *Chlamydia trachomatis*. Antimicrob Agents Chemother. 1987;31(5):811-2.
95. Heifets L, Mor N, Vanderkolk J. *Mycobacterium avium* strains resistant to clarithromycin and azithromycin. Antimicrob Agents Chemother. 1993;37(11):2364-70.
96. Manzella JP. Quinupristin-dalfopristin: a new antibiotic for severe gram-positive infections. Am Fam Physician. 2001;64(11):1863-6.
97. Soltani M, Beighton D, Philpott-Howard J, Woodford N. Mechanisms of resistance to quinupristin-dalfopristin among isolates of *Enterococcus faecium* from animals, raw meat, and hospital patients in Western Europe. Antimicrob Agents Chemother. 2000;44(2):433-6.
98. Sum PE, Petersen P. Synthesis and structure-activity relationship of novel glycylcycline derivatives leading to the discovery of GAR-936. Bioorg Med Chem Lett. 1999;9(10):1459-62.
99. Livermore DM. Tigecycline: what is it, and where should it be used? J Antimicrob Chemother. 2005;56(4):611-4.
100. Brickner SJ, Hutchinson DK, Barbachyn MR, Manninen PR, Ulanowicz DA, Garmon SA, et al. Synthesis and antibacterial activity of U-100592 and U-100766, two oxazolidinone antibacterial agents for the potential treatment of multidrug-resistant gram-positive bacterial infections. J Med Chem. 1996;39(3):673-9.
101. Meka VG, Gold HS. Antimicrobial resistance to linezolid. Clin Infect Dis. 2004;39(7):1010-5.
102. Ednie LM, Jacobs MR, Appelbaum PC. Comparative antianaerobic activities of the ketolides HMR 3647 (RU 66647) and HMR 3004 (RU 64004). Antimicrob Agents Chemother. 1997;41(9):2019-22.
103. Tait-Kamradt A, Davies T, Appelbaum PC, Depardieu F, Courvalin P, Petitpas J, et al. Two new mechanisms of macrolide resistance in clinical strains of *Streptococcus pneumoniae* from Eastern Europe and North America. Antimicrob Agents Chemother. 2000;44(12):3395-401.
104. Wright G. Antibiotics: An irresistible newcomer. Nature. 2015;517(7535):442-4.

105. Weinstein L. The complications of antibiotic therapy. Bull N Y Acad Med. 1955;31(7):500-18.
106. Rantz LA. Consequences of the widespread use of antibiotics. Calif Med. 1954;81(1):1-3.
107. Forbes GB. Infection with penicillin-resistant staphylococci in hospital and general practice. Br Med J. 1949;2(4627):569-71.
108. Dowling HF, Lepper MH, Jackson GG. Observations on the epidemiological spread of antibiotic-resistant staphylococci, with measurements of the changes in sensitivity to penicillin and aureomycin. Am J Public Health Nations Health. 1953;43(7):860-8.
109. Saez Nieto JA, Vazquez JA, Marcos C. Meningococci moderately resistant to penicillin. Lancet. 1990;336(8706):54.
110. Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet. 2022;399(10325):629-55.
111. (WHO) WHO. Antibiotic Resistance [Fact Sheet]. [Available from: https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance.
112. (WHO) WHO. Global Antimicrobial Resistance and Use Surveillance System (GLASS) 2022 [Available from: https://www.who.int/initiatives/glass.
113. (WHO) WHO. Global antimicrobial resistance and use surveillance system (GLASS) report 2021. Geneva: World Health Organization (WHO); 2021.
114. Evaluation OoTASfPa. The National Action Plan for Combating Antibiotic-Resistant Bacteria (CARB), 2020-2025. In: Services USDoHHaH, editor. Washington (DC): National Academies Press (US); 2020.
115. CDC. Antibiotic Resistance Threats in the United States, 2019. In: (CDC) CfDC, editor. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019.
116. Government A. Responding to the threat of antimicrobial resistance: Australia's First National Antimicrobial Resistance Strategy 2015–2019. 2015.
117. Government A. Australia's National Antimicrobial Resistance Strategy – 2020 and Beyond In: Health Do, editor. 2019.
118. Coombs GW, Daley DA, Mowlaboccus S, Pang S, Australian Group on Antimicrobial R. Australian Group on Antimicrobial Resistance (AGAR) Australian *Staphylococcus aureus* Sepsis Outcome Programme (ASSOP) Annual Report 2019. Commun Dis Intell (2018). 2020;44.
119. Santos JA, Lamers MH. Novel Antibiotics Targeting Bacterial Replicative DNA Polymerases. Antibiotics (Basel). 2020;9(11):776.
120. Epand RM, Walker C, Epand RF, Magarvey NA. Molecular mechanisms of membrane targeting antibiotics. Biochim Biophys Acta. 2016;1858(5):980-7.
121. Capasso C, Supuran CT. Sulfa and trimethoprim-like drugs - antimetabolites acting as carbonic anhydrase, dihydropteroate synthase and dihydrofolate reductase inhibitors. J Enzyme Inhib Med Chem. 2014;29(3):379-87.
122. Balemans W, Vranckx L, Lounis N, Pop O, Guillemont J, Vergauwen K, et al. Novel antibiotics targeting respiratory ATP synthesis in Gram-positive pathogenic bacteria. Antimicrob Agents Chemother. 2012;56(8):4131-9.
123. Liu Y, Breukink E. The Membrane Steps of Bacterial Cell Wall Synthesis as Antibiotic Targets. Antibiotics (Basel). 2016;5(3):28.

124. Tipper DJ, Strominger JL. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci U S A. 1965;54(4):1133-41.
125. Yocum RR, Waxman DJ, Rasmussen JR, Strominger JL. Mechanism of penicillin action: penicillin and substrate bind covalently to the same active site serine in two bacterial D-alanine carboxypeptidases. Proc Natl Acad Sci U S A. 1979;76(6):2730-4.
126. Cooper MA, Williams DH. Binding of glycopeptide antibiotics to a model of a vancomycin-resistant bacterium. Chem Biol. 1999;6(12):891-9.
127. Nieto M, Perkins HR. Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex-formation with vancomycin. Biochem J. 1971;123(5):789-803.
128. Shoji S, Dambacher CM, Shajani Z, Williamson JR, Schultz PG. Systematic chromosomal deletion of bacterial ribosomal protein genes. J Mol Biol. 2011;413(4):751-61.
129. Proshkin S, Rahmouni AR, Mironov A, Nudler E. Cooperation between translating ribosomes and RNA polymerase in transcription elongation. Science. 2010;328(5977):504-8.
130. Pato ML. Tetracycline inhibits propagation of deoxyribonucleic acid replication and alters membrane properties. Antimicrob Agents Chemother. 1977;11(2):318-23.
131. Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev. 2001;65(2):232-60 ; second page, table of contents.
132. Norrby R. Linezolid--a review of the first oxazolidinone. Expert Opin Pharmacother. 2001;2(2):293-302.
133. Garcia-Prats AJ, Schaaf HS, Draper HR, Garcia-Cremades M, Winckler J, Wiesner L, et al. Pharmacokinetics, optimal dosing, and safety of linezolid in children with multidrug-resistant tuberculosis: Combined data from two prospective observational studies. PLoS Med. 2019;16(4):e1002789.
134. Verma AK, Bauer C, Yajjala VK, Bansal S, Sun K. Linezolid Attenuates Lethal Lung Damage during Postinfluenza Methicillin-Resistant *Staphylococcus aureus* Pneumonia. Infect Immun. 2019;87(10):e00538-19.
135. Bassetti M, Baguneid M, Bouza E, Dryden M, Nathwani D, Wilcox M. European perspective and update on the management of complicated skin and soft tissue infections due to methicillin-resistant *Staphylococcus aureus* after more than 10 years of experience with linezolid. Clin Microbiol Infect. 2014;20:3-18.
136. Livermore DM. Linezolid in vitro: mechanism and antibacterial spectrum. J Antimicrob Chemother. 2003;51:ii9-16.
137. World Health Organization. Critically important antimicrobials for human medicine, 6th revision. Switzerland: AGISAR; 2019.
138. Vijayan P, Srinivas D, Siddaiah N, Bahubali VKH. Device-Associated Meningitis by Linezolid-Resistant *Staphylococcus haemolyticus* in a Vancomycin- Hypersensitive Patient. J Neurosci Rural Pract. 2019;10(4):718-20.
139. Yue J, Dong BR, Yang M, Chen X, Wu T, Liu GJ. Linezolid versus vancomycin for skin and soft tissue infections. Cochrane Database Syst Rev. 2016(1):CD008056.
140. Garrabou G, Soriano A, Lopez S, Guallar JP, Giralt M, Villarroya F, et al. Reversible inhibition of mitochondrial protein synthesis during linezolid-related hyperlactatemia. Antimicrob Agents Chemother. 2007;51(3):962-7.
141. Vinh DC, Rubinstein E. Linezolid: a review of safety and tolerability. J Infect. 2009;59:S59-74.

142. Swaney SM, Aoki H, Ganoza MC, Shinabarger DL. The oxazolidinone linezolid inhibits initiation of protein synthesis in bacteria. Antimicrob Agents Chemother. 1998;42(12):3251-5.
143. Wilson DN, Schluenzen F, Harms JM, Starosta AL, Connell SR, Fucini P. The oxazolidinone antibiotics perturb the ribosomal peptidyl-transferase center and effect tRNA positioning. Proc Natl Acad Sci U S A. 2008;105(36):13339-44.
144. Ippolito JA, Kanyo ZF, Wang D, Franceschi FJ, Moore PB, Steitz TA, et al. Crystal structure of the oxazolidinone antibiotic linezolid bound to the 50S ribosomal subunit. J Med Chem. 2008;51(12):3353-6.
145. Roberts MC. Resistance to macrolide, lincosamide, streptogramin, ketolide, and oxazolidinone antibiotics. Mol Biotechnol. 2004;28(1):47-62.
146. Lee H, Popodi E, Tang H, Foster PL. Rate and molecular spectrum of spontaneous mutations in the bacterium *Escherichia coli* as determined by whole-genome sequencing. Proc Natl Acad Sci U S A. 2012;109(41):E2774-83.
147. Tsui HC, Feng G, Winkler ME. Negative regulation of mutS and mutH repair gene expression by the Hfq and RpoS global regulators of *Escherichia coli* K-12. J Bacteriol. 1997;179(23):7476-87.
148. Sniegowski P. Evolution: bacterial mutation in stationary phase. Curr Biol. 2004;14(6):R245-6.
149. Aubry-Damon H, Soussy CJ, Courvalin P. Characterization of mutations in the rpoB gene that confer rifampin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 1998;42(10):2590-4.
150. Morrow TO, Harmon SA. Genetic analysis of *Staphylococcus aureus* RNA polymerase mutants. J Bacteriol. 1979;137(1):374-83.
151. Davidovich C, Bashan A, Yonath A. Structural basis for cross-resistance to ribosomal PTC antibiotics. Proc Natl Acad Sci U S A. 2008;105(52):20665-70.
152. Ettayebi M, Prasad SM, Morgan EA. Chloramphenicol-erythromycin resistance mutations in a 23S rRNA gene of *Escherichia coli*. J Bacteriol. 1985;162(2):551-7.
153. Willingham-Lane JM, Berghaus LJ, Berghaus RD, Hart KA, Giguere S. Effect of Macrolide and Rifampin Resistance on the Fitness of *Rhodococcus equi*. Appl Environ Microbiol. 2019;85(7):e02665-18.
154. Revuelta JL, Serrano-Amatriain C, Ledesma-Amaro R, Jimenez A. Formation of folates by microorganisms: towards the biotechnological production of this vitamin. Appl Microbiol Biotechnol. 2018;102(20):8613-20.
155. Flensburg J, Skold O. Massive overproduction of dihydrofolate reductase in bacteria as a response to the use of trimethoprim. Eur J Biochem. 1987;162(3):473-6.
156. Pao SS, Paulsen IT, Saier MH, Jr. Major facilitator superfamily. Microbiol Mol Biol Rev. 1998;62(1):1-34.
157. Costa SS, Viveiros M, Amaral L, Couto I. Multidrug Efflux Pumps in *Staphylococcus aureus*: an Update. Open Microbiol J. 2013;7:59-71.
158. Ubukata K, Yamashita N, Gotoh A, Konno M. Purification and characterization of aminoglycoside-modifying enzymes from *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob Agents Chemother. 1984;25(6):754-9.
159. Murphy E. Nucleotide sequence of a spectinomycin adenyltransferase *aad(9)* determinant from *Staphylococcus aureus* and its relationship to *aad(3") (9)*. Mol Gen Genet. 1985;200(1):33-9.
160. Bruckner R, Matzura H. Regulation of the inducible chloramphenicol acetyltransferase gene of the *Staphylococcus aureus* plasmid pUB112. EMBO J. 1985;4(9):2295-300.

161. Horinouchi S, Weisblum B. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J Bacteriol. 1982;150(2):815-25.
162. Chervaux C, Sauvonnet N, Le Clainche A, Kenny B, Hung AL, Broome-Smith JK, et al. Secretion of active beta-lactamase to the medium mediated by the *Escherichia coli* haemolysin transport pathway. Mol Gen Genet. 1995;249(2):237-45.
163. Brook I. The role of beta-lactamase-producing bacteria in the persistence of streptococcal tonsillar infection. Rev Infect Dis. 1984;6(5):601-7.
164. Donhofer A, Franckenberg S, Wickles S, Berninghausen O, Beckmann R, Wilson DN. Structural basis for TetM-mediated tetracycline resistance. Proc Natl Acad Sci U S A. 2012;109(42):16900-5.
165. Li W, Atkinson GC, Thakor NS, Allas U, Lu CC, Chan KY, et al. Mechanism of tetracycline resistance by ribosomal protection protein Tet(O). Nat Commun. 2013;4(1):1477.
166. Skinner R, Cundliffe E, Schmidt FJ. Site of action of a ribosomal RNA methylase responsible for resistance to erythromycin and other antibiotics. J Biol Chem. 1983;258(20):12702-6.
167. Saito R, Nonaka S, Nishiyama H, Okamura N. Molecular mechanism of macrolide-lincosamide resistance in *Moraxella catarrhalis*. J Med Microbiol. 2012;61(Pt 10):1435-8.
168. Thakker-Varia S, Ranzini AC, Dubin DT. Ribosomal RNA methylation in *Staphylococcus aureus* and *Escherichia coli*: effect of the "MLS" (erythromycin resistance) methylase. Plasmid. 1985;14(2):152-61.
169. PubChem Compound Summary for CID 6087, Methicillin [Internet]. 2020 [cited August 28, 2020]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Methicillin.
170. Hartman BJ, Tomasz A. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J Bacteriol. 1984;158(2):513-6.
171. Leski TA, Tomasz A. Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *Staphylococcus aureus*: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. J Bacteriol. 2005;187(5):1815-24.
172. Utsui Y, Yokota T. Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother. 1985;28(3):397-403.
173. Long KS, Vester B. Resistance to linezolid caused by modifications at its binding site on the ribosome. Antimicrob Agents Chemother. 2012;56(2):603-12.
174. Xiong L, Kloss P, Douthwaite S, Andersen NM, Swaney S, Shinabarger DL, et al. Oxazolidinone resistance mutations in 23S rRNA of *Escherichia coli* reveal the central region of domain V as the primary site of drug action. J Bacteriol. 2000;182(19):5325-31.
175. Fulle S, Saini JS, Homeyer N, Gohlke H. Complex long-distance effects of mutations that confer linezolid resistance in the large ribosomal subunit. Nucleic Acids Res. 2015;43(16):7731-43.
176. Schwarz S, Werckenthin C, Kehrenberg C. Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. Antimicrob Agents Chemother. 2000;44(9):2530-3.
177. Giessing AM, Jensen SS, Rasmussen A, Hansen LH, Gondela A, Long K, et al. Identification of 8-methyladenosine as the modification catalyzed by the radical SAM methyltransferase Cfr that confers antibiotic resistance in bacteria. RNA. 2009;15(2):327-36.
178. Long KS, Poehlsgaard J, Kehrenberg C, Schwarz S, Vester B. The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. Antimicrob Agents Chemother. 2006;50(7):2500-5.

179. Flamm RK, Mendes RE, Hogan PA, Streit JM, Ross JE, Jones RN. Linezolid Surveillance Results for the United States (LEADER Surveillance Program 2014). Antimicrob Agents Chemother. 2016;60(4):2273-80.
180. Pakula KK, Hansen LH, Vester B. Combined Effect of the Cfr Methyltransferase and Ribosomal Protein L3 Mutations on Resistance to Ribosome-Targeting Antibiotics. Antimicrob Agents Chemother. 2017;61(9):e00862-17.
181. Liu Y, Wang Y, Schwarz S, Li Y, Shen Z, Zhang Q, et al. Transferable multiresistance plasmids carrying *cfr* in *Enterococcus* spp. from swine and farm environment. Antimicrob Agents Chemother. 2013;57(1):42-8.
182. Wang Y, He T, Schwarz S, Zhou D, Shen Z, Wu C, et al. Detection of the staphylococcal multiresistance gene *cfr* in *Escherichia coli* of domestic-animal origin. J Antimicrob Chemother. 2012;67(5):1094-8.
183. Wang X, Zhu Y, Hua X, Chen F, Wang C, Zhang Y, et al. F14:A-:B- and IncX4 Inc group *cfr*-positive plasmids circulating in *Escherichia coli* of animal origin in Northeast China. Vet Microbiol. 2018;217:53-7.
184. Tian Y, Li T, Zhu Y, Wang B, Zou X, Li M. Mechanisms of linezolid resistance in staphylococci and enterococci isolated from two teaching hospitals in Shanghai, China. BMC Microbiol. 2014;14:292.
185. Nguyen LTT, Roman F, Morikawa K, Trincado P, Marcos C, Rojo-Martin MD, et al. Prevalence of pSCFS7-like vectors among *cfr*-positive staphylococcal population in Spain. Int J Antimicrob Agents. 2018;52(2):305-6.
186. Liu BH, Lei CW, Zhang AY, Pan Y, Kong LH, Xiang R, et al. Colocation of the Multiresistance Gene *cfr* and the Fosfomycin Resistance Gene *fosD* on a Novel Plasmid in *Staphylococcus arlettae* from a Chicken Farm. Antimicrob Agents Chemother. 2017;61(12):e01388-17.
187. Diaz L, Kiratisin P, Mendes RE, Panesso D, Singh KV, Arias CA. Transferable plasmid-mediated resistance to linezolid due to *cfr* in a human clinical isolate of *Enterococcus faecalis*. Antimicrob Agents Chemother. 2012;56(7):3917-22.
188. Shore AC, Brennan OM, Ehricht R, Monecke S, Schwarz S, Slickers P, et al. Identification and characterization of the multidrug resistance gene *cfr* in a Panton-Valentine leukocidin-positive sequence type 8 methicillin-resistant *Staphylococcus aureus* IVa (USA300) isolate. Antimicrob Agents Chemother. 2010;54(12):4978-84.
189. Gopegui ER, Juan C, Zamorano L, Perez JL, Oliver A. Transferable multidrug resistance plasmid carrying cfr associated with *tet*(*L*), *ant*(4')-*Ia*, and *dfrK* genes from a clinical methicillin-resistant *Staphylococcus aureus* ST125 strain. Antimicrob Agents Chemother. 2012;56(4):2139-42.
190. Zeng ZL, Wei HK, Wang J, Lin DC, Liu XQ, Liu JH. High prevalence of Cfr-producing *Staphylococcus* species in retail meat in Guangzhou, China. BMC Microbiol. 2014;14:151.
191. Liu XQ, Wang J, Li W, Zhao LQ, Lu Y, Liu JH, et al. Distribution of cfr in *Staphylococcus spp*. and *Escherichia coli* Strains from Pig Farms in China and Characterization of a Novel *cfr*-Carrying F43:A-:B- Plasmid. Front Microbiol. 2017;8:329.
192. Mendes RE, Deshpande L, Rodriguez-Noriega E, Ross JE, Jones RN, Morfin-Otero R. First report of Staphylococcal clinical isolates in Mexico with linezolid resistance caused by *cfr*: evidence of in vivo *cfr* mobilization. J Clin Microbiol. 2010;48(8):3041-3.

193. Morales G, Picazo JJ, Baos E, Candel FJ, Arribi A, Pelaez B, et al. Resistance to linezolid is mediated by the *cfr* gene in the first report of an outbreak of linezolid-resistant *Staphylococcus aureus*. Clin Infect Dis. 2010;50(6):821-5.
194. Mendes RE, Deshpande LM, Castanheira M, DiPersio J, Saubolle MA, Jones RN. First report of *cfr*-mediated resistance to linezolid in human staphylococcal clinical isolates recovered in the United States. Antimicrob Agents Chemother. 2008;52(6):2244-6.
195. O'Connor C, Powell J, Finnegan C, O'Gorman A, Barrett S, Hopkins KL, et al. Incidence, management and outcomes of the first *cfr*-mediated linezolid-resistant *Staphylococcus epidermidis* outbreak in a tertiary referral centre in the Republic of Ireland. J Hosp Infect. 2015;90(4):316-21.
196. Toh SM, Xiong L, Arias CA, Villegas MV, Lolans K, Quinn J, et al. Acquisition of a natural resistance gene renders a clinical strain of methicillin-resistant *Staphylococcus aureus* resistant to the synthetic antibiotic linezolid. Mol Microbiol. 2007;64(6):1506-14.
197. Locke JB, Zuill DE, Scharn CR, Deane J, Sahm DF, Denys GA, et al. Linezolid-resistant *Staphylococcus aureus* strain 1128105, the first known clinical isolate possessing the *cfr* multidrug resistance gene. Antimicrob Agents Chemother. 2014;58(11):6592-8.
198. Guglielmini J, Neron B, Abby SS, Garcillan-Barcia MP, de la Cruz F, Rocha EP. Key components of the eight classes of type IV secretion systems involved in bacterial conjugation or protein secretion. Nucleic Acids Res. 2014;42(9):5715-27.
199. Llosa M, Zunzunegui S, de la Cruz F. Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. Proc Natl Acad Sci U S A. 2003;100(18):10465-70.
200. Garcillan-Barcia MP, Jurado P, Gonzalez-Perez B, Moncalian G, Fernandez LA, de la Cruz F. Conjugative transfer can be inhibited by blocking relaxase activity within recipient cells with intrabodies. Mol Microbiol. 2007;63(2):404-16.
201. Guasch A, Lucas M, Moncalian G, Cabezas M, Perez-Luque R, Gomis-Ruth FX, et al. Recognition and processing of the origin of transfer DNA by conjugative relaxase TrwC. Nat Struct Biol. 2003;10(12):1002-10.
202. Lucas M, Gonzalez-Perez B, Cabezas M, Moncalian G, Rivas G, de la Cruz F. Relaxase DNA binding and cleavage are two distinguishable steps in conjugative DNA processing that involve different sequence elements of the nic site. J Biol Chem. 2010;285(12):8918-26.
203. Ramsay JP, Kwong SM, Murphy RJ, Yui Eto K, Price KJ, Nguyen QT, et al. An updated view of plasmid conjugation and mobilization in *Staphylococcus*. Mob Genet Elements. 2016;6(4):e1208317.
204. Leon-Sampedro R, Novais C, Peixe L, Baquero F, Coque TM. Diversity and Evolution of the Tn*5801-tet(M)*-Like Integrative and Conjugative Elements among *Enterococcus*, *Streptococcus*, and *Staphylococcus*. Antimicrob Agents Chemother. 2016;60(3):1736-46.
205. O'Brien FG, Yui Eto K, Murphy RJ, Fairhurst HM, Coombs GW, Grubb WB, et al. Origin-of-transfer sequences facilitate mobilisation of non-conjugative antimicrobial-resistance plasmids in *Staphylococcus aureus*. Nucleic Acids Res. 2015;43(16):7971-83.
206. Blokesch M. Natural competence for transformation. Curr Biol. 2016;26(21):R1126-R30.
207. Bergh O, Borsheim KY, Bratbak G, Heldal M. High abundance of viruses found in aquatic environments. Nature. 1989;340(6233):467-8.
208. Jackson EN, Jackson DA, Deans RJ. EcoRI analysis of bacteriophage P22 DNA packaging. J Mol Biol. 1978;118(3):365-88.

209. Miwa T, Matsubara K. Identification of sequences necessary for packaging DNA into lambda phage heads. Gene. 1982;20(2):267-79.
210. Chen J, Quiles-Puchalt N, Chiang YN, Bacigalupe R, Fillol-Salom A, Chee MSJ, et al. Genome hypermobility by lateral transduction. Science. 2018;362(6411):207-12.
211. Giovanetti E, Brenciani A, Morroni G, Tiberi E, Pasquaroli S, Mingoia M, et al. Transduction of the *Streptococcus pyogenes* bacteriophage Phim46.1, carrying resistance genes *mef*(A) and *tet*(O), to other Streptococcus species. Front Microbiol. 2014;5:746.
212. Mirold S, Rabsch W, Rohde M, Stender S, Tschape H, Russmann H, et al. Isolation of a temperate bacteriophage encoding the type III effector protein SopE from an epidemic *Salmonella typhimurium* strain. Proc Natl Acad Sci U S A. 1999;96(17):9845-50.
213. Kaneko J, Kamio Y. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci Biotechnol Biochem. 2004;68(5):981-1003.
214. Chiang YN, Penades JR, Chen J. Genetic transduction by phages and chromosomal islands: The new and noncanonical. PLoS Pathog. 2019;15(8):e1007878.
215. Novick RP. Mobile genetic elements and bacterial toxinoses: the superantigen-encoding pathogenicity islands of *Staphylococcus aureus*. Plasmid. 2003;49(2):93-105.
216. Roberts AP, Chandler M, Courvalin P, Guedon G, Mullany P, Pembroke T, et al. Revised nomenclature for transposable genetic elements. Plasmid. 2008;60(3):167-73.
217. Stewart PR, Dubin DT, Chikramane SG, Inglis B, Matthews PR, Poston SM. IS257 and small plasmid insertions in the *mec* region of the chromosome of *Staphylococcus aureus*. Plasmid. 1994;31(1):12-20.
218. Byrne ME, Gillespie MT, Skurray RA. 4',4'' Adenyltransferase activity on conjugative plasmids isolated from *Staphylococcus aureus* is encoded on an integrated copy of pUB110. Plasmid. 1991;25(1):70-5.
219. Yui Eto K, Firth N, Davis AM, Kwong SM, Krysiak M, Lee YT, et al. Evolution of a 72-Kilobase Cointegrant, Conjugative Multiresistance Plasmid in Community-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates from the Early 1990s. Antimicrob Agents Chemother. 2019;63(11):e01560-19.
220. Rowland SJ, Dyke KG. Tn*552*, a novel transposable element from *Staphylococcus aureus*. Mol Microbiol. 1990;4(6):961-75.
221. Shearer JE, Wireman J, Hostetler J, Forberger H, Borman J, Gill J, et al. Major families of multiresistant plasmids from geographically and epidemiologically diverse staphylococci. G3 (Bethesda). 2011;1(7):581-91.
222. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. Clin Microbiol Rev. 2018;31(4):e00088-17.
223. Carroll AC, Wong A. Plasmid persistence: costs, benefits, and the plasmid paradox. Can J Microbiol. 2018;64(5):293-304.
224. Mahillon J, Chandler M. Insertion sequences. Microbiol Mol Biol Rev. 1998;62(3):725-74.
225. Mikl M, Pilpel Y, Segal E. High-throughput interrogation of programmed ribosomal frameshifting in human cells. Nat Commun. 2020;11(1):3061.
226. Meydan S, Klepacki D, Karthikeyan S, Margus T, Thomas P, Jones JE, et al. Programmed Ribosomal Frameshifting Generates a Copper Transporter and a Copper Chaperone from the Same Gene. Mol Cell. 2017;65(2):207-19.

227. Lopatina A, Tal N, Sorek R. Abortive Infection: Bacterial Suicide as an Antiviral Immune Strategy. Annu Rev Virol. 2020;7(1):371-84.
228. Bernheim A, Sorek R. The pan-immune system of bacteria: antiviral defence as a community resource. Nat Rev Microbiol. 2020;18(2):113-9.
229. Cheng K, Wilkinson M, Chaban Y, Wigley DB. A conformational switch in response to Chi converts RecBCD from phage destruction to DNA repair. Nat Struct Mol Biol. 2020;27(1):71-7.
230. McClelland M. The effect of sequence specific DNA methylation on restriction endonuclease cleavage. Nucleic Acids Res. 1981;9(22):5859-66.
231. Murray NE. Type I restriction systems: sophisticated molecular machines (a legacy of Bertani and Weigle). Microbiol Mol Biol Rev. 2000;64(2):412-34.
232. Loenen WA, Dryden DT, Raleigh EA, Wilson GG, Murray NE. Highlights of the DNA cutters: a short history of the restriction enzymes. Nucleic Acids Res. 2014;42(1):3-19.
233. Rao DN, Dryden DT, Bheemanaik S. Type III restriction-modification enzymes: a historical perspective. Nucleic Acids Res. 2014;42(1):45-55.
234. Loenen WA, Raleigh EA. The other face of restriction: modification-dependent enzymes. Nucleic Acids Res. 2014;42(1):56-69.
235. Adhikari S, Curtis PD. DNA methyltransferases and epigenetic regulation in bacteria. FEMS Microbiol Rev. 2016;40(5):575-91.
236. Waldron DE, Lindsay JA. Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. J Bacteriol. 2006;188(15):5578-85.
237. Seeber S, Kessler C, Gotz F. Cloning, expression and characterization of the Sau3AI restriction and modification genes in *Staphylococcus carnosus* TM300. Gene. 1990;94(1):37-43.
238. Xu SY, Corvaglia AR, Chan SH, Zheng Y, Linder P. A type IV modification-dependent restriction enzyme SauUSI from *Staphylococcus aureus* subsp. *aureus* USA300. Nucleic Acids Res. 2011;39(13):5597-610.
239. Monk IR, Shah IM, Xu M, Tan MW, Foster TJ. Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. mBio. 2012;3(2):e00277-11.
240. Monk IR, Foster TJ. Genetic manipulation of Staphylococci-breaking through the barrier. Front Cell Infect Microbiol. 2012;2:49.
241. Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol. 2002;43(6):1565-75.
242. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007;315(5819):1709-12.
243. Wheatley RM, MacLean RC. CRISPR-Cas systems restrict horizontal gene transfer in Pseudomonas aeruginosa. ISME J. 2021;15(5):1420-33.
244. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, et al. Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol. 2011;9(6):467-77.
245. Carte J, Wang R, Li H, Terns RM, Terns MP. Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes. Genes Dev. 2008;22(24):3489-96.
246. Nunez JK, Kranzusch PJ, Noeske J, Wright AV, Davies CW, Doudna JA. Cas1-Cas2 complex formation mediates spacer acquisition during CRISPR-Cas adaptive immunity. Nat Struct Mol Biol. 2014;21(6):528-34.

247. Yosef I, Goren MG, Qimron U. Proteins and DNA elements essential for the CRISPR adaptation process in Escherichia coli. Nucleic Acids Res. 2012;40(12):5569-76.
248. Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, Brouns SJJ, et al. Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. Nat Rev Microbiol. 2020;18(2):67-83.
249. Zhao X, Yu Z, Xu Z. Study the Features of 57 Confirmed CRISPR Loci in 38 Strains of *Staphylococcus aureus*. Front Microbiol. 2018;9:1591.
250. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, et al. In vivo genome editing using *Staphylococcus aureus* Cas9. Nature. 2015;520(7546):186-91.
251. Li Q, Xie X, Yin K, Tang Y, Zhou X, Chen Y, et al. Characterization of CRISPR-Cas system in clinical *Staphylococcus epidermidis* strains revealed its potential association with bacterial infection sites. Microbiol Res. 2016;193:103-10.
252. De Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, et al. Bergey's manual of systematic bacteriology: Volume three: The firmicutes. New York: Springer; 2009.
253. Parte AC, Sarda Carbasse J, Meier-Kolthoff JP, Reimer LC, Goker M. List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. Int J Syst Evol Microbiol. 2020;70(11):5607-12.
254. Wertheim HF, Verveer J, Boelens HA, van Belkum A, Verbrugh HA, Vos MC. Effect of mupirocin treatment on nasal, pharyngeal, and perineal carriage of *Staphylococcus aureus* in healthy adults. Antimicrob Agents Chemother. 2005;49(4):1465-7.
255. Kirby WM. Extraction of a Highly Potent Penicillin Inactivator from Penicillin Resistant Staphylococci. Science. 1944;99(2579):452-3.
256. Rountree PM, Beard MA. Hospital strains of *Staphylococcus aureus*, with particular reference to methicillin-resistant strains. Med J Aust. 1968;2(26):1163-8.
257. Saravolatz LD, Markowitz N, Arking L, Pohlod D, Fisher E. Methicillin-resistant *Staphylococcus aureus*. Epidemiologic observations during a community-acquired outbreak. Ann Intern Med. 1982;96(1):11-6.
258. Pearman JW, Christiansen KJ, Annear DI, Goodwin CS, Metcalf C, Donovan FP, et al. Control of methicillin-resistant *Staphylococcus aureus* (MRSA) in an Australian metropolitan teaching hospital complex. Med J Aust. 1985;142(2):103-8.
259. Udo EE, Pearman JW, Grubb WB. Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. J Hosp Infect. 1993;25(2):97-108.
260. Gardam MA. Is methicillin-resistant *Staphylococcus aureus* an emerging community pathogen? A review of the literature. Can J Infect Dis. 2000;11(4):202-11.
261. Mediavilla JR, Chen L, Mathema B, Kreiswirth BN. Global epidemiology of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA). Curr Opin Microbiol. 2012;15(5):588-95.
262. Munckhof WJ, Schooneveldt J, Coombs GW, Hoare J, Nimmo GR. Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infection in Queensland, Australia. Int J Infect Dis. 2003;7(4):259-64.
263. van Hal SJ, Steinig EJ, Andersson P, Holden MTG, Harris SR, Nimmo GR, et al. Global Scale Dissemination of ST93: A Divergent *Staphylococcus aureus* Epidemic Lineage That Has Recently Emerged From Remote Northern Australia. Front Microbiol. 2018;9(1453):1453.

264. Peleg AY, Munckhof WJ, Kleinschmidt SL, Stephens AJ, Huygens F. Life-threatening community-acquired methicillin-resistant *Staphylococcus aureus* infection in Australia. Eur J Clin Microbiol Infect Dis. 2005;24(6):384-7.
265. Coombs GW, Daley DA, Mowlaboccus S, Lee YT, Pang S, Australian Group on Antimicrobial R. Australian Group on Antimicrobial Resistance (AGAR) Australian *Staphylococcus aureus* Sepsis Outcome Programme (ASSOP) Annual Report 2018. Commun Dis Intell (2018). 2020;44.
266. Shuping LL, Kuonza L, Musekiwa A, Iyaloo S, Perovic O. Hospital-associated methicillin-resistant *Staphylococcus aureus*: A cross-sectional analysis of risk factors in South African tertiary public hospitals. PLoS One. 2017;12(11):e0188216.
267. Roberts SS, Kazragis RJ. Methicillin-resistant *Staphylococcus aureus* infections in U.S. service members deployed to Iraq. Mil Med. 2009;174(4):408-11.
268. Jimenez-Truque N, Saye EJ, Soper N, Saville BR, Thomsen I, Edwards KM, et al. Association Between Contact Sports and Colonization with *Staphylococcus aureus* in a Prospective Cohort of Collegiate Athletes. Sports Med. 2017;47(5):1011-9.
269. El-Sharif A, Ashour HM. Community-acquired methicillin-resistant Staphylococcus aureus (CA-MRSA) colonization and infection in intravenous and inhalational opiate drug abusers. Exp Biol Med (Maywood). 2008;233(7):874-80.
270. Maree CL, Eells SJ, Tan J, Bancroft EA, Malek M, Harawa NT, et al. Risk factors for infection and colonization with community-associated methicillin-resistant *Staphylococcus aureus* in the Los Angeles County jail: a case-control study. Clin Infect Dis. 2010;51(11):1248-57.
271. Tacconelli E, De Angelis G, Cataldo MA, Pozzi E, Cauda R. Does antibiotic exposure increase the risk of methicillin-resistant *Staphylococcus aureus* (MRSA) isolation? A systematic review and meta-analysis. J Antimicrob Chemother. 2008;61(1):26-38.
272. Australia GoW. Decolonisation treatment for MRSA. In: Health Do, editor. 2022.
273. Australia. GoW. Management of CA-MRSA. In: Health Do, editor.: Public Health; 2022.
274. Brown NM, Goodman AL, Horner C, Jenkins A, Brown EM. Treatment of methicillin-resistant *Staphylococcus aureus* (MRSA): updated guidelines from the UK. JAC Antimicrob Resist. 2021;3(1):dlaa114.
275. Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2000;44(6):1549-55.
276. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements. Classification of staphylococcal cassette chromosome mec (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother. 2009;53(12):4961-7.
277. Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc Natl Acad Sci U S A. 2002;99(11):7687-92.
278. Ray MD, Boundy S, Archer GL. Transfer of the methicillin resistance genomic island among staphylococci by conjugation. Mol Microbiol. 2016;100(4):675-85.
279. Morikawa K, Takemura AJ, Inose Y, Tsai M, Nguyen Thi le T, Ohta T, et al. Expression of a cryptic secondary sigma factor gene unveils natural competence for DNA transformation in *Staphylococcus aureus*. PLoS Pathog. 2012;8(11):e1003003.

280. Scharn CR, Tenover FC, Goering RV. Transduction of staphylococcal cassette chromosome mec elements between strains of *Staphylococcus aureus*. Antimicrob Agents Chemother. 2013;57(11):5233-8.
281. Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res. 2018;3:124.
282. Jolley KA, Maiden MC. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics. 2010;11(1):595.
283. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004;186(5):1518-30.
284. Dossett JH, Kronvall G, Williams RC, Jr., Quie PG. Antiphagocytic effects of staphylococcal protein A. J Immunol. 1969;103(6):1405-10.
285. Frenay HM, Bunschoten AE, Schouls LM, van Leeuwen WJ, Vandenbroucke-Grauls CM, Verhoef J, et al. Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. Eur J Clin Microbiol Infect Dis. 1996;15(1):60-4.
286. Kaya H, Hasman H, Larsen J, Stegger M, Johannesen TB, Allesoe RL, et al. SCCmecFinder, a Web-Based Tool for Typing of Staphylococcal Cassette Chromosome mec in *Staphylococcus aureus* Using Whole-Genome Sequence Data. mSphere. 2018;3(1):e00612-17.
287. Urushibara N, Aung MS, Kawaguchiya M, Kobayashi N. Novel staphylococcal cassette chromosome mec (SCC*mec*) type XIV (5A) and a truncated SCC*mec* element in SCC composite islands carrying *speG* in ST5 MRSA in Japan. J Antimicrob Chemother. 2020;75(1):46-50.
288. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis. 2003;9(8):978-84.
289. Voyich JM, Otto M, Mathema B, Braughton KR, Whitney AR, Welty D, et al. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? J Infect Dis. 2006;194(12):1761-70.
290. Lina G, Piemont Y, Godail-Gamot F, Bes M, Peter MO, Gauduchon V, et al. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis. 1999;29(5):1128-32.
291. Goerke C, Pantucek R, Holtfreter S, Schulte B, Zink M, Grumann D, et al. Diversity of prophages in dominant *Staphylococcus aureus* clonal lineages. J Bacteriol. 2009;191(11):3462-8.
292. Treangen TJ, Ondov BD, Koren S, Phillippy AM. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol. 2014;15(11):524.
293. Rolo J, Miragaia M, Turlej-Rogacka A, Empel J, Bouchami O, Faria NA, et al. High genetic diversity among community-associated *Staphylococcus aureus* in Europe: results from a multicenter study. PLoS One. 2012;7(4):e34768.
294. Xie X, Bao Y, Ouyang N, Dai X, Pan K, Chen B, et al. Molecular epidemiology and characteristic of virulence gene of community-acquired and hospital-acquired methicillin-resistant *Staphylococcus aureus* isolates in Sun Yat-sen Memorial hospital, Guangzhou, Southern China. BMC Infect Dis. 2016;16:339.

295. Knight GM, Budd EL, Whitney L, Thornley A, Al-Ghusein H, Planche T, et al. Shift in dominant hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones over time. J Antimicrob Chemother. 2012;67(10):2514-22.
296. Bouchiat C, El-Zeenni N, Chakrakodi B, Nagaraj S, Arakere G, Etienne J. Epidemiology of *Staphylococcus aureus* in Bangalore, India: emergence of the ST217 clone and high rate of resistance to erythromycin and ciprofloxacin in the community. New Microbes New Infect. 2015;7:15-20.
297. Steinig EJ, Duchene S, Robinson DA, Monecke S, Yokoyama M, Laabei M, et al. Evolution and Global Transmission of a Multidrug-Resistant, Community-Associated Methicillin-Resistant Staphylococcus aureus Lineage from the Indian Subcontinent. mBio. 2019;10(6):e01105-19.
298. Song JH, Hsueh PR, Chung DR, Ko KS, Kang CI, Peck KR, et al. Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. J Antimicrob Chemother. 2011;66(5):1061-9.
299. Chuang YY, Huang YC. Molecular epidemiology of community-associated meticillin-resistant *Staphylococcus aureus* in Asia. Lancet Infect Dis. 2013;13(8):698-708.
300. Johnson AP. Methicillin-resistant *Staphylococcus aureus*: the European landscape. J Antimicrob Chemother. 2011;66 Suppl 4:iv43-iv8.
301. DeLeo FR, Kennedy AD, Chen L, Bubeck Wardenburg J, Kobayashi SD, Mathema B, et al. Molecular differentiation of historic phage-type 80/81 and contemporary epidemic Staphylococcus aureus. Proc Natl Acad Sci U S A. 2011;108(44):18091-6.
302. Arias CA, Reyes J, Carvajal LP, Rincon S, Diaz L, Panesso D, et al. A Prospective Cohort Multicenter Study of Molecular Epidemiology and Phylogenomics of *Staphylococcus aureus* Bacteremia in Nine Latin American Countries. Antimicrob Agents Chemother. 2017;61(10):e00816-17.
303. Blomfeldt A, Eskesen AN, Aamot HV, Leegaard TM, Bjornholt JV. Population-based epidemiology of *Staphylococcus aureus* bloodstream infection: clonal complex 30 genotype is associated with mortality. Eur J Clin Microbiol Infect Dis. 2016;35(5):803-13.
304. David MZ, Daum RS, Bayer AS, Chambers HF, Fowler VG, Jr., Miller LG, et al. *Staphylococcus aureus* bacteremia at 5 US academic medical centers, 2008-2011: significant geographic variation in community-onset infections. Clin Infect Dis. 2014;59(6):798-807.
305. Monecke S, Coombs G, Shore AC, Coleman DC, Akpaka P, Borg M, et al. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS One. 2011;6(4):e17936.
306. Chen H, Yin Y, van Dorp L, Shaw LP, Gao H, Acman M, et al. Drivers of methicillin-resistant *Staphylococcus aureus* (MRSA) lineage replacement in China. Genome Med. 2021;13(1):171.
307. Coombs GW, Daley DA, Mowlaboccus S, Lee YT, Pang S, Australian Group on Antimicrobial R. Australian Group on Antimicrobial Resistance (AGAR) Australian *Staphylococcus aureus* Sepsis Outcome Programme (ASSOP) Annual Report 2019. Commun Dis Intell (2018). 2020;44.
308. (ACSQHC). ACoSaQiHC. AURA 2019: third Australian report on

antimicrobial use and resistance in human health. In: ACSQHC, editor. Sydney2019.
309. Harkins CP, Pichon B, Doumith M, Parkhill J, Westh H, Tomasz A, et al. Methicillin-resistant *Staphylococcus aureus* emerged long before the introduction of methicillin into clinical practice. Genome Biol. 2017;18(1):130.

310. Oliveira DC, Tomasz A, de Lencastre H. Secrets of success of a human pathogen: molecular evolution of pandemic clones of meticillin-resistant *Staphylococcus aureus*. Lancet Infect Dis. 2002;2(3):180-9.
311. Oliveira D, Santos-Sanches I, Mato R, Tamayo M, Ribeiro G, Costa D, et al. Virtually all methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the largest Portuguese teaching hospital are caused by two internationally spread multiresistant strains: the 'Iberian' and the 'Brazilian' clones of MRSA. Clin Microbiol Infect. 1998;4(7):373-84.
312. Holden MT, Lindsay JA, Corton C, Quail MA, Cockfield JD, Pathak S, et al. Genome sequence of a recently emerged, highly transmissible, multi-antibiotic- and antiseptic-resistant variant of methicillin-resistant *Staphylococcus aureus*, sequence type 239 (TW). J Bacteriol. 2010;192(3):888-92.
313. Diekema DJ, Richter SS, Heilmann KP, Dohrn CL, Riahi F, Tendolkar S, et al. Continued emergence of USA300 methicillin-resistant *Staphylococcus aureus* in the United States: results from a nationwide surveillance study. Infect Control Hosp Epidemiol. 2014;35(3):285-92.
314. Strauss L, Stegger M, Akpaka PE, Alabi A, Breurec S, Coombs G, et al. Origin, evolution, and global transmission of community-acquired *Staphylococcus aureus* ST8. Proc Natl Acad Sci U S A. 2017;114(49):E10596-E604.
315. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol. 2003;41(11):5113-20.
316. Frisch MB, Castillo-Ramirez S, Petit RA, 3rd, Farley MM, Ray SM, Albrecht VS, et al. Invasive Methicillin-Resistant *Staphylococcus aureus* USA500 Strains from the U.S. Emerging Infections Program Constitute Three Geographically Distinct Lineages. mSphere. 2018;3(3):e00571-17.
317. Melendez AG, Satola SW, Crispell EK, Farley MM. Characteristics of USA500/Iberian Methicillin-Resistant *Staphylococcus aureus* (MRSA) Invasive Disease. Open Forum Infect Dis. 2014;1:S115-S.
318. Benson MA, Ohneck EA, Ryan C, Alonzo F, 3rd, Smith H, Narechania A, et al. Evolution of hypervirulence by a MRSA clone through acquisition of a transposable element. Mol Microbiol. 2014;93(4):664-81.
319. Boyle-Vavra S, Li X, Alam MT, Read TD, Sieth J, Cywes-Bentley C, et al. USA300 and USA500 clonal lineages of *Staphylococcus aureus* do not produce a capsular polysaccharide due to conserved mutations in the *cap5* locus. mBio. 2015;6(2):e02585-14.
320. Walther B, Monecke S, Ruscher C, Friedrich AW, Ehricht R, Slickers P, et al. Comparative molecular analysis substantiates zoonotic potential of equine methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol. 2009;47(3):704-10.
321. Cuny C, Friedrich A, Kozytska S, Layer F, Nubel U, Ohlsen K, et al. Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in different animal species. Int J Med Microbiol. 2010;300(2-3):109-17.
322. Haenni M, Chatre P, Dupieux-Chabert C, Metayer V, Bes M, Madec JY, et al. Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus* in Horses, Cats, and Dogs Over a 5-Year Period in France. Front Microbiol. 2017;8(2493):2493.
323. Vincze S, Stamm I, Kopp PA, Hermes J, Adlhoch C, Semmler T, et al. Alarming proportions of methicillin-resistant *Staphylococcus aureus* (MRSA) in wound samples from companion animals, Germany 2010-2012. PLoS One. 2014;9(1):e85656.

324. Sato T, Usui M, Konishi N, Kai A, Matsui H, Hanaki H, et al. Closely related methicillin-resistant *Staphylococcus aureus* isolates from retail meat, cows with mastitis, and humans in Japan. PLoS One. 2017;12(10):e0187319.
325. Waters AE, Contente-Cuomo T, Buchhagen J, Liu CM, Watson L, Pearce K, et al. Multidrug-Resistant *Staphylococcus aureus* in US Meat and Poultry. Clin Infect Dis. 2011;52(10):1227-30.
326. Li M, Diep BA, Villaruz AE, Braughton KR, Jiang X, DeLeo FR, et al. Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. Proc Natl Acad Sci U S A. 2009;106(14):5883-8.
327. Jansen van Rensburg MJ, Whitelaw AC, Elisha BG. Genetic basis of rifampicin resistance in methicillin-resistant *Staphylococcus aureus* suggests clonal expansion in hospitals in Cape Town, South Africa. BMC Microbiol. 2012;12:46.
328. Perovic O, Iyaloo S, Kularatne R, Lowman W, Bosman N, Wadula J, et al. Prevalence and Trends of *Staphylococcus aureus* Bacteraemia in Hospitalized Patients in South Africa, 2010 to 2012: Laboratory-Based Surveillance Mapping of Antimicrobial Resistance and Molecular Epidemiology. PLoS One. 2015;10(12):e0145429.
329. Axon JE, Carrick JB, Barton MD, Collins NM, Russell CM, Kiehne J, et al. Methicillin-resistant *Staphylococcus aureus* in a population of horses in Australia. Aust Vet J. 2011;89(6):221-5.
330. Raby E, Coombs GW, Pang S, Pearson J, Robinson OJ, Dyer J. Zoonotic transmission of the methicillin-resistant *Staphylococcus aureus* ST612-IV equine strain2016 25/10/2018; (17):[4-6 pp.]. Available from: https://www.asainc.net.au/newsletters/.
331. Haag AF, Fitzgerald JR, Penades JR. *Staphylococcus aureus* in Animals. Microbiol Spectr. 2019;7(3).
332. Devriese LA, Hommez J. Epidemiology of methicillin-resistant *Staphylococcus aureus* in dairy herds. Res Vet Sci. 1975;19(1):23-7.
333. Weese JS. Methicillin-resistant *Staphylococcus aureus* in animals. ILAR J. 2010;51(3):233-44.
334. Hall S, Kearns A, Eckford S. Livestock-associated MRSA detected in pigs in Great Britain. Vet Rec. 2015;176(6):151-2.
335. Fitzgerald JR. Human origin for livestock-associated methicillin-resistant *Staphylococcus aureus*. mBio. 2012;3(2):e00082-12.
336. Aires-de-Sousa M. Methicillin-resistant *Staphylococcus aureus* among animals: current overview. Clin Microbiol Infect. 2017;23(6):373-80.
337. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, Andersen PS, et al. *Staphylococcus aureus* CC398: host adaptation and emergence of methicillin resistance in livestock. mBio. 2012;3(1):e00305-11.
338. Larsen J, Petersen A, Sorum M, Stegger M, van Alphen L, Valentiner-Branth P, et al. Meticillin-resistant *Staphylococcus aureus* CC398 is an increasing cause of disease in people with no livestock contact in Denmark, 1999 to 2011. Euro Surveill. 2015;20(37):30021.
339. Olsen JV, Calvo-Artavia FF, Sandoe P, Toft N. Modeling the cost of eradicating livestock-associated methicillin-resistant *Staphylococcus aureus* in countries with a high proportion of positive herds. Prev Vet Med. 2018;158:97-105.
340. Bond MP. Guidelines for Prescribing, Authorising and Dispensing Veterinary Medicines (2005). 4 ed: Australian Veterinary Association Ltd; 2013.
341. (ASTAG) ASaTAGoA. Importance Ratings and Summary of Antibacterial Uses in Humans in Australia. 2014.

342. Voss A, Loeffen F, Bakker J, Klaassen C, Wulf M. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerg Infect Dis. 2005;11(12):1965-6.
343. Armand-Lefevre L, Ruimy R, Andremont A. Clonal comparison of Staphylococcus aureus isolates from healthy pig farmers, human controls, and pigs. Emerg Infect Dis. 2005;11(5):711-4.
344. van Loo I, Huijsdens X, Tiemersma E, de Neeling A, van de Sande-Bruinsma N, Beaujean D, et al. Emergence of methicillin-resistant *Staphylococcus aureus* of animal origin in humans. Emerg Infect Dis. 2007;13(12):1834-9.
345. van Cleef BA, Monnet DL, Voss A, Krziwanek K, Allerberger F, Struelens M, et al. Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Europe. Emerg Infect Dis. 2011;17(3):502-5.
346. Wolff Sönksen U, Bager F, Bortolaia V, Ellis-Iversen J, Hendriksen RS, Borck Høg B, et al. DANMAP 2016 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Kgs. Lyngby: Statens Serum Institut og Technical University of Denmark; 2017.
347. Chuang YY, Huang YC. Livestock-associated meticillin-resistant *Staphylococcus aureus* in Asia: an emerging issue? Int J Antimicrob Agents. 2015;45(4):334-40.
348. Wertheim HFL, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in Staphylococcus aureus infections. The Lancet Infectious Diseases. 2005;5(12):751-62.
349. Solberg CO. A study of carriers of *Staphylococcus aureus* with special regard to quantitative bacterial estimations. Acta Med Scand Suppl. 1965;436:1-96.
350. Pirolo M, Visaggio D, Gioffre A, Artuso I, Gherardi M, Pavia G, et al. Unidirectional animal-to-human transmission of methicillin-resistant *Staphylococcus aureus* ST398 in pig farming; evidence from a surveillance study in southern Italy. Antimicrob Resist Infect Control. 2019;8:187.
351. Reynaga E, Navarro M, Vilamala A, Roure P, Quintana M, Garcia-Nunez M, et al. Prevalence of colonization by methicillin-resistant *Staphylococcus aureus* ST398 in pigs and pig farm workers in an area of Catalonia, Spain. BMC Infect Dis. 2016;16(1):716.
352. van Cleef BA, van Benthem BH, Verkade EJ, van Rijen M, Kluytmans-van den Bergh MF, Schouls LM, et al. Dynamics of methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *Staphylococcus aureus* carriage in pig farmers: a prospective cohort study. Clin Microbiol Infect. 2014;20(10):O764-71.
353. Sahibzada S, Hernandez-Jover M, Jordan D, Thomson PC, Heller J. Emergence of highly prevalent CA-MRSA ST93 as an occupational risk in people working on a pig farm in Australia. PLoS One. 2018;13(5):e0195510.
354. Rosen K, Roesler U, Merle R, Friese A. Persistent and Transient Airborne MRSA Colonization of Piglets in a Newly Established Animal Model. Front Microbiol. 2018;9:1542.
355. Kluytmans JA. Methicillin-resistant *Staphylococcus aureus* in food products: cause for concern or case for complacency? Clin Microbiol Infect. 2010;16(1):11-5.
356. Sieber RN, Larsen AR, Urth TR, Iversen S, Moller CH, Skov RL, et al. Genome investigations show host adaptation and transmission of LA-MRSA CC398 from pigs into Danish healthcare institutions. Sci Rep. 2019;9(1):18655.
357. Larsen J, Petersen A, Larsen AR, Sieber RN, Stegger M, Koch A, et al. Emergence of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Bloodstream Infections in Denmark. Clin Infect Dis. 2017;65(7):1072-6.

358. Nielsen RT, Kemp M, Holm A, Skov MN, Detlefsen M, Hasman H, et al. Fatal Septicemia Linked to Transmission of MRSA Clonal Complex 398 in Hospital and Nursing Home, Denmark. Emerg Infect Dis. 2016;22(5):900-2.
359. Casey JA, Curriero FC, Cosgrove SE, Nachman KE, Schwartz BS. High-density livestock operations, crop field application of manure, and risk of community-associated methicillin-resistant *Staphylococcus aureus* infection in Pennsylvania. JAMA Intern Med. 2013;173(21):1980-90.
360. Feßler A, Kadlec K, Wang Y, Zhang W-J, Wu C, Shen J, et al. Small Antimicrobial Resistance Plasmids in Livestock-Associated Methicillin-Resistant Staphylococcus aureus CC398. Front Microbiol. 2018;9:2063-.
361. Kadlec K, Fessler AT, Hauschild T, Schwarz S. Novel and uncommon antimicrobial resistance genes in livestock-associated methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect. 2012;18(8):745-55.
362. Groves MD, Crouch B, Coombs GW, Jordan D, Pang S, Barton MD, et al. Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus* Isolated from Australian Veterinarians. PLoS One. 2016;11(1):e0146034.
363. Worthing KA, Abraham S, Pang S, Coombs GW, Saputra S, Jordan D, et al. Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* Isolated from Australian Animals and Veterinarians. Microb Drug Resist. 2018;24(2):203-12.
364. Williamson DA, Bakker S, Coombs GW, Tan H, Monecke S, Heffernan H. Emergence and molecular characterization of clonal complex 398 (CC398) methicillin-resistant *Staphylococcus aureus* (MRSA) in New Zealand. J Antimicrob Chemother. 2014;69(5):1428-30.
365. Goncalves da Silva A, Baines SL, Carter GP, Heffernan H, French NP, Ren X, et al. A phylogenomic framework for assessing the global emergence and evolution of clonal complex 398 methicillin-resistant *Staphylococcus aureus*. Microb Genom. 2017;3(1):e000105.
366. Sahibzada S, Abraham S, Coombs GW, Pang S, Hernandez-Jover M, Jordan D, et al. Transmission of highly virulent community-associated MRSA ST93 and livestock-associated MRSA ST398 between humans and pigs in Australia. Sci Rep. 2017;7(1):5273.
367. Sahibzada S, Hernández-Jover M, Jordan D, Thomson PC, Heller J. Emergence of highly prevalent CA-MRSA ST93 as an occupational risk in people working on a pig farm in Australia. PLoS One. 2018;13(5):e0195510-e.
368. Danish Agriculture & Food Council. Statistics 2019 Pigmeat. Axelborg Copenhagen: Landbrug & Fødevarer F.m.b.A.; 2020.
369. Korsgaard HB, Ellis-Iversen J, Hendriksen RS, Borck Høg B, Ronco T, Attauabi M, et al. DANMAP 2019 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Kgs. Lyngby: Statens Serum Institut og Technical University of Denmark; 2020.
370. Lindsay JA, Holden MT. *Staphylococcus aureus*: superbug, super genome? Trends Microbiol. 2004;12(8):378-85.
371. Didelot X, Maiden MC. Impact of recombination on bacterial evolution. Trends Microbiol. 2010;18(7):315-22.
372. Everitt RG, Didelot X, Batty EM, Miller RR, Knox K, Young BC, et al. Mobile elements drive recombination hotspots in the core genome of *Staphylococcus aureus*. Nat Commun. 2014;5(1):3956.

373. Varga M, Kuntova L, Pantucek R, Maslanova I, Ruzickova V, Doskar J. Efficient transfer of antibiotic resistance plasmids by transduction within methicillin-resistant *Staphylococcus aureus* USA300 clone. FEMS Microbiol Lett. 2012;332(2):146-52.
374. Humphrey S, San Millan A, Toll-Riera M, Connolly J, Flor-Duro A, Chen J, et al. Staphylococcal phages and pathogenicity islands drive plasmid evolution. Nat Commun. 2021;12(1):5845.
375. Schierup MH, Hein J. Consequences of recombination on traditional phylogenetic analysis. Genetics. 2000;156(2):879-91.
376. Didelot X, Falush D. Inference of bacterial microevolution using multilocus sequence data. Genetics. 2007;175(3):1251-66.
377. Vos M, Didelot X. A comparison of homologous recombination rates in bacteria and archaea. ISME J. 2009;3(2):199-208.
378. Hedge J, Wilson DJ. Bacterial phylogenetic reconstruction from whole genomes is robust to recombination but demographic inference is not. mBio. 2014;5(6):e02158.
379. Abadi S, Azouri D, Pupko T, Mayrose I. Model selection may not be a mandatory step for phylogeny reconstruction. Nat Commun. 2019;10(1):934.
380. Kwong SM, Ramsay JP, Jensen SO, Firth N. Replication of Staphylococcal Resistance Plasmids. Front Microbiol. 2017;8(2279):2279.
381. Novick RP. Staphylococcal plasmids and their replication. Annu Rev Microbiol. 1989;43:537-65.
382. Firth N, Skurray RA. Genetics: accessory elements and genetic exchange. Gram-Positive Pathogens. 2006:413-26.
383. Goering RV, Ruff EA. Comparative analysis of conjugative plasmids mediating gentamicin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 1983;24(3):450-2.
384. Rossi F, Diaz L, Wollam A, Panesso D, Zhou Y, Rincon S, et al. Transferable vancomycin resistance in a community-associated MRSA lineage. N Engl J Med. 2014;370(16):1524-31.
385. MacWilliams MP, Liao M. Luria broth (lb) and luria agar (la) media and their uses protocol. [Internet]. American Society for Microbiology; 2006 [Available from: https://www.asmscience.org/content/education/protocol/protocol.3031.
386. Kreiswirth BN, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, et al. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature. 1983;305(5936):709-12.
387. Costa SK, Donegan NP, Corvaglia AR, Francois P, Cheung AL. Bypassing the Restriction System To Improve Transformation of *Staphylococcus epidermidis*. J Bacteriol. 2017;199(16):e00271-17.
388. Dummler A, Lawrence AM, de Marco A. Simplified screening for the detection of soluble fusion constructs expressed in *E. coli* using a modular set of vectors. Microb Cell Fact. 2005;4:34.
389. Norrander J, Kempe T, Messing J. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene. 1983;26(1):101-6.
390. Okonechnikov K, Golosova O, Fursov M, team U. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics. 2012;28(8):1166-7.
391. Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res. 2017;27(5):722-36.
392. Hunt M, Silva ND, Otto TD, Parkhill J, Keane JA, Harris SR. Circlator: automated circularization of genome assemblies using long sequencing reads. Genome Biol. 2015;16(1):294.

393. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754-60.
394. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078-9.
395. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19(5):455-77.
396. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One. 2014;9(11):e112963.
397. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, et al. Protein Identification and Analysis Tools on the ExPASy Server. In: Walker JM, editor. The Proteomics Protocols Handbook. Totowa, NJ: Humana Press; 2005. p. 571-607.
398. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671-5.
399. Bringans S, Eriksen S, Kendrick T, Gopalakrishnakone P, Livk A, Lock R, et al. Proteomic analysis of the venom of Heterometrus longimanus (Asian black scorpion). Proteomics. 2008;8(5):1081-96.
400. Ramsay JP. High-throughput β-galactosidase and β-glucuronidase Assays Using Fluorogenic Substrates. Bio-Protocol. 2013;3(14):e827.
401. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10.
402. Schijffelen MJ, Boel CH, van Strijp JA, Fluit AC. Whole genome analysis of a livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of human endocarditis. BMC Genomics. 2010;11:376.
403. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20.
404. Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 2015;31(10):1674-6.
405. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30(14):2068-9.
406. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012;67(11):2640-4.
407. Mellmann A, Friedrich AW, Rosenkotter N, Rothganger J, Karch H, Reintjes R, et al. Automated DNA sequence-based early warning system for the detection of methicillin-resistant *Staphylococcus aureus* outbreaks. PLoS Med. 2006;3(3):e33.
408. Couvin D, Bernheim A, Toffano-Nioche C, Touchon M, Michalik J, Neron B, et al. CRISPRCasFinder, an update of CRISRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. Nucleic Acids Res. 2018;46(W1):W246-W51.
409. Information. NCfB. BLAST® Command Line Applications User Manual Bethesda (MD)2008 [Available from: https://www.ncbi.nlm.nih.gov/books/NBK569841/.
410. Novick RP, Ram G. Staphylococcal pathogenicity islands-movers and shakers in the genomic firmament. Curr Opin Microbiol. 2017;38:197-204.

411. Novick R. Properties of a cryptic high-frequency transducing phage in Staphylococcus aureus. Virology. 1967;33(1):155-66.
412. Krawczyk PS, Lipinski L, Dziembowski A. PlasFlow: predicting plasmid sequences in metagenomic data using genome signatures. Nucleic Acids Res. 2018;46(6):e35.
413. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792-7.
414. Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, et al. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic Acids Res. 2017;45(D1):D200-D3.
415. Higgins DG, Thompson JD, Gibson TJ. Using CLUSTAL for multiple sequence alignments. Computer Methods for Macromolecular Sequence Analysis. Methods in Enzymology. 266: Academic Press; 1996. p. 383-402.
416. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol. 2018;35(6):1547-9.
417. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 1993;10(3):512-26.
418. Chua KY, Seemann T, Harrison PF, Monagle S, Korman TM, Johnson PD, et al. The dominant Australian community-acquired methicillin-resistant *Staphylococcus aureus* clone ST93-IV [2B] is highly virulent and genetically distinct. PLoS One. 2011;6(10):e25887.
419. Price MN, Arkin AP. PaperBLAST: Text Mining Papers for Information about Homologs. mSystems. 2017;2(4):e00039-17.
420. Moodley A, Oosthuysen WF, Duse AG, Marais E, South African MSG. Molecular characterization of clinical methicillin-resistant *Staphylococcus aureus* isolates in South Africa. J Clin Microbiol. 2010;48(12):4608-11.
421. Antiabong JF, Kock MM, Maphanga TG, Salawu AM, Mbelle NM, Ehlers MM. Trends in the Genetic Background of Methicillin-Resistant *Staphylococcus Aureus* Clinical Isolates in a South African Hospital: An Institutional-Based Observational Study. Open Microbiol J. 2017;11:339-51.
422. Perovic O, Singh-Moodley A, Govender NP, Kularatne R, Whitelaw A, Chibabhai V, et al. A small proportion of community-associated methicillin-resistant *Staphylococcus aureus* bacteraemia, compared to healthcare-associated cases, in two South African provinces. Eur J Clin Microbiol Infect Dis. 2017;36(12):2519-32.
423. Dailey L, Coombs GW, O'Brien FG, Pearman JW, Christiansen K, Grubb WB, et al. Methicillin-resistant *Staphylococcus aureus*, Western Australia. Emerg Infect Dis. 2005;11(10):1584-90.
424. Murphy RJT, Lee YT, Pang S, Bastholm TR, Crow JE, Davis AM, et al. Complete Genome Sequence of a *Staphylococcus aureus* Sequence Type 612 Isolate from an Australian Horse. Microbiol Resour Announc. 2018;7(4):e00869-18.
425. Coombs GW, Monecke S, Pearson JC, Tan HL, Chew YK, Wilson L, et al. Evolution and diversity of community-associated methicillin-resistant *Staphylococcus aureus* in a geographical region. BMC Microbiol. 2011;11:215.
426. Oosthuysen WF, Orth H, Lombard C, Sinha B, Wasserman E. *In vitro* characterization of representative clinical South African *Staphylococcus aureus* isolates from various clonal lineages. New Microbes New Infect. 2014;2(4):115-22.

427. Viana D, Blanco J, Tormo-Mas MA, Selva L, Guinane CM, Baselga R, et al. Adaptation of *Staphylococcus aureus* to ruminant and equine hosts involves SaPI-carried variants of von Willebrand factor-binding protein. Mol Microbiol. 2010;77(6):1583-94.
428. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. PHAST: a fast phage search tool. Nucleic Acids Res. 2011;39(Web Server issue):347-52.
429. van Alen S, Ballhausen B, Kaspar U, Kock R, Becker K. Prevalence and Genomic Structure of Bacteriophage phi3 in Human-Derived Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates from 2000 to 2015. J Clin Microbiol. 2018;56(9):e00140-18.
430. Wang L, Safo M, Archer GL. Characterization of DNA sequences required for the CcrAB-mediated integration of staphylococcal cassette chromosome mec, a *Staphylococcus aureus* genomic island. J Bacteriol. 2012;194(2):486-98.
431. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25(17):3389-402.
432. Ito T, Ma XX, Takeuchi F, Okuma K, Yuzawa H, Hiramatsu K. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. Antimicrob Agents Chemother. 2004;48(7):2637-51.
433. Cortes MF, Botelho AM, Almeida LG, Souza RC, de Lima Cunha O, Nicolas MF, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* from ST1 lineage harboring a new SCCmec IV subtype (SCCmec IVm) containing the *tetK* gene. Infect Drug Resist. 2018;11:2583-92.
434. Haroche J, Allignet J, Buchrieser C, El Solh N. Characterization of a variant of *vga(A)* conferring resistance to streptogramin A and related compounds. Antimicrob Agents Chemother. 2000;44(9):2271-5.
435. Wright LD, Grossman AD. Autonomous Replication of the Conjugative Transposon Tn*916*. J Bacteriol. 2016;198(24):3355-66.
436. Bastos MC, Murphy E. Transposon Tn*554* encodes three products required for transposition. EMBO J. 1988;7(9):2935-41.
437. Kehrenberg C, Schwarz S. Florfenicol-chloramphenicol exporter gene *fexA* is part of the novel transposon Tn*558*. Antimicrob Agents Chemother. 2005;49(2):813-5.
438. Koch M, Willi J, Pradere U, Hall J, Polacek N. Critical 23S rRNA interactions for macrolide-dependent ribosome stalling on the ErmCL nascent peptide chain. Nucleic Acids Res. 2017;45(11):6717-28.
439. Sato S, Miyanaga A, Kim SY, Kuzuyama T, Kudo F, Eguchi T. Biochemical and Structural Analysis of FomD That Catalyzes the Hydrolysis of Cytidylyl (S)-2-Hydroxypropylphosphonate in Fosfomycin Biosynthesis. Biochemistry. 2018;57(32):4858-66.
440. Imae K, Saito Y, Kizaki H, Ryuno H, Mao H, Miyashita A, et al. Novel Nucleoside Diphosphatase Contributes to *Staphylococcus aureus* Virulence. J Biol Chem. 2016;291(36):18608-19.
441. Zeman M, Maslanova I, Indrakova A, Siborova M, Mikulasek K, Bendickova K, et al. *Staphylococcus sciuri* bacteriophages double-convert for staphylokinase and phospholipase, mediate interspecies plasmid transduction, and package *mecA* gene. Sci Rep. 2017;7:46319.
442. Gargis AS, Heath LS, Heath HE, LeBlanc PA, Sloan GL. Characterization of pACK4, a mobilizable plasmid from *Staphylococcus simulans* biovar *staphylolyticus*. Plasmid. 2009;62(3):201-5.

443. Slifierz MJ, Friendship RM, Weese JS. Methicillin-resistant *Staphylococcus aureus* in commercial swine herds is associated with disinfectant and zinc usage. Appl Environ Microbiol. 2015;81(8):2690-5.
444. Noack S, Chapman HD, Selzer PM. Anticoccidial drugs of the livestock industry. Parasitol Res. 2019;118(7):2009-26.
445. Butaye P, Devriese LA, Haesebrouck F. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. Clin Microbiol Rev. 2003;16(2):175-88.
446. Rutkowski J, Brzezinski B. Structures and properties of naturally occurring polyether antibiotics. Biomed Res Int. 2013;2013:162513.
447. Nilsson O, Myrenas M, Agren J. Transferable genes putatively conferring elevated minimum inhibitory concentrations of narasin in *Enterococcus faecium* from Swedish broilers. Vet Microbiol. 2016;184:80-3.
448. Australian Pesticides and Veterinary Medicines Authority. Report on the Quantity of Antimicrobial Products Sold for Veterinary Use in Australia: July 2005 to June 2010 [Internet]. Kingston, ACT: Australian Pesticides and Veterinary Medicines Authority; 2014 [73]. Available from: https://apvma.gov.au/node/11816.
449. Monk IR, Tree JJ, Howden BP, Stinear TP, Foster TJ. Complete Bypass of Restriction Systems for Major *Staphylococcus aureus* Lineages. mBio. 2015;6(3):e00308-15.
450. Cao L, Gao CH, Zhu J, Zhao L, Wu Q, Li M, et al. Identification and functional study of type III-A CRISPR-Cas systems in clinical isolates of *Staphylococcus aureus*. Int J Med Microbiol. 2016;306(8):686-96.
451. Stinear TP, Holt KE, Chua K, Stepnell J, Tuck KL, Coombs G, et al. Adaptive change inferred from genomic population analysis of the ST93 epidemic clone of community-associated methicillin-resistant *Staphylococcus aureus*. Genome Biol Evol. 2014;6(2):366-78.
452. Jamrozy DM, Fielder MD, Butaye P, Coldham NG. Comparative genotypic and phenotypic characterisation of methicillin-resistant *Staphylococcus aureus* ST398 isolated from animals and humans. PLoS One. 2012;7(7):e40458.
453. Pirolo M, Sieber RN, Moodley A, Visaggio D, Artuso I, Gioffre A, et al. Local and Transboundary Transmissions of Methicillin-Resistant *Staphylococcus aureus* Sequence Type 398 through Pig Trading. Appl Environ Microbiol. 2020;86(13):e00430-20.
454. Wendlandt S, Kadlec K, Fessler AT, van Duijkeren E, Schwarz S. Two different *erm(C)*-carrying plasmids in the same methicillin-resistant *Staphylococcus aureus* CC398 isolate from a broiler farm. Vet Microbiol. 2014;171(3-4):382-7.
455. Bosch T, Witteveen S, Haenen A, Landman F, Schouls LM. Next-Generation Sequencing Confirms Presumed Nosocomial Transmission of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* in the Netherlands. Appl Environ Microbiol. 2016;82(14):4081-9.
456. Coombs GW. The Australian Community Methicillin Resistant *Staphylococcus aureus* Endemic: Clonal Spread or Multiple Evolutionary Events. [dissertation on the Internet] Bentley, WA: Curtin University; 2012.
457. Chanchaithong P, Perreten V, Am-In N, Lugsomya K, Tummaruk P, Prapasarakul N. Molecular Characterization and Antimicrobial Resistance of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates from Pigs and Swine Workers in Central Thailand. Microb Drug Resist. 2019;25(9):1382-9.

458. Ruiz-Ripa L, Belles A, Garcia M, Torres C. Detection of a *cfr*-positive MRSA CC398 strain in a pig farmer in Spain. Enferm Infecc Microbiol Clin. 2021;39(3):139-41.
459. Kadlec K, Ehricht R, Monecke S, Steinacker U, Kaspar H, Mankertz J, et al. Diversity of antimicrobial resistance pheno- and genotypes of methicillin-resistant *Staphylococcus aureus* ST398 from diseased swine. J Antimicrob Chemother. 2009;64(6):1156-64.
460. Argudin MA, Tenhagen BA, Fetsch A, Sachsenroder J, Kasbohrer A, Schroeter A, et al. Virulence and resistance determinants of German *Staphylococcus aureus* ST398 isolates from nonhuman sources. Appl Environ Microbiol. 2011;77(9):3052-60.
461. Fessler A, Scott C, Kadlec K, Ehricht R, Monecke S, Schwarz S. Characterization of methicillin-resistant *Staphylococcus aureus* ST398 from cases of bovine mastitis. J Antimicrob Chemother. 2010;65(4):619-25.
462. Lienen T, Schnitt A, Hammerl JA, Maurischat S, Tenhagen BA. Genomic Distinctions of LA-MRSA ST398 on Dairy Farms From Different German Federal States With a Low Risk of Severe Human Infections. Front Microbiol. 2020;11:575321.
463. Moreno LZ, Dutra MC, Moreno M, Ferreira TS, Silva GF, Matajira CE, et al. Vancomycin-intermediate livestock-associated methicillin-resistant *Staphylococcus aureus* ST398/t9538 from swine in Brazil. Mem Inst Oswaldo Cruz. 2016;111(10):659-61.
464. Bortolami A, Verin R, Chantrey J, Corro M, Ashpole I, Lopez J, et al. Characterization of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* CC398 and mecC-positive CC130 from Zoo Animals in the United Kingdom. Microb Drug Resist. 2017;23(7):908-14.
465. Zhou W, Li X, Shi L, Wang HH, Yan H. Novel SCC*mec* type XII methicillin-resistant *Staphylococcus aureus* isolates identified from a swine production and processing chain. Vet Microbiol. 2018;225:105-13.
466. Fessler AT, Calvo N, Gutierrez N, Munoz Bellido JL, Fajardo M, Garduno E, et al. Cfr-mediated linezolid resistance in methicillin-resistant *Staphylococcus aureus* and *Staphylococcus haemolyticus* associated with clinical infections in humans: two case reports. J Antimicrob Chemother. 2014;69(1):268-70.
467. Ruiz-Ripa L, Bellés-Bellés A, Fernández-Fernández R, García M, Vilaró A, Zarazaga M, et al. Linezolid-resistant MRSA-CC398 carrying the cfr gene, and MRSA-CC9 isolates from pigs with signs of infection in Spain. Journal of Applied Microbiology. 2021;n/a(n/a).
468. Jones JM, Yost SC, Pattee PA. Transfer of the conjugal tetracycline resistance transposon Tn*916* from *Streptococcus faecalis* to *Staphylococcus aureus* and identification of some insertion sites in the staphylococcal chromosome. J Bacteriol. 1987;169(5):2121-31.
469. Kehrenberg C, Aarestrup FM, Schwarz S. IS*21-558* insertion sequences are involved in the mobility of the multiresistance gene *cfr*. Antimicrob Agents Chemother. 2007;51(2):483-7.
470. Cuny C, Arnold P, Hermes J, Eckmanns T, Mehraj J, Schoenfelder S, et al. Occurrence of *cfr*-mediated multiresistance in staphylococci from veal calves and pigs, from humans at the corresponding farms, and from veterinarians and their family members. Vet Microbiol. 2017;200:88-94.
471. Wang Y, He T, Schwarz S, Zhao Q, Shen Z, Wu C, et al. Multidrug resistance gene *cfr* in methicillin-resistant coagulase-negative staphylococci from chickens, ducks, and pigs in China. Int J Med Microbiol. 2013;303(2):84-7.

472. Wang Y, Zhang W, Wang J, Wu C, Shen Z, Fu X, et al. Distribution of the multidrug resistance gene *cfr* in *Staphylococcus* species isolates from swine farms in China. Antimicrob Agents Chemother. 2012;56(3):1485-90.
473. Li SM, Zhou YF, Li L, Fang LX, Duan JH, Liu FR, et al. Characterization of the Multi-Drug Resistance Gene cfr in Methicillin-Resistant *Staphylococcus aureus* (MRSA) Strains Isolated From Animals and Humans in China. Front Microbiol. 2018;9(2925):2925.
474. Thomas DJ, Morgan JA, Whipps JM, Saunders JR. Transfer of plasmid pBC16 between *Bacillus thuringiensis* strains in non-susceptible larvae. FEMS Microbiol Ecol. 2002;40(3):181-90.
475. Polak J, Novick RP. Closely related plasmids from *Staphylococcus aureus* and soil bacilli. Plasmid. 1982;7(2):152-62.
476. Government. A. Australian Pigmeat Industry, Report no. 35. In: Commission P, editor. Melbourne2005.
477. Tan TY, Ng SY. Comparison of Etest, Vitek and agar dilution for susceptibility testing of colistin. Clin Microbiol Infect. 2007;13(5):541-4.
478. Challand MR, Salvadori E, Driesener RC, Kay CW, Roach PL, Spencer J. Cysteine methylation controls radical generation in the Cfr radical AdoMet rRNA methyltransferase. PLoS One. 2013;8(7):e67979.
479. Gupta P, Kannan K, Mankin AS, Vazquez-Laslop N. Regulation of gene expression by macrolide-induced ribosomal frameshifting. Mol Cell. 2013;52(5):629-42.
480. Smialowski P, Doose G, Torkler P, Kaufmann S, Frishman D. PROSO II--a new method for protein solubility prediction. FEBS J. 2012;279(12):2192-200.
481. LaMarre JM, Locke JB, Shaw KJ, Mankin AS. Low fitness cost of the multidrug resistance gene cfr. Antimicrob Agents Chemother. 2011;55(8):3714-9.
482. Denoya CD, Dubnau D. Site and substrate specificity of the ermC 23S rRNA methyltransferase. J Bacteriol. 1987;169(8):3857-60.
483. Ntokou E, Hansen LH, Kongsted J, Vester B. Biochemical and Computational Analysis of the Substrate Specificities of Cfr and RlmN Methyltransferases. PLoS One. 2015;10(12):e0145655.
484. Koutmou KS, Schuller AP, Brunelle JL, Radhakrishnan A, Djuranovic S, Green R. Ribosomes slide on lysine-encoding homopolymeric A stretches. Elife. 2015;4:e05534.
485. Kime L, Randall CP, Banda FI, Coll F, Wright J, Richardson J, et al. Transient Silencing of Antibiotic Resistance by Mutation Represents a Significant Potential Source of Unanticipated Therapeutic Failure. mBio. 2019;10(5):e01755-19.
486. Hutchinson DK. Oxazolidinone antibacterial agents: a critical review. Curr Top Med Chem. 2003;3(9):1021-42.
487. Zhao Q, Wang Y, Wang S, Wang Z, Du XD, Jiang H, et al. Prevalence and Abundance of Florfenicol and Linezolid Resistance Genes in Soils Adjacent to Swine Feedlots. Sci Rep. 2016;6:32192.
488. Wasserman S, Louw G, Ramangoaela L, Barber G, Hayes C, Omar SV, et al. Linezolid resistance in patients with drug-resistant TB and treatment failure in South Africa. J Antimicrob Chemother. 2019;74(8):2377-84.
489. Wasserman S, Meintjes G, Maartens G. Linezolid in the treatment of drug-resistant tuberculosis: the challenge of its narrow therapeutic index. Expert Rev Anti Infect Ther. 2016;14(10):901-15.

490. Groves MD, O'Sullivan MV, Brouwers HJ, Chapman TA, Abraham S, Trott DJ, et al. *Staphylococcus aureus* ST398 detected in pigs in Australia. J Antimicrob Chemother. 2014;69(5):1426-8.
491. Sahibzada S, Pang S, Hernandez-Jover M, Jordan D, Abraham S, O'Dea M, et al. Prevalence and antimicrobial resistance of MRSA across different pig age groups in an intensive pig production system in Australia. Zoonoses Public Health. 2020;67(5):576-86.
492. Sahibzada S. Epidemiology and molecular characterisation of methicillin resistant *Staphylococcus aureus* in the Australian pig industry. Australia: Charles Sturt University; 2018.
493. Coombs GW, Daley D, Shoby P, Yee NWT, Robinson JO, Murray R, et al. Genomic Characterisation of CC398 MRSA Causing Severe Disease in Australia. Int J Antimicrob Agents. 2022:106577.
494. Australian Pesticides and Veterinary Medicines Authority. Qantity of antimicrobial products sold for veterinary use in Australia: July 2005 to June 2010. Kingston ACT: APVMA; 2014.
495. Cutler R, Gleeson B, Page S, Norris J, Browning G, Australian Veterinary Association L, et al. Antimicrobial prescribing guidelines for pigs. Aust Vet J. 2020;98(4):105-34.
496. Australian Strategic and Technical Advisory Group on Antimicrobial Resistance. Importance Ratings and Summary of Antibacterial Uses in Humans and Animal Health in Australia [Internet]. Australian Strategic and Technical Advisory Group; 2018 [36]. Available from: https://www.amr.gov.au/resources/importance-ratings-and-summary-antibacterial-uses-human-and-animal-health-australia.
497. Australian Pesticides and Veterinary Medicines Authority. Substances not permitted for use on food-producing animals in Australia [Internet]. Australian Pesticides and Veterinary Medicines Authority; 2014 [Available from: https://apvma.gov.au/node/11626.
498. Kehrenberg C, Schwarz S. Distribution of florfenicol resistance genes *fexA* and *cfr* among chloramphenicol-resistant *Staphylococcus* isolates. Antimicrob Agents Chemother. 2006;50(4):1156-63.
499. Trott DJ, Turnidge J, Kovac JH, Simjee S, Wilson D, Watts J. Comparative macrolide use in humans and animals: should macrolides be moved off the World Health Organisation's critically important antimicrobial list? J Antimicrob Chemother. 2021;76(8):1955-61.
500. Murphy RJT, Ramsay JP, Lee YT, Pang S, O'Dea MA, Pearson JC, et al. Multiple introductions of methicillin-resistant *Staphylococcus aureus* ST612 into Western Australia associated both with human and equine reservoirs. Int J Antimicrob Agents. 2019;54(6):681-5.
501. Morice P, Allano M, Provost C, Fairbrother JH, Gagnon CA, Sauve F. Whole genome sequencing of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* isolated from 4 horses in a veterinary teaching hospital and its ambulatory service. Can J Vet Res. 2021;85(3):218-23.
502. Amoako DG, Somboro AM, Abia ALK, Allam M, Ismail A, Bester L, et al. Genomic analysis of methicillin-resistant *Staphylococcus aureus* isolated from poultry and occupational farm workers in Umgungundlovu District, South Africa. Sci Total Environ. 2019;670:704-16.
503. Moremi N, Claus H, Vogel U, Mshana SE. The role of patients and healthcare workers *Staphylococcus aureus* nasal colonization in occurrence of surgical site infection among patients admitted in two centers in Tanzania. Antimicrob Resist Infect Control. 2019;8(1):102.
504. Papp JR, Rowlinson MC, O'Connor NP, Wholehan J, Razeq JH, Glennen A, et al. Accuracy and reproducibility of the Etest to detect drug-resistant *Neisseria gonorrhoeae* to contemporary treatment. J Med Microbiol. 2018;67(1):68-73.

Supplementary 1. Media, buffer and reagents

Table 7-1 - S1 Media

Media	Ingredients
Lysogeny broth (LB)	5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl
LB agar	16 g/L agar added to LB
Mueller Hinton Broth (MH)	21 g/L MH powder
Mueller Hinton Agar	16g/L agar added to MH
Tryptic soy broth (TSB)	30 g/L tryptone soy broth powder
Tryptic soy agar (TSA)	16 g/L agar added to TSB
Super Optimal Broth (SOB)	20 g/L tryptone, 5 g/L yeast extract, 0.584 g/L NaCl, 0.186 g/L KCl, 2.4 g/L $MgSO_4$

Media were prepared in distilled water and autoclaved at 121 C and 15 psi for 15 min.

Table 7-2 - S2 Buffers

Buffer/reagent	Components
1 x TAE buffer	40 mM Tris, 20 mM acetic acid, 1 mM EDTA
4x SDS loading buffer	50 mM Tris pH 6.8, 100 mM DTT, 2% (v/v) Sodium dodecyl sulphate (SDS), 0.1% (w/v) bromophenol blue, 30% (v/v) glycerol
1x Protein gel running buffer	25 mM Tris pH 8.3, 192 mM glycine, 0.1% (v/v) SDS
Amylose column buffer	20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA
Coomassie blue stain	2.5 g/L Coomassie Brilliant Blue R250, 30% (v/v) ethanol, 10% (v/v) acetic acid
Coomassie blue destaining solution	10% (v/v) acetic acid, 30% (v/v) ethanol

Nickel-affinity binding buffer	50 mM NaH_2PO_4 pH 7.5, 1 M NaCl, 10% (v/v) glycerol, 25 mM imidazole
Size-exclusion column buffer	50 mM NaH_2PO_4 pH 7.5, 300 mM NaCl, 5% (v/v) glycerol

Supplementary 2. Plasmids detected in Australian Pigs

Table 7-3 - Plasmids of Australian ST93 and ST398 MRSA

Plasmid name	Size	Prevalence (ST93 / ST398)	ORFs	Length (aa)	Predicted function	Homologue (Identity %)
pPTFIP3	2,365 bp	76% / 46%	*repL*	158	Plasmid replication	ETO50985.1 (100)
			ermC	244	Macrolide resistance	AIU96746.1 (100)
pPTDrAP2a	4,203 bp	4% / 4%	*repB*	199	Plasmid replication	WP_012816703.1 (100)
			ermC	244	Macrolide resistance	AIU96746.1 (97.23)
			pre	396	Plasmid recombination enzyme	WP_031917739.1 (100)
			fomD	169	fosfomycin biosynthesis	WP_031915809.1 (100)
			cop-6	55	Copy number control	ADA79934.1 (100)
pRGMX-2993	2,993 bp	18% / 46%	*rep_1*	334	Plasmid replication	WP_078370069.1 (100)
			rgmX	273	23s RNA guanine methyltransferase	PPJ84487.1 (100)
pRGMX-3050	3,050 bp	9% / 15%	*rep_1*	311	Plasmid replication	WP_109028160.1 (100)
			rgmX	273	23s RNA guanine methyltransferase	PPJ84487.1 (100)
pH15	4,757 bp	18% / 11%	*rep_1*	334	Plasmid replication	AAA84919.1 (99.7)
			tetL	458	Tetracycline resistance	WP_047342058.1 (95.51)
			pre	419	Plasmid recombination enzyme	WP_064136911.1 (99.76)

Plasmid name	Size	Prevalence (ST93 / ST398)	ORFs	Length (aa)	Predicted function	Homologue (Identity %)
pH4	5,685 bp	4% / 4%	*rep_1*	334	Plasmid Replication	AAA84919.1
			aadD	256	Kanamycin resistance	ANW07957.1
			tetL	428	Tetracycline resistance	WP_047342058.1 (95.51)
			pre	420	Plasmid recombination enzyme	WP_046207705.1 (100)
pPTGrAP5	3,620 bp	9% / 4%	*repC*	314	Plasmid replication	WP_150862132.1 (100)
			mobC	138	plasmid mobilisation relaxasome protein	WP_172693035.1 (95.65)
			mobA	369	plasmid mobilisation	WP_150862131.1 (100)
pP3330	3,595 bp	0% / 38%	*rep7*	312	Plasmid replication	WP_150868142.1 (100)
			mobC	129	Plasmid mobilisation relaxasome protein	WP_012779611.1 (100)
			mobA	325	Conjugative nickase	WP_150861597.1 (100)
			hyp	226	Unknown	ACN21627.1 (99)
pE008	2,608 bp	9% / 4%	*rep_1*	311	Plasmid replication	AGK82339.1 (99.67)
			hyp	57		AGK82340.1 (100)
			qacC	106	Quaternary ammonium resistance	WP_015740450.1 (100)
			cop-6	53	Copy number regulator	AGK82337.1 (100)

Plasmid name	Size	Prevalence (ST93 / ST398)	ORFs	Length (aa)	Predicted function	Homologue (Identity %)
pH14	5,756 bp	2% / 0%	*repC*	314	Plasmid replication	WP_031892478.1 (100)
			tetK	459	Tetracycline resistance	EVJ05357.1 (100)
			mobC	127	Plasmid mobilisation relaxasome protein	WP_054189139.1
			mobA	283	Plasmid mobilisation	WP_031879624.1 (100)
			mobB	169	Plasmid mobilisation	SLC84770.1 (95.87)
			mobB	111	Plasmid mobilisation	NAN71198.1 (100)
pPTGrBP5	4,754 bp	0% / 4%	*repC*	279	Plasmid replication	WP_000277654.1 (100)
			tetK	459	Tetracycline resistance	EVJ05357.1 (100)
			pre	413	Plasmid recombination enzyme	QBK46667.1 (99.76)
pE026	3,357 bp	0% / 8%	*rep_1*	306	Plasmid replication	WP_151131050.1 (99.67)
			hsdS	370	Restriction methyltransferase specificity subunit	WP_151131049.1 (100)

Table 7-4 - Plasmid pP221 of ST93 MRSA

Plasmid name	Size	Prevalence (ST93 / ST398)	ORFs	Length (aa)	Predicted function	Homologue (Identity %)
pP221	27, 186 bp	0% / 23%	*rep_1*	280	Plasmid replication	WP_049948378.1 (100)
			IS257	224	Transposase	WP_116020504.1 (95.55)
			Hypothetical	296	Replication protein	WP_078100104.1 (100)
			DUF536 protein	247	Unknown	WP_031794970.1 (100)
			Hypothetical	97	Replication protein	EAE5933115.1 (100)
			Hypothetical	88	Unknown	EZW90202.1 (100)
			DUF4260	114	Unknown	WP_031797807.1 (100)
			Hypothetical	87	Unknown	WP_031797806.1 (100)
			Hypothetical	94	Unknown	WP_165885998.1 (77.91)
			ABC-F ATP-binding cassette domain protein	539	Putative polyketide exporter	WP_114668475.1 (100)
			Tetronasin resistance protein	535	Tetronasin resistance	WP_031794962.1 (100)
			ABC transporter ATP-binding protein	308	ABC transporter ATP-binding protein	WP_031794961.1 (100)
			tetR	229	AcrR family transcriptional regulator	WP_202112068.1 (100)
			Hypothetical	74	Unknown	EZR82204.1 (100)

Plasmid name	Size	Prevalence (ST93 / ST398)	ORFs	Length (aa)	Predicted function	Homologue (Identity %)
			Hypothetical	64	Recombinase (truncated)	EZR82213.1 (100)
			ydhK DUF1541	180	unknown	WP_031794957.1 (100)
			mco	477	Multicopper oxidase	WP_031794956.1 (100)
			copA	693	Cu^{2+} transporter ATPase	WP_164711974.1 (100)
			hypothetical	42	Unknown	PTI59527.1 (81.48)
			hypothetical	51	Unknown	SKR87959.1 (100)
			Tn*552* invertase	192	Recombinase	WP_031794966.1 (99.48)
			hypothetical	50	Unknown	WP_191239256.1 (100)
			hypothetical	240	Unknown	WP_192947932.1 (99.58)
			repA_N	318	Plasmid replication	WP_031794967.1 (100)
			hypothetical	56		EZT73249.1 (100)
			cadD	205	cadmium binding protein	WP_031869318.1 (100)
			cadX	115	Transcriptional regulator	WP_115265151.1 (99.12)

Supplementary 3. Restriction-Modification in ST93

Table 7-5 - S3 Presence of JKD6159 Restriction-Modification loci in ST93

	JKD6159 RM Loci				
Strain	**93_1**	**93_2**	**93_3**	**93_4**	**93_5**
ST93_E008				y	
ST93_E017		y			
ST93_E021				y	
ST93_ETDrA		y		y	y
ST93_H11		y		y	
ST93_H13		y		y	y
ST93_H14		y	y	y	
ST93_H15	y	y	y	y	
ST93_H16	y	y	y	y	y
ST93_H17	y	y	y		
ST93_H18	y	y	y	y	
ST93_H19	y	y	y	y	
ST93_H2	y	y	y	y	y
ST93_H24	y	y	y	y	
ST93_H25	y	y	y	y	
ST93_H26	y	y	y	y	y
ST93_H27	y	y	y	y	y
ST93_H29	y	y		y	
ST93_H3	y	y	y	y	y
ST93_H31	y	y	y	y	
ST93_H4	y		y	y	
ST93_H6				y	
ST93_H7		y			y

Strain	93_1	93_2	93_3	93_4	93_5
ST93_H8		y			y
ST93_H9			y		
ST93_HT13		y			y
ST93_HT3			y		y
ST93_HW15					y
ST93_HW2					y
ST93_HW24					y
ST93_P271					y
ST93_P306					
ST93_P325					
ST93_PTDrAP4					y
ST93_PTDrBP4					y
ST93_PTF1P3					y
ST93_PTGrAP5					y
ST93_PTPgP1					y
ST93_W1Bb4					
ST93_W1Dr3					
ST93_W1Fi5					
ST93_W1Fi9					
ST93_W1FPb22					
ST93_W1Gr24					
ST93_WWeU6					

Table 7-6 - S4 Presence of PTDrAP2 Restriction-Modification loci in ST398

	PTDrAP2 RM loci		
Strain	**398_1**	**398_2**	**398_3**
ST398_E026	y		
ST398_ETWeP		y	y
ST398_H20	y	y	
ST398_H21		y	y
ST398_H23		y	y
ST398_H33		y	y
ST398_HT19		y	y
ST398_HW-31	y	y	y
ST398_P1N1		y	y
ST398_P216	y	y	y
ST398_P221		y	y
ST398_P223		y	
ST398_P236		y	
ST398_P244	y		y
ST398_P257	y	y	y
ST398_P330		y	y
ST398_PTDrAP2	y	y	y
ST398_PTDrBP5		y	
ST398_PTGrAP4	y	y	y
ST398_PTGrBP1	y		y
ST398_PTWeP5		y	y
ST398_W1FPB20	y		y
ST398_W1FPa4		y	y
ST398_W1FSb2			y
ST398_W1Gr12			y
ST398_WweU9	y		