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Analysis of Integrase and Transposase-Mediated Mechanisms for Transfer of Mobile Genetic Elements

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Abstract

Mobile genetic elements (MGE) are DNA segments most of which are capable of their own self mobilisation through genes either intracellularly (transposase or integrase genes) or intercellularly (conjugation genes), thus both contributing in bacterial evolution. This thesis investigated the functions of two distinct integrase genes in the mobilisation of two metal resistance GIs, G08 and G62, of *Acinetobacter baumannii*. G8*int* encodes a novel family of phage-type integrases whereas the G62*int* gene encodes a classic phage-type integrase. In addition, the role of transposon-mobilised resistance genes have been addressed in a number of *Staphylococcus epidermidis* clinical isolates, conferring resistance to biocides by way of the metabolic *ileS* and *fabI* genes. Real-time PCR demonstrated integrase dependent GI excision, utilising IPTG-inducible integrase genes in plasmid-based mini-GIs in *E. coli*. In *A. baumannii* integrase-dependent excision of the original chromosomal GIs could be observed after mitomycin C induction. In both *E. coli* and *A. baumannii* the rate of excision and circularisation was found to be dependent on the expression level of the recombinases. Susceptibility testing in *A. baumannii* strains ATCC 17978, A424 and their respective ΔG62 and ΔG08 mutants confirmed the contribution of the GI-encoded efflux transporters to heavy metal resistance mainly for copper. In *S. epidermidis*, the distribution and phenotypes of the insertion-sequence mobilised *ileS2* gene on mupirocin resistance and *sh-fabI* gene on tricolsan resistance is described in this study and the novel type of mobilization of the *sh-fabI* carrying TnSha1 element was investigated. In summary, the data evidence the functionality and essential contribution of two integrases in the mobilisation of the two *A. baumannii* heavy metal resistance GIs G08 and G62 in *E. coli* as well as when chromosomally located in their natural host. Moreover, the contribution of additional laterally transferred genes, *ileS2* and *sh-fabI* in reducing the susceptibility to mupirocin and triclosan, respectively was confirmed in the current study. The different transfer mechanisms in both elements result in

genomes plasticity participating in the dissemination of resistance determinants in *A. baumannii* and *Staphylococcus* species.

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Abbreviations

λ	Lambda
µg	Microgram
µl	Microliters
mM	Milliomolar
bp	Base pair
CFU	Colony forming units
Ct	Cycle threshold
DCO	Double cross-over
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DR	Direct repeat
FRT	Flp recombinase target
g	grams
gDNA	Genomic DNA
GI	Genomic island
HGT	Horizontal Gene Transfer
ICE	Integrative conjugative elements
IPTG	Isopropyl-**β**-Dthiogalactopyranoside
IS	Insertion Sequence
kb	Kilobase pair
L	Litres
LA	Lysogeny broth agar
LB	Lysogeny broth
LF	Left homologous flank
M	Molar
MCS	Multiple cloning site
mg	milligram
MGE	Mobile genetic element
MLST	Multilocus sequence typing
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
nH_2O	Nanopure, PCR-grade water
OD	Optical Density
°C	Degrees Celsius
ORF	Open reading frame
PAI	Pathogenicity island
PATE	palindrome-associated transposable elements
PCR	Polymerase chain reaction
qPCR	Quantitative real time polymerase chain reaction
RF	Right homologous flank
SCC	Staphylococcal chromosomal complex
SCO	Single crossover
tRIP-PCR	tRNA interrogation of pathogenicity islands- polymerase chain reaction
TE	Transposable element

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Chapter 1 Introduction

1.1 Horizontal gene transfer

1.1.1 Contribution to evolution of bacterial species

The diversity and adaptation of the bacterial species is a continuous and on-going process by which microbial genomes evolve mainly by two different mechanisms. The first route is through internal changes occurring in the genetic contents such as mutations and rearrangements (Koonin and Wolf, 2008). The second mechanism is through horizontal gene transfer (HGT) by obtaining a foreign DNA from external sources, either by homologous recombination events on the core genome, or through acquisition of mobile genetic elements (MGE) (de la Cruz and Davies, 2000). This mechanism of recombination is widely used during processes of transformation, conjugative transfer of a chromosomal region, and sequences transferred by generalized transducing phages (Thomas and Nielsen, 2005). It is a mechanism utilized by DNA segments to incorporate their DNA in the host chromosome lacking the necessary features for integration and excision or transposition. The DNA sequence of the donor and the recipient requires segments with a high degree of homology, whereby the exchange of the DNA sequence occurs. That is to say, homologous recombination, takes place between related species (Lawrence and Hendrickson, 2003).

On the other hand, HGT is a mode that ensures adaptive evolution is not confined to accumulating point mutations and genetic rearrangements, resulting in acquisition of novel genotypes (Ochman et al., 2000, Redfield, 2001a, Thomas and Nielsen, 2005). For example, horizontally acquired genes that carry selective advantages to the host or mobile genetic elements (MGE) carrying their own transfer and maintenance functions tend to rapidly spread within a bacterial population (Thomas and Nielsen, 2005). In the early 1990s, scientists and population geneticists believed that the rates of recombination events in the bacterial chromosomes were very low and the major differences between the sequences of isolates were due to the continuous mutation events (Redfield, 2001b). This could be valid for particular species, like *Mycobacterium tuberculosis* complex

(MTBC), where the rate of genetic exchange is extremely low (Gutacker et al., 2002) and that the members of this complex have probably evolved by a bottleneck from a single ancestor more than 30,000 years ago (Gutierrez et al., 2005).

In *Haemophilus influenzae* and *Neisseria meningitides* genetic variation is driven by the hypermutations in the “contingency loci” which are short DNA tandem repeats of 1-8 bp sequence (Bayliss et al., 2001, Moxon, 2011). However, following the advancement of multi-locus sequence typing (MLST), it was established that the homologous recombination that accounted for most of the variations among strains of certain species (Feil et al., 2001). For example, in *Streptococcus pneumoniae, Neisseria meningitides* and *Staphylococcus aureus* occurrence of recombination is far more frequent than mutations at the housekeeping loci (neutral mutations), resulting in significant evolutionary modifications that could not be detected by phylogenetic signals (Feil et al., 2001). The events of recombination are frequently occurring between various species as well as within isolates of the same species such that the phylogenies produced are incongruent (Smith et al., 1991). For instance, sequences of DNA of genes from closely related bacteria have shown mosaic structure of these genes in which the regions of high similarity were interspersed with regions with up to 20% nucleotide differences, indicating the most likely occurrence of frequent local recombination events in hundreds of base pairs. A well-known example of such recombination events is the penicillin resistance in *Streptococcus pneumonia*e (Smith et al., 1991).

The acquisition of foreign DNA through HGT is a well-adapted process by which various bacterial species evolve and acquire new traits and functions (Boyd, 2002, Recchia and Hall, 1997), whereas mutations can modify existing genes resulting in few changes at the cellular level (Lawrence, 1999). Interestingly, HGT can occur even between distantly related organisms like between bacteria and plants or between bacteria and fungi (Heineman and Sprague, 1989). For instance, phylogenetic analysis showed significant similarities between the glycosyl hydrolases of

the rumen fungi *Orpinomyces joyonii* and the rumen bacteria *Fibrobacter succinogenes* was strongly suggestive of HGT (Garcia-Vallve´ et al., 2000). In 1997, the genome-based biology was born and a new biological age was established by Koonin and colleagues (Koonin et al., 1997). The era of whole genome sequencing has allowed the evolutionary biologists to elaborate on the concept of HGT to explain the inter-species evolutionary discrepancies, where abundance of available genomes provided sufficient evidence for such event (Doolittle, 1998). It was shown that the HGT had contributed to about 18% in subsequent evolution of the *Escherichia coli* genome (Lawrence and Ochman, 1998). In addition, it was estimated that the similarity between the genes of bacteria hyperthermophile *Thermotoga maritima* and the archaeal genes was 24% (Nelson et al., 1999), which is the highest percentage of gene similarity to archaeal genes. Another evidence of clustering of 81 archaeal-like genes in 15 regions of *T. maritima* genes with conservation of gene order between these two thermophilic Eubacteria and Archaea highly suggest the occurrence of horizontal gene transfer (Nelson et al., 1999). Horizontal gene transfer is achieved by three major process; conjugation, transduction and transformation (Figure 1.1). In addition, vesicle trafficking and intercellular nanotubes were mentioned as possible mechanisms for transfer of DNA between prokaryotes and will be discussed in the following section.

1.2 Mechanisms of HGT

1.2.1 Conjugation

The process of conjugation is defined as the direct transfer of genetic material between two bacteria (Wozniak and Waldor, 2010). Various MGE transfer via this mechanism including plasmids (Lederberg and Tatum, 1953), conjugative transposons (e.g Tn*916*) (Clewell, 1981) and integrative and conjugative elements (ICEs) (Burrus et al., 2002). The conjugal transfer is mediated by the type IV secretion system (T4SS) in which a single stranded DNA is transferred. For the conjugation process to take place, a relaxase enzyme (encoded by *mob* genes) is needed to

bind with a *cis* configured origin of transfer (*oriT*) element, forming a relaxome complex which subsequently nicks the DNA separating the two DNA strands (Grohmann et al., 2003).

The vast majority of large conjugative plasmids almost always harbour multi-resistance determinants as in the case of *streptococci* and *enterococci* and these show abroad host range, thus referred to as broad-host-range plasmids (Clewell, 1990, Schaberg and Zervos, 1986). On the other hand, a smaller size type of conjugative plasmids limited to the genus *Staphylococcus* and conferring a broad spectrum of antibiotic resistance (Macrina and Archer, 1993).

1.2.2 Transduction

Transduction is a bacteriophage-mediated DNA transfer from the infected cell to the recipient cell. This mechanism of genetic exchange was discovered by Zinder and Lederberg in *Salmonella typhimurium* in 1952 (Zinder and Lederberg, 1952). During this process, the phage enters the cell by attaching to extracellular receptors located at the host cell surface. Upon entry to the host cell, the lytic phage starts replicating producing many copies of their genomes and leaves the cell resulting in the host cell lysis. Another type of phage, is the lysogenic phage in which it can incorporate its genome within the host chromosome and remain dormant, so that it could replicate as part of the host cell (Zaneveld et al., 2008).

Under certain circumstances, such as DNA damage the lysogenic prophages can exit the host cell by excision followed by lysing the cell. However, sometimes errors in prophage excision can result in packaging of host DNA instead or along with the phage DNA. In a second instance, this phage containing the foreign DNA from the previous host could insert into another host and then this DNA incorporates into the genome of the new host. Generalized transducing phages have been described in which a broad host-range phage could infect across genera or even classes (Figure 1.1 green dotted line) (Jensen et al., 1998). By contrast, a specialized transducing phage could only integrate within a preferable

attachment site in the host genome, so the transferred genes are often limited (Figure 1.1, red dotted line) (Canchaya et al., 2003).

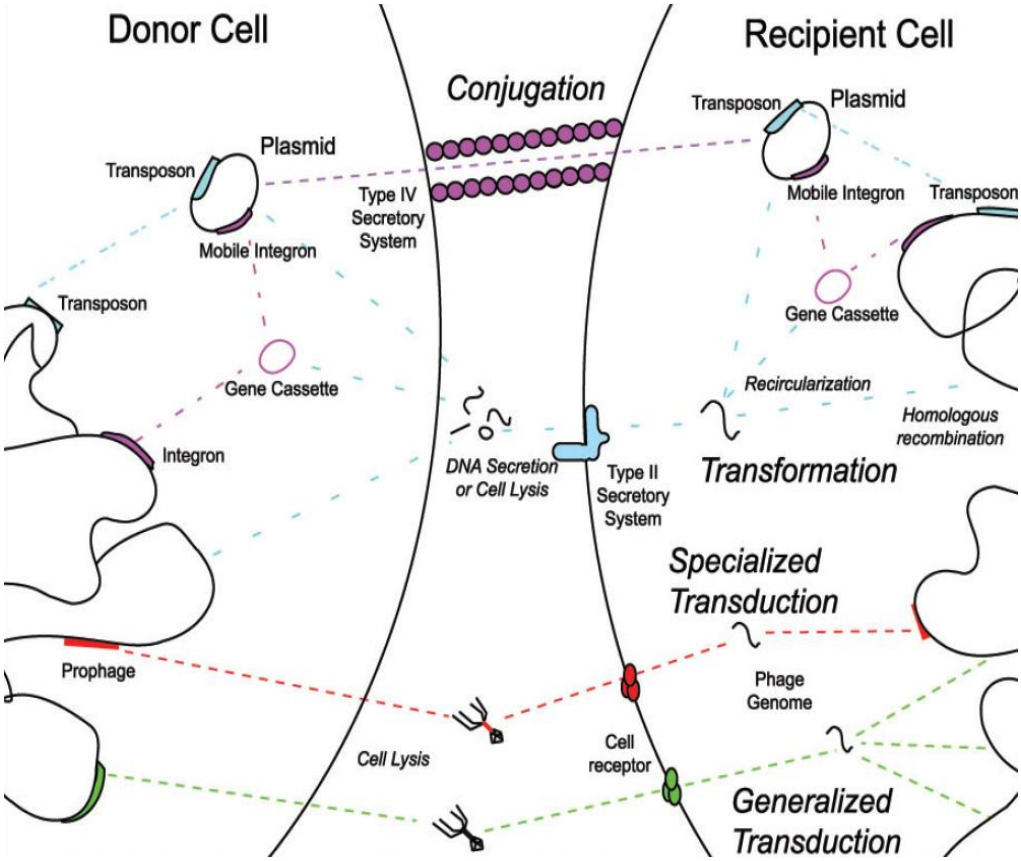


Figure 1.1 Major mechanisms of gene transfer.

Processes are shown in italics. Notably, several types of mobile element may move by more than one mechanism of gene transfer. Conversely, particular mechanisms of gene transfer can mobilize multiple types of element. Each mechanism is indicated with a different coloured dotted line as follows: red dotted line indicates the specialized transduction pathway. Green dotted line denotes the generalized transduction pathway. Blue dotted line indicates the various pathways for transformation. Purple dotted line explains the pathway of integrons (See text for details of each mechanism). Figure adapted from (Zaneveld et al., 2008).

For example, these phages and phage-like elements as well as insertion sequences (IS) are major sources of diversity in various *Streptococcus pyogenes* isolates, a major cause of wound infections and rheumatic fever. The scarlet fever toxin is one of the secreted proteins encoded by prophages involved in human-bacterium interactions (Smoot et al., 2002).

Another prophage of *Streptococcus pyogenes* carries the macrolide efflux resistance genes *mef*(A)/*msr*(D) which is capable of conjugal transfer among streptococci (Iannelli et al., 2014).

1.2.3 Transformation

The process by which the bacterial cell is capable of natural uptake of exogenous DNA from the environment and incorporate it to its own chromosome. This is one of the main mechanisms of acquiring foreign DNA from the environment (Figure 1.1, blue dotted line), and in some occasions it is a defense mechanism the bacteria utilize to acquire adaptive traits and trigger competence in response to antibiotics and vaccines (Charpentier et al., 2012, Prudhomme et al., 2006). Under certain growth conditions, some bacteria are naturally transformable like *Acinetobacter baylyi* and *Streptococcus pneumoniae* while others are not (Bacher et al., 2006, Engelmoer et al., 2013). For instance, *A. baylyi* ADP1 can be transformed with a foreign DNA easily as its highly competent to natural transformation (Vaneechoutte et al., 2006, Young et al., 2005). It has been also shown that some strains of *A. baumannii* can also undergo natural transformation, thus contributing to the emergence of multi-drug resistance phenotypes (Ramirez et al., 2011). Other examples of naturally competent bacteria include for example *S. pneumoniae* where the phenotypes conferred by its high level of natural competence led to the discovery of DNA as hereditary material (Avery et al., 1979).

1.2.4 Membrane vesicles

One of the mechanisms by which many Gram-negative bacteria exchange antimicrobial factors, toxins, and DNA by packaging molecules into extracellular membrane vesicles (MVs) (Mashburn-Warren and Whiteley, 2005). These MVs are produced during bacterial growth and are composed of outer membrane proteins, lipopolysaccharide, periplasmic proteins, phospholipids, DNA, and RNA (Nudleman et al., 2005, Yaron et al., 2000). Examples of the virulence factors which have been isolated from these vesicles include proteases, phospholipases, hemolysins, autolysins and Shiga toxins (Beveridge, 1999, Kadurugamuwa and

Beveridge, 1995) (Kolling and Matthews, 1999, Li et al., 1998). In addition, many vesicle-producing bacteria were found to contain DNA such as *Pseudomonas aeruginosa* (Kadurugamuwa and Beveridge, 1995) and *Neisseria gonorrhoeae* (Dorward et al., 1989). Moreover, vesicles of *Escherichia coli* O157:H7 harbored both chromosomal and bacteriophage-associated virulence genes (Kolling and Matthews, 1999). Previous studies have confirmed the role of these vesicles in antibiotic resistance not only through the physical spread of the resistance genes carried on plasmids but also through the so called preformed antibiotic inactivating enzymes (Beveridge, 1999, Dorward et al., 1989).

1.2.5 Intercellular nanotubes

An additional type of molecular exchange that involves physical interactions between neighboring bacterial cells is through a network of intercellular membrane nanotubes which enable long-distance communication between cells (Dubey and Ben-Yehuda, 2011). This is analogous to the mechanisms described in plants and mammalian cells, in which tubular conduits and synaptic junctions are used to transfer nutrients, signals and proteins (Heinlein and Epel, 2004, Lucas et al., 2009). In prokaryotes, these nanotubes act as tunnels for exchange of intracellular contents as well as viruses (Belting and Wittrup, 2008, Hurtig et al., 2010, Schara et al., 2008). Recent research evidenced that nanotubes in the form of intercellular connections or as extending tubes both can exist at low cell density (Dubey et al., 2016). These nanotubes are formed within minutes and are composed of chains of continuous membranous segments (Dubey et al., 2016). Electron and fluorescence microscopy studies showed that these nanotubes connect bacterial cells and enabling intercellular transfer of cytoplasmic materials including genetic material such as plasmids, chemical signals and proteins (Pande et al., 2015).

1.3 Mobile genetic elements

1.3.1 MGE as tools for HGT

Mobile genetic elements (MGE) have a prime role in the evolution of the genomes of their host in various ways. This has been attributed to different mechanisms like the rearrangement of the plasmids and chromosomes, gene activation, sequestration, transmission and mutations. Major mechanisms for mobilisation of of MGE are summarised in figure 1.1. To study mobile elements many bioinformatic and statistical procedures have been developed to identify genes acquired via HGT (Garcia-Vallve´ et al., 2000). This method is based on analysing the G+C content, the codon usage, amino acid usage and gene position. It was found that the pathogenic bacteria had lower percentage of horizontally transferred genes as compared to the nonpathogenic and archaeal ones (Garcia-Vallve´ et al., 2000). Most of these genes were part of a mobile element including prophages, pathogenicity islands, or integrases, transposases and recombinases. The set of genes transferred by the HGT are variable in terms of function and under certain environmental and growth conditions they can be useful for the cells (Juhas et al., 2009). To be maintained within a population, horizontally acquired regions must provide a great enough benefit or low enough fitness cost to avoid loss due to genetic drift or selection (Baltrus, 2013). However, evidence showed that recently acquired regions often function inefficiently within their new genomic backgrounds so that, despite great evolutionary benefits, they can be energetically or physiologically costly to the cells (Chou et al., 2011, Park and Zhang, 2012).

1.3.2 Genomic islands

Most of the genomic islands (GIs) are found as DNA segments forming blocks of genes within the chromosome. The GIs were originally known as Pathogenicity Islands (PAIs) by Hacker et al in late 1980s, when they were examining the genetic virulence mechanisms in *Escherichia coli* (Hacker et al., 1997). The term pathogenicity emerged from the study of virulence related factors (Blum et al., 1994, Dallas and Falkow, 1980,

Hacker and Kaper, 2000). However, the concept of GIs is broader than pathogenicity and includes other functional characteristics than the pathogenicity including symbiosis (Sullivan et al., 2002), metabolism (metabolic islands) (Gaillard et al., 2006), metal resistance (resistance islands) and siderophore synthesis (Larbig et al., 2002).

1.3.2.1 General features of GIs

Most of the known GIs share a number of the following features that will be discussed in this section (Figure 1.2). They are a discrete DNA segments with a size of 10 to 200 kb usually detected during comparative genomic analysis of different closely related strains (Juhas et al., 2009). However, some of the GIs reported to be as small as 4 kb, to which they were referred to as genomic islets instead (Hacker and Kaper, 2000). The GIs are usually different from the rest of the chromosome in GC content as well as other nucleotide statistics including cumulative GC skew, tetranucleotide frequencies or codon usage (Juhas et al., 2009). In addition, most but not all GIs have an insertion hot spot such as tRNA encoding genes like in the case of integrative and conjugative elements (ICEs). Another important feature of the GIs is that they are usually flanked by perfect or imperfect direct repeats (DR) of variable sizes between 16-20 bp. These DRs usually are formed as a result of site-specific integration event of the GIs occurring at the target site and can be recognized by the enzymes responsible for integration and excision of the GIs (Schmidt and Hensel, 2004).

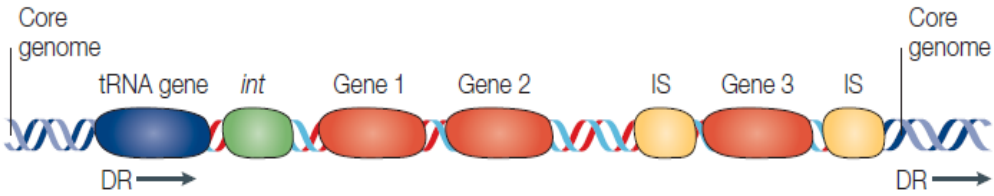


Figure 1.2 General characteristics of genomic islands.

Genomic islands are horizontally acquired DNA regions that are usually chromosomally inserted in the vicinity of tRNA genes. A typical GI is flanked by direct repeat (DR) structures and carries several genes encoding traits that may increase bacterial adaptability or fitness under certain growth conditions (represented here by genes 1–3). Typically, GIs carry multiple functional and fragmented insertion sequence (IS) elements and other mobility-related genes, as well as a functional integrase (*int*) gene, the product of which is involved in insertion and deletion of the DNA region that is flanked by DR structures. (Dobrindt et al., 2004).

The GIs mostly harbor genes coding for an integrase and in some cases a recombination directionality factor (RDF) responsible for the mobilisation of the GI. Other GIs could also carry insertion sequences (IS) or transposons that are responsible for the DNA mobilisation of the element (Buchrieser et al., 1998, Gal-Mor and Finlay, 2006). GIs could also harbor genes which are advantageous to the host and they have been named accordingly. This could be exemplified by the xenobiotic-degradation islands coding for genes that have evolved with time enabling the bacteria to resist the potential toxic effects of xenobiotic compounds and to exploit them as a nutrient source (Dobrindt et al., 2004).

1.3.2.2 Evolutionary origin of GIs

Despite most of the GIs fit into the definition and characteristic features discussed above, this is not always the case as there are some GIs which lack some of these features. The vast majority of our knowledge of GIs are derived from the genome sequencing and comparative analyses of closely related species. However, few studies solely focused on study of how the HGT is regulated, what kind of environmental signals involved in triggering this process and how the bacteria maintain stability of their

chromosomes after all the events of recombination, deletion and insertion, and so many evolutionary information is lacking and need further studies (Dobrindt et al., 2004).

Evolutionary studies based on the phylogeny analyses of GIs within specific genus have found that GIs could be structurally distinct and cannot be grouped based on their phylogenetic relatedness (Juhas et al., 2009, Vernikos and Parkhill, 2008). In the last few years, when only a few complete genome sequences were available, it was thought that there were multiple evolutionary origins of the GIs that have arisen in parallel, as some of these carried phages while others contained genes related conjugative plasmids. Moreover, not all GIs contain the same functional modules as some of them could have evolved through time and acquired other genes making it hard to categorize them within a certain type of GIs (Frost et al., 2005, Leplae et al., 2010, Toussaint and Merlin, 2002). For instance, prophages at variable stages of functionality can be found integrated in the chromosome, suggestive of possible acquisition of non-phage genes at different time points following the cointegration of the phage in the chromosome (Dobrindt et al., 2004, Klockgether et al., 2003).

Some studies have shown that few hybrid GIs have formed originally from plasmid transfer genes, as the case with the SXT element of *Vibrio cholerae* (Beaber et al., 2002), the CTn*4371* biphenyl transposon of *Ralstonia oxalatica* (Toussaint et al., 2003) and for the *Mesorhizobium loti* R7A symbiosis island (Ramsay et al., 2006, Sullivan et al., 2002). By contrast, studies of the ICEs have shown experimentally that these elements were fully capable of excision and integration from the host and their evolutionary origin is not related to any known plasmid. Upon the best studied examples of these ICEs are ICEH*in1056* from *Haemophilus influenzae*, pKLC102 from *Pseudomonas aeruginosa* and ICE*clc* from *Pseudomonas* sp. strain B13 (Dimopoulou et al., 2002, Gaillard et al., 2006, Juhas et al., 2009, Klockgether et al., 2003, van der Meer and Sentchilo, 2003). These are known as a separate family of ICEs harboring type IV secretion systems.

1.3.3 Contribution of MGE in antimicrobial resistance

Antibiotic-resistance determinants are often associated with mobile or transferable genetic elements such as plasmids, integrons and complex transposons (Paulsen et al., 2003). With the increasing evidence of discovery of pathogenicity genes as well as other functional genes transferred horizontally between different bacterial genera (Hacker and Kaper, 2000) and the plasmid-mediated resistance which was first addressed in the early 1960s (Watanabe and Fukasawa, 1961), this area has become of a great concern for scientists and medical professionals (Carattoli, 2013). Most of the available data on resistance genes have looked at each mobile element individually, whereas a particular attention should've been given to the collective role of these elements as a pack (Salyers and Amabile-cuevas, 1997). Various ICE elements were found to harbor genes encoding for antibiotic and metal resistance (Boltner et al., 2002, Davies et al., 2009, Rice, 1998, Whittle et al., 2002). The contribution of MGE to antimicrobial resistance will be addressed in context with the various types in each section.

1.3.4 Integrative and conjugative elements

The integrative and conjugative elements (ICEs) are defined as self-transmissible MGEs that carry the full machinery for integration and excision as well as the regulatory system to control their conjugative transfer from the chromosome (Burrus and Waldor, 2004, Saylers et al., 1995). They are carried in both Gram-positive and Gram-negative bacteria but ICEs also encompass the conjugative transposons which were originally found in Gram-positive bacteria and could target multiple integration sites (Burrus et al., 2002). After integration ICEs replicate as part of the host chromosome. The ICEs form circular intermediates intracellularly, but cannot be maintained extrachromosomally as some of them lack the ability of autonomous replication. However, a number of ICEs were observed to be capable of autonomous replication after being activated. This could be exemplified by the first conjugative transposon

ever identified; Tn*916* which can replicate autonomously via a rolling circle mechanism (Wright and Grossman, 2016). This replication was found to be driven by a relaxase encoding gene along with the Tn*916* origin of transfer; *oriT(916)* acting as an origin of replication. This rolling circle mechanism of replication was also facilitated by a functional single-strand origin of replication (*sso*) in Tn*916* that was predicted to prime second-strand synthesis (Wright and Grossman, 2016).

Despite almost all ICEs having similar modules for conjugal transfer, individual ICEs have variable phenotypes depending on the many other genes carried on the element.

1.3.5 Insertion sequences (IS) and transposons

1.3.5.1 Definition of IS

The general definition of IS is a short DNA segment between 0.7 to 2.5 kb encoding only enzymes that are needed for transposition and are capable of insertions in various sites within the chromosome utilising mechanisms that do not require large regions of DNA homology between the IS and the target (Chandler and Mahillon, 2002) (Figure 1.3, A). The structure of a classical IS usually occurs with one to two open reading frames (*orfs*), occupying the whole IS which terminates in the flanking imperfect inverted repeats (IR) (Chandler et al., 2013). In addition, the *orfs* constitute of transposase enzyme catalyzing the cleavage and transfer of the DNA strand, resulting in the IS mobilisation.

1.3.5.2 History of IS

Historically, the first insertion sequences had been described about 79 years ago the amazing discovery of transposable elements in maize/corn by Barbara McKlintock, when the author had presented evidence that homozygous minute deficiencies of specific regions of chromosomes in maize are responsible for the appearance of readily recognizable modified phenotypes of various coloureful corn cobs (McClintock, 1944). The following studies have identified short DNA segments either in association

with the control region of the galactose operon or bacteriophage λ (Fiandt et al., 1972, Hirsch et al., 1972). Shortly afterwards, the IS have been identified in multiple copies in *E. coli* chromosome (Saedler and Heiss, 1973) and involved in deletions in the gal operon (Saedler and Reif, 1974) and activation of gene expression (Reif and Saedler, 1975). Figure 1.3 below explains the basic structure of IS and transposons.

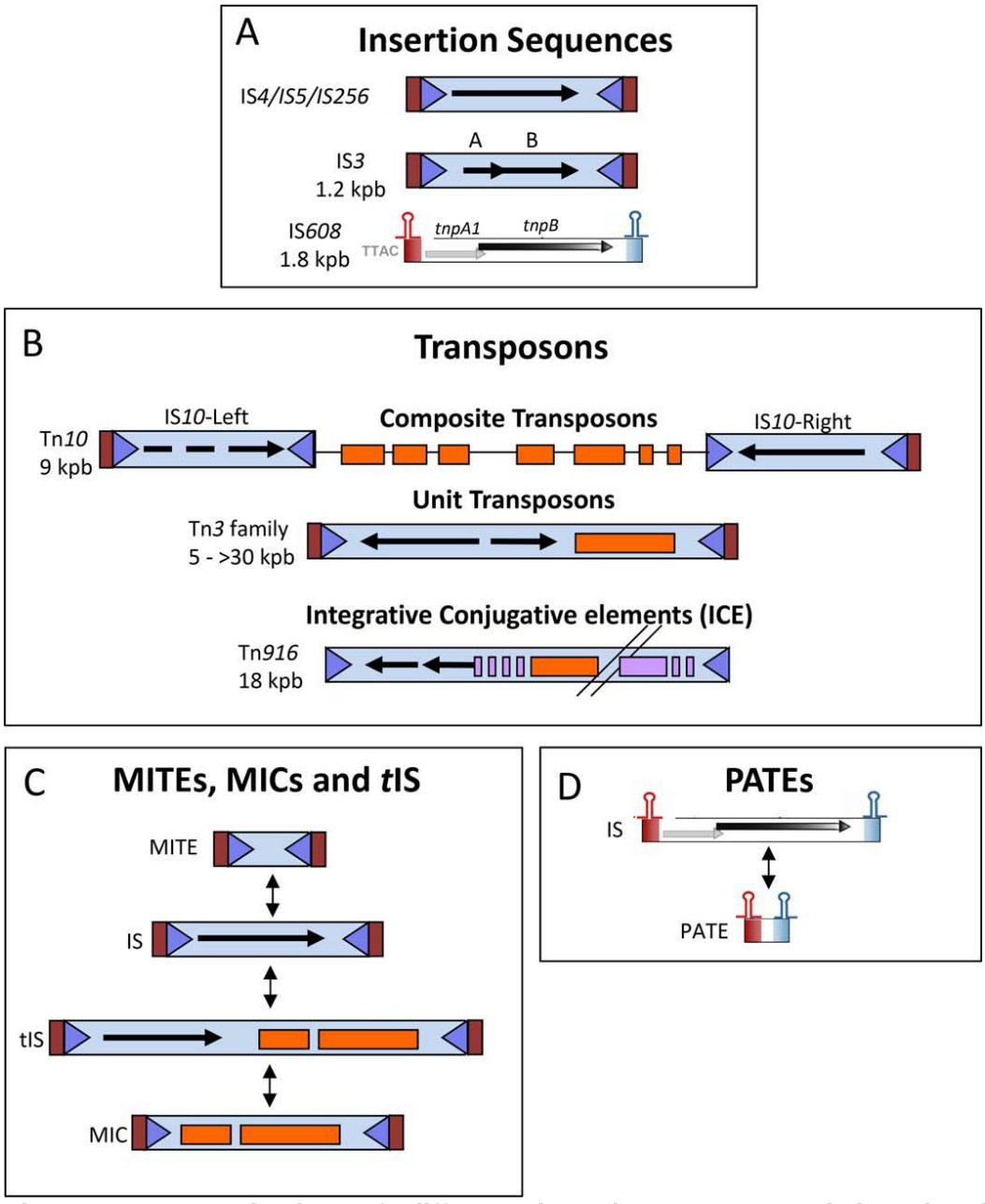


Figure 1.3 Organization of different insertion sequence (IS) -related derivatives.

IS with DDE transposases (Tpases) and their derivatives are shown as blue boxes, terminal inverted repeats as light blue triangles and flanking direct target repeats as red boxes. The Tpase orfs are shown as black horizontal arrows. Passenger genes are shown as orange boxes and transfer functions (in the case

of ICE) are shown as purple boxes. The single-strand IS are indicated with their left (red) and right (blue) subterminal secondary structures indicated. (A) IS organization. From top to bottom: a typical IS with a single Tpase orf; an IS in which the Tpase reading frame is distributed over two reading phases and requires frameshifting for expression; and the organization of a typical member of the single- strand IS family IS*200*/IS*605*. (B) Different IS-related TE. From top to bottom: composite transposon Tn*10* with inverted flanking copies of IS*10* (note that the left IS*10* copy is not autonomously transposable); a unit transposon of the Tn*3* family; and an integrative conjugative element (ICE). (C) Relationship between IS, miniature inverted repeat transposable elements (MITE), transporter IS (tIS) and mobile insertion cassettes (MIC). (D) Generation of palindrome-associated transposable elements (PATE) from IS200/IS605 family members (Figure adapted from (Siguier et al., 2015).

Later on, the IS elements were identified in *E. coli* plasmids by electron microscopy studies (Hu et al., 1975). Several studies from the same time had described that various antibiotic resistance genes were transposed by plasmids (Barth et al., 1976, Hedges and Jacob, 1974, Heffron et al., 1975).

It was then recognized that IS elements and transposons both belonged to genetic entities referred to as mobile genetic elements (MGE) or transposable elements (TE) and that DNA segments carrying various genes could be transposable when flanked by two inverted repeats of the IS elements (Arber, 1979, So et al., 1979). Other studies had also identified the presence of IS elements in high copy number (Nyman et al., 1981, Ohtsubo et al., 1981). However, it was the later studies that have brought into our attention the fact that IS elements were very abundant and widely distributed among bacteria and that they play an extremely vital role in the shaping of the genomes (Aziz et al., 2010).

There is an increasing knowledge of the mobilome and its diverse groups of elements (MGE includes TE, ICE, GI, plasmids and phages). There are some issues in distinguishing the IS from other TE as the borders has become unclear. Previously, the IS is defined as a structure devoid of the transposition genes that facilitates the translocation of the elements, whereas the TE include the passenger gene in addition to others like antibiotic resistance or virulence genes. However, this is no longer

applicable as many of the transposition genes are harbored within the IS. On the other hand, some typical TE lack the passenger genes within their structure. (Figure 1.3) (Siguier et al., 2015).

It is vital to note that the nomenclature of TE is quiet complex due to the fact there is a huge overlap in the literature about the description of mechanism, structure and function of these elements (Siguier et al., 2015). In addition, there are various genome annotations issues in public databases that resulted in confusion regarding the nomenclature. For example, transposase versus recombinase. Several reviews are available addressing these issues (He et al., 2015, Hickman and Dyda, 2015).

As the number of IS elements is expanding dramatically, there was a strong need to pool all the IS into the ISfinder database (www-is.biotoul.fr). Since 1998, this centralized database has provided a framework for the basic nomenclature and classification of the major IS groups. Previously, naming the IS was simply by assigning a number, however, this has now been changed to include the first letter of the genus followed by the first two letters of the species and a number (for example, IS*Bce1* for *Bacillus cereus*) (Chandler and Mahillon, 2002). Currently the ISfinder have more than 4,000 different IS (Siguier et al., 2015). Moreover, the IslandPath is a programme which can annotate transposases found near genes putatively involved in HGT or genomic islands, thus could identify novel TE (Hsiao et al., 2003).

On the other hand, transposons are MGE that are similar to ISs in their composition, I.e. transposes and inverted repeats. They tend to be larger than IS as they carry other genes in between. Composite transposons are formed when two ISs inserted next to each other on the chromosome (Galimand et al., 2005) (figure 1.3; B). In this case, there are two different sets of inverted repeats, but the outermost IRs are usually the target for the transposase enzymes and the intervening IRs are transferred with the ISs. As a result, composite transposons have accumulated a variety of genes in the intervening region such as antibiotic resistance genes

(Galimand et al., 2005), metal detoxification and resistance (Liebert et al., 1999) and genes involved in catabolism of compounds (Top and Springael, 2003). Non-composite transposons are simply composed of a transposase enzyme with flanking inverted repeats IRS but no ISs structure (Schaefer and Kahn, 1998).

1.3.6 Integrons

This class of MGEs was first identified in late 1980s in association with antibiotic resistance genes (Stokes and Hall, 1989). These integrons consist of three essential parts; the integrase gene (*int*), a promoter (*pC*), and a recombination site (*attI*). They usually facilitate the mobilisation of gene cassettes as they lack the ability for self-mobilisation, and thus considered non-autonomous. The gene cassettes are promoterless open reading frames with 59 bp attachment site (*attC*). The integrons mobilized within MGEs have significantly variable (*attC*) and the gene cassettes vary in number between one to ten.

There are various classes of integrons, with class 1 being strongly associated with the spread of antibiotic resistance (Fluit and Schmitz, 2004). Another four classes of integrons has been identified in plasmids or transposons and were associated with transfer of resistance determinants (Mazel, 2006). On the other hand, a set of integrons have been recognized by whole-genome sequencing to be located in the chromosome of a wide range of bacteria (Fluit and Schmitz, 2004, Nemergut et al., 2004, Rowe-Magnus and Mazel, 2002). These were found to be less involved in the spread of antibiotic resistance determinants as they are less transferred horizontally. In addition, their associated gene cassettes have homogenous recombination sites (*attC*).

1.4 Mechanisms of mobilisations of MGE

1.4.1 Site-specific recombination

Site-specific recombination is the mechanism by which mobile genetic elements move into and out of the chromosomes of their host cells. Site-specific recombination occurs by two different mechanisms, conservative and transpositional site-specific recombination. These two processes require specialized recombination enzymes to occur, integrases and transposases, respectively. In this PhD thesis, both of these types of site-specific recombination are investigated involving different mobile genetic elements. Integrases will be discussed in reference to *Acinetobacter baumannii* and transposases in will be explored in various *Staphylococcus* species in the proceeding chapters.

Site-specific recombination mediates a variety of processes including: integration and excision of bacteriophages from bacterial chromosomes, the control of circular replicon inheritance, the processing of the initial products of genetic transposition, and the mediation of genetic 'switches' through inversion or deletion of specific DNA segments (Stark et al., 1992); (Kilbride et al., 1999, Landy, 1993, Nash, 1996). For example, the phase-variable Type I restriction-modification system (SpnD39III) in *Streptococcus pneumoniae* has a six alternative rearrangements with distinct methylation patterns and each displaying a unique expression profile (Manso et al., 2014).

In the conservative site-specific recombination short heteroduplex joints are produced and require identical DNA sequences in both the host and recipient (Alberts et al., 2002). On the other hand, the transpositional site-specific recombination proceeds via breakage reactions of two ends of mobile DNA sequence, and integration of these ends at different non-homologous target DNA sites. No heteroduplex is formed in the latter type of recombination.

1.4.2 Integration and excision

The excision from the host chromosome and integration into the recipient genome, or reinsertion into the same chromosome, are vital processes by which many MGE use as a mechanism for HGT such as the integron cassettes, ICEs and prophages (Dobrindt et al., 2004). Generally, a site-specific integrase mediates the integration step into the chromosome, or plasmid, at specific sites. These sites are named *attB* in the case of phage integrases. The location of the *attB* sites are variable, and are usually possessed within the region coding for the anticodon loop of tRNA or tmRNA genes (Williams, 2002). These two genes are favorable in most cases for integration over the other protein-coding genes for the two main reasons. The first one is their reliability as the tRNA genes are present in multiple copies in the bacterial genome, thus providing multiple potential integration sites (Cheetham and Katz, 1995). In addition, other studies showed that the divergence rate per base pair in the tRNA gene sequences is 6 folds in average, lower than other protein-coding genes (Lynch, 1997, Ochman et al., 1999). The *attB* sequence in tDNA is relatively stable therefore this may improve long term survival of genetic element as well as provide wider range of distribution among host bacteria. Another factor of tRNA genes being preferred integration hotspot is their small size will make the amount of host DNA to be recognized by the *attP* in order to reconstitute the target gene upon integration to be minimal, otherwise this fragments to be captured in *attP* within a protein-coding gene would have been much larger and impractical for restoration (Williams, 2002).

Other genetic structures known as unconventional circularizable structures (UCSs) which lack their own recombinase genes but are capable of excision in circular form due to the presence of (DRs) flanking the DNA segments undergoing excision have been described in a number of bacteria mostly carrying antibiotic resistance genes (Palmieri et al., 2013). Moreover, UCSs can undergo both integration and transposition. The DRs of the UCSs are usually imperfect and much longer than those

of the conventional MGEs (Frost et al., 2005). These elements are also carried within other MGEs which might be of a mutual benefit enabling continuous change in the genetic pool of the host (Palmieri et al., 2013).

There have been several reports on another novel mechanism of transposition of IS through the formation of a translocatable unit (TU), carrying the internal DNA segment of a composite transposon and one copy of a flanking IS. This mode of movement has been described in IS*26*-based composite transposon, a member of the IS*6* family occurring through two distinct mechanisms (Harmer et al., 2014, Tansirichaiya et al., 2016). The first one is via cointegrate formation with IS*26* duplication and target duplication is generated. The second one is through incorporation of TU at an existing IS*26* (Harmer et al., 2014).

1.4.3 Homologous recombination

This mechanism of recombination is widely used during processes of transformation, conjugative transfer of a chromosomal region, and sequences transferred during by generalized transducing phages (Thomas and Nielsen, 2005). It is a mechanism utilized by DNA segments to incorporate their DNA in the host chromosome lacking the necessary features for integration and excision or transposition. The DNA sequence of the donor and the recipient requires segments with a high degree of homology, whereby the exchange of the DNA sequence occurs. That is to say, homologous recombination, takes place between related species (Lawrence and Hendrickson, 2003). The key enzyme in this process is RecA which binds single stranded DNA and scans double stranded DNA for homologous regions to allow for recombination (Chen et al., 2008).

1.4.4 Transposition

Transposition is another mechanism for transfer of the genetic material, however, as a process it does not involve intercellular transfer of DNA and thus is not considered a HGT mechanism (Kleckner, 1981). Transposition could occur intracellularly from the chromosome, to another position in the

chromosome, to a plasmid or a phage, thus facilitating mobilisation of these immobile genes to the other cells. Furthermore, transposable elements from outside the cells carrying foreign DNA could recognize the homologous sequences on the transposable element inside the cell and subsequently integrates via homologous recombination (Kleckner, 1981).

1.5 Classification of major mobilisation-mediated genes

1.5.1 Classification of major recombinases

The MGE are essentially mobilized by proteins encoded by genes of either transposases or integrases broad categories which will be discussed in this section along with brief description of their mechanisms of action. Integrases (recombinases) are enzymes that mediate unidirectional and site-specific recombination between two DNA molecules after recognition of specific target sequences. Integrases are categorized into two, not evolutionary related, main families based on amino acid sequence homology and catalytic residues; the serine recombinases and the tyrosine recombinases (Groth and Calos, 2004). This diversity is reflected by the fact that the Protein family database Pfam reports multiple entries when queried with the term “integrase”, serine recombinase or tyrosine recombinase (http://pfam.xfam.org/). Figure 1.4 shows the structure of the major classes of tyrosine and serine recombinases.

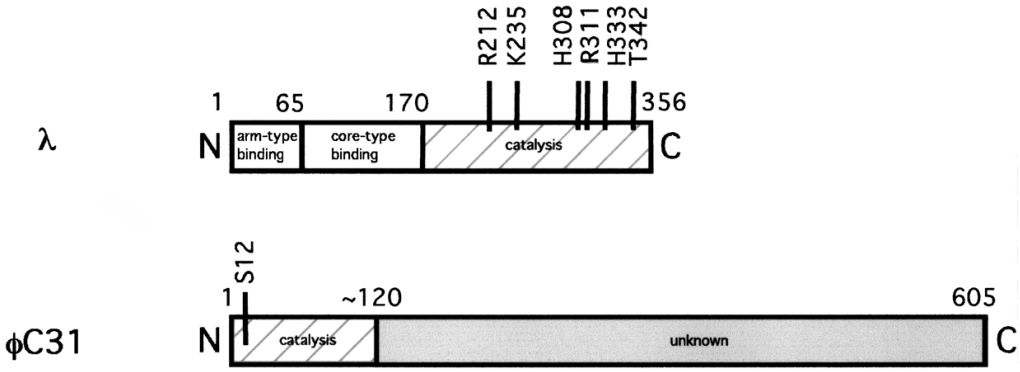


Figure 1.4 Protein domain structure.

The domain structure of the best studied λ integrase from the tyrosine family and ϕC31 from the serine family, is shown. Other family members can be aligned by sequence homology with these prototypical members and are thus believed to have similar domain structures. The lined region represents the catalytic domain, while the open region depicts the DNA binding domains. In l, the DNA-binding domain is further classified into the region that binds core-type sites and the region that binds arm-type sites. The shaded region represents domains of unknown function. The conserved catalytic residues are shown. Figure is adapted from (Groth and Calos, 2004).

1.5.1.1 Serine recombinases

Serine recombinase family members include the resolvases γδ and Tn*3*, the invertase Gin and the phage integrases ϕC31, R4 and TP901-1 (Smith and Thorpe, 2002). The resolvase/invertase family utilizes a serine nucleophile to mediate a concerted double strand cleavage and re-joining reaction at nucleotide phosphates separated by 2 base pairs (Thorpe and Smith, 1998). During the process of site-specific recombination mediated by both serine and tyrosine families, four recombinase molecules bound to two ~30 bp recombination core sites catalyze the breaking and rejoining of four DNA phosphodiester bonds (Thorpe and Smith, 1998).

1.5.1.2 Tyrosine recombinases

There are four major members of the integrase family of tyrosine recombinases: the lambda (λ) Int protein (Enquist et al., 1979), Cre proteins of phage P1 (Austin et al., 1981), XerC and XerD protein of *E. coli* (Abremski and Hoess, 1984) and FLP invertase from yeast (Andrews et al., 1985). The λ integrase family enzymes use a tyrosine nucleophile to

mediate sequential pairs of strand exchanges that are positioned 6–8 bp apart (Hosahalli S.Subramanya, 1997). The recombinase XerD along with its related partner XerC, function in the stable inheritance of the *E. coli* chromosome and multicopy circular plasmids, apparently by converting to monomers the circular multimers that can arise by homologous recombination (Blakely et al., 1993, Sherratt et al., 1995). In XerD and XerC, the two conserved arginines and the tyrosine are required for DNA cleavage while the conserved histidine is required for DNA re-joining (Arciszewska et al., 1997, Blakely et al., 1993). It is likely that the two families of integrases have evolved separately due to the differences in structure and mechanism of action (Groth and Calos, 2004).

The translated protein sequence of XerC contains two regions which are homologous to the two conserved domains of the λ integrase family of site-specific recombinases (Colloms et al., 1990). The second domain (Domain 2) of the XerC sequence has three totally conserved amino acids; histidine, arginine, and tyrosine, as well as other less conserved amino acids. The XerC sequence has 32% amino acid identity to the *E. coli* proteins FimB and FimE in an alignment covering about 160 amino acids (Colloms et al., 1990). These two proteins are involved in inverting a segment of the *E. coli* chromosome to switch fimbrial antigens (Klemm, 1986). Within the conserved domain 2, the XerC sequence shows considerable similarity (66% identity) to an integrase-like inferred protein sequence from plasmid R46 (Hall and Vockler, 1987).

Many proteins have been assigned to the tyrosine family based on amino acid sequence homology and conservation of catalytic residues, but their functions have not been experimentally studied (Groth and Calos, 2004). By homology and/or experimental data, it appears that at least 10–15% of tyrosine recombinases are integrases from phage or cryptic prophage. These phage integrases use a tyrosine to attack the DNA backbone during cleavage (Groth and Calos, 2004).

Phage integrases are enzymes that mediate unidirectional site-specific recombination between two short DNA recognition sites, the phage attachment site *attP*, and the bacterial attachment site *attB*. Once the phage genome integrates, it will be flanked by two hybrid sites, *attL* and *attR,* each consists of half from each of the *attP* and *attB* sequences. The hybrid att become the substrates for another recombination process in which the same sequence can be excised by integrase and an additional phage protein called excionase, sometimes with the help of other factors (Groth and Calos, 2004). The role of phage integrases in recombining two short DNA sequences has significant applications in genetic engineering (O'Gorman et al., 1991, Sauer and Henderson, 1988, Sauer and Henderson, 1990).

1.5.1.3 Mechanism of recombination of tyrosine recombinases

Recombination is initiated when the conserved tyrosine hydroxyl attacks the scissile phosphate, forming a 3`phosphotyrosyl–DNA complex and a free 5` hydroxyl (Lee and Jayaram, 1993). In the second step, a 5` hydroxyl from the adjacent partner duplex attacks the phosphor-tyrosine to form a Holliday junction intermediate (Van Dyne, 2002). The recombination reaction is completed by the exchange of the second pair of strands, using the same mechanism, 6–8 bp away from the site of the initial strand exchange (Van Dyne, 2002).

1.5.2 Classification of major transposases

The principal classification of IS is mainly based on the type of their transposase enzyme (Siguier et al., 2015). There are two major classes based on the breaking and re-joining of the DNA during the translocation of the transposable elements: the DDE (and DEDD) transposases and the HUH transposases (Siguier et al., 2015).

1.5.2.1 DDE transposases

Insertion sequences with DDE transposases are the most abundant types in the public database (Siguier et al., 2015). DDE enzymes are called so because of a conserved Asp, Asp, Glu triad of amino acids. These amino acids control the essential metal ions and use the hydroxyl group (OH) as a nucleophile in a transesterification reaction (Mizuuchi and Baker, 2002). No covalent Tpase-DNA intermediate is formed during the transposition process. The Tpase share a functional similarity with the retroviral integrase mainly at the catalytic site (Khan et al., 1991, Kulkosky et al., 1992). The Tpase of transposable elements triad of amino acids DDE, where the distance between the second D and the E is separated by 35 residues. However, this distance is variable among different Tpases. Crystallographic studies for IS*50* have shown that the second D and E were separated by either α-helical (Hickman et al., 2000) or β-strand structures (Hickman et al., 2005). These are larger “insertion domains” of which their function still not very known (Siguier et al., 2015).

1.5.2.2 Transposition chemistry of DDE transposases

Generally, the transposition event is very similar among various types of TE. However, there may be a simple variation in the insertion intermediate formation step, which leads to shedding of the donor DNA in the flanking region (Hickman and Dyda, 2015). In almost all the DDE transposases, the initial step includes enzymatically activating the cleavage of a single DNA strand, creating a free 3’ OH which acts as a nucleophile attacking the DNA phosphate backbone at the target site. This process generates the so-called transferred strand (Siguier et al., 2015). On the other hand, the processing of the non-transferred strand (the second strand) is variable in different IS (Figure 1.5) (Curcio and Derbyshire, 2003, Turlan and Chandler, 2000).

In the first type of second strand processing of certain IS such as Tn*3* and IS*6,* there is no cleavage involved but co-integrates are formed by the fusion of the donor and the target molecules from a directly repeated copy at each junction. This process is known as target primed replicative

transposition (Figure 1.5, 1st column) (Hickman and Dyda, 2015). In the second pathway, the non-transferred strand can be processed by cleaving several bases within the IS followed by the cleavage of the transferred strand, such as the case of IS*630* and T*c1* (Figure 1.5, 2nd column) (Feng and Colloms, 2007, Plasterk, 1996).

A third pathway as in IS*4* can occur in which the cleavage of the first strand results in 3' OH which is used to attack the second strand generating a hairpin intermediate at the IS ends. This latter hairpin structure release the IS from the flanking DNA and further hydrolyzed regenerating 3' OH. This type of transposition is called conservative or cut-and-paste transposition (Figure 1.5, 3rd column) (Haniford and Ellis, 2015). In the fourth pathway, 3' OH from the transferred strand of the IS targets the other end forming a donor molecule and replicated to generate a double strand circular intermediate, thus regenerating the donor molecule in a copy out-paste in manner or referred to as donor primed transposon replication, such as IS*3* (Figure 1.5, 4th column) (Rousseau et al., 2010).

In the last group of IS, which has only been described for eukaryotic TE of hAT family and in V (D)J recombination (Zhou et al., 2004), the 3' OH at the end of the non-transferred strand attacks the second strand to generate a hairpin at the DNA flanks and a 3' oh at the transferred strand.

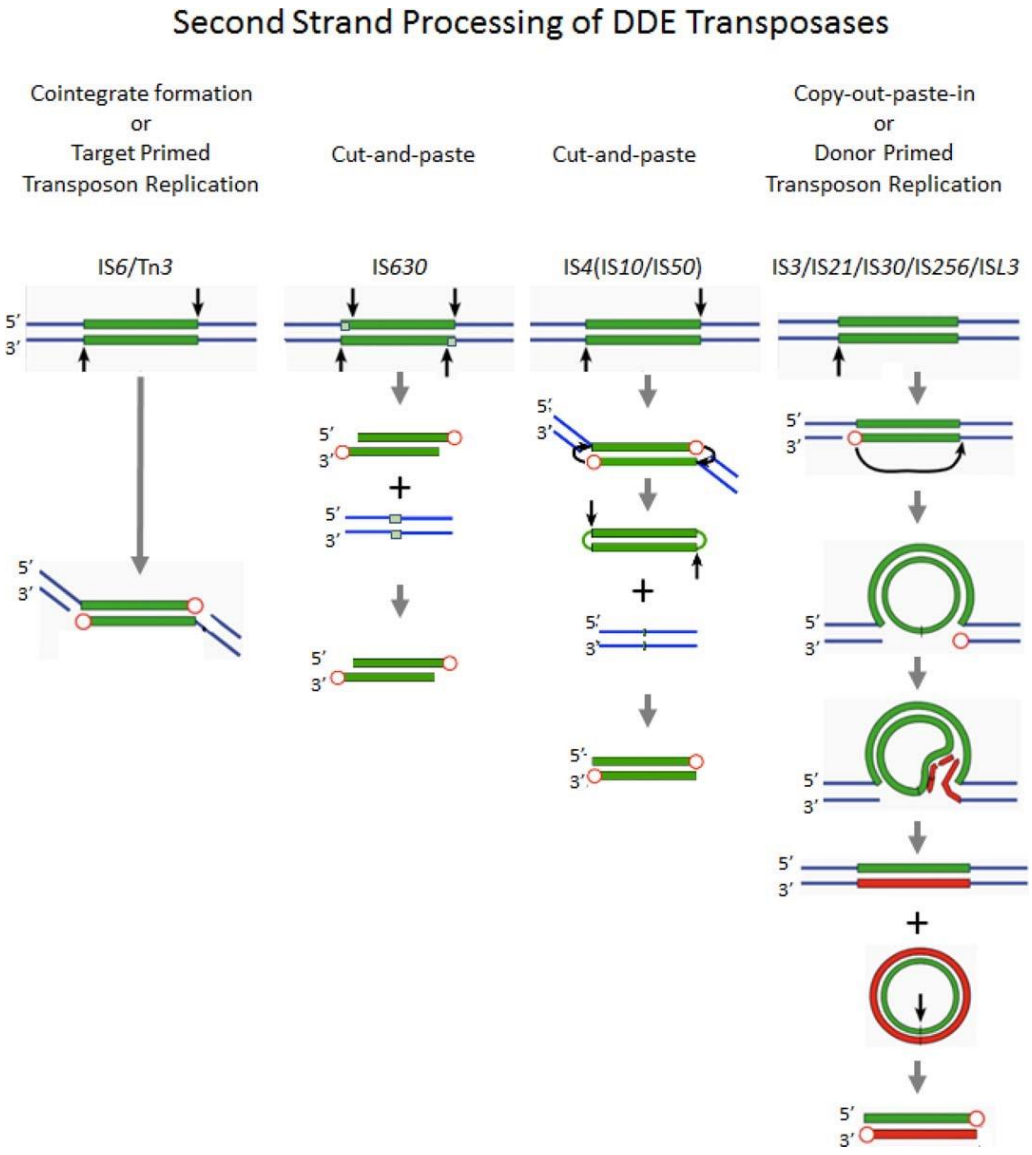


Figure 1.5 Insertion sequence (IS) families with DDE transposases are distinguished by how the second ("nontransferred") strand is processed.

IS are shown in green, flanking DNA in blue. Cleavage is shown as bold vertical arrows. 3′ OH residues are shown as red circles, replicated DNA is indicated in red. The first column shows initial cleavages which generate the 3′OH of the transferred strand and are subsequently used to attack target DNA (not shown) without prior liberation from the flanking donor DNA. This is also called target primed transposon replication (e.g Tn*3* and IS*6*). The second column shows a pathway adopted by the IS*630* family in which the nontransferred strand is cleaved two bases within the TE (light green square) before cleavage of the transferred strand, which generates the 3′ OH. This is a cut-and-paste mechanism without TE replication. The third column represents transposition using a hairpin intermediate in which the transferred strand is first cleaved and the resulting 3′ OH then attacks the opposite strand to form a hairpin at the TE ends liberating the TE from flanking donor DNA. This is a cut-and paste mechanism without TE replication. The fourth column shows a "copy out-paste"

in mechanism adopted by a large number of IS families. It involves cleavage of one IS end and attack of the opposite end by the liberated 3′ OH, the TE then undergoes replication using the 3′ OH in the donor DNA, a process that is also called donor primed transposon replication. This generates a double-strand DNA transposon circle and regenerates the donor molecule. The circle then undergoes cleavage and insertion. Figure adapted from (Siguier et al., 2015)

1.5.2.3 DEDD transposases

This is also known as DEDD Tpase, a transposase which is closely related to the Holliday junction resolvase RuvC, in the structure of the catalytic site (Choi et al., 2003). Despite the chemistry of both DDE and DEDD enzymes being predicted to be similar, the mechanism of transposition may be different due to the variability in the order of their functional domains. Moreover, the IS in this group do not possess well defined IR and insertion usually does not result in producing direct repeats (Siguier et al., 2015). Only one family so far belongs to this category of Tpases; the IS*110*.

1.5.2.4 HUH transposases

The HUH Tpases are single-stranded endonucleases that contain two conserved His residues separated by the (U) hydrophobic amino acid. It uses tyrosine residue to generate a covalent 5’ tyrosine-DNA as a transient molecule (Chandler et al., 2013).

They are essentially containing two types of functional proteins. The Rep proteins which are needed for plasmid and bacteriophage rolling circle replication and Mob proteins (relaxsases) responsible for the conjugative transfer of plasmids (Chandler et al., 2013). The HUH enzymes are the less common than DDE transposases, and are found in only IS*91* and IS*200*/IS*605* prokaryotic TE (He et al., 2015), and one eukaryotic TE (Helitron) (Thomas et al., 2010).

The chemistry of transposition of HUH enzymes is completely different from the DDE transposases. The cleavage of the DNA is mediated by the

formation of transitory bond between the enzyme phosphate and tyrosine of the DNA (Siguier et al., 2015). Furthermore, unlike the other transposons with the IR at the ends, the associated IS includes additional secondary structures at the terminal regions instead of the IR (He et al., 2015). The HUH Tpases are categorized into two main groups based on presence of one or two Y residues at the catalytic site: Y1 and Y2 enzymes (Chandler et al., 2013). However, the transposition chemistry of these two Tpases are not similar.

1.5.2.5 Serine transposases

This type of transposase is less abundant and includes only one IS family, IS607 (Siguier et al., 2015). It is similar to the site-specific serine recombinase and uses serine residue in a similar way. The serine is used to form a transitory 5' phospho-serine bond between the enzyme and the substrate DNA (Smith and Thorpe, 2002).

1.5.2.6 Tyrosine transposases

These Tpases use a catalytic tyrosine (Y) residue to generate a 5' phospho-tyrosine bond between the enzyme and the substrate DNA resembling the site-specific tyrosine recombinases (Lee and Jayaram, 1993, Taylor and Churchward, 1997). The site-specific tyrosine recombinases of the bacteriophage integrase (Int) are considered to be Tpases because they are usually associated with the integrative and conjugative transposons (ICE). However, thus far no IS family is utilising this type of enzyme (Siguier et al., 2015).

1.6 *Acinetobacter* the species and the clinic

1.6.1 Acinetobacter species

The *Acinetobacter* species can be found free living and with the appropriate enrichment media they can be easily isolated and grown from water, soil, food and sewage (Towner, 1997). Similarly, these organisms are ubiquitous in clinical settings and can be readily obtained as commensals from skin of medical professionals as well as patients admitted to hospitals (Bergogne-Berezin and Towner, 1996). Initially, the Acinetobacter was referred to as *Micrococcus calcoaceticus,* by the Dutch microbiologist, Beijerinck. The word "*calcoaceticus*" pertains to calcium acetate which he used in the enrichment medium to isolate the organism (Baumann, 1968). Unfortunately, the scientific and medical literature from the past decades carry a tremendous amount of confusing taxonomy with these *Acinetobacter* organisms, rendering the interpretation of these data to be difficult (Bergogne-Berezin and Towner, 1996). Members of this genus have undergone significant taxonomic changes, partly due to misidentification and diagnostic uncertainties, and have been referred to as "unknown" micro-organism (Towner, 1997).

Despite being recognized as commensals, it is now well established that the *Acinetobacter* species, particularly *Acinetobacter baumannii* are causing a huge threat in terms of treatment in hospitals (Fishbain and Peleg, 2010). It was noticed that the outbreaks of *A. baumannii* infections was mainly occurring due to spread of a local strain in intensive care units (ICUs) among ventilated patients with pneumonia resulting in severe infections mostly affecting vulnerable and immunocompromised patients (Bello et al., 1997, Levi and Rubinstein, 1996). Moreover, the potential capability of these organisms in causing community-acquired infections which in some occasions are life threatening has been shown in some studies (Howard et al., 2012).

The *Acinetobacter* virulence was attributed to their ability to thrive for prolonged periods in hospital environment (Catalano et al., 1999). The

burden of treating *Acinetobacter* infections was significantly increasing over the last few years, due to their Multi-drug resistance to broad spectrum antibiotics (Fournier et al., 2006). Both of these two factors have resulted in exerting even more selective pressure due to antibiotic overuse or misuse (Peleg et al., 2008). Many of the clinical isolates were exceptionally resistant to antibiotics such as tigecycline due to expressing increased efflux activity as shown by a recent study (Hornsey and Wareham, 2018).

In the following sections of this chapter, a brief description of the epidemiology of *Acinetobacter* species will be discussed putting into context the rapid spread of Multi-Drug resistance (MDR). The role of efflux systems in antibiotics and metal resistance in *A. baumannii* will be briefly discussed. In the rest of the chapter, I will describe the various types of genomic islands and their role in dissemination of resistance determinants. The last part will give the overall aims and objectives of this thesis.

1.6.2 Ecology and epidemiology of *Acinetobacter*

Acinetobacter is a ubiquitous organism thus it can be grown from different environmental samples after culturing in enrichment media (Baumann, 1968). The most commonly described natural habitats of acinetobacters are soil, surface water and wetland (Anandham et al., 2010, Nemec et al., 2010, Nishimura et al., 1988). Other *Acinetobacter* species have been recovered from wastewater and sewage, activated sludge and chemicals dump sites (Carr et al., 2003, Kim et al., 2008, Malhotra et al., 2012, Vaz-Moreira et al., 2011). A range of animal sources as well as plants and floral nectars were also had various Acinetobacter species (Alvarez-Perez et al., 2013, Li et al., 2014, Li et al., 2015, Li et al., 2013). According to the Centre for Disease Control (CDC) 2004 report, *A. baumannii* accounts for about 80% of all reported Acinetobacter infections (www.cdc.gov/ncidod/dhqp/ar| acinetobacter.html).

A. baumannii infections are no longer confined to hospitals as many

outbreaks has also been associated with wars and natural disasters like the Marmara earthquake in 1999 (Camp and Tatum, 2010). A significant increase in *A. baumannii* isolated from ICU patients from 7.3% before the earthquake to 31.2% after the earthquake in the GATA Haydarpasa training Hospital (Oncul et al., 2002). *A. baumannii* have been isolated from wounds and soft tissue injuries from returning military soldiers from Iraq and Afghanistan after war (Scott et al., 2007).

The *Acinetobacter* species are part of the skin flora of healthy individuals in which the carriage rates can be up to 75% without causing serious illness (Seifert et al., 1997). Critically-ill patients and immunocompromised, elderly and premature babies, presence of underlying cause such as major surgery, burns, chronic alcoholism, cancer, bronchopulmonary infections can be predisposing factors to *A. baumannii* infections (Kempf and Rolain, 2012). A wide range of infections are associated with this pathogen including ventilation-associated pneumonia, skin and soft tissue infections, urinary tract infections, meningitis, bacteremia and sepsis, surgical site infection, central line infections and abdominal infections (Gaynes and Edwards 2005).

Back in 1960s, the carriage of *A. baumannii* and the other *Acinetobacter* species was not a concern as it was susceptible to most of the available antibiotics (Kempf and Rolain, 2012). *A. baumannii* is considered one of the pathogens of ESKAPE group; *Enterococcus faecuim, Stapylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, pseudomonas aeruginosa* and *Enterobacter* species, the leading cause of nosocomial infections (Rice, 2008). This name has been given by Rice and colleagues to emphasize that the vast majority of these pathogens cause infections that “ecsape” the effective antibiotic treatment. In the last few decades, there was a huge threat of *A. baumannii* outbreaks worldwide. The rapid development of resistance to various antibiotics grouped as multidrug resistance (MDR), pan-drug resistance (PDR) and extended drug resistance (XDR) is alarming around the globe. The CDC defines the

MDR as the resistance to more than two classes of the following antimicrobials: antipseudomonal cephalosporins, β-lactams (including sulbactams and carbapenems), fluroquinolones and aminoglycosides. PDR is the resistance to all first-line antimicrobials used for *A. baumannii* treatment including polymyxin and tigecycline (Peleg et al., 2008). However, in some cases even last line of treatment was inefficient in treating life threatening *A. baumannii* infections. A recent cohort study has shown that even combination therapy of high-dose colistin and standard dose of tigecycline to treat carbapenem-resistant *A. baumannii* induced bacteraemia did not result in reduced mortality of critically ill patients (Amat et al., 2017).

The empirical use of broad spectrum antibiotics before obtaining a positive culture hindered the effectiveness of the antibiotic therapy (Davies and Davies, 2010, Wright, 2007). According to a survey conducted by the European Centre for Disease Prevention and Control (ECDC) in 2012, MDR was reported in 81% of *A. baumannii* isolates (Zarb et al., 2012). There is an increasing evidence on the significant correlation between MDR *A. baumannii* infections and clinical mortality as shown by (Falagas et al., 2006) meta-analysis, where ICU patients' mortality rate (43%) is higher than the other hospitalized patients (23%).

1.6.3 Virulence and molecular resistance mechanisms of *Acinetobacter*

The major issue faced by all the clinicians and hospitals nowadays is the Multi-Drug resistance of *A. baumannii.* These pathogens have been burdened with massive use of broad spectrum antibiotics for decades. The presence of *A. baumannii* with closely related Gram-negative bacteria in clinical settings has contributed to the development to new resistance mechanisms on top of their own intrinsic factors (Camp and Tatum, 2010). *A. baumannii* and the other Gram-negative pathogens are known of their genome plasticity and capable of evolving and acquiring new virulence determinants (Bergogne-Be´re´zin et al., 2008). Class1 integrons were observed to be present in 88% of biofilm forming *A. baumannii*

(Rajamohan et al., 2009). Moreover, class 1 integron were associated with strains isolated from military hospitals treating soldiers from the USA and the United Kingdom (Turton et al., 2006).

A. baumannii harbours a set of intrinsic abilities and mechanisms of resistance including porins and a wide array of efflux pumps (Fournier et al., 2006). Porins are Outer Membrane Proteins (OMP) that permeate the access of essential nutrients and metabolites into the cells (Schirmer, 1998). Researchers suggest that *A. baumannii* is less permeable and contains smaller size of porins, which allows little or no permeability for antibiotics to enter the cells through the outer membrane (Obara and Nakae, 1991). A study by Del Mar Tom'as and colleagues 2005, have shown that certain *A. baumannii* isolates were intrinsically resistance to imipenem due to the absence of three porins from the outer membrane (del Mar Tomas et al., 2005). Another interesting mechanism of resistance in *A. baumannii* is the wide range of efflux systems which are capable of pumping out a spectrum of antimicrobials from the cells (Maragakis and Perl, 2008). The co-existence of porins and efflux pumps provide a very powerful resistance mechanism for the removal of antimicrobial from the bacterial cells (Maragakis and Perl, 2008).

One of the most common resistance mechanisms in *A. baumannii* and the other Gram-negative pathogens is acquisition of genes coding for certain enzymes through the transfer from one cell to another. A well-known enzyme is β-lactamase, which confers resistance to penicillins, cephalosporins and carbapenems (Bonomo and Szabo, 2006, Naas et al., 2006, Thomson and Bonomo, 2005). The most common resistance mechanism to β-lactams is through the ADC genes (Table 1.1) (Hujer et al., 2006). The VIM genes has a hydrolyzing abilities and could transfer to other Gram-negative bacteria, which could be a new threat in a clinical setting (Docquier, 2003). Similarly, the IMP genes were observed to rapidly spread to other Gram-negative pathogens like *Pseudomonas aeruginosa* through clonal outbreaks in some studies (Koh et al., 2007,

Landman et al., 2002). So far, there are more than 15 oxacillinase (OXA) genes which are grouped into four major classes and yet more to be discovered (Koh et al., 2007, Landman et al., 2002). Studies have shown that TEM-1 has been associated with several mutations in its active site resulting in acquiring resistance to more β-lactams antibiotics (De Wals et al., 2008, Hujer et al., 2006). SHVs are considered to be the most prevalent Extended-spectrum β-lactamases (ESBL) (Hujer et al., 2006, Tzouvelekis and Bomono, 1999). A list of most resistance coding genes are shown in table 1.1.

Another set of modifying enzymes include acetyltransferases, phosphotransferases and nucleotidyltransferases confer resistance to fluroquinolones and aminoglycosides (Hujer et al., 2006, Nemec et al., 2004, Riccio et al., 2000). *aadB* is a gene cassette sometimes found to be associated with class 1 integrons (Jones et al., 2005, Nemec et al., 2004, Shaw et al., 1993). Both *aacC1* and *aacC2* are involved in nosocomial epidemics usually caused by enteric pathogens and can potentially spread between Gram-negatives (Shaw et al., 1993). By contrast, *aphA6* is primarily associated with Acinetobacter species and rarely found in other Gram-negative bacteria (Shaw et al., 1993). Another mechanism of resistance is a mutation in certain genes which could alter the target site of the bacteria for the antimicrobial resulting in reduced affinity for the drugs. For instance, a point mutation in the *gyrA* and *parC* leads to increased resistance to quinolones and if the point mutation occurs in both genes the bacteria will be resistant to all fluroquinolones (Bonomo and Szabo, 2006). Hujer and colleagues reported that 88% of *A. baumannii* strains had one or both genes (Hujer et al., 2006).

Despite the various resistance mechanisms in *A. baumannii*, there are still some potentially effective antibiotic treatments available for the ICU setting including monobactams, carbapenems, some aminoglycosides, sulbactams, fluroquinolones, polymixins and glycylcyclines (Towner, 2009). The standard therapy for the MDR *A. baumannii* strains is

carbapenems, in spite of different documented cases of resistance by carbapenems-hydrolyzing β-lactamases (Afzal-Shah et al., 2001, Bergogne-Berezin and Towner, 1996, Livermore et al., 2002, Poirel and Nordmann, 2006).

Table 1.1 Genes conferring antibiotic resistance and resistance mechanisms in *A. baumannii*

Enzyme group, gene name	Description	Antimicrobial resistance	Reference(s)
β-lactamase genes			
ADC	Chromosomally integrated cephalosporinase	Extended spectrum cephalosporins	(Hujer et al., 2006)
VIM	Acquired metallo-β-lactamase	All β-lactams except monobactams	(Hujer et al., 2006); (Docquier, 2003)
IMP	Stronger carbapenems activity than OXA	Carbapenem resistance	(Koh et al., 2007)
OXA	Carbapenem-hydrolyzing oxallinases	Carbapenem resistance	(Koh et al., 2007)
TEM	A broad-spectrum enzyme	Narrow-spectrum cephalosporins, all penicillins except temocillin	(Hujer et al., 2006)
SHV	Plasmid-mediated. Includes SHV-1 & at least 23 variants	Extended-spectrum cephalosporins, ampicillin	(Hujer et al., 2006)
Aminoglycosides-Modifying Enzymes			
aadB	Enzymatic inactivation by adenylation	Kanamycin, tobramycin & gentamicin	(Nemec et al., 2004)
aacC1	Enzymatic inactivation by acetylation	Gentamicin, apramicin, lividomicin	(Shaw et al., 1993)
aacC2	Enzymatic inactivation by acetylation	some aminoglycosides	(Shaw et al., 1993)
aphA6	Enzymatic inactivation by phosphorylation	Kanamycin, neomycin, gentamicin, gentamicin B, paromomycin, amikacin & other	(Shaw et al., 1993)
aadA1	Modifies 3"-hydroxyl position of streptomycin and 9"- hydroxyl position of spectinomycin	streptomycin and spectinomycin	(Shaw et al., 1993)
Gene-encoding Efflux Pumps			
adeABC	AdeA, AdeB & AdeC proteins (Rameckers et al.)	Aminoglycosides, quinolones, tetracyclines & trimethoprim	(Marchand et al., 2004)
abeM	AbeM (MATE)	norfloxacin, ofloxacin, ciprofloxacin, gentamicin, DAPI, triclosan, doxorubicin, ethidium bromide	(Vila et al., 2007)
tet(A)/ tet(B)	Tet (A)/Tet (B) (MFS)	Tetracycline/ minocycline	(Vila et al., 2007)
cmlA	CmlA	Chloramphenicol	(Vila et al., 2007)
Point mutations			
gyrA	Point mutation at ser83	Quinolones	(Hujer et al., 2006)
parC	Point mutation at Ser80	Quinolones	(Hujer et al., 2006)

Table adapted and modified from (Camp and Tatum, 2010)

Therefore, determining the susceptibility profile for each isolate is essential to ensure proper management of the infection and avoiding unnecessary prescriptions (Erbay et al., 2009). No significant new antibiotic discoveries have been made in the twenty years, apart from some trials. This puts the whole microbiology community alarmed about the seriousness of the issue, and stimulated the “antibiotic action” group to take place in the United Kingdom. This initiative brings into the attention of the government and the media all about the antibiotic resistance issues.

1.6.4 GIs in *A. baumannii*

1.6.4.1 AbaR resistance GIs

The first GI identified in *A. baumannii* was a large GI of 86 Kbp in size, during the sequencing of the epidemic strain AYE in France, and was named “AbaR1” harboring 45 resistance determinants (Fournier et al., 2006). There is a number of similar GIs in *A. baumannii* and named from AbaR0 to AbaR27 (Kochar et al., 2012, Zhu et al., 2013). Whole-genome sequencing has greatly facilitated the discovery of a wide range of variable GIs in *A. baumannii*, comprising different other MGE such as integrons, transposons and IS. In addition, the genetic content of these GI is diverse and includes various antibiotic and virulence determinants. A recent evidence has shown an interspecies transfer of AbaR-like GIs (Kim and Ko, 2015). This latter finding will probably widen our current knowledge about these GIs as it showed that these AbaR-like elements are no longer confined to *A. baumannii*.

The AbaR1 type of GI, are structurally related composite transposons, and they all have an integrated transposon in their backbone such as Tn*6019,* Tn*6021* and Tn*6022*, which mostly flanked by two copies of Tn*6018* (Post et al., 2010, Seputiene et al., 2012). The Tn*6019* features genes involved in transposition (*tniA, tniB*), an arsenate resistance operon, a universal stress protein gene (*uspA*), and a sulphate permease gene (Gutierrez et al., Post et al., 2010). The Tn*6018* encodes for heavy metal resistance determinants and the two copies of this transposon

enclose a multiple-antibiotic resistance region (MARR) inserted within *uspA* gene, making Tn*6018*- MARR-Tn*6018* compound transposon (Post and Hall, 2009).

Despite being similar in the backbone structure, AbaR-like GIs are often variable in terms of size and genetic composition. For example, the MARR region of the AbaR GI harbor a set of genes encoding antibiotic, heavy metal, antiseptic resistance and efflux determinants (Kochar et al., 2012). In the case of AbaR6 and AbaR7 GIs, they only have one copy of the Tn*6018* as a result of large deletions of the transposons (Post et al., 2010, Seputiene et al., 2012). The diversity in AbaR GI compositions are as a result of several events of recombination like integration, excision and rearrangements (Post and Hall, 2009). The role IS*26* elements in the evolution and shaping of the TnAbaR GI is highlighted by the study of (Krizova et al., 2011). Comparative genomic analysis showed that the IS*26* were present in high frequency in these GIs, with variable deleted regions in between is strongly suggestive of recombination events (Krizova et al., 2011).

Detailed analysis of the AbaR transposons revealed that they share a common ancestor to Tn*7* transposon (Kochar et al., 2012, Rose, 2010), thus the AbaR GI was suggested to be renamed as TnAbaR in (Kochar et al., 2012). The AbaR GIs usually integrate within the *comM* gene as a hot spot in a site and orientation specific manner (Adams et al., 2008). However, other integration sites than the *comM* gene has been described where also more than one copy of AbaR GI could be identified in one strain (Adams et al., 2008, Rose, 2010, Smith et al., 2007).

In the (Di Nocera et al., 2011), a comparative genomic analysis was performed, where genomic DNA sequences of different sequence type strains of *A. baumannii* were compared. This analysis showed extensive synteny and identified 3068 coding regions which are conserved, at the same chromosomal position, in all *A. baumannii* genomes. Genome alignments also identified 63 DNA regions, ranging in size from 4 - 126 kb, all defined as genomic islands due to exhibiting certain features of GIs

such as IS present at one end, different GC content, tRNA insertions, non-coding RNA genes and target site duplications (TSD) resulted upon integration events (Dobrindt et al., 2004, Sridhar et al., 2010). Generally, these GIs can be present in some genomes, while missing in others of a closely related strain, or otherwise replaced with non-homologous DNA sequences.

Comparative genomic analysis of *A. baumannii* isolates representing the most frequently occurring sequence types (STs) revealed the presence of diverse groups of resistance GIs with different genes encoding resistance to antibiotics and heavy metals (Di Nocera et al., 2011) which are grouped in clusters (Figure 1.6). The *aadA1* (streptomycin-resistance encoding) gene, flanked by *satR* (streptothricin-resistance encoding) and *dhfr* (trimethoprim-encoding resistance) genes are found in GIs in clusters. Genes involved in resistance to mercury (*merRCAD* cluster) are found to be located in a separate cluster, and a 4.5 kb DNA segment containing *feoAB* (ferrous iron transport operon), *czc* (tricomponent proton/cation antiporter efflux system) and *ars* (arsenite transporters) genes are found as agroup, next to the *cus* (copper resistance) genes conserved in the same chromosomal locations of certain GIs (Figure 1.7), however, these genes differ in sequence and overall arrangement from other homologous GIs in *A. baumannii*. This supports the notion that the set of accessory genes had been independently acquired by the different strains. Additional resistance genes found in GIs include aminoglycoside phosphotransferase genes, dihydropteroate synthase gene, and an ABC-type multidrug transport system, conserved in some GIs.

1.6.4.2 Other GIs in *A. baumannii*

The accessory DNA found in *A. baumannii* is composed of a diverse group of genes that have been acquired horizontally. Apart from resistance GIs, *A. baumannii* carry other GIs encoding several functions such as genes for surface components and transport system. These include O-antigen biosynthetic genes, gene operons involved in the assembly of fimbriae (type I pili) by the chaperone/usher pathway (Nuccio

and Baumler, 2007). Moreover, many surface adhesion protein (Vallenet et al., 2008), filamentous haemagglutinins (FHA) (Locht et al., 1993) and cyclopropane fatty acids (CFA) (Zhang and Rock, 2008) are encoded in some *A. baumannii* associated- GIs.

In addition, many GIs carry genes encoding proteins involved in specific metabolic pathways such as, *mph* (multi component phenol hydroxylase) gene complex (Rocco et al., 2009) and genes involved in the catabolism of 3HPP (3-hydroxyphenylpropionic acid) and PP (phenyl- propionic acid). Some GIs also encodes 4 proteins (tartrate dehydratase subunits alpha and beta, a MFS transporter and a transcriptional regulator) possibly involved in the metabolism of tartrate (Di Nocera et al., 2011).

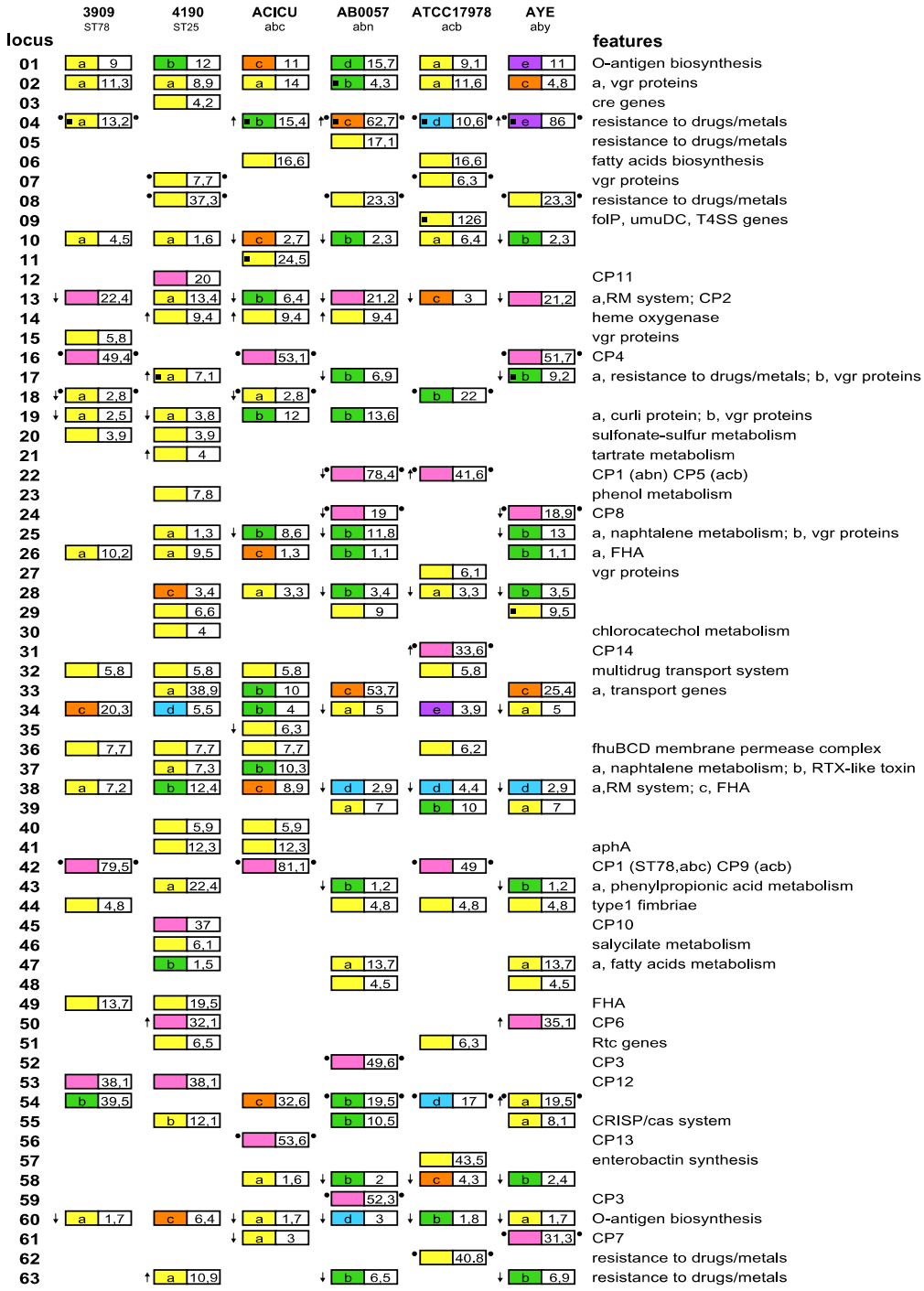


Figure 1.6 Variable regions in *A. baumannii* genomes. Each line corresponds to a chromosomal locus.

Different GIs inserted at the same locus in different strains are marked by different colours and lower case letters. Sizes of GIs are given in kb. Black boxes within GIs denote mobile sequences, down and up arrows to the left indicate that the GI G+C content is lower than 36% or higher than 42%, respectively. Dots flanking GIs denote TSDs. The strain names and relative acronyms used throughout the text are given at the top. Acronyms below complete genomes are those used at Kyoto Encyclopaedia of Genes and Genomes (KEGG). Figure adapted from (Di Nocera et al., 2011).

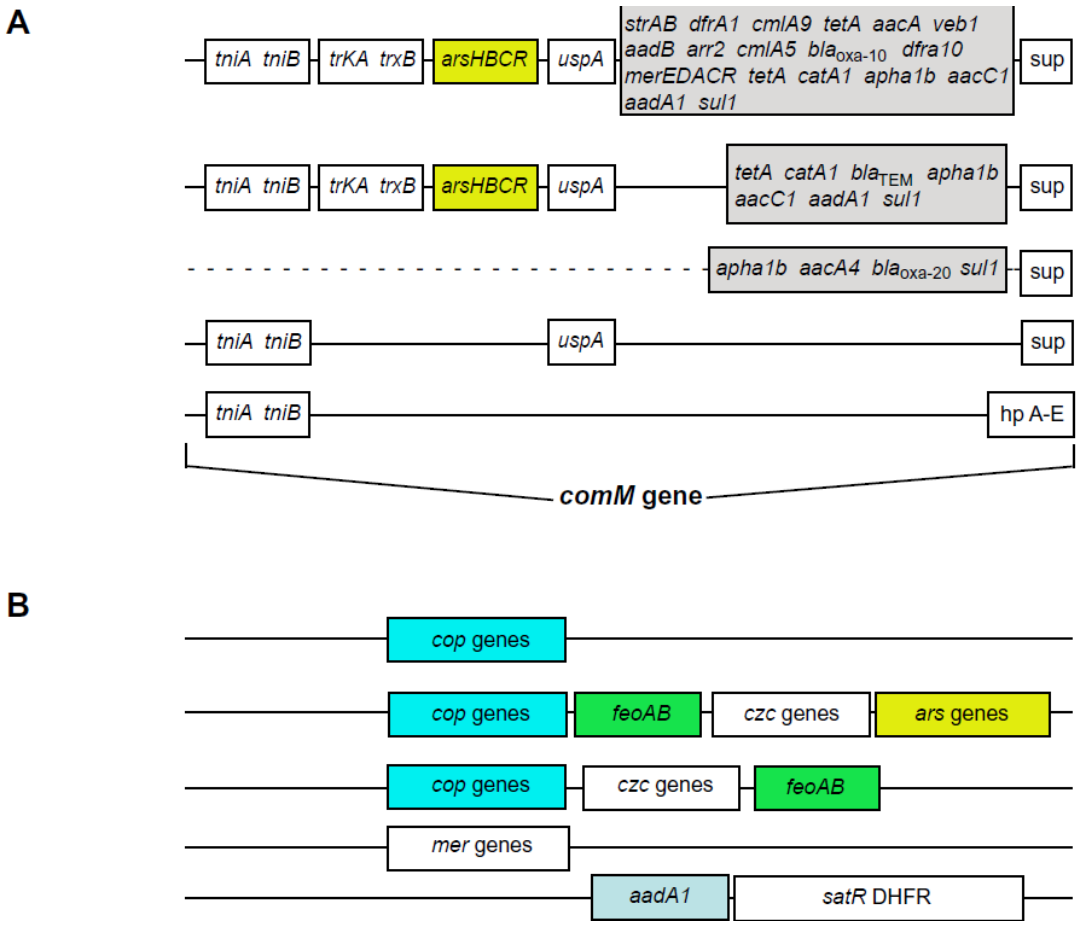


Figure 1.7 Resistance gene islands.

(A) Diagrammatic representation of AbaR-like GIs in *A. baumannii* with different gene contents. Grey boxes represent MARR. Deleted DNA is marked by a dotted line. B) Resistance genes in other GEIs. (Di Nocera et al., 2011)

Genome analysis of *A. baumannii* isolates in (Di Nocera et al., 2011) showed that many variable genomic regions are relatively large (19 to 82 kb) DNA blocks, could potentially encode phage products. These regions have all been classified as cryptic prophages Clements et al. (2007). CPs characteristically carries an integrase gene at one end, and many are defined by flanking TSDs produced upon insertion.

1.7 *Staphylococcus*, the species and the clinic

1.7.1 *Staphylococcus*, the species history

The term "*Staphylococcus*" was first introduced by Ogston in 1882, where the first closely linked *Staphylococcus*-like microorganisms with wound infections (Ogston, 1880, Ogston, 1882). This was followed by the work of Rosenbach, a German surgeon, who recovered abscess from animal experiments and gave the designations to two different species "*Staphylococcus pyogenes aureus*" and "*Staphylococcus pyogenes albus*" (Becker et al., 2014). In the late 1890s, Welch, a pathologist from the United States described "*Staphylococcus epidermidis albus*" as a colonizer of the human skin (Peacock, 2010). Old scientific literature indicated various methods to distinguish the different staphylococcal species depending on their pathogenicity or the pigment production. The principle of coagulase production was introduced by Fairbrother, as a technique for differentiating staphylococcal species (Fairbrother, 1940). In the late 1970s, 10 newly identified species were added, and the number dramatically increased onwards to more than 50 validly described species.

According to the widely accepted medical classification, staphylococci are mainly devided into coagulase-positive *Staphylococcus* (CoPS), and coagulase-negative *Staphylococcus* (CoNS). The CoPS almost includes *S. aureus* exclusively. Regarding the CoNS, it comprises the two most prevalent species *S. epidermidis* and *S. haemolyticus*, as well other known species of *S. capitis*, *S. hominis*, *S. simulans*, and *S. warneri. S. saprophyticus,* is a known pathogen causing acute arthritis as well as other infections. All of the above mentioned staphylococcal species in addition to *S. pettenkoferi* and *S. massiliensis* are clinically defined as "*S. epidermidis* group" (Becker et al., 2014). *S. lugdunensis* has a recently been added to the CoNS, displaying clinical characteristics between *S. aureus* and *S. epidermidis* group (Figure 1.8).

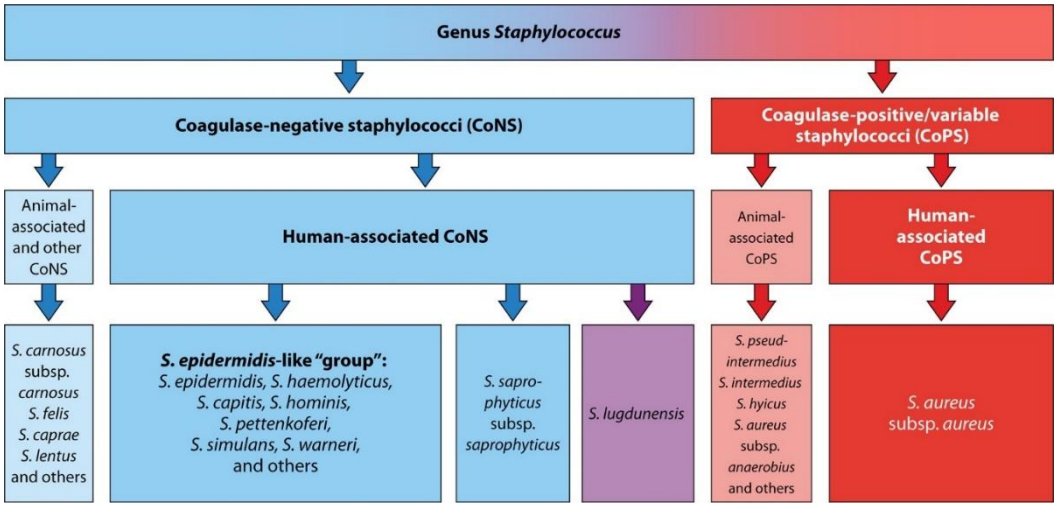


Figure 1.8 Clinical and epidemiological schema of staphylococcal species.

The diagram is based on the categorization of coagulase as a major virulence factor and its resulting impact on human health. (Becker et al., 2014).

1.7.2 Ecology of human-associated Staphylococci

Staphylococcus is an essential part of the microflora in the skin and mucus membranes of both human and animals (Becker et al., 2014). Traditional approaches like cultures showed that staphylococci are usually cultivated from moist surfaces such as axilla, popliteal folds, gluteal region, umbilicus, toe webs and humid mucus membranes including the anterior nares (Wos-Oxley et al., 2010).

S. epidermidis is the most frequently isolated staphylococcus in humans. This species is also predominantly recovered from moist surfaces (Schleifer and Kloos, 1975). The other species in the *S. epidermidis* group such as *S. haemolyticus* and *S. hominis* are often recovered from areas high in sweat glands such as the axillae and the genital area (Kloos and Musselwhite, 1975, Schleifer and Kloos, 1975), whereas *S. capitis* is found in sebaceous glands-rich areas like on the forehead and scalp after the age of puberty (Kloos and Schleifer, 1975). *S. auricularis* is almost exclusively isolated from the human external ear (Kloos and Schleifer, 1983).

1.7.3 Clinical manifestations of Staphylococci

Despite the fact that staphylococcal species are part of the normal microbiota of the human and natural food surfaces, these bacteria have the capability of causing a wide spectrum of infections. Even the less virulent species could potentiate infection in the case of presence of a foreign body or immunosuppression (von Eiff et al., 2006). *S. aureus* could cause diseases as a result of the release of superantigen and exfoliative toxins such as toxic shock syndrome (TSS), staphylococcal scalded skin syndrome (SSSS), and staphylococcal food poisoning (SFP). However, no evidence so far could confirm that *S. epidermidis* release any of these toxins (Becker and Skaar, 2014).

On the other hand, *S. epidermidis* have been strongly involved with device-associated health care-associated infections (DA HAIs). This type of infection often starts with colonization of the foreign body by the bacteria and followed by seeding of the causative organism elsewhere in the body resulting in various types of infections including sepsis, meningitis, endocarditis, arthritis and others.

In addition, *S. aureus* and *S. epidermidis* were shown to be the main causes of infections in implanted devices such as prosthetic valve infective endocarditis and prosthetic joint infections (20% and 31%, respectively) according to the statistics from the U.S. nationwide Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) database (Wisplinghoff et al., 2003). Moreover, cases of infective endocarditis resulting from non-sterile drug injections among addicts, were reported to be caused by *S. aureus* (51.3%) and streptococcal species (23.1%), *S. epidermidis* (15.4%) (Carozza et al., 2006). Several reports found that Staphylococcal species can cause invasive infections in neonates and preterm infants particularly those with foreign body implants (Becker et al., 2014) and references therein. Regarding the bacteremia and septicemia in neutropenic patients

undergoing chemo-therapy treatment, *S. epidermidis* is still the most common causative organism (Gonzfilez-Barca et al., 1996, Horasan et al., 2011).

1.7.4 Pathogenicity and virulence in Staphylococci

Staphylococcal species have the capacity to colonize and infect the human body due to the presence of several specific factors that enable adhesion, aggression, invasion, persistence, and/or evasion of both innate and adaptive immunity. These factors include adherence to surfaces especially foreign bodies and multilayered biofilm formation which is mediated by several proteins (Hussain et al., 1993, Mack et al., 1996, Peters et al., 1981). A recent study found that increased virulence in CA-MRSA, inducing skin abscess formation in animal model was correlated to the quantity of wall teichoic acid (WTA), an essential cell wall component of the *S. aureus* (Wanner et al., 2017).

It has been shown by some studies that *S. aureus* could be ingested by human host cells and remain hidden within nonprofessional phagocytes, thus escaping the antimicrobial therapy and subsequent evasion of the patient's immune system. It was shown that Atl autolysin mediates *S. epidermidis* adhesion to human endothelial cells and internalization within these cells (Hirschhausen et al., 2010).

A specific phenotype named the "small colony variant" (SCV), have been shown to be associated with chronic, relapsing and recurrent type of infection in *S. aureus* and *S. epidermidis* (Proctor et al., 2006). They are tiny colonies localize within specific sites inside the cellular host thus could survive antibiotic therapy and host immune response (Tuchscherr et al., 2011, von Eiff et al., 2001). These are pleomorphic "G" form colonies that are switching from the normal wild-type phenotype, reflecting various changes at the cellular level, including metabolism and modifications of the transcriptome and proteome (Besier et al., 2007, Kriegeskorte et al., 2011, Seggewiss et al., 2006, von Eiff et al., 2006).

Recent research evidenced that adaptive persistence traits was associated with single nucleotide mutations in the *mprF* genes as detected by whole genome sequencing data (Richards et al., 2015). These SNPs resulted in increased bacterial fitness and enhanced immune evasion. Other studies have shown that polymorphisms in the promoter of Toll-like receptor (TLR)-9, and changes in gene expression are involved in the persistence of *S. aureus* nasal carriage (Nurjadi et al., 2018). In addition, Research showed that staphylococcal protein A (SpA), a B cell superantigen is essentially needed for persistence of colonization of *S. aureus* in the nasopharynx in mice (Sun et al., 2018).

Unlike the aggressiveness of *S. aureus*, that produces a number of cytotoxins, *S. epidermidis* uses a milder strategy based on passive defense mechanisms to evade the human immune which are usually mediated by enzymatic activities, such as the protease SepA that cleaves AMPs (Cheung et al., 2010). Other toxins include pyrogenic toxin superantigen (PTSAg) family, comprising staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1) (Valle et al., 1991).

1.7.5 Horizontal gene transfer in *Staphylococcus*

S. aureus has always been significantly pathogenic bacteria even before the era of antibiotics causing a range of infections, some of which are mild like skin infections while others can be lethal if not treated promptly like endocarditis, osteomyelitis and septicemia (Corvaglia et al., 2010, Moskowitz and Wiener-Kronish, 2010). However, the intermittent or persistent carriage rate of *S. aureus* among the human population is as high as 30%. The most alarming threat associated with this pathogen was due to the emergence of Methicillin- resistant *S. aureus* (MRSA). The MRSA has acquired resistance to a broad-spectrum of antibiotics and is a main cause of hospital-acquired infections. Moreover, the emergence of a highly resistant and virulent MRSA strains resulted in serious illness in healthy people in the community (Schijffelen et al., 2010).

There are a variety of factors contributed to the virulence of *S. aureus*,

some of which are due to a number of genes encoding toxins which are either located in the chromosome or acquired horizontally (Li et al., 2009, Wang et al., 2007). The other issue in tackling the *S. aureus* infections was the antibiotic resistance due to genes carried on horizontally acquired mobile elements including transposons, plasmids, GIs and ICEs (Ito et al., 2003, Juhas et al., 2009, Queck et al., 2009).

1.7.6 SCC*mec* in Staphylococci

Among these resistance determinants in *S. aureus*, is the gene encoding methicillin resistance, *mecA.* is carried on a genomic island called the staphylococcal cassette chromosome *mec* (SCC*mec*) (Figure 1.9) (Ito et al., 2003, Katayama et al., 2000). The methicillin resistance determinant (*mec*) was identified in *S. aureus* in 1970s (Kuhl et al., 1978, Sjöström et al., 1975). Methicillin-susceptible *S. aureus* do not have any allelic equivalent of the *mec* gene. The *mecA* gene was found to have an additional protein domain which has low affinity for binding all β-lactam antibiotics, called the penicillin-binding protein (PBP2a) (Archer et al., 1994, Kornblum et al., 1981). PBP2a is a class B transpeptidase which has a high molecular weight and catalyzes the formation of cross-bridges in the peptidoglycan of the bacterial cell wall (Berger-Bachi and Rohrer, 2002, Goffin and Ghuysen, 1998). When *S. aureus* is exposed to β-lactam antibiotics, the normally occurring PBP is usually inactivated by ligating the antibiotic. However, in the presence of *mecA*, the function of cell wall biosynthesis is taken over by a transglycosylase enzyme from the native PBP2 (Pinho et al., 2001).

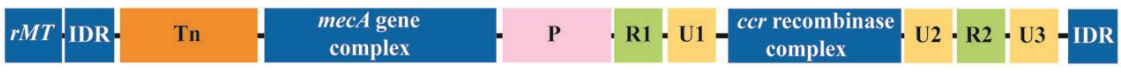


Figure 1.9 Schematic representation of the staphylococcal cassette chromosome *mec* (SCC*mec*).

The figure depicts the main features of the genomic island SCC*mec* associated with multidrug resistance acquisition by *Staphylococcus aureus* and spread of methicillin-resistant *S. aureus* (MRSA) worldwide. All SCC*mec* types sequenced so far share common features which are highlighted blue: 1. *mecA* gene complex conferring resistance to methicillin 2. *ccr* recombinase complex responsible for mobilisation 3. terminal inverted and direct repeats (IDR) and 4.

insertion site in a conserved ribosomal methyltransferase (rMT). In addition, SCC*mec* islands usually encode a wide variety of other factors, like transposons (Tn), integrated plasmids (P), resistance genes (R) and open reading frames of unknown function (U). Figure is adapted from (Juhas, 2015).

mecA gene is carried on a genomic island called the staphylococcal cassette chromosome *mec* (SCC*mec*) (Figure 1.9) (Ito et al., 2003, Katayama et al., 2000). The SCC*mec* GI carry a wide spectrum of other genes encoding resistance to antibiotics including tetracycline, kanamycin, streptogramin, bleomycin, lincosamide and tobramycin (Deurenberg et al., 2007, Ito et al., 2003). Other studies by (Ito et al., 2004, Ito et al., 2003, Smyth et al., 2011) have identified subtypes of the SCC*mec* GI with variable sizes ranging between 21 to 70 kb.

The SCC*mec* GIs are classified into subgroups based on the recombinase encoded in the island and the genetic composition of *mecA* gene complex, the transposons as well as the *ccr* complex (cassette chromosome recombinase as *ccrA/B* or *ccrC*. , (Ito et al., 2001, Katayama et al., 2001, Okuma et al., 2002, Wisplinghoff et al., 2003). The genetic exchange of genes through the SCC has been well-established by *S. aureus* under stressful conditions (Katayama et al., 2003). The integration of SCC occurs at a specific site on the bacterial chromosome, *attB*SCC, which is a 15 bp sequence and usually located near the origin of replication of *S. aureus*. Upon integration of the SCC on the chromosome, the *attB*SCC is duplicated at the junctional region. At the right end flanking region, one of the two repeat sequences is present within the SCC*mec*, whearas another incomplete inverted repeats are located at both borders of the SCC*mec* element (Ito et al., 2001). During the integration and excision of the SCC*mec*, these repeats are recognized by the SCC*mec* encoded recombinases (Hiramatsu et al., 2001).

For the mobilisation of the SCC*mec*, *ccr* recombinases are encoded by specific genes within the SCC*mec* and they catalyze the excision and the integration of the element from and to the chromosome (Katayama et al., 2000, Wang and Archer, 2010). There are four different homologous pairs

of *ccrAB* genes and one *ccrC* gene have been described (Ito et al., 2004, Katayama et al., 2000, Oliveira and Lencastre, 2002). Recombinases of the invertase/resolvase family have a characteristic Ccr catalytic motif at the N-terminal domain (Abdel-Meguid et al., 1984). In addition, the all the Ccr proteins have a conserved catalytic serine residue at the recombination active site (Ito et al., 2001).

The specific types of CcrAB and CcrC are unique, however, they could be related to certain site-specific integrases such as integrases from bacteriophage TP901-1 of *Lactococcus lactis* (Christiansen et al., 1996), an integrase of *Bacillus cereus* bacteriophage TP21 ply21 (Leossner et al., 1997), a site-specific recombinase SpoIVCA from *Bacillus subtilis* (Ken-ichi Takemaru et al., 1995), and a transposase TnpX from *Clostridium perfringens* (Bannam et al., 1995).

For the integration process to occur, a recombination process is mediated by the CcrB and the CcrA, the circular form of SCC*mec* has *attS* as site-specific sequence will recognize the *attB* attachment site in the chromosome (Wang and Archer, 2010). Upon excision of the element, the SCC*mec* has to be in a linear form where two specific attachment sequences are located at both ends, the *attL* the *attR*. Once the SCC*mec* is excised, the *attS* on the circular intermediate and the *attB* on the chromosome both will be restored to their original sequences (Wang and Archer, 2010). The rate of frequency and efficiency of integration of the SCC*mec* GI depends on the flanking sequences of the att sites (Wang et al., 2012). Recently, it has been shown that the insertion site of SCC*mec* occurs on a gene encoding a ribosomal methyltransferase (Boundy et al., 2013).

According to some studies, the SCC*mec* was predicted to be originally derived from *Staphylococcus epidermidis* or *Staphylococcus fleuretti* during which the acquisition of SCC*mec* occurred via HGT resulting in the evolution of *S. aureus* harboring the island (Aslantas et al., 2012, Deurenberg et al., 2007). A study by (Bloemendaal et al., 2010) performed sequence analysis of methicillin-susceptible and resistant *S.*

aureus isolates (MSSA and MRSA) and *S. epidermidis*, in that order and have isolated a MRSA strain from a patient undergoing antibiotic treatment. The analysis revealed a possible transfer of SCC*mec* between the two different species during the treatment. A multiplex-PCR analysis of another study showed that both *S. aureus* and S*. epidermidis* isolates comprised a common small number of SCC*mec* IV subtypes (Smyth et al., 2011). In addition, analysis by (Fluit et al., 2013) have shown that 98% of the *ccrB* alleles from MRSA isolates were identical to coagulase-negative staphylococcal *ccrB* alleles. All of the above studies are suggestive of possible interspecies HGT of SCC*mec* from *S. epidermidis* to *S. aureus*, however, none of these studies have clearly demonstrated the SCC*mec* transfer to *S. aureus*.

1.7.6.1 SCC*mec* subtypes

The SCC*mec* were classified into four major types (I–V) and named according to the different combination of two parts: the *ccr* complex and the *mec* complex (Hiramatsu et al., 2001, Ito et al., 2004, Katayama et al., 2001) (Figure 1.10). There are five allotypes of the *ccr* gene complex named as *ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrAB4* and *ccrC* (Ito et al., 2001, Ito et al., 2004, Oliveira and Lencastre, 2002). Five classes of the *mec* gene complex (A–E) have been described (Katayama et al., 2001, Lim et al., 2003). The various SCC*mec* types are called SCC*mec* type I (class B *mec* gene complex and *ccrAB* type 1); SCC*mec* type II (class A *mec* gene complex and *ccrAB* type 2); SCC*mec* type III (class A *mec* gene complex and *ccrAB* type 3); SCC*mec* type IV (class B *mec* gene complex and *ccrAB* type 2), and SCC*mec* type V (class C2 *mec* gene complex and *ccrC*) (Figure 1.10) (Hanssen and Ericson Sollid, 2006).

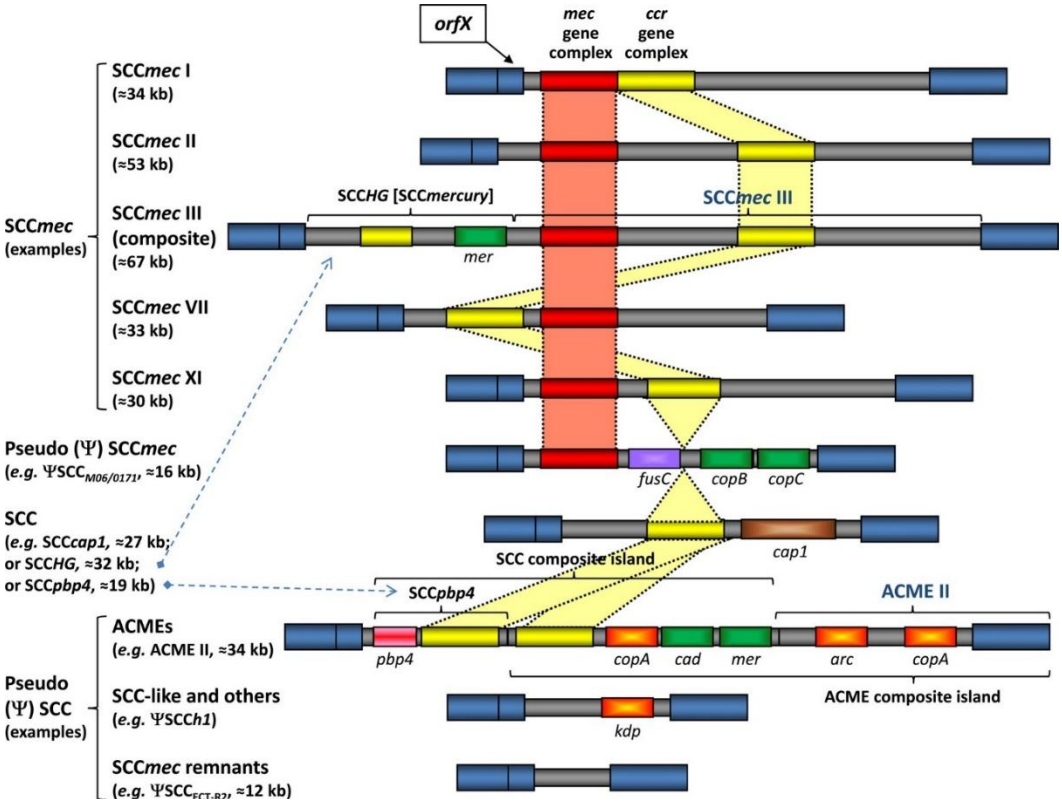


Figure 1.10 Highly simplified schema of the composition of the SCC family integrated into the *S. aureus* chromosome (blue).

Some examples of various SCC types, including those organized in composite islands, are given. Basic structures comprise the *mec* gene complex (red), the *ccr* gene complex (yellow), and the joining regions. Some SCCs additionally contain (i) resistance operons acting against antibiotic agents (violet), such as fusidic acid (*fusC*); (ii) resistance operons for metalloids and transition metals with toxic/bactericidal properties (green), such as arsenic (*ars*), cadmium , copper (*copB* and *copC*), and mercury (*mer*); (iii) virulence genes (brown), such as the capsule polysaccharide gene (*cap1*); (iv) other genes (orange), such as the genes composing the arginine catabolic mobile element (*arc*), the copper-translocating P-type ATPase gene (*copA*), and the potassium-transporting ATPase genes (*kdp*); (v) further penicillin-binding protein 4 (PBP4) genes , such as *pbp4*; and (vi) pseudogenes. Figure is taken from (Becker et al., 2014).

In addition, SCC*mec* islands usually encode a wide variety of other factors, like transposons (Tn), integrated plasmids (P), resistance genes (R) and open reading frames of unknown function (U) (Juhas, 2015). For example, type IA variant of SCC*mec* contain an integrated copy of pUB110, whereas SCC*mec* type IIIA is marked by the absence of pT181 and its flanking IS*431* elements (Oliveira and Lencastre, 2002). pUB*110* is

flanked by a pair of IS*431* elements (McKenzie et al., 1986), and encodes kanamycin and tobramycin (*aadD*)/bleomycin (*ble*) resistance (Ito et al., 1999). With the exception of *mecA*, SCC*mec* types I, IV and V lack any antibiotic resistance genes, (Okuma et al., 2002). SCC*mec* type II harbours erythromycin (*ermA*) and spectinomycin (*spc)* resistance encoding transposon, Tn*554* (Murphy et al., 1985), whereas SCC*mec* subtype IVc contain Tn*4001* encoding bifunctional AAC/APH protein (*aacA-aphD*), conferring resistance to most of the aminoglycosides (Gillespie et al., 1987). SCC*mec* type III contains Tn*554*, pseudo ¥Tn*554* encoding cadmium resistance, pUB*110*, an integrated copy of pT181 (*tetK*, tetracycline resistance) and pI*258*, encoding mercury resistance (Ito et al., 1999). SCC*mec* type V encodes a type I restriction- modification system, *hsdR, hsdS and hsdM*, which may contribute to the stability of the element (Ito et al., 2004).

There are at least one to four copies of IS*431*in *Staphylococci*, one of which is located downstream of *mecA* (IS*431mec*) (Archer et al., 1994). IS*431* elements are reservoirs for many resistance determinants as well as trap for related IS elements through homologous recombination (Hanssen and Ericson Sollid, 2006). This could explain the high prevalence of multiple drug resistance phenotype of staphylococci. IS*431mec* also contains an open reading frame of a putative transposase gene and 14–22bp terminal inverted repeats as a consensus (Kobayashi et al., 2001).

1.7.6.2 Distribution of SCC*mec* types

To understand the mechanisms of resistance and how they spread, it is vital to highlight the basics of staphylococci at the genetic level. The two essential routes for the spread of resistance determinants are the through clonal spread of isolates or the horizontal spread of genes. *mec* is only observed to be horizontally transferred and various details on the mechanism of spread are still missing.

The SCC*mec* is distributed to a few clonal complexes of MRSA

(Katayama et al., 2005). SCC*mec* IV is predominantly found among the community (CA-MRSA) and to a lesser extent among health professionals (H-MRSA) (Ito et al., 2003, Okuma et al., 2002). Epidemic outbreaks of H-MRSA belong usually to SCC*mec* types I, II or III (Enright et al., 2002, Ito et al., 2001). The majority of SCC*mec* of unkown types are found in CA-MRSA (Berglund et al., 2005). In methicillin-resistant *Staphylococcus epidermidis* (MRSE), 10 various structural types of SCC*mec* have been described (Miragaia et al., 2007). A study by (Wisplinghoff et al., 2003) reported hospital isolates of *S. epidermidis*, 36% were SCC*mec* type IV, 34% SCC*mec* type II, 28% SCC*mec* type III and only 2% SCC*mec* type I.

1.7.7 Staphylococcal non-SCC*mec* MGE

1.7.7.1 Plasmids and transposons-mediated resistance

Plasmids are DNA molecules which are capable of auto-replication. Staphylococcal plasmids are usually variable in size and classified to three main groups. The first type are small plasmids with multiple copy number either cryptic or carry one resistance determinant. The second type of plasmids are larger in size ranging from 15 to 30 kb and are low in copy number, carrying several resistance determinants. The third type is the conjugative plasmids with multi-resistance determinants (Berg et al., 1998). As *S. aureus* is naturally low in competence, plasmids often transfer via conjugation or transduction (Morikawa et al., 2003). Upon entry to the host cell, the plasmids can either remain as a circular form or integrate within the bacterial DNA.

Antibiotic treatment of *S. aureus* infections with penicillin was very effective initially, however, more than 90% of *S. aureus* are now resistant to penicillin (Olsen et al., 2006). Penicillin resistance in *S. aureus* is mediated by β-lactamase which is encoded by the *blaZ* and the regulatory genes *blaI* and *blaR* (Hackbarth and Chambers, 1993). The latter genes are located in plasmids, transposons or even in the chromosome (Sidhu et al., 2002), (Olsen et al., 2006).

Very few effective antibiotic treatment options are available against multi-

drug resistant *S. aureus* like vancomycin. However, a number of cases of MRSA have emerged were also vancomycin resistant (VRSA) (Weigel et al., 2003). Following reports showed evidence of *in vitro* HGT of vancomycin resistant genes from other species like *Enterococcus* to *S. aureus* from the same patients. These reports have demonstrated the spread of *vanA*, a vancomycin resistance encoding gene, from *Enterococcus faecalis* and *Enterococcus faecium* to *S. aureus.* It was shown experimentally that VRSA isolates resulted from the acquisition of an Inc*18* family plasmid, carrying *vanA*-encoding transposon Tn1*546* from the vancomycin-resistant *Enterococcus* strains (de Niederhausern et al., 2011, Severin et al., 2004, Zhu et al., 2008). Tn*1546* transposon encodes the *vanA* operon, comprising other genes, *vanA, vanH, vanX, vanS, vanR, vanY and vanZ* (Saha et al., 2008, Weigel et al., 2003). In the second VRSA isolate reported in the US (VRSA-2), the van operon was found to be located in a very large plasmid of 120-kbp in size (Tenover et al., 2003). Various plasmid-encoded antibiotic resistance determinants are summarised in table 1.2. Furthermore, *S. aureus* plasmids carry a wide array of other metabolic and resistance encoding genes, such as heavy metal resistance determinants such as cadmium, mercury and arsenate (Table 1.2).

Transposons in *S. aureus* usually carry genes encoding antibiotic resistance (Table 1.2). The transposons which are small in size are usually presented in multiple copies in the staphylococcal genome, either inserted into the chromosome or into MGEs, such as SCC or plasmids (Malachowa and DeLeo, 2010). This group includes Tn*554* and Tn*552*, which encode resistance to MLSB antibiotics and spectinomycin or penicillinase, respectively (Ito et al., 2003, Jensen and Lyon, 2009, Philips and Novick, 1979). By contrast, larger transposons more than 18 kbp in size, are usually present in single copies and encode antibiotic resistance genes including tetracycline (Soge et al., 2008), trimethoprim (Rouch and Skurray, 1989), aminoglycosides (Byrne et al., 1991, Lange et al., 2003), or vancomycin (Ballard et al., 2005, Weigel et al., 2003, Zhu et al., 2008).

Table 1.2 Resistance determinants encoded on non-SCC*mec* staphylococcal MGEs

MGE/Genes	Antibiotic/metal	Mechanism	Reference/s
plasmids			
aadD	Neomycin, kanamycin, paromomycin, and tobramycin	Aminoglycoside adenyltransferase	(Byrne et al., 1991); (Kadlec and Schwarz, 2009)
ant4'	Tobramycin	Aminoglycoside nucleotidyltransferase	(Lelie`vre et al., 1999)
arsRBC	Arsenate	Efflux ATPase	(Broer et al., 1993)
blaZ, blaI, blaR1	Penicillin (β-lactam antibiotics)	β-lactamase	(Hou et al., 2007)
ble	bleomycin	Bleomycin-binding protein prevents DNA damage by binding bleomycin	(Gennimata et al., 1996)
cadA,B	Cadmium resistance and probably zinc	Cadmium efflux ATPase	(Crupper et al., 1999)
cadD,X	Cadmium resistance	Efflux	(Massidda et al., 2006)
cat	Chloramphenicol	Chloramphenicol acetyltransferase	(Projan et al., 1988)
cfr	Chloramphenicol, florfenicol, and clindamycin	Methylation of 23S subunit of bacterial ribosome	(Kehrenberg and Schwarz, 2006)
dfrA, dfrK	Trimethoprim	Dihydrofolate reductase	(Tennent et al., 1988)
ermB,C	MLSB resistance (macrolides: erythromycin, lincosamides: clindamycin, streptogramin B)	Methylation of 23S subunit of bacterial ribosome	(Otsuka et al., 2007)
fusB	Fusidic acid	Ribosome protection mechanism	(Jappe et al., 2008)
ileS-2	High-level mupirocin resistance	Isoleucyl RNA synthetase	(de Oliveira et al., 2009)
mer operon	mercury	Reduction of mercury ions to elementary Hg	(Laddaga et al., 1987)
mphBM	macrolides	Putative phosphorylase	(Matsuoka et al., 1998)
msrA	macrolides	Active efflux	(Matsuoka et al., 1998)
mupA	High-level mupirocin resistance	Novel isoleucyl RNA synthetase	(Antonio et al., 2002)
qacA,B and smr (qacC/D)	Quaternary ammonium compounds, biocides	Drug efflux pump	(Littlejohn et al., 1991)
str	Streptomycin	Streptomycin adenyltransferase	(Projan et al., 1988)
tetK, tetL	Tetracyclines	Active efflux of tetracycline	(Bismuth et al., 1990)
vat	Streptogramins type A	Acetylation of the antibiotic	(Korczynska et al., 2007)
vga	Streptogramins type A, lincosamides, and pleuromutilins	Efflux	(Kadlec and Schwarz, 2009)
vgb	Streptogramins type B	Inactivation by virginiamycin B lyase	(Mukhtar et al., 2001)
Transposon *aacA-aphD*	Gentamycin, kanamycin,	Antibiotic modification by	(Lange et al., 2003)

	tobramycin	aminoglycoside acetyltransferase and aminoglycoside phosphotransferase	
blaZ, blaI, blaR1	β-Lactams	Hydrolysis of -βlactam ring	(Rowland and Dyke, 1990)
cadB, cadC	Cadmium resistance	efflux	(Dubin et al., 1992)
ermA,B	MLSB resistance (macrolides: erythromycin, lincosamides: clindamycin, streptogramin B)	Methylation of 23S subunit of bacterial ribosome	(Westh et al., 1995)
fexA	Florfenicol, chloramphenicol	efflux	(Kehrenberg and Schwarz, 2006)
merA, B	inorganic and organic mercury resistance, respectively	Ion transport	(Babich et al., 1991)
sat4	Streptothricin	Streptothricin acetyltransferase	(Schwarz et al., 2004)
spc(ant9)	Spectinomycin	Spectinomycin adenyltransferase	(Lelie`vre et al., 1999)
tetM	Tetracycline, minocycline	Protection of ribosome binding site for tetracycline	(Trzcinskia et al., 2000)
vanRSHAXYZ[a]	vancomycin	Production of low affinity pepdydoglican precursor with terminal D-Ala-D-Lac	(Weigel et al., 2003)
SCC*476* *far1*	Fusidic acid resistance		(Holden et al., 2004)
SCC*mercury*			
mer operon	mercury	Ion transport	(Chongtrakool et al., 2006)

[α] Vancomycin resistance is encoded on Tn*1546* transposon but transferred by conjugative plasmid

1.8 Contribution of MGE on antibiotic resistance and virulence in staphylococci

There are various mobile genetic elements associated with the spread of resistance and virulence in staphylococci. These include genomic islands, bacteriophages, pathogenicity islands, chromosomal cassettes, plasmids, insertion sequences and transposons (Baba et al., 2002, Gill et al., 2005, Holden et al., 2004, Ito et al., 2003, Lindsay and Holden, 2004). There is a huge concern regarding the antimicrobial treatment of different *S. aureus* infections, as the SCC*mec* GI encodes resistance to a wide variety of antibiotics.

Other important genomic islands frequently associated with *S. aureus* are

S. aureus pathogenicity islands (SaPIs). These islands vary in size between 14 to 27 kb and encode virulence factors and toxins in addition to the core mobilizing genes, the integrase and excisionase (U´beda et al., 2006). These SaPIs are usually transmissible via integration, excision and replication. In addition, they are capable of transducing and re-integration after induction by a prophage helper phage (Novick et al., 2010, Ubeda et al., 2009). Another interesting feature of the SaPIs is their capability of transduction between various species as well as genera thus contributing to the evolution of other organisms including *Listeria monocytogenes* (Chen and Novick, 2009).

The most significant encoding enterotoxins are B and C, and toxic shock syndrome toxin (TSS) (Baba et al., 2002, Holden et al., 2004, Lindsay and Holden, 2004). Bacteriophage (ϕSa1– ϕSa5) with their integrated copies also constitute mobile elements carrying, for example, Panton–Valentine leukocidine (PVL), enterotoxin A (sea), or exfoliative toxin A (Baba et al., 2002, Holden et al., 2004, Ito et al., 2003, Lindsay and Holden, 2004).

In addition to carrying the antibiotic resistance-encoding genes, genomic islands such as SCC*mec* have evolved, to include other additional virulence genes like the *psm-mec* gene that encodes a cytolytic peptide (Wang et al., 2008). This could provide a bridge connecting between the antibiotic resistance and virulence determinants in *S. aureus* (Queck et al., 2009). Interestingly, some studies also showed the contribution of MGE in spreading not only virulence and resistance encoding genes, but further included house-keeping genes and functional genes involved in metabolism and membrane transport (Chan et al., 2011).

1.9 Aims and objectives of this study

This project was focused on understanding how integrases and transposes are involved in excision and mobilisation of the GIs and transposons, respectively. Based on existing knowledge of the role of recombinases in integration and excision of MGEs, it was hypothesized that under particular conditions, integrases of two distinct types are capable of inducing excision of two GIs in *A. baumannii*. Similarly, transposases could possibly mobilize ISs and transposons in staphylococcal species. Since it has been shown by several studies that exposure to stressful environment or to certain substances like antibiotics and Mitomycin C can encourage the activation of these MGEs, it was deemed important to establish how these mechanisms may operate in significant nosocomial pathogens, as *A. baumannii and Staphyloccoci*. Through the elucidation of this regulation, this study could help to ascertain how these MDR strains maintain and evolve antibiotic resistance, and may possibly influence clinical guidelines in the treatment of such pathogens.

1. To survey a large group of *A. baumannii* clinical isolates for the presence of G08 and G62 integrases

   A diverse range of *A. baumannii* clinical isolates will be selected for screening of the presence of integrases. These strains will primarily consist of the laboratory strain collection, which were isolated from the local hospital University Hospital Leicester NH Trust, and also from the Sultan Qaboos University Hospital (Oman). The selection of these strains may have some bias towards a particular clonal complex as they were derived from the same hospital at the same time-point during an epidemic outbreak of *A. baumannii* in Muscat, Oman in 2013 and these specimens were obtained during screening of all admitted patients, However, the lab collection from Leicester are sourced from different geographical locations and times. The diversity of their clonal lineage will not be assessed in this study.

Integrase analysis will start with bioinformatics analysis addressing the phylogenetic relationship between various integrases of *A. baumannii* GIs. To expand the number of integrases analysed, these analyses will also be completed on *A. baumannii* strains that have available genome sequences on NCBI.

This will be followed by PCR screening for integrase genes, such as the integrase G08 and G62. Subsequently, the GI structures and their internal regions will be determined by PCR mapping and whole-genome sequencing.

2. To quantify integrase activity within *A. baumannii*

   Analysis of integrase activity will be addressed through the design and use of molecular tools such as cloning and plasmid construction that allow the artificial induction of integrase activity under experimental conditions. The activity will be compared in both *A. baumannii* and *E. coli* backgrounds, and will be compared with integrase activity without induction by means of qPCR Fitness costs will be approximated via growth curves.

3. To investigate the phenotypes of G08 and G62 in both wild type strains and their G08/G62 deletion mutants.

   Both of G08 and G62 harbour genes conferring resistance to a variety of heavy metals. This analysis is first starts with the description of allelic exchange tool which is used for the creation of the mutants. Phenotypic assays are done by susceptibility testing using two different methods, the broth microdilution and disc diffusion method.

4. To characterize IS-element mediated transposase mobilisation of resistance determinants, namely transposon mediated mupirocin resistance and triclosan resistance determinants in *S. epidermidis* clinical isolates.

These analyses will include mutation determining changes in IleS and FabI, mutations affecting the *ileS* and *fabI* promoter regions. The presence of the *ileS2* gene and how it impacts the phenotype of mupirocin susceptibility will be covered. The final part will include testing the excision and mobilisation of the Tnsha1 and TnSha2 elements using various molecular tools like real-time qPCR as well as cloning.

Chapter 2 Materials and methods

2.1 Bacterial strains

2.1.1 *Acinetobacter baumannii* isolates

The *A. baumannii* strains used in this study were obtained from two different sources. 100 strains were collected from clinical samples at Sultan Qaboos University Hospital (SQUH, Oman) between 2012 to 2013. These strains were collected from various body sites of patients admitted in the internal medical wards in SQUH. The rest of the strains were from the collection of Kumar Rajakumar Department of infection, immunity and inflammation, University of Leicester. In both cases the strains were stored at -80°C in brain heart infusion broth with 30 % glycerol. The strains were streaked out for single colony into Lysogeny broth (LB) agar plates and incubated at 37°C for 16 to 24 hrs, unless otherwise specified. Next day, single colonies from each strain were picked and sub-cultured in LB broth overnight at 37°C at 200rpm. The *A. baumannii* strains were routinely grown in media supplemented with 40 µg/ml kanamycin, 30 µg/ml chloramphenicol or 200 µg/ml gentamicin when required, unless otherwise specified. Bacterial strains used in this study are listed in table 2.1.

Table 2.1 List of *A. baumannii* and *E. coli* strains.

Strain	Relevant characteristics	Reference
Acinetobacter baumannii WT		
A424	Clinical isolate from Croatia	Lab 212 (University of Leicester)
AYE	Epidemic MDR type strain, France	(Fournier et al., 2006)
AB0057	MDR type strain	(Adams et al., 2008)
ATCC 17978		(Smith et al., 2007)
ATCC1960 6	Type strain	(Peleg et al., 2012)
ACICU	Clone of an outbreak from Rome	(Iacono et al., 2008)
KR3815		SQUH, Oman
KR3831		SQUH, Oman
KR4132		Lab 212 (University of Leicester)
Acinetobacter baumannii mutants		Lab 212 (University of Leicester)
A424 ΔG08	*G08::aacC1*	This study
ATCC1797 8 ΔG62	*G08::aacC1*	This study
Escherichia coli		
DH5α	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$^-$	Lab 212 (University of Leicester)
S17.1λ*pir*	*TpR SmR recA, thi, pro*, hsdR-M+RP4: 2-Tc:Mu: Km Tn7 *λpir*	(De Lorenzo and Timmis, 1994)
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1 λpir*	(Herrero et al., 1990)

2.1.2 *Staphylococcus* isolate

Staphylococcal species used in this thesis are *S. aureus, S. epidermidis and S. haemolyticus.* A collection of 25 *S. epidermidis* clinical isolates collected between November 2007 and February 2014, from blood cultures of patients admitted in the ICU of Aberdeen Royal Infirmary (Aberdeen, UK). The strains were collected by Felicity Abbott and Ian Gould (Department of Medical Microbiology, Aberdeen Royal Infirmary, Aberdeen, UK) and initial analysis was performed by Karolin Hijazi (School of Medicine and Dentistry, University of Aberdeen, Aberdeen,

UK). This analysis included DNA preparation for genome sequencing, PCR for *qacAB* and MIC determination for chlorhexidine and ethidium bromide (Hijazi et al., 2016). *S. aureus* and *S. haemolyticus* species used in this work, comprising the isolates listed in table 2.2 are from a world-wide collection of Quotient Bioresearch transferred in the past to the Department of Genetics of the University of Leicester as part of a separate project (Ciusa et al., 2012, Furi et al., 2016).

Staphylococcal species were routinely recovered from the frozen cryotubes and streaked on Trypticase soy agar (TSA) or LB agar and incubated at 37°C for 16 to 24 hrs, unless otherwise specified. Next day, single colonies were picked and sub-cultured in TSB or LB overnight at 37°C at 200 x *g*.

2.2 Whole Genome Sequencing of *S. epidermidis* isolates

Genomic DNA was quantified on a Qubit Fluorimeter (Thermo Fisher Scientific, Waltham, MO) and quality assessed on a Tapestation (Agilent Technologies, Santa Clara, CA) with genomic DNA screentapes (DIN 8.4 – 9.7). Dual indexed TruSeq libraries were prepared from 200ng gDNA using the TruSeq Nano DNA library preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions using a Bioruptor Pico (Diagenode, Seraing, Belgium) for fragmentation to 550bp. Libraries were quantified by qPCR, pooled at equimolar concentrations and 14pM of the pool was sequenced on a MiSeq using version 3 chemistry and 300bp paired end reads (Illumina, San Diego, CA), with 29.3M pass filter reads generated. Library construction and genome sequencing was performed by the Centre for Genome-Enabled Biology and Medicine of the University of Aberdeen)

Table 2.2 List of *Staphylococcus* strains.

Species and strain name	comments	Reference
Staphylococcus aureus		
PB4-11	PVL positive	Julie Morrissey
RN4220		Lab 121 (UoL)
1222		Lab 121 (UoL)
Staphylococcus haemolyticus		
CN1197		Lab 121 (UoL)
Staphylococcus epidermidis		
STAPH 54	Clinical isolate	Hijazi et al 2016
STAPH 56	Clinical isolate	Hijazi et al 2016
STAPH 58	Clinical isolate	Hijazi et al 2016
STAPH 59	Clinical isolate	Hijazi et al 2016
STAPH 60	Clinical isolate	Hijazi et al 2016
STAPH 61	Clinical isolate	Hijazi et al 2016
STAPH 62	Clinical isolate	Hijazi et al 2016
STAPH 63	Clinical isolate	Hijazi et al 2016
STAPH 64	Clinical isolate	Hijazi et al 2016
STAPH 66	Clinical isolate	Hijazi et al 2016
STAPH 67	Clinical isolate	Hijazi et al 2016
STAPH 68	Clinical isolate	Hijazi et al 2016
STAPH 69	Clinical isolate	Hijazi et al 2016
STAPH 70	Clinical isolate	Hijazi et al 2016
STAPH 73	Clinical isolate	Hijazi et al 2016
STAPH 74	Clinical isolate	Hijazi et al 2016
STAPH 75	Clinical isolate	Hijazi et al 2016
STAPH 77	Clinical isolate	Hijazi et al 2016
STAPH 78	Clinical isolate	Hijazi et al 2016
STAPH 79	Clinical isolate	Hijazi et al 2016
STAPH 83	Clinical isolate	Hijazi et al 2016

2.3 Sequence analyses

The 25 *S. epidermidis* clinical isolates were sequenced by whole genome sequencing (WGS). The data files of the sequences were analysed using SPECTRE (special Computational Teaching and Research Environment) as follow. Sequences were trimmed in Trimmomatic which is a multithreaded command line tool used to trim and crop Illumina FASTQ data (Bolger et al., 2014). The trimmed, paired-end Illumina data were next assembled using the SPades module (St. Petersburg genome assembler) (Nurk et al., 2013). The data was then run in QUAST to investigate and assess the quality of the assemblies produced by Spades (Gurevich et al., 2013). The contig files generated from the latter step were uploaded in the Centre of Genomic Epidemiology server (http://cge.cbs.dtu.dk/services/MLST) for Multi-locus Sequence Typing (MLST) analysis.

2.4 Genomic DNA extraction

2.4.1 Kit-based DNA extraction

2ml overnight cultures of the bacterial strains were inoculated by picking single colonies and incubated for ~16-18 hours at 37°C with vigorous shaking at 200 x*g*. The genomic DNA extraction was performed following the protocol of the 5 PRIME Archive Pure DNA Purification Kit (VWR) for gram negative bacterial culture. Plasmid DNA extraction was performed using GenElute Plasmid MiniPrep Kit (Sigma-Aldrich). All kits were used according to manufacturer's instructions.

2.4.2 Manual extraction by phenol-chloroform method

Phenol-chloroform DNA extraction was performed according to protocols obtained from (Green and Sambrook, 2017). Briefly, a single colony of the required strain was inoculated in 5 ml of LB and incubated overnight at 37°C at 200 x *g* shaking incubator. 1.5ml of culture was then centrifuged for 5 minutes at 13,000 x *g* and the supernatant was removed. The pellet

was re-suspended in buffer1 (20% sucrose, 1M pH8 Tris-HCl, 0.5M pH8 EDTA) and 100µg/ml of lysostaphin. The mixture was incubated at 37°C until the cells have lysed forming a viscous solution. Then 20 mg/ml proteinase K and 10% SDS was added and incubated at 37°C for 25 minutes. 5M NaCl and 24:1 chloroform/isoamyl alcohol was added and incubated at room temperature with frequent inversions for and centrifuged at 4500 x *g* for 10 minutes. The aqueous phase was transferred to a new Eppendorf tube. Equal volume of isopropanol was added to precipitate DNA and centrifuged at maximum speed (≥13000 x *g*) for 10 minutes. The supernatant was then removed and then washed with 70% ethanol and centrifuged at maximum speed for 10 minutes. The supernatant was removed and the pellet was left to air-dry. The pellet was re-suspended in 50-100 µl of nano-pure H_2O.

2.4.3 Manual Alkaline lysis method

This method is usually used to extract plasmids or small sized circular DNA elements (Feliciello and Chinali, 1993). 5 ml culture of LB with a single bacterial colony was inoculated and incubated in 37°C shaker overnight. The culture was next transferred into 1.5 ml Eppendorf tube and microfuged for 1 minute. The supernatant was removed and another aliquot of culture was added to the tube. The latter step was repeated until the entire 5 ml culture was spun down in one tube. The pellet was re-suspended in 100 µl of solution I containing 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 50 mM glucose and dH_2O. 20 µl of 10 mg/ml lysozyme solution was added containing 0.01 g lysozyme and 0.250 M Tris pH 8.0 and the mixture was incubated at room temperature for 2 minutes. 200 µl of Solution II iwas added to the tube which constitutes of: 50 µl 20% SDS, 20 µl 10 N NaOH, 930 µl dH_2O, mixed well and placed on ice for 5 minutes. 150 µl of Solution III was added containing 5 M KOAc, glacial acetic acid (to pH5.5) and dH_2O (approximately 12 ml, 2.3 ml and 5.7 ml). The mixture was vortexed gently to form small white clumps and placed in ice for 5 minutes. The above mixture was then microfuged for 5 minutes in cold microfuge. The supernatant was transferred to new tube. 400 µl of

phenol: chloroform was added and vortexed and microfuged for 2 minutes. The aqueous phase was transferred to a new tube. 1 ml of ethanol was added to the aqueous layer and mixed well and incubated at room temperature for 2 minutes. The tubes were then microfuged for 5 minutes in cold microfuge. The ethanol was poured off and the pellet was left to dry completely. The pellet was re-suspended in 50 µl of TE/ RNase (20 µg/ml) and incubated at 37°C for 30 minutes. The gDNA was used directly or stored at -20°C for future experiments.

2.4.4 Ethanol precipitation

Ethanol precipitation is often used to purify and concentrate the DNA before being used for transformation or cloning experiments. 1 volume of DNA sample was re-suspended in 2.5 volumes of 100% ethanol, 0.1volume of 3M sodium acetate (pH 5.6). The mixture was kept on ice for 10 minutes and then centrifuged at 13,000 x *g* for 8 minutes. The supernatant was discarded and the pellet was re-suspended in ice-cold 70% ethanol and centrifuged for 2 minutes. Ethanol was then discarded and the samples were dried by vacuum pump to evaporate any remaining ethanol. The pellet was eluted in nano-pure dH_2O and stored at -20°C.

2.5 Gel electrophoresis, amplicon purification and DNA sequencing

2.5.1 Gel electrophoresis

Gel electrophoresis was routinely performed for visualizing DNA fragments using 0.8% agarose gels containing 1× TAE buffer and 0.5 µg/ml ethidium bromide. DNA marker used is Hyperladder or GeneRuler^{TM} (Fermentas) to estimate the size of DNA fragments. PCR amplified fragments were gel extracted and purified using a PCR DNA purification kit (Geneflow).

2.5.2 DNA sequencing

Sequencing used in this work was performed according to the sequencing facility used. For Sanger sequencing (The Protein Nucleic Acid Chemistry Laboratory PNACL, University of Leicester) and illumina sequencing at microbesNG (https://microbesng.uk, University of Birmingham), protocols provided by the respective facility was followed where genomic DNA was prepared in the lab as per the above mentioned protocols and then sent for sequencing. The sequences are then retrieved from the websites and analysed accordingly.

2.6 Oligonucleotides design and synthesis

The primers used in this study were designed using the Primer3 Plus tool (Untergasser et al., 2012), and checked for secondary structures and the primer annealing temperature using the Oligo Calc software (Kibbe, 2007). The designed primers was further assessed by *in-silico* PCR amplification software (http://insilico.ehu.es/PCR/) (Bikandi et al., 2004). The primers were synthesized by Sigma-Aldrich, dissolved in nanopure H_2O to 10 pmol/µL and stored at -20°C. The primers used in this study are listed in tables 2.7 and 2.8.

2.7 Polymerase chain reaction (PCR)

2.7.1 Conventional PCR

The PCR assays in this study were carried out using *Go-taq* DNA polymerase (Promega Ltd.). Long range PCR products were amplified using Phusion High-Fidelity DNA Polymerase (Thermo-Scientific). KOD Hot Start II DNA polymerase (Novagen) was used when high fidelity amplification was required for cloning purposes. All the enzymes were used according to the manufacturer's protocols. The PCR cycling conditions were adjusted depending on the amplicon size and the primer's melting temperature. The cycling conditions for each enzyme are represented in table 2.3.

Table 2.3 PCR Cycling conditions for *Gotaq* and KOD polymerases

Step	temperature	Time
Initial Denaturation	95°C	2 min
Denaturation	95°C	30 s
Annealing	(Lowest Tm)°C	30 s
Extension	72°C -72°C	10 s 1 min / kb
Hold	4°C	

2.7.2 Colony PCR

A single colony was picked from a plate and re-suspended in 30 µl of nanopure H_2O, and then heated for 10 minutes at 100 °C in a heat block. Cells were then pelleted at 16000 × *g* for 1 minute and 1 µl of the supernatant was used as template for PCR. The preparation of the PCR mastermix was as per manufacturer's instructions similar to that of the genomic DNA PCR except that Dimethyl sulphoxide (DMSO) is added for the colony PCR mastermix to facilitate the annealing of the primers to the template, therefore it enhances the amplification. Colony PCR for staphylococci was carried out similarly with the addition of lysostaphin to facilitate cell lysis.

2.7.3 Splicing by overlap extension-PCR (SOE-PCR)

This PCR was used to create hybrid DNA fragment for mutant allele construction required for allelic exchange mediated recombination (Heckman and Pease, 2007). The upstream flank (UF) and downstream flank (DF) of the region intended for deletion of about 500 bp in size were amplified with an antibiotic cassette in between (Figure 2.1). Thermocycling conditions for the SOE-PCR is summarised in table 2.4.

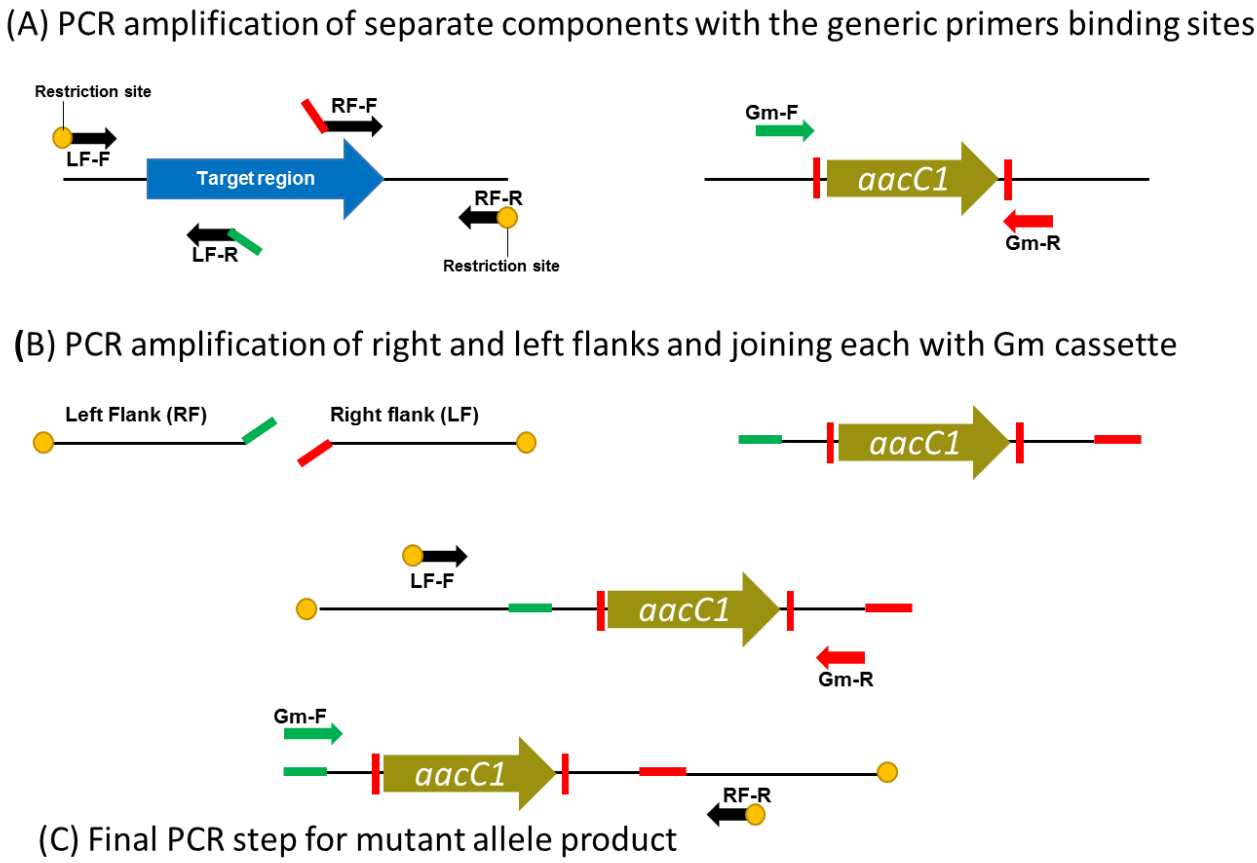


Figure 2.1 SOE-PCR consists of three sequential steps.

In **(A)** fragments corresponding to the left flank (LF) and right flank (RF) (represented as a black line) of the conserved region targeted for allelic exchange and a selectable antibiotic resistance cassette (as olive filled arrows), in this case *aacC1* conferring resistance to gentamicin are amplified by PCR separately. The green line and red line on internal primers RF-F and LF-R correspond to 5' overlaps where sequence corresponds to the reverse complement of Gm-F and Gm-R, respectively. The target region is shown as blue filled arrow. Restriction sites are shown as yellow filled circles **(B)** the LF and RF fragments are spliced to the *aacC1* cassette using SOE-PCR to create the LF-Gm and RF-Gm cassettes. In **(C)** the final SOE-PCR assembly takes place by splicing LF-Gm and RF-Gm to produce the desired mutant allele.

Table 2.4 Thermocycling conditions for SOE-PCR

Steps	Temperature	Number of cycles	Duration
Polymerase activation	95°C	1	2 min
Initial thermocycles without primers			
Denaturation	95°C	14	20 s
Anealing	2°C below the lowest Tm of overlapping region		30 s
Extension	70°C		10-25 s/kb
Thermocycles after adding primers			
Initial denaturation	95°C	1	20 s
Denaturation	95°C	20-40	20 s
Annealing	2°C below the lowest Tm of primers		30 s
Extension	70°C		10-25 s/kb
Final extension	70°C	1	5 min
Final hold	15°C	1	

2.7.4 tRIP-PCR

The *tR*IP PCR stands for *tRNA* interrogation of pathogenicity islands (He et al., 2007). This approach investigated the contents and contexts of bacterial *tRNA* and *tmRNA* genes, which are known insertion hotspots for genomic islands (GIs). In this study, 2 kb upstream and downstream regions flanking the locus 8 were extracted. These core chromosomal sequences immediately flanking the direct repeats (DR) in *A. baumannii* genomes investigated were shown to be highly conserved in almost all the strains examined. Primer pairs 1F_08 and 1R_08, and 2F_08 and 2F_08 were designed to amplify the internal regions within upstream flank and downstream flank, respectively as shown in figure 2.1. Similar approach was used for the flanks of G62 using the primer pairs 1F_62 and 1R_62 for the UF, and 2F_62 and 2F_62 for the DF.

2.8 Genetic manipulation

2.8.1 Construction of plasmids

Details for the plasmid construction will be discussed in chapter 3.

2.8.2 Preparation and transformation of electro-competent bacteria

2.8.2.1 *Escherichia coli* and *A. baumannii* electro-competent cells

A single colony was inoculated in 5 ml of LB, and grown overnight at 37^{o}C at shaking incubator at 200 x *g* for 16 hrous. The culture was used to inoculate fresh LB at a ratio of 1:100 which was grown to an OD_{600nm} of 0.4 – 0.5. The cells were placed on ice for cooling to 4°C for 20 minutes. The cells were then harvested by centrifugation (3000 × *g*, 15 minutes, 4^{o}C) and gently washed three times with smaller volumes of ice cold 10 % (v/v) glycerol. After the final wash, the cells were resuspended in a volume of ice cold 10 % (v/v) glycerol equal to 1/100th of the initial culture volume and stored as 50 µl aliquots at -80^{o}C.

Transformation by electroporation was completed using 0.2 cm cuvettes in the Bio-Rad Gene Pulser system (Bio-Rad) at the following settings: 25 kV/cm, 25 µF and 200 Ω. 950 µl of SOC (Section 2.16) was added immediately after transformation, and cells were incubated at 37^{o}C for 1 hour. Transformed cells were then plated onto LB agar containing appropriate antibiotics and incubated at 37^{o}C, unless otherwise specified. This method was employed both for the preparation of electrocompetent *E. coli* and *A. baumannii.*

2.8.2.2 *Staphylococcus* species electro-competent cells

Staphylococcus species including *S. aureus, S. epidermidis* and *S. haemolyticus* strains were prepared using the same protocol. Strains required are taken from frozen stock at -80°C streaked on Trypticase Soy Agar (TSA) from (Difco) and incubated at 37°C for 24 hrs. Next day, single colonies were picked from each strain and inoculated in 5-10 ml of

Tryticase Soy broth (TSB) from (Difco) and incubated overnight at 37°C at 200 x *g* shaking incubator. The overnight liquid cultures were diluted 1/50 in fresh TSB, grown in a shaking incubator at 37°C until OD_{600nm} = 0.4 was reached. The cultures were centrifuged down at 4°C at 4000 x *g* for 10 minutes. The supernatant was discarded and the pellet was re-suspended in equal volume of 0.5M Sucrose and centrifuged at 4°C at 4000 x *g* for 10minutes. The supernatant was discarded and re-suspended in ½ volume of 0.5 M Sucrose the suspension was then kept on ice for 15-30 minutes. The cultures were centrifuged at 4°C at 4000 x *g* for 10minutes and re-suspended in 0.1 volume of 0.5M Sucrose. The electro-competent cells were either used directly or 50 µl were aliquoted into Eppendorf tubes and stored in -80°C for future use. The cells can be saved for up to three months with high efficiency.

2.8.3 Transformation by electroporation of *S. aureus*

The DNA (PCR product or plasmid) was filtered by dialysis membrane for 30 minutes. 5-30 µl of DNA (depending on the DNA concentration) was mixed with 50 µl of electro-competent cells. The mixture was electroporated at 25 µF, 2.5 V and 200 Ω (Time constant ~4.8) and 900 µl SMMP50 media was immediately added to recover the cells and placed in universal tubes in shaking incubator 200 x *g* at 37°C for 2 hours. The culture was centrifuged at 3,000 x *g* for 5 minutes and 800 µl of the broth was discarded. 100 µl was plated out onto TSA plates containing selection drug and control plates were incubated accordingly at 37°C.

2.8.4 Restriction enzyme digestion of DNA

Restriction digestion by enzymes (Promega, NEB and Roche) were carried out according to manufacturer's instructions. The reaction volume was routinely between 10 – 20 µl and was adjusted according to the total mass of DNA and length of time for digestion. The enzymes were inactivated by heat at 95 °C for five minutes followed by cooling on ice. The reaction was incubated at 37 °C for one to three hours and cleaned for further use.

2.8.5 Dephosphorylation of DNA

Plasmid DNA linearized by one restriction enzyme is prone to self- ligation in subsequent ligation reactions. This can be prevented by adding alkaline phosphatase which removes the 5' phosphate groups from DNA. Thermosensitive alkaline phosphatase (Antarctic phosphatase by New England Biolabs) was added to plasmid restriction digests at 1 U/µg DNA. When the reaction was complete, TSAP was inactivated at 70°C for 15 minutes.

2.8.6 Ligation of DNA fragments

Ligation reactions were performed in a final volume of 10 – 20 µl containing 3 U of T4 DNA ligase (Promega) and 1 – 2 µl of 10 × ligation buffer. The reaction was incubated for 60 minutes and then overnight at 4°C or 15°C, and subsequently transformed into *E. coli*.

2.8.7 Suicide vector-based allelic exchange

Allelic exchange method is a conjugation based method to introduce a suicide vectors containing a mutated allele sequence into a target bacterial species (*A. baumannii* in this case) as illustrated in figure 4.3. This method has been employed to support deletion of large sequences or sequences spanning multiple operons (Reyrat et al., 1998). Other methods include lambda red recombination systems (utilising a lambda red phage to rescue linear DNA fragments) Datsenko and Wanner system 2000 (Datsenko and Wanner, 2000) and Anthony Poteete system (Poteete and Fenton, 2000). Allelic exchange method utilises competent *E. coli* cells to introduce the constructed suicide vector to the target *A. baumannii* by conjugation (thus increasing efficiency). After conjugation, a single cross over event occurred where the vector was inserted in the genome alongside the target sequence. Or a much less commonly, a double cross over event, where allelic exchange will take place and the mutated sequence replaced the target sequence. Counter selection was utilized at this point to select for the double cross over event.

An antibiotic marker was inserted into the target locus to be replaced by allelic exchange using a conjugative suicide plasmid bearing the resistance marker gene flanked by locus-specific targeting sequences. The suicide vector is a plasmid which is unable to replicate in the strain targeted for mutagenesis. The latter composite DNA fragment was synthesized by Splice Overlap Extension PCR (SOE-PCR) (Figure 2.1). The upstream and downstream flanks of the desired locus were amplified from their respective strains and an intervening FRT-flanked antibiotic resistance marker was separately amplified by PCR. After amplifying each flank and the cassette, one-step SOE-PCR was carried out to join the upstream and the downstream flanks with antibiotic cassette, following the PCR conditions in table 2.4. The final SOE product was gel purified and quantified by nanodrop machine. The constructed allele as well as the suicide vector of choice (in this study pJTOOL-3) were both digested with the respective restriction enzyme designed at each flank. After confirming the digestion by PCR, the digested products were ligated using T4 ligase at 4°C for 3 hours.

2.8.8 Transformation

The overnight ligation reaction was used to transform an aliquot of electro-competent bacteria. Firstly, *E. coli* CC118λpir strain was used as it is more competent and frequently used as a host for replication as it increases the copy number of plasmids (Herrero et al., 1990). Secondly, the resultant transformation product was electroporated into *E. coli* SI17λpir which is less competent but it's a conjugative strain used in the following step. The electroporated mixture was immediately recovered with super optimal broth with catabolite repression (SOC) and re-incubated at 37°C shaking incubator for 1 hour. The mixture was finally plated on chloramphenicol (30 µg/ml) and gentamicin (25 µg/ml) plates. Controls were run alongside the transformation reaction with uncut plasmid (pJTOOL-3). Control competent cells were also electroporated without any ligation mixture to exclude any contamination as well as to test the ability of competent cells to recover form electroporation process.

The colonies of uncut pJTOOL-3 of both competent cells grew on chloramphenicol (30 µg/ml) but not on gentamicin (25 µg/ml) plates. The ligation reaction with the suicide vector yielded colonies on gentamicin but no colonies on chloramphenicol plates (30 µg/ml). Colony PCR of the colonies growing on the gentamicin plates was performed. After successful confirmation of the colonies carrying the correct suicide vector (growing on both chloramphenicol (30 µg/ml) and gentamicin (25 µg/ml), a verification steps was carried out as follows: plasmid minipreps were performed to check for the correct suicide vector. Colony PCRs were done to check for the selection marker (*sacB*), gentamicin cassette and Upstream flanks using F1 and R3 (GmR), downstream flanks using F3 (GmF) and R2 and the entire SOE product using F1 and R2. The confirmed plasmid from *E. coli* CC118ʎpir was prepared according to kit's instruction and then transformed into *E. coli* SI17ʎpir. Similarly, *E. coli* SI17ʎpir were subjected through the same confirmation steps as before. The conjugation donor SI17ʎpir were ready for the conjugation process.

2.8.9 Conjugation

The *E. coli* SI17ʎpir with the new construct transformed with the suicide vector was grown in 5 ml LB broth with gentamicin (25µg/ml) in order to preserve the plasmid overnight at 37°C. The recipient *A. baumannii* strain A424 or ATCC 17978 strain was grown overnight at 37°C. 1:50 or 1:100 was taken from the previous two cultures and re-inoculated in 5 ml of fresh LB broth to OD_{600nm} 0.6-0.8. 1 ml from each of the previous two cultures were mixed, pelleted and re-suspended in 20 µl of $MgSO_4$. The mixture was then transferred into nitrocellulose filter papers and left overnight on LB agar.

2.9 Artificial induction methods

2.9.1 IPTG induction

5 ml overnight culture of *A. baumannii or E. coli* was diluted at 1:100 into fresh LB and then incubated at 37°C in the shaking incubator at 200 x*g*, until it reached OD_{600nm} = 0.2. IPTG was then added to induce the strains

to a concentration of 1.0 mM, and the cultures were then incubated, with 500 µl of the culture removed at time points 4, 8 and 24 hours for crude DNA preparation as described before. The supernatant was used as template for qPCR analysis.

2.9.2 Mitomycin C induction

5 ml of overnight cultures of *A. baumannii or S. aureus* strains were sub-cultured in 1:100 fresh Mueller-Hinton broth (MHB) and allowed to grow until mid-exponential phase (OD_{600nm} 0.6). Mitomycin C induction assay for the GIs excision was carried out as per a modified protocol for integrative elements excision (Farrugia et al., 2015). The bacterial sub-cultures were treated with sub-lethal concentrations of mitomycin C MIC (0.75×MIC) for 2 hours. Mitomycin C MIC of AYE, A424, KR3831 and ATCC 17978 was found to range from 32-64 µg/ml. Non-induced cultures were run alongside in each occasion under identical conditions. For the purposes of detection of circular forms from excised GIs by real-time PCR, 500 µl of each culture was taken and prepared for PCR as described previously (Yang et al., 2009). Briefly, cells were pelleted from 500 µl of the cultures by centrifugation at maximum speed for 2 minutes, and then washed twice with 1ml ultrapure water. The cells were then re-suspended in fresh 500 µl ultrapure water and then boiled by heating to 100°C for 2 minutes. The suspension was centrifuged at 13000 × *g* for 3 minutes to pellet the cells, and the supernatant was separated to be used as a template in qPCR reactions, and stored at -20°C for further analysis. qPCR analysis was completed as outlined in section 3.5; Chapter 3. Isolation of DNA using a manual derivative of the alkaline lysis method, conventionally used to isolate plasmid DNA, was required to enable detection of the excised GI through conventional PCR for some strains. This method is described under section 2.4.3 in this chapter.

2.10 RNA-related techniques and methods

2.10.1 RNA extraction

Following antibiotic induction, 5ml of cells were harvested by centrifugation for 5 min at 4000 x *g*, and resuspended in 1 ml RNALater (Invitrogen) and stored at 4°C. The total RNA was extracted the next day using an amended version of the Geneflow total RNA purification kit protocol (Norgen) as described below. The pellet was re-suspended in 200 µl TE buffer containing 1 mg/ml lysozyme and incubated at room temperature for 5 minutes with vortexing every two minutes. 300 µl RL buffer (Norgen; 1/10 volume of ß-mercaptoethanol was added to buffer RL before use) and the solutions were mixed well by vigorous vortexing for at least 10 seconds. 200 µl of 100 % ethanol was added and vortexed for 10 seconds and 600 µl of RNA was fixed to the binding spin column (Norgen) by centrifugation for 1 minute at 3500 x *g* in a benchtop microfuge and the flow-through discarded. DNA in the sample was digested by pipetting 80 µl buffer RDD containing 10 µl DNaseI stock solution (Qiagen) onto the column and incubating it at room temperature for 15 minutes. The DNA was washed off the membrane by adding 400 µl wash solution A and centrifuging the column for another 1 minute at 3500 × *g*. The sample was again washed by addition of 400 µl Wash Solution A (Norgen) onto the column and centrifugation for 1 minute at 3500 × *g*. Another 400 µl wash solution A was pipetted onto the column and it was centrifuged for 2 minutes at 3500 x *g*, the flow-through was discarded and it was centrifuged for 1 minute further at 9000 x *g*. The RNA was eluted from the column into a 1.5 ml microcentrifuge tube by addition of 30 µl RNase-free water and centrifugation for 1 minute at 9000 × *g*. RNA solutions were stored at -20°C until needed.

2.10.2 Reverse Transcription

Total RNA was quantified by spectrophotometry at A260 (Nanodrop 2000; Fisher ThermoScientific), and cDNA was created by taking 1 µg of RNA per each reverse transcription reaction which was 20 µl, and the

procedure was completed according to the High Capacity RNA-to-cDNA kit (applied Biosystems). In each case, a simultaneous reaction was set up without the reverse transcriptase enzyme to provide a negative control for gDNA contamination.

2.11 Quantitative PCR

2.11.1 Real-time PCR conditions and quantification standards

The standard curve method has been largely implemented in qPCR data analysis being a simple and reliable alternative in comparison to other methods (Larionov et al., 2005). The reference genes used in this study were the relatively stable (*gyrB* and 16S) in both *E. coli* and *A. baumannii*. Five-fold serial dilutions were used to obtain good standard curves for both reference genes as well as the target elements. All standards were run in minimum of triplicates to insure precision of the assays. The serial dilutions of the gDNA were plotted in logarithmic scale against the threshold cycles and the best fit line was drawn.

The real-time PCR reactions had a total volume of 20 µL containing 5 µL of template DNA, 10 µl of the SensiMixPlus SYBR Green mastermix (Bioline), 0.5 µL of each 15 µM primer (F-G8-exc and R-G8-circ) or (F-G62-exc) and (R-G62-circ) (Table 2.8). Since there was no positive control used, every run included a negative control without target DNA, and all reactions were performed in triplicate. The reactions were performed in an Applied Biosystems Prism model7500HT Sequence Detection System with the following settings: 40 cycles of 20 s at 95°C and 1 minute at 60°C. Determinations of cycle threshold (Ct), or the PCR cycle where fluorescence first occurred, were performed automatically by the Sequence Detection Systems software of the instrument (version 2.3; Applied Biosystems).

To generate standard curves, serial dilutions of crude DNA boilates of the strains carrying the plasmids were performed in molecular grade water in

a concentration range from 1 ng to 10 fg DNA µL-1. Each dilution (7×10^5 to 10^7 genome equivalents per 5 µL target in the reaction) was used for quantification standards. For quantification of *A. baumannii* in experimental samples, one aliquot of 1 ng/L standard DNA was serially diluted to create the standard curve in each real-time PCR run. Using this method, the logarithm of the standard DNA concentration series was plotted against the Ct values. The slope of the standard curve generated for each PCR run was used in the following equation to estimate the reaction efficiency:

$E = 10^{-1/\text{slope}} - 1$. R^2 value is an efficiency parameter, which was used in these experiments to determine how the x and y values correlate to one another. R^2 of >0.99 was considered as a good confidence for the correlation between the two values.

2.12 Growth curves

The tested strains were streaked for single colonies in LB agar and incubated at 37°C overnight. Next day, single colonies were picked from the plates and inoculated into a LB liquid broth and incubated at 37°C overnight in a shaking incubator at 200 x *g*. The overnight broth culture of the strains was diluted at 1:100 in broth medium. 200 µl aliquot of the prepared suspension were taken and added to 96 well polystyrene flat bottom plates (NUNC, Roskilde, Denmark). The 96-well plate was incubated in a shaking Varioskan® flash spectral scanning multimode reader with continuous shaking at 37°C for 24 hour measuring the absorbance at 600 nm every 10 minutes. (It should be noted that any compound needed for the growth curve should be added in the required concentration prior to adding the inoculum). The size of the initial inoculum was determined by plating the appropriate dilution of the bacterial suspension on agar media and counting the CFU the following day. Data obtained saved as excel sheet and analysed by graphPad Prism6 software. The mean value ± standard deviations (error bars) was calculated for three independent wells for each strain.

2.13 Bioinformatics

Various programmes have been used for sequence analysis in this study. All software programmes are listed in the table 2.5. The protein and nucleotide sequences of the G08 associated integrases were aligned using the CLUSTAL-Omega web-based tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). The protein sequences of the integrases were further examined with InterPro tool for the domains functions. An automated protein homology modelling server called Swiss Model was used to generate tertiary protein structures for the integrases (Biasini et al., 2014). Finally, the putative integrase proteins sequences were aligned separately against the Protein Data Bank (PDB) using Blast Protein (blastP). The protein sequences were uploaded to PDB website and checked for the similar reported proteins and matched for protein structures with minimum identity. The results of the above mentioned analysis are summarised in chapter 3 in section 3.3.2 (Figure 3.2). This method is normally followed by Structural Biology Labs to get structures for putative/ unknown proteins (Backer and Sali, 2001).

Table 2.5 List of bioinformatics tools used in this work

Tool	Function	reference
BLAST	Basic local alignment search tool	(Altschul et al., 1997)
Clustal Omega	Multiple sequence alignments	(Sievers et al., 2011)
Primer3 Plus	Primer design	(Untergasser et al., 2012)
Clone manager	Visualisation of DNA sequences	Clone-manager.software.informer.com
InSilico-PCR	Insilico check for PCR amplicon size	(Bikandi et al., 2004)
Oligocalc	Primer annealing temperatures	(Kibbe, 2007)
A-plasmid Editor (ApE)	Visualisation of DNA sequences	Biologylabs.utah.edu /Jorgensen/wayned/ape/
Artemis	visualization and analysis of high-throughput sequences	(Carver et al., 2011)
Easyfig	Multiple sequences alignment and visulaisation tool	(Sullivan et al., 2011)
CARD	Antibiotic resistance database	(McArthur et al., 2013)

2.13.1 CARD database for identification of resistance genes

Analysis of unannotated genomes for presence of putative antibiotic resistance genes was performed using the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013). The database was accessed online (https://card.mcmaster.ca/home). Resistance gene identifier (RGI) was used for the analysis by selecting perfect and strict hits only option (as a default). To use the database, a single FASTA file was generated for each sequenced strain and saving the file as .txt format. The nucleotide sequence of each resistance gene was then used to search for the location of the gene in each strain utilising BLASTn alignment tool.

2.13.2 Phylogenetic tree construction

The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Twelve integrases derived from GIs are named as G followed by a number indicating their location in order. Other integrase sequences obtained by BLAST search of the above genomes were indicated only by strain and accession numbers.

2.14 Heavy metal compound preparation

For testing of susceptibility to the heavy-metal salts, analytical-grade salts of $CdCl_2.H_2O$, $CoCl_2.6H_2O$, $NiSO_4.6H_2O$, and $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$ and $AsSO_3$ were used to prepare 1.0 M stock solutions, which were filter-sterilized and added to the medium at final concentrations of 1 mM. The concentrations were adjusted for the different assays. Distilled water was used to dissolve these metal compounds.

2.15 Susceptibility testing to antimicrobials

2.15.1 Broth microdilution method

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) to mupirocin (Sigma-Aldrich, Gillingham, Dorset, UK) was performed as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines using broth microdilution method (Barry et al., 1999). Briefly, starting inocula of 1×10^5 CFU/ml of all *S. epidermidis* strains was aliquoted in 96-well plates containing serial dilutions of mupirocin in the range 0.5-1024 µg/ml using Mueller-Hinton broth (Oxoid Ltd., Basingstoke, UK).

MICs of triclosan were determined by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines, except for the way triclosan was added to the cultures. Stock solutions of triclosan (Irgasan; Sigma, Steinheim, Germany) were prepared at 102,400 g/L in methanol. Due to the high hydrophobicity of triclosan, serial 16-fold diluted substocks in methanol where prepared from which to prepare subsets of three dilutions in the microtitre plate. This approach was necessary to avoid serial two-fold dilutions in microplates thus minimising the absorption of triclosan to the plastic and to decrease the chances of triclosan precipitating out of solution when triclosan in methanol was added to water in the media. MBCs were determined by sub-culturing 10 µL from each well without visible bacterial growth on Mueller–Hinton agar plates (Biotec, Grosseto, Italy). After 24 hours of incubation at 37°C, the dilution yielding three colonies or less was scored as the MBC, as described by the CLSI for starting inocula of 1×10^5 CFU/mL. No neutralisation step was included in the MBC assay as initial experiments verified that triclosan carry-over did not occur when 10 µL was inoculated onto agar.

2.15.2 Disc diffusion method

This method is a well-known approach otherwise called Kirby–Bauer antibiotic testing for determining antibiotic susceptibility. In this method, plates with the strains to be tested were prepared and streaked for single colonies. At least four morphologically similar colonies were touched with a sterile loop and transferred into a Mueller-Hinton broth that has been shown not to interfere with the test. The broth was incubated with shaking at 37°C until the visible turbidity was equal to or greater than the 0.5 McFarland standard (OD_{600nm} of approximately 0.5) and suspensions were used within 10 minutes. Using an aseptic technique, sterile swab was placed into the broth culture of a specific organism and then the excess liquid was gently removed by gently pressing or rotating the swab against the inside of the tube 90° and repeated three times. The plate was then allowed to dry for approximately 5 minutes. Plain sterile filter discs obtained from filter papers were loaded with 10 µl of 1M solutions of each metal compound and allowed to dry for 15 minutes. Filter discs were then firmly applied to the surface of the agar plate which had previously been dried. A distance of 20-35 mm between discs on each plate was kept to avoid overlapping between zones of inhibition. Plates were incubated within 15 minutes of disc application to prevent larger zone of inhibition. Then plates were incubated overnight at 37°C. After 18 hours in the incubator, the sensitivity of mutants and their parental strains to heavy metals was determined by measurement of the inhibition zones. At least three independent experiments were performed. Statistical significance was tested by the Student's t test (two-tailed, unpaired) and p values below 0.05 were considered significant.

2.16 Media preparation

Table 2.6 Media preparation and uses

Media	Use	Preparation
SMM buffer	*Staphylococcus* post-transformation and enhancement of cell wall recovery after electroporation	SMM buffer = 1 M sucrose, 0.04 M Maleic acid and 0.04 M $MgCl_2$ adjusted to pH media to 6.5 and autoclaved
SMMP50 broth	*Staphylococcus* post-	5.5 parts SMM buffer, 4 parts

	transformation and enhancement of cell wall recovery after electroporation	Pennesay (Difco antibiotic medium 3, BD) and 0.5 parts 10% BSA (filter sterilized). Store at 4˚C and filter sterilize before use
Lysogeny Broth (LB)	Standard liquid (LB) and solid growth (LA) medium for bacterial cultures	4 g of tryptone, 2 g of yeast extract and 2 g of NaCl dissolved in distilled water to a final volume of 400 ml
Brain Heart Infusion (BHI) broth and 30 % glycerol	Storage of bacterial stocks at -20 and -80°C	47 g of broth powder (Oxoid) was dissolved in a final volume of 1 l of distilled water containing 30 % (v/v) glycerol, autoclaved before use
Simmon's Citrate Agar (SCA)	Selection for *A. baumannii* during conjugation experiments	9.2 g of powder (Oxoid) was dissolved in a final volume of 400 ml distilled water, media autoclaved before use
Trypticase Soy broth (TSB)	For standard liquid growth of *Staphylococcus* species	30.4 g of powder (Difco) was dissolved in a final volume of 1 L of distilled water, media autoclaved before use
Mueller- Hinton broth (MHB)	For susceptibility assays by broth microdilution methods	22 g of powder (Oxoid) was dissolved in a final volume of 1 L of distilled water, media autoclaved before use
Super Optimal broth with Catabolite repression (SOC)	For non-selective outgrowth of bacteria post-transformation and enhancement of cell wall recovery after electroporation	5 g of tryptone, 2.5 g of yeast extract and 5 g of NaCl into 200 ml of distilled water. After autoclaving, 50 µl of 2 M $MgCl_2$ (filter sterilized) and 200 µl of 1M glucose (filter sterilized) were added to 1 ml of medium
Tris-EDTA (TE) buffer	Various genomic DNA extraction methods	10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0.
Tris-acetate-EDTA (TAE) buffer	Various genomic DNA extraction methods	2 M Tris-HCl 2 M Acetic acid, 50 mM EDTA
1 $MgSO_4$	Isotonic solution for washing the bacterial suspension	24.6 g $MgSO_4.7H_2O$ Distilled water to 100 ml
0.9% NaCl	Normal saline	9 g NaCl (154 mM final; 0.9% w/v) Distilled water to 1 litre Filter sterilize

Table 2.7 List of primers used for PCR mapping of G08 and G62

name	5'-3' sequence (Forward)	5'-3' sequence (reverse)	Reference
G08_map1	GTATCGCCCCAATCAGATGC	ATCAGTGTAGCCCCAATGATGGA	This study
G08_map2	TCCATCATTGGGGCTACACTGAT	ATCCATTTGAGATGAATACACTC	This study
G08_map3	GAGTGTATTCATCTCAAATG	GCTTATCTTTAAGGTCTGCC	This study
G08_map4	CGGAAGACAGGACGATAGGC	GCGACTATGGTTGTAGCTGC	This study
G08_map5	GTGTACTTGCCTTTCTTAAG	TGGTGATTATCTAAAGCAAG	This study
G62_map1	TGATGTTTCTATCGAATTAA	AACTTAATCTTTAAAGTTATT	This study
G62_map2	AATAACTTTAAAGATTAAGTT	TTCTCTTTCCATTCATCTCC	This study
G62_map3	AAAAGCTTATTGAGTATGGC	GTATTAAGACTTTTACTATC	This study
G62_map4	ATCCTACTTTTAAAGTTTTG	CATTAATCTTTAAAGTTATTG	This study
G62_map5	GATAGTAAAAGTCTTAATAC	AGTAGTCGATGAAATAGTTG	This study
G62_map6	GATATTAAGTGGGCTGAAGG	CGAATAAATATGTCGCCATG	This study
G62_map7	CGCGTTAATTCCTGCTATGG	AACAGGATGCCAAAGTGTTG	This study
G62_map8	GCTGTTTGCCGTTCATTTGC	TGGTAAGTTTGTTGCACCTC	This study
G62_map9	TGTTCTTGTCCTTTACTGTG	GAAGAGGGTAAATCTTCTCC	This study
G62_map10	TAACGGTCAAAGGTAACGTG	ATCAATTCCCACTACAGTTG	This study
G62_map11	TGTTGTAGGAATATTACTCG	ATGCATATTCTTTTTACACC	This study
G62_map12	AAATCGGAAGAACAGCCAGC	ATGAGAAGTCGGTAAGGGGC	This study
G62_map13	GAATGTTTTTTCCCGAGGTG	ATCTGCCCTTCTTCACATTC	This study

Table 2.8 List of primers

Name	Sequence (forward)	Sequence (Reverse)	comments	Reference
1_08	TGGATGGACCATGACTTGCC	GAAAGGGCAGCATTATACGG	Upstream flank of G08	This study
2_08	AGGCAGCCATTGTTGTATCC	CAGCAGACTCAAGCGCAATA	Downstream flank of G08	This study
1_62	ATTTGCGCCGCTTTGTAGTG	CGCGTGAATATGTCGTGCTG	Upstream flank of G62	This study
2_62	TAAGGCATTTGGCCGCATTG	GTTTCTGCCAAAAGCGCCAT	Downstream flank of G62	This study
int_08	CATCAATGAGGCGTAGCACC	ACATTTGGCTGAGCATGTTG	integrase gene in G08	This study
int_62	TTAAAACCACAAGACAAACC	CCATTGAAGCATAGAGCGTC	integrase gene in G62	This study
Bla_oxa_51	CTAATAATTGATCTACTCAAG	CCAGTGGATGGATGGATAGATTATC	*bla*-$_{oxa}$_51 like gene	Queenan et al 2007
alx_UG08	CTGCAGGTGATAGGCTTCACGGCCAA	TGGTACTTCAAGATCCCCAATTCGTAATAAAACCAT	UF of G08 with restriction site for allelic exchange	This study
Alx_DG08	GCTTTTGAAGCTAATTCGTAATGGCTGCATAAA	GTCGACTGCGGGCTATTTTTGGTAAGCTGG	DF of G08 with restriction site for allelic exchange	This study
Gm	CGAATTAGCTTCAAAAGCGCTCTGA	CGAATTGGGGATCTTGAAGTTCCT	Amplification of FRT-gentamicin-FRT cassette	Lab 212 (UOL)
lam_F8	CATGTACAACAAAGTGAGTACAACCTGTTGCT	AGCTGGCTCGGCATTAGGGATTTTTGC	SOE amplicon of UF+aacC1 cassette +DF of G08	This study
gyrB_Ab	CACCCTGAAGAAGGGGTTTC	AACACCTACGCCGTGTAAGC	*gyrB* gene for qPCR	This study
16S	ACTCCTACGGGNGGCNGCA	GTATTACCGCNNCTGCTGGCAC	16S gene for qPCR	This study
G08-exc	ATGTTTAGCTGCGTAAATCG	TTTGGTTGCTGGTGTGGACAA	G08 junction by qPCR	This study
G08-circ	TTCAGAATAAGCTGGATAGC	TAAGCGTTAGGTGTTGGGTC	G08 excision by qPCR	This study
G62-exc	AACAAATATGAACTAATTGC	TTGTTGAAGACTATTAATG	G62 junction by qPCR	This study
G62-circ	CAAGACAACCTGCTTTTACG	CGATATGGTTTGTCTTGTGG	G62 excision by qPCR	This study
G08-X-Seq	TTGTTGTTTCATTGCGAGCT	CCAAGTATTTATCTATGGCA	Sequencing primers for G08 circular forms	This study
G08-J-Seq	GGCAGCCTATATTCAAGTTG	GTTTGGCCCTGAATCGACAG	Sequencing primers for G08 junctions	This study
G62-X-Seq	GATCATTGTGCACTCCTTTG	CCAGATAACCGTATTTTATT	Sequencing primers for G62 circular forms	This study
G62-J-Seq	TAAAGATGGGCAACCTGTTTACT	GTAGCGGTGGTACTTCTCGG	Sequencing primers for G62 circular forms	This study
G08-int-clon	CGAGAGCTCAATAAATGTTAAAAGATACC	GTGTGTCGACTTAAGTGACCTCCTCATGC	Cloning of G08int	This study
G62_int_clon	AAAAGCTTATTGAGTATGGCAAAGCACCG	CTTTAACTGCATATGTAGGATGCCGACTG	Cloning of G62int	This study
A424-DCO	TCGCCATAACCTATGCTGGC	CAACAACAGAAGCGGCAGCC	DCO G08 mutant check	This study
17978-DCO	GTCACGATTCACGAAGATGA	GCTGCCTGACGCATAATCAC	DCO G62 mutant check	This study
AB57-int	ATGAAGTTGGGAGCGAATACGG	CCAAGATTCGGCACACCTTTC	Screening G08 int	This study
17978-int	CATCGATAA CGGTCTGGCATG	AAGTACCGGTGAAACGACAGC	Screening G62 int	This study
PR3136/PR3137	CGGGATCCAATGCAGGCAAGTTGATTCC	CGGGATCCCGTTCGGGTCTTTCATGTCT	Ori from pWH1266 for cloning into pWSK129	Lab 212
gyrBEc	GCTGCGGAATGTTGTTGGTA	AGAACAAAACGCCGATCCAC	qPCR *gyrB* gene in *E coli*	This study
ColE1	GGCGCTTTCTCATAGCTCAC	AGTCGTGTCTTACCGGGTTG	qPCR ColEl in *E coli*	This study
LF-RS-G08	CTGCAGGTGATAGGCTTCACGGCCAA	GTACTTCAAGATCCCCAATTCGTAATAAAACCATTG	LF G08 Mutant validation colony PCR	This study
RF-RS-G08	GCTTTTGAAGCTAATTCGTAATGGCTGCATAAA	GTCGACTGCGGGCTATTTTTGGTAAGCTGG	RF G08 Mutant validation colony PCR	This study
LF-RS-G62	CGAATTGGGGATCTTGAAGTACCTATTCCG	CGAATTAGCTTCAAAAGCGCTCTGAAGTTCC	LF G62 Mutant validation colony PCR	This study
RF-RS-G62	CATGTACAACAAAGTGAGTACAACCTGTTGCT	AGCTGGCTCGGCATTAGGGATTTTTGC	RF G62 Mutant validation colony PCR	This study
Gm-S	CATTAAACTGCTCATTCCAG	TAAGGCATTTGGCCGCATTG	aacC1 screening in SCO of allelic exchange	This study

G08-S	TTAAAACCACAAGACAAACCTCAGCCC	CCATTGAAGCATAGAGCGTCCCAGTG	SCO screening in allelic exchange of G08	This study
G62-S	TCACCGAGGACTCCTTCTTC	GGCGCAATACGTCTGATCTC	SCO screening in allelic exchange of G62	This study
LF-08	TGATGTTTCTATCGAATTAATGTCATG	AACTTAATCTTTAAAGTTATT GCTA	Cloning of UF in mini islands of G08 in pUC18	This study
RF-08	AATAACTTTAAAGATTAAGTTACTG	ATCCTCTTTTAAAGTTTTGACTA	Cloning of DF in mini islands of G08 in pUC18	This study
LF-08	AGTAGTCGATGATGAAATAGTTGATGC	CGCGTTAATTCCTGCTATGGTCC	Cloning of LF in mini islands of G62 in pUC18	This study
RF-62	AAAAGCTTATTGAGTATGGCACTGC	CCTTTAACTGCATATGTAGGCCGGTA	Cloning of RF in mini islands of G62 in pUC18	This study
F/RG08int	CATTAATCTTTAAAGTTATTGCATC	GATAGTAAAAAGTCTTAATACTGACT	Cloning of G08 integrase gene into pUC18	This study
F/RG62int	CGAGTCGACAATAAATGTTAAAAGATACC	GTGTGGATCCTTAAGTGACCTCCTCATGC	Cloning of G62 integrase gene into pUC18	This study
EXC8-F/R	TGTTCTTGTCCTTTACTGTG	GAAGAGGGTAAATCTTCTCC	Detection of circular forms of G08 from chromosome	This study
JUNC8-F/R	TAACGGTCAAAGGTAACGTG	ATCAATTCCACTACAGTTG	G08 target Junction from chromosome	This study
EXC62-F/R	TGTTGTAGGAATATTACTCG	ATGCATATTCTTTTTACACC	Detection of circular forms of G62 from chromosome	This study
JUNC62-F/R	AAATCGGAAGAACAGCCAGC	ATGAGAAGTCGGTAAGGGC	G62 target Junction from chromosome	This study
pWSK-EXC8	GGAAAAGGTTGAGGTCTTGC	CAGCGAAGTCGAGGCATT	Detection of circular forms of G08 from pWSK129 plasmids	This study
pWSK-JUNC8	CAAAATGGCTCCTCCGTTTA	GGCCAATTTTATTGCCTTGA	G08 target Junction from pWSK129 plasmids	This study
pWSK-EXC62	AATGTGACCTGCGTTGTGTC	TCAGAAAGAAGTTCCCCTTTGA	Detection of circular forms of G62 from pWSK129 plasmids	This study
pWSK-JUNC62	TCGTCAAATATTTTTGGTCTGC	CACCCTGAAGAAGGGGTTTC	G62 target Junction from pWSK129 plasmids	This study
pWSKori	GGCGCTTTCTCATAGCTCAC	AGTCGTGTCTTACCGGGTTG	Detection of ori for control pWSK129 plasmids	This study
PS-F/R	TTACCCAAGACTTCGCCTCT	CCCGATAACTCCATTCTTCG	Loss of pJTOOL-3 backbone	This study
2LF-08/2RR-08	AATTGGGGATCTTGAAGTTCCT	GATGGCGTAAATCGTGGTA	Flank1 *aacC1* in mutant alleles of G08	This study
1LF-08/1RR-08	CAACTTTAGCGATTTCTGG	CTTTAGCAAACATGACCTACC	Flank2 *aacC1* in mutant alleles of G08	This study
2LF-62/2RR-62	TACACCCGGGTTAATCGT	GCGCTTCAAAATCTGATGTA	Flank1 *aacC1* in mutant alleles of G62	This study
1LF-62/1RR-62	GCGTATATTTTGTTTCCATTC	GACCTTTCTTATCACAACGA	Flank2 *aacC1* in mutant alleles of G62	This study

Chapter 3 Assessment of integrase activity in *Acinetobacter baumannii*

3.1 Background

Genomic islands usually encode for many functional proteins for antimicrobial resistance and virulence as well as hypothetical proteins, most of which are of unknown function, thus could act like as reservoir for genetic diversity (Hsiao et al., 2005). Many GIs integrate within a transfer RNA or transfer-messenger RNA (tmRNA) encoding genes (Boyd et al., 2009, Williams, 2002). tmRNA is a small molecule of stable ribonucleic acid that is found in all bacteria usually involved in rescuing stalled ribosome (Shpanchenko et al., 2005). The use of tRNA and tmRNA as hot spots is not a feature to all GIs, as in other cases it has been established that GIs could use protein coding genes as integration hotspots (Ogier et al., 2010) like GMP synthases (Song et al., 2012) or *dusA/dusB* genes (Farrugia et al., 2015).

Previous comparative genomic analysis of *A. baumannii* explored the chromosomal loci of 63 GIs including the two GIs object of this study, G08 and G62, within seven strains belonging to different genotypes ST1, ST2, ST25, ST77 and ST78. The genomic alignment revealed GIs of various functions such as those encoding for surface components and transport systems as well as resistance to drugs and heavy metals. More recently, data of a pan-genome analysis of 50 *A. baumannii* isolates derived from military patients and 249 previously sequenced *A. baumannii* strains were compiled (Chan et al., 2015). The dataset in this study confirmed the diversity of gene pools found within the GIs identified and described it as an adaptative response of the *A. baumannii* strains to facilitate their survival in a nutrient-deficient environment. This was followed by bioinformatic analysis conducted on *A. baumannii* and *Pseudomonas* sp. genomes previously sequenced by several studies (Farrugia et al., 2013, Loper et al., 2012, Paulsen et al., 2005, Roy et al., 2010) which established for the first time that some GIs integrases could insert within the dihydrouridine sythetase A (*dusA*) gene, thus considering the relative integrase as belonging to a novel family of integrases (Farrugia et al., 2015). The *dusA* gene encodes the tRNA-dihydrouridine synthase A

enzyme, which catalyses the post-transcriptional reduction of uridine to dihydrouridine in tRNA. In that study, BLAST search revealed putative integrase in the genomes of more than 200 genomes of many bacterial species was identified at a single site within the 5′ end of *dusA*. The GIs from these Prtoeobacteria were highly diverse in terms of function and the only universal shared feature was the presence of the integrase gene, and thereby named as dusA-associated integrases (DAI). The majority of these DAI were predominantly present in the three Gammaproteobacterial genera *Acinetobacter*, *Escherichia* and *Pseudomonas* as shown by phylogenetic analysis. In each individual case, the 3' end of DAI was flanked by a gene cluster which typically indicates a horizontally transferred origin, such as the presence of different trinucleotide content, and that these clusters are not conserved in the relevant strains.

An essential aspect for the study of GIs is the assessment of integrase activity and its impact on the host cell. Screening for integrase genes associated with GIs is crucial and has been addressed in several studies (Hocquet et al., 2012). In addition, it has been well-established that various types of mobile genetic elements including GIs in *E. coli* as well as other Gram-negative bacteria are capable of spontaneous excision from the host chromosome and thus promoting the spread of antibiotic and heavy metal resistance, via horizontal gene transfer (Bille et al., 2005, Doublet et al., 2005). In most cases, however, the rate of excision from the chromosome occurs at a very low frequency so that it is not possible to detect the circular forms except by using a nested PCR approach both for integrons (Hocquet et al., 2012) and ICEs elements (Lin et al., 2008). Several attempts have been made to amplify spontaneously excised circular forms by conventional nested PCR from total gDNA extraction methods, however, was not always successful and in some cases prone to contamination (Hocquet et al., 2012). On the other hand, the excision of various MGEs like prophages and prophage remnants (Canchaya et al., 2002), integron cassettes (Guerin et al., 2009), and integrative and conjugative elements (ICEs) (Bellanger et al., 2007) in both Gram-positive and -negative bacteria was shown to occur at detectable frequencies after

induction of an SOS response typically using antimicrobial agents like mitomycin C which induce DNA damage or replication stress.

Alternatively, as addressed in this chapter, integrase gene can be cloned under the control of an inducible promoter in a low copy number plasmid. A wide variety of plasmids with inducible promoter that can induce gene expression by addition of a compound such as tetracycline (Blau and Rossi, 1999), arabinose (Guzman et al., 1995) and IPTG (Hansen et al., 1998). In this study, IPTG inducible promoter was utilised after cloning of mini-islands of the relative integrases of G08 and G62.

3.2 Aims and objectives

1. To analyse the integrases associated with *A. baumannii* genomic islands G08 and G62 in the context of other related integrase proteins present in a selected group of strains.
2. To analyse the integrase activity of Int-G08 and Int-G62 by construction of G08 and G62 mini-islands in IPTG-inducible integrase-expression plasmids in *E. coli*.
3. To generate shuttle vectors for the transfer of the constructs to an *A. baumannii* background.
4. To quantify the integrase activity under IPTG-induction by PCR assays in the original host.
5. To explore in the original host, the possibility of GIs excision and detection of circular intermediates after mitomycin C induction.
6. To detect the circular form by PCR and sequence of these amplicons to define the attachment *att* sites.
7. To conduct bioinformatic analyses of the integration/excision sites and build a predictive model for excision and integration.
8. To quantify the excision of the GIs in both *E. coli* and *A. baumannii* backgrounds by qPCR.

3.3 Results

3.3.1 Phylogenetic analysis of integrases of genomic islands in *A. baumannii* strains

A. baumannii GIs encode site specific recombinases of either the tyrosine recombinase class (phage integrases) or transposases. Analysis of the genomes of strains AB0057, AYE, A424 and ATCC17978 allowed to identify phage integrases for the genomic islands G08, G13, G22, G24, G50, G52, G59 and G61 and transposase for islands G04, G17, G25 and G29. Strains AB0057, AYE and ATCC 17978 harboured a total of 31, 28 and 26 GIs, respectively, but for many genomic islands present in these genomes no dedicated site specific recombinase could be identified. To visualise similarity of recombinases, a phylogenetic tree analysis was performed using the amino acid sequences of all the integrases in strains AB0057, AYE, A424 and ATCC 17978. The protein sequences were extracted from NCBI and summarised in table 3.1.

The sequenced genomes of the *A. baumannii* AB0057 and AYE strains assigned them to ST1 and strain ATCC 17978 to ST77. MLST analysis showed that A424 is a single locus variant of AYE, and as a result most of A424 integrases were found to be identical to AYE integrases, therefore these were excluded from the analysis. Transposase sequences have been also excluded from the analysis and interrupted or partial sequences have been “repaired” prior to the analysis. Additional three non-annotated integrase protein orthologues found by BLASTx search in these genomes were included in the final analysis.

In total, 21 integrase protein sequences were pulled-out and were aligned by CLUSTALW with a cut-off value of 30% and 500 bootstrap replicates were set, followed by tree construction using the MEGA6 software (Figure 3.1). The tree was inferred using the Neighbour-joining method (Saitou and Nie, 1987) and drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Table 3.1 Integrases and transoposases of *A. baumannii* GIs

GIs*	AYE**	AB0057	ATCC 17978	domains	size	features
G01						O-antigen biosynthesis
G02		AB57_0129		transposase	402	*vgr* proteins
G03						*cre* genes
G04	ABAYE3667	AB57_0245	A1S0210	transposase	236	resistance to drugs/metals
G05		AB57_0564		transposase	236	resistance to drugs/metals
G06						fatty acids biosynthesis
G07						*vgr* proteins
G08	ABAYE3221	AB57_0644		integrase/recombinase	382	resistance to drugs/metals
G09			A1S0661-2	phage integrase	400	*folP*, *umuDC*, T4SS genes
G10						not annotated
G11						
G12				phage integrase	351	CP11***
G13	ABAYE2904	AB57_0959		integrase	427	RM system, CP2
G14						heme oxygenase
G15						
G16	ABAYE2683			integrase	465	CP4
G17	ABAYE2649			transposase	148, 177	resistance to drugs/metals
G18						not annotated
G19						curli protein, vgr proteins
G20						sulfonate-sulfur metabolism
G21						tartrate metabolism
G22		AB57_1310	A1S1175	phage integrase	401, 374	CP1, CP5
G23						phenol metabolism
G24	ABAYE2495	AB57_1406		integrase/recombinase	308	CP8
G25	ABAYE2452			transposase		*vgr* proteins, naphtalene metabolism
G26						FHA
G27						*vgr* proteins
G28						
G29	ABAYE2350			transposase	189	
G30						chlorocatechol metabolism
G31			A1S1580	phage integrase	143	CP14
G32						multidrug transport system
G33						transport genes
G34						
G35						
G36						*fhuBCD* membrane permease
G37						naphtalene metabolism, toxin
G38						RM system, FHA
G39						
G40						
G41						*aphA*
G42			A1S 2040	phage integrase	349	CP1(ST78, abc) CP9 (abc)
G43						phenylpropionic metabolism
G44						type 1 fimberiae
G45						CP10
G46						salycilate metabolism
G47						fatty acid metabolism
G48						
G49						
G50	ABAYE1225			integrase/recombinase	339	CP6
G51						*rtc* genes
G52		AB57_2742		phage integrase	89	CP3
G53				phage integrase	398	CP12
G54						
G55						CRISP/cas system
G56						CP13
G57			A1S 2550	transposase	245	enterobactin synthesis
G58						
G59		AB57_3187		integrase/recombinase	414	CP3
G60						O-antigen biosynthesis
G61	ABAYE0532			integrase/recombinase	355	CP7
G62			A1S 2927	phage integrase	425	resistance to drugs/metals
G63				phage integrase	178	resistance to drugs/metals

cera et al., 2011); **locus tag of GenBank file; ***CP cryptic prophage

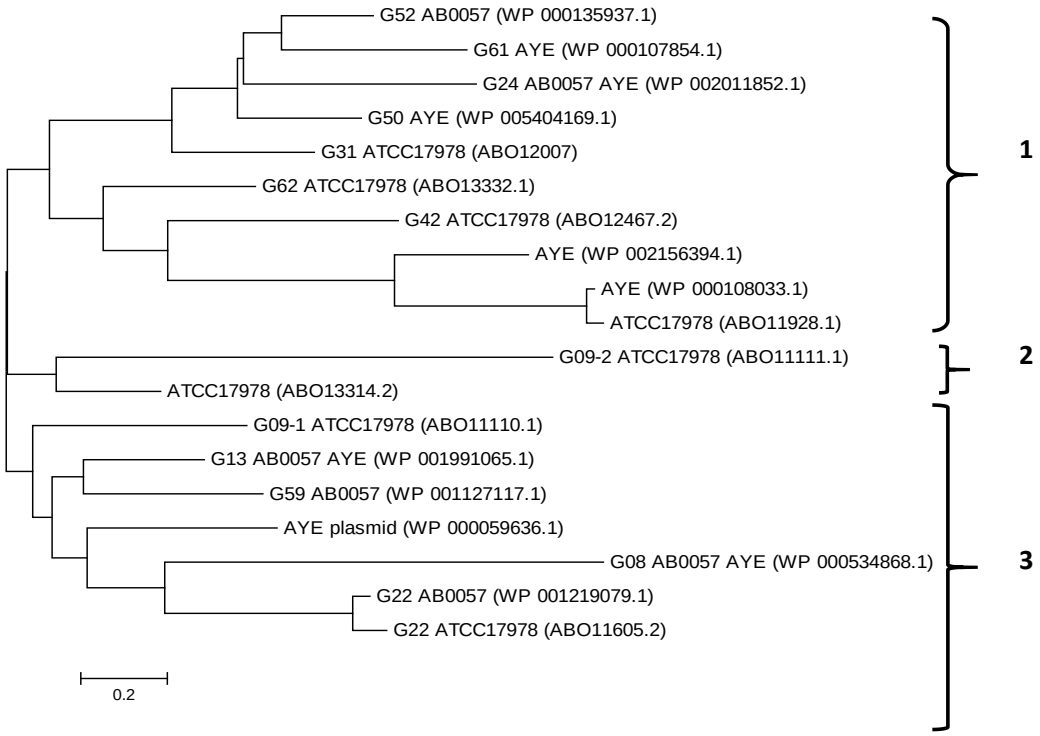


Figure 3.1 . Phylogenetic tree of tyrosine recombinases from strains ATCC17978, AB0057 and AYE.

The evolutionary relationship between phage integrase family proteins (NCBI Reference Sequence) detected in three strains of *A. baumannii* was inferred using the Neighbor-Joining method (Saitou and Nie, 1987). Integrases were labelled according to the genomic island number, strain name followed by Refseq accession numbers. Identical proteins in different genomes which yield identical Refseq numbers were shown only once. The three major branches are indicated by numbers on the right side of the figure.

The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 19 amino acid sequences. All positions containing gaps and missing data were eliminated automatically by the software. The protein sequences of all integrases included in this analysis are shown in appendix 1.

Overall, the outcome from the analysis showed three main branches of integrases. Within each branch, clustering of certain GIs into groups was noticed. A cluster of phage-type integrases belonging to GIs G09-2, G24, G31, G42, G50, G52, G61 and G62 derived from cryptic prophages (CPs) and GIs encoding for drug and metal resistance, while G59 and G22 phage integrases were in a different cluster as shown in 3.1. Interestingly,

the DAI of the G08 is phylogenetically distinct from the G62 phage integrase as shown in figure 3.1.

3.3.2 The integrases of the G08 and G62 genomic islands.

The integrases of two genomic islands, G08 and G62, linked to heavy metal resistance were analysed for their function. The sequences of the integrases associated with G08 in strains AYE (NC_010404.1) and AB0057 (NC_011586.2) were obtained from GenBank. The sequence of the G08 integrase of strain A424 was published (Kochar et al., 2012), and the sequence for strain 3831 was obtained by whole genome sequencing in this study. The G08 integrase genes (1,149 bp) and the respective proteins (383 aa) are 100% identical in the four strains AYE, AB0057, A424 and 3831 used in this study. The G62 gene and protein sequences (1,278 bp; 425 aa) were obtained for the deposited ATCC 17978 sequence (CP000521.1).

A web based functional analysis performed using Pfam (Bateman et al., 2004) and InterPro (Apweiler et al., 2003) showed that the G08 integrase proteins are phage integrases (Figure 3.2). As for the lambda integrase protein, the G08 integrase consists of two domains: The N-terminal domain which includes residues 1-64 is responsible for binding the arm-type sites of the *attP*, and the C-terminal domain (CTD), that binds the lower affinity core-type sites and contains the catalytic site. The CTD can be further divided into: (a) core-type binding domain (residues 65-169) and (b) catalytic core domain (residues 170-356) (Figure 3.2; A). The most likely tertiary structures was illustrated using Swiss model (Guex and Peitsch, 1997) and indicated that integrase proteins of AYE, AB0057 and A424 are identical. Therefore, for illustration purposes, the integrase protein structure of the AB0057 strain only is shown in figure (3.2; B). This analysis confirmed that the annotated ORFs were encoding for a genuine integrase.

Similarly, the analysis for G62 integrase revealed that it is a phage integrase consisting of two major domains: The N-terminal domain which

includes residues 1-89 and is responsible for binding the arm-type sites of the *attP*, and the C-terminal domain, that binds the lower affinity core-type sites and contains the catalytic site. The CTD can be further divided into: (a) core-type binding domain (residues 100-210) and (b) catalytic core domain (residues 211-418).

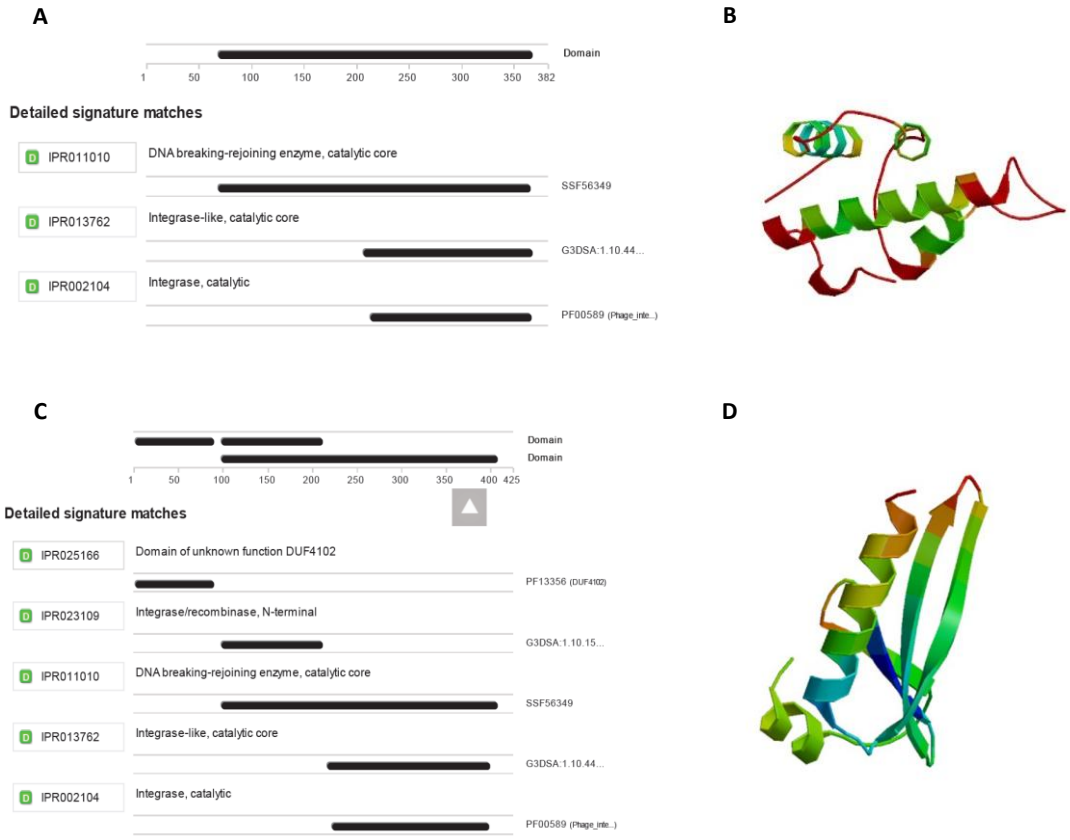


Figure 3.2 Analysis of the G08 and G62 integrase proteins.

(A) and (C) The matches between the integrase protein of G08 of strain AB0057 (accession WP_00534868.1) and G62 of strain ATCC 17978 integrase protein (accession ABO13332.1), respectively, were examined against the Protein Data Base (PDB). The analysis identified three sub-domains; the catalytic core of the DNA breaking-rejoining enzyme, the integrase catalytic core and the integrase. (B) and (D) The tertiary protein structures of the integrases generated by Swiss Model software for the G08-int of AB0057 and G62-int of ATCC 17978, respectively.

3.3.3 Survey of G08 and G62 in clinical *A. baumannii* isolates

Comparative genomic analysis studies have increased the number of whole genomes sequenced in the public databases and have facilitated

retrospective search for MGE including GIs (Turton et al., 2006). Many of these studies have focused on the epidemiology of certain GIs and thus better understanding of the spread of these elements (Hsiao et al., 2005). Likely wise, in this study, screening of G08 and G62 was performed to explore the prevalence of these elements in various clinical as well as laboratory isolates collected at different time points. In this study, screening of the two GIs by PCR included 175 *A. baumannii* in total, 100 of which were clinical isolates obtained from Sultan Qaboos University Hospital, Oman collected between 2011-2013 as part of routine swab screening. Another set of samples included screening of 45 lab strains (Dr Kumar Rajakumar, Department of Infection, immunity and inflammation, University of Leicester). All isolates were screened for G08 and G62 using primers mapping of conserved sequences flanking the target region (Table 2.7; Chapter 2).

PCR screening has been successfully used as a tool to predict for any possible integration of genomic islands in certain locations within the core chromosome, an approach known as *t*RIP-PCR which stands for tRNA interrogation of pathogenicity islands (He et al., 2007). To perform a survey of the presence of the GIs in the available strains, briefly, primers are designed to amplify conserved flanking regions from the bacterial host chromosome on either side. Positive PCR means that no band was detected by gel electrophoresis which could indicate an insertion of the GIs which cannot be seen due to its large size (>10 Kb). Primer pair 01-FG08 and 02-RG08 for G08 screening, and primers set 01-FG62 and 02-RG62 for screening of G62 (Table 2.8, Chapter 2; Figure 3.3). A schematic of this type of PCR is shown in figure 3.3 for demonstration. The flanking region in both sides of the GIs were consereved in all strains of *A. baumannii*.

The PCR screening analysis which have failed to yield a PCR amplicon could be possibly occupied with a G08 in only one clinical isolate (strain 3831) and confirmed the presence of G08 in the sequenced strains AYE, AB0057 and A424. For G62, only a single lab reference strain was found

harbouring the GI which is ATCC 17978 and this G62 could not be identified in any other lab or clinical isolate and therefore the negative PCR results are not shown. As the next step PCR mapping was performed to check if the G08 was identical in all positive strains as discussed in the next section.

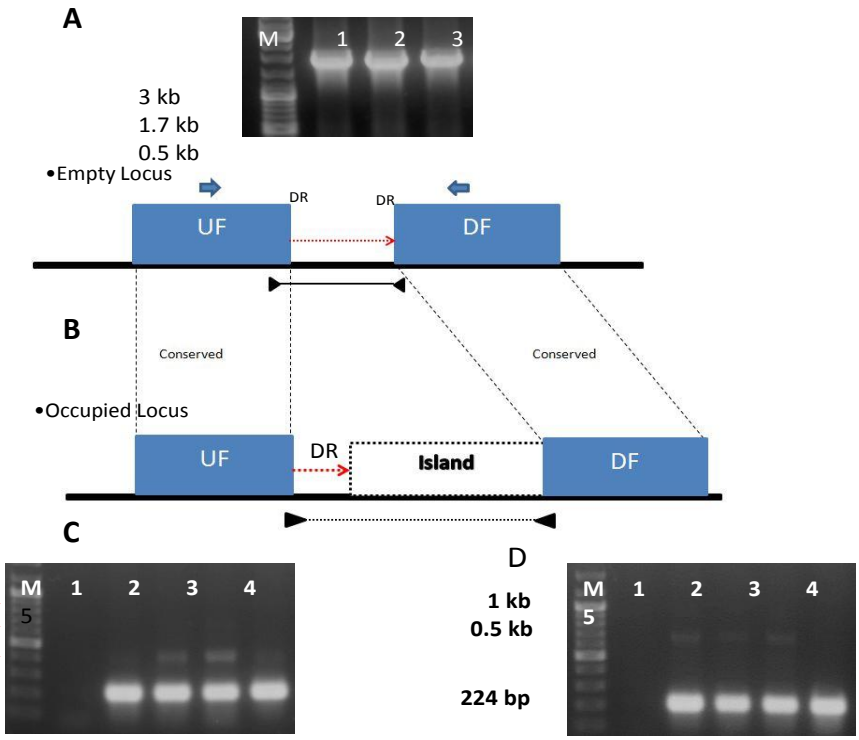


Figure 3.3 Schematic representation of tRIP-PCR for G08 screening in clinical isolates of *A .baumannii.*

The strains names are indicated by the numbers (3830, 3825, 3756, 3775). For simplicity only four strains are shown labeled with their lab numbers as these are not sequenced. The conserved upstream flank (UF) and downstream flank (DF) regions flanking DR are shown as dark blue filled boxes. DR represented as dark green boxes. GIs, where present, are indicated as broken boxes to emphasize the relatively large size of these regions. The solid line between the arrowheads shown in (A) indicates a likely successful *in vitro* PCR amplification; while the dotted line in (b) indicates a successful e-PCR-based 'amplification' that would typically yield a product of size far in excess of that could be generated through standard *in vitro* PCR. (B) PCR amplicon of an unoccupied locus yielding a PCR product of 1.7 kb, M gene ruler; 1- 3830; 2- 3825;3- 3756. (C) 260 bp PCR amplicon of UF of G08, M gene ruler; 1- 3830; 2- 3825;3- 3756; 4- 3775. (D) (C) 224 bp PCR amplicon of DF of G08, M gene ruler; 1- 3830; 2- 3825;3- 3756; 4- 3775.

3.3.4 PCR mapping of G08 and G62

Initially, due to unavailability of full genome sequencing, PCR mapping was used to first confirm identity and size of the G08 in strain in the

clinical isolate 3831 using AB0057 as control. Mapping approach by PCR aims to define the specific gene clusters present in the locus in all the *A. baumannii* positive strains for the putative GI under study and to compare these in relevant to reference strains. The schematic in figure 3.4 represent the genetic maps of G08 and G62, which shows the gene clusters and orientation of individual genes with the primers and amplicons for PCR mapping also indicated. All the PCR fragments showed similar amplicon sized in both 3831 clinical isolate and the reference strain AB0057.

Whole genome sequencing of strain 3831 identified the G08 on two separate contigs with a contig break in between. Overall the genetic content of the G08 is similar in 3831 when compared to AB0057 reference strain. The genetic map for G62 is shown for only ATCC 17978 as no strain was harbouring this island (Figure 3.4).

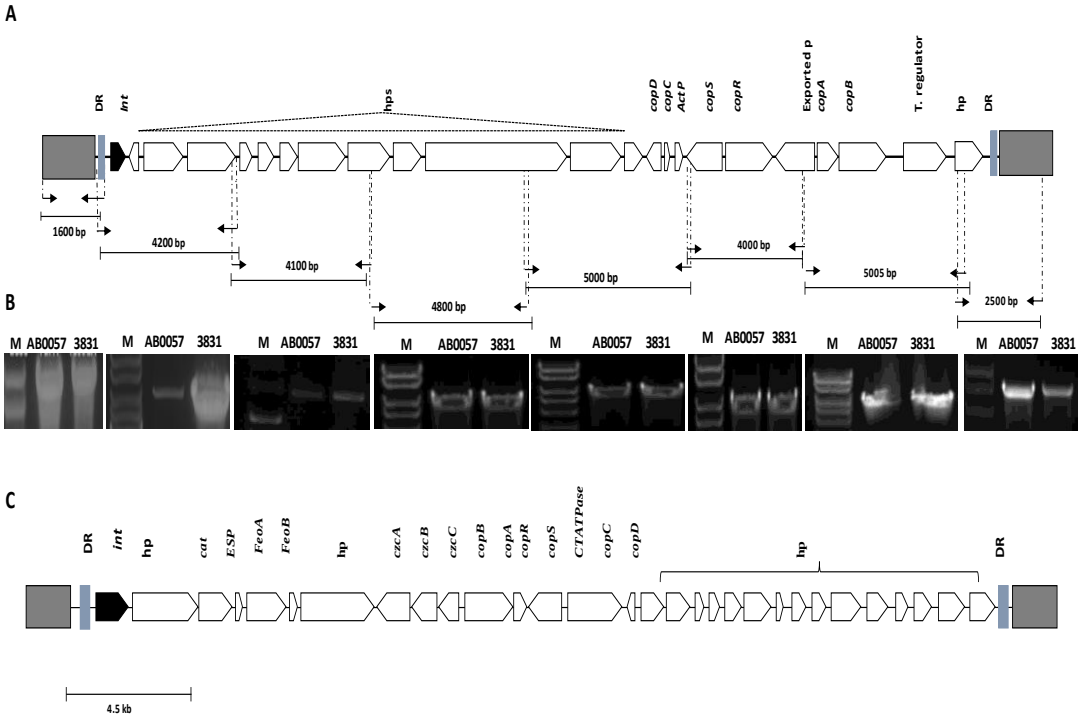


Figure 3.4 Schematic representation of PCR mapping of the G08 island.

(A)The schematic map shows the ORF of the G08 and G62 genomic islands of *A. baumannii* strain AB0057 (A) and ATCC 17978 (C). Below the maps the PCR fragments for mapping are shown (B) for AB0057 and 3831 as indicated above the PCR gel electrophoresis images, M, indicated the gene marker used Hyperladder. The primers used for the PCR mapping are listed in table along with summary of the genes amplified (Table 2.7; Chapter 2).

3.3.5 Study of GI excision using mini-islands and inducible integrase genes

3.3.5.1 Construction of G08 and G62 mini-islands in *E. coli*

A simple approach to study the spontaneous excision of a GI is by a conventional PCR using primer pairs facing outwards to check any circular intermediates. However, in this study attempts have been made to detect spontaneous excision mediated by the integrases by patching more than 1000 colonies and then performing simple PCR, were not successful. Therefore, alternatively, this experiment was designed to clone the integrases of the GIs of interest into IPTG-inducible plasmids to demonstrate their functionality.

This approach was used for similar purpose of creating mini-islands in *S. aureus* and to demonstrate excision for larger GIs in *Streptomyces lividans* 66 (Ubeda et al., 2005); (He et al., 2007). In this approach, a mini-island is generated by creating a smaller circular molecule with precise site-specific excision via *attL/attR*, and integrase coding gene cloned in a plasmid usually under an inducible promoter. In this study, to examine whether the integrase genes of G08 and G62 are functional in excising their respective GIs, two plasmid constructs were created. Two “mini-island” were constructed containing the integrase genes as well as the upstream and downstream flanks from their original chromosomal GIs, G08 and G62 (Figure 3.6). The attachment sites *attL*/*attR* required for excision are included within the left and right flanking regions to be cloned into the new constructs. The new fragment generated was later cloned into pUC18 vector (Yanisch-Perron et al., 1985). This pUC18 is a pMB1-derived plasmid that carries an *E. coli* origin of replication and *bla* gene which confers ampicillin resistance.

The primer pairs F-LF-08/R-LF-08 and F-RF-08/ R-RF-08 (Table 2.8) were used to amplify the left and right G08 flanking regions including the

att sites from strain A424. Similarly, the left and right flanking regions including the *att* sites of G62 GI were amplified from the strain ATCC 17978 using the primer pairs F-LF-62/R-LF-62 and F-RF-62 and R-RF-62 (Table 2.8), respectively (Figure 3.6). The two integrase genes G08*int* (A424_1287 from A424) and G62*int (*A1S-2927 from ATCC17978*)* were separately amplified by PCR using primer pairs F-G08int/R-G08int and F-G62int and R-G62int. In addition, the amplicon containing the integrases was ligated in the HindIII within the MCS to be expressed under the *lacZ* promoter in the final recipient vector (Figure 3.5). The three PCR fragments (LF, RF and integrase) were finally joined by fusion PCR resulting in a recombinant DNA product.

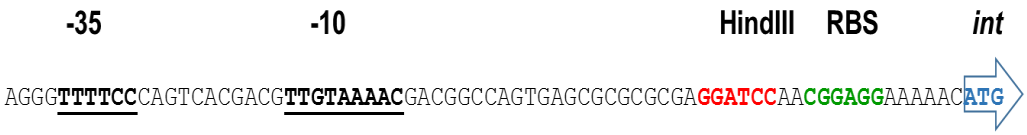


Figure 3.5 Nucleotide sequence of the start of the A424_1287 *int* gene and the region of pUC18 directly upstream of the MCS site.

Once the digested fragments were ligated. (see the text below for details). The start codon of the A424_1287 gene is marked in bold blue colour. The -35 and -10 sequences of the *lacZ* promoter are indicated in bold black colour and underlined. The restriction enzyme site HindIII was used in the directional cloning of the insert is indicated in bold red colour. The RBS region is shown in green bold colour.

3.3.5.2 Construction of IPTG-inducible plasmids in *A. baumannii*

The vectors pUC18-G08*int* and pUC18-G62*int* carrying the min-islands of G08 and G62 respectively were sub-cloned into pWSK129. pWSK129 is a low-copy-number plasmid carrying aminoglycoside 3'-phosphotransferase (*aphA1)* gene conferring kanamycin-resistance (Km^R) and has 16 unique cloning sites flanked by T*7* and T*3* RNA polymerase promoters positioned within the *lacZ*α gene (Wang and Kushner, 1991). This pWSK129, a pSC101 based plasmid is compatible with pBM1-derived plasmids like pUC18, thus insuring the sub-cloning will be efficient (Peterson and Phillips, 2008).

These mini-island-carrying vectors pUC18-G08*int* and pUC18-G62*int* were ligated into BamHI and SalI sites of pWSK129 generating

pWSK129-G08*int* and pWSK129-G62*int* as shown in figure 3.7. The resultant plasmids were transformed into the strain DH5α *E.coli* by electroporation and plated on kanamycin 40 µg/ml (to select for the pWSK129) and ampicillin 100 µg/ml (to select for the mini-islands). These two constructs were made to test for the excision of the mini-island in *A. baumannii* background. However, this attempt was not successful, due to the incompatibility of these plasmids with *A. baumannii* strains. Therefore, pWH1266 shuttle vector was used to optimise the excision in *A. baumannii* which is described in the next section.

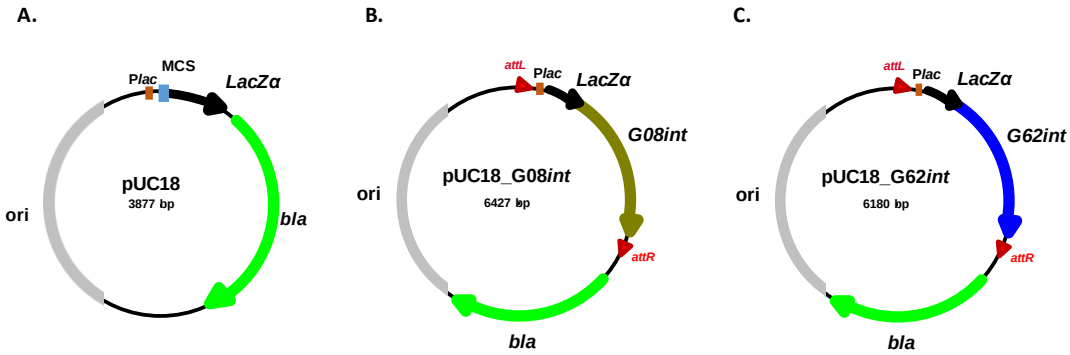


Figure 3.6 Plasmid construction for IPTG inducible excision of G08 and G62.

(A) pUC18 used as a vector harbouring *bla* gene conferring ampicillin resistance (green), *E. coli* origin of replication *ori* (grey), *lacZ* gene (black arrow) and MCS shown as light blue rectangle, to clone mini-GIs containing upstream and downstream flanking borders with *attL/attR* sites (red) of the respective islands as well as integrase genes (B) G08*int* (olive colour) and (C) G62*int* (blue colour) into MCS. Coupling G08 and G62 mini-islands to the IPTG-inducible P_{lac} promoter of *lacZ*α of the new constructs were used for integrase assays.

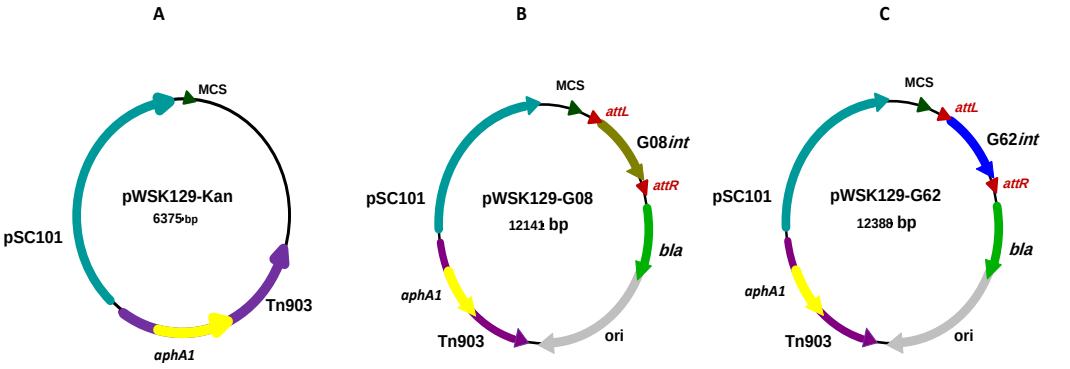


Figure 3.7 Plasmids used to assess integrase activity in *A. baumannii*.

These were constructed by coupling G08 and G62 mini-islands-containing plasmids pUC18-G08*int*/pUC18-G62*int* with pWSK129 In (A, B, C), (yellow)

aphA1 conferring kanamycin resistance, (purple) from Tn903, (siam) similar to pSC101, light green Rep (pLG339 replication initiation protein), (red) upstream and downstream flanks including the *attL/attR* sites.

3.3.5.3 Optimization of plasmids by transferring the *A. baumannii* compatible *oriR*

In the previous section, plasmids were generated to see if both integrase genes G08*int* and G62*int* were functional in *E. coli,* and this part, explores whether the integrases are functioning in similar manner in *A. baumannii* background. As it was established that the origin of replication in pWSK129 cannot be maintained in *A. baumannii* strains, here the construction of a new conjugative plasmid which is compatible with *A. baumannii* strains is described. This was achieved by PCR amplification of the origin of transfer from pWH1277, a cryptic plasmid from an *A. lwoffii* strain fragment of pWH1266 (an *Acinetobacter – E. coli* shuttle plasmid (Hunger et al. 1990, kindly donated by Philip N. Rather, Emory University, USA), using the primer pair PR3136 and PR3137 (Table 2.8). This is a 1752 bp fragment that contains a putative origin of transfer (*OriT*), which could be recognized by some *Acinetobacter* species, as well as TA system. Both pWSK129-kan and the PCR fragment were digested with BamHI, ligated forming the new plasmid pWSK129-WH (Figure 3.8). This latter construct was afterwards transformed into competent *A. baumannii* strains (A424 and ATCC 17978) by electroporation.

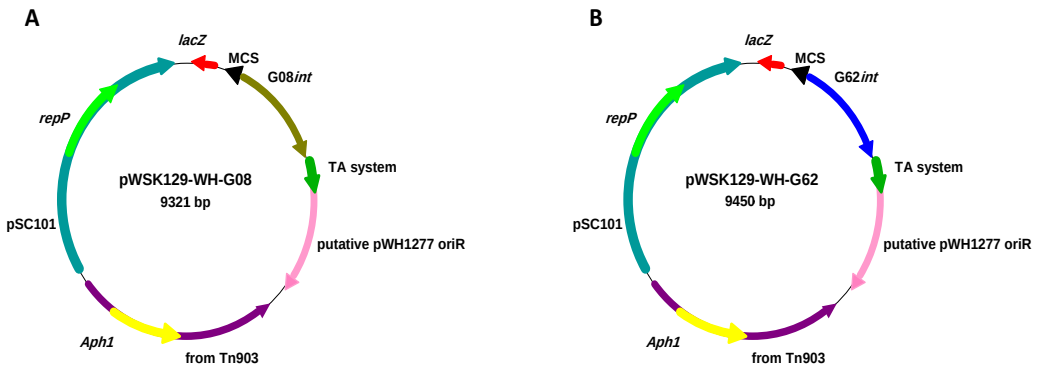


Figure 3.8 Schematic representation of the construction of the pWSK129-WH-based plasmids.

(A) pWSK129-WH-G608and pWSK129-WH-G62 (B) optimised vector compatible with A*. baumannii* strains. pWSK129-kan original plasmid which has IPTG-inducible Plac promoter used to clone the *Acinetobacter* origin of Replication (oriR) from pWH1266 (pink colour) resulting in pWSK129-WH-derived new constructs compatible with *A. baumannii* strains. Rep; pLG339 replication initiation protein (light green), putative TA system (dark green), Aph1; Kanamycin-resistance conferring protein (yellow), similar to pSC101 (siam), lacZ (red), multiple cloning site (black).

3.3.5.4 The IPTG induction of G08*int*/G62*int* results in significant fitness costs in all backgrounds tested, with most severe effects shown in *A. baumannii* suggest possible occurrence of DNA damage

Prior to conducting integrase assays, growth curves of strains transformed with the pWH/pWSK129 were performed to assess any fitness cost on the growth of recombinant strains. In this study, the growth curves of three different strains *A. baumannii* A424 and ATCC 17978, and *E. coli* DH5α with or without the plasmids and IPTG induction were carried out with eight replicates for each strain and the results are shown in figure 3.9. In all cases, the growth cultures were supplemented with kanamycin to ensure the maintenance of pWSK129-WH plasmids. The data evidence that the addition of 1 mM IPTG to the cell cultures with plasmids for integrase overexpression has a statistically significant effect on the growth of all strains as compared to the strains without the plasmids with or without IPTG induction (Figure 3.9). The effect of fitness after IPTG induction is more pronounced in *E. coli* DH5α due to lack of DNA repair

mechanisms. There is no significant difference in the growth dynamics between the strains alone or with the plasmids without IPTG induction.

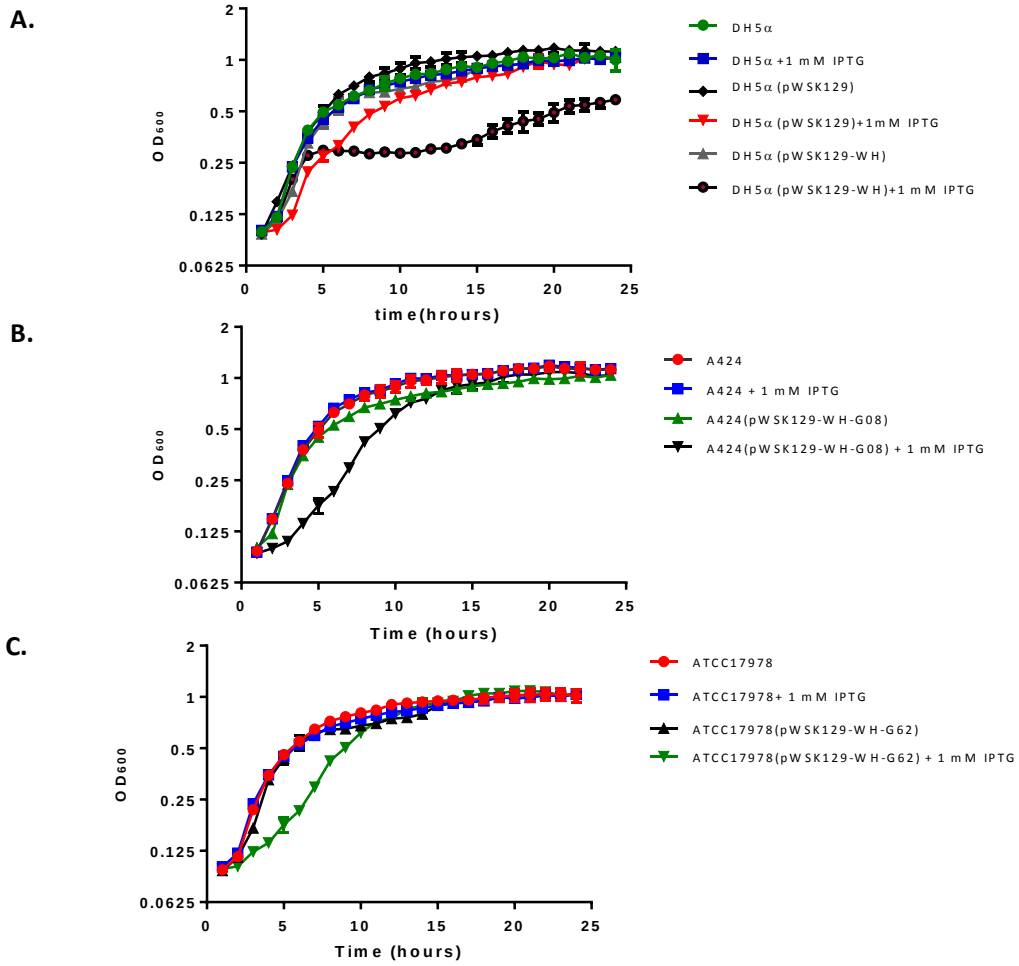


Figure 3.9 Growth curves showing the effects of IPTG on the cells with and without the pWSK129/pWSG129-WH plasmids.

The effects of the plasmids were tested on *E. coli* – DH5α (A) and two strains of *A. baumannii*, A4424 (B) and ATCC 17978 (C). In the three strains, cells without IPTG induction and with 1 mM IPTG are shown with different colours and shapes as indicated in the right upper side of each figure. Standard error bars are shown and were calculated from eight replicates.

The integrase overexpression by IPTG resulted in significant defect in the growth of DH5α at an early exponential phase and the growth impairment (increased doubling time) was evident as the strain could not catch-up to the wild type strain until the end of 24 hours. In comparison, the *A. baumannii* strains A424 and ATCC 17978 carrying the overexpressed pWSK129-WH plasmids had growth impairment throughout the whole exponential phase however, showed a similar behavior during early

stationary phase to their non-plasmid bearing counterparts after 15 hours of growth.

3.3.5.5 IPTG- inducible excision in *E. coli* DH5α and *A. baumannii*

As it was hypothesized that the integrase as well as the *attR/attL* are the two vital elements for the GIs excision, primers were designed facing outwards to enable the detection of circular forms of the mini-islands after being excised from their location in the plasmid facilitated by the integrase activity. This type of assay has been performed previously in similar studies of integron cassette excisions (Collis and Hall, 1992, Hocquet et al., 2012).

To test this, the two *E. coli* DH5α (carrying the pWSK129-G08 and pWSK129-G62) were exposed to 1.0 mM of IPTG. As shown in (Figure 3.10; F and G) the circular elements can be visibly detected by gel electrophoresis after 2 hours, and more evident in the IPTG-induced cultures as compared to the non-induced ones. In addition, the PCR fragments are specific and becomes increasingly visible overtime. The intensity of the band is almost similar at T=8 and T=24 since the bacterial cells have reached the end of the exponential phase at T=8 and thus no further circular elements were generated. The PCR product was sent for sequencing and the results are demonstrated in (Figure 3.10; D and E.), confirming the correct circular forms sequence as well as the location of the primers. This experiment was required as a validation step for the feasibility of the test, however this evidence needed further experiments to provide a quantification of the circular forms present at each time point by qPCR as described in the next section.

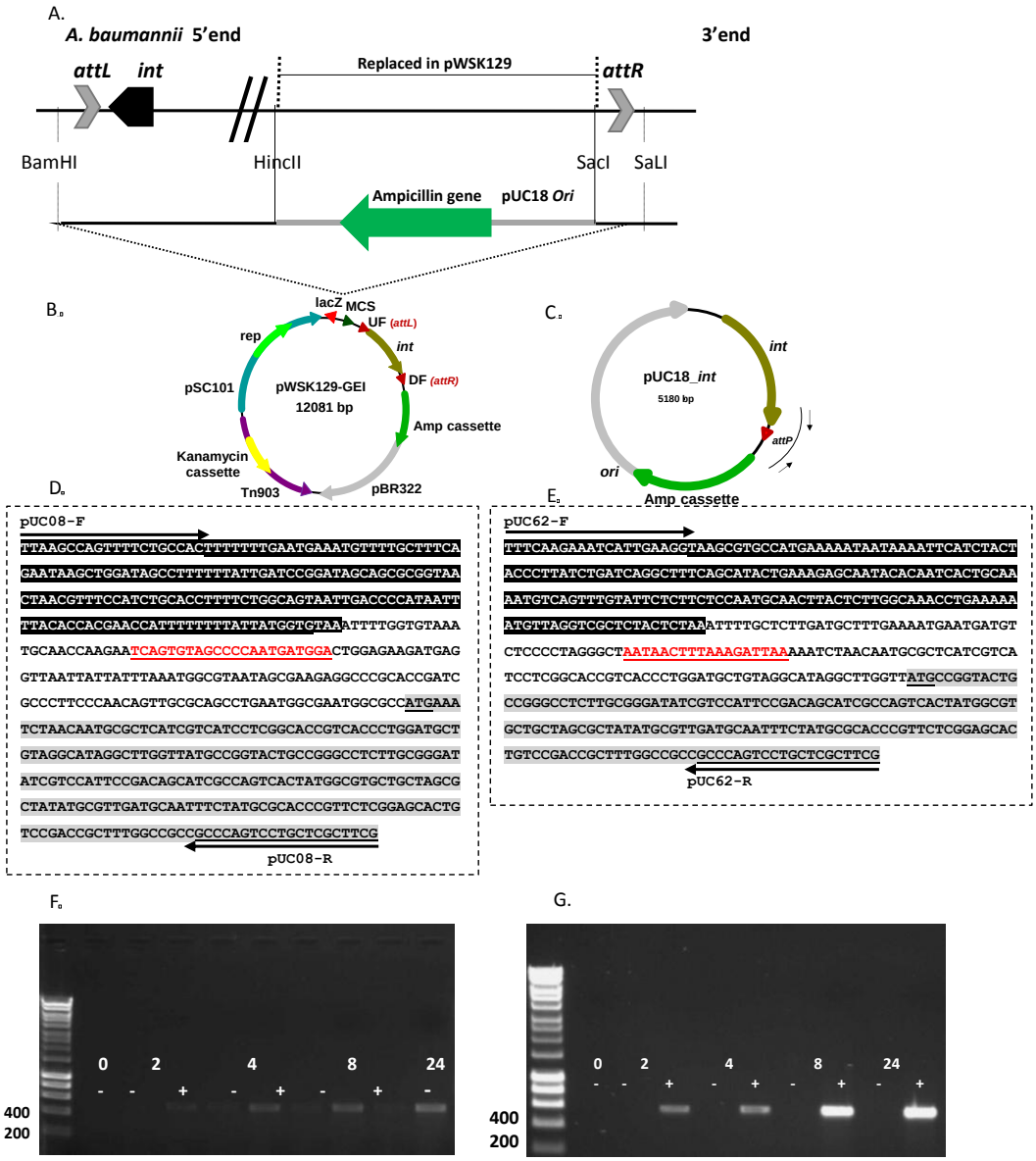


Figure 3.10 A. Schematic representation of the G08/G62 mini-island used in this study and its cognate wild-type island in the chromosome of *A. baumannii*.

The *attL* and *attR* boundaries are shown as grey triangles, *A. baumannii* DNA as black lines or arrows, and plasmid DNA or mini-island specific components (*bla* gene and pUC18 *ori*) as grey lines or arrows. Dashed lines indicate restriction sites outside the mini-island, while solid lines denote restriction sites within the boundaries of the mini-island. **C.** Map of pUC18-int showing the positions of primer binding sites for P-F and P-R (short arrows). D and E show sequencing results of the PCR product using the primers pUC08-F and pUC08-R, pUC62-F and pUC62-R, with their position shown as black arrows, which amplified part of the circular product spanning the 3' end if the integrase shaded in black and its stop codon underlined and bold. The *attP* sequence is in red bold and underlined. The coding region of the 5' end of the ampicillin cassette is shaded in grey and its start codon is in bold and underlined. **F and G.** Gel images of circularized G08 and G62 mini-islands after IPTG induction at different time

points. The gel electrophoresis showing PCR results of crude DNA preparation from a culture of pWSK129_G08 generating a 567 bp product(D) and pWSK129_G62 with 489 bp PCR product (E) in *E. coli* DH5α, taken at time points shown, with and without induction of 1 mM IPTG as indicated by a – or + sign. At T=0 IPTG was added so only one cell suspension was prepared. The marker is Gene Ruler 1 kb (bp).

3.3.6 Mitomycin C induction of GI excision in *A. baumannii*

Mitomycin C is a potent DNA-damaging agent used as chemotherapy for cancer, and is well known to induce an SOS response in bacteria through its cross-linking action resulting in increased expression of certain genes including integrases (Canchaya et al., 2002, Guerin et al., 2011). It was hypothesized that mitomycin C can facilitate the excision of G08 and G62 directly from the chromosome and enable detection the circular intermediates by PCR. Conventional PCR attempts in this study failed to amplify both the target junction and the excised elements from colonies patched in media containing selective antibiotics without any induction methods. To test excision and reconstitution of the chromosomal target site, the G08-positive AB005&, AYE and 3831 strains as well as the G62-positive strain ATCC 17978, were grown to mid log phase and exposed to 38 µg/ml of mitomycin C for 2 hours. One sample for each strain was induced, DNA extracted and amplified. Negative controls of uninduced samples were included for all strains. The reconstituted target site as well as the circular intermediates could be amplified for all three G08-positive strains (Figure. 3.11; A) using the primers pairs EXC8-F and EXC8-R for the circles and primers (JUNC8-F and JUNC8-R) for the junctions. In the case of the G62-positive strain ATCC17978 both the reconstituted target and the circular forms could be amplified (Figure. 3.11; B) using primers EXC62-F EXC62-R, JUNC62-F and JUNC62-R for the circular forms and chromosomal junction at the G62, respectively, produced 389 bp PCR amplicon as (Figure 3.11; B). Primers used in this assay are listed in table 2.8.

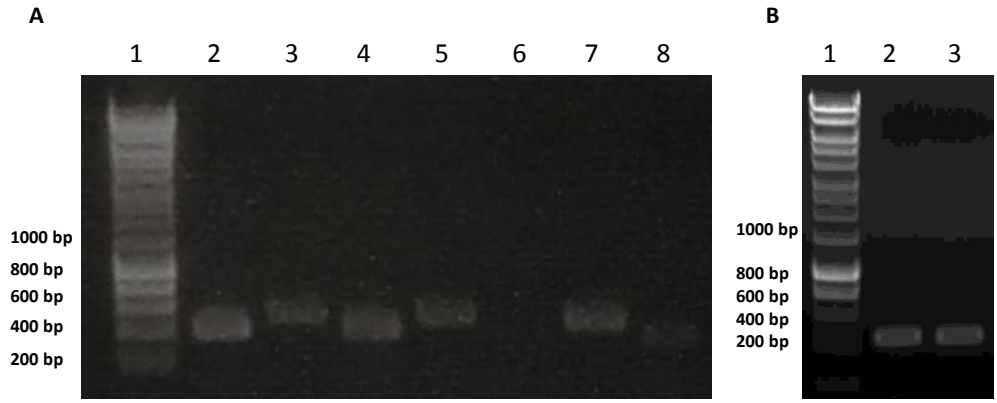


Figure 3.11 PCR detection of excised (A) *dus*-associated GI (G08) and restored *dusA* chromosomal junctions in *A. baumannii*.

The strains used (AB0057, AYE and 3831) (circular forms) 425 bp (lane 2 (AB0057), lane 4 (AYE), lane 8 (3831) and (junction) 500 bp, (lane 3 (AB0057), lane 5 (AYE), lane 7 (3831) (J), lane 6 (negative control). (B) G62 GI excision 389 bp (lane 2) and restored junction 389 bp (lane 3). Molecular weight standard on both gels is Hyperladder (shown in lane 1) in (A) and (B).

3.3.7 Predicted mechanism of excision and circularisation

Following mitomycin C induction (discussed above), a modified PCR protocol was used to detect the circular intermediates forming as a result of the integrase activity, which catalyzes the excision of the G08 from its chromosomal locus. The circular forms were detectable by PCR after two-hour collection of the bacterial culture as shown in figure 3.11 in the previous section. The PCR amplicons of both the circular forms as well as the chromosomal junctions were sequenced to understand the dynamics of integration and excision and define the *att* site recombination. The chromosomal junction of the reference strain ATCC 17978, known to have an empty G08 locus as evidenced by previous *t*RIP PCR data, was sequenced as a control. Figure (3.12) illustrates the predicted model of excision and is described below.

Initial sequencing data obtained in this study has shown some interesting finding that the empty chromosomal G08 junction is actually a combination of the two neighboring genes, *dusA* and *MFS* as shown in figure 3.12. This data showed that in the case of unoccupied G08 junction, the 5'-end of original chromosomal *dusA* gene is forming almost half of

the junction and the other half of the junction is formed by a major facilitator superfamily gene encoding for an MFS transporter protein. The initial data in this study was suggesting that the insertion *att* sites of G08 are found within the *dusA* gene. The findings in this work was confirmed in a study in which it was shown that the G08 integrase of *A. baumannii* as well as integrases from other *proteobacteria* predominantly, *Pseudomonas* and *Escherichia* could insert within the 5' end of *dusA* genes thus interrupting the transcription of the gene (Farrugia et al., 2015). However, as the G08 integrates at the 5' end of the *dusA* gene, it replaces that portion with a different 5'-end, thus pushing the original *dusA* at the other end forming the outer downstream boundary of the GI. Once the excision of the GI occurs, the 5'portion of the *dusA* at the chromosomal junction will be reformed again to its original wild-type *dusA* sequence. The empty Locus of ATCC 17978 G08 is shown in figure (3.12; B), whereas the G08 junction of AYE strain with occupied locus after excision is demonstrated in figure (3.12; A). The sequencing data was used to map the putative attachment sites in different strains, precisely as well as the island boundaries at both flanks which are summarised in figure 3.13.

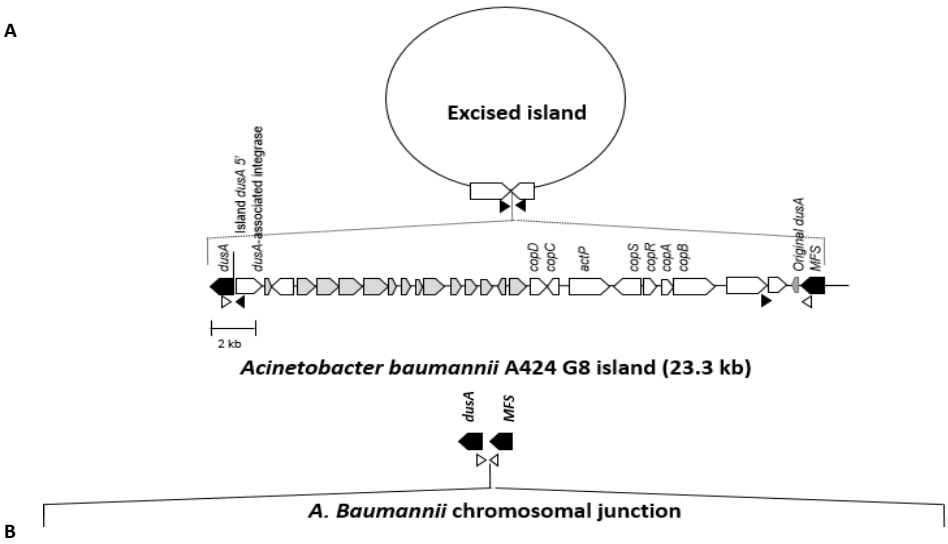


Figure 3.12 Excision and integration of the *A. baumannii* A424 dusA-specific GI.

(A) The *dusA*-specific G08 encodes an integrase that putatively catalyses excision of the GI as a circular intermediate, as well as its integration into the 5' end of the chromosomal tRNA-dihydrouridine synthase A (*dusA*) gene (black). The 5' portion of the *dusA* gene affected by the integration is replaced by a new 5' end (white) provided by the GI, with the original *dusA* 5' end (dark grey) forming the outer boundary of the island. G08 encodes several hypothetical proteins (light grey). The *dusA*-associated G08 is flanked by a gene encoding a MFS transporter (black). This figure is drawn to scale with the exception of the excised island, which is displayed at 60% of scale. (B) The nucleotide sequence of the G08 junction of strain ATCC 17978, after amplifying the empty target by PCR. The sequence showed that the *dus*-associated G08 GI was excised and the *dusA* chromosomal junction was restored. In panel B, the *MFS* sequence is shown in blue and the *dusA* sequence is shown in green. The *attB* in the junction is underlined.

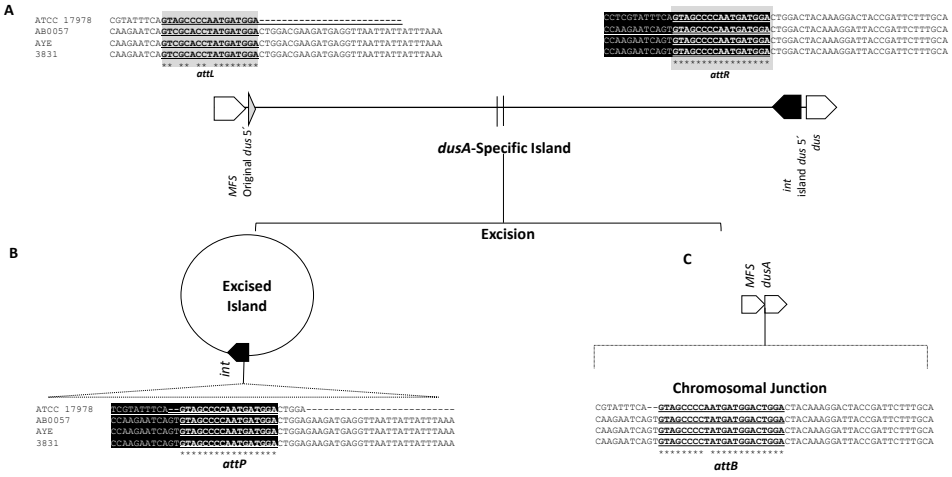


Figure 3.13 Predicted mechanism of GI excision in *A. baumannii* strains AYE, AB0057 and 3831.

The integrated forms of GIs (A) are predicted to excise as a closed circular molecule (B) from the chromosome, resulting in the restoration of the native *dusA* sequence (C; also Fig 3.12; B). The chromosomal gene flanking the island is shown as MFS (white). The shading of the nucleotide sequences represents their origin as either from the circularized GI (black and dark grey) or chromosome (white). The underlined sequences represent the putative attachment sites involved in GI excision and integration (*attP* and *attB*), which upon integration generate a pair of imperfect direct repeats (*attL* and *attR*). Nucleotides in bold are identical in the *attP/attB* and *attL/attR* pairs of their respective organisms. The shaded (light grey) boxes represent the 17 bp consensus *attL/attR* sequences present in *dusA*-specific GIs.

The *attL* and *attR* are 17 bp in size and differ by a single nucleotide. In both AYE and 3831 upon excision the the *attP* site of the circular form shows the A SNP of the *attL* site and the chromosomal *attB* shows the C SNP of the *attR* site. In contrast AB0057 shows two different SNPs at *attB* site and *attP*, once an A SNP like the other two strains and in the other instance as C SNP which is similar to *attP.* To rule out any sequencing error, the *attB* site sequencing of AB0057 had been repeated at least three times. However, in all cases the sequencing data were showing similar variabilities suggesting the presence of two different alleles of the *attB* site in AB0057. This could be explained by the fact that the *attB* sites show two different allelic variations as evidenced by BLAST search of *A. baumannii* G08 empty strains as shown in figure 3.14. *attB* sites in *dusA*

gene of ATCC 17978 and AB307-0294 showed unoccupied G08 junction with two different alleles and SNPs.

Figure 3.14 Allelic variation in the *attB* site of *A. baumannii* strains.

(A) shows the variability of *att* sequences in AB0057 and AYE. The att sites sequenced in this study in abbreviated as (seq). *attR*_AB0057, *attP* seq AYE, *attP* seq AB0057 and *attB* seq AYE are similar and shown in blue font, whearas *attL* and *attB* of AB0057 are similar and shown in red font. (B) shows two variable alleles of *attB* sequences in *dusA* in *A. baumannii strains* ATCC 17978 and AB307-0294. These two sequences were obtained by BLAST search.

3.3.8 Relative quantifications of excision and circularisation of GIs by qPCR

As shown above, the integrases have catalyzed the excision and integration of the GIs G08 and G62. While excision and circularisation of the whole element was not detectable in the original host, excision of mini-islands could be detected upon integrase overexpression in both *E. coli* and *A. baumannii* and the excision and circularisation of the whole element in *Acinetobacter* could be detected following mitomycin C induction. Those data did not provide a quantification of the rate of excision. Therefore, qPCR assays were designed to quantify the events of excision and circularisation.

The qPCR assays are highly sensitive and beneficial in this context to provide a relative quantification of both the excision and circularisation

events. In the coming assays the term circularisation will be referring to the circular intermediates detected in the crude DNA extracts and excision will be pertaining to the junction of the GIs after the elements have been excised either from the plasmids or chromosomal DNA. Variation between samples was corrected by using two reference genes for normalisation, *gyrB* and 16SrRNA for the non-induced as well as IPTG-induced qPCR assays, whereas with mitomycin C-induced assays only 16S was used for normalisation. This is because it was found that the *gyrB* expression will be altered as a result of mitomycin C being added to the bacterial culture, thus affecting the reliability of this normalisation. Sample to sample variation between different strains of G08 was also corrected by normalising the results to the AB0057, which was used as a reference strain throughout this section, and its expression was arbitrary set to be at 1 (equivalent to 100%). The levels of detection of circularisation and excision are expressed as fold change. The primers efficiencies were checked by performing up to 5 serial dilutions of the gDNA and plotted against the cycle threshold (Ct) values. The standard curves of all primer pairs are shown in figure 3.15.

3.3.8.1 Relative quantifications of spontaneous excision and circularisation of GIs by qPCR

This assay focussed on amplifying both the excision and circularisation occurring spontaneously, i.e., without any induction method utilising the advantage of qPCR being highly sensitive and could detect relatively small amounts of mobilised elements that conventional PCR failed to amplify (Figure 3.16). The quantitative fold change of three strains harbouring G08 (AYE, A424 and 3831) were compared in each qPCR experiment to the reference strain AB0057. The fold change in excision and circularisation of AYE was almost similar to AB0057 (*P* value >0.05), while A424 and 3831 have slightly lower level of mobilisation (i.e. excision and circularisation) as compared to AB0057 (*P* value <0.01).

On the other hand, the G62 integrase activity was only assessed in one strain ATCC 17978, and hence no statistical analysis has been performed further apart from normalisation to *gyrB* and 16S. The results are expressed as fold change and shown in graphs (3.17).

3.3.8.2 Relative quantifications of excision and circularisation of GIs of 8-hour IPTG-induced cultures by qPCR

As seen in section (3.3.5.4), where the integrase activity has been assessed by IPTG induction of the different inducible vectors. The same crude DNA extracts were used to run qPCR assays discussed in this section. The primers, however where redesigned to amplify shorter fragments of about 200 bp to optimize the detection by qPCR.

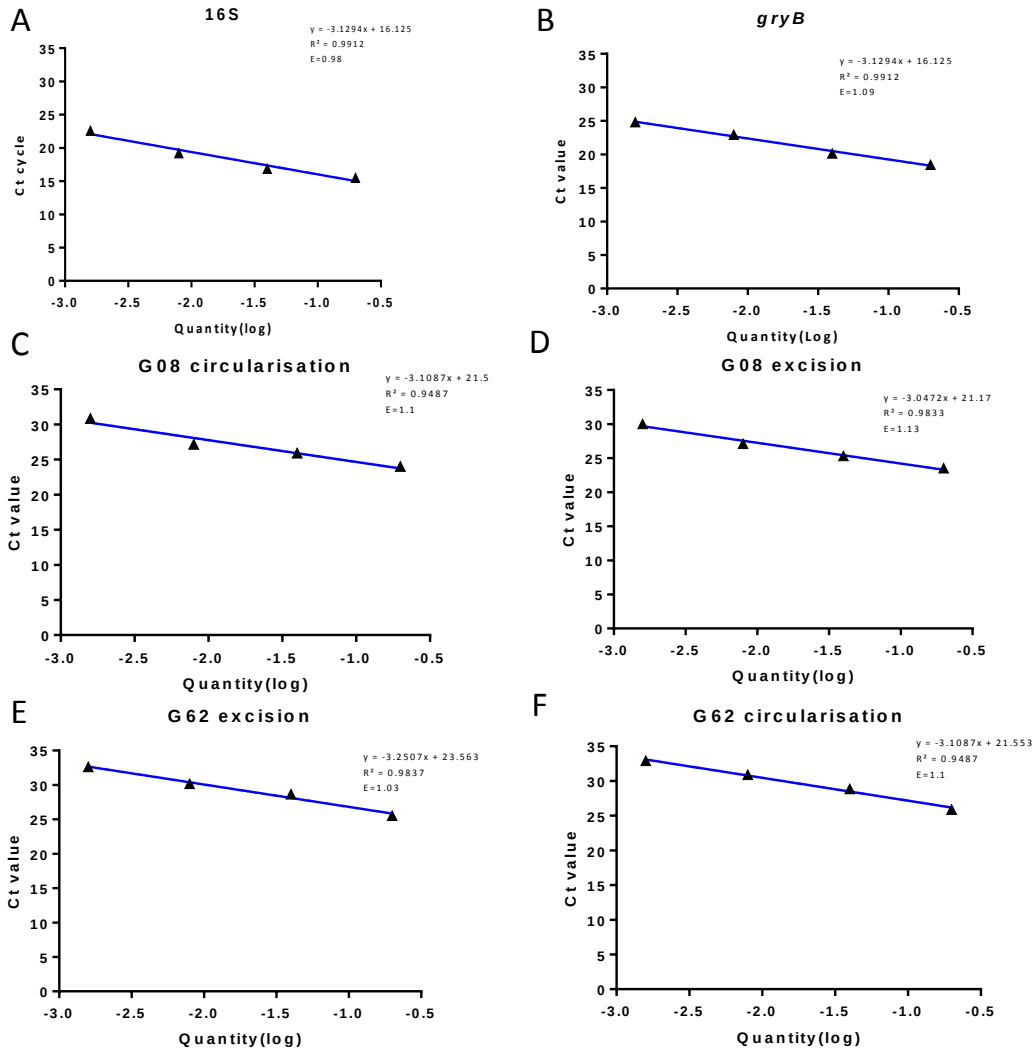


Figure 3.15 Standard curves for the qPCR. The log dilutions of DNA are plotted versus the cycle threshold (Ct) values.

The equation of the regression line and its R^2 values are displayed in the upper right corner of each graph. For each qPCR assay a standard curve was generated using four 5-fold dilutions of gDNA. All these assays were conducted in *A. baumannii* backround (primer pairs used in these curves are: (A)16S-F/R, (B) gyrB-F/R (C) G08-EXC F/R, (D) G08-JUN F/R, (E) G62-JUN F/R, (E) G62-EXC F/R (Listed in table 2.8, Chapter 2).

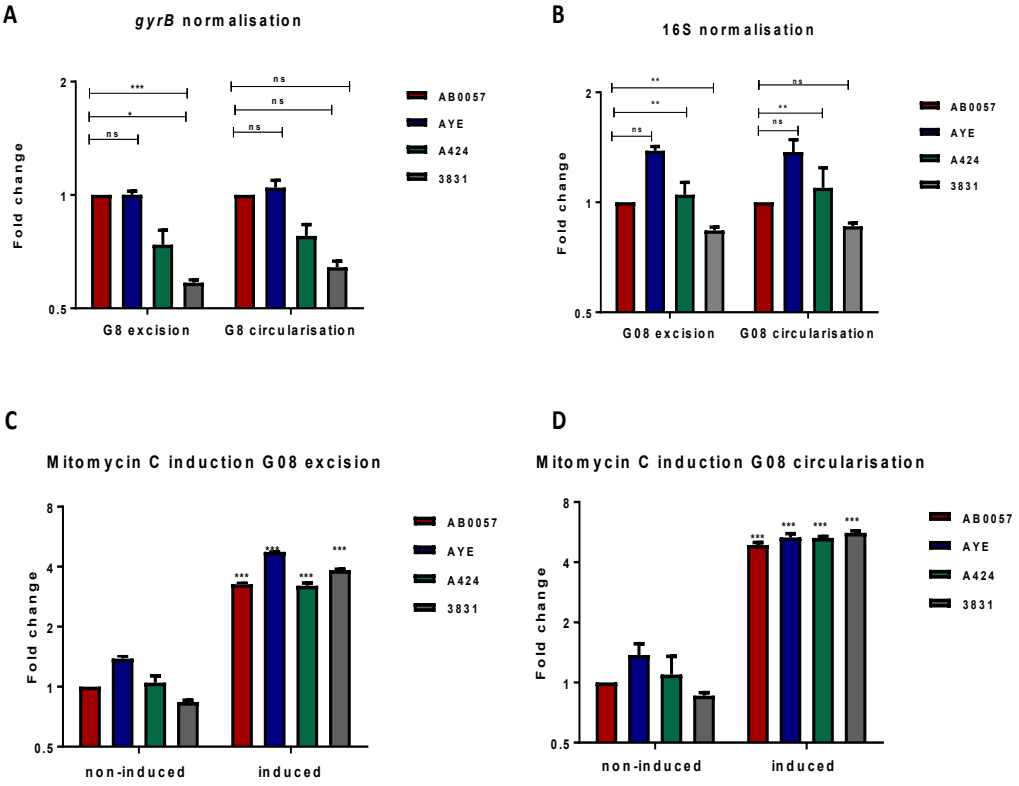


Figure 3.16 Histograms of relative quantity of circular and excised elements of G08 GI in various *A. baumannii* strains with and without mitomycin C induction.

Each bar chart represents comparison of strains harbouring the G08, after normalisation with AB0057 strain. Two reference genes are used here to normalise the data without induction: (A) and (B) showing the rate of G08 GI excision and circularisation when normalised to 16S and *gyrB*, respectively. (C) and (D) showing the change in the rate of G08 excision and circularisation after mitomycin C induction (0.75×MIC) for 2 hrs when normalised to *gyrB*. There is significant increase in the fold detection of circular forms after Mitomycin C induction in all strains. The asterisks represent the significance of change in each strain after mitomycin C induction when compared to AB0057 before induction. The statistical analysis done by Two-way ANOVA. Error bars represent SEM of three independent replicates. Ns: not significant, P >0.05 *: *P* <0.05; **: *P* <0.01, ***: *P*<0.001.

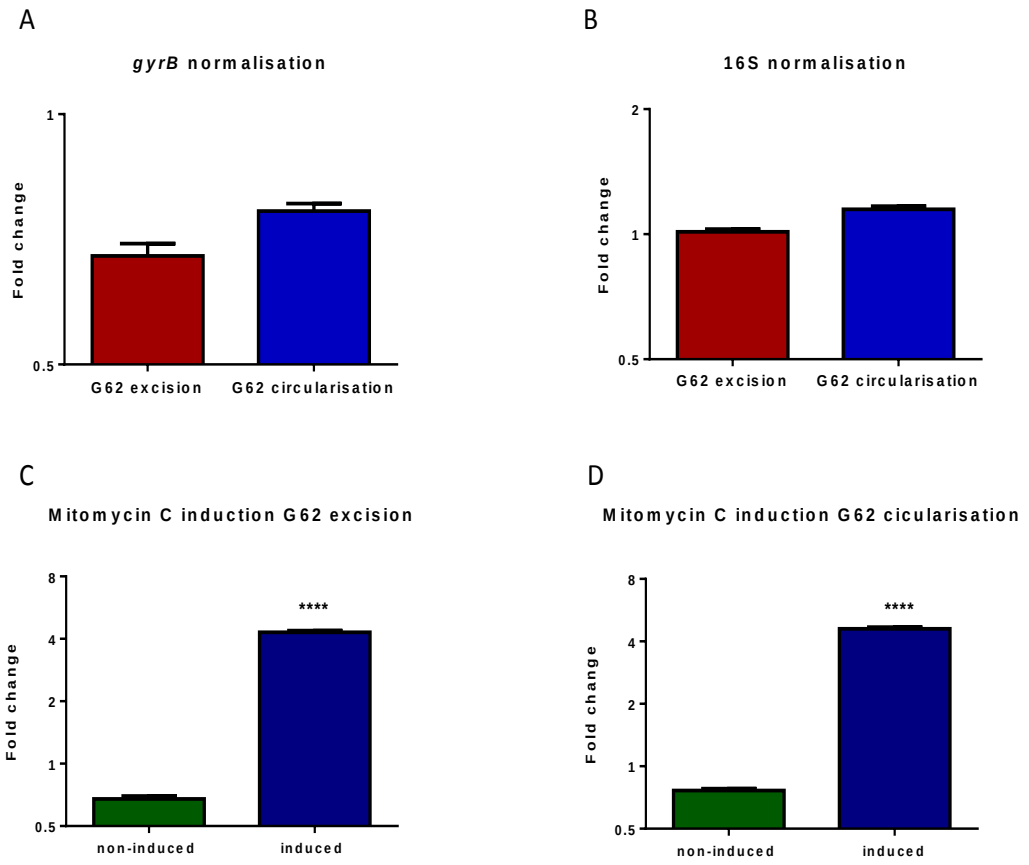


Figure 3.17 Bar charts representing the rate of G62 excision and circularisation in ATCC 17978 *A. baumannii* strain when normalised to 16S as well as *gyrB* without any induction methods.

The pattern of G62 excision and circularisation in ATCC 17978 *A. baumannii* strain, is similar to that of G08 when comparing the fold change in excised and circular elements with and without mitomycin C induction. Excision after mitomycin C induction is similarly significantly increased as expected, in comparison to the non-induced strains. Error bars indicate the SEM of three independent replicates in each experiment. (***: *P* <0.001 ****: *P* value <0.0001) as analysed by two-way ANOVA test.

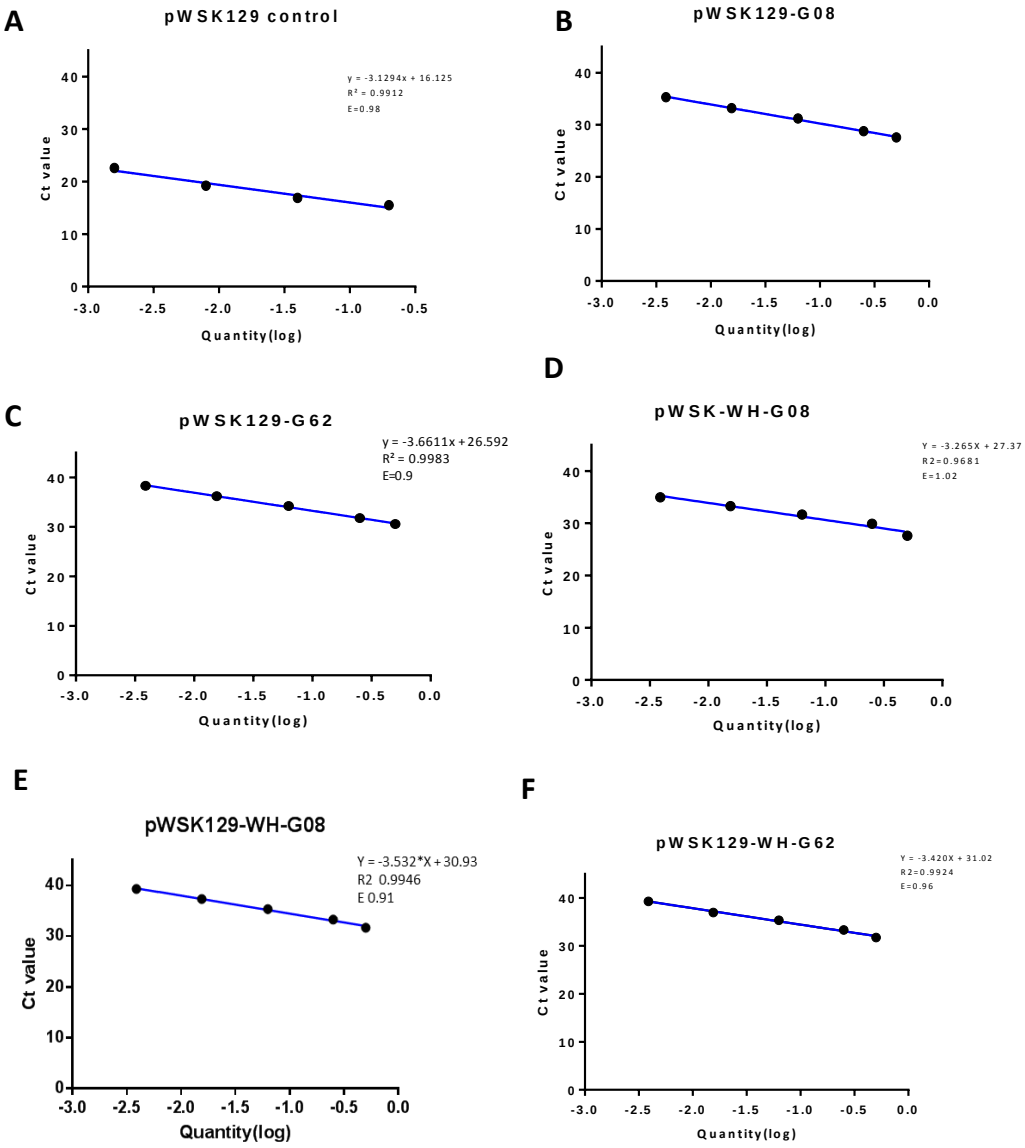


Figure 3.18 Standard curves for the qPCR primers to detect circular G08 and G62 mini-islands.

Data are in IPTG-inducible plasmids pWSK129-kan and its derivatives in *E. coli* DH5α panels and pWSH129-WH derivatives in *A. baumannii* background panels (D-F). Primer pair used in the generation of these standard curves are (shown in table 2.8) (A) pWSKori-F/pWSKori-R, (B, D, E) pWSKEXC8-F/R, (C, F) pWSKEXC62-F/R. The log concentrations of DNA are plotted versus the Ct values. The equation of the regression line and its R^2 values are displayed in the upper right side of each graph. For each qPCR assay a standard curve was generated using 4-5 5log dilutions of gDNA.

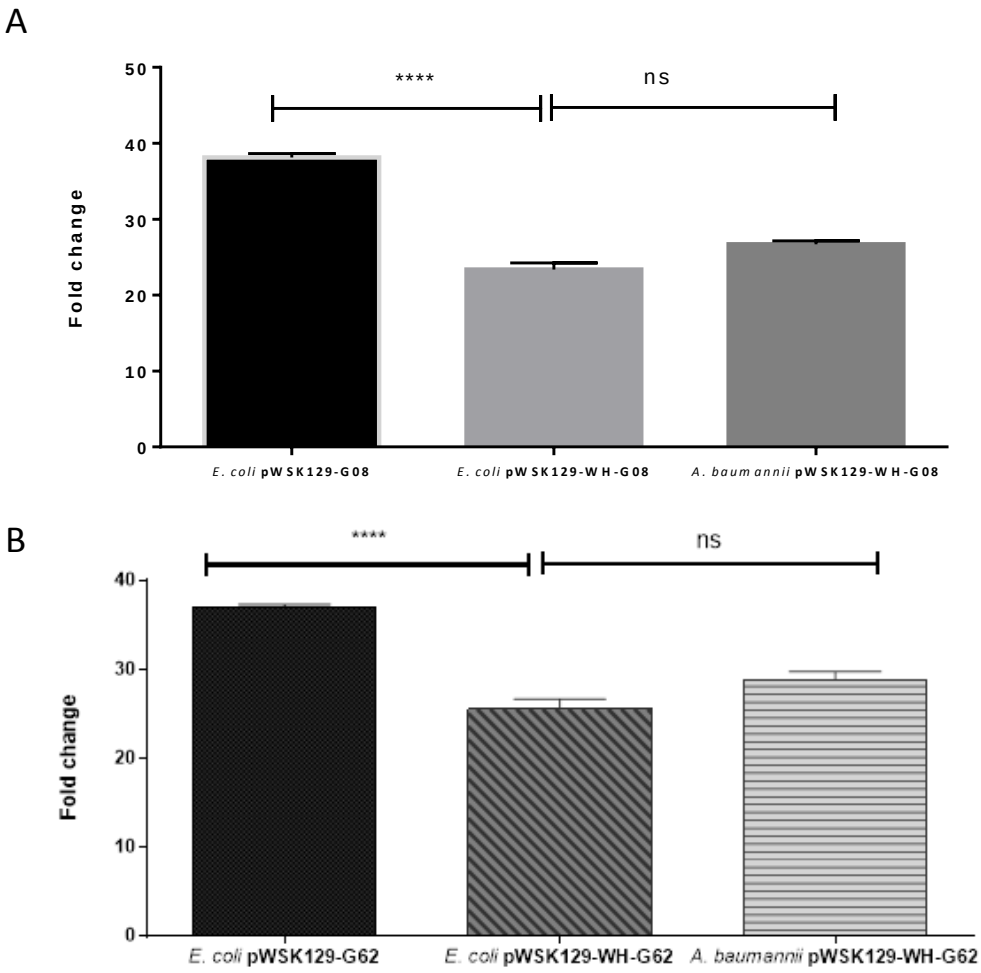


Figure 3.19 Bar chart showing the relative quantification of pWSK129-derived plasmids in the strains indicated, when normalised to their native plasmid pWSK129 (without the insert).

Error bars represent SEM calculated from three independent replicates. Statistical analysis was completed with a two-way ANOVA, and no significant differences in the fold change in pWSK129-WH between *E. coli* and *A. baumannii* backgrounds in both GIs G08 (A) and G62 (B), while there is significant difference between *E. coli* pWSK129 and pWSK129-WH. It seems that the WH *oriR* added to the plasmid has affected the plasmid copy number Error bars indicate the SEM of three independent replicates in each experiment. (ns: not significant, ****: *P* value <0.0001) as analysed by two-way ANOVA test.

3.4 Discussion

Previous surveys of integrases have found many integrases are non-functional in various species due to frameshift and nonsense-mutations (Cambray et al., 2011a, Gillings et al., 2005, Nemergut et al., 2008). In this work the aim was to check whether the G08 and G62 integrases are functional and contribute to excision of the islands. A recent study has found that the G08 phage integrases were identified to target a novel site within *dusA* and hence referred to as *dusA*-associated integrases (Farrugia et al., 2015). This is a novel class of a protein coding gene that the integrase could integrate into its 5' end, resulting in changes in the chromosomal junctional site.

Phylogenetic analysis of integrases has been demonstrated to be an effective approach to determine the evolutionary history and relationships among MGEs (Boyd et al., 2009). In the current work, a total of 19 integrases encoded by GIs of four as well as orthologues form the public database Genkbank from *A. baumannii* strains were analysed. A phylogenetic analysis was performed using a range of optimality criteria including neighbour-joining and maximum likelihood, on the integrases homologs (Figure 3.1). This analysis revealed that the phage integrases form the majority of tyrosine recombinases found within GIs in the *A. baumannii* isolates examined and most likely originate from cryptic prophages. As proposed by previous detailed studies, some GIs cannot be considered as degenerate remnants of integrated plasmids or prophages and are unrelated to these elements as shown by phylogenetic analysis (Boyd et al., 2009). These studies have found that phage integrases are not evolutionarily related to island-associated integrases. Interestingly, the G08*int* and G62*int* are not related to each of which belong to a separate clade within the tyrosine recombinases in the phylogenetic tree despite that both GIs carry genes for drugs and metal resistance. However, since the analysis included only a small number of integrases of interest, only a snapshot of the full extent of (functional)

diversity of GIs is described and a conclusion as to whether these evolutionarly not-related cannot be withdrawn.

It is noteworthy that in natural conditions the excision and integration events of some GIs occur at extremely low frequencies. In this study, initial experiments to amplify the circular excised forms of the GIs using conventional PCR, have failed to yield any PCR product which can be visualized by gel electrophoresis. In these experiments, crude DNA preps were obtained from colonies patched on selective antibiotics. As in previous studies, these basic conventional protocols sometimes fail to amplify the circular intermediates (Rose, 2010). Therefore, alternative methods such as a modified protocol for alkaline lysis were used to extract the DNA circular fragments usually used for plasmid extraction the assay to detect circular intermediates has been attempted several times. This approach has been previously employed in other *A. baumannii* studies (Rose, 2010) as well as other GIs circularisation and have successfully demonstrated excision after the use of modified protocols (Dominguez et al., 2011, Doublet et al., 2005, Rameckers et al., 1997). Despite mitomycin C induction, the gel electrophoresis bands were faint. This could be explained by very low level of excision frequency, most likely below 10^{-7}. Similar observations were reported on other *dusA/dusB* associated integrases (Farrugia et al., 2015).

The PCR amplicons obtained were sequenced to determine the *att* sites at the flanking regions of both G08 and G62 both at junction sites and circular forms. It was found that in all examined strains, G08 was flanked by two 17 bp semiconserved attachment sequences (Figure 3.13), suggesting that the *dusA*-specific integrases target a conserved region within the *dusA* gene. The sequence of the attachment sites was almost conserved, allowing the coordinates of G08 in different *A. baumannii* strains to be approximated. However, few issues were encountered during the analysis of these sequences including finding variability in the *attR/attL* and *attP/attB* for strains AYE and 3831 by a single nucleotide (A SNP) and by two SNPs (A and C) in the strain AB0057 (Figure 3.14). This

was initially thought to be due to either a sequencing or a technical error and therefore repeated several times using different conditions each time like using nested PCR approach and changing the PCR cycling conditions. Thorough BLAST search have shown the occurrence two variable alleles of the *attB* sites in *dusA* gene of two different strains ATCC 17978 and AB307-0294, both of which are harbouring an empty G08. Given the possibility of having multiple *att* sites, the excision and integration reactions can lead to having multiple insertions occurring at different frequencies depending on the most prevalent or preferable sites. It could also be argued that the importance of such variability in attachment sites is probably minor, due to the low excision frequencies under laboratory conditions.

In the light of these findings it is also possible to re-interpret the data from the study of Farrugia et al 2015 which used the same approach to determine the *att* sites within *dusA*-associated integrases however not in only *A. baumannii* but in various other species with insertions within the *dusA* gene. The data from the latter study showed that the putative attachment sites in *A. baumannii* D1279779 and *Pseudomonas protegens* Pf-5 range between 26 to 28 bp, while the length of the *att* sites is 19 bp in *A. baumannii* ACICU. Further detailed analysis of 92 other *dusA*-specific GIs allowed the identification of direct repeats in these sequences obtained from almost complete genome sequences from the public databases (Farrugia et al., 2015). These GIs were flanked by a pair of 21 bp semi-conserved attachment sites, which is strongly indicating that even in the case of variable integrase protein sequences, *dusA*-specific integrases target a conserved sequence within the *dusA* gene (Farrugia et al., 2015).

This work shows that low copy number plasmids with inducible promoters such as P*lac* promoter can successfully promote integrases to mediate excision of mini-islands that have been cloned in these vectors. Similar system has been exploited experimentally and used as the basis of shuttle vectors, allowing robust expression of integrase genes carried on

various vectors (Ausubel et al., 2004). Moreover, pWSK129-WH plasmids created in this study have incorporated the origin of replication that could be maintained in *A. baumannii* thus facilitates the study of integrase assays in other species of *Acinetobacter*. These two examples of plasmids generated in this work could also be easily utilized further for integration assays under controllable experimental conditions. This could possibly be explained by the fact that the latter *A. baumannii* strains have an intact *recA* thus allowing DNA repair and recombination machinery to cure the DNA damage caused by IPTG.

Several PCR assays were conducted to test for possibly detectable circular forms of G08 and G62 in *A. baumannii* have failed and therefore led us to question whether these integrases were functional. Initial work in this study have demonstrated that pWSK129-derived plasmids are efficient for assessing integrase activity in *E. coli* background. This was evidenced by the successful detection of the circular mini-islands after IPTG induction in a time-dependent manner. This plasmid could also allow us to test for integrase activity in compatible species that have not been studied such as other Gram negative species.

Studies in which integrase activity was assessed in *A. baumannii* background are limited, most of which were performed in integron studies in the closely related non-pathogenic species *Acinetobacter baylyi* due to the ease of genetic modification and transformations (Domingues et al., 2012, Starikova et al., 2012, Vaneechoutte et al., 2006). However, these studies showed experimentally that the integrase activity is controlled under negative selection in *A. baylyi* and overtime is subjected to mutations (Domingues et al., 2012, Starikova et al., 2012). In this study, the integrase activity was assessed in *A. baumannii* which could shed light on the fitness cost of these integrases in the species of interest. This data showed that pWSK-WH-derived plasmids are efficient for integrase activity in *A. baumannii* background.

Previous studies showed that the horizontal gene transfer of certain mobile genetic elements like integrons affects the fitness cost of *A. baumannii* strains, suggesting that the presence of integrase genes affects population dynamics (Harms et al., 2013, Starikova et al., 2012). This would also suggest that *A. baumannii* strains growth will be more affected than *E. coli* by the overexpression of integrase genes. However, this is not the case since the *E. coli* strain used in this experiment is DH5α, has been genetically modified to have *recA* mutation and hence will have a reduced homologous recombination events by less than 10,000 fold in comparison to its parent *E. coli* strain K-12. In addition, DH5α will accumulate mutations when subjected to DNA damaging conditions and this would render the strain to be less fit when a recombinase is transformed (Casali, 2003).

The comparative analysis of the fold change after IPTG induction showed significantly higher circular forms in pWSK129-based plasmids than the pWSK129-WH-based plasmids in *E. coli*. This could be explained by the fact that pWSK129 plasmid is better maintained in *E. coli* and can be better induced without having the origin of replication for *A. baumannii* which is present in the other pWSK129-WH plasmid. However, since the integrase expression has not been tested in both *E. coli* and *A. baumannii* backgrounds, and the fact that each plasmid has a different origin of replication, this observation may not be relevant. This could also explain the statistically insignificant difference between the level of detection of circular intermediates in *A. baumannii* and *E. coli* pWSK129-WH plasmids.

It should be noted that cloning of the integrase along with their mini-islands of G08 and G62 directly into pWH1266 was attempted to avoid the negative effect that could be exerted by the presence of two origins of replications in the same shuttle vector and to ensure that the assays can be run in *A. baumannii* instantly. However, these attempts have resulted in various deletion on the integrase gene after being cloned into pWH1266 that could be due to the deleterious or toxic effect of integrase insertion.

These data were consequently not considered comparable from which reliable conclusions could be drawn for the integrase assay. For this reason, these results have not been shown here.

Bacterial species have adapted variable pathways in response to environmental stresses, including the SOS response which has been known to be activated for the repair of DNA damage (Weinert and Hartwell, 1988). In addition, the role of SOS response in increasing the mobility of MGE including integrons and ICEs has been recently investigated via the LexA which acts as a global regulator for more than 40 genes within the cell (Beaber et al., 2002, Butala et al., 2009, Guerin et al., 2009, Justin Courcelle et al., 2001), including integrase expression (Guerin et al., 2011).

The classical SOS response was first identified in *E. coli* in response to DNA damage and have been extensively studied (Gudas and Pardee, 1975). In a typical SOS response, RecA surrounds single-stranded DNA building up at stalled replication forks which then mediates proteolytic self-cleavage of the LexA resulting in expression of the genes downstream of the binding box (Rao and Radding, 1995). This mechanism ensures that expression of any damaging and harmful proteins can be minimized such as recombination mediating proteins or error-prone polymerases. However, these enzymes can be protective for the cells under stressful conditions therefore will be activated accordingly.

Combating DNA damage is one of the main triggers at which this response is particularly activated. In addition, a number of other factors and DNA damaging conditions can induce an SOS response such as desiccation, UV irradiation and exposure to certain antibiotics such as quinolones (Phillips et al., 1987). Once the involvement of SOS response in increasing expression of enzymes like integrases of MGEs has been discovered, the role of antimicrobial chemotherapy in the spreading of multidrug resistance have been realized (Guerin et al., 2009). Since *A. baumannii* as other *Moraxellaceae* family lack a LexA homologue, the role

of RecA in regulating the DNA damage response has been addressed by several studies (Aranda et al., 2011, Aranda et al., 2013, Hare et al., 2006, Peter J. G. Rauch et al., 1996).

The SOS in response to mitomycin C, has been studied and research has shown that UmuDAb acts as a direct putative regulator of the DNA damage SOS response in *A. baumannii* as a result of mitomycin C exposure (Aranda et al., 2013). After mitomycin C being added, 39 genes were induced, including the *umuD* gene in which its product specifically binds to a palindromic sequence in the promoter region as demonstrated by the electrophoretic mobility shift assays (Aranda et al., 2013). In the latter study found that at least eight genes are essential components of the UmuDAb regulon. In addition, when *umuDAb* gene was inactivated, all of the induced genes after DNA damage were deregulated (Aranda et al., 2013).

The data presented here have confirmed that the use of mitomycin C can effectively induce the excision of the GIs G08 and G62 via the visualization of the bands in gel electrophoresis. This have also facilitated the sequencing of the circular intermediates and the chromosomal junctions after excision, which was not possible without mitomycin C induction. The qPCR data discussed in section (3.5.1), demonstrate that both islands excise also without induction however, at a lower frequency as compared to the mitomycin C induced GIs.

Our understanding of these stress responses is crucial for developing strategies on how to prevent the spread of the global MDR pathogens. Proper planning of prescription guidelines in future settings should take into account these important findings. Controlled and restricted persecutions of DNA-damaging agents and antibiotics involved in mediating SOS-response could facilitate in slowing the rates of the genetic events resulting in mobilisation of TE.

The level of detection of circular intermediates were taken on crude DNA

preparations of cultures at 8 hours after subculture, which was the time at which readings were taken for the other experiments in this study. Other studies have established that DNA was extracted at 20 h, a time point at which copy number of plasmids are at maximum levels in *E. coli* (Skulj et al., 2008) . However, in this study, DNA extraction at this time point was avoided as many dead cells would have interfered with the results of the assay. Moreover, the assays in this study are relatively quantifying the circular forms rather than the absolute copy number of plasmids. Nevertheless, it could be argued that the results would have been variable if the experiments were performed at other time points.

The growth curves of both plasmids pWSK129 and pWSK129-WH in *E. coli* DH5α background have shown differences in the pattern of growth after IPTG induction (Figure 3.9). This could be due to the presence of origin of replication from pWH1266 which may have affected the growth in *E. coli* DH5α negatively resulting in problems during cell division and replication. However, the lack of evidence that *E. coli* DH5α is capable of recognizing pWH1266 *oriR* and the presence of two origins of replications in some shuttle vectors like pFLP2 (Hoang et al 1998), could lead us to further explore other possibilities to explain such differences.

During the construction of pWSK129-WH plasmids, caution was taken when introducing the pWH1266 *oriR* so that it did not interfere with function of the plasmid or affect the host strain in any way. The pWH1266 *oriR* was inserted downstream of the integrase gene ensuring that it does not affect any neighboring genes. In addition, the increased size of the plasmid by 1758 bp due to insertion of the new *oriR* was still in the acceptable range and considered as a small plasmid of less than 10 kb not resulting in any instability of the insert (Ausubel et al., 2004).

Chapter 4 Construction of mutant alleles and suicide vectors for mutagenesis of targeted *A. baumannii* genomic islands G08 and G62 for metal efflux analysis

4.1 Background

4.1.1 Efflux systems in *A. baumannii*

There are five major families of the efflux pumps, which are proteins responsible for extruding the toxic substances from the cell into the external environment. The main groups are as follows: Major Facilitator Superfamily (MFS), Multidrug and Toxic Efflux (MATE), the Tripartite Resistance-Nodulation-Division (RND), Small Multidrug Resistance (SMR) and ATP Binding Cassette (ABC) (Figure 4.1) (Piddock, 2006). These efflux systems use different sources of energy for their activity such as the ATP hydrolysis used by the ABC transporters, the proton- motive force fueling the MFS, RND, and SMR and the Na+/H+ drug antiport systems of the MATE transporters (Piddock, 2006). Some efflux systems have a specific substrate for binding such as the Tet(A)/Tet(B) specific for the efflux of tetracycline, whereas other systems have a broad range of substrates extruding a wide range of antibiotics and compounds such as the AdeABC efflux pump (Vila et al., 2007) (Table 1.3, Chapter 1). The AdeABC efflux pump belongs to the resistance-nodulation-cell division family and it is a multi-drug efflux system, which can pump out several antibiotic groups such as aminoglycosides, tetracyclines, fluoroquinolones, chloramphenicol, β-lactams, macrolides and trimethoprim (Marchand et al., 2004). The is of particular concern, as often overexpression of these pumps result in antibiotic resistance of more than one class as well as some dyes, detergents and disinfectants including some commonly used biocides (Marchand et al., 2004).

These pumps are originally not antibiotic-efflux pumps, but are involved in the elimination of waste products, in addition to extrusion of regulation or defense molecules produced by the host. These MDR pumps have evolved later on to mediate resistance to various classes of antibiotics which poses a huge concern for antibiotic therapy especially when they have a wide substrate specificity (Piddock, 2006). Studies have also

described that bacteria can minimize the entry of antibiotics into the cell by reducing the number of targets such as porins in the cell surface (Vila et al., 2007). Replacement of the susceptible antimicrobial targets and acquiring novel pumps for extruding antimicrobial agents from cell have also been described as common resistance mechanisms in bacteria (Andersson and Hughes, 2010). Efflux pumps belonging to the Major Facilitator Super-family (MFS) such as tetracycline repressor protein class A and class B (TetA/B), chloramphenicol efflux protein (CmlA), the RND such as AdeABC and the MATE, like AbeM have been well studied for their function and substrate in various *A. baumannii* strains (Table 1.2, chapter 1) (Vila et al., 2007).

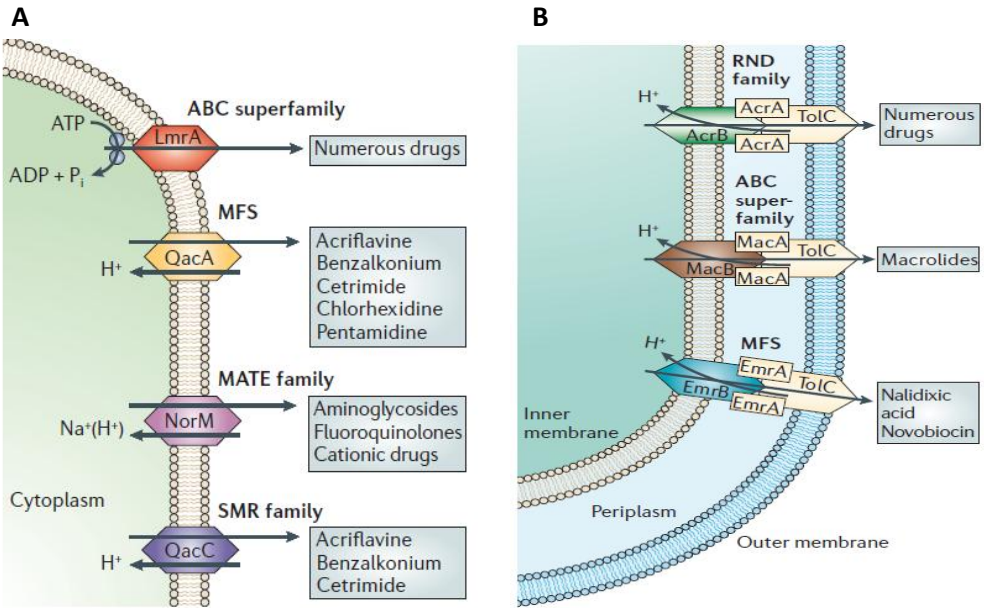


Figure 4.1 Multidrug-resistance efflux pumps.

There are five families of multidrug-resistance efflux pumps: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division family (RND). A diagrammatic representation of the structure and membrane location of efflux pumps from each of these families is shown, (A) for Gram-positive bacteria. Common examples of the individual proteins that form each class of efflux pump are indicated. Antibiotic substrates and examples of other substrates are also listed for each class of efflux pump. (B) Multidrug-resistance efflux pumps expressed by Gram-negative bacteria usually have several components, and the outer membrane protein is typically TolC. Pi, inorganic phosphate. (Piddock, 2006).

The G08 and G62 GIs harbour a set of heavy metal resistance genes that are identical (as described in chapter 3). Importantly, both GIs are of interest in this study as they were hypothesized to confer resistance phenotypes related to heavy metals (Di Nocera et al., 2011). The G62, carries, with respect to G08, additional set of genes encoding for metal resistance as shown in the reference *A. baumannii* strain ATCC 17978. By the time of writing this thesis, only a few studies have described the G62 and the presence of similar resistance island had been shown in an *A. baumannii* hyper-virulent and outbreak-associated isolate, LAC-4 in China (Ou et al., 2015). In this LAC-4 clinical isolate, the G62 was referred to as copper resistance gene cluster and was sandwiched between two copies of IS*Aba26* element. The ATCC 17978 genome has been extensively analysed in previous studies (Smith et al., 2007) and 13 putative zinc/copper resistance efflux pumps have been identified, including the efflux pumps present in G08 and G62 (Hassan et al., 2017) (Figure 4.2).

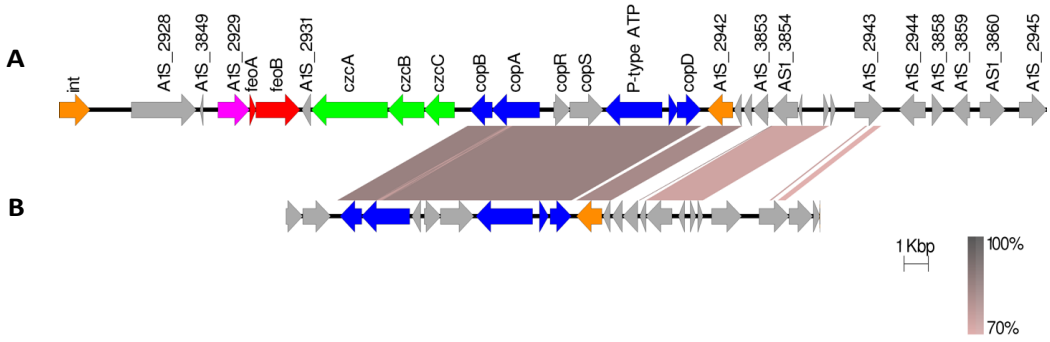


Figure 4.2 Putative efflux systems identified in *A. baumannii* strains genomic islands G62 in (A) (ATCC 17978) and G08 in (B) (AB0057).

The annotated sequences were extracted from GenBank and subsequently aligned and visualised by the Easyfig tool (Sullivan et al., 2011). The genes involved in encoding copper efflux systems are shown in blue, all *czc*-like genes *czcA*, *czcB* and *czcC* encoding cadmium, zinc and cobalt resistance are represented in green colour, genes encoding ferrous iron transport proteins iron shown in red (*feoA* and *feoB*), a putative further heavy metal efflux system A1S_2929 is shown in pink, genes encoding possible mobilisation of the DNA are coloured in orange and all other genes in grey. The image is drawn in scale and the percentage of DNA identity between various regions is shown by gradient shading.

Using transcriptional analysis, it has been observed that several transporters were upregulated in response to addition of either copper or zinc or both when used in combination (Hassan et al., 2017). This identification of the putative copper/zinc efflux pumps was not only defined for strain ATCC 17978, but also extended by comparative genomic analysis to other *A. baumannii* strains (Hassan et al., 2017). Comparative analysis of putative zinc and/or copper efflux systems in *A. baumannii* and *A. baylyi* ADP1 other than the ones in G08 and G62, identified a number of genes ranging between eight (strain SDF) and 18 (strain AB6870155) in each of the strains examined, all of which were chromosomally located (Eijkelkamp et al., 2014, Hassan et al., 2017). Further BLAST search of revealed that five strains harbored more than ten genes encoding putative zinc and/or copper efflux components, including ATCC 17978, ATCC 19606T, AB0057, AB6870155, and ACICU. The chromosomal region harbouring these genes are likely to have been acquired laterally on mobile genetic elements, with the G62 of ATCC 17978 and LAC-4 being the largest of these elements (Ou et al., 2015). Strains AB0057 and AB6870155 encode the related G08 island, which has inserted into the *dusA* locus (Farrugia et al., 2015).

In previous studies, the main focus was on identifying the efflux systems with their phenotypes, however, no mutants in the single transport systems were constructed to confirm the substrate specificity of the efflux pumps. Moreover, several studies have extensively investigated the mechanisms involved in acquiring transition metal ions such as iron and zinc (Dorsey et al., 2003, Eijkelkamp et al., 2011, Gaddy et al., 2012, Hood and Skaar, 2012, Mortensen et al., 2014, Nwugo et al., 2011, Penwell et al., 2012, Zimbler et al., 2009). However, there is less literature available on understanding the metal efflux in *A. baumannii* especially in response to extracellular metal ion stress and intoxication. These metals play a crucial role in the viability of the bacteria and are essential for many cellular processes such as the role of zinc in cellular adhesions to the host

cells (Hood and Skaar, 2012, Mortensen et al., 2014, Smani et al., 2012). Bacterial cells have certain processes by which the metals are tightly regulated in such a way that their abundance intracellularly would not cause any intoxication resulting in uncontrolled binding of these metals to the non-cognate binding sites such as metalloproteins Fe-S clusters (Bruins et al., 2000, Macomber and Imlay, 2009, Xu and Imlay, 2012).

Interestingly, metals have been used in ancient ages for treating certain infections due to their antibacterial properties (Becker and Skaar, 2014), including the use of silver (Ag) for treating cholera as well as incorporating the modern use of Ag in healthcare applications such as Foley's catheters (Edwards-Jones, 2009). In addition, copper (Cu) has been used by several cultures for hygienic and antimicrobial purposes thousands of years ago, including the use of Cu for sterilizing water and wounds (Samanovic et al., 2012).

Metal efflux is one of the most distinct mechanisms facilitating the bacteria to prevent metal toxicity among other several mechanisms (Hantke, 2001, Nies, 2003). Cu is mainly regulated by the P-type ATPase efflux systems, such as CopA from *E. coli*, which exports this metal from the cytoplasm (Drees et al., 2015). Cu resistance is mediated through a number of proteins in various species such as the CopB outer membrane protein (OMP) and periplasmic multi-copperoxidases, such as CopA of *Pseudomonas syringae* and CueO of *E. coli* (Cha and Cooksey, 1991, Grass and Rensing, 2001). Cu and Zn can also be exported by the Heavy Metal Efflux (HME) family of transporters, which are a subclass of the RND family of efflux pumps in Gram-negative bacteria. The HME transporters are multimembrane-spanning protein complexes, comprising of inner membrane proteins, periplasmic membrane fusion proteins and OMPs (Alvarez-Ortega et al., 2013, Yamaguchi et al., 2015). These protein complexes act as a group to export metal ions into the extracellular environment across the outer membrane, thus preventing intracellular toxicity. Both metal Zn and Cu are substrates of the HME efflux systems, which could be exemplified by the well-characterized

CusCFBA from *E. coli* as a Cu-exporting HME transporter (Fung et al., 2013).

Another efflux system of metals is the CDF family of transporters, which includes members for exporting Zn and/or Cu. An example of a well characterized CDF is YiiP from *E. coli*, with detailed x-ray crystal structure of the protein provides better insights into the metal ion pathway of these efflux systems (Lu and Fu, 2007). Despite these systems being well characterized in a wide array of bacterial species, the mechanisms by which *A. baumannii* responds to metal toxicity, especially those mediated by Zn, have not been elucidated (Hassan et al., 2017).

The investigation of metal toxicity in *A. baumannii* is a very important aspect in which will provide better insights in how the bacteria adapt for the translocation of these metals to achieve survival in stressful environments. However, as the studies in this part are few, the metal toxicity in *A. baumannii* remained poorly understood. To this date, no published reports have assessed similar metal phenotypes in deletion mutants in *A. baumannii.* It was therefore deemed necessary to shed light on the effect of exposure to the various metal compounds in our study by understanding the contribution of the G08 and G62 islands in the metal efflux in *A. baumannii* isolates.

Table 4.1 Annotations of the predicted ORFs found on the 32.3 kb G08 *A. baumannii* strains

Locus tags			
AB0057	AYE	A424	annotation
AB57_0642	-		hypothetical protein
AB57_0644	ABAYE3221	A424_1287	integrase/recombinase
AB57_0643	ABAYE3220	A424_1288	hypothetical protein
AB57_0645	ABAYE3219	A424_1289	hypothetical protein
AB57_0646	ABAYE3218	A424_1290	hypothetical protein
AB57_0647	ABAYE3217	A424_1291	hypothetical protein
AB57_0648	ABAYE3216	A424_1292	hypothetical protein
AB57_0649	ABAYE3215	A424_1293	hypothetical protein
AB57_0650	ABAYE3214	A424_1294	hypothetical protein
AB57_0651	ABAYE3213	A424_1295	hypothetical protein
AB57_0652	ABAYE3212	A424_1296	exported protein
AB57_0653	ABAYE3210	A424_1297	hypothetical protein
AB57_0654	ABAYE3209	A424_1298	exported protein
AB57_0655	-	A424_1299	hypothetical protein
AB57_0656	ABAYE3208	A424_1300	phage/plasmid-related protein
AB57_0657	ABAYE3207	A424_1301	*copD*, copper resistance protein
AB57_0658	ABAYE3206	A424_1302	*copC*, copper resistance protein
AB57_0659	ABAYE3205	A424_1303	*actP*, copper- P-type ATPase
AB57_0660	ABAYE3204	A424_1304	*copS*, sensor kinase
AB57_0661	ABAYE3203	A424_1305	*copR*, regulator
AB57_0662	ABAYE3202	A424_1306	exported protein
AB57_0663	ABAYE3201	A424_1307	*copA*, copper resistance protein
AB57_0664	ABAYE3200	A424_1308	*copB*, copper resistance protein
AB57_0665	ABAYE3199	A424_1309	transcriptional regulator
AB57_0666	ABAYE3198	A424_1310	hp

Table 4.2 Annotation of G62 associated ORFs

ATCC17978 locus tags		annotation
A1S_ 2927	425	phage integrase
A1S_ 2928	869	Restriction endonuclease
A1S_ 2929	437	cation efflux system p
AS1_3850	86	*feoA*, ferrous iron transport protein A
A1S_ 2930	516	*feoB*, ferrous iron transport protein B
A1S_ 2931	86	hypothetical protein
A1S_ 2932	976	*czcA*, Heavy metal efflux pum
A1S_ 2933	445	*czcB*, heavy metal RND efflux membrane fusion p
A1S_ 2934	339	*czcC*, heavy metal RND efflux outer membrane p
A1S_ 2935	264	*copB*, copper resistance p B precursor
A1S_ 2936	635	*copA*, copper resistance p A precursor
A1S_ 2937	175	*copR*, heavy metal response regulator
A1S_ 2938	430	*copS*, heavy metal sensor kinase
A1S_ 2939	716	ATPase E1-E2 type: Copper-translocating P-type ATPase
A1S_ 2940	107	*copC*, copper resistance protein
A1S_ 2941	297	*copD*, Copper resistance D
A1S_ 2942	312	hypothetical protein
AS1_3851	98	hypothetical protein
AS1_3852	109	hypothetical protein
AS1_3853	208	hypothetical protein
AS1_3854	357	hypothetical protein
AS1_3855	48	hypothetical protein
AS1_3856	72	hypothetical protein
AS1_3857	67	hypothetical protein
A1S_ 2943	391	hypothetical protein
A1S_ 2944	282	hypothetical protein
AS1_3858	163	hypothetical protein
AS1_3859	239	hypothetical protein
AS1_3860	351	hypothetical protein
A1S_ 2945	340	hypothetical protein

To validate this claim, mutant construction with deleted G08 and G62 by means of allelic exchange will be addressed in this chapter. Many genetic tools have been developed for introducing mutations in bacterial chromosomes and have been described in detail (Martinez-Morales et al., 1999, Murphy et al., 2000, Yu et al., 2000). One of the most useful tools are suicide vectors-based allelic exchange. There are two major types of suicide plasmids that have been engineered. The first type is temperature-sensitive plasmids like pSC101 (Cornet et al., 1994), pKO3 and their derivative plasmids (Link et al., 1997). Second are plasmids carrying the replication origin of R6K, such as pCVD441 and its derivative plasmids

(Donnenberg and Kaper, 1991) .

To ensure the occurrence of successful homologous recombination at both right and left flanking regions, the following two steps are crucial. Initially, the suicide vector is integrated into the chromosome of the target strain resulting in single cross-over strains (meroploids). Then, a second homologous recombination event takes place resulting in either abortive allelic exchange in which both the suicide vector and the mutant allele are lost, or a successful allelic exchange occurs in which the suicide vector and the original native allele are lost. In the latter case, a knock-out mutant strain is created figure (4.3).

For the second cross-over homologous recombination to occur, the suicide vector usually carries a counter-selectable marker to select the clones without the suicide vector. The application of allelic exchange technique has been employed in a wide range of bacterial species and has been described in many publications (Aranda et al., 2011, Ausubel et al., 2004, Blomfietd et al., 1991, Reyrat et al., 1998, Rosen et al., 2008, Struve et al., 2008). The recombinant mutant alleles are composed of an antibiotic cassette of selection, flanked by sequences that are homologous with the upstream and downstream flanking regions of the target gene/locus to be deleted. The construction of the recombinant allele is described in detail in methods chapter 2 (Section 2.9.7). In most cases published in the literature, the counter-selectable marker *sacB* conferring sucrose-sensitivity to a number of Gram-negative bacteria, had been used by researchers (Blomfietd et al., 1991, Gay et al., 1985).

4.2 Aims and objectives

The aims and objectives in this chapter are as follows:

- To describe the generation of two deletion mutants using the well-known method for genetic manipulation, suicide-vector based allelic exchange. This approach used for generation of mutants by replacing the target GIs, G08 and G62 with an antibiotic marker.

- To investigate the phenotypes of G08 and G62 in both wild type strains and their G08/G62 deletion mutants. The investigation of the phenotypes of the resultant mutant strains is well known as reverse genetics analysis. The phenotypic assays carried out to test possible differences in heavy metal resistance of A424 and ATCC 17978 wild type strains and their respective G08 and G62 deletion mutants will utilise two techniques of susceptibility testing of the MIC/MBC by broth microdilution and disc diffusion method.

4.3 Results

4.3.1 Suicide vector-based allelic exchange for mutant construction in *A. baumannii*

For the construction of deletion mutants of the GIs in *A. baumannii,* the pJTOOL-3 suicide vector was used (van Aartsen and Rajakumar, 2011). pJTOOL-3 is based on a combination of the *E. coli* replicon pDS132 and the standard *E. coli* plasmid pBluescript II KS+ (Alting-Mees and Short, 1989, Philippe et al., 2004) and contains a multiple cloning site. The plasmids pJTOOL-3-G08 and pJTOOL-3-G62 used for the allelic exchange were constructed to contain the flipase recognition target sites (FRT) flanking the *aacC1* gentamicin gene. These are short sequences that would allow Flp-FRT recombination of Flp recombinase-mediated site-specific excision of the gene cassette, and thus allowing for the generation of marker-less mutants. However, marker-less mutants were not made in this study due to time limit and only the knock out strains carrying the resistance cassette were analysed.

A schematic drawing of the double-crossover mediated deletion of the G08 element using suicide vector pJTOOL-3 is presented in Figure 4.3 as an example. Firstly, the plasmid pJTOOL-3 was transformed into the *E. coli* CC118*λpir* strain as a host for replication. Subsequently, pJTOOL-3 was transformed into A424 by conjugation with a conjugative donor strain *E. coli* S17-1*λpir*. The S17-1*λpir* donor strain carries the transfer genes of the broad host range IncP–type plasmid RP4 integrated in its

chromosomes. RP4 can utilize many Gram-negative bacteria as a recipient for conjugative DNA transfer (Simon et al., 1983). The S17-1*λpir* strain is susceptible to gentamicin, chloramphenicol and kanamycin.

The protocol and the vector used for this deletion that was previously described for the deletion of the TnAbaR23 (Kochar et al., 2012) and discussed in chapter 2 of this thesis. Initially, *E. coli* S17-1*λpir* was used to conjugate pJTOOL-3 into *A. baumannii* A424 and ATCC 17978 recipient strains. After overnight incubation, conjugation mixes were plated onto Simmons's Citrate medium agar (SCA) supplemented with gentamicin (6 µg/ml) to select against *E. coli* S17-1*λpir*, to select the single cross-over (SCO) mutants. Various single-crossover merodiploids possessing integrated copies of pJTOOL-3 were readily obtained. Three putative merodiploids were examined and confirmed to have the expected resistance phenotype using patch-plating (gentamicin-resistant and chloramphenicol-resistant). In addition, all three colonies showed positive PCR bands for the single cross-over alleles using primer pair G08-SF and G08-SR for *G08*::*aacC1* primers Gm-SF/G08-SR and primers G62-SF and G62-SR (Table 2.8) for *G62::aacC1,* the mutant allele present on pJTOOL-3 (Figure 4.4).

Next, serial dilutions containing 10^4 to 10^9 CFU of merodiploid *G08*::pJTOOL-3 as well as *G62*::pJTOOL-3 were plated on 6% sucrose containing SCA medium and incubated overnight at 37°C to select clones that had lost the gene (sucrose levansucrase *sacB* gene), and had therefore also lost pJTOOL-3. Colonies obtained after *sacB*-counter-selection were screened for gentamicin (6 µg/ml) and chloramphenicol (30 µg/ml) sensitivity by passaging in parallel in plates with the various selections and analysed by PCR for loss of the suicide vector backbone (primers PS-F and PS-R) and retention of the mutant allele (*aacC1* primers 1LF-F and 1RR-R). The expected genotype was obtained in all three randomly selected colonies that possessed the expected chloramphenicol-sensitive and gentamicin-resistant phenotype (Figure

4.4). Finally, *G08*::*aacC1* and was *G62*::*aacC1* mapped to the G08 and G62 loci by PCR using primers 2LF-F and 2RR-R). (Figure 4.4).

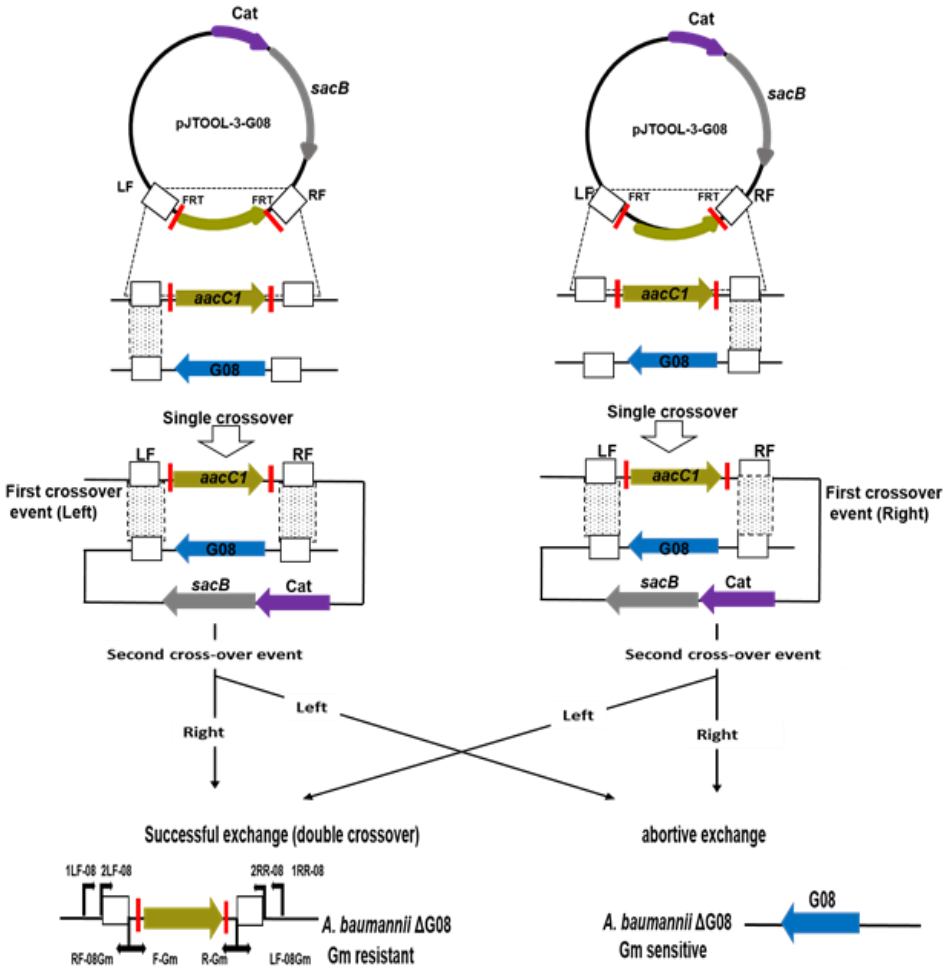


Figure 4.3 Schematic representation of double cross-over mediated deletion of G08 and G62 loci of *A. baumannii* strains using pJTOOL-3 suicide vector (accession number JF756693.1).

In step 1, merodiploids arising out of loss of the free plasmid and selection of clones harbouring a chromosomally-integrated form of the suicide plasmid arising following left- or right-sided single-crossover homologous recombination are shown. After *sacB* counter-selection on plates, putative double-crossover mutants are identified by a chloramphenicol-sensitive, gentamicin-resistant phenotype and verified by colony PCR analysis. (I) Genetic configurations arising from Left/Right or Right/Left (1st crossover/2nd crossover events) homologous recombination mediated successful allelic exchange. (II) Abortive allelic exchange resulting from Left/Left or Right/Right-sided recombination events. Abbreviations: *cat*, chloramphenicol acetyltransferase for chloramphenicol resistance; *aacC1*, aminoglycoside acetyltransferase C1 conferring gentamicin resistance.

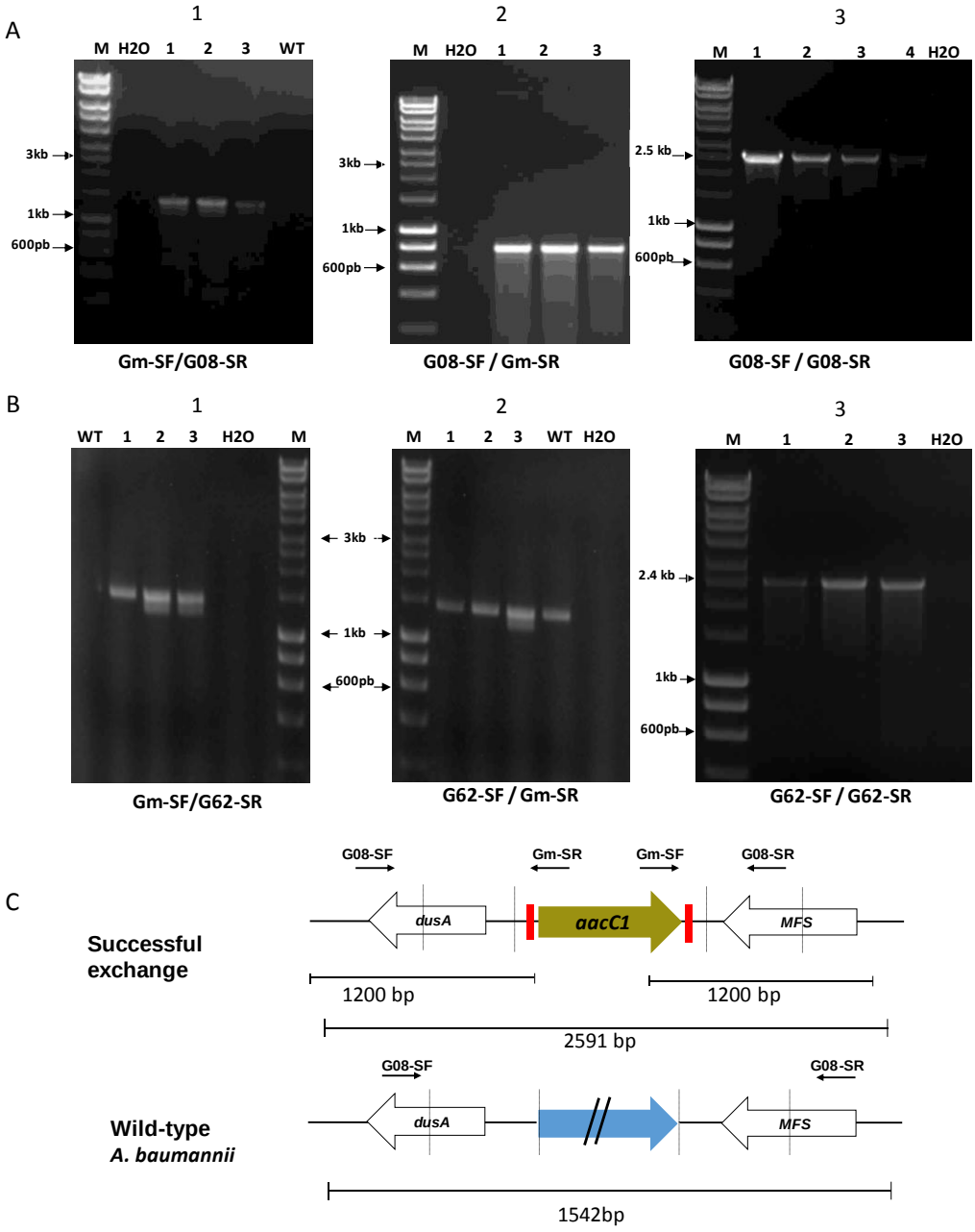


Figure 4.4 Analysis of double cross-over recombinants.

Gel electrophoresis images showing the results of PCR assays to confirm successful allelic exchange events and genotype of putative mutants G08::Gm (panel A) and G62::Gm (panel B). in (A1) PCR assays to confirm the genotype of putative meroploids (SCO), (A2) and (A3) PCR assays to confirm the genotype of putative mutants G08::*aacC1*. In (B1) PCR assays to confirm the genotype of putative meroploids (SCO), (B2) and (B3) PCR assays to confirm the genotype of putative mutants G62::*aacC1*. (C) Schematic representation of the expected sizes for PCR products of all the PCRs are shown below the gel images (G08::*aacC1* mutant is used as an example), with primers indicated by black arrows, FRT sites by red boxes and the flanking regions involved in the allelic exchange indicated by dashed vertical lines.

4.3.2 Growth dynamics A424 ATCC 17978 mutants

The growth curves of A424ΔG08 and ATCC17978ΔG62 mutants with deleted GIs were compared to their A424 and ATCC 17978 wild type counterparts to examine whether there is any change in growth dynamic of the mutant strains following the deletion of the islands. As apparent in the growth curves in figure (4.5), the deletion of the GIs did not have any significant impact on the growth of the mutated strains.

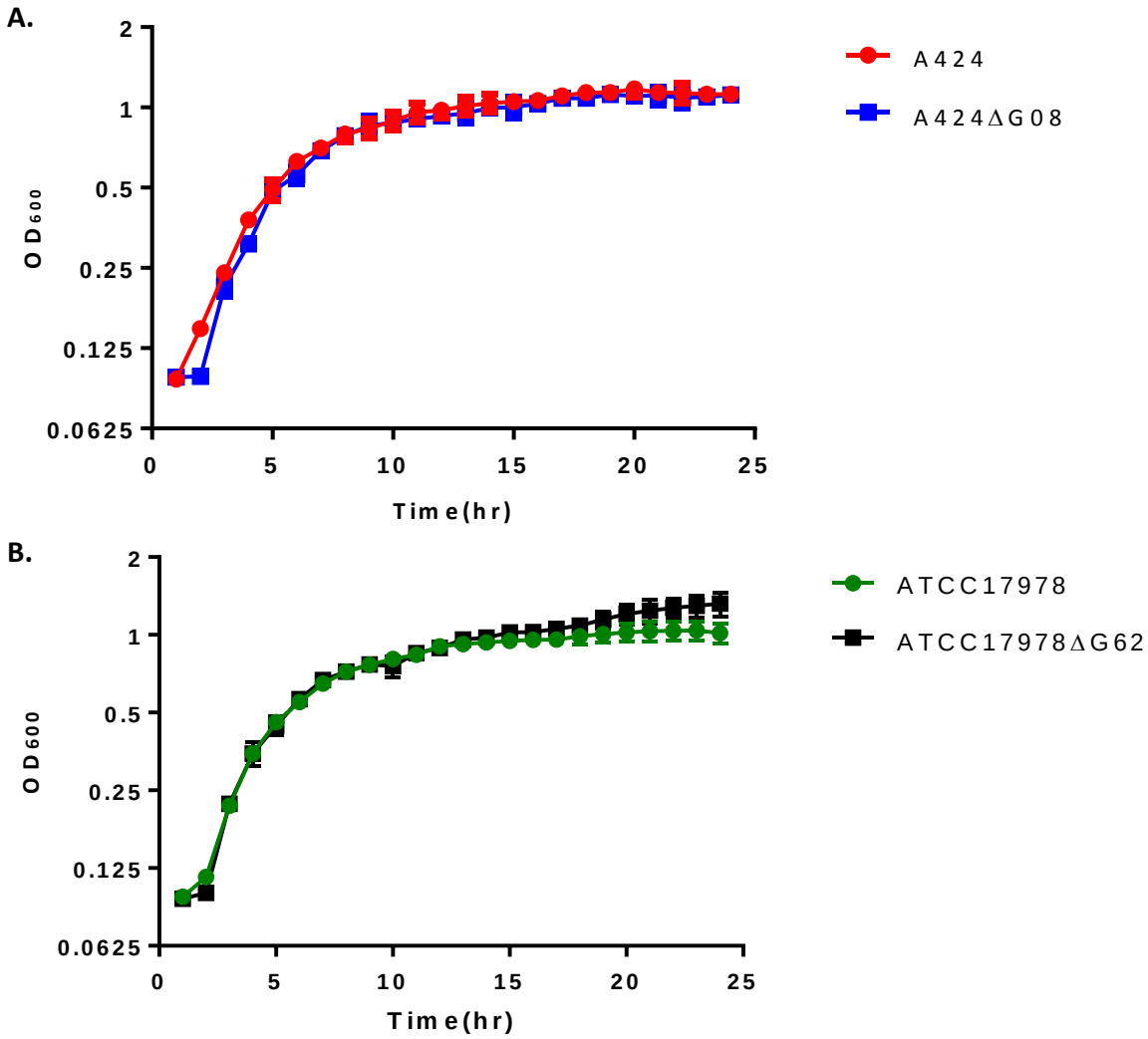


Figure 4.5 Growth curve of wild-type and G08 and G62 deletion mutants.

Data for A424 and its A424ΔG08 mutant are shown in panel A and those of ATCC17978 and its ΔG62 mutant in panel B. The data shown represents the mean value ± standard deviations (error bars) for eight independent replicates. No significance difference in the growth dynamics was observed between A424 WT and its G8 mutant (A). Similarly, with ATCC 17978 WT and its mutant (B).

Bacteria were diluted 1:100, grown in 200 ul in a microtiter plate and the OD values recorded every 10 minutes by a Varioskan® flash spectral scanning multimode reader spectrophotometer.

4.3.3 Phenotypic assays of heavy metals

4.3.3.1 Growth curves of A424ΔG08 and ATCC17978ΔG62 in various heavy metals

The growth dynamics of *A. baumannii* strains A424 and ATCC 17978 and their mutants A424ΔG08 and ATCC17978ΔG62 were assessed in the presence of different metals. Several transition metals were used in the experiments including copper (Cu), zinc (Zn), iron (Fe), manganese (Mn), nickel (Ni), cobalt (Co), cadmium (Cd) and arsenic (As). For each strain and mutant, four biological replicate clones, originally isolated from different transformant colonies, were tested. Initially, the highest concentration tested was 1 mM. However, this concentration was not high enough to determine the MIC for some of the metals tested. Thus, it was necessary to increase the maximum concentrations to 10 mM, starting from an initial stock solution of 1 M. For each strain tested, decreasing concentrations of metals were used (10, 5, 2.5, 1.25, 0.6, 0.15, 0.08, 0.04 mM) by serially diluting the compounds in 96-well microtiter plates. The bacterial growth was monitored by incubation at 37°C in a spectrophotometer which measures the OD_{600} every 10 minutes. As in standard MIC testing, cultures without metals were used as positive controls and media without bacterial inoculum as negative control and run in parallel in each plate. This assay allows monitoring the growth curves to observe any changes in the growth dynamics and to determine the MIC and MBC for each metal.

Initial growth curves showed variable responses to the various metals tested. It was observed that ATCC 17978 was sensitive to extracellular zinc resulting in growth delay starting at 0.6 mM. Further reduction in the ATCC 17978 growth was seen at higher concentrations with growth delay of 1-1.5 hrs at 1.25 mM (Figure 4.6 A1). In the ATCC 17978 ΔG62 mutant the effect on the growth was more pronounced as the growth delay was observed at 0.3 mM of zinc lasting more than 7 hrs throughout the log

phase and the overall growth was much less even after 16 hrs at 1.25 mM (Figure 4.6 A2). The MIC of Zn in ATCC 17978 ΔG62 was 2.5 mM, that is one dilution (2-fold) less than its parent strain, which was inhibited at 5 mM (Table 4.4, Figure 4.7). When the strain ATCC 17978 was challenged with cobalt, the growth was delayed at 0.625 mM and was inhibited at 5 mM, whereas the growth delay of ATCC 17978ΔG62 was observed at 1.25 mM and complete inhibition at 2.5 mM which is one dilution less than the wild type strain (Table 4.4, Figure 4.6 B2 and B2, Figure 4.7). With the addition of cadmium to the ATCC 17978 wild type, the growth delay was less than that seen in cobalt and zinc and the inhibition of growth was evident at 2.5 mM whereas in ATCC 17978ΔG62 again the inhibition was occurring at one dilution less than the wild type which was 1.25 mM (Table 4.4, Figure 4.6 C1 and C2, Figure 4.7). In the case of nickel, there was inhibition at the highest concentration of 10 mM and 5 mM in the ATCC 17978 WT and ATCC 17978ΔG62, respectively. This unexpectedly high level of resistance could be explained by the fact that active export of nickel encoded by genes outside or in addition to those in G62. Interestingly, all the three above mentioned metals zinc, cobalt and cadmium are co-transported by the same efflux system, the *czc*-like transporter which explains the phenotype observed (Nies, 2003). On the other hand, iron, manganese and arsenic had similar phenotype in both ATCC 17978 wild type and ATCC 17978ΔG62 mutants with minimal inhibitory concentrations at 5, 10 and 2.5 mM, respectively (Table 4.4, Figure 4.7).

The effect of heavy metals on the growth phenotypes of *A. baumannii* strain A424 ΔG08 and wild type strain A424 was less evident. There was no significant difference in MICs between A424 wild type and its mutant A424 ΔG08 in all the heavy metals tested, except for manganese which showed 2-fold lower MIC in the A424 ΔG08 mutant (Table 4.4, Figure 4.7). In contrast, copper stress had no apparent effect on the phenotype of both ATCC 17978 wild type and its mutant ATCC 17978ΔG62 at all the concentrations tested (Figure 4.8), which shows that this *A. baumannii* isolate is highly resistant to copper with an MIC of > 10 mM (Table 4.4,

Figure 4.7). This finding is consistent to a previous study (Williams et al., 2016) which showed limited effect of 1 mM copper on *A. baumannii* tested on nutrient deficient media. In both ATCC 17978 and A424 wild-type and mutants, the MBC readings were either the same or one dilution higher than the MIC (Table 4.4).

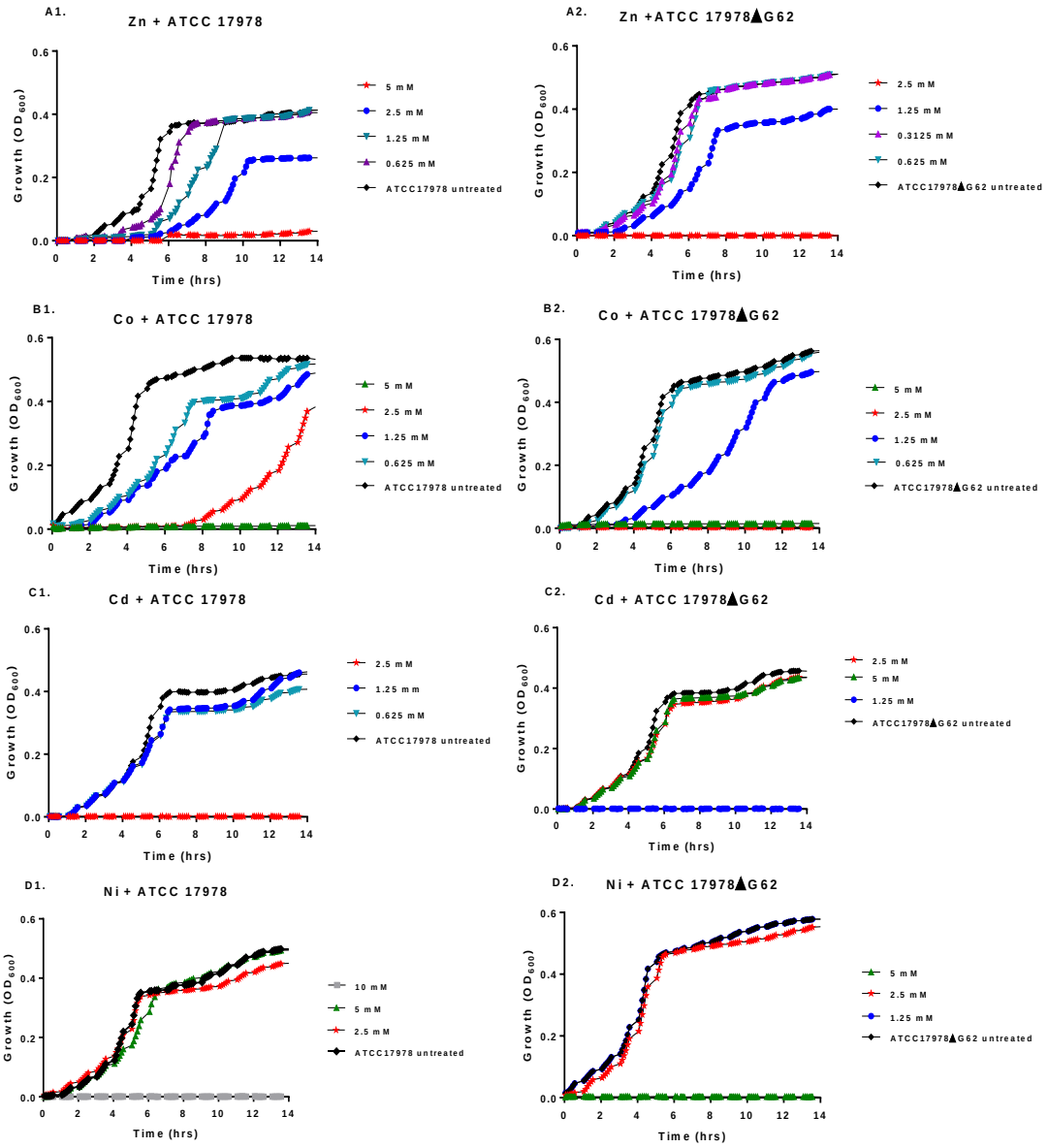


Figure 4.6 Growth curves of *A. baumannii* strains in presence of metals.

Growth curves are shown for ATCC17978 wild-type (panels A1-D1) and ATCC 17978ΔG62 (panelsA2-D2) when exposed to serial dilutions of heavy metals (A) zinc Zn, (B) cobalt Co, (C) cadmium Cd and (D) manganese Mn. The concentration of the metals used are indicated with colours and the legends are shown below the graphs. The positive control is the strain without any metal added and labelled as “untreated” (black filled circle). The assay is performed in at least four biological replicates for each strain. The graphs shown are only one replicate representative of the four independent biological replicates. Bacteria

were grown in a microtiter plate in LB medium and turbidity was monitored every 10 minutes in an Eon Bioteck spectrophotometer.

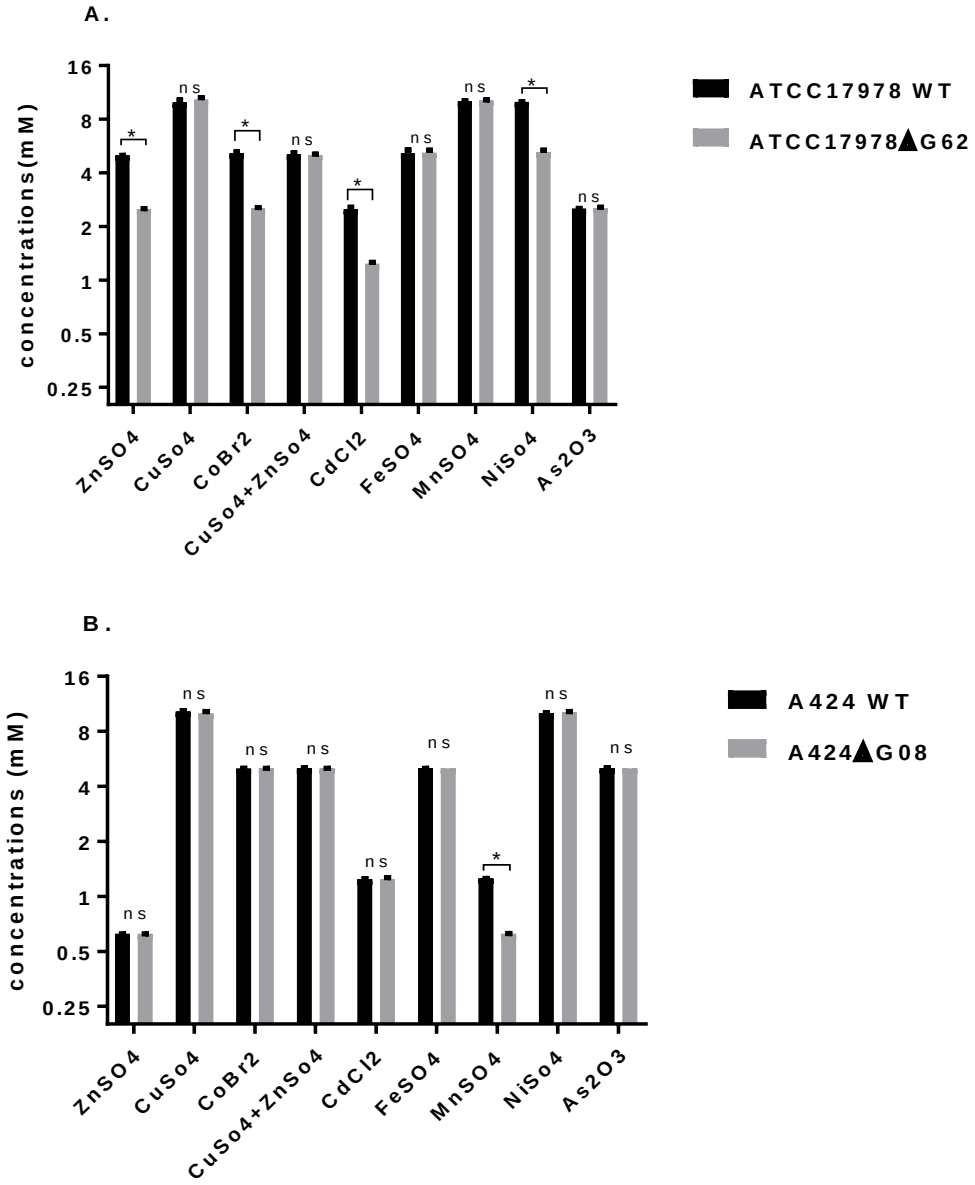


Figure 4.7 Minimal inhibitory concentrations (MICs) of different heavy metals.

A. baumannii strains ATCC 17978 (A) and A424 (B) wild type (black) and mutants (grey) were tested using four independent colonies of wild type (form the same stock) and four independent transformant mutants for both ATCC17978ΔG62 and A424ΔG08. Statistical analysis was performed using two-way ANOVA, where * *P* value <0.05 and ns *P* value >0.05 (not significant).

4.3.3.2 Zinc and copper combination have synergetic effect in impairment of growth

Treatment with both zinc and copper showed a synergistic impact on *A. baumannii* ATCC 17978 growth as the amount of growth delay was greater than that observed for the individual treatments with zinc or copper (Figure. 4.8). A similar effect was observed with A424 however, to a lesser extent in comparison to ATCC 17978. This data is consistent with a previous study that has examined the impact of the Zn and Cu combination on the growth of various *A. baumannii* strains (Hassan et al., 2017). In that work, data showed that ATCC 17978 as well as the SDF strains were strongly affected by combined Zn and Cu stress (Hassan et al., 2017). This contrasts with the effect of solid brass (Zn + Cu) on *A. baumannii* survival, which was shown in a previous study to have a more moderate antimicrobial effect as compared to Cu alone (Williams et al., 2016).

The combination of copper with other transition metal ions, manganese, cobalt, iron and nickel was also attempted to see if there was any significant impact on susceptibility phenotypes. The combination of copper with nickel resulted in MIC of 2.5 mM in ATCC 17978 ΔG62 and A424 ΔG08, a one dilution less (log2) than their wild type strains ATCC 17978 and A424 (Table 4.4). The other combination of copper and cobalt had also a one log lower MIC of 1.25 mM in ATCC 17978 ΔG62 as compared to ATCC 17978 which showed an MIC of 2.5 mM, whereas the MIC remained unchanged at 1.25 mM for both A424 wild type and A424 ΔG08 for the same metal combination of copper and cobalt (Table 4.4). Interestingly, both of the previous metal combinations resulted in lower MICs than the MICs of nickel and cobalt alone in both ATCC 17978 and A424 and their mutants (Table 4.4), which was showed minor statistical significance (P<0.05). Previous data measuring the intracellular metal concentration in *A. baumannii* strain ATCC 17978 after zinc and copper combination treatment, showed reduced copper accumulation was specific to copper alone and less for other combinations of metals (Hassan et al., 2017). In that study however, no data were shown regarding the impact of these various metals combination on the growth dynamics or MICs.

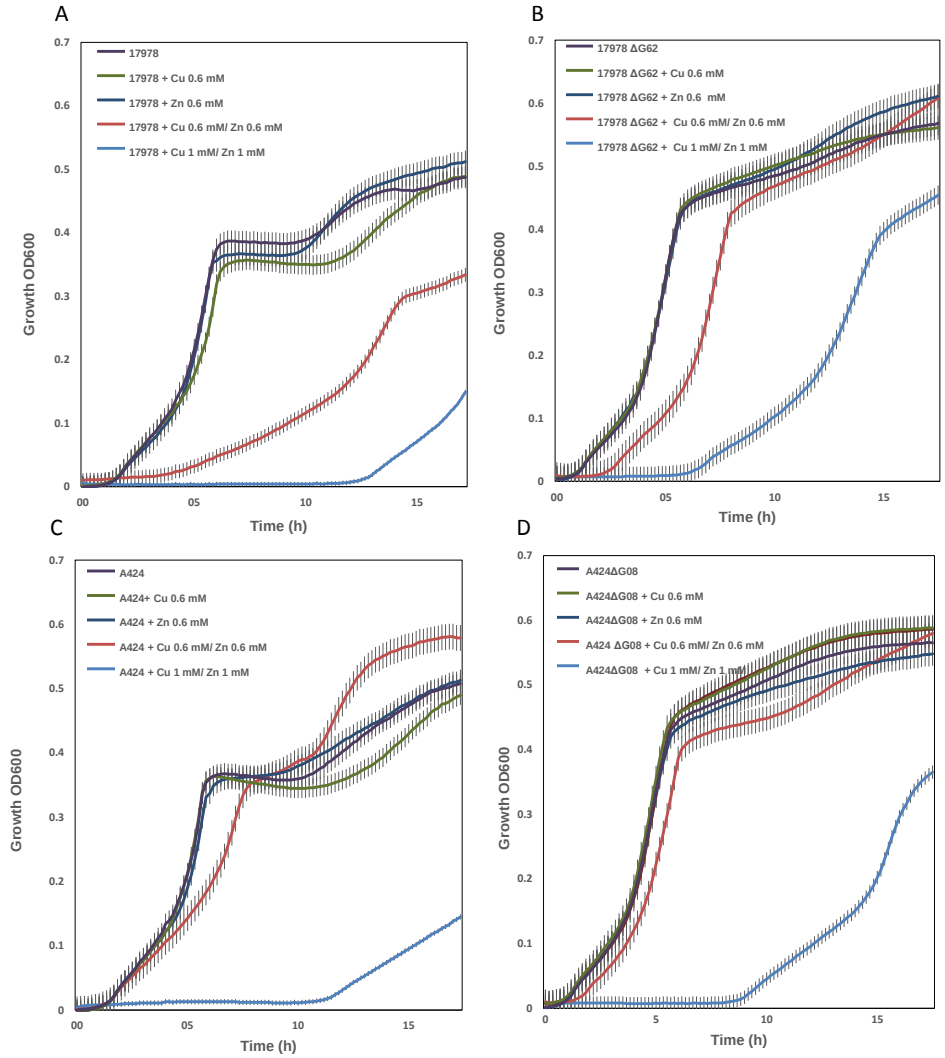


Figure 4.8 The effect of zinc and copper stress on *A. baumannii* growth.

Growth as determined by measuring the optical density at 600 nm (OD600) of *A. baumannii* strain (A) ATCC 17978 and (B) ATCC 17978 ΔG62 (C) A424 and (D) A424 ΔG08 mutants under 0.6 mM and 1 mM concentrations of Zn, Cu or Zn + Cu (n≥4) as indicated by the different colours in the legend in the left upper side of each image. The data are the mean of at least four biological replicates (±SEM).

4.3.4 Metal susceptibility testing by disc diffusion

The disc diffusion method was used to evaluate the susceptibility of the *A. baumannii* strains A424 and ATCC 17978 and their mutants to various heavy metals. This assay was performed using two different methods for

comparison. Initially, the empty disc papers were placed in a petri dish uniformly inoculated with the strain to be tested. 10 µl of 1M metal compound is dropped in the centre of the blank disc and allowed to dry before incubation. However, this approach has resulted in overlapping between the zones of inhibition of the various compounds rendering the reading to be difficult. This was avoided by dropping only 5 µl of the 1 M metal compound to enable better recording of the diameter. In the second method, the filter paper discs were impregnated with the 5 µl of the 1 M metal to be tested and allowed to dry before application to the agar plate. The sensitivity to the compounds is judged according to the diameter of the zone of inhibition as described by CLSI protocols. Preliminary experiments were performed to examine the discrepancies between the readings taken from the two different approaches, and have shown that they were very minimal and therefore considered to be insignificant (Table 3.4, Figure 4.9 and Figure 4.10). The second method was chosen as a standard approach in this study due to less overlap between inhibition zones thus enabling better interpretation of the MIC data, and the assay was repeated at least three times for confirmation. In this method, filter paper discs are impregnated with the metal stock solution to be tested and disks are allowed to dry before application to the agar plate. The susceptibility to the compounds is judged according to the diameter of the zone of inhibition as per the CLSI protocols. The relatedness between these methods usually is dependent on various factors like the antimicrobial compound and the species to be tested with 70% to 90% consistency in MIC between broth microdilution and disc diffusion (Dickert et al., 1981) .

The disc diffusion method results are shown in figure 4.9. The measurements of the zones of inhibition are summarised in table 4.4. Generally, the order of susceptibility was observed to be the same in both ATCC 17978 and A424 at the following ascending order: cadmium (22 mm), nickel (17 mm), zinc=cobalt (15± 3 mm), iron (12 mm), copper (10 mm) and Manganese (9 mm). As shown in the figure 4.9, cadmium, cobalt and nickel resulted in the largest zone of inhibition values of 24 mm and

22 mm for cadmium and 15 mm and 19 mm for cobalt, in ATCC 17978 and A424 wild type strains respectively. In the mutant strains ATCC 17978 ΔG62 and A424ΔG08 the zone of inhibition for cadmium has increased to 26 mm and 23 mm, respectively. For cobalt, the zone of inhibition of has increased from 15 to 22 mm in the ATCC 17978 ΔG62 mutant. Similarly, for nickel it has increased from 17 to 22 mm. By contrast, the zone of inhibition for cobalt remained the same at 19 mm for the A424 ΔG08 mutant. All the remaining metals (zinc, copper, manganese, arsenic and iron) had minimal zones of inhibition and the strains could be considered resistant for the metal concentrations used in both mutants and wild type strains of A424 and ATCC 17978.

Table 4.3 Metal susceptibility testing for *A. baumannii* ATCC17978 and A424 and their mutants ATCC17978ΔG62 and A424ΔG08 compared using two approaches

	Zone diameters (mm) by dry disc				Zone diameters (mm) wet disc			
	ATCC17978	**ATCC17978 ΔG62**	**A424**	**A424 ΔG08**	**ATCC17978**	**ATCC17978 ΔG62**	**A424**	**A424 ΔG08**
$ZnSO_4$	12	12	12	12	12	12	14±1	15±1
$CuSO_4$	9	9	9	9	9	9	9	9
$CdCl_2$	22	21	24	26	25	24	26	26
$MnSO_4$	8	8	9	7	9	8	7	8
$FeSO_4$	9	8	9	9	9	9	8±1	8±1
$CoBr_2$	16	16	18	15	16	17	16	17
$NiSO_4$	18	17	17	15	18	18	15	15
As_2O_3	9	9	9	9	9	9	9	9

There was a clear overlap between cadmium and nickel's zones of inhibition in ATCC 17978 ΔG62, A424ΔG08 and their parental strains ATCC 17978 and A424 (Figure 4.9). In addition, there was slight overlap between cobalt and nickel zones of inhibition in ATCC 17978 parent strain and ATCC 17978 ΔG62, which was not observed in the parent wild type strain A424, however shown in A424 ΔG08. Preliminary disc diffusion assays also revealed overlapping of inhibition zones for almost all of the metal compounds tested (Figure 4.10). There is a possibility that these metals are a co-transported by the same efflux systems, however this claim needs to be extensively explored to be validated.

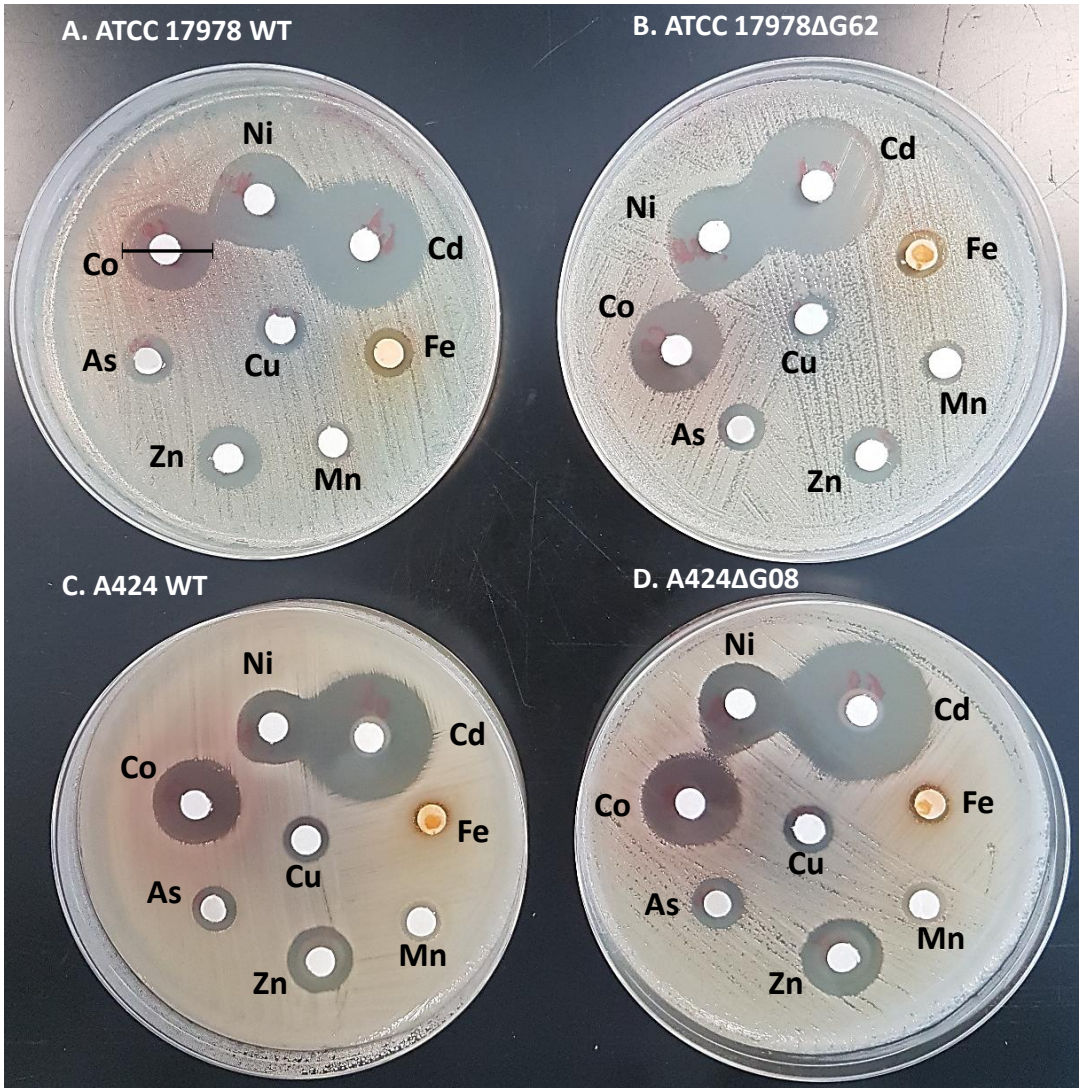


Figure 4.9 Disc diffusion method (dried discs) testing the susceptibility of A424 and ATCC 17978 and their ATCC 17978 ΔG62 and A424ΔG08 mutants to various metals.

Each disc and the represented metal tested are labelled in each picture. The zone of inhibition was recorded by measuring the diameter for each zone as shown in (A) cobalt as an example. The pictures shown are representative of three independent replicates. All discs contained 1 µg of metal (5µl of the metal stock at 1 M).

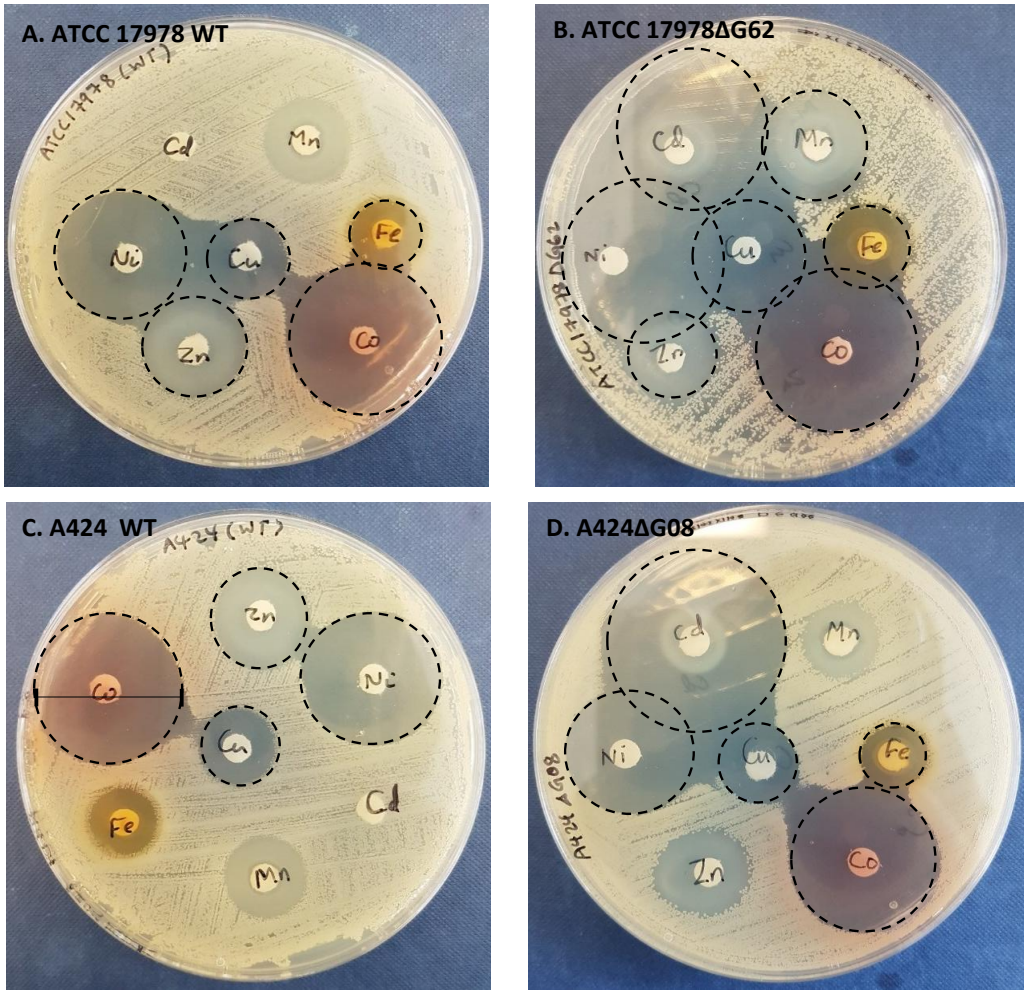


Figure 4.10 Disc diffusion method (wet discs) testing the susceptibility of A424 and ATCC 17978 and their ATCC 17978 ΔG62 and A424ΔG08 mutants to various metals.

Each disc and the represented metal tested are labelled in each picture. The zone of inhibition was recorded by measuring the diameter for each zone as shown in (C) cobalt as an example. The pictures shown are representative of three independent replicates. All discs contained 1 µg of metal (5µl of the metal stock at 1 M).

Table 4.4 Metal susceptibility testing for *A. baumannii* ATCC17978 and A424 and their mutants ATCC17978ΔG62 and A424ΔG08

	Zone diameters (mm)				MIC (mM)				MBC (mM)			
	ATCC 17978	ATCC17978 ΔG62	A424	A424 ΔG08	ATCC 17978	ATCC17978 ΔG62	A424	A424 ΔG08	ATCC 17978	ATCC17978 ΔG62	A424	A424 ΔG08
$ZnSO_4$	**15**	**15**	**15**	**15**	**5**	**2.5**	**0.625**	**0.625**	**10**	**5**	**1.25**	**1.25**
$CuSO_4$	**10**	**10**	**10**	**10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
$CdCl_2$	**24**	**26**	**22**	**23**	**2.5**	**1.25**	**1.25**	**1.25**	**5**	**5**	**1.25**	**1.25**
$MnSO_4$	**9**	**9**	**9**	**9**	**10**	**10**	**1.25**	**0.625**	**>10**	**>10**	**2.5**	**2.5**
$FeSO_4$	**12**	**12**	**12**	**12**	**5**	**5**	**5**	**5**	**10**	**10**	**10**	**10**
$CoBr_2$	**15**	**22**	**19**	**19**	**5**	**2.5**	**5**	**5**	**10**	**5**	**10**	**10**
$NiSo_4$	**17**	**22**	**17**	**17**	**10**	**5**	**10**	**10**	**>10**	**10**	**>10**	**>10**
As_2O_3	**15**	**15**	**15**	**15**	**2.5**	**2.5**	**5**	**5**	**5**	**2.5**	**5**	**5**
$CuSo_4$+ $NiSO_4$					**5**	**2.5**	**5**	**2.5**	**5**	**5**	**10**	**10**
CuSo+ $CoBr_2$					**2.5**	**1.25**	**1.25**	**1.25**	**5**	**5**	**5**	**5**

4.4 Discussion

Metal ions are essentially required in all bacteria for a range of cellular functions. The widely varying concentrations encountered in nature and the risk of heavy metal intoxication however, necessitates the bacteria to tightly regulate the cellular levels of ions to avoid detrimental effects of metal overload. To test the contribution of transporters encoded by the islands G08 and G62 of *A. baumannii*, the heavy metal susceptibility was assessed in *A. baumannii* strains ATCC 17978 and A424 and their mutants ATCC17978 ΔG62 and A424 ΔG08 by using two different methods: broth microdilution to test the MIC and MBC, and disc diffusion method to confirm variations in susceptibility and evidence interactions between metals. The correlation between the phenotypes observed and the presence of putative metal ion efflux systems was assessed.

Initially, the growth curves of *A. baumannii* strain ATCC 17978 showed delay in the exponential phase when grown with zinc. Similar phenotype was reported in Hassan et al 2017 in six other clinical *A. baumannii* species from different clonal groups, as well as in the environmental closely related strain *A. baylyi* ADP1 (Hassan et al., 2017). The most striking phenotypic observation is *A. baumannii* strains were highly resistant to copper, which has also been observed in *A. baumannii* strains except for strain SDF which is lacking the *copAB* genes (Hassan et al., 2017). It the latter study, when the cellular metal ion abundance was examined in strain ATCC 17978, it showed that under zinc or copper stress, neither of these metals was accumulating at significantly higher levels. This could indicate that *A. baumannii* either prevents their uptake or is able to efficiently efflux the ions from the cell (Hassan et al., 2017).

The interpretation of the susceptibility data from broth microdilution method showed that MIC and MBC are both consistent with the MBC data being identical or one dilution higher than the MIC. Generally, the order of susceptibility was maintained in both the mutants and their parent strains

of *A. baumannii* ATCC 17978 and A424. The confidence interval above which a difference is considered statistically significant was (P<0.05). The MIC/MBC data obtained by broth microdilution showed that *A. baumannii* strain ATCC 17978 and ATCC17978 ΔG62 mutant were susceptible to the heavy metals tested in the following order of susceptibility: arsenic/cadmium (MIC 2.5 mM), cobalt/zinc/iron (MIC 5 mM), nickel/manganese (MIC 10 mM), and copper (MIC >10 mM). For A424 and the mutant A424 ΔG08 the MIC/MBC was almost similar to that of ATCC17978 with only a slight difference in the following ascending order: A424: zinc (MIC 0.625 mM), cadmium/manganese (MIC 1.25 mM), arsenic/cobalt/iron (MIC 5mM), nickel (MIC 10mM) and copper (MIC >10 mM). Interestingly, the most statistically significant discrepancy in MIC between the two *A. baumannii* strains was observed for the two metals manganese (MIC 10 mM and 1.25 mM) and zinc (MIC 5 mM and 0.625 mM), for ATCC 17978 and A424, respectively. However, the high resistance to manganese in ATCC 17978 was expected however, cannot be solely explained by the MIC data alone described above. Studies of metal efflux in *E. coli* showed higher MIC of 20 mM for manganese, which was contributed by CDF efflux transporter (Nies, 1999, Nies, 2003). MIC/MBC data for A424 wild-type and mutant were slightly different but statistically insignificant from those of ATCC 17978/ ATCC 17978 ΔG62 as the readings were only one dilution less (P>0.05) for arsenic and cadmium (MIC 2.5 and 1.25 mM, 2.5mM and 5mM for ATCC 17978 and A424, respectively).

The measured MIC of zinc previously reported in *P. aeruginosa* as 24 to 48 mM (De Vicente et al., 1999) and 8 mM (Teitzel and Parsek, 2003), both are higher than zinc MIC in *A. baumannii*. By contrast, the MIC of copper was 2 mM in *P. aeruginosa* (Teitzel and Parsek, 2003) and 0.1 mM for *P. syringae*, which is significantly lower than copper MIC in this study. In the recent study the *A. baumannii* SDF strain was found to have *copA* and *copB* genes disrupted by IS elements and hence showed no growth in the presence of copper (Hassan et al., 2017). These data suggest that CopA/CopB- type copper resistance mechanisms have a

prominent role in copper reduced tolerance in *A. baumannii* species. However, the genomic and phenotypic analyses do not reveal a specific efflux system or type of efflux pathway as being a major determinant of zinc resistance.

Putative efflux pumps for copper and zinc were analysed in *A. baumannii* ATCC 17978 by TransAAP (Elbourne et al., 2017). 13 efflux systems were identified that belong to either the CDF family, P-type ATPase family, CorA metal ion transporter family, HME family of RND transporters, or CopB-type family of Cu exporters. Transcriptional data by qPCR revealed that four of the 13 putative efflux genes were induced by addition of zinc by a range of (2.9-13.7 folds), and five of the 13 putative Zn or Cu transport systems were up-regulated by extracellular copper by a range of 2.4-80 folds, some of which were zinc-induced. Furthermore, the presence of both Zn and Cu stress induced most, but not all, of the efflux systems observed to be up-regulated under the individual stresses of Zn or Cu (Hassan et al., 2017).

The MIC/MBC data of broth microdilution were identical in both A424 and ATCC 17978 wild-type and mutants for copper, cobalt, iron and nickel. In the disc diffusion method, the inhibition zones of these metals were variable with both cobalt and nickel having larger diameters than iron and copper. On the other hand, the MICs of cobalt, nickel, copper and zinc in *E. coli* were previously reported at 1mM which are generally lower than MICs of *A. baumannii* (Nies, 1999, Nies, 2003). The *Ralstonia metallidurans* (previously *Achromobacter xylosoxidans*) strains however, were observed to have higher MICs for cobalt, zinc, nickel and copper (15 mM, 10 mM, 8 mM and 8 mM, respectively). The cadmium MICs in both *E. coli* and *R. metallidurans* were found to be similar at 0.5 mM, both of which are higher than cadmium MIC in *A. baumannii* in this study (2.5-1.25 mM) and (22± 4 mm) inhibition zone. This observation was partially explained by the presence of *cnrCBA* mediating resistance to cobalt-nickel as well as *czcCBA* genes which are cobalt-zinc-cadmium resistance determinants in this bacterial strain (Legatzki et al., 2003, Nies,

2003). The CnrA belonging to RNA subtype is a nickel/cobalt export transporter in *Alcaligenes eutrophus* as well as NccA is a nickel/cobalt/cadmium co-transporter in *Alcaligenes xylosoxidans* (Nies, 1995, Nies and Silver, 1989). Similar iron susceptibility data in both wild-type and mutants of ATCC 17978 and A424 could be due to the presence of putatively non-functional FeoB in ATCC 17978, and the tolerance could be attributed to another iron efflux transporters.

The disc diffusion data of both *A. baumannii* wild-type strains and their parent strains showed similar order of susceptibility with both wet and dried filter papers (as explained in the results section 4.3.5), despite the minor discrepancies in the measurements of the inhibition zones between the two techniques tested. However, the order was slightly variable than that of the broth microdilution method. Surprisingly, the high level of resistance observed for nickel revealed by the broth microdilution MIC/MBC (10 mM), was slightly less pronounced to the observations in the disc diffusion test, especially with the dried filter discs. In addition, the manganese MIC/MBC in A424 wild-type and mutant were in the susceptible range when tested by the broth microdilution, in contrast to the disc diffusion where it showed the smallest zones of inhibition. However, manganese showed comparable susceptibility results in both assays for ATCC 17978, with high MIC/MBC of 10 mM and (9 ± 1 mm) inhibition zone.

The metal disc diffusion susceptibility data for zinc, iron, copper, cobalt, cadmium and arsenic were comparable to the data obtained by MIC/MBC in both ATCC 17978 and A424. The discrepancy between the data in the disc diffusion it-self led us to the conclusion that MIC/MBC determination with broth microdilution method is better suited to describe the susceptibility of the metals. This could be due to several reasons some of which were technical issues. In addition, the reliability of the disc diffusion test depends on the settings by which the assay was conducted and the reading vary considerably each time the conditions change. One of issues were whether to impregnate the discs with the metal compound prior to

attaching them to the petri dishes or to add the metals drops with the desired concentration directly on the empty filter discs. Both of these protocols were previously described by several papers addressing metal susceptibility assays. Another issue was determining the distance between the discs in such a way so that the overlapping between zones of the inhibition will not interfere with the readings of the diameters. This sometimes resulted in huge overlaps thus hindering the borders between the inhibition zones.

Table 4.5 Metal toxicity.

Metal	Toxicity	Reference/s
Cobalt (Co)	Fe-S proteins inactivation, sulfur metabolism and Fe-S cluster biogenesis. Formation of reactive oxygen species	(Eitinger, 2013)
Nickel (Ni)	Fe replacement in enzymes Binding to cysteine or histidine or negatively charged residues in active sites of non-metal enzymes	(Eitinger, 2013)
Zinc (Zn)	Transition metal ion homeostasis Impairment of oxidative stress response mechanisms Disruption of central carbon metabolism	(Turner et al., 2015), (McDevitt et al., 2011), (Ong et al., 2015)
Copper (Cu)	Redox activity by binding to Fe-S Formation of reactive oxygen species	(Macomber and Imlay, 2009)
Iron (Fe)	Formation of hydroxyl radicals reacting with biomolecules such as DNA.	(Braun, 1997)
Manganese (Mn)	intracellular competition of Mg2+ for binding to biological molecules such as Mg2+-requiring enzymes, ATP and nucleic acids	(Blackwell et al., 1998)
Cadmium (Cd)	Enzyme inhibition binding to the ligands of phosphate and the cysteinyl and histidyl groups of proteins	(Vig et al., 2003)

The data in this study collectively show that *A. baumannii* ATCC 17978 possesses a wide range of efflux systems associated with resistance to

heavy metals, particularly zinc and/or copper. The resistance profiles showed overlap between the different metals, such as zinc and copper could have indicated that these metal ion efflux systems that may have poly-specific cation efflux properties. However, it is also possible that one or both of these pathways may be specific to a certain metal and have been activated inappropriately under another metal stress, such as the presence of extracellular zinc resulting in the copper-depletion phenotype observed even in a previous study (Hassan et al., 2017).

There are several limitations in the metal analysis which should be addressed. As the main focus of this work was to examine the contribution of G08 and G62 putative efflux systems in transportation of heavy metals, the phenotypes were tested in only two mutants with targeted deletion of these GIs. The analysis of the susceptibility data of the various metals revealed slight contribution of these GIs suggestive of other efflux transporters present elsewhere in the chromosome of the tested *A. baumannii* isolates, which was confirmed by the recent study of (Hassan et al., 2017). Further analysis, is therefore mandatory to explore the role of all efflux metals in ATCC 17978 and A424 other than the genes within G08 and G62. In addition, introducing mutations for such efflux systems with vital role in the various biological processes in the bacteria could be lethal and one should be cautious. Studies of efflux systems tend to overexpress these pumps to examine their phenotypic changes, an area which could be further addressed in this study.

Collectively, this work reveals that metal resistance is one of the strategies utilized by *A. baumannii* to survive metal ion stress, allowing it to thrive in diverse environments. In addition, evidence from recent studies have shown that zinc and copper play crucial roles in management of oxidative stress and the membrane composition of *A. baumannii* as well as the distinct role of zinc and copper in macrophage-mediated killing of this pathogen. Moreover, collectively with this work and the previous study of (Hassan et al., 2017), both show evidence that the cellular levels of copper can be depleted by zinc overload, this can be

exploited as a novel strategy to target metal ion homeostasis mechanisms in *A. baumannii* as effective antimicrobial therapy, to tackle this MDR pathogen.

Chapter 5 Transposase-mediated mobilisation of resistance genes; epidemiology and mechanisms of transposition in *Staphylococcus epidermidis*

5.1 Background

Site-Specific Recombination is the mechanism by which mobile genetic elements move into and out of the chromosomes of their host cells. The mechanisms of site-specific recombination have been described in detail in the introduction chapter. The first mechanism is the conservative site-specific recombination in which short hetero-duplex joints are produced and require identical DNA sequences in both the host and recipient (Alberts et al., 2002). This type is an integrase-mediated as discussed in chapter three of this study. The second type is referred to as a trans-positional site-specific recombination which proceeds via breakage reactions of two ends of mobile DNA sequence, and integration of these ends at different non-homologous target DNA sites. No hetero-duplex is formed in this type of recombination. This process of site-specific recombination requires specialized recombination enzymes; the transposases.

I have co-authored two studies characterising the modes of transposition and the distribution of a novel transposase-mobilised element in Staphylococci (Furi et al., 2016, Hijazi et al., 2016). The previous epidemiological work, focused on disinfectant/biocide resistance of *Staphylococcus epidermidis* strains to chlorhexidine (Hijazi et al., 2016). This work has identified the *qacA/B* genes in *S. aureus* and *S. epidermidis* clinical isolates from patients with bacteraemia and admitted to the intensive care unit in Aberdeen (Scotland). These patients have been exposed to chlorhexidine on a regular basis as part of the routine care of an infection control measure. The study found that the *qacA/B* carriage in the *S. epidermidis* strains was associated with reduced chlorhexidine susceptibility as compared to *qacA/B* negative isolates. The majority of these strains carried *qacA/B* alleles that were identical to the full-length q*acA* gene, except three strains having single nucleotide polymorphisms (SNPs) resulting in single amino acid substitutions present in *qacB* (C455T, G871A and T1139C). These genes did neither match fully to the

qacA gene, nor to the *qacB* genes deposited in Genbank and hence were re-named *qacAB.*

My contribution to this work started when whole genome sequence data of this starin collection became available. Multi-locus sequence typing from the whole genome sequences showed that these *S. epidermidis* isolates were clonally variable with 65% of them belonging to ST-2, which is the most prevalent multi-drug resistant (MDR) clone in hospital-acquired infections (Widerstrom et al., 2012). The two *S. epidermidis* isolates showing the highest occurrence of SNPs were found to belong to ST-83 clone. The other *qac*-positive *S. epidermidis* isolates belonged to various sequence types; ST5, ST559, ST59 and ST48.

The whole genome sequencing of these *S. epidermidis* strains facilitated also the detection of further antimicrobial resistance related genes as the mupirocin-resistance associated isoleucyl-tRNA synthetase gene (*ileS)* in 10 of the strains. Mupirocin (pseudomonic acid A) is a polyketide antibiotic that is naturally produced by *Pseudomonas fluorescens* strain NCIMB 10586 (Sutherland et al., 1985) and it has been widely used as a topical antibacterial agent to eradicate the nasal carriage of Methicillin-resistant *staphylococcus aureus* (MRSA) and reduce certain types of surgical site infections (Pujol et al., 1996). Mupirocin's epoxide chain is structurally similar to that of isoleucine and thus can bind to the isoleucine-specific binding site of isoleucyle-tRNA synthetase (*ileRS*). This latter enzyme normally converts isoleucine and tRNA to isoleucyl-tRNA. The inhibition of isoleucyl-tRNA synthetase is mediated by mupirocin thus preventing protein and RNA synthesis leading to cellular death eventually (Nakama et al., 2001).

There is an important concern, however, of increasing rates of mupirocin resistance (Mup^r), which has been classified into two types: low-level Mup^r (MIC, 8-256 µg/ml) and high-level Mup^r (MIC, >256 µg/ml) (Coates et al., 2009). The low-level Mup^r is usually attributed to the presence of mutations in the isoleucyle-tRNA synthetase chromosomal gene (*ileS*),

the V588F point mutation being the most commonly described (Antonio et al., 2002).

The V588F mutation is known to disrupt the hydrophobic pocket within the mupirocin-binding Rossman fold, which has a significant effect on the conformation of IleS, leading to low-level Mup^r (Antonio et al., 2002). On the other hand, the high-level Mup^r is mediated by the acquisition of pSK41/pGO1-like conjugative plasmids carrying the *ileS2* gene, or alternatively called *mupA* (originating form a *Bacillus* spp*)*, which encodes for an alternate isoleucyle-tRNA synthetase and has facilitated the dissemination of this resistance mechanism (Ramsey et al., 1996). Generally, the *ileS2* gene is flanked by two copies of insertion sequence IS*257* (Figure 5.1) which have played a key role in the transfer of resistance genes among bacterial strains (Perez-Roth et al., 2011). A new high-level Mup^r determinant, *mupB*, has been described, sharing 65.5% sequence identity with *mupA* but only 45.5% identity with *ileS* (Seah et al., 2012). Moreover, the IS*257* insertion sequence has also been reported to mediate the transposition of Tn*4003* element harbouring the *dfrA* gene that confers resistance to trimethoprim (Needham and Noble, 1995).

In addition, the NADH-dependent *trans*-2-enoyl-acyl carrier protein reductase *sh-fabI*, an enzyme involved in the bacterial fatty acid biosynthesis pathway and conferring resistance to triclosan has been detected in eight of the sequenced *S. epidermidis* strains (Ciusa et al., 2012). Our recent study (Furi et al., 2016) have shown that this *sh-fabI;* a plasmid carried element is originally mobilised from *Staphylococcus haemolyticus,* was found in various *Staphylococcus aureus* as well as *S. epidermidis* strains (Ciusa et al., 2012). However, this is not always the case as the *sh-fabI* can be present as well in *qac*-negative isolates as reported in one of the *S. epidermidis* in (Hijazi et al., 2016). A recent study concerning the IS*1272* element have shown however, that although that IS*1272* was first found and characterized in *S. haemolyticus* (Archer et al., 1996), it could well be that this IS*1272* have evolved through adaptation to *S. aureus* as evidenced by the multi-IS*1272* copy system detected during

cluster analysis (Wan et al., 2017). The tricolsan susceptibility testing was not performed in (Hijazi et al., 2016) study and hence will be covered in this work.

Interestingly also the *sh-fabI* resistance mechanism to the biocide triclosan is mediated by IS-mediated transposition by the TnSha1 and TnSha2 elements, described in the second manuscript co-authored by me (Furi et al., 2016).

The FabI protein is not only the target for triclosan, but actively investigated as target for the development of new antibiotics (Lu and Tonge, 2008, Payne et al., 2001). However, the wide use of this biocide may be of concern potentially driving the development and spread of resistant *fabI* alleles or even strains with fatty acid pathway deletions (Furi et al., 2016, Gloux et al., 2017). In the latter study, a significant number of *S. aureus* isolates were resistant to tricolsan upon exposure to fatty acids and subsequently resulting in emergence of resistant and non-culturable variants, a finding that is very worrisome (Gloux et al., 2017).

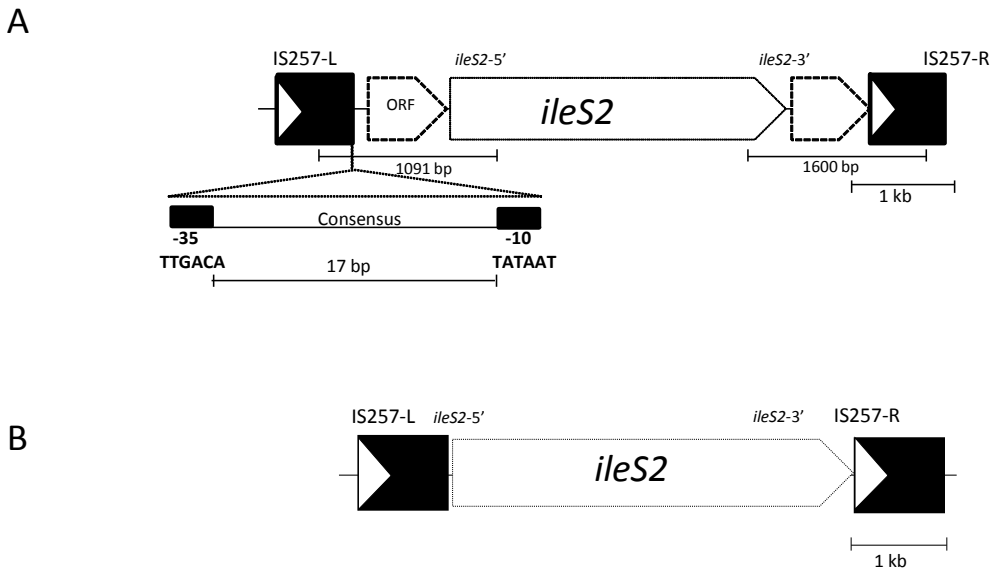


Figure 5.1 Structural organization of *ileS2* of the region encompassing *ileS2* gene.

(A)The schematic map of *ileS2* in pGO400 plasmid (accession KT780705.1) and *S. aureus* strain (HUNSC491) (accession NC_013653.1) (B). (A) Upstream (IS*257*-L) and downstream (IS*257*-R) IS*257* elements flanking the *ileS2* gene are represented by solid boxes. The white arrows indicate the direction of IS*257*

transposase transcription. The *ileS2* genes and the predicted ORF upstream and downstream are represented by arrows indicating their orientation. The position of the -10 and -35 sequences of the putative promoter are shown below the figure. The spacer region between -10 and -35 is variable in different plasmids so this is shown as an example.

In addition to the mutation in the promoter region or coding sequences of *fabI* as a resistance mechanism to triclosan, there is another novel determinant in which there is a different copy of *fabI* acquired from *S. haemolyticus (sh-fabI)* (Ciusa et al., 2012). The *sh-fabI* gene is found within a transposon which is 3022 bp in size and could be mobilised by a single copy of insertion sequence IS*1272* (figure 5.2, GenBank accession no. JQ712986) (Ciusa et al., 2012). Previous studies (Archer et al., 1994, Archer et al., 1996, Tonouchi et al., 2014) had originally identified the IS*1272 in sh-fabI* gene of *S. epidermidis* has about 84% nucleotide similarity and 91% identity to *sa-fabI* of *S. aureus* (Ciusa et al., 2012). This transposon integrates between two short direct repeats in the loop of a hairpin with an 18 bp inverted repeat stem. This *sh-fabI* gene originates from the core chromosome of *S. haemolyticus*, which does not carry any other *fabI* genes.

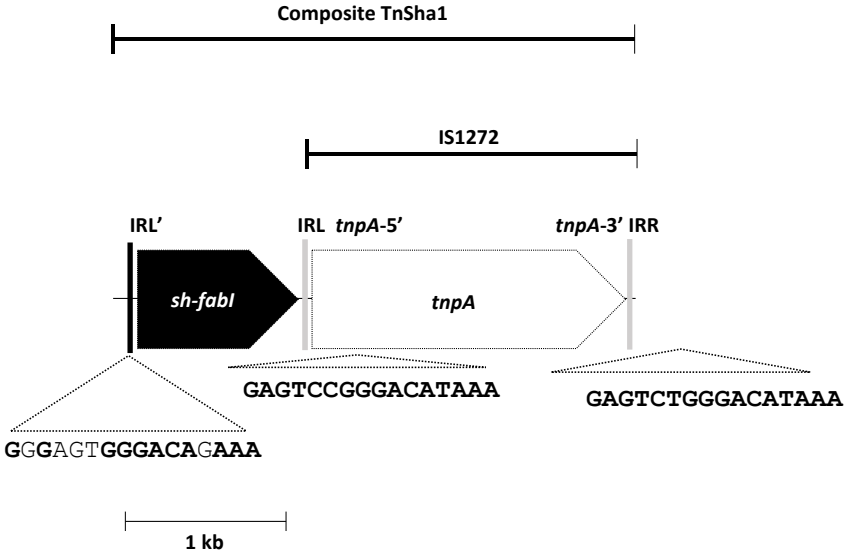


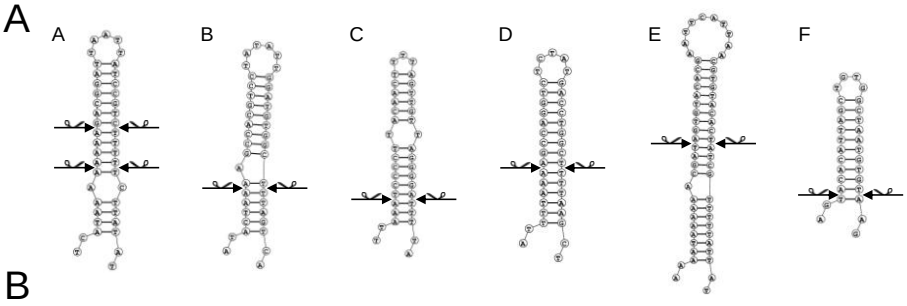
Figure 5.2 Structure of TnSha1 transposon.

The schematic map of the composite transposon TnSha1 in which the sh-fabI gene (black) precedes the IS*1272* element. The updated IS*1272* sequences contains six SNPs (four of which indels) with respect to U35635 and encodes for a single transposase gene without a stop codon within the IS element. The

sequence of the prototypeTnSha1 element corresponds to position 3908 to 887 of Genbank accession JQ712986 relative to strain QBR-102278-1619. The inverted repeat sequences of IS*1272* (named IRL and IRR) aligned to the IRL' upstream of sh-fabI which shows 69% identity are shown. A 1 kb scale is given below the figure.

The presence of an alternative inverted repeat (IRL') in the *S. haemolyticus* chromosome upstream of *sh-fabI* with a high degree of similarity to the inverted repeats of IS*1272* (Figure 5.2) could provide a potential aid of the transposition of this unit. The updated composite TnSha1 is composed of IS*1272* and a single transposase within the IS element without a stop codon (Furi et al., 2016).

Our work (Furi et al., 2016) investigated the epidemiology and distribution of Tnsha1 elements in ten *S. aureus* isolates which have been previously sequenced and shown to have the *sh-fabI* (Ciusa et al., 2012). Seven different integration sites have been identified in just ten *S. aureus* strains, named as A to G (Figure 5.3) (Furi et al., 2016). Further analysis of another 63 Tnsha1 elements of published *S. aureus* sequences have identified 12 target sites, suggestive of little or no preferable integration sites (Furi et al., 2016). Interestingly, the detailed analysis of the insertion sites in *S. aureus* reference strains COL and MW2 have revealed that the TnSha1 integrates at a stem loop structures resulting in partial deletion of the target site of insertion (Furi et al., 2016). This was recognized as a novel mode of integration of IS elements (Siguier et al., 2015). All of the seven insertion sites had inverted repeats that could potentially form secondary hairpins and upon integration of the TnSha elements, cause blunt end cleavage which subsequently, results in target duplications (Furi et al., 2016).




```
A)    S. aureus COL        149148 TTTTCATAAAAAAAA------gacggattaatt atccgtc-------TTTTTCTTATATCTTT
A2)   S. aureus COL        149148 TTTTCATAAAA------aaaagacggattaatt atccgtctttt-------TCTTATATCTTT
B)    S. aureus COL       2545016 CTGGAACGATACTAA--aagccacgtcctatatt ggatgtggct---TTAGTCATACTTATAT
C)    S. aureus COL       1097865 CTAAATTAATTTAAA-tccccttacaacttt agttgttaggggga--TTTTATGCATGTGCTC
D)    S. aureus JKD6159   2563232 TTTAATGATTTTAAA----agccaggtctctat gacctggct-----TTTAAGCTTTTTCCTG
E)    S. aureus MW2       2638638 AAAATAAAAAACGAT-agtgtacacgaattcattaacgtgtacact-ATCGTTTTTATTATGA
F)    S. aureus MW2       1678951 TTCCTATTTTTAAGT----acacattagctgtggctaatgtgt----AAGAACCACTACATAA
G)    S. aureus MW2       2109277 AATAACAAAGAGAATgttattactataacaatagttataacagtaaggaatttttttattaaGA

P)    S. haemol. p25-44      5401 TTAGGTTAAAAGAAA---ggagctaacctttagggttagctcc----TTTCTGTGTTCTTTAT
V)    S. aureus pTW20_1     13941 CTTAATAATAAAAAA-gtagaaccatttaaaattaatggttctac--TTTTTAACTATTACTA
W)    S. aureus pWGB745     22280 ATTTTACAAATAAAA---aagaaagtgattatatcactttctt----TCCTTAACTATACGAT
X)    S. aureus pSA1379     20646 TACTTAATAATAAAAaagtagaaccatttaaaattaatggttctactTTTTAACTATTACTAA
Y)    S. epid. pSAP108A     12204 TATTCAAAAAAAAATaatgtattcacgcctaaaggcgtgaatacattATTTTCTCTAAAAAGA
Z)    S. aureus pUSA03       1076 TTTCAAAAAAAAGAG---attatacacctaaaaaggtgtataat---CTTTACGGTTCTGTTG
```

Figure 5.3 Insertion sites of TnSha1 in bacterial chromosomes and plasmids.

The seven target sites found in the 10 *S. aureus* strains sequenced in Furi et al 2016, have been named from A to F. Staphylococcal plasmid insertion sites are named P to Z. Panel **(A)** shows the hairpin structures formed by the transposon target sites and the scissors indicate the position of sequence breaks generated upon TnSha1 insertion. Secondary structure predictions were made using RNA structure web-based software (Reuter and Mathews, 2010). Two independent insertions into target **(A)** have been identified and both cleavage sites are shown which in each case generate blunt-end structures in the stem of the hairpin. The sequences in both panels **(A, B)** refer to TnSha1-free target sites in the chromosomes (COL GenBank accession CP000046 and MW2 GenBank accession BA000033) and plasmids. The target sequences in panel **(B)** report in lower case the sequence deleted upon insertion and the inverted repeats underlined. (Furi et al., 2016)

The other *sh-fabI* element is TnSha2, which is essentially a TnSha1 element inserted within the terminator of the *traQ* gene of a 8.7 kb plasmid (Furi et al., 2016). The plasmid can in same cases be found integrated into the chromosome by recombination of IS*1272* creating a composite element of 11kb flanked by two IS*1272* elements (Furi et al., 2016).

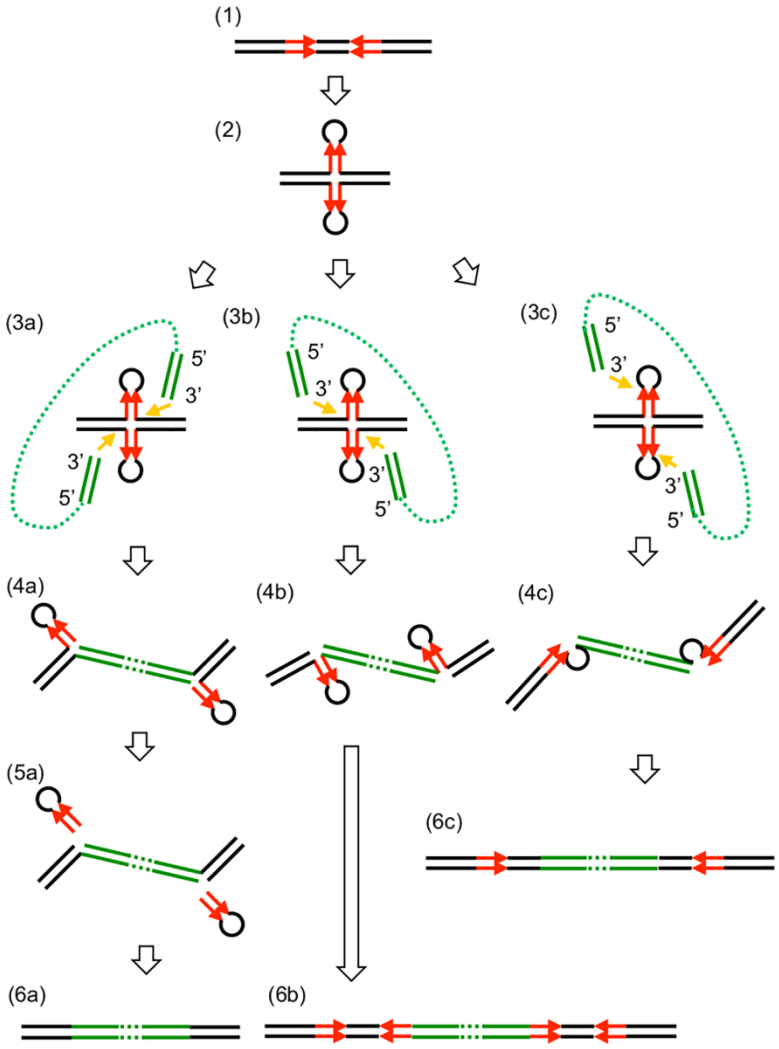


Figure 5.4 Replacement by structure-dependent transposition (RST) or stem-loop replacement: A possible model for the transposition mechanism of IS*1272*.

Palindromic sequences (red) are present in donor and recipient sequences. Palindromic sequences form a cruciform. (3a-4a) The 3' ends of IS*1272* (stem-loop) attack and are joined to the target DNA at the 3' feet of two stem-loops. (5a) The stem-loops on recipient sequences are removed. (6a) IS is inserted, replacing a stem-loop sequence. (3b-4b) The 3' ends of IS attack and are joined to the target DNA at the 5' feet of the two stem-loop. (6b) The sequence forming the stem-loop is duplicated at both ends of IS. (3c-4c) The 3' ends of IS attack and are joined to target DNA at the 5' of two loops. (6c) The sequence corresponding to the two loops is duplicated at both ends of IS. Red arrows, palindromic sequences; green lines, IS DNA.(Wan et al., 2017)

It should be noted that by the time of writing of this thesis, a study was published by Wan and colleagues (Wan et al., 2017) investigating the genome of a PVL-positive *S. aureus* strain isolated from a 4-year old boy with skin abscesses. Detailed analysis of all IS*1272* elements was

conducted in the latter study with evidence suggesting that IS*1272* transposes through an IR-replacing mechanism (Figure 5.4) with an irreversible process unlike that of “canonical” transpositions, in which the sequence data was showing potential stem-loop-replacement of the IS*1272*. This mechanism of transposition resulted in genomic variations, and that, among the familial strains analysed, the patient strain has strong virulence potential based on community-associated virulence factors (Wan et al., 2017). Therefore, the data on transposition presented here was all based on our previous knowledge prior to the recent hypothesis by Wan and colleagues.

5.2 Aims and objectives

This chapter aims at the characterization of IS-element mediated transposase mobilisation of resistance determinants by analysing a series of *S. epidermidis* clinical strains. The analysis will include the following points:

1. Characterization of classical resistance genes carried on elements that are mobilised by integrase-like genes in *S. epidermidis* isolates using bioinformatics tools. This also involves testing the excision and mobilisation of the TnSha1 and TnSha2 elements using various molecular tools like real-time qPCR as well as cloning.
2. Characterization of the transposon mediated mupirocin resistance determinants and phenotypes
3. Characterization of the transposon mediated triclosan resistance determinants and phenotypes
4. Multi locus sequence typing of TnSha1 and TnSha2 carrying isolates using available staphylococcus sequenced strains form the NCBI database.

5.3 Results

Some of the results presented here are a continuation of the work which has been published in two recent papers with myself as co-author (Furi et

al., 2016, Hijazi et al., 2016). Collection of strains, routine species identification, PCR and partial sequence of *qac* genes and chlorhexidine and ethidium bromide MIC was performed by Felicity Abbott and Ian Gould (Department of Medical Microbiology, Aberdeen Royal Infirmary, Aberdeen, UK) and by Karolin Hijazi (School of Medicine and Dentistry, University of Aberdeen, Aberdeen, UK). This initial analysis did not allow for epidemiological or phylogenetic typing of strains and the limited sequences did not allow for differentiation of *qacA* and *qacB* genes. This initial work claimed high occurrence of biocide resistance in *S. epidermidis*, but no information was provided on the transposon mobilised biocide resistance determinants present in *S. epidermidis*. To allow proper epidemiologic classification and in depth molecular characterization of resistance determinants, the whole genome sequences of these clinical isolates were analysed.

5.3.1 Characterization of classical resistance genes carried by MGE in *S. epidermidis* isolates

5.3.1.1 Identification of resistance genes in *S. epidermidis* clinical isolates using CARD

In *Staphylococcus* species, a number of protein biosynthesis inhibitors (PBI) resistance genes have been found to be part of non-conjugative transposons, such as *erm*(A) + *spc* (Tn*554*), *vga*(A) (Tn*5406*), *fexA* (Tn*558*), *erm*(B) (Tn*917*/Tn*551*), *aacA-aphD* (Tn*4001*), *aadE* + *sat* + *aphA3* (Tn*5405*), and *erm*(A) + *spc* + *vga*(E) (Tn*6133*) (Schwarz et al., 2011). Other resistance determinants were also carried on variable sized plasmids ranging between single resistance genes to multi-resistance plasmid carriers.

The Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013) was utilised to search for the resistance genes found in our collection of unannotated *S. epidermidis* strains. The resistance gene identifier (RGI) allows the detection of putative resistance genes under three different algorithms; perfect, strict and loose. For the purpose of analysis in this chapter the "perfect and strict" criteria was used as a

default. The perfect paradigm detects the perfect matches to the known curated sequences of the antibiotic resistance genes in the CARD, whereas, the strict algorithm allows broader search for unknown variants of antimicrobial resistance genes as well as an additional search for key SNPs and mutations within the genes. The final results displayed a list of the putative resistance genes with their corresponding *E*-values.

All the *S. epidermidis* isolates were analysed and all the antibiotic resistance genes detected are listed in table 5.1 and table 5.2 provides the relevant genes, their coding enzyme, and the associated resistance phenotypes. The majority of these genes were found to be plasmid-borne and mediate resistance to inhibitors of protein biosynthesis at various target sites (Table 5.1). The tetracycline conferring resistance; *tetK* gene was detected in three *S. epidermidis* strains 51, 56 and 68 carried in a plasmid. It encodes for a membrane-efflux protein belonging to the major facilitator superfamily (MFS) (Schwarz et al., 2011). Fifteen *S. epidermidis* isolates had another plasmid-borne gene; *erm*(C), coding for combined resistance to macrolides, lincosamides and streptogramin B. In *Staphylococcus*, at least six plasmid-carried *erm* genes categorised as A, B, C, T, Y, and 33 had been identified in previous studies (Kadlec, 2010 #506)(Matsuoka, 2002 #507)(Schwarz, 2002 #508)(Roberts et al., 1999).

The ABC transporter protein encoded by *vga*(B) gene conferring resistance to streptogramin A was found in strain 54 only. Typically, *vgb*(B) was found to be carried in 4.9 kb small plasmid along with streptogramin A *vat*(C) in staphylococcal species (Allignet et al., 1996). The gene *mph*(C) encodes a macrolide phosphotransferase (Hauschild and Schwarz, 2010, Matsuoka et al., 1998) and was identified in three strains (68, 77 and 79) whereas *lnu*(A) codes for lincosamide nucleotidyltransferase (Brisson-Noel and Courvalin, 1986, Lüthje and Schwarz, 2007) and was found in strain (71) only.

lnu(A) gene was found to be carried either solely or in duplications into plasmid along with *rep* gene. *Vga*(B), *Mph*(C) and *lnu*(A) genes confer resistance to streptrogramin B, macrolides and lincosamides respectively,

by enzymatic inactivation (Allignet et al., 1996, Allignet et al., 1998). Multiple aminoglycoside resistance genes were detected. The aminoglycosides resistance *aac(6')-Ie-aph(2")-Ia* gene was detected in ten *S. epidermidis* strains (Table 5.1). It codes for a bifunctional enzyme with acetyltransferase and phosphotransferase functions, confering resistance to gentamicin, kanamycin, tobramycin, and amikacin when overexpressed (Ferretti et al., 2001, Torres García et al., 1996). *ant(4')-Ib* is another gene encoding for aminoglycoside adenylyltransferase, conferring aminoglycosides resistance detected in four isolates; 61, 63, 69 and 83.

The aminoglycosides resistance *aac(6')-Ie-aph(2")-Ia* gene is of particular interest as it is carried in a Tn*4001* composite transposon, flanked by two copies of IS*256* elements situated in opposite orientations (Rouch et al., 1987) (Figure 5.5). The structure of the Tn*4001* transposon is very similar to the FabI element described earlier in this chapter. This transposon was originally identified on the 28 kb multi-resistance plasmid pSK1 (Lyon and Skurray, 1987, Rouch et al., 1987) . The Tn*4001* is about 4.6 kb transposon, with a transposase gene at the the upstream flank of the IS*256.* Complete Tn*4001* elements have been located on various elements including plasmids and mosaic SCC*mec* element, in a SCC*mec* type IVc cassette as well as different chromosomal locations. evidence of complete Tn*4001* elements in the chromosomal DNA were identified by whole genome sequencing of *S. haemolyticus* and S*. epidermidis* (Gill et al., 2005, Takeuchi et al., 2005), whereas Truncated Tn*4001* elements in which the terminal IS*256* sequences were partially deleted have been located on multi-resistance plasmids in *S. aureus* and in avian CoNS isolates, including *S. warneri* and *S. sciuri*. (Caryl and O'Neill, 2009, Lange et al., 2003).

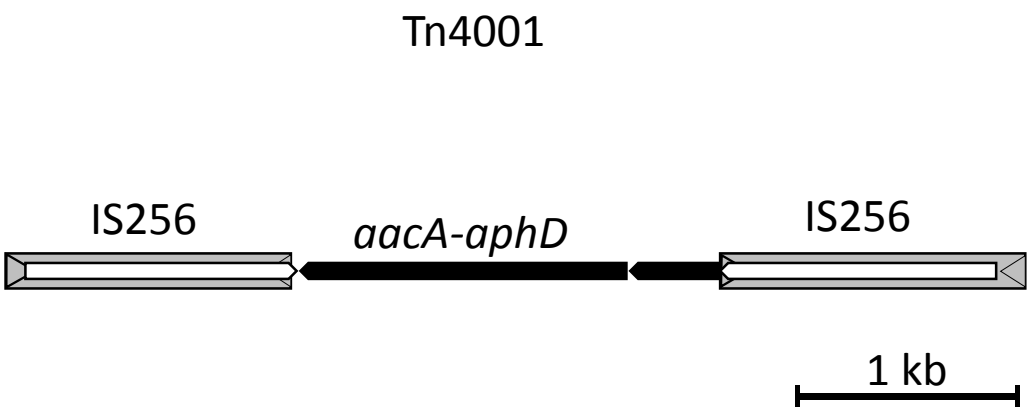


Figure 5.5 Schematic map of transposon Tn*4001* of *Staphylococcus aureus*.

Tn*4001* (4,581 bp; GenBank accession AB682805) (Rouch et al., 1987) is composed of two copies of IS*256* (grey) flanking the bifunctional *aacA-aphD* gene (black), which is co-transcribed with an upstream putative acetyltransferase (black) and the transposase gene (white) of the right copy of IS*256* (grey). The inverted repeats of the IS elements (grey) are shown as grey triangles. A scale bar is shown below the map.

Moreover, the three resistance genes *aphA-3+sat +aadE* were observed in a plasmid in one *S. epidermidis* isolate 26, via the integration of the transposon Tn*5405*. The 12 kb transposon Tn*5405* is from *S. aureus* and is similar to Tn*4001*, which is also a composite transposon. Tn*5405* has a central resistance gene region encoding the streptomycin resistance gene *aadE* (also known as *ant* (*6*), the streptothricin acetyltransferase gene *sat4*, and the kanamycin/neomycin resistance gene *aphA3* (also known as *aph*(*3*′)). The central region is flanked by the insertion sequences IS*1182L* and IS*1182R*; an insertion sequence of the type IS*1181* is integrated in the terminal part of IS*1182R* (Schwarz et al., 2011).

Table 5.1 Resistance genes identified in the Comprehensive Antibiotic Resistance Database

strain	Antibiotic resistance genes*	Biocide resistance genes
42	*blaZ, erm*(C), *fusB*	
48	*mecA, blaZ, erm(C), dfrC*	*qacA*
49	*mecA, blaZ, erm(C), dfrC, fusB, aac6*	*qacA*
51	*mecA, blaZ, erm(C), tetK*	*qacAB*
56	*mecA, blaZ, aad6, aphIII, cat, tetK*	*qacA*
59	*mecA*, *blaZ* , *fusB*	*qacAB*
60	*mecA*, *blaZ*, *fusB*, *aac(6')-Ie-aph(2'')-Ia*	*qacA*
61	*mecA*, *blaZ*, *fusB*, *aac(6')-Ie-aph(2'')-Ia, ant(4')-Ib*	*qacA*
62	*mecA*, *blaZ*, *fusB*, *aac(6')-Ie-aph(2'')-Ia*	*qacA*
63	*mecA*, *blaZ*, *erm(C), fusB*, *aac(6')-Ie-aph(2'')-Ia, ant(4')-Ib*	*qacA*
64	*mecA*, *blaZ, fusB*, *aac(6')-Ie-aph(2'')-Ia*	*qacA*
66	*blaZ, aac(6')-Ie-aph(2'')-Ia*	
67	*blaZ*, *erm(C), dfrG*	
68	*blaZ*, *erm(C), tetK, mph(C)*	
69	*mecA*, *blaZ, ant(4')-Ib*	*qacA*
70	*mecA*, *blaZ*	*qacA*
71	*mecA*, *blaZ, erm(C), lnu(A)*	
73	*blaZ, erm(C), fusB*	
74	*erm(C)*	
75	*mecA*, *blaZ, erm(C), fusB, aac(6')-Ie-aph(2'')-Ia*	*qacA*
77	*mecA*, *blaZ*, *erm(C), mph(C), aac(6')-Ie-aph(2'')-Ia, aph(3')-IIIa, dfrG*	*qacA* (fragment)***
78	*mecA*, *blaZ, erm(C), fusB, aac(6')-Ie-aph(2'')-Ia*	*qacA*
79	*mecA*, *blaZ, erm(C), fusB, mph(C)*	*qacA*
83	*mecA*, *blaZ, erm(C), aac(6')-Ie-aph(2'')-Ia, ant(4')-Ib*	*qacA*

* Search parameters set for perfect and strict hits only

** *norA* efflux pump not listed as it is a chromosomal gene present in most strains

*** Truncated *qacA* sequence (only 3′ 450-bp fragment)

Table 5.2 Resistance gene localization

Gene*	Enzyme	Resistance	Location
cat	Chloramphenicol acetyl transferase	Chloramphenicol	plasmid carried or integrated plasmid often by use of IS element
blaZ	Beta-lactamase	Beta-lactams	plasmid carried
mecA	Pbp2a transpeptidase	Beta-lactams	chromosomal island with two site specific recombinases
erm(C)	23S rRNA methylase	Macrolides, lincosamides, streptogramin B	Plasmid-borne integrated via IS*257*
tetK	Active efflux (major facilitator superfamily)	Tetracycline	Plasmid-carried gene (integrated via IS*257*)
dfrC			
fusB		Fusidic acid	Plasmid-carried co-located with *blaZ* (truncated Tn*552*)
aph(3')-IIIa	Aminoglycoside 3' phospho-transferase		in IS*1595* flanked by two IS*257*
Aad(6), ant(4)-Ia	Aminoglycoside 6-adenyltransferase	Streptomycin	flanked by two IS*257*
aac(6')-Ie-aph(2'')-Ia	bifunctional 6′-aminoglycoside acetyltransferase 2''-aminoglycoside phosphotransferase	aminoglycosides	Tn4001 flanked by two IS*256*
Sat4	streptothricin acetyl transferase	streptothricin	Tn5405 with *aphA-3*+ *aadE*
mph(C)	Macrolide 2'-phosphotransferase C	Macrolides	Plasmid carried
vga(B)	ABC Efflux transporter	Streptogramin A	Plasmid carried in association with methylase genes
ant(4')-Ib	aminoglycoside adenylyltransferase	aminoglycoside	Plasmid-carried
lnu(A)	lincosamide nucleotidyl transferase	lincosamides	Plasmid-carried

Beta-lactams conferring resistance genes *blaZ* was identified in all the *S. epidermidis* strains expect strain 74 which belongs to a novel sequence type. Similarly, *mecA gene* conferring methicillin resistance was found in the majority of the *S. epidermidis* isolates, 19 isolates were methicillin-

resistant (MRSE) whereas the remaining five strains were methicillin-susceptible (MSSE). It was observed that, 13 of the MRSE isolates (65%) of the 25 isolates belonged to the multidrug- resistant (MDR) clone ST2, which is the most prevalent in hospital-acquired infections (Roach et al., 2015, Schoenfelder et al., 2010), while the MSSE was found in clonally diverse strains belonging to various sequence types (ST-19, ST-210, ST-54 and ST-204).

5.3.2 Characterization of the transposon mediated mupirocin resistance determinants and phenotypes

5.3.2.1 Susceptibility of clinical isolates to mupirocin

To obtain phenotypic data on susceptibility to mupirocin, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) data were generated from the serious of 25 *S. epidermidis* clinical isolates previously characterized for their profiles of susceptibility to chlorhexidine (Hijazi et al., 2016). Susceptibility data are shown in Figure 5.6. The susceptibility profile of these isolates is classified into three main groups: susceptible; as shown in 13 strains, low-level resistance in 6 out of the 25 and high-level resistance in 5 isolates (Table 5.3). To further understand the observed phenotypes, the MIC/MBC data will be discussed into the context of the molecular basis of mupirocin resistance determinants.

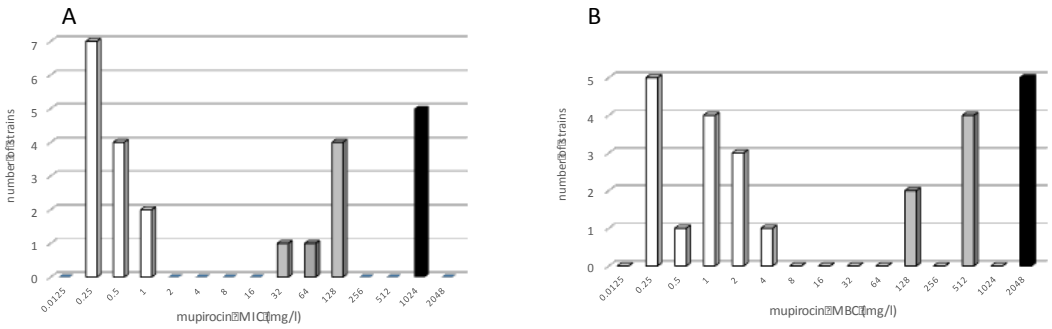


Figure 5.6 Distribution of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of mupirocin.

The MIC values of the *S. epidermidis* clinical strains are represented as bar charts. The x-axis represents the MIC/MBC value as interpreted by the broth microdilution method. The Y-axis shows the number in strains in each group. The shading indicated strains with a wt *ileS* gene (white), a mutation in *ileS* (grey or those having an additional *ileS2* gene (black). The MIC data are shown in panel A and the MBC data in panel B.

Table 5.3 Susceptibility of 25 *S. epidermidis* isolates to mupirocin

MIC(µg/ml)	isolate	Remark
<8	54, 58, 60, 61, 66, 67, 68, 70, 73, 74, 77, 78, 79	susceptible
16-32	62*	Low-level resistance
64-128	48, 49, 63, 64, 75	
>256	51, 53, 56, 59, 69	High-level resistance

*STAPH 62 strain had Low-level mup-resistance due to *ileS2* being located in the chromosome

5.3.2.2 Mutation determining changes in IleS

Out of the total isolates, the 13 mupirocin-susceptible isolates (MIC, ≤1 µg/ml) (Cookson, 1998), all lack the mup resistance determinant, the *ileS* gene. Six isolates showed low-level mupirocin resistance (MIC, 16-128 µg/ml). The *ileS* gene with a missense V588F mutation was found in 5 out of these 6 isolates. However, interestingly STAPH62 strain was the only one which had low-level resistance (MIC, 32 µg/ml) to mupirocin and did not have the V588F mutation in the *ileS* gene. High-level mupirocin resistance (MIC, > 256 µg/ml) (Patel et al., 2009) was observed in five *S. epidermidis* isolates (51, 53, 56, 59, 69). The MIC values for each of *S.*

epidermidis strains and the presence of mutations in IleS are indicated in table 5.4.

A particular attention was given to STAPH62 low-level resistance phenotype, and it was hypothesized that this could be attributed to presence of variable populations that may or may not harbour the V588F mutation. To investigate the possibility that this strain could have a mixed population, twenty-five colonies of STAPH62 strain were patched from a plate without mupirocin into a plate with 50 µg/ml and a control plate without antibiotic. It was observed that 28% of the patched colonies were able to grow on the Mup 50 µg/ml plate. To further confirm this finding, a PCR was performed to amplify the *ileS* region with the V588F mutation and sent for sequencing. The nucleotide sequence obtained confirmed that the *ileS* was present in that population.

Additionally, various single nucleotide polymorphisms (SNPs) in IleS were identified in this study including N250K, N257D, A270E, V296I, all of which co-exist in 11 out of the 25 total isolates. R536C, P606S and R406K were also SNPs that were detected in the IleS, however none of these mutations were previously reported to be associated to mupirocin resistance, as these polymorphisms were present in both Mup resistant and Mup susceptible isolates as shown in table 5.4.

5.3.2.3 Mutations affecting the *ileS* promoter region

The mutations occurring in the promoter region of the *ileS* region were identified in addition to the SNPs of the IleS to explore whether any of the detected SNPs could result in changing the phenotype of the isolate. For this analysis, the sequence of *S. epidermidis* strain ATCC 12228 was used as a reference (Accession: NC_004461.1).

Using reg-precise and BPROM web-based tools to carry out the *ileS* promoter region analysis, three main SNPs were detected in only seven isolates in total as shown in figure 5.7. G73A was detected in two isolates

(51 and 59) and interestingly, both of these isolates belong to the same ST-83 and were showing high-level Mup resistance (MIC > 1024 μg/ml). G278C was identified in five isolates (67, 68, 73, 79 and 83) and G285A in 5 isolates (68, 73, 74, 77 and 79). Both of the latter SNPs were not associated with any significant impact on the Mup phenotype as all of the isolates carrying these substitutions are susceptible to Mup, except for STAPH75 which had low-level Mup resistance due to the presence of V588F mutation as previously mentioned (Table 5.4). No SNPs were detected in the ribosomal binding sites or -10 or -35 of the promoter region of *ileS* in any of the *S. epidermidis* strains (Figure 5.7).

Table 5.4 Isoleucine synthase related genotypes and phenotypes

Isolate ID	ST	*ileS* N250K[a]	ileS N257D	*ileS* A270E	*ileS* V296I	*ileS* V588F	*ileS* R536C	*ileS* P606S	*ileS* R406K	*ileS2*	MIC	MBC	SNPs in promoter region
STAPH 48	559	+		+		+				-	128	512	-
STAPH 49	2	+	+	+	+	+					64	128	-
STAPH 51	83									+	>1024	>1024	1
STAPH 53	5									+	>1024	>1024	-
STAPH 54	5										0.5	2	
STAPH 56	2	+	+	+	+			+	+	+	>1024	>1024	-
STAPH 58	2	+		+							0.5	1	-
STAPH 59	83									+	>1024	>1024	1
STAPH 60	2	+	+	+	+						0.5	2	-
STAPH 61	2	+	+	+	+						<0.5	<0.5	-
STAPH 62	2	+	+	+	+	+[b]					32[b]	128	-
STAPH 63	2	+	+	+	+	+					128	512	-
STAPH 64	2	+	+	+	+	+					128	512	-
STAPH 66	19	+		+							0.25	1	-
STAPH 67	210	+					+				0.25	0.5	1
STAPH 68	54										0.25	1	2
STAPH 69	2									+	>1024	>1024	-
STAPH 70	2	+		+							<0.5	<0.5	-
STAPH 73	204	+					+				<0.5	<0.5	2
STAPH 74	novel	+									<0.5	<0.5	1
STAPH	2	+	+	+	+	+					128	512	-

75 STAPH 77	59					0.5	1	1
STAPH 78	2	+	+	+	+	<0.5	<0.5	-
STAPH 79	48	+	+	+	+	1	2	2
STAPH 83	2	+	+	+	+	1	4	1

[a] IleS amino acid changes in St*aphylococcus epidermidis* ATCC 12228 (Genbank accession number NC_004461.1).

[b] mutation detected in resistant subclone. MIC, minimum inhibitory concentration of mupirocin. MBC, minimum bactericidal concentration, SNPs, single nucleotide polymorphism

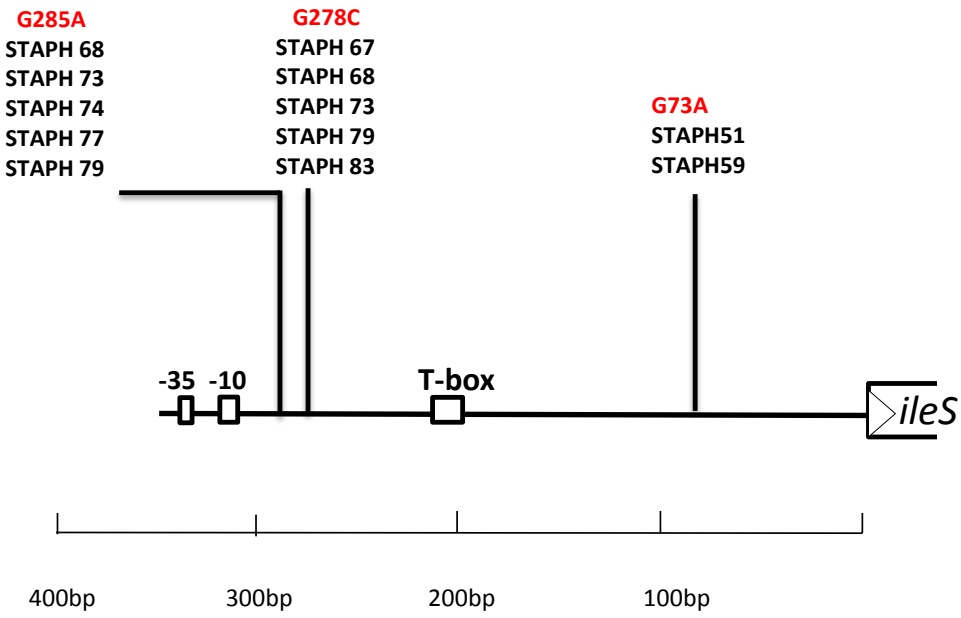


Figure 5.7 Schematic map of the intergenic region upstream of *ileS*.

SNPs in the promoter region of *ileS* gene of *S. epidermidis* clinical isolates are shown. The numbering initiates at the nucleotide in front of the start codon of *ileS* and counts from right to left. The putative promoter consensus is shown as -10 and -35. The promoter of the ATCC 12228 reference strain is used as a reference (Accession: NC_004461.1). The T-BOX for RNA binding upstream the *ileS* gene was predicted by Reg-precise (http://regprecise.lbl.gov/RegPrecise/regulon.jsp?regulon_id=26969). The putative -10 and -35 consensus was predicted by BPROM (http://linux1.softberry.com/cgi-bin/programs/gfindb/bprom.pl).

5.3.2.4 Presence of the *ileS2* gene

The contribution of *ileS2* for conferring high-Mup resistance levels have been addressed in several studies. The *S. epidermidis* isolates were screened for the presence of *ileS2* gene, which has been shown to be

associated with high-level of Mup resistance. The *ileS2* was identified in five strains (51, 53, 56, 59 and 69). All of these five isolates were high-mup resistant (MIC >1 1024 μg/ml) (Table 5.4). Two of these isolates belong to ST-2, two isolates belong to ST-83 and one isolate is ST-5.

The location of *ileS2* whether in the chromosome or plasmid-borne has been shown to be equally important in determining the susceptibility phenotype of staphylococcal strains. All of the *S. epidermidis* strains harbouring *ileS2* carried this gene in a plasmid (51, 53, 56 and 59) except one strain, STAPH 69 where *ileS2* was located on the chromosome. In this strain, the *ileS2* gene was found to be sandwiched between two copies of insertion sequence IS*257* indicating that integration had occurred by homologous recombination on the IS element. These findings correspond to previous data published that in the vast majority of Hi-mupirocin resistant strains, the *ileS2* gene is plasmid-borne, and rarely being reported to be in the chromosome (Perez-Roth et al., 2011). In the cases where the *ileS2* gene is chromosomally located, the isolates conferred lower levels of mupirocin resistance (Seah et al., 2012).

All in all, the molecular determinants for mup resistance should be addressed collectively as in some occasions more than one mechanism is present in the same isolates. In addition to amino acid substitutions, SNPs in promoter region and presence of additional gene like *ileS2*, other determinants could also exist like *mupB* gene conferring high-mup resistance (Seah et al., 2012). There might also further mechanisms of resistance yet to be discovered and the need for newer antibiotics is mandatory. The following part of this chapter will look into another transposon-mediated resistance, the FabI conferring reduced triclosan susceptibility.

5.3.3 Characterization of the transposon mediated triclosan resistance determinants and phenotypes

5.3.3.1 Triclosan susceptibility phenotypes

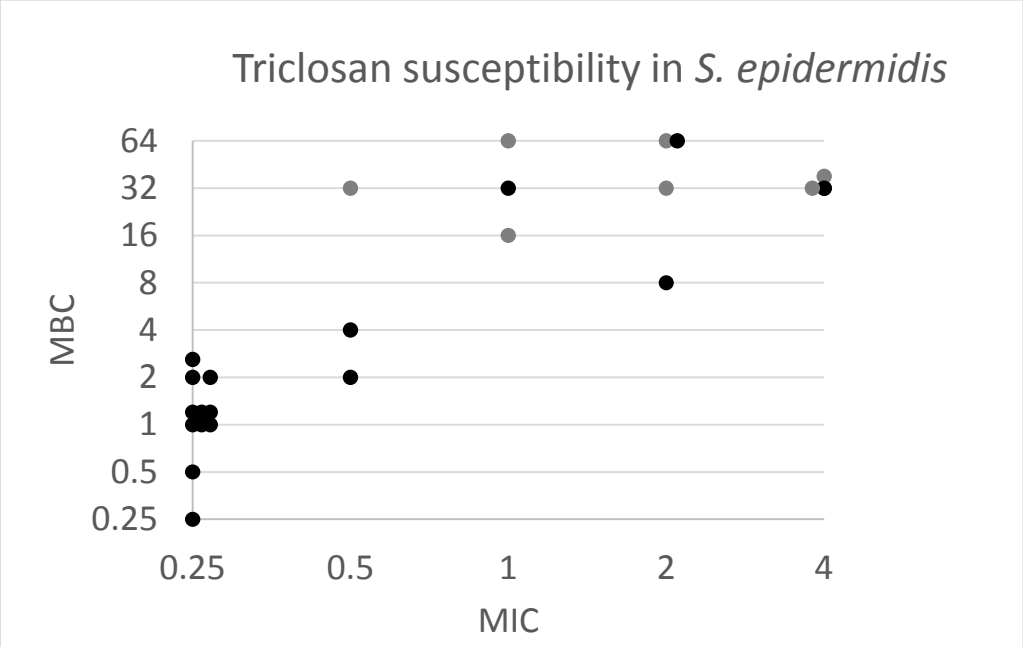
In this work, the phenotypes associated to increased MIC values, which exceed the epidemiological cut-off (ECOFF) (Morrissey et al., 2014) will be reported as reduced susceptibility. This is due to the fact that for biocides and disinfectants no clinical breakpoint values exist which can define the term “resistance”. The analysis of MICs and MBCs of *S. epidermidis* are summarised in table 5.5 and figure 5.8. The MICs for the 25 strains tested varied from 0.25 to 4 mg/l, while the MBCs readings were, as expected, higher, and ranging between 0.25 and 64 mg/l. The MBC values for most of the isolates are more than two dilutions higher than the MICs. The MIC and MBC both showed a continuous pattern in their distribution.

This is in contrast to what have been shown previously in triclosan susceptibility testing in *S. aureus* where only MIC values were continuously distributed whereas the MBCs were discontinuous (Ciusa et al., 2012). Statistical analysis showed that MIC and MBC values of triclosan of clinical strains were moderately correlated (ρ = 0.56; P >0.001). However, the MBC values are better capable of separating triclosan-non-susceptible strains than the MIC. The data suggests an epidemiological cut-off MBCs (ECOFF) of ≤1 mg/l as susceptible and ≥4 mg/l as non-susceptible.

Table 5.5 NADH-dependent trans-2-enoyl-acyl (ACP) reductase-related genotypes and phenotypes

Isolate ID	ST	G23S	H60Q	H61Y	S78A	V84L	F204L	*fabI*	*Sh-fabI*	MIC	MBC	SNPs in promoter region
STAPH48	559		+	+				mut[b]	+	1	64	7
STAPH 49	2							wt	-	0.25	2	4
STAPH 51	83						+	mut	+	2	64	4
STAPH 53	5							wt	+	4	32	-
STAPH 54	5	+						mut	-	1	32	-
STAPH 56	2							wt	+	2	32	4
STAPH 58	2							wt	+	4	32	4
STAPH 59	83						+	mut	-	2	8	4
STAPH 60	2							wt	-	0.25	0.25	4
STAPH 61	2							wt	-	0.25	1	4
STAPH 62	2							wt	-	0.25	0.5	4
STAPH 63	2							wt	-	0.25	2	4
STAPH 64	2							wt	-	0.25	1	4
STAPH 66	19			+	+	+		mut	-	2	64	5
STAPH 67	210				+			mut	+	0.5	32	5
STAPH 68	54							wt	-	0.5	2	4
STAPH 69	2							wt	-	0.25	1	4
STAPH 70	2							mut	+	1	16	4
STAPH 73	204							wt	-	0.25	1	4
STAPH 74	novel				+			mut	-	4	32	5
STAPH 75	2							wt	-	0.25	1	4
STAPH 77	59							wt	+	4	32	4
STAPH 78	2							wt	-	0.25	2	4
STAPH 79	48							wt	-	0.25	1	4
STAPH 83	2							wt	-	0.5	4	4

[a] FabI amino acid changes in St*aphylococcus epidermidis* ATCC 12228 (GenBank accession number AFJ97287.1|). [b]mut refers to mutated FabI, MIC, minimum inhibitory concentration of triclosan, MBC, minimum bactericidal concentration, SNPs, single nucleotide polymorphism



ibility of *S. epidermidis* strains to triclosan.

The triclosan MICs are shown in relation to their respective MBCs. Each strain is represented with a circle symbol, where the grey symbols indicate the strains carrying an additional *sh-fabI* gene, whereas black circles represent strains with only one copy of *fabI*.

5.3.3.2 Mutation determining changes in FabI

It has been previously shown that mutations upstream of the *fabI* or the promoter region of *fabI* in *S. aureus* clinical isolates have resulted in increased gene expression of the *fabI* (Grandgirard et al., 2015). In the latter study, 18% of triclosan resistant clinical isolates had mutations in the *fabI* promoter. In addition, FabI mutations (C34T, T101G and A101C) were present in strains with upregulated *fabI*, in both laboratory mutants and/or clinical isolates. Similar studies also demonstrated that mutations in the promoter region of *norA* were associated with elevated expression of this efflux gene, thus inducing resistance to various compounds including quaternary ammonium compounds and bisbiguanides (Furi et al., 2013).

The protein sequence of FabI of clinical strains of *S. epidermidis* was aligned against the reference FabI protein of the triclosan susceptible

reference strain ATCC 12228 and the results are summarised in table 5.5. The analysis of the amino acid substitutions was carried out in a similar manner as shown before for the IleS protein. In this analysis, eleven out of the twenty-five clinical isolates harboured genes encoding for proteins with the identical FabI amino acid sequence as ATCC 12228. The remaining fourteen strains had one or more non-synonymous mutations, causing changes in the predicted amino acid sequence. Isolates STAPH48 had a H60Q and H61Y mutation, STAPH51 and STAPH59 had a substitution of phenylalanine for a valine at position 204 (F204V), isolates STAPH66 had a S78A, H61Y and V84L and STAPH67 had a S78A mutation only. Mutation at F204V position has previously been described in *fabI* of *S. aureus* with reduced susceptibility to triclosan (Ciusa et al., 2012). In this study, strains with this mutation showed reduced triclosan susceptibility (MIC 2μg/ml), which is similar to previously published data in *S. aureus* (Ciusa et al., 2012). Isolate STAPH54 had a single mutation in the G23S position and showed reduced susceptibility to tricolsan as evidenced by the MBC (MIC 1 μg/ml, MBC 32 μg/ml). This G23S substitution could be a potential novel mutation as this isolate does not have any other resistant determinant for triclosan. It should be noted however, that the interpretation of all these mutations has to be considered carefully, and cannot be separated from other molecular mechanisms of triclosan resistance including mutations in the *fabI* promoter region or the presence of an additional *sh-fabI*, which will be addressed in the following sections of this chapter.

5.3.3.3 Mutations affecting the *fabI* promoter region

The *S. epidermidis* isolates were further analysed for the presence of any SNPs in the promoter region of the *fabI* gene. In addition of the mutations in the FabI protein, several nucleotide polymorphisms in the putative promoter region have been identified (Figure 5.9). Data are in part reported in (Hijazi et al., 2016). In total, 18 SNPs were distributed at different positions including the -10 and -35 and the putative shine-Dalgarno (SD) sequence of the promoter region. The majority of the

isolates had least 4 to 7 SNPs in the sequence analysed, except for two isolates which had no SNPs and their promoter sequence was identical to the reference strain ATCC 12228 (STAPH53 and STAPH54).

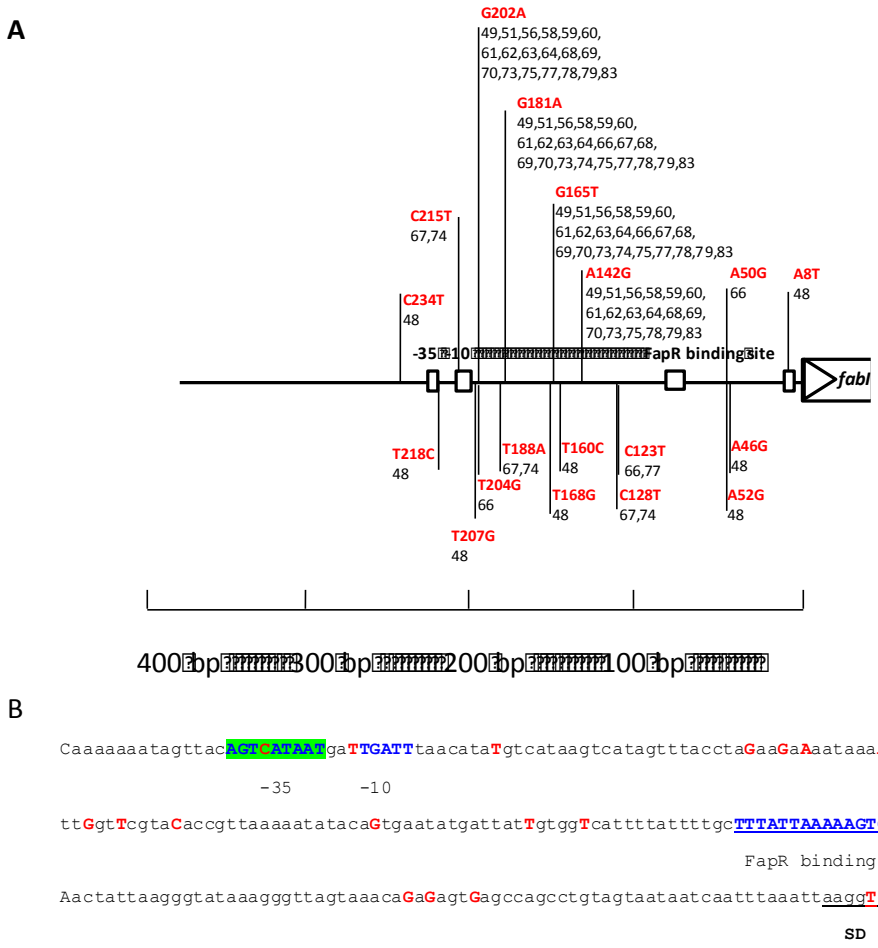


Figure 5.9 Schematic map of SNPs in the *S. epidermidis* fabI gene.

(A) SNPs detected in clinical isolates are mapped above and below the sequence. The *fabI* upstream region from the *S. epidermidis* ATCC 12228 genome (GenBank Accession: NC_004461.1) is reported. The positions of the SNPs are reported with respect to the first nucleotide preceding the start codon of *fabI* and numbered backwards. The nucleotide substitution is described above/below the polymorphic position together with the number of the clinical isolates (in black). The putative −35 and −10 consensus sequences were identified by BPROM. The consensus of the FapR regulator binding site is reported as mapped in RegPrecise. In panel (A) amino acid substitutions are shown in bold red upper-case letters. In panel B the SNPs are shown again in red upper case, the predicted binding site for the FapR regulator and the -10 and -35 sequences are shown in blue bold upper-case letters. The shine-Dalgarno sequence of *fabI* is indicated as SD.

The occurrence of high numbers of SNPs in such number of strains did not appear to have any significant impact on the triclosan susceptibility as a sole factor, due to the presence of other determinants that are known for

inducing reduced tolerance such as the FabI amino acid substitutions as well as additional *sh-fabI* in the majority of these *S. epidermidis* isolates. The latter resistance conferring *sh-fabI* gene will be discussed in the next section.

5.3.3.4 Presence of the *sh-fabI* gene

To further address the molecular basis of reduced triclosan susceptibility, the genome data were searched for any additional *sh-fabI* gene, mobilised by TnSha1 or TnSha2 from *S. haemolyticus* and a known genotype conferring reduced susceptibility to triclosan (Ciusa et al., 2012). Eight of the strains were found to harbour an additional *sh-fabI* gene and four of these eight *sh-fabI* positive strains had a mutated *fabI*, whereas the other four strains harboured a wild type *fabI* gene. In seven of the isolates, *sh-fabI* was found in the intergenic region of the *S. epidermidis* chromosomes, whilst the gene was located in a plasmid in one strain only (STAPH 53). Five of the eight *sh-fabI* strains were positive for MDR efflux determinant *qac*, as shown in table 5.5 (Hijazi et al., 2016). The clinical isolates with reduced susceptibility to triclosan showed significant association with either a mutated *fabI* or an additional *sh-fabI* as evidenced by the data analysed in table 5.5.

5.3.4 Multi locus sequence typing of TnSha1 and TnSha2 carrying isolates

The work presented in this section focused on the analysis of triclosan resistant staphylococcus isolates. Data on the epidemiology of the *sh-fabI* carrying transposons TnSha1 and TnSh2 (Furi et al., 2016) has been explored by analysing the genomes of ten *S. aureus* strains that were sequenced in a previous work (Ciusa et al., 2012). The latter paper had identified the presence of an additional *fabI* allele acquired from *S. haemolyticus* (*sh-fabI*) resulting in the duplication of the *fabI* target gene, and thus reduced triclosan susceptibility in those strains. Further analysis of those 10 *S. aureus* isolates has led to the identification of the novel composite transposons TnSha1 and TnSha2 involved in the transposition of *sh-fabI*. My contribution to that work was to search the Genbank database (Furi et al., 2016) and extract the genomes of TnSha1 and TnSha2 staphylococcal positive strains and to perform the MLST analysis on those species. This data aimed to address the dynamics of TnSha transposition between various species.

The TnSha1 element was identified in various *Staphylococcal* species and plasmids including *S. aureus, S. argenteus, S. haemolyticus, S. epidermidis, S. saprophyticus,* and *S. warneri* by searching the Genbank database (Furi et al., 2016). In the TnSha1 plasmid-containing Staphylococcal species, the element was found to be integrated between *repA* and IS*257* in pUSA03-like plasmids (Furi et al., 2016). The target site for the integration of the TnSha elements was referred to as (Loeza-Lara et al.), where Z-integration site is particularly interesting as the element was found to be inserted in two different orientations.

Multi locus sequence typing (MLST) of the 109 *Staphylococcus* strains (62 *S. aureus* and 47 *S. epidermidis*) showed that TnSha1 was predominantly found in *S. aureus* sequence type 8 (n=23) and ST-5 (n=18). The occurrences of the other sequence types of *S. aureus* was ST239 (n = 6), and ST290 (n = 6) strains and in *S. epidermidis* in ST59 (n = 10) and ST2 (n = 6) strains (Figure 5.10). The TnSha1 elements from

the *S. aureus* isolates sequenced in previous work of this lab (Ciusa et al., 2012, Furi et al., 2016) were aligned and found to have a pattern of TnSha1-SNPs clustering. This clustering of SNPs in the same sequence types is consistant of TnSha1 being inserted at the same integration sites followed by clonal expansion in almost all of the TnSha1 insertion sites, except for A2 and Z sites in which the same TnSha1 element was observed to be integrated in strains that belong to different STs which is suggestive of possiblely being transferred horizontally (Furi et al., 2016). Interestingly, the TnSha2 was found to mainly belong to different Sequence Types in various *Staphylococcus* speices; ST3 n = 6 in *S. haemolyticus*, and ST2 n = 21 in *S. epidermidis* and ST5 n=1 in *S. aureus*). This pattern of clonal diversity is suggestive of various dissemination mechanisms in different species (Furi et al., 2016). As previously mentioned in this study, the ST2 *S. epidermidis* are of a hospital origin (Roach et al., 2015, Schoenfelder et al., 2010), whereas ST3 *S. haemolyticus* are all clinical isolates without any indication of community or hospital origin of the infection (Furi et al., 2016).

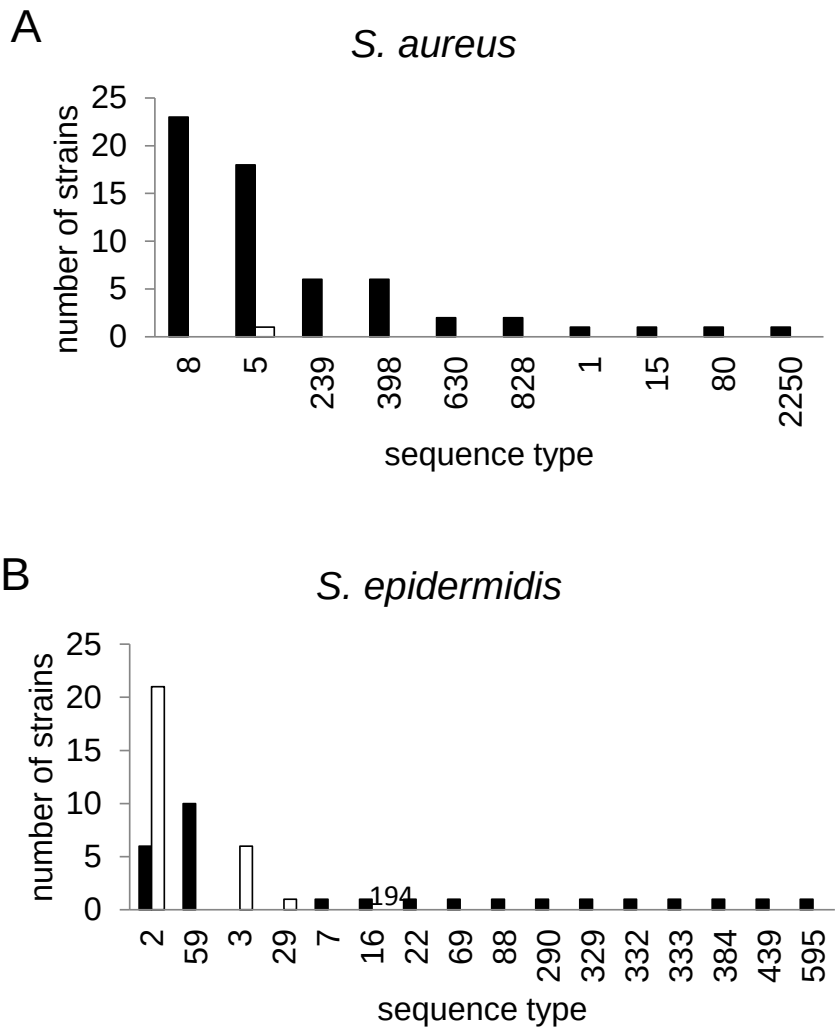


Figure 5.10 MLST profiles of staphylococcal strains carrying TnSha1 and TnSha2.

The sequence type (ST) of 62 *S. aureus* (panel A) and 47 *S. epidermidis* strains (panel B) which carry the *sh-fabI* elements TnSha1 (filled bars) and TnSha2 (open bars) are shown. Data are from staphylococcal genomes accessed in GenBank in December 2015 and sequence types were defined using the MLST web-service of the Center for Genomic Epidemiology (Larsen et al., 2012).

5.3.5 Testing for the excision and mobilisation of the TnSha1 element by qPCR

For one of the TnSha1 palindromic target sequences (Furi et al., 2016), two different transposition events could be detected by genome analysis, as they had led to deletions of different sizes in the same target site (named as A and A2; Figure 5.3). This led us to postulate that the "A" site is a frequent or preferred site of integration and was therefore chosen to test functionality of the TnSha1 element.

An experiment was designed to detect the circular form of the IS*1272* or the composite transposon TnSha1 containing IS*1272* and *sh-fabI* upon excision from the chromosome. Three sets of primers were designed as follows: a set of divergent primers on TnSha1, another set on IS*1272*, and a third set of primers targeting the "A" insertion site (Figure 5.11). The FAM labelled TaqMan probe and the qPCR primers used are shown in (Table 5.6). Detection of strains carrying TnSha1 in either the A or B integration sites via real-time PCR amplification was performed in a LightCycler 480 system (Roche Diagnostics, Germany) using primers annealing between the integration site and the transposon (Table 5.6); (Isola et al., 2005, Oggioni et al., 2002, Yesilkaya et al., 2006). Primers LF_30 and LF_31 were used to detect the presence of bacteria with a transposon free integration site A (Table 5.6). Unfortunately, no excision events producing a circular intermediate could be observed when two *S. aureus* strains were tested with or without the presence of triclosan in the

culture medium, and after induction with mitomycin C. Several attempts with new primers, adjusted qPCR conditions as well as *S. epidermidis* and *S. haemolyticus* isolates STAPH66 and CN1197 respectively, failed to yield any qPCR amplicons. It was concluded that either the element was not mobile in these conditions, in all strains tested or that neither the chromosome nor the excised element went through covalent re-ligation of the DNA filaments. Formation of hairpin loops as shown by RNA structure in figure 5.3 (Furi et al., 2016). An alternative method was used to demonstrate the mobilisation of the element is discussed in the next section.

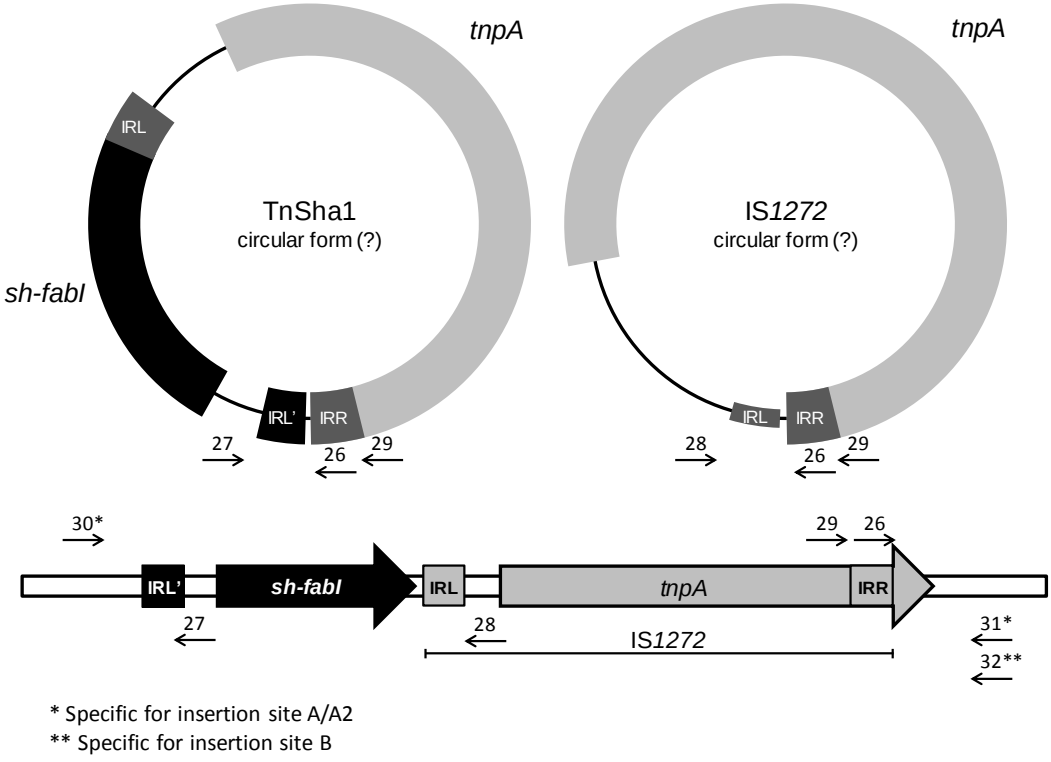


Figure 5.11 Schematic representation of the positions of the different primers used to amplify the circular intermediates of the TnSha1 and IS*1272* by qPCR assays.

The nucleotide sequences and the GenBank ID of positions of primers are shown in table 5.6 below.

Table 5.6 List of primers used to amplify the circular forms of TnSha1 and IS1272

Name	Position (GenBank ID)	Sequence
LF_26	Taqman probe for TnSha1 (JQ712986)	6FAM-TTCACTTATCCAAGAACTTTATGTCCCGGA-BHQ-1
LF_27	IRL' of TnSha1 (JQ712986)	ATCCTTGCCGGGGTAATACAAC
LF_28	IRL of TnSha1 (JQ712986)	AAAGCGAGCCAACAATACGGAGTA
LF_29	IRR of TnSha1 (JQ712986)	TAGTAGCTCAACGAGCTGAAAATAATC
LF_30	Upstream region flanking the TnSha1 integration site A/A2 (NC_002951)	TTGATTTATTTCCCAGCCTATTCTTTTCA
LF_31	Downstream region flanking the TnSha1 integration site A/A2 (NC_002951)	AGGATGTCGATTTGATTTATATTTTTTGTACAT
LF_32	Downstream region flanking the TnSha1 integration site B (NC_002951)	ATCATTTCGTTTATATATAGCAGACATGATAGA

5.3.5.1 Testing for the excision and mobilisation of the TnSha1 element using a reporter plasmid with a transposon trap

In our previous work it was shown that the TnSha1 transposon could insert into various chromosomal sites referred to as A to Z insertion sites (Furi et al., 2016). That work showed that the Z target-site is of interest because we could identify multiple insertions of TnSha1 from various strains into the *repA* terminator of plasmids (Figure 5.12) (Furi et al., 2016). The aligned sequences obtained from Genbank search show at least four independent insertions in two opposite orientations of TnSha1 into the *repA* terminator in plasmids homologous to plasmid pUSA03 (target Z). The Genbank IDs of the aligned sequences are mentioned in the legend of figure 5.12.

```
LPIH6008      TAAATTTTCAAAAAAAAGAG***GGGAGTGGGNNNNNNNNCCCGGACTC***CTTTACGGTTCTGTTGCAAA
F77919        TAAATTTTCAAAAAAAAGAG***GGGAGTGGGNNNNNNNNCCCGGACTC***CTTTACGGTTCTGTTGCAAA
W21479        TAAATTTTCAAAAAAAAGAG***GGGAGTGGGNNNNNNNNCCCGGACTC***CTTTACGGTTCTGTTGCAAA
HOAG6084      TAAATTTTCAAAAAAAAG--***GGGAGTGGGNNNNNNNNCCCGGACTC***-TTTACGGTTCTGTTGCAAA

F70077        TAAATTTTCAAAAAAAAGA-***GAGTCCGGGNNNNNNNNCCCACTCCC***CTTTACGGTTCTGTTGCAAA
F12753        TAAATTTTCAAAAAAAAGA-***GAGTCCGGGNNNNNNNNCCCACTCCC***CTTTACGGTTCTGTTGCAAA
F12753        TAAATTTTCAAAAAAAAGA-***GAGTCCGGGNNNNNNNNCCCACTCCC***-TTTACGGTTCTGTTGCAAA

target        TAAATTTTCAAAAAAAAGAGATTATACACCTAAAAAGGTGTATAATCTTTACGGTTCTGTTGCAAA
              ******************                                  ******************
```

Figure 5.12 . Z insertion site of TnSha1 in plasmids.

This panel shows evidence for multiple insertions of TnSha1 in target Z in the *repA* terminator of plasmids. The aligned sequences show at least four independent insertions in two opposite orientations of TnSha1 into the *repA* terminator in plasmid pUSA03 (target Z). TnSha1 sequences are shown in bold italics upper case, and the *repA* terminator hairpin underlined. Note that the

upper four TnSha1 sequences are in one orientation while the lower three in the opposite orientation. The *S. aureus* strain names are given on the left (Furi et al., 2016). Strains Genbank IDs are: LPIH6008 (ID: JDXZ01000007), HOAG6084 (ID: JBSH01000015), F77919 (ID: JEEN01000028), W21479 (ID: JEOI01000012), F70077 (ID: JEQM01000030), F12753 (ID: JGFR01000050).

The aim of this experiment was to demonstrate and explore the transposition of the *sh-fabI* carrying TnSha1 or of the IS*1272* element alone in various staphylococcal species. To this scope, a construct of a reporter strain with a transposon trap was made. The approach chosen, envisaged cloning of the hairpin constituting the Z target-site upstream a promoter-less aminoglycoside phosphotransferase III (*aphIII)* gene conferring kanamycin resistance (Figure 5.13). This was aimed to construct a synthetic target and "transposon trap" upstream a reporter gene. The construct was done in the *Escherichia coli-Bacillus subtilis* shuttle vector pMK4 carrying a chloramphenicol resistance gene for selection in *Staphylococcus* (Sullivan et al., 1984). After cloning in *E. coli*, the recombinant vector was electroporated in *S. aureus* RN4220 to facilitate further transfer to *S. aureus*, *S. epidermidis and S. haemolyticus* strains harbouring ThSha1 or TnSha2 (Furi et al., 2016). As the transposase gene of IS*1272* is extending outside of the IS element, an integration of the IS element into the Z-target hairpin was expected to delete the hairpin and drive expression of the promoter-less *aphIII* gene; at least when inserted in one of the two possible orientations.

In these experiments, pMK4 (Sullivan et al., 1984), a low copy number shuttle vector that is compatible with *S. aureus* strains was used. pMK4 has *bla* and *cat* genes conferring ampicillin and chloramphenicol resistance, respectively. Initially, several *S. aureus* lab strains were screened for an empty Z-site, i.e. a Z insertion site that is not occupied by a TnSha1 transposon. Several sequenced *S. aureus* lab strains were searched by Blastn for any possible empty Z target-site. Strain PB4 (kind gift of Julie Morrissey, lab 121, University of Leicester), a methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from a patient with persistent bacteraemia, was found to have an empty Z target-site has

been used for the cloning experiment figure 5.13. For primer design, the contig containing the Z-site (sequenced by Julie Morrissey's group, unpublished) was uploaded on RAST web tool for annotation (Aziz et al., 2008). The primer pair (F-repA and R-Z1) were used to amplify a 538 bp fragment that includes part of the upstream *repA* gene along with the 55 bp Z-site. The size of the PCR product was decided in order to facilitate detection by gel electrophoresis and subsequent cloning into the plasmid pMK4. The reverse primers used to amplify the Z-site were designed to include three stop codons to avoid fusion of any genes arising from the upstream of the gene, as well as a ribosomal binding site consensus at the 5' end. The Z-site was amplified separately and then ligated by SOEin-PCR (Heckman and Pease, 2007) with a promoter-less, kanamycin-resistance *aphIII* gene. The final PCR product was ligated into the pMK4 vector after both being digested with EcoRI enzyme resulting in pMK4-Zkan.The cloning experiment is illustrated in figure 5.13 below.

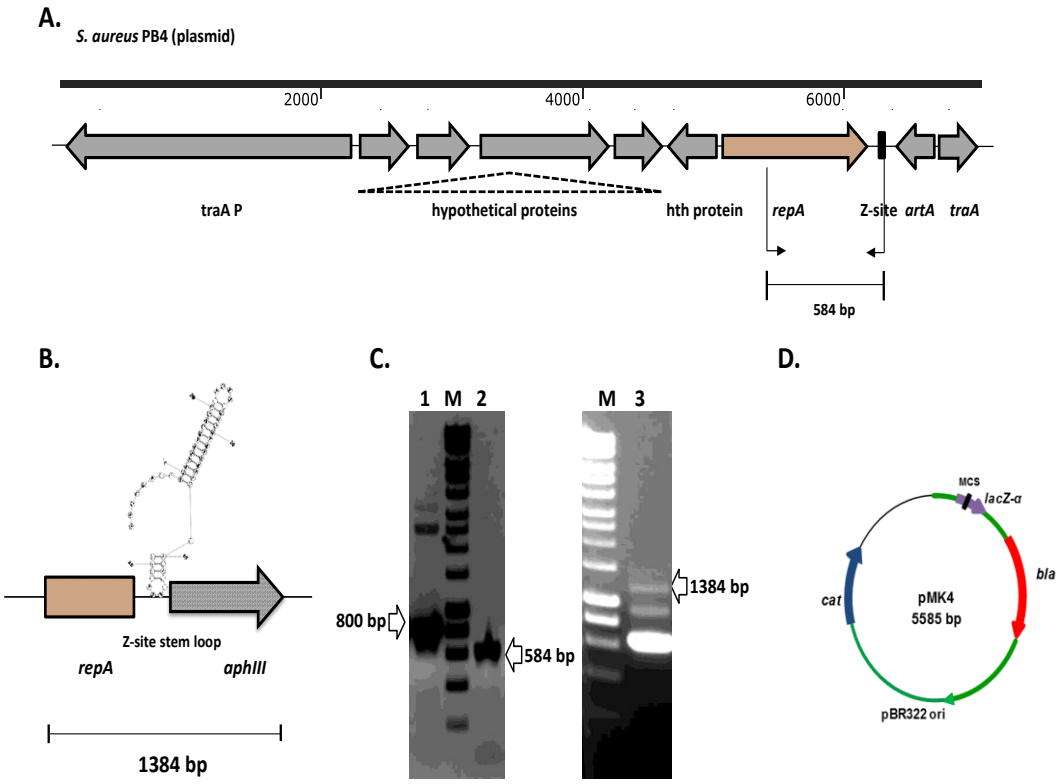


Figure 5.13 Cloning of the Z target-site from *S. aureus* PB4 into pMK4 shuttle vector.

(A) 584 bp region was amplified by PCR (primers F-repP/R-Z1) including the empty Z-site and with part of the upstream gene *repA* (red striped). (B) the Z-site and *repA* PCR product was joined with an 800 bp *aphIII* cassette resulting in a recombinant product of 1384 bp. (C) gel electrophoresis images of the PCR products, indicating the size in bp: lane 1 *aphIII* cassette, lane M hyper-ladder gene marker, lane 2 *repA*+ Z-site stem loop, lane 3 recombinant allele resulting from PCR 1+2. (D) pMK4 shuttle vector used to ligate the recombinant allele into the EcoRI site after both plasmid and PCR product digestion with restriction enzyme EcoRI.

In the first instance, the new construct pMK4-Zkan was transformed into *E. coli* DH5α by electroporation and the transformation mixture was plated in LB agar containing 100 µg/ml ampicillin for selection and incubated at 37°C for 24 hours. Positive controls which comprise original pMK4 without insert and negative controls as competent DH5α cells with dH_2O were transformed in parallel. Out of the 200 colonies counted the next day, 10 colonies were passaged on ampicillin 100 µg/ml plates. The same colonies were screened by colony PCR to confirm the kanamycin cassette in plasmid pMK4-Zkan using the primer pair F-kan2/R-kan and the gel electrophoresis images are shown in figure 5.14.

The new pMK4-Zkan was then isolated by plasmid mini prep kit for a second electroporation into RN4220, a *S. aureus* laboratory strain, which is frequently used in *S. aureus* experiments as it is characterized by a mutation in the *sau1 hsdR* gene, making it deficient in the SauI restriction system and hence an ideal intermediate cloning host (Kreiswirth et al., 1983). The transformation protocol for all staphylococcal species differ from that of *E. coli* species as discussed in the methods chapter 2. In strain RN4220 the selection for the transformation colonies was with chloramphenicol 10 µg/ml. The transformant colonies were then passaged in the selection media and confirmed by colony PCR screening of the *aphIII* cassette using the primer pair F-kan2/R-kan as shown in figure 5.14. The pMK4-Zkan was extracted and the insert was further confirmed by sequencing of the whole insert.

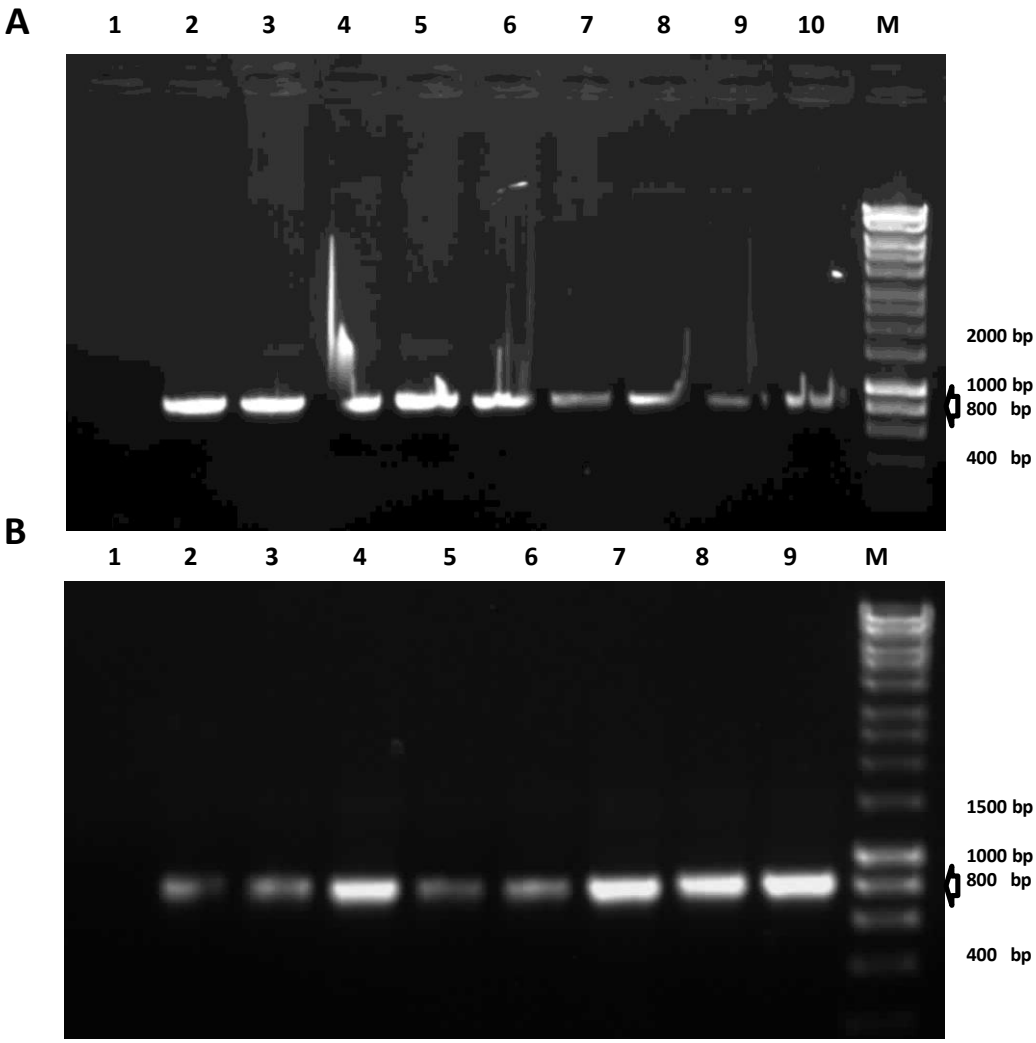


Figure 5.14 Gel electrophoresis images of colony PCR.

The PCR data are confirming the presence of *aphIII* kanamycin cassette using primers F-kan1 and R-kan in both *E. coli* DH5α transformant cells (A) as well as *S. aureus* RN4220 colonies (B). in both panels A and B the lanes are labelled and represent the following: 1: negative control dH2O; 2: positive control kanamycin purified cassette from pUC19; lanes (2-10) in (A) and (2-9) in (B) are confirmed colonies with the right *aphIII* cassette amplifying 800 bp PCR product and lane M is hyper-ladder marker.

With the recombinant reporter plasmid harbouring the empty Z-site in front of the promoter-less resistance gene as a hot spot for insertion, the next step was to check whether the TnSha1 element or IS*1272* alone could mobilise into the empty site. Electro-competent cells were prepared from three *Staphylococcus* species strains positive for TnSha1 (*S. aureus* 25923) or TnSha2 (*S. epidermidis* STAPH 66 and *S. haemolyticus* CN1197). The competent cells were subsequently transformed with pMK4-Zkan. All strains were selected on TSA plates containing

chloramphenicol 10 µg/ml (selection for the vector). Colonies count was performed after 24 hours. To test for transposition into the Z-site, colonies were then passaged into kanamycin 75 µg/ml plates. This is a vital step as it allows selection for kanamycin positive colonies which should harbour recombinant plasmids with the IS element in front of the promoter-less resistance gene. These clones had to be screened for the presence of the TnSha1 transposon in order to confirm that the insertion had occurred. The number of colonies in each of the transformation steps above are summarised in table 5.7.

Table 5.7 Colony count achieved in transformation of pMK4 and its pMK4-Zkan derivative in *Staphylococcus* species

strain	vector	Colony counts		
		amp	cm	km
S. aureus 25923	**pMK4-Zkan from RN4220**		10^3	44/50*
S. epidermidis STAPH 66	**pMK4-Zkan from RN4220**	-	10	8
S. haemolyticus CN1197	**pMK4-Zkan from RN4220**		0	0

*44 colonies out of the 50 colonies passaged in kanamycin 75 µg/ml plates

Based on the planning of the reporter construct, it was assumed that the kanamycin-resistant colonies were the (rare) ones in which TnSha1 or IS*1272* would have transposed into the Z target-site and by inactivating the hairpin drive expression of the promoter-less *aphIII* gene by generation of a transcriptional fusion with the transposase gene of the IS element. Therefore, these colonies were screened by PCR using the primers LF-29/IF-kan to amplify 5'-end of the IS1272 and part of the *aphIII* cassette yielding a 600 bp PCR product, as shown in figure 5.14.

To further confirm that these colonies are the desired product after IS*1272* insertion, several colonies were sent for sequencing using the same primers for PCR screening (LF-29/IF-kan). The sequencing would be expected to yield IS*1272* sequence upstream the promoter-less *aphIII* cassette, resulting from the transposition of the element within the target

trap. However, nucleotide sequencing results did not show any related sequence to the expected insert and only showed the *aphIII* sequence. Therefore, it was assumed that this could have resulted from leaky expression of the promoter-less *aphIII* cassette kanamycin.

Nevertheless, this data also shows that whilst IS*1272* could not insert within the trap, it is possible that the occurrence of such event under natural conditions are very rare and at very low frequency.

5.4 Discussion

In staphylococci of human origin, a large variety of resistance genes have been described, some of which are found intrinsically while other may be either acquired by *de novo* mutations or via horizontal transfer. One of the main vehicles for the horizontal spread of resistance genes are plasmids, which could either function as carriers of plasmid-borne resistance genes or act as vectors for transposon-borne resistance determinants (Schwarz et al., 2011). There is an expanding knowledge on the role of transposons in dissemination of resistance to antimicrobials and biocides among staphyloccoci.

In this work, a thorough screening for the resistance genes was conducted using the CARD web-based tool, with specific focus on the ones which are plasmid-borne or transposon-borne. The search showed that the numerous number of these genes are located on these mobile elements, like small PBI resistance plasmids carrying a wide array of genes conferring resistance to various antibiotics including (*tetK*), a tetracycline resistance gene that is widely spread among staphylococci (Schwarz and Noble, 1994, Schwarz and Wang, 1993) typically carried in pT181. Moreover, macrolides, lincosamides and streptogramin B (MLS) resistance determinants *emrC* was carried on plasmids in most of our *S. epidermidis* isolates. This gene is typically found in a 2.3 -4 kb pE194-pSES22–like plasmids. A number of plasmids acting as vectors of transposon-borne PBI resistance genes were also found in these *S.*

epidermidis strains such as the Tn*4001-carried aac(6')-Ie-aph(2'')-Ia* conferring aminoglycosides resistance and Tn*5405*–borne *aphA-3-sat4-aadE* conferring streptothricin resistance. Both Tn*4001* and Tn*5405* have been found either completely or partially integrated on staphylococcal plasmids or in chromosomal DNA (Schwarz et al., 2011).

Current evidence strongly suggests that staphylococci can acquire resistance genes like *erm*B and *erm*T by plasmids or plasmid-carried transposons not only from other *Staphylococcus* species but also from different genera including *Streptococcus*, *Enterococcus* and *Lactobacillus* (Schwarz et al., 2011). In addition, the IS mediated transfer of metabolic resistance genes, in our case *sh-fabI* genes and triclosan resistance, is a highly relevant mechanism for the acquisition and spread of antibiotic resistance. The *ileS2* and *fabI* genes are similarly bracketed by IS elements that enable their fast spread among other species. Analysis of the large plasmid pBR9 from *S. aureus* and *ileS2*-carrying plasmids in other staphylococcal species revealed the role of IS*257* in the dissemination of resistance genes especially in mediating mupirocin resistance (Perez-Roth et al., 2011). It has been shown that IS*257* mediated integration of pT181 plasmids into larger plasmids in *S. aureus* (Needham and Noble, 1995) as well as *S. epidermidis* (Werckenthin et al., 1996) where in both cases the IS*257* integration occurred upstream of *repC* gene separating the coding sequence of the gene from its promoter. Similarly, the integration/recombination processes involving IS*257* have been described in *S. haemolyticus* and *S. warneri* plasmids (Werckenthin et al., 1996). Studies have also shown evidence of presence of copies of IS*431*, which is related to IS*257* in most of the SCC*mec* element downstream of the *mecA* gene (Kondo et al., 2007, Shore et al., 2005, Stewart et al., 1994).

FabI, the IS-mediated resistance determinant which is an important target for drugs including isoniazid, used for treatment of tuberculosis and has been recently reconsidered for the discovery of new antimicrobial therapies targeting type II fatty acid synthesis pathways (FASII) (Escaich

et al., 2011). This includes using triclosan, a known disinfectant and biocide as an antibacterial target for staphylococcus infections (Lu and Tonge, 2008, Wang et al., 2013). A recently identified chromosomal element of IS*1272* harbouring the *S. haemolyticus* originally-derived *sh-fabI* gene, has been detected in a large number of *S. aureus* isolates and has been renamed as Tnsha1 (Ciusa et al., 2012, Furi et al., 2016). Moreover, it has been found that the *sh-fabI* could also be mobilised by a larger plasmid named as TnSha2, which is composed of the composite TnSha1 and two flanking IS*1272* resulting from the integration into a chromosomal IS*1272* (Furi et al., 2016).

In our study (Furi et al., 2016), BLAST searches of 4800 staphylococcus sequences was performed to check the co-existence of the known IS-mediated metabolic genes of *dfrA-thyE* genes (Rouch and Skurray, 1989), conferring trimethoprim resistance, and *ileS2* genes (Needham and Noble, 1995), resulted in 279 hits in *S. aureus* and 134 in *S. epidermidis* for *dfrA-thyE*, whereas *ileS2* yielded 207 hits in *S. aureus* and 18 in *S. epidermidis*. This shows well the relative occurrence of 65 TnSha1/2 elements in *S. aureus* and 50 in *S. epidermidis* in the same study (Furi et al., 2016).

In this work, careful analysis of 25 *S. epidermidis* clinical isolates for the triclosan and mupirocin susceptibility phenotype and genotype was carried out. To determine the underlying molecular basis for tricolsan and mupirocin reduced susceptibility, mutations in the amino acids and SNPs in the promoter region of the *fabI* were analysed in addition to screening of any *sh-fabI* genes. Similar analyses were performed for detection of mutations in the *ileS* conferring *mup* resistance. The presence of the missense mutation V558F was associated with low level of *mup* resistance, which is similar to what have been found in previous studies (Ciusa et al., 2012, Grandgirard et al., 2015), whereas, isolates with high-mup resistance were harbouring an additional *ileS2* gene mostly of which was plasmid-carried. It is well established that the position of point mutations may have significant impact on transcription level if it occurred

at the -10 or -35 of the promoter region. On the other hand, mutations affecting translation of the genes when occurring at the ribosomal binding sites. Therefore, the relatedness of the SNPs found in this study to the phenotypes should be evaluated in that context. In a similar way, all of the strains showing high MBCs had mutations in the coding region of *fabI* and the isolates with the highest MBCs were found to have an additional *sh-fabI* gene (MBC > 64 μg/ml). Studies have demonstrated that triclosan resistance can be manifested by increasing the target with cloning of the *fabI* into a plasmid (Slater-Radosti et al., 2001). Similarly, the presence of original *fabI* with an additional *sh-fabI* could increase the target by heterologous target duplications thus creating a new level of resistance (Ciusa et al., 2012). This work in *S. epidermidis* confirms the findings of previous studies on *S. aureus*, that the chromosomal mutations of *fabI* is the main mechanism of triclosan resistance as well as evidenced that presence of the horizontally transferred gene *sh-fabI* is driving an additional resistance mechanism , (Morrissey et al., 2014, Oggioni et al., 2015). This study also shows that the detection of *sh-fabI* in the *S. epidermidis* isolates indicates that it is rapidly spreading between staphylococcus species. The *sh-fabI* elements was detected in 14% of *S. epidermidis* isolates, which is a significant number of isolates carrying this resistance element. This could indicate the abusive use of triclosan as disinfectant for decontamination. However, the lower sample size and the lack of clinical information on these isolates may render this observation irrelevant.

Interestingly, the MLST data on the distribution of both TnSha elements showed a species specific pattern with TnSha1 being more prevalent in *S. aureus* while Tnsha2 was almost exclusively carried in *S. haemolyticus* and *S. epidermidis* were found to harbour both elements (Figure 5.10). Similar observations were seen in the *dfrA* gene conferring trimethoprim resistance which was mobilised between various staphylococcal species (Dale et al., 1995, Leelaporn et al., 1996). The study of transposition of the elements in the three staphylococcal species was therefore attempted

by the detection of circular forms in this study. This would have helped us to understand the epidemiology of these elements, however, these attempts were not successful.

The integration mechanism of IS*1272* originally described, have demonstrated that these IS elements did not appear to produce target duplications upon insertion (Archer et al., 1996). Further studies also showed that IS*1182* family, of which IS*1272* is a member, use palindromic sequences as target sites (Siguier et al., 2015). This work goes in line with these observations that both TnSha1 and IS*1272* elements seem to have a novel mechanism of integration, targeting hairpin loops, producing blunt-end cuts and upon insertion of the element, part of the target will be deleted (Furi et al., 2016). Another important observation is the absence of primary sequence consensus as multiple TnSha1 insertions have been detected in two different orientations. In addition, there is no insertions observed into a rolling circle plasmids in the previous study (Furi et al., 2016). Both of these observations indicate the that there is no bias for the orientation in which elements could insert into a replication fork as previously shown with other IS (Siguier et al., 2014).

The mechanisms by which DDE transposases integrate into their target sites are variable in the various subtypes as discussed in chapter 1 (Figure 1.4). One of these mechanisms is the transposition through cut-and paste mechanism and formation of hairpin intermediate without TE replication (Figure 1.4 column 3). During this process, after cleavage of the transferred strand the free 3'OH attacks the opposite strand forming a hairpin at each end of the IS element thus generating an intermediate that if free of attachment with the donor DNA. In this case, the hairpin intermediate formation could be the most likely hypothesis leading to very few events or single event that the detection of such circular elements by qPCR was not possible. This could also explain the absence of any positive colonies for the TnSha1 element in the trap cloning experiment.

During the writing of this thesis, however, a study was published by Wan et al 2017, demonstrating the precise structure, transposition and model of IS*1272* (Wan et al., 2017). The mode of transposition predicted by the latter study suggests a possible IR-replacing mechanism and a potential stem-loop-replacing transposition mechanism of IS*1272* (Wan et al., 2017). The detailed replacement mechanism is illustrated in figure 5.4 taken from the (Wan et al., 2017).

On the other hand, the case of TnSha2 transposition might be different as is the case for other transposons utilising various integration mechanisms like Tn*7* (Siguier et al., 2014). As previously shown, few available data showed that TnSha2 integration mechanism is similar to IS*1272* (Furi et al., 2016) and the mobilisation of TnSha2 could occur by the hypothetical ORFs leading to duplications and co-integrate formation as shown by the few genomic data available (Needham and Noble, 1995). As TnSha2 also exists as a plasmid and harbours genes for mobilisation, this could indicate possibility of HGT of the element. However, there is still not enough molecular evidences to allow us to better understand the epidemiology of these TnSha elements in staphylococcal species as in the case of *dfrA* genes (Dale et al., 1995, Leelaporn et al., 1996). Detection of both TnSha1 and TnSha2 element in relatively high number from the readily available sequences in the database have contributed to expand our knowledge of these two mobile elements and investigate the generation of this widely spreading element.

In conclusion, this study shows the significance of IS mediated transposition of metabolic genes as a growing antimicrobial resistance phenomenon. In addition to the well-described Tn*4003* element, which mobilizes *dfrA* by IS*257* thus conferring trimethoprim resistance (Rouch and Skurray, 1989), and the IS*257* mediated transposition of *ileS2* conferring mupirocin resistance (Needham and Noble, 1995), this data adds more to the existing knowledge by introducing few new concepts on TnSha1 and TnSha2 utilising IS transposition and integration. Furthermore, the plasmid mobilisation allowing the transfer of the *fabI*

gene of *S. haemolyticus* to different staphylococci thus contributing to triclosan resistance. With open access public databases, the analysis of similar IS mediated transposition may invest in more detailed investigations.

Chapter 6 General discussion and conclusions.

This thesis has investigated the mobilisation by site specific recombination of genomic islands by exploring both examples of integrase and transposase mediated mechanisms. For the former mechanism, I investigated in detail the features and genetic context of the integrases of two heavy metal resistance related genomic islands, G08 and G62, in *A. baumannii* and for the latter mechanisms I investigated the mobilisation of two IS-mediated transposable elements conferring reduced susceptibility to biocides in *Staphylococcus.* In both cases of these MGEs, serve as vehicles spreading various resistance determinants between pathogens by HGT.

Genomic islands are dynamic and distinct class of ancient integrative elements in which within each subset of islands, the genetic content is considerably variable. There are subsets of GIs which can be readily transmissible under standard laboratory conditions and with sufficient evidence that they have been recently acquired. Examples of mobile GIs include the well-known 500 kb symbiosis island in *Mesohrizobium loti* (Sullivan et al., 2002), SXT GI in *Vibrio cholerae* (Hochhut and Waldor, 1999) and *clc* GI of *Pseudomonas* sp. strain B-13 (Sentchilo et al., 2003). By contrast, many GIs which are chromosomally integrated seem to have lost their transmissibility. Several of these 'immobile' structures exhibit highly mosaic content, indicating the likely occurrence of multiple recombination events (Hsiao et al., 2003, Mantri and Williams, 2004). Despite that integrase mediated mobilisation occurs site-specifically in the chromosome, illegitimate recombination events at non-canonical sites have been demonstrated resulting in potentially deleterious effects on the cells (Cambray et al., 2011b). In addition, recombination frequencies can widely vary between species, but that in "non-stressful" conditions the occurrence of such recombination events are rare and the tendency for these events are likely only under stressful environmental conditions.

In the first part of this study, a combination of epidemiological survey on a substantial set of clinical isolates as well as experimental data to confirm

the functionality of integrases, has given an insight into the dynamics of these mobile elements. By closely analysing the integrase genes carried in these GIs, their structure and phylogeny, it was evident that these integrases are relatively related in the *A. baumannii* examined. This study also highlighted the various targeted PCR assays for the detection of evident mobilisation of circular intermediates, furthermore, new tools for studying integrase activity in a range of strain backgrounds were created.

Detection of genetic composition of GIs through *t*RIP-PCR, PCR mapping and whole genome sequencing yielded a total of identification of G08 in 1 clinical isolate and 3 lab strains, while G62 was only found in one reference strain and no clinical isolates. The sequencing data showed contig breaks in all G08 detected resulting from the GI insertion. A new set of integrase-targeting PCR primers outlined in chapter 3 give an alternative PCR assay to encompass a larger section of the integrase gene, which would only give a positive result when all the regions coding for the respective GI would be present. Through this new amplicon, the detection of false positive GIs (which failed to yield a band in initial *t*RIP-PCR which actually contain unoccupied locus may be circumvented. In addition, this thesis demonstrated through the use of modified PCR protocols that many circular excised GI are being undetected by conventional PCR assays (Rose, 2010). The mobile elements which with undetectable excision may still contribute to multidrug resistance phenotypes but their frequency of excision occur at extremely low frequencies. However, these issues would become easier to tackle, and the full picture of all GIs surveyed carried by different strains will be easily determined by the availability of whole genome sequencing alone which is now becoming economically viable for large scale screening (Chan et al., 2015, Di Nocera et al., 2011).

Growth curves of strains with and without an overexpressed integrase gene were used in this thesis to investigate the fitness cost of these elements in *A. baumannii*. These experiments were completed using a novel *A. baumannii*- compatible plasmid bearing an integrase under the

control of P_{lac}, which allowed the IPTG induction of the integrase. The plasmid also contained a mini-island from pUC18, which constitutes the circular form of which could be easily detected by PCR, in order to quantify the integrase activity. These assays showed that the integrase was biologically active in both an *A. baumannii* and *E. coli* backgrounds.

These plasmids can demonstrate how feasible it is to overexpress integrases in a controlled manner, for the purpose of investigating integrase activity or fitness cost. With any desired modifications of these plasmids to contain appropriate antibiotic resistance genes, this system may facilitate the investigation of integrase activity in a wider range of other Gram-negative pathogens including some MDR strains. Furthermore, the integrase can be placed under its natural promoter on a plasmid, therefore facilitating the analysis of integrase activity under the natural conditions.

Preliminary conventional PCR assays looking at integrase activity by detecting the excised circular intermediates in crude DNA obtained from wild-type *A. baumannii* clinical isolates failed to yield any PCR amplicons. The same assays were repeated with qPCR showed that Integrase-mediated GI excision of G08 and G62 was occurring at extremely low frequency only after 40 cycles of qPCR. As the previous studies suggested that *A. baumannii* was capable of expressing a biologically functional integrase (Aranda et al., 2011, Rieck et al., 2012, Shaikh et al., 2009) it was concluded that failure of detecting excised forms was due to absence of appropriate conditions that result in the expression of integrases and therefore these conditions can be adjusted experimentally. As evidenced in other studies, that DNA-damaging conditions could trigger a classical “SOS response”, which subsequently activates the expression of a number of genes, including integrases (Guerin et al., 2011, Hocquet et al., 2012), thus in this work *A. baumannii* clinical isolates were also exposed to mitomycin C, to test whether integrase activity could be induced. Whilst quantitative real-time PCR analysis showed that there were few detectable circular forms as well as chromosomal junctions

without any induction methods, the three strains tested along with the reference strains had significant increase in the level of circular intermediates and junctions detected of after 2 hours of exposure to DNA-damaging conditions (38-64 µg/ml mitomycin C).

The G08 and G62 genomic islands consists a set of heavy metal resistance mediated genes that are identical. Importantly, both genomic islands are hypothesized to confer resistance phenotypes related to heavy metals (Di Nocera et al., 2011). The ATCC 17978 genome has been extensively analysed and 13 putative zinc/copper resistance efflux pumps have been identified, including the efflux pumps present in G08 and G62 (chapter 4, figure 4.2). In this work, the phenotypes of G08 and G62 in both wild type strains and their G08/G62 deletion mutants was investigated by susceptibility testing using various metal compounds. Growth curves showed a significant growth delay due to the synergetic effect of adding both zinc and copper in the media, a finding that goes in the line with similar observation of a recent study (Hassan et al., 2017). The MIC assays confirmed the contribution of GI-encoded efflux transporters to heavy metal resistance mainly for copper and to a lesser extent arsenic and cadmium. During the writing of this thesis no study so far has published data on the role of efflux transporters tested in deletion mutants. However, the differences in the phenotypes observed in this study between wild types and mutant strains of *A. baumannii* are very minor and not reach the acceptable level of statistical significance. The need for more studies to address the role of these efflux systems in mediating resistance to metals and probably antibiotics is vital.

In the second part of this thesis, attention was given to another class of TE, the IS or transposons that are mostly carried within plasmids. These included IS-carried metabolic genes, *ileS* and *fabI* encoding for mupirocin and triclosan resistance, respectively (Furi et al., 2016, Nakama et al., 2001). These are plasmid-borne elements and in some occasion are found in several large plasmids that also harbour *qac* genes conferring efflux mediated resistance to quaternary ammonium compounds and

ethidium bromide (Ciusa et al., 2012), other operons confer cadmium and heavy metal determinants (Schwarz et al., 2011). This study has addressed if the presence of mutations either in the promoter region or in the coding sequences of these genes, have an impact on the level of resistance to both mupirocin and triclosan. Moreover, the clinical isolates of *S. epidermidis* harbouring additional copies of *ileS2 or sh-fabI* can even add the resistance mechanism of target duplication and thus reduce the susceptibility to mupirocin and triclosan, respectively (Ciusa et al., 2012, Furi et al., 2016). A recent study by (Wan et al., 2017) have established that the IS1272 mediating the mobilisation of triclosan resistance determinant, occurs via an IR-replacement mechanism, which could also explain the unsuccessful attempt to demonstrate the circular elements of the IS1272 or TnSha in this study. As with antibiotic misuse, continuous use of biocides is suggested to drive selection for resistance genes and reduced susceptibility (Morrissey et al., 2014, Oggioni et al., 2015, Tansirichaiya et al., 2017).

It is not easy to unequivocally establish which selective force is responsible for causing selection of a specific mechanism of resistance, especially when these genetic determinants confer simultaneous resistance to different drugs or when several different resistance elements are associated in the same gene transfer element (Morrissey et al., 2014, Oggioni et al., 2015, Tansirichaiya et al., 2017). For instance, in disinfectants that can provoke cross-resistance to antibiotics, it is difficult to know whether the selective agent has been the biocide or the antibiotic itself. FabI enzyme is targeted only by triclosan in *S. aureus*. The resistance mechanism identified to triclosan acting by heterologous target duplication excludes other antimicrobials as being selective forces. This finding is a direct evidence that the biocide triclosan produces a selective pressure on *S. aureus* and other staphylococci and is the first clear demonstration that utilisation of biocides can drive development of biocide resistance in clinical isolates.

These results demonstrate in specific selected cases that GIs and IS

mobilisation is possible under the control of integrase and transposase expression in the different species backgrounds tested. In particular, this study highlights how the influence of the movement and transposition mechanisms may impact the host maintenance, structure and evolution of mobile genetic elements. Whilst these results suggest that mobilisation genes, integrases of *A. baumannii* or transposases of *Staphylococcus* can be induced in response to adaptation to changing environments, they nevertheless contribute to its MDR phenotype in both species.

Since it was established that antibiotics such as quinolones can themselves cause bacterial DNA damage, in depth understanding of the mechanism for how mobilisation enzymes of MGEs can be activated is highly crucial for highlighting their contribution to acquiring antimicrobial resistance determinants. Such knowledge could influence the planning of antibiotic prescription guidelines in future. Many studies have demonstrated that various MGEs can also be activated by the SOS response including the *V. cholerae* CTXφ prophage (Quinones et al., 2005), and the SXT integrative conjugative element also from *V. cholerae* (Beaber et al., 2002) and integrons (Guerin et al., 2009). This is of a huge concern, since it means that antimicrobials intended for therapeutic use may in themselves encourage the spread of antibiotic resistance within and across important pathogenic bacterial species.

Other clinical practices have also contributed to the antibiotic resistance such as the use of sterilizing agents in medical settings such as UV light irradiation (Riley and Edward, 1989), biocides and disinfectants (Aiello et al., 2007, McDonnell and Russell, 1999, Morrissey et al., 2014, Oggioni et al., 2015, Tansirichaiya et al., 2017), alcohol hand rubs (Edwards et al., 2007), in addition to the antibiotics (Beaber et al., 2002, Guerin et al., 2009) actually could promote persistence and mutagenesis of clinical isolates, as well as induce lateral gene transfer, if not used appropriately.

By bringing this information to light, this thesis adds to the growing evidence that adherence to antibiotic prescription guidelines as well as

controlled use of biocides globally, would be the most effective way to combat the global problem of antibiotic resistance. The capability of *Staphylococcus* species as well as Gram-negative pathogens including *Acinetobacter* in adapting to environmental changes using their existing resistance genes as well as developing novel resistance mechanisms seems to be inevitable and precautionary measures in proper use of antimicrobials can only slow these processes to controllable levels.

Appendix 1

Protein sequences of all the integrases used to construct the phylogenetic tree (Figure 3.1).

AYE

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ATCC ATCC17978

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ALADAIVERGAPATAVHVREIVLQVFRWAIERGQKVENPAELVRPTSIARFEPRDRALTPEEIGLMYQYMERV
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LVPLSKQSIEILEKVYQYSGDCDFVFPSLNNKRIFISENTPNDALRRMGYTKEEISFHGFRALARSALGEMSI
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>O1 (ABO11928.1)
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References

Abdel-Meguid, S., Grindley, N. D. F., Tempelton, N. S. & Steitz, T. A. 1984. Cleavage of the site-specific recombination proteiny 6 resolvase: The smaller of two fragments binds DNA specifically. *J Biochemistry,* 81**,** 2001-2005.

Abremski, K. & Hoess, R. 1984. Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. *Journal of Biological Chemistry,* 259**,** 1509-1514.

Adams, M. D., Goglin, K., Molyneaux, N., Hujer, K. M., Lavender, H., Jamison, J. J., MacDonald, I. J., Martin, K. M., Russo, T., Campagnari, A. A., Hujer, A. M., Bonomo, R. A. & Gill, S. R. 2008. Comparative genome sequence analysis of multidrug-resistant Acinetobacter baumannii. *J Bacteriol,* 190**,** 8053-64.

Afzal-Shah, M., Woodford, N. & Livermore, D. M. 2001. Characterization of OXA-25, OXA-26, and OXA-27, molecular class D beta-lactamases associated with carbapenem resistance in clinical isolates of Acinetobacter baumannii. *Antimicrob Agents Chemother,* 45**,** 583-8.

Aiello, A. E., Larson, E. L. & Levy, S. B. 2007. Consumer Antibacterial Soaps: Effective or Just Risky? *Clin Infect Dis,* 45**,** S137-S147.

Alberts, B., Johnson, A. & Lewis, J. 2002. *Molecular Biology of the Cell, Site-Specific Recombination* [Online]. New York: Garland Science, Taylor & Francis. Available: http://www.ncbi.nlm.nih.gov/books/NBK26845/ [Accessed 31/05 2016].

Allignet, E., Aubert, S., Morvan, A. & El Solh, N. 1996. Distribution of Genes Encoding Resistance to Streptogramin A and Related Compounds among Staphylococci Resistant to These Antibiotics. *Antimicrob Agents Chemother,* 40.

Allignet, J., Liassine, N. & El Solh, N. 1998. Characterization of a Staphylococcal Plasmid Related to pUB110 and Carrying Two Novel Genes, vatC and vgbB, Encoding Resistance to Streptogramins A and B and Similar Antibiotics. *Antimicrob Agents Chemother,* 42**,** 1794–1798.

Alting-Mees, M. A. & Short, J. M. 1989. pBluescript II:gene mapping vectors. *Nucleic Acids Res,* 17**,** 9494.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res,* 15**,** 3389–3402.

Alvarez-Ortega, C., Olivares, J. & Martinez, J. L. 2013. RND multidrug efflux pumps: what are they good for? *Front Microbiol,* 4**,** 7.

Alvarez-Perez, S., Lievens, B., Jacquemyn, H. & Herrera, C. M. 2013. Acinetobacter nectaris sp. nov. and Acinetobacter boissieri sp. nov., isolated from floral nectar of wild Mediterranean insect-pollinated plants. *Int J Syst Evol Microbiol,* 63**,** 1532-9.

Amat, T., Gutiérrez-Pizarraya, A., Machuca, I., Gracia-Ahufinger, I., Elena-Pérez-Nadales, Torre-Giménez, A., Garnacho-Montero, J., Cisneros, J. M. & Torre-Cisneros, J. 2017. The Combined Use of Tigecycline with High-Dose Colistin Might Not be Associated With Higher Survival in Critically ill Patients with Bacteraemia due to Carbapenem-Resistant Acinetobacter baumannii *Clin Microbiol Infect,* 9.

Anandham, R., Weon, H. Y., Kim, S. J., Kim, Y. S., Kim, B. Y. & Kwon, S. O. 2010. Acinetobacter brisounii sp. nov., isolated from a wetland in Korea. *J Microbiol,* 48**,** 36-39.

Andersson, D. I. & Hughes, D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol,* 8**,** 260-71.

Andrews, B. J., Proteau, G. A., Beatty, L. G. & Sadowski, P. D. 1985. The FLP recombinase of the 2μ circle DNA of yeast: interaction with its target sequences. *Cell,* 40**,** 795-803.

Antonio, M., McFerran, N. & Pallen, M. J. 2002. Mutations Affecting the Rossman Fold of Isoleucyl-tRNA Synthetase Are Correlated with Low-Level Mupirocin Resistance in Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy,* 46**,** 438-442.

Apweiler, R., Attwood, T. K., Bairoch, A., Bateman, A., Birney, E., Biswas, M., Bucher, P., Cerutti, L., Corpet, F., Croning, M. D. R., Falquet, L., Durbin, R., Fleischmann, W., Gouzy, J., Hermjakob, H., Hulo, N., Jonassen, I., Kahn, D., Kanapin, A., Karavidopoulou, Y., Lopez, R., Marx, B., Mulder, N. J., Oinn, T. M., Pagni, M., Servant, F., Sigrist, C. J. A. & Zdobnov, E. M. 2003. InterPro—an integrated documentation resource for protein families, domains and functional sites. *Bioinformatics,* 16**,** 1145–1150.

Aranda, J., Bardina, C., Beceiro, A., Rumbo, S., Cabral, M. P., Barbe, J. & Bou, G. 2011. Acinetobacter baumannii RecA protein in repair of DNA damage, antimicrobial resistance, general stress response, and virulence. *J Bacteriol,* 193**,** 3740-7.

Aranda, J., Poza, M., Shingu-Vazquez, M., Cortes, P., Boyce, J. D., Adler, B., Barbe, J. & Bou, G. 2013. Identification of a DNA-damage-inducible regulon in Acinetobacter baumannii. *J Bacteriol,* 195**,** 5577-82.

Arber, W. 1979. Promotion and limitation of genetic exchange. *Cell Mol Life Sci,* 35**,** 287-293.

Archer, G. L., Niemeyer, D. M., Thanassi, J. A. & Pucci, M. J. 1994. Dissemination among Staphylococci of DNA Sequences Associated with Methicillin Resistance. *Antimicrob Agents Chemother,* 38**,** 447-454.

Archer, G. L., Thanassi, J. A., Niemeyer, D. M. & Pucci, M. J. 1996. Characterization of IS1272, an Insertion Sequence-Like Element from Staphylococcus haemolyticus. *Antimicrob Agents Chemother,* 40**,** 924–929.

Arciszewska, L. K., Grainge, I. & Sherratt, D. J. 1997. Action of site-specific recombinases XerC and XerD on tethered Holliday junctions. *The EMBO journal,* 16**,** 3731-3743.

Aslantas, Ouml, zkan, Uuml, Rkyilmaz, S., uuml, heyla, Yilmaz, M. A., Erdem, Z. & Demir, C. 2012. Isolation and Molecular Characterization of Methicillin-Resistant Staphylococci from Horses, Personnel and Environmental Sites at an Equine Hospital in Turkey. *Journal of Veterinary Medical Science,* 74**,** 1583-1588.

Austin, S., Ziese, M. & Sternberg, N. 1981. A novel role for site-specific recombination in maintenance of bacterial replicons. *Cell,* 25**,** 729-736.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D., Seidman, J. G. & Struhl, K., . 2004. *Current Protocols in Molecular Biology*, John Wiley & Sons.

Avery, S. T., Macleod, C. M. & McCarthy, M. 1979. Studies on the chemical nature of the substance inducing transformation of Pneumoccocal types. Induction of transformation by desoxyribonucleic acid fraction isolated from Pneumococcustype III. *J Exp Med,* 149**,** 297-326.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A. & Zagnitko, O. 2008. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics,* 9**,** 75.

Aziz, R. K., Breitbart, M. & Edwards, R. A. 2010. Transposases are the most abundant, most ubiquitous genes in nature. *Nucleic Acids Res,* 38**,** 4207-17.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K.-i., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K. & Hiramatsu, K. 2002. Genome and virulence determinants of high virulence community-acquired MRSA. *The Lancet,* 359**,** 1819-1827.

Babich, K., Engle, M., Skinner, J. S. & Laddaga, R. A. 1991. Deletion mutant analysis of the Staphylococcus aureus plasmid pI258 mercury-resistance determinant. *Canadian J Micro.,* 37**,** 624-631.

Bacher, J. M., Metzgar, D. & de Crecy-Lagard, V. 2006. Rapid evolution of diminished transformability in Acinetobacter baylyi. *J Bacteriol,* 188**,** 8534-42.

Backer, D. & Sali, A. 2001. Protein Structure Prediction and Structural Genomics. *Science,* 294**,** 93-96.

Ballard, S. A., Pertile, K. K., Lim, M., Johnson, P. D. & Grayson, M. L. 2005. Molecular characterization of vanB elements in naturally occurring gut anaerobes. *Antimicrob Agents Chemother,* 49**,** 1688-94.
Baltrus, D. A. 2013. Exploring the costs of horizontal gene transfer. *Trends in Ecology Evolution* 28**,** 489-495.
Bannam, T. L., Crellin, P. K. & Rood, J. I. 1995. Molecular genetics of the chloramphenicol-resistance transposon Tn4451from Clostridium perfringens: the TnpX site-specific recombinase excises a circular transposon molecule. *Molecular Microbiology,* 16**,** 535–551.
Barry, A. L., Craig, W. A., Nadler, H., Reller, L. B., Sanders, C. C. & Swenson, J. M. 1999. Methods for determining bactericidal activity of antimicrobial agents; approved guideline. *NCCLS document M26-A,* 19.
Barth, P. T., Datta, N., Hedges, R. W. & Grinter, N. J. 1976. Transposition of a Deoxyribonucleic Acid Sequence Encoding Trimethoprim and Streptomycin Resistances from R483 to Other Replicons. *J Bacteriol,* 125**,** 800-810.
Bateman, A., Coin, L., Durbin, R., Finn, R. D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E. L., Studholme, D. J., Yeats, C. & Eddy, S. R. 2004. The Pfam protein families database. *Nucleic Acids Res,* 32**,** D138-41.
Baumann, P. 1968. Isolation of Acinetobacter from Soil and Water. *J Bacteriol,* 96**,** 39-42.
Bayliss, C. D., Field, D. & Moxon, E. R. 2001. The simple sequence contingency loci of Haemophilus influenzae and Neisseria meningitidis. *J Clin Invest,* 107**,** 657-62.
Beaber, J. W., Hochhut, B. & Waldor, M. K. 2002. Genomic and Functional Analyses of SXT, an Integrating Antibiotic Resistance Gene Transfer Element Derived from Vibrio cholerae. *Journal of Bacteriology,* 184**,** 4259-4269.
Becker, K., Heilmann, C. & Peters, G. 2014. Coagulase-negative staphylococci. *Clin Microbiol Rev,* 27**,** 870-926.
Becker, K. W. & Skaar, E. P. 2014. Metal limitation and toxicity at the interface between host and pathogen. *FEMS Microbiol Rev,* 38**,** 1235-49.
Bellanger, X., Morel, C., Decaris, B. & Guedon, G. 2007. Derepression of excision of integrative and potentially conjugative elements from Streptococcus thermophilus by DNA damage response: implication of a cI-related repressor. *J Bacteriol,* 189**,** 1478-81.
Bello, H., Gonzalez, G., Dominguez, M., Zemelman, R., Garcia, A. & Mella, S. 1997. Activity of selected β-lactams, ciprofloxacin, and amikacin against different< i> Acinetobacter baumannii</i> biotypes from chilean hospitals. *Diagnostic microbiology and infectious disease,* 28**,** 183-186.
Belting, M. & Wittrup, A. 2008. Nanotubes, exosomes, and nucleic acid–binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: implications in health and disease. *J Cell Biol,* 183**,** 1187–1191.
Berg, T., Firth, N., Apisiridej, S., Hettiaratchi, A., Leelaporn, A. & Skurray, R. A. 1998. Complete Nucleotide Sequence of pSK41: Evolution of Staphylococcal Conjugative Multiresistance Plasmids. *J Bacteriol,* 180**,** 4350–4359.
Berger-Bachi, B. & Rohrer, S. 2002. Factors influencing methicillin resistance in staphylococci. *Arch Microbiol,* 178**,** 165-71.
Berglund, C.,¨lling, P. M.,¨berg, L. S. &¨derquist, B. S. 2005. Predominance of staphylococcal cassette chromosome mec (SCCmec) type IV among methicillin-resistant Staphylococcus aureus (MRSA) in a Swedish county and presence of unknown SCCmec types with Panton-Valentine leukocidin genes. *Clinical Microbiology and Infection, ,* 11
Bergogne-Be´re´zin, E. n., Friedman, H. & Bendinelli, M. 2008. Acinetobacter Biology and Pathogenesis. springer.
Bergogne-Berezin, E. & Towner, K. J. 1996. Acinetobacter spp. as Nosocomial Pathogens: Microbiological, Clinical, and Epidemiological Features. *Clin Microbiol Rev,* 9**,** 148–165.

Besier, S., Ludwig, A., Ohlsen, K., Brade, V. & Wichelhaus, T. A. 2007. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of Staphylococcus aureus. *International Journal of Medical Microbiology,* 297**,** 217-225.

Beveridge, T. J. 1999. Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles. *J Bacteriol,* 181**,** 4725–4733.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Gallo Cassarino, T., Bertoni, M., Bordoli, L. & Schwede, T. 2014. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res,* 42**,** W252-8.

Bikandi, J., San Millan, R., Rementeria, A. & Garaizar, J. 2004. In silico analysis of complete bacterial genomes: PCR, AFLP-PCR and endonuclease restriction. *Bioinformatics,* 20**,** 798-9.

Bille, E., Zahar, J. R., Perrin, A., Morelle, S., Kriz, P., Jolley, K. A., Maiden, M. C., Dervin, C., Nassif, X. & Tinsley, C. R. 2005. A chromosomally integrated bacteriophage in invasive meningococci. *J Exp Med,* 201**,** 1905-13.

Bismuth, R., Zilhao, R., Sakamoto, H., Guesdon, j.-L. & Courvalin, P. 1990. Gene Heterogeneity for Tetracycline Resistance in Staphylococcus spp. *Antimicrob Agents Chemother,* 34**,** 1611-1614.

Blackwell, K. J., Tobin, J. M. & Avery, S. V. 1998. Manganese toxicity towards Saccharomyces cerevisiae : Dependence on intracellular and extracellular magnesium concentrations. *Appl Microbiol Biotechnol,* 49**,** 751-757.

Blakely, G., May, G., McCulloch, R., Arciszewska, L. K., Burke, M., Lovett, S. T. & Sherratt, D. J. 1993. Two related recombinases are required for site-specific recombination at< i> dif</i> and< i> cer</i> in E. coli K12. *Cell,* 75**,** 351-361.

Blau, H. M. & Rossi, F. M. V. 1999. Tet B or not tet B: Advances in tetracycline-inducible gene expression. *PNAS,* 96**,** 797–799.

Bloemendaal, A. L. A., Brouwer, E. C. & Fluit, A. C. 2010. Methicillin Resistance Transfer from Staphylocccus epidermidis to Methicillin-Susceptible Staphylococcus aureus in a Patient during Antibiotic Therapy. *PLoS One,* 5**,** e11841.

Blomfietd, I. C., Vaughn, V., Rest, R. F. & Eisenstein, B. I. 1991. Allelic exchange in Escherichia coli using the Baciiius subtiiis sacB gene and a temperature-sensitive pSC101 replicon. *Mol Microbiol,* 5**,** 1447-1457.

Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschape, H. & Hacker, J. 1994. Excision of Large DNA Regions Termed Pathogenicity Islands from tRNA-Specific Loci in the Chromosome of anEscherichia coli Wild-Type Pathogen. *Infect Immun,* 62**,** 606-614.

Bolger, A. M., Lohse, M. & Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics,* 30**,** 2114-20.

Boltner, D., MacMahon, C., Pembroke, J. T., Strike, P. & Osborn, A. M. 2002. R391: a Conjugative Integrating Mosaic Comprised of Phage, Plasmid, and Transposon Elements. *Journal of Bacteriology,* 184**,** 5158-5169.

Bonomo, R. A. & Szabo, D. 2006. Mechanisms of Multidrug Resistance in Acinetobacter Species and Pseudomonas aeruginosa. *J Clinical Infectious Diseases,* 43**,** S49–56.

Boundy, S., Safo, M. K., Wang, L., Musayev, F. N., O'Farrell, H. C., Rife, J. P. & Archer, G. L. 2013. Characterization of the Staphylococcus aureus rRNA methyltransferase encoded by orfX, the gene containing the staphylococcal chromosome Cassette mec (SCCmec) insertion site. *J Biol Chem,* 288**,** 132-40.

Boyd, E. F., Almagro-Moreno, S. & Parent, M. A. 2009. Genomic islands are dynamic, ancient integrative elements in bacterial evolution. *Trends Microbiol,* 17**,** 47-53.

Boyd, E. F. a. B. s., Harald 2002. Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. *RENDS in Microbiology,* 10**,** 521-529.

Braun, V. 1997. Avoidance of iron toxicity through regulation of bacterial iron transport. *Biol chem,* 378**,** 779-786.

Brisson-Noel, A. & Courvalin, P. 1986. Nucleotide sequence of gene linA encoding resistance to lincosamides in Staphylococcus haemolyticus. *Gene,* 43**,** 247-253.
Broer, S., Ji, G., Broer, A. & Silver, S. 1993. Arsenic Efflux Governed by the Arsenic Resistance Determinant of Staphylococcus aureus Plasmid p1258. *J Bacteriol,* 175**,** 3480-3485.
Bruins, M. R., Kapil, S. & Oehme, F. W. 2000. Microbial resistance to metals in the environment. *Ecotoxicol Environ Saf,* 45**,** 198-207.
Buchrieser, C., Brosch, R., Bach, S., Guiyoule, A. & Carniel, E. 1998. The high-pathogenicity island of Yersinia pseudotuberculosis can be inserted into any of the three chromosomal asn tRNA genes. *Molecular Microbiology* 30**,** 965–978.
Burrus, V., Pavlovic, G., Decaris, B. & Guédon, G. r. 2002. Conjugative transposons: the tip of the iceberg. *Molecular Microbiology* 46**,** 601–610.
Burrus, V. & Waldor, M. K. 2004. Shaping bacterial genomes with integrative and conjugative elements. *Res Microbiol,* 155**,** 376-86.
Butala, M., Zgur-Bertok, D. & Busby, S. J. 2009. The bacterial LexA transcriptional repressor. *Cell Mol Life Sci,* 66**,** 82-93.
Byrne, M. E., Gillespie, M. T. & Skurray, R. A. 1991. 4',4' Adenyltransferase activity on conjugative plasmids isolated from Staphylococcus aureus is encoded on an integrated copy of pUB110. *In Plasmid,* 25**,** 70-75.
Cambray, G., Sanchez-Alberola, N., Campoy, S., Guerin, E. m., Da Re, S., González-Zorn, B., Ploy, M.-C. c., Barbé, J., Mazel, D. & Erill, I. 2011a. Prevalence of SOS-mediated control of integron integrase expression as an adaptive trait of chromosomal and mobile integrons. *Mobile DNA,* 2.
Cambray, G., Sanchez-Alberola., Campoy, S., Guerin, G., Da Re, S., González-Zorn, B., Ploy, M., Barbé, J., Didier, M. & Ivan, E. 2011b. Prevalence of SOS-mediated control of integron integrase expression as an adaptive trait of chromosomal and mobile integrons. *Mobile DNA,* 2.
Camp, C. & Tatum, O. L. 2010. A Review ofAcinetobacter baumanniias a Highly Successful Pathogen in Times of War. *Laboratory Medicine,* 41**,** 649-657.
Canchaya, C., Desiere, F., McShan, W. M., Ferretti, J. J., Parkhill, J. & Brussow, H. 2002. Genome analysis of an inducible prophage and prophage remnants integrated in the Streptococcus pyogenes strain SF370. *Virology,* 302**,** 245-58.
Canchaya, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M.-L. & Brüssow, H. 2003. Phage as agents of lateral gene transfer. *Current Opinion in Microbiology,* 6**,** 417-424.
Carattoli, A. 2013. Plasmids and the spread of resistance. *Int J Medical Microbilogy,* 303**,** 298–304.
Carozza, A., De Santo, L. S., Romano, G., Della Corte, A., Ursomando, F., Scardone, M., Caianiello, G. & Cotrufo, M. 2006. Infective Endocarditis in Intravenous Drug Abusers: Patterns of Presentation and Long-Term Outcomes of Surgical Treatment. *J Heart Valve Disease,* 15**,** 125-131.
Carr, L. E., Kampfer, P., Patel, B. K. C., Gulter, V. & Seviour, R. J. 2003. Seven novel species of Acinetobacter isolated from activated sludge. *Int J systematic evolutionary microbiol,* 53**,** 953-963.
Carver, T., Harris, S. R., Berriman, M., Parkhill, J. & McQuillan, J. A. 2011. Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. *Bioinformatics,* 28**,** 464–469.
Caryl, J. A. & O'Neill, A. J. 2009. Complete nucleotide sequence of pGO1, the prototype conjugative plasmid from the Staphylococci. *Plasmid,* 62**,** 35-8.
Casali, N. 2003. Escherichia coli Host Strains. *In:* CASALI, N. & PRESTON, A. (eds.) *Methods in Molecular Biology.* Totowa, NJ: Humana Press Inc.
Catalano, M., Quelle, L. S., Jeric, P. E., Di Martino, A. & Maimone, S. M. 1999. Survival of Acinetobacter baumannii on bed rails during an outbreak and during sporadic cases. *J Hospital Infection,* 42**,** 27–35.

Cha, J. S. & Cooksey, D. A. 1991. Copper resistance in Pseudomonas syringae mediated by periplasmic and outer membrane proteins. *Proc Natl Acad Sci U S A,* 88**,** 8915-9.
Chan, A. P., Sutton, G., DePew, J., Krishnakumar, R., Choi, Y., Huang, X. Z., Beck, E., Harkins, D. M., Kim, M., Lesho, E. P., Nikolich, M. P. & Fouts, D. E. 2015. A novel method of consensus pan-chromosome assembly and large-scale comparative analysis reveal the highly flexible pan-genome of Acinetobacter baumannii. *Genome Biol,* 16**,** 143.
Chan, C. X., Beiko, R. G. & Ragan, M. A. 2011. Lateral transfer of genes and gene fragments in Staphylococcus extends beyond mobile elements. *J Bacteriol,* 193**,** 3964-77.
Chandler, M., de la Cruz, F., Dyda, F., Hickman, A. B., Moncalian, G. & Ton-Hoang, B. 2013. Breaking and joining single-stranded DNA: the HUH endonuclease superfamily. *Nat Rev Microbiol,* 11**,** 525-38.
Chandler, M. & Mahillon, J. 2002. Insertion sequences revisited. *In:* CRAIG, N., CRAIGIE, R., GELLERT, M. & LAMBOWITZ, A. (eds.) *Microbial genetics and molecular biology.* Washington DC: ASM press, Washington DC.
Charpentier, X., Polard, P. & Claverys, J. P. 2012. Induction of competence for genetic transformation by antibiotics: convergent evolution of stress responses in distant bacterial species lacking SOS? *Curr Opin Microbiol,* 15**,** 570-6.
Cheetham, B. F. & Katz, M. E. 1995. A role for bacteriophages in the evolution and transfer of bacterial virulence determinants. *Mol Microbiol,* 18**,** 201-208.
Chen, J. & Novick, R. P. 2009. Phage-mediated intergeneric transfer of toxin genes. *Science,* 323**,** 139-41.
Chen, Z., Yang, H. & Pavletich, N. P. 2008. Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. *nature,* 453**,** 489-494.
Cheung, G. Y. C., Rigby, K., Wang, R., Queck, S. Y., Braughton, K. R., Whitney, A. R., Teintze, M., DeLeo, F. R. & Otto, M. 2010. Staphylococcus epidermidis Strategies to Avoid Killing by Human Neutrophils. *PLoS Pathog,* 6**,** e1001133.
Choi, S., Ohta, S. & Ohtsubo, E. 2003. A Novel IS Element, IS621, of the IS110/IS492 Family Transposes to a Specific Site in Repetitive Extragenic Palindromic Sequences in Escherichia coli. *Journal of Bacteriology,* 185**,** 4891-4900.
Chongtrakool, P., Ito, T., Ma, X. X., Kondo, Y., Trakulsomboon, S., Tiensasitorn, C., Jamklang, M., Chavalit, T., Song, J. H. & Hiramatsu, K. 2006. Staphylococcal cassette chromosome mec (SCCmec) typing of methicillin-resistant Staphylococcus aureus strains isolated in 11 Asian countries: a proposal for a new nomenclature for SCCmec elements. *Antimicrob Agents Chemother,* 50**,** 1001-12.
Chou, H. H., Chiu, H. C., Delaney, N. F., Segre, D. & Marx, C. J. 2011. Diminishing returns epistasis among beneficial mutations decelerates adaptation. *Science,* 332**,** 1190-2.
Christiansen, B., Brondsted, L., Vogensen, F. K. & Hammer, K. 1996. A Resolvase-Like Protein Is Required for the Site-Specific Integration of the Temperate Lactococcal Bacteriophage TP901-1. *J Bacteriol,* 178**,** 5164–5173.
Ciusa, M. L., Furi, L., Knight, D., Decorosi, F., Fondi, M., Raggi, C., Coelho, J. R., Aragones, L., Moce, L., Visa, P., Freitas, A. T., Baldassarri, L., Fani, R., Viti, C., Orefici, G., Martinez, J. L., Morrissey, I., Oggioni, M. R. & Consortium, B. 2012. A novel resistance mechanism to triclosan that suggests horizontal gene transfer and demonstrates a potential selective pressure for reduced biocide susceptibility in clinical strains of Staphylococcus aureus. *Int J Antimicrob Agents,* 40**,** 210-20.
Clements, A., Tull, D., Jenney, A. W., Farn, J. L., Kim, S.-H., Bishop, R. E., McPhee, J. B., Hancock, R. E., Hartland, E. L. & Pearse, M. J. 2007. Secondary acylation of Klebsiella pneumoniae lipopolysaccharide contributes to sensitivity to antibacterial peptides. *Journal of Biological Chemistry,* 282**,** 15569-15577.
Clewell, D. B. 1981. Plasmids, drug resistance, and gene transfer in the genus Streptococcus. *Microbiol Rev,* 45**,** 409-36.
Clewell, D. B. 1990. Movable Genetic Elements and Antibiotic Resistance in Enterococci. *European Society of Clinical Microbiology and Infectious Diseases,* 9**,** 90-102.

Coates, T., Bax, R. & Coates, A. 2009. Nasal decolonization of Staphylococcus aureus with mupirocin: strengths, weaknesses and future prospects. *J Antimicrob Chemother,* 64**,** 9-15.
Collis, C. M. & Hall, R. M. 1992. Site-Specific Deletion and Rearrangement of Integron Insert Genes Catalyzed byt heI ntegron DNA Integrase. *J Bacteriol,* 174**,** 1574-1585.
Colloms, S., Sykora, P., Szatmari, G. & Sherratt, D. 1990. Recombination at ColE1 cer requires the Escherichia coli xerC gene product, a member of the lambda integrase family of site-specific recombinases. *Journal of bacteriology,* 172**,** 6973-6980.
Cookson, B. D. 1998. The emergence of mupirocin resistance: a challenge to infection control and antibiotic prescribing practice. *Antimicrob Agents Chemother,* 41**,** 11–18.
Cornet, F., Mortier, I., Pattie, J. & Louren, J.-M. 1994. Plasmid pSC101 Harbors a Recombination Site,psi, Which Is Able To Resolve Plasmid Multimers and To Substitute for the Analogous Chromosomal Escherichia coli Site dif. *J Bacteriol,* 176**,** 3188-3195.
Corvaglia, A. R., Francois, P., Hernandez, D., Perron, K., Linder, P. & Schrenzel, J. 2010. A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical Staphylococcus aureus strains. *Proc Natl Acad Sci U S A,* 107**,** 11954-8.
Crupper, S. S., Worrell, V., Stewart, G. C. & Iandolo, J. J. 1999. Cloning and Expression of cadD, a New Cadmium Resistance Gene of Staphylococcus aureus. *J Bacteriol,* 181**,** 4071–4075.
Curcio, M. J. & Derbyshire, K. M. 2003. The outs and ins of transposition: From Mu to Kangaroo. *Nat Rev Microbiol,* 4**,** 1-13.
Dale, G. E., Broger, C., Hartman, P. G., Langen, H., Page, M. G. P., Then, R., L. & Stu¨ber, D. 1995. Characterization of the Gene for the Chromosomal Dihydrofolate Reductase (DHFR) of Staphylococcus epidermidis ATCC 14990: the Origin of the Trimethoprim-Resistant S1 DHFR from Staphylococcus aureus? *J Bacteriol,* 177**,** 2965–2970.
Dallas, W. S. & Falkow, S. 1980. Amino acid sequence homology between cholera toxin and Escerichia coli heat-labiloe toxin. *naure* 288**,** 499-501.
Datsenko, K. A. & Wanner, B. L. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proceedings of the National Academy of Sciences,* 97**,** 6640-6645.
Davies, J. & Davies, D. 2010. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev,* 74**,** 417-33.
Davies, M. R., Shera, J., Van Domselaar, G. H., Sriprakash, K. S. & McMillan, D. J. 2009. A Novel Integrative Conjugative Element Mediates Genetic Transfer from Group G Streptococcus to Other -Hemolytic Streptococci. *Journal of Bacteriology,* 191**,** 2257-2265.
de la Cruz, F. & Davies, a. J. 2000. Horizontal gene transfer and the origin of species: lessons from bacteria. *trends in Microbiology,* 8**,** 128-133.
De Lorenzo, V. & Timmis, K. N. 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5 and Tn10-derived minitransposons. *Methods in Ezymology,* 44.
de Niederhausern, S., Bondi, M., Messi, P., Iseppi, R., Sabia, C., Manicardi, G. & Anacarso, I. 2011. Vancomycin-resistance transferability from VanA enterococci to Staphylococcus aureus. *Curr Microbiol,* 62**,** 1363-7.
de Oliveira, N. E., Cavalcanti, E. D., Laport, M. S., Bastos Mdo, C. & Giambiagi-deMarval, M. 2009. Constitutive expression of the ileS-2 gene responsible for high-level mupirocin resistance in Staphylococcus aureus. *J Med Microbiol,* 58**,** 1582-4.
De Wals, P.-Y., Doucet, N. & Pelletier, J. N. 2008. High tolerance to simultaneous active-site mutations in TEM-1 β-lactamase: Distinct mutational paths provide more generalized β-lactam recognition. *Protein Science***,** NA-NA.
del Mar Tomas, M., Beceiro, A., Perez, A., Velasco, D., Moure, R., Villanueva, R., Martinez-Beltran, J. & Bou, G. 2005. Cloning and functional analysis of the gene encoding the

33- to 36-kilodalton outer membrane protein associated with carbapenem resistance in Acinetobacter baumannii. *Antimicrob Agents Chemother,* 49**,** 5172-5.
Deurenberg, R. H., Vink, C., Kalenic, S., Friedrich, A. W., Bruggeman, C. A. & Stobberingh, E. E. 2007. The molecular evolution of methicillin-resistant Staphylococcus aureus. *Clin Microbiol Infect,* 13**,** 222-35.
Di Nocera, P. P., Rocco, F., Giannouli, M., Triassi, M. & Zarrilli, R. 2011. Genome organization of epidemic Acinetobacter baumannii strains. *BMC Microbiol,* 11**,** 224.
Dickert, H., Machka, K. & Braveny, I. 1981. *Infection and immunity,* 9.
Dimopoulou, I. D., Russell, J. E., Mohd-Zain, Z., Herbert, R. & Crook, D. W. 2002. Site-Specific Recombination with the Chromosomal tRNALeu Gene by the Large Conjugative Haemophilus Resistance Plasmid. *Antimicrobial Agents and Chemotherapy,* 46**,** 1602-1603.
Dobrindt, U., Hochhut, B., Hentschel, U. & Hacker, J. 2004. Genomic islands in pathogenic and environmental microorganisms. *Nat Rev Microbiol,* 2**,** 414-24.
Docquier, J. D. 2003. On functional and structural heterogeneity of VIM-type metallo-beta-lactamases. *Journal of Antimicrobial Chemotherapy,* 51**,** 257-266.
Domingues, S., da Silva, G. J. & Nielsen, K. M. 2012. Integrons: Vehicles and pathways for horizontal dissemination in bacteria. *Mob Genet Elements,* 2**,** 211-223.
Dominguez, N. M., Hackett, K. T. & Dillard, J. P. 2011. XerCD-mediated site-specific recombination leads to loss of the 57-kilobase gonococcal genetic island. *J Bacteriol,* 193**,** 377-88.
Donnenberg, M. S. & Kaper, J. B. 1991. Construction of an eae Deletion Mutant of Enteropathogenic Escherichia coli by Using a Positive-Selection Suicide Vector. *Infect Immun,* 59**,** 4310-4317.
Doolittle, W. F. 1998. You are what you eat: a gene transfer ratchet could account for bacterial genes in eukaryotic nuclear genomes. *Trends in Genetics,* 14.
Dorsey, C. W., Beglin, M. S. & Actis, L. A. 2003. Detection and Analysis of Iron Uptake Components Expressed by Acinetobacter baumannii Clinical Isolates. *Journal of Clinical Microbiology,* 41**,** 4188-4193.
Dorward, D. W., Garon, C. F. & Judd, R. C. 1989. Export and Intercellular Transfer of DNA via Membrane Blebs of Neisseria gonorrhoeae. *J Bacteriol,* 171**,** 2499-2505.
Doublet, B., Boyd, D., Mulvey, M. R. & Cloeckaert, A. 2005. The Salmonella genomic island 1 is an integrative mobilizable element. *Mol Microbiol,* 55**,** 1911-24.
Drees, S. L., Beyer, D. F., Lenders-Lomscher, C. & Lubben, M. 2015. Distinct functions of serial metal-binding domains in the Escherichia coli P1 B -ATPase CopA. *Mol Microbiol,* 97**,** 423-38.
Dubey, G. P. & Ben-Yehuda, S. 2011. Intercellular Nanotubes Mediate Bacterial Communication. *Cell,* 144**,** 590–600.
Dubey, G. P., Mohan, G. B. M., Dubrovsky, A., Amen, T., Tsipshtein, S., Rouvinski, A., Rosenberg, A., Kaganovich, D., Sherman, E., Medalia, O. & Ben-Yehuda, S. 2016. Architecture and Characteristics of Bacterial Nanotubes. *Cell Press,* 36**,** 453–461.
Dubin, D. T., Chikramane, S. G., Inglis, B., Mathews, P. R. & Stewart, P. R. 1992. Physical mapping of the rnec region of an Australian methicillin-resistant Staphylococcus aureus lineage and a closely related American strain. *J General Microbiology,* 138**,** 169- 180.
Edwards, J., Patel, G. & Wareham, D. W. 2007. Low concentrations of commercial alcohol hand rubs facilitate growth of and secretion of extracellular proteins by multidrug-resistant strains of Acinetobacter baumannii. *J Med. Microbiol.,* 56**,** 1595–1599.
Edwards-Jones, V. 2009. The benefits of silver in hygiene, personal care and healthcare. *Lett Appl Microbiol,* 49**,** 147-52.
Eijkelkamp, B., Stroeher , U., Hassan, K., Paulsen, I. & Brown, M. 2014. Comparative analysis of surface-exposed virulence factors of Acinetobacter baumannii. *BMC Genomics,* 15.

Eijkelkamp, B. A., Hassan, K. A., Paulsen, I. T. & Brown, M. H. 2011. Investigation of the human pathogen Acinetobacter baumannii under iron limiting conditions. *BMC Genomics,* 12**,** 126.

Eitinger, T. 2013. Transport of nickel and cobalt in Prokaryotes. *In:* CULOTTA, V. & SCOOT, R. A. (eds.) *Metals in Cells* Chichester: John Wiley & Sons.

Elbourne, L. D., Tetu, S. G., Hassan, K. A. & Paulsen, I. T. 2017. TransportDB 2.0: a database for exploring membrane transporters in sequenced genomes from all domains of life. *Nucleic Acids Res,* 45**,** D320-D324.

Engelmoer, D. J., Donaldson, I. & Rozen, D. E. 2013. Conservative sex and the benefits of transformation in Streptococcus pneumoniae. *PLoS Pathog,* 9**,** e1003758.

Enquist, L., Kikuchi, A. & Weisberg, R. The role of λ integrase in integration and excision. Cold Spring Harbor symposia on quantitative biology, 1979. Cold Spring Harbor Laboratory Press, 1115-1120.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H. & Spratt, B. G. 2002. The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). *PNAS* 99**,** 7687–7692.

Erbay, A., İdil, A., Gözel, M. G., Mumcuoğlu, İ. & Balaban, N. 2009. Impact of early appropriate antimicrobial therapy on survival in Acinetobacter baumannii bloodstream infections. *International Journal of Antimicrobial Agents,* 34**,** 575-579.

Escaich, S., Prouvensier, L., Saccomani, M., Durant, L., Oxoby, M., Gerusz, V., Moreau, F., Vongsouthi, V., Maher, K., Morrissey, I. & Soulama-Mouze, C. 2011. The MUT056399 inhibitor of FabI is a new antistaphylococcal compound. *Antimicrob Agents Chemother,* 55**,** 4692-7.

Fairbrother, R. W. 1940. Coagulase production as a criterion for the classification of the staphylococci. *J Pathology,* 50**,** 83-88.

Falagas, M. E., Bliziotis, I. A. & Siempos, II 2006. Attributable mortality of Acinetobacter baumannii infections in critically ill patients: a systematic review of matched cohort and case-control studies. *Crit Care,* 10**,** R48.

Farrugia, D. N., Elbourne, L. D., Mabbutt, B. C. & Paulsen, I. T. 2015. A novel family of integrases associated with prophages and genomic islands integrated within the tRNA-dihydrouridine synthase A (dusA) gene. *Nucleic Acids Res,* 43**,** 4547-57.

Farrugia, D. N., Elbourne, L. D. H., Hassan, K. A., Eijkelkamp, B. A., Tetu, S. G., Brown, M. H., Shah, B. S., Peleg, A. Y., Mabbutt, B. C. & Paulsen, I. T. 2013. The Complete Genome and Phenome of a Community- Acquired Acinetobacter baumannii. *PLoS One,* 8**,** e58628.

Feil, E. J., Holmes, E. C., Bessen, D. E., Chan, M.-S., Day, N. P. J., Enright, M. C., Goldstein, R., Hood, D. W., Kalia, A., Moore, C. E., hou, J. Z. & Spratt, B. G. 2001. Recombination within natural populations of pathogenic bacteria: Short-term empirical estimates and long-term phylogenetic consequences. *PNAS,* 98 182–187.

Feliciello, I. & Chinali, G. 1993. A modified alkaline lysis method for the preparation of highly purified plasmid DNA from Escherichia coli. *Analytical Biochemistry,* 212**,** 394-401.

Feng, X. & Colloms, S. D. 2007. In vitro transposition of ISY100, a bacterial insertion sequence belonging to the Tc1/mariner family. *Molecular Microbiology,* 65**,** 1432-1443.

Ferretti, J. J., McShan, W. M., Ajdic, D., Savic, D. J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A. N., Kenton, S., Lai, H. S., Shao Ping Lin, Qian, Y., Jia, H. G., Najar, F. Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S. W., Roe, B. A. & McLaughlin, R. 2001. Complete genome sequence of an M1 strain of Streptococcus pyogenes. *PNAS* 98**,** 4658–4663

Fiandt, M., Szybalski, W. & Malamy, M. H. 1972. Polar Mutations in lac, gal and Phage k Consist of a Few IS-DNA Sequences Inserted with Either Orientation. *molecular genetics and genomics,* 119**,** 223--231.

Fishbain, J. & Peleg, A. Y. 2010. Treatment of Acinetobacter infections. *Clin Infect Dis,* 51**,** 79-84.

Fluit, A. C., Carpaij, N., Majoor, E. A., Bonten, M. J. & Willems, R. J. 2013. Shared reservoir of ccrB gene sequences between coagulase-negative staphylococci and methicillin-resistant Staphylococcus aureus. *J Antimicrob Chemother,* 68**,** 1707-13.
Fluit, A. C. & Schmitz, F. J. 2004. Resistance integrons and super-integrons. *Clin Microbiol Infect,* 10**,** 272-88.
Fournier, P. E., Vallenet, D., Barbe, V., Audic, S., Ogata, H., Poirel, L., Richet, H., Robert, C., Mangenot, S., Abergel, C., Nordmann, P., Weissenbach, J., Raoult, D. & Claverie, J. M. 2006. Comparative genomics of multidrug resistance in Acinetobacter baumannii. *PLoS Genet,* 2**,** e7.
Frost, L. S., Leplae, R., Summers, A. O. & Toussaint, A. 2005. Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol,* 3**,** 722-32.
Fung, D. K., Lau, W. Y., Chan, W. T. & Yan, A. 2013. Copper efflux is induced during anaerobic amino acid limitation in Escherichia coli to protect iron-sulfur cluster enzymes and biogenesis. *J Bacteriol,* 195**,** 4556-68.
Furi, L., Ciusa, M. L., Knight, D., Di Lorenzo, V., Tocci, N., Cirasola, D., Aragones, L., Coelho, J. R., Freitas, A. T., Marchi, E., Moce, L., Visa, P., Northwood, J. B., Viti, C., Borghi, E., Orefici, G., Consortium, B., Morrissey, I. & Oggioni, M. R. 2013. Evaluation of reduced susceptibility to quaternary ammonium compounds and bisbiguanides in clinical isolates and laboratory-generated mutants of Staphylococcus aureus. *Antimicrob Agents Chemother,* 57**,** 3488-97.
Furi, L., Haigh, R., Al Jabri, Z., Morrisey, I., Ou, H.-Y., León-Sampedro, R., Martinez, J. L., Coque, T. M. & Oggioni, M. R. 2016. Dissemination of novel antimicrobial resistance mechanisms through the insertion sequence mediated spread of metabolic genes. *Frontiers in Microbiology,* 7.
Gaddy, J. A., Arivett, B. A., McConnell, M. J., Lopez-Rojas, R., Pachon, J. & Actis, L. A. 2012. Role of acinetobactin-mediated iron acquisition functions in the interaction of Acinetobacter baumannii strain ATCC 19606T with human lung epithelial cells, Galleria mellonella caterpillars, and mice. *Infect Immun,* 80**,** 1015-24.
Gaillard, M., Vallaeys, T., Vorholter, F. J., Minoia, M., Werlen, C., Sentchilo, V., Puhler, A. & van der Meer, J. R. 2006. The clc element of Pseudomonas sp. strain B13, a genomic island with various catabolic properties. *J Bacteriol,* 188**,** 1999-2013.
Gal-Mor, O. & Finlay, B. B. 2006. Pathogenicity islands: a molecular toolbox for bacterial virulence. *Cell Microbiol,* 8**,** 1707-19.
Galimand, M., Sabtcheva, S., Courvalin, P. & Lambert, T. 2005. Worldwide Disseminated armA Aminoglycoside Resistance Methylase Gene Is Borne by Composite Transposon Tn1548. *Antimicrob Agents Chemother,* 49**,** 2949–2953.
Garcia-Vallve´, S., Romeu, A. & Palau, J. 2000. Horizontal Gene Transfer in Bacterial and Archaeal Complete Genomes. *Genome research,* 10**,** 1719–1725.
Gay, P., Coq, D. L., Steinmetz, M., Berkelman, T. & Kadoi, C. I. 1985. Positive Selection Procedure for Entrapment of Insertion Sequence Elements in Gram-Negative Bacteria. *J Bacteriol,* 164**,** 918-921.
Gaynes, R. & Edwards , J. R. 2005. Overview of Nosocomial Infections Caused by Gram-Negative Bacilli. *Health Care epidemiology,* 41**,** 848-854.
Gennimata, D., Davies, J. & Tsiftsoglou, A. S. 1996. Bleomycin resistance in Staphylococcus aureus clinical isolates. *Antimicrob Agents Chemother,* 37**,** 65-75.
Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., Deboy, R. T., Ravel, J., Paulsen, I. T., Kolonay, J. F., Brinkac, L., Beanan, M., Dodson, R. J., Daugherty, S. C., Madupu, R., Angiuoli, S. V., Durkin, A. S., Haft, D. H., Vamathevan, J., Khouri, H., Utterback, T., Lee, C., Dimitrov, G., Jiang, L., Qin, H., Weidman, J., Tran, K., Kang, K., Hance, I. R., Nelson, K. E. & Fraser, C. M. 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain. *J Bacteriol,* 187**,** 2426-38.
Gillespie, M. T., Lyon, B. R., Messeroiti, L. J. & Skurray, R. A. 1987. Chromosome- and plasmid-mediated gentamicin resistance in Staphylococcus aureus encoded by

Tn4007. *J Med. Microbiol.,* 24**,** 139-144.
Gillings, M. R., Holley, M. P., Stokes, H. W. & Holmes, A. J. 2005. Integrons in Xanthomonas: A source of species genome diversity. *PNAS,* 102**,** 4419–4424.
Gloux, K., Guillemet, M., Soler, C., Morvan, C., Halpern, D., Pourcel, C., Vu Thien, H., Lamberet, G. & Gruss, A. 2017. Clinical Relevance of Type II Fatty Acid Synthesis Bypass in Staphylococcus aureus. *Antimicrob Agents Chemother,* 61.
Goffin, C. & Ghuysen, J. M. 1998. Multimodular Penicillin-Binding Proteins: An Enigmatic Family of Orthologs and Paralogs. *Microbiol Molec Bio Rev,.* 62**,** 1079–1093.
Gonzfilez-Barca, E., Fernfindez-Sevilla, A., Carratalgt, J., Grafiena, A. & Gudiol, E. 1996. Prospective Study of 288 Episodes of Bacteremia in Neutropenic Cancer Patients in a Single Institution. *Eur. J. Clin. Microbiol. Infect. Dis* 15**,** 291-296.
Grandgirard, D., Furi, L., Ciusa, M. L., Baldassarri, L., Knight, D. R., Morrissey, I., Largiader, C. R., Leib, S. L. & Oggioni, M. R. 2015. Mutations upstream of fabI in triclosan resistant Staphylococcus aureus strains are associated with elevated fabI gene expression. *BMC Genomics,* 16**,** 345.
Grass, G. & Rensing, C. 2001. CueO is a multi-copper oxidase that confers copper tolerance in Escherichia coli. *Biochem Biophys Res Commun,* 286**,** 902-8.
Green, M. R. & Sambrook, J. 2017. Isolation of High-Molecular-Weight DNA Using Organic Solvents. *Cold Spring Harb Protoc,* 2017**,** pdb prot093450.
Grohmann, E., Muth, G. & Espinosa, M. 2003. Conjugative Plasmid Transfer in Gram-Positive Bacteria. *Microbiology and Molecular Biology Reviews,* 67**,** 277-301.
Groth, A. C. & Calos, M. P. 2004. Phage Integrases: Biology and Applications. *Journal of Molecular Biology,* 335**,** 667-678.
Gudas, L. J. & Pardee, A. 1975. Model for Regulation of Escherichia coli DNA Repair Functions (recA-, lex- mutants/cell division/DNA binding proteins). *PNAS,* 72**,** 2330-2334.
Guerin, É., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Da Re, S., Gonzalez-Zorn, B., Barbé, J., Ploy, M.-C. & Mazel, D. 2009. The SOS Response Controls Integron Recombination. *Science,* 324**,** 1034-1034.
Guerin, E., Jove, T., Tabesse, A., Mazel, D. & Ploy, M. C. 2011. High-level gene cassette transcription prevents integrase expression in class 1 integrons. *J Bacteriol,* 193**,** 5675-82.
Guex, N. & Peitsch, M. C. 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis,* 18**,** 2714-23.
Gurevich, A., Saveliev, V., Vyahhi, N. & Tesler, G. 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics,* 29**,** 1072-1075.
Gutacker, M. M., Smoot, J. C., Migliaccio, C. A. L., Ricklefs, S. M., Hua, S., Cousins, D. V., Graviss, E. A., Shashkina, E., Kreiswirth, B. N. & Musser, J. M. 2002. Genome-Wide Analysis of Synonymous Single Nucleotide Polymorphisms in Mycobacterium tuberculosis Complex Organisms: Resolution of Genetic Relationships Among Closely Related Microbial Strains. *Genetics,* 162 1533–1543
Gutierrez, M. C., Brisse, S., Brosch, R., Fabre, M., Omais, B., Marmiesse, M., Supply, P. & Vincent, V. 2005. Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. *PLoS Pathog,* 1**,** e5.
Guzman, L., Belin, D., Carson, M. J. & Beckwith, J. 1995. Tight Regulation, Modulation, and High-Level Expression by Vectors Containing the Arabinose PBAD Promoter. *J Bacteriol,* 177**,** 4121–4130.
Hackbarth, C. J. & Chambers, H. F. 1993. blaI and blaRi Regulate ,B-Lactamase and PBP 2a Production in Methicillin-Resistant Staphylococcus aureus. *Antimicrob Agents Chemother,* 37**,** 1144-1149.
Hacker, J., Blum-Oehler, G., Muhldorfer, I. & Tschape, H. 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol Microbiol,* 23**,** 1089-97.
Hacker, J. & Kaper, J. B. 2000. Pathogenicity islands and the evolution of microbes. *Annual Review of Microbiology* 54**,** 641.

Hall, R. M. & Vockler, C. 1987. The region of the incN plasmid R46 coding for resistance to β-lactam antibiotics, streptomycin/spectinomycin and sulphonamides is closely related to antibiotic resistance segments found In IncW plasm ids and in Tn21-like transposons. *Nucleic acids research,* 15**,** 7491-7501.
Haniford, D. B. & Ellis, M. J. 2015. Transposons Tn10 and Tn5. *In:* CRAIG, N., CRAIGIE, R., GELLERT, M., LAMBOWITZ, A., RICE, P. & SANDMEYER, S. (eds.) *Mobile DNA III.* Washington, DC: ASM Press.
Hansen, L. H., Knudsen, S. & Sørensen, S. J. 1998. The Effect of the lacY Gene on the Induction of IPTG Inducible Promoters, Studied in Escherichia coli and Pseudomonas fluorescens. *Current Microbiology,* 36**,** 341–347.
Hanssen, A. M. & Ericson Sollid, J. U. 2006. SCCmec in staphylococci: genes on the move. *FEMS Immunol Med Microbiol,* 46**,** 8-20.
Hantke, K. 2001. Bacterial zinc transporters and regulators. *BioMetals,* 14**,** 239–249.
Hare, J. M., Perkins, S. N. & Gregg-Jolly, L. A. 2006. A constitutively expressed, truncated umuDC operon regulates the recA-dependent DNA damage induction of a gene in Acinetobacter baylyi strain ADP1. *Appl Environ Microbiol,* 72**,** 4036-43.
Harmer, C. J., Moran, R. A. & Hall, R. M. 2014. Movement of IS26-Associated Antibiotic Resistance Genes Occurs via a Translocatable Unit That Includes a Single IS26 and Preferentially Inserts Adjacent to Another IS26. *mBio,* 5**,** e01801-14.
Harms, K., Starikova, I. & Johnsen, P. J. 2013. Costly Class-1 integrons and the domestication of the the functional integrase. *Mob Genet Elements,* 3**,** e24774.
Hassan, K. A., Pederick, V. G., Elbourne, L. D., Paulsen, I. T., Paton, J. C., McDevitt, C. A. & Eijkelkamp, B. A. 2017. Zinc stress induces copper depletion in Acinetobacter baumannii. *BMC Microbiol,* 17**,** 59.
Hauschild, T. & Schwarz, S. 2010. Macrolide resistance in Staphylococcus spp. from free-living small mammals. *Vet Microbiol,* 144**,** 530-1.
He, S., Hickman, A. B., Varani, A., Patricia Siguier, Chandler, M., Dekker, J. P. & Dyda, F. 2015. Insertion Sequence IS26 Reorganizes Plasmids in Clinically Isolated Multidrug-Resistant Bacteria by Replicative Transposition. *mBio,* 6**,** e00762-15.
He, X., Ou, H. Y., Yu, Q., Zhou, X., Wu, J., Liang, J., Zhang, W., Rajakumar, K. & Deng, Z. 2007. Analysis of a genomic island housing genes for DNA S-modification system in Streptomyces lividans 66 and its counterparts in other distantly related bacteria. *Mol Microbiol,* 65**,** 1034-48.
Heckman, K. L. & Pease, L. R. 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc,* 2**,** 924-32.
Hedges, R. W. & Jacob, A. E. 1974. Transposition of Ampicillin Resistance from RP4 to Other Replicons. *Mol Genetics and Genomics,* 132**,** 31-40
Heffron, F., Sublett, R., Hedges, R. W., Jacob, A. E. & Falkow, S. 1975. Origin of theTEM Beta-Lactamase Gene Foundon Plasmids. *J Bacteriol,* 122**,** 250-256.
Heineman, J. & Sprague, G. F. 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. *nature,* 340**,** 205-209.
Heinlein, M. & Epel, B. L. 2004. Macromolecular Transport and Signaling Through Plasmodesmata. *International Review of Cytology,* 235**,** 93-164.
Herrero, M., De Lorenzo, V. & Timmis, K. N. 1990. Transposon Vectors Containing Non-Antibiotic Resistance Selection Markers for Cloning and Stable Chromosomal Insertion of Foreign Genes inGram-Negative Bacteria. *J Bacteriol,* 172**,** 6557-6567.
Hickman, A. B. & Dyda, F. 2015. Mechanisms of DNA Transposition. *microb spectrum,* 3**,** MDNA3-0034-2014.
Hickman, A. B., Li, Y., Mathew, S. V., May, E. W., Craig, N. L. & Dyda, F. 2000. Unexpected Structural Diversity in DNA Recombination: The Restriction Endonuclease Connection. *Molecular Cell,* 5**,** 1025–1034,.
Hickman, A. B., Perez, Z. N., Zhou, L., Musingarimi, P., Ghirlando, R., Hinshaw, J. E., Craig, N. L. & Dyda, F. 2005. Molecular architecture of a eukaryotic DNA transposase. *Nat Struct Mol Biol,* 12**,** 715-21.

Hijazi, K., Mukhopadhya, I., Abbott, F., Milne, K., Al-Jabri, Z. J., Oggioni, M. R. & Gould, I. M. 2016. Susceptibility to chlorhexidine amongst multidrug-resistant clinical isolates of Staphylococcus epidermidis from bloodstream infections. *Int J Antimicrob Agents*.
Hiramatsu, K., Cui, L., Kuroda, M. & Ito, T. 2001. The emergence and evolution of methicillin-resistant Staphylococcus aureus. *trends in Microbiology,* 9**,** 486-493.
Hirsch, H.-J., Starlinger, P. & Brachet, P. 1972. Two Kinds of Insertions in Bacterial Genes. *Mol Genetics and Genomics,* 119**,** 191--206.
Hirschhausen, N., Schlesier, T., Schmidt, M. A., Gotz, F., Peters, G. & Heilmann, C. 2010. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. *Cell Microbiol,* 12**,** 1746-64.
Hochhut, B. & Waldor, M. K. 1999. Site-specifc integration of the conjugal Vibrio cholerae SXT element into prfC. *Mol Microbiol,* 32**,** 99-110.
Hocquet, D., Llanes, C., Thouverez, M., Kulasekara, H. D., Bertrand, X., Plesiat, P., Mazel, D. & Miller, S. I. 2012. Evidence for induction of integron-based antibiotic resistance by the SOS response in a clinical setting. *PLoS Pathog,* 8**,** e1002778.
Holden, M. T., Feil, E. J., Lindsay, J. A., Peacock, S. J., Day, N. P., Enright, M. C., Foster, T. J., Moore, C. E., Hurst, L., Atkin, R., Barron, A., Bason, N., Bentley, S. D., Chillingworth, C., Chillingworth, T., Churcher, C., Clark, L., Corton, C., Cronin, A., Doggett, J., Dowd, L., Feltwell, T., Hance, Z., Harris, B., Hauser, H., Holroyd, S., Jagels, K., James, K. D., Lennard, N., Line, A., Mayes, R., Moule, S., Mungall, K., Ormond, D., Quail, M. A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Sharp, S., Simmonds, M., Stevens, K., Whitehead, S., Barrell, B. G., Spratt, B. G. & Parkhill, J. 2004. Complete genomes of two clinical Staphylococcus aureus strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A,* 101**,** 9786-91.
Hood, M. I. & Skaar, E. P. 2012. Nutritional immunity: transition metals at the pathogen-host interface. *Nat Rev Microbiol,* 10**,** 525-37.
Horasan, E. S., Ersoz, G., Tombak, A., Naci, T. & Ali, K. 2011. Bloodstream infections and mortality-related factors in febrile neutropenic cancer patients
. *Med Sci Monit,* 17**,** CR304-309.
Hornsey, M. & Wareham, D. W. 2018. Effects of In vivo Emergent Tigecycline Resistance on the Pathogenic Potential of Acinetobacter baumannii. *Sci Rep,* 8**,** 4234.
Hosahalli S.Subramanya, L. K. A., Rachel A.Baker, Louise E.Bird, David J.Sherratt, Dale B.Wigley 1997. crystal structure of of the site-specific recombinase XerD. *The EMBO Journal,* 16**,** 5178–5187.
Hou, Z., Meng, J. R., Zhao, J. R., Hu, B. Q., Liu, J., Yan, X. J., Jia, M. & Luo, X. X. 2007. Inhibition of beta-lactamase-mediated oxacillin resistance in Staphylococcus aureus by a deoxyribozyme. *Acta Pharmacol Sin,* 28**,** 1775-82.
Howard, A., O'Donoghue, M., Feeney, A. & Sleator, R. D. 2012. Acinetobacter baumannii: an emerging opportunistic pathogen. *Virulence,* 3**,** 243-50.
Hsiao, W., Wan, I., Jones, S. J. & Brinkman, F. S. L. 2003. IslandPath: aiding detection of genomic islands in prokaryotes. *Bioinformatics,* 19**,** 418-420.
Hsiao, W. W. L., Ung, K., Aeschliman, D., Bryan, J., Finlay, B. B. & Brinkman, F. S. L. 2005. Evidence of a Large Novel Gene Pool Associated with Prokaryotic Genomic Islands. *PloS Genetics,* 1**,** e62.
Hu, S., Ohtsubo, E., Davidson, N. & Saedler, H. 1975. Electron Microscope Heteroduplex Studies of Sequence Relations Among Bacterial Plasmids: Identification and Mapping of the Insertion Sequences IS1 and IS2 in F and R
Plasmids. *J Bacteriol,* 122**,** 764-775.
Hujer, K. M., Hujer, A. M., Hulten, E. A., Bajaksouzian, S., Adams, J. M., Donskey, C. J., Ecker, D. J., Massire, C., Eshoo, M. W., Sampath, R., Thomson, J. M., Rather, P. N., Craft, D. W., Fishbain, J. T., Ewell, A. J., Jacobs, M. R., Paterson, D. L. & Bonomo, R. A. 2006. Analysis of Antibiotic Resistance Genes in Multidrug-Resistant Acinetobacter sp. Isolates from Military and Civilian Patients Treated at the Walter

Reed Army Medical Center. *Antimicrobial Agents and Chemotherapy,* 50**,** 4114-4123.
Hurtig, J., Chiu, D. T. & Önfelt, B. 2010. Intercellular nanotubes: insights from imaging studies and beyond. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.,* 2**,** 260–276.
Hussain, M., Wilcox, M. H. & White, P. J. 1993. The slime of coagulase-negative staphylococci: biochemistry and relation to adherence. . *fems Microbiol rev,* 10**,** 191–207.
Iacono, M., Villa, L., Fortini, D., Bordoni, R., Imperi, F., Bonnal, R. J., Sicheritz-Ponten, T., De Bellis, G., Visca, P., Cassone, A. & Carattoli, A. 2008. Whole-genome pyrosequencing of an epidemic multidrug-resistant Acinetobacter baumannii strain belonging to the European clone II group. *Antimicrob Agents Chemother,* 52**,** 2616-25.
Iannelli, F., Santagati, M., Santoro, F., Oggioni, M. R., Stefani, S. & Pozzi, G. 2014. Nucleotide sequence of conjugative prophage Phi1207.3 (formerly Tn1207.3) carrying the mef(A)/msr(D) genes for e ffl ux resistance to macrolides in Streptococcus pyogenes. *Front Microbiol,* 5**,** 687.
Isola, D., Pardini, M., Varaine, F., Niemann, S., Rusch-Gerdes, S., Fattorini, L., Orefici, G., Meacci, F., Trappetti, C., Rinaldo Oggioni, M., Orru, G. & group, L.-D. s. 2005. A Pyrosequencing assay for rapid recognition of SNPs in Mycobacterium tuberculosis embB306 region. *J Microbiol Methods,* 62**,** 113-20.
Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, K., Tiensasitorn, C. & Hiramatsu, K. 2001. Structural Comparison of Three Types of Staphylococcal Cassette Chromosome mec Integrated in the Chromosome in Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy,* 45**,** 1323-1336.
Ito, T., Katayama, Y. & Hiramatsu, K. 1999. Cloning and Nucleotide Sequence Determination of the Entire mec DNA of Pre-Methicillin-Resistant Staphylococcus aureus N315. *Antimicrob Agents Chemother,* 43**,** 1449–1458.
Ito, T., Ma, X. X., Takeuchi, F., Okuma, K., Yuzawa, H. & Hiramatsu, K. 2004. Novel Type V Staphylococcal Cassette Chromosome mec Driven by a Novel Cassette Chromosome Recombinase, ccrC. *Antimicrobial Agents and Chemotherapy,* 48**,** 2637-2651.
Ito, T., Okuma, K., Ma, X. X., Yuzawa, H. & Hiramatsu, K. 2003. Insights on antibiotic resistance of Staphylococcus aureus from its whole genome: genomic island SCC. *Drug Resistance Updates,* 6**,** 41-52.
Jappe, U., Heuck, D., Strommenger, B., Wendt, C., Werner, G., Altmann, D. & Witte, W. 2008. Staphylococcus aureus in dermatology outpatients with special emphasis on community-associated methicillin-resistant strains. *J Invest Dermatol,* 128**,** 2655-64.
Jensen, E. C., Schrader, H. S., Rieland, B., Thompson, T. L., Lee, K. W., Nickerson, K. W. & Kokjohn, T. A. 1998. Prevalence of Broad-Host-Range Lytic Bacteriophages of Sphaerotilus natans, Escherichia coli, and Pseudomonas aeruginosa. *Appl Environ Microbiol,* 64**,** 575–580.
Jensen, S. O. & Lyon, B. R. 2009. Genetics of antimicrobial resistance in Staphylococcus aureus. *Future Microbiol,* 4**,** 565-82.
Jones, L. A., McIver, C. J., Kim, M. J., Rawlinson, W. D. & White, P. A. 2005. The aadB gene cassette is associated with blaSHV genes in Klebsiella species producing extended-spectrum beta-lactamases. *Antimicrob Agents Chemother,* 49**,** 794-7.
Juhas, M. 2015. Horizontal gene transfer in human pathogens. *Crit Rev Microbiol,* 41**,** 101-8.
Juhas, M., van der Meer, J. R., Gaillard, M., Harding, R. M., Hood, D. W. & Crook, D. W. 2009. Genomic islands: tools of bacterial horizontal gene transfer and evolution. *FEMS Microbiol Rev,* 33**,** 376-93.
Justin Courcelle, Arkady Khodursky, Brian Peter, Brown, P. O. & Hanawalt, P. C. 2001. Comparative Gene Expression Profiles Following UV Exposure in Wild-Type and SOS-Deficient Escherichia coli. *Genetics,* 158: **,** 41–64.

Kadlec, K. & Schwarz, S. 2009. Novel ABC transporter gene, vga(C), located on a multiresistance plasmid from a porcine methicillin-resistant Staphylococcus aureus ST398 strain. *Antimicrob Agents Chemother,* 53**,** 3589-91.
Kadurugamuwa, J. L. & Beveridge, T. J. 1995. Virulence Factors Are Released from Pseudomonas aeruginosa in Association with Membrane Vesicles during Normal Growth and Exposure to Gentamicin: a Novel Mechanism of Enzyme Secretion. *J Bacteriol,* 177**,** 3998–4008.
Katayama, Y., Ito, T. & Hiramatsu, K. 2000. A New Class of Genetic Element, Staphylococcus Cassette Chromosome mec, Encodes Methicillin Resistance in Staphylococcus aureus. *Antimicrob Agents Chemother,* 44**,** 1549–1555.
Katayama, Y., Ito, T. & Hiramatsu, K. 2001. Genetic organization of the chromosome region surrounding mecA in clinical staphylococcal strains: role of IS431-mediated mecI deletion in expression of resistance in mecA-carrying, low-level methicillin-resistant Staphylococcus haemolyticus. *Antimicrob Agents Chemother,* 45**,** 1955-63.
Katayama, Y., Robinson, D. A., Enright, M. C. & Chambers, H. F. 2005. Genetic background affects stability of mecA in Staphylococcus aureus. *J Clin Microbiol,* 43**,** 2380-3.
Katayama, Y., Takeuchi, F., Ito, T., Ma, X. X., Ui-Mizutani, Y., Kobayashi, I. & Hiramatsu, K. 2003. Identification in Methicillin-Susceptible Staphylococcus hominis of an Active Primordial Mobile Genetic Element for the Staphylococcal Cassette Chromosome mec of Methicillin-Resistant Staphylococcus aureus. *Journal of Bacteriology,* 185**,** 2711-2722.
Kehrenberg, C. & Schwarz, S. 2006. Distribution of florfenicol resistance genes fexA and cfr among chloramphenicol-resistant Staphylococcus isolates. *Antimicrob Agents Chemother,* 50**,** 1156-63.
Kempf, M. & Rolain, J. M. 2012. Emergence of resistance to carbapenems in Acinetobacter Baumannii in Europe: clinical impact and theaputic options. *Int J Antimicrob Agents,* 39**,** 105-114.
Ken-ichi Takemaru, Motoki Mizuno, Tsutomu Sato, Takeuchi, M. & Kobayashi, Y. 1995. Complete nucleotide sequence of a skin element excised by DNA rearrangement during sporulation in Bacillus subtilis. *microb* 323-327.
Khan, E., Mack, J. P. G., Katz, R. A., Kulkosky, J. & Skalka, A. M. 1991. Retroviral integrase domains: DNA binding and the recognition of LTR sequences. *Nucleic Acids Res,* 19**,** 851-860.
Kibbe, W. A. 2007. OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Res,* 35**,** W43-6.
Kilbride, E., Boocock, M. R. & Stark, W. M. 1999. Topological selectivity of a hybrid site-specific recombination system with elements from Tn< i> 3 res</i>/resolvase and bacteriophage P1< i> loxP</i>/Cre. *Journal of molecular biology,* 289**,** 1219-1230.
Kim, D., Baik, K. S., Kim, M. S., Park, S. C., Kim, S. S., Kwak, Y. S. & Seong, C. N. 2008. Acinetobacter soli sp. nov., isolated from forest soil. *J Microbiol,* 46**,** 396-401.
Kim, D. H. & Ko, K. S. 2015. AbaR-type genomic islands in non-baumannii Acinetobacter species isolates from South Korea. *Antimicrob Agents Chemother,* 59**,** 5824-6.
Kleckner, N. 1981. Transposable elements in prokaryotes. *Annu Rev Genet,* 15**,** 341-404.
Klemm, P. 1986. Two regulatory fim genes, fimB and fimE, control the phase variation of type 1 fimbriae in Escherichia coli. *The EMBO journal,* 5**,** 1389.
Klockgether, J., Reva, O., Larbig, K. & Tummler, B. 2003. Sequence Analysis of the Mobile Genome Island pKLC102 of Pseudomonas aeruginosa C. *Journal of Bacteriology,* 186**,** 518-534.
Kloos, W. E. & Musselwhite, M. S. 1975. Distribution and Persistence ofStaphylococcus and Micrococcus Species and Other Aerobic Bacteria on
Human Skin. *appl microbiol,* 30**,** 381-395.
Kloos, W. E. & Schleifer, K. H. 1975. Isolation and Characterization of Staphylococci from Human Skin. Descriptions of Four New Species: Staphylococcus warneri, Staphylococcus capitis, Staphylococcus horninis, and Staphylococcus simulans. *int j systematic bactriology,* 25**,** 62-79.

Kloos, W. E. & Schleifer, K. H. 1983. Staphylococcus auricularis sp. nov. : an Inhabitant of the Human External Ear. *int j systematic bactriology,* 33**,** 9-14.
Kobayashi, N., Alam, M. & Urasawa, S. 2001. Analysis on distribution of insertion sequence IS431 in clinical isolates of staphylococci. *Diagnostic Microbiology and Infectious Disease,* 39**,** 61–64.
Kochar, M., Crosatti, M., Harrison, E. M., Rieck, B., Chan, J., Constantinidou, C., Pallen, M., Ou, H. Y. & Rajakumar, K. 2012. Deletion of TnAbaR23 Results in both Expected and Unexpected Antibiogram Changes in a Multidrug-Resistant Acinetobacter baumannii Strain. *Antimicrobial Agents and Chemotherapy,* 56**,** 1845-1853.
Koh, T. H., Sng, L.-H., Wang, G. C. Y., Hsu, L.-Y. & Zhao, Y. 2007. IMP-4 and OXA β-lactamases in Acinetobacter baumannii from Singapore. *Journal of Antimicrobial Chemotherapy,* 59**,** 627-632.
Kolling, G. L. & Matthews, K. R. 1999. Export of Virulence Genes and Shiga Toxin by Membrane Vesicles of Escherichia coli O157:H7. *Appl Environ Microbiol,* 65**,** 1843–1848.
Kondo, Y., Ito, T., Ma, X. X., Watanabe, S., Kreiswirth, B. N., Etienne, J. & Hiramatsu, K. 2007. Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. *Antimicrob Agents Chemother,* 51**,** 264-74.
Koonin, E. V., Mushegian, A. R., Galperin, M. Y. & Walker, D. R. 1997. Comparison of archaeal and bacterial genomes: computer analysis of protein sequences predicts novel functions and suggests a chimeric origin for the archaea. *Molecular Microbiology* 25**,** 619–637.
Koonin, E. V. & Wolf, Y. I. 2008. Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. *Nucleic Acids Res,* 36**,** 6688-719.
Korczynska, M., Mukhtar, T. A., Wright, G. D. & Berghuis, A. M. 2007. Structural basis for streptogramin B resistance in Staphylococcus aureus by virginiamycin B lyase
. *PNAS,* 104**,** 10388–10393.
Kornblum, J., Hartman, B. J., Novick, R. P. & Tomasz, A. 1981. Conversion of a Homogeneously Methicillin-Resistant Strain of Staphylococcus aureus to Heterogeneous Resistance by Tn551-Mediated Insertional Inactivation. *Eur. J. Clin. Microbiol.,* 5**,** 714-718.
Kreiswirth, B. N., Lofdhal, S., Betley, M. J., O'Reilly, M., Schilievert, P. M., Bergdoll, M. S. & Novick, P. R. 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature,* 305**,** 709-712.
Kriegeskorte, A., Konig, S., Sander, G., Pirkl, A., Mahabir, E., Proctor, R. A., von Eiff, C., Peters, G. & Becker, K. 2011. Small colony variants of Staphylococcus aureus reveal distinct protein profiles. *Proteomics,* 11**,** 2476-90.
Krizova, L., Dijkshoorn, L. & Nemec, A. 2011. Diversity and Evolution of AbaR Genomic Resistance Islands in Acinetobacter baumannii Strains of European Clone I. *Antimicrobial Agents and Chemotherapy,* 55**,** 3201-3206.
Kuhl, S. A., Pattee, P. A. & Baldwin, J. N. 1978. Chromosomal Map Location of the Methicillin Resistance Determinant in Staphylococcus aureus. *J Bacteriol,* 135**,** 460-465.
Kulkosky, J., Jones, K., Katz, R., Mack, J. P. G. & Skalka, A. M. 1992. Residues Critical for Retroviral Integrative Recombination in a Region That Is Highly Conserved among Retroviral/Retrotransposon Integrases and Bacterial InsertionSequence Transposases. *molecular cellular biology,* 12**,** 2331-2338.
Laddaga, R. A., Chu, L., Misra, T. K. & Silver, S. 1987. Nucleotide sequence and expression of the mercurial-resistance operon from Staphylococcus aureus plasmid pI258. *biochem* 84**,** 5106-5110.
Landman, D., Quale, J. M., Mayorga, D., Adedeyo, A., Vangala, K., Ravishankar, J., Flores, C. & Brooks, S. 2002. Citywide Clonal Outbreak of Multiresistant Acinetobacter baumannii and psuedomonas aeroginosa in Brooklyn, NY.
The Preantibiotic eara Has Returned. *JAMA Internal Medicine,* 162**,** 1515-1520.

Landy, A. 1993. Mechanistic and structural complexity in the site-specific recombination pathways of Int and FLP. *Current opinion in genetics & development,* 3**,** 699-707.
Lange, C. C., Werckenthin, C. & Schwarz, S. 2003. Molecular analysis of the plasmid-borne aacA/aphD resistance gene region of coagulase-negative staphylococci from chickens. *J Antimicrob Chemother,* 51**,** 1397-401.
Larbig, K. D., Christmann, A., Johann, A., Klockgether, J., Hartsch, T., Merkl, R., Wiehlmann, L., Fritz, H. J. & Tummler, B. 2002. Gene Islands Integrated into tRNAGly Genes Confer Genome Diversity on a Pseudomonas aeruginosa Clone. *Journal of Bacteriology,* 184**,** 6665-6680.
Larionov, A., Krause, A. & Miller, W. 2005. A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics,* 6**,** 62.
Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., Jelsbak, L., Sicheritz-Ponten, T., Ussery, D. W., Aarestrup, F. M. & Lund, O. 2012. Multilocus sequence typing of total-genome-sequenced bacteria. *J Clin Microbiol,* 50**,** 1355-61.
Lawrence, J. G. & Hendrickson, H. 2003. Lateral gene transfer: when will adolescence end? *Molecular Microbiology,* 50**,** 739-749.
Lawrence, J. G. & Ochman, H. 1998. Molecular archaeology of the Escherichia coli genome. *PNAS,* 95**,** 9413–9417.
Lawrence, J. G. L. 1999. Gene transfer, speciation, and the evolution of bacterial genomes. *Current opinion in microbiology,* 2**,** 519–523.
Lederberg, J. & Tatum, E. L. 1953. Sex in bacteria: Genetic studies, 1945-1952. *Science,* 118**,** 169-175.
Lee, J. & Jayaram, M. 1993. Mechanism of site-specific recombination. Logic of assembling recombinase catalytic site from fractional active sites. *J Biol Chem,* 268**,** 17564-70.
Leelaporn, A., Neville, F., Paulsen, I. T. & Skurray, R. A. 1996. IS257-Mediated Cointegration in the Evolution of a Family of Staphylococcal Trimethoprim Resistance Plasmids. *J Bacteriol,* 178**,** 6070–6073.
Legatzki, A., Grass, G., Anton, A., Rensing, C. & Nies, D. H. 2003. Interplay of the Czc System and Two P-Type ATPases in Conferring Metal Resistance to Ralstonia metallidurans. *Journal of Bacteriology,* 185**,** 4354-4361.
Lelie`vre, H., Lina, G., Jones, M. E., Olive, C., Oise Forey, F., Roussel-Delvallez, M., Nicolas-Chanoine, M.-H. l. n., Be´be´ar, C. c. M., Antoine Andremont, V. J., Vandenesch, F. O. & Etienne, J. 1999. Emergence and Spread in French Hospitals of Methicillin- Resistant Staphylococcus aureus with Increasing Susceptibility to Gentamicin and Other Antibiotics. *j clinical micro,* 37**,** 3452–3457.
Leossner, M. J., Maier, S. K., Daubek-Puza, H., Wendlinger, G. & Scherer, S. 1997. Three Bacillus cereus Bacteriophage Endolysins Are Unrelated but Reveal High Homology to Cell Wall Hydrolases from Different Bacilli. *J Bacteriol,* 179**,** 2845–2851.
Leplae, R., Lima-Mendez, G. & Toussaint, A. 2010. ACLAME: a CLAssification of Mobile genetic Elements, update 2010. *Nucleic Acids Res,* 38**,** D57-61.
Levi, I. & Rubinstein, E. 1996. Acinetobacter infections—overview of clinical features. *Acinetobacter: microbiology, epidemiology, infections, management***,** 101-115.
Li, M., Diep, B. A., Villaruza, A. E., Braughtona, K. R., Jiangb, X., DeLeoa, F. R., Chambersc, H. F., Lub, Y. & Otto, M. 2009. Evolution of virulence in epidemic community-associated methicillin-resistant Staphylococcus aureus. *PNAS,* 106**,** 5883–5888.
Li, W., Zhang, D., Huang, X. & Qin, W. 2014. Acinetobacter harbinesis sp. nov., isolated from river water. *Int J systematic evolutionary microbiol,* 64**,** 1507-1513.
Li, Y., Chang, J., Guo, L., Wang, H., Xie, S., Piao, C. & He, W. 2015. Description of Acinetobacter populi Sp. nov. isolated from symptomatic bark of Populus euramericana canker. *Int J systematic evolutionary microbiol,* 65**,** 4461-4468.
Li, Y., Piao, C., Ma, Y., He, W., Wang, H., Chang, J., Guo, L., Wang, X. & Xie, S. 2013. Acinetobacter puyangensis sp. nov., isolated from the healthy and diseases part of Populus xeuramericana canker bark. *Int J systematic evolutionary microbiol,* 63**,** 2963-2969.

Li, Z., Clarke, A. J. & Beveridge, T. J. 1998. Gram-Negative Bacteria Produce Membrane Vesicles Which Are Capable of Killing Other Bacteria. *J Bacteriol,* 180**,** 5478–5483.
Liebert, C. A., Hall, R. M. & Summers, A. O. 1999. Transposon Tn21, flagship of the floating genome. *Microbiol Mol Biol Rev,* 63**,** 507-22.
Lim, T. T., Chong, F. N., O'brien, F. G. & Grubb, W. B. 2003. Are all community methicillin-resistant Staphylococcus aureus related? A comparison of their mec regions. *Pathology,* 35**,** 336-343.
Lin, T. L., Lee, C. Z., Hsieh, P. F., Tsai, S. F. & Wang, J. T. 2008. Characterization of integrative and conjugative element ICEKp1-associated genomic heterogeneity in a Klebsiella pneumoniae strain isolated from a primary liver abscess. *J Bacteriol,* 190**,** 515-26.
Lindsay, J. A. & Holden, M. T. 2004. Staphylococcus aureus: superbug, super genome? *Trends Microbiol,* 12**,** 378-85.
Link, A. J., Phillips, D. & CHURCH, G. M. 1997. Methods for Generating Precise Deletions and Insertions in the Genome of Wild-Type Escherichia coli: Application to Open Reading Frame Characterization. *J Bacteriol,* 179**,** 6228–6237.
Littlejohn, T. G., DiBerardino, D., Messerotti, L., Spiers, S. J. & Skurray, R. A. 1991. Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in Staphylococcus aureus. *Gene,* 101**,** 59-66.
Livermore, D. M., James, D., Reacher, M., Graham, C., Nichols, T., Stephens, P., Johnson, A. P. & George, R. C. 2002. Trends in Fluoroquinolone (Ciprofloxacin) Resistance in Enterobacteriaceae from Bacteremias, England and Wales, 1990–1999. *Emerging Infectious Diseases,* 8**,** 473-478.
Locht, C., Berlin, P., Menozzi, F. D. & Renauld, G. 1993. The filamintous haemagglutinin, a multifaceted adhesin produced by virulent Brodetella spp. *Mol Microbiol,* 9**,** 653-660.
Loeza-Lara, P. D., Soto-Huipe, M., Baizabal-Aguirre, V. M., Ochoa-Zarzosa, A., Valdez-Alarcon, J. J., Cano-Camacho, H. & Lopez-Meza, J. E. 2004. pBMSa1, a plasmid from a dairy cow isolate of Staphylococcus aureus, encodes a lincomycin resistance determinant and replicates by the rolling-circle mechanism. *Plasmid,* 52**,** 48-56.
Loper, J. E., Karl A. Hassan, Dmitri V. Mavrodi, Edward W. Davis, Chee Kent Lim, Brenda T. Shaffer, Liam D. H. Elbourne, Virginia O. Stockwell, Sierra L. Hartney, Katy Breakwell, Marcella D. Henkels, Tetu3, S. G., Lorena I. Rangel, Teresa A. Kidarsa, N. L. W., Judith E. van de Mortel, Chunxu Song, Rachel Blumhagen, Diana Radune, Jessica B. Hostetler, Lauren M. Brinkac, A. Scott Durkin, Daniel A. Kluepfel, W. Patrick Wechter, Anne J. Anderson, Young Cheol Kim, Leland S. Pierson,, Elizabeth A. Pierson, S. E. L., Donald Y. Kobayashi, & Jos M. Raaijmakers, D. M. W., Linda S. Thomashow, Andrew E. Allen16, Ian T. Paulsen 2012. Comparative Genomics of Plant-Associated Pseudomonas spp.: Insights into Diversity and Inheritance of Traits Involved in Multitrophic Interactions. *PloS Genetics,* 8**,** e1002784.
Lu, H. & Tonge, P. J. 2008. Inhibitors of FabI, an Enzyme Drug Target in the Bacterial Fatty Acid Biosynthesis Pathway. *accounts of chemical research,* 41**,** 11-20.
Lu, M. & Fu, D. 2007. Structure of the zinc transporter YiiP. *Science,* 317**,** 1746-8.
Lucas, W. J., Ham, B. K. & Kim, J. Y. 2009. Plasmodesmata - bridging the gap between neighboring plant cells. *Trends Cell Biol,* 19**,** 495–503.
Lüthje, P. & Schwarz, S. 2007. Molecular basis of resistance to macrolides and lincosamides among staphylococci and streptococci from various animal sources collected in the resistance monitoring program BfT-GermVet. *International Journal of Antimicrobial Agents,* 29**,** 528-535.
Lynch, M. 1997. Mutation Accumulation in Nuclear, Organelle, and Prokaryotic Transfer RNA Genes. *mol biol evol.,* 14**,** 914-925.
Lyon, B. R. & Skurray, R. 1987. Antimicrobial Resistance of Staphylococcus aureus: Genetic Basis. *microb reviews,* 51**,** 88-134.
Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H. & Laufs, R. 1996. The Intercellular Adhesin Involved in Biofilm Accumulation of Staphylococcus

epidermidis Is a Linear -1,6-Linked Glucosaminoglycan: Purification and Structural Analysis. *J Bacteriol,* 178**,** 175–183.
Macomber, L. & Imlay, J. A. 2009. The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proc Natl Acad Sci U S A,* 106**,** 8344-9.
Macrina, F. L. & Archer, G. L. 1993. Conjugation and Broad Host Range Plasmids in Streptococci and Staphylococci. *In:* CLEWELL, D. B. (ed.) *bacterial conjugation.* Boston: Springer, Boston, MA.
Malachowa, N. & DeLeo, F. R. 2010. Mobile genetic elements of Staphylococcus aureus. *Cell Mol Life Sci,* 67**,** 3057-71.
Malhotra, J., Anand, S., Jindal, S., Rajagopal, R. & Lal, R. 2012. Acinetobacter indicus sp. nov., isolated from a hexachlorocyclohexane dump site. *International Journal of Systematic and Evolutionary Microbiology,* 62**,** 2883-2890.
Manso, A. S., Chai, M. H., Atack, J. M., Furi, L., De Ste Croix, M., Haigh, R., Trappetti, C., Ogunniyi, A. D., Shewell, L. K., Boitano, M., Clark, T. A., Korlach, J., Blades, M., Mirkes, E., Gorban, A. N., Paton, J. C., Jennings, M. P. & Oggioni, M. R. 2014. A random six-phase switch regulates pneumococcal virulence via global epigenetic changes. *Nat Comm***,** 5:5055.
Mantri, Y. & Williams, K. P. 2004. Islander: a database of integrative islands in prokaryotic genomes, the associated integrases and their DNA site specificities. *Nucleic Acids Res,* 32**,** D55-8.
Maragakis, L. L. & Perl, T. M. 2008. Acinetobacter baumannii: epidemiology, antimicrobial resistance, and treatment options. *Clin Infect Dis,* 46**,** 1254-63.
Marchand, I., Damier-Piolle, L., Courvalin, P. & Lambert, T. 2004. Expression of the RND-type efflux pump AdeABC in Acinetobacter baumannii is regulated by the AdeRS two-component system. *Antimicrob Agents Chemother,* 48**,** 3298-304.
Martinez-Morales, F., Borges, A. C., Martinez, A., Shanmugam, K. T. & Ingram, L. O. 1999. Chromosomal Integration of Heterologous DNA in Escherichia coli with Precise Removal of Markers and Replicons Used during Construction. *J Bacteriol,* 181**,** 7143–7148.
Mashburn-Warren, L. & Whiteley, M. 2005. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature Letters,* 437**,** 422-425.
Massidda, O., Mingoia, M., Fadda, D., Whalen, M. B., Montanari, M. P. & Varaldo, P. E. 2006. Analysis of the beta-lactamase plasmid of borderline methicillin-susceptible Staphylococcus aureus: focus on bla complex genes and cadmium resistance determinants cadD and cadX. *Plasmid,* 55**,** 114-27.
Matsuoka, M., Endou—, K., Kobayashi, H., Inoue˜, M. & Nakajima, Y. 1998. A plasmid that encodes three genes for resistance to macrolide antibiotics in Staphylococcus aureus. *fems Microbiol letters* 221-227.
Mazel, D. 2006. Integrons: agents of bacterial evolution. *Nat Rev Microbiol,* 4**,** 608-620.
McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., Bhullar, K., Canova, M. J., De Pascale, G., Ejim, L., Kalan, L., King, A. M., Koteva, K., Morar, M., Mulvey, M. R., O'Brien, J. S., Pawlowski, A. C., Piddock, L. J., Spanogiannopoulos, P., Sutherland, A. D., Tang, I., Taylor, P. L., Thaker, M., Wang, W., Yan, M., Yu, T. & Wright, G. D. 2013. The comprehensive antibiotic resistance database. *Antimicrob Agents Chemother,* 57**,** 3348-57.
McClintock, B. 1944. The Relation Of Homologous Deficiencies To Mutations And Allelic Series In Maize. *Genetics,* 29**,** 478-502.
McDevitt, C. A., Ogunniyi, A. D., Valkov, E., Lawrence, M. C., Kobe, B., McEwan, A. G. & Paton, J. C. 2011. A Molecular Mechanism for Bacterial Susceptibility to Zinc. *PloS Pathogens,* 7**,** e1002357.
McDonnell, G. & Russell, D. 1999. Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Review,* 12.
McKenzie, T., Hoshino, T., Tanaka, T. & Sueoka, N. 1986. The nucleotide sequence of pUB110: Some salient features in relation to replication and its regulation. *In Plasmid,* 15**,** 93-103.

Miragaia, M., Thomas, J. C., Couto, I., Enright, M. C. & de Lencastre, H. 2007. Inferring a population structure for Staphylococcus epidermidis from multilocus sequence typing data. *J Bacteriol,* 189**,** 2540-52.

Mizuuchi, K. & Baker, T. 2002. Chemical mechanism for mobilizing DNA. *In:* CRAIG, N., CRAIGIE, R., GELLERT, M. & LAMBOWITZ, A. (eds.) *Mobile DNA II.* Washington, DC: ASM Press.

Morikawa, K., Inose, Y., Okamura, H., Maruyama, A., Hayashi, H., Takeyasu, K. & Ohta, T. 2003 A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. *Genes to Cells* 8**,** 699–712.

Morrissey, I., Oggioni, M. R., Daniel Knight, Curiao, T., Coque, T. M., Kalkanci, A., Martinez, J. L. & Consortium, B. 2014. Evaluation of Epidemiological Cut-Off Values Indicates that Biocide Resistant Subpopulations Are Uncommon in Natural Isolates of Clinically-Relevant Microorganisms. *PLoS One,* 9**,** e86669.

Mortensen, B. L., Rathi, S., Chazin, W. J. & Skaar, E. P. 2014. Acinetobacter baumannii response to host-mediated zinc limitation requires the transcriptional regulator Zur. *J Bacteriol,* 196**,** 2616-26.

Moskowitz, S. M. & Wiener-Kronish, J. P. 2010. Mechanisms of bacterial virulence in pulmonary infections. *Curr Opin Crit Care,* 16**,** 8-12.

Moxon, E. R. 2011. Darwin, microbes and evolution by natural selection. *Adv Exp Med Biol,* 697**,** 77-86.

Mukhtar, T. A., K., K. P., Hughes, D. W. & Wright, G. D. 2001. Vgb from Staphylococcus aureus Inactivates Streptogramin B Antibiotics by an Elimination Mechanism Not Hydrolysis. *Biochemistry J* 40**,** 40, 8877-8886.

Murphy, E., Huwyler, L. & Bastos, M. d. C. d. F. 1985. Transposon Tn554: complete nucleotide sequence and isolation of transposition-defective and antibiotic-sensitive mutants. *EMBO journal,* vol.4 3357-3365,.

Murphy, K. C., Campellone, K. G. & Poteete, A. R. 2000. PCR-mediated gene replacement in Escherichia coli. *Gene,* 246**,** 321–330.

Naas, T., Coignard, B., Carbonne, A., Blanckaet, K., Bajolet, O., Bernet, C., Verdeil, X., Astagneau, P., J.C., D. & Nordmann, P. 2006. VEB-1 Extended Spectrum B-lactamase-producing *Emerging Infectious diseases,* 12**,** 1214-1222.

Nakama, T., Nureki, O. & Yokoyama, S. 2001. Structural basis for the recognition of isoleucyl-adenylate and an antibiotic, mupirocin, by isoleucyl-tRNA synthetase. *J Biol Chem,* 276**,** 47387-93.

Nash, H. A. 1996. Site-specific recombination: integration, excision, resolution, and inversion of defined DNA segments. *Escherichia coli and Salmonella: cellular and molecular biology,* 2**,** 2363-2376.

Needham, C. & Noble, W. C. a. D., K. G. 1995. The staphylococcal insertion sequence IS257 is active. *Plasmid,* 34**,** 198-205.

Nelson, K. E., Clayton, R. A., Gill, S. R., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Nelson, W. C., Ketchum, K. A., McDonald, L., Utterback, T. R., Malek, J. A., Linher, K. D., Garrett, M. M., Stewart, A. M., Cotton, M. D., Matthew S. Pratt, Phillips, C. A., Richardson, D., Heidelberg, J., Sutton, G. G., Fleischmann, R. D., Eisen, J. A., White, O., Salzberg, S. L., Smith, H. O., Venter, J. C. & Fraser, C. M. 1999. Evidence for lateral gene transfer between Archaea and Bacteria from genome sequence of Thermotoga maritima. *Nat Rev Microbiol,* 399**,** 323-329.

Nemec, A., Dolzani, L., Brisse, S., van den Broek, P. & Dijkshoorn, L. 2004. Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among strains of pan-European Acinetobacter baumannii clones. *J Med Microbiol,* 53**,** 1233-40.

Nemec, A., Musilek, M., Sedo, O., De Baere, T., Maixnerova, M., van der Reijden, T. J., Zdrahal, Z., Vaneechoutte, M. & Dijkshoorn, L. 2010. Acinetobacter bereziniae sp. nov. and Acinetobacter guillouiae sp. nov., to accommodate Acinetobacter genomic species 10 and 11, respectively. *Int J Syst Evol Microbiol,* 60**,** 896-903.

Nemergut, D. R., Martin, A. P. & Schmidt, S. K. 2004. Integron Diversity in Heavy-Metal-Contaminated Mine Tailings and Inferences about Integron Evolution. *Applied and Environmental Microbiology,* 70**,** 1160-1168.
Nemergut, D. R., Robeson, M. S., Kysela, R. F., Martin, A. P., Schmidt, S. K. & Knight, R. 2008. Insights and inferences about integron evolution from genomic data. *BMC Genomics,* 9**,** 261.
Nies, D. H. 1995. The Cobalt, Zinc, and Cadmium Efflux System CzcABC from Alcaligenes eutrophus Functions as a Cation-Proton Antiporter in Escherichia coli. *J Bacteriol,* 177**,** 2707–2712.
Nies, D. H. 1999. Microbial heavy-metal resistance. *Appl Microbiol Biotechnol,* 51**,** 730-50.
Nies, D. H. 2003. Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiology Reviews,* 27**,** 313-339.
Nies, D. H. & Silver, S. 1989. Metal ion uptake by a plasmid-free metal-sensitive Alcaligenes eutrophus strain. *J Bacteriol,* 171**,** 4073-5.
Nishimura, Y., Ino, T. & Iizuka, H. 1988. Acinetobacter radioresistens sp. nov. Isolated from Cotton and Soil. *International J Ssystematic Bacteriology,* 38**, 209-211**.
Novick, R. P., Christie, G. E. & Penades, J. R. 2010. The phage-related chromosomal islands of Gram-positive bacteria. *Nat Rev Microbiol,* 8**,** 541-51.
Nuccio, S. P. & Baumler, A. J. 2007. Evolution of the chaperone/usher assembly pathway: fimbrial classification goes Greek. *Microbiol Mol Biol Rev,* 71**,** 551-75.
Nudleman, E., Wall, D. & Kaiser, D. 2005. Cell-to-Cell Transfer of Bacterial Outer Membrane Lipoproteins. *Science,* 309 125-127.
Nurjadi, D., Heeg, K., Weber, A. N. R. & Zanger, P. 2018. Toll-like receptor (TLR)-9 promoter polymorphisms and gene expression are associated with persistent Staphylococcus aureus nasal carriage. *Clin Microbiol Infect,* 2.
Nurk, S., Bankevich, A., Antipov, D., Gurevich, A. A., Korobeynikov, A., Lapidus, A., Prjibelski, A. D., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., Clingenpeel, S. R., Woyke, T., McLean, J. S., Lasken, R., Tesler, G., Alekseyev, M. A. & Pevzner, P. A. 2013. Assembling Single-Cell Genomes and Mini-Metagenomes From Chimeric MDA Products. *Journal of Computational Biology,* 20**,** 714-737.
Nwugo, C. C., Gaddy, J. A., Zimbler, D. L. & Actis, L. A. 2011. Deciphering the iron response in Acinetobacter baumannii: A proteomics approach. *J Proteomics,* 74**,** 44-58.
Nyman, K., Nakamura, K., Ohtsubo, H. & Ohtsubo, E. 1981. Distribution of the insertion sequence IS1 in Gram-negative bacteria. *nature,* 289**,** 609-612.
O'Gorman, S., Fox, D. T. & Wahl, G. M. 1991. Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science,* 251**,** 1351-1355.
Obara, M. & Nakae, T. 1991. Mechanisms of resistance to fMactam antibiotics in Acinetobacter calcoaceticus. *J Antimicrob Chemother,* 28**,** 791-800.
Ochman, H., Elwyn, S. & Moran, N. A. 1999. Calibrating bacterial evolution. *PNAS,* 96**,** 12638–12643
Ochman, H., Lawrence, J. G. & Groisman, E. A. 2000. Lateral gene transfer and the nature of bacterial innovation
. *Nat Rev Microbiol,* 405**,** 299-304.
Oggioni, M. R., Coelho, J. R., Furi, L., Knight, D. R., Viti, C., Orefici, G., Martinez, J.-L., Freitas, A. T., Coque, T. M., Morrissey, I. & consortium, o. b. o. t. B. 2015. Significant Differences Characterise the Correlation Coefficients between Biocide and Antibiotic Susceptibility Profiles in Staphylococcus aureus. *Current Pharm Des,* 21**,** 2054-2057.
Oggioni, M. R., Meacci, F., Carattoli, A., Ciervo, A., Orru, G., Cassone, A. & Pozzi, G. 2002. Protocol for Real-Time PCR Identification of Anthrax Spores from Nasal Swabs after Broth Enrichment. *Journal of Clinical Microbiology,* 40**,** 3956-3963.
Ogier, e.-C., Calteau, A., Forst, S., Goodrich-Blair, H., Roche, D., Rouy, Z., Suen, G., Zumbihl, R., Givaudan, A., Tailliez, P., Médigue, C. & Gaudriault, S. 2010. Units of plasticity in bacterial genomes: new insight from the comparative genomics of two bacteria interacting with invertebrates, Photorhabdus and Xenorhabdus. *BMC Genomics,* 11**,** 568.

Ogston, A. 1880. Ueber abscesse. *Arch. Klin. Chir. ,* 25**,** 588.
Ogston, A. 1882. Micrococcus Poisoning. *J anatomy physiology,* 17**,** 24-28.
Ohtsubo, E., Nyman, K., Doroszkiewicz, W. & Ohtsubo, H. 1981. Multiple copies of iso-insertion sequences of IS1 in Shigella dysenteriae chromosome. *nature,* 292**,** 640-643.
Okuma, K., Iwakawa, K., Turnidge, J. D., Grubb, W. B., Bell, J. M., O'Brien, F. G., Coombs, G. W., Pearman, J. W., Tenover, F. C., Kapi, M., Tiensasitorn, C., Ito, T. & Hiramatsu, K. 2002. Dissemination of New Methicillin-Resistant Staphylococcus aureus Clones in the Community. *Journal of Clinical Microbiology,* 40**,** 4289-4294.
Oliveira, D. C. & Lencastre, H. d. 2002. Multiplex PCR Strategy for Rapid Identification of Structural Types and Variants of the mec Element in Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy,* 46**,** 2155-2161.
Olsen, J. E., Christensen, H. & Aarestrup, F. M. 2006. Diversity and evolution of blaZ from Staphylococcus aureus and coagulase-negative staphylococci. *J Antimicrob Chemother,* 57**,** 450-60.
Oncul, O., Keskin, O., Acar, H. V., Kucukardali, Y., Evrenkaya, R., Atasoyy, E. M., Top, C., Nablant, S., Ozkani, S., Emekdas, G., Cavuslu, S., Us, M. H. & Gokben, M. 2002. Hospital-acquired infections following the 1999 Marmara earthquake. *J Hospital Infection,* 51**,** 47-51.
Ong, C.-l. Y., Walker, M. J. & McEwan, A. G. 2015. Zinc disrupts central carbon metabolism and capsule biosynthesis in Streptococcus pyogenes. *Sci Rep,* 5**,** 10799.
Otsuka, T., Zaraket, H., Takano, T., Saito, K., Dohmae, S., Higuchi, W. & Yamamoto, T. 2007. Macrolide–lincosamide–streptogramin B resistance phenotypes and genotypes among Staphylococcus aureus clinical isolates in Japan. *European Society of Clinical Microbiology and Infectious Diseases,* 13**,** 325–327.
Ou, H. Y., Kuang, S. N., He, X., Molgora, B. M., Ewing, P. J., Deng, Z., Osby, M., Chen, W. & Xu, H. H. 2015. Complete genome sequence of hypervirulent and outbreak-associated Acinetobacter baumannii strain LAC-4: epidemiology, resistance genetic determinants and potential virulence factors. *Sci Rep,* 5**,** 8643.
Palmieri, C., Mingoia, M. & Varaldo, P. E. 2013. Unconventional Circularizable Bacterial Genetic Structures Carrying Antibiotic Resistance Determinants. *Antimicrob Agents Chemother,* 57**,** 2440–2441.
Pande, S., Shitut, S., Freund, L., Westermann, M., Bertels, F., Colesie, C., Bischofs, l. B. & Kost, C. 2015. Metabolic cross-feeding via intercellular nanotubes among bacteria. *Nature Communications***,** 10.1038.
Park, C. & Zhang, J. 2012. High expression hampers horizontal gene transfer. *Genome Biol Evol,* 4**,** 523-32.
Patel, J. B., Gorwitz, R. J. & Jernigan, J. A. 2009. Mupirocin resistance. *Clin Infect Dis,* 49**,** 935-41.
Paulsen, I. T., Banerjei, L., Myers, G. S. A., Nelson, K. E., Seshadri, R., Read, T. D., Fouts, D. E., Eisen, J. A., Gill, S. R., Heidelberg, J. F., Tettelin, H., Dodson, R. J., Umayam, L., Beanan, B., L. , Beanan, M., Daugherty, S., DeBoy, R. T., Durkin, S., Kolonay, J., Madupu, R., Nelson, W., Vamathevan, J., Tran, B., Upton, J., Hansen, T., Shetty, J., Khouri, H., Utterback, T., Radune, D., Ketchum, K. A., Dougherty, B. A. & Fraser, C. M. 2003. Role of Mobile DNA in the Evolution of Vancomycin-Resistant Enterococcus faecalis. *Science,* 299 2071-2074.
Paulsen, I. T., Press, C. M., Ravel, J., Kobayashi, D. Y., Myers, G. S. A., Mavrodi, D. V., DeBoy, R. T., Seshadri, R., Ren, Q., Madupu, R., Dodson, R. J., Durkin, A. S., Brinkac, L. M., Daugherty, S. C., Sullivan, S. A., Rosovitz, M. J., Gwinn, M. L., Zhou, L., Schneider, D. J., Cartinhour, S. W., Nelson, W. C., Weidman, J., Watkins, K., Tran, K., Khouri, H., Pierson, E. A., Pierson, L. S., Thomashow, L. S. & Loper, J. E. 2005. Complete genome sequence of the plant commensal Pseudomonas fluorescens Pf-5. *Nature Biotechnology,* 23**,** 873-878.

Payne, D. J., Warren, P. V., Holmes, D. J., Ji, Y. & Lonsdale, J. T. 2001. Bacterial fatty-acid biosynthesis: a genomics-driven target for antibacterial drug discovery. *theraputic focus reviews,* 6**,** 537-544.

Peacock, S. J. 2010. *Staphyloccocus*, Topley and Wilson's, Microbiology and Microbial Infections.

Peleg, A. Y., de Breij, A., Adams, M. D., Cerqueira, G. M., Mocali, S., Galardini, M., Nibbering, P. H., Earl, A. M., Ward, D. V., Paterson, D. L., Seifert, H. & Dijkshoorn, L. 2012. The success of acinetobacter species; genetic, metabolic and virulence attributes. *PLoS One,* 7**,** e46984.

Peleg, A. Y., Seifert, H. & Paterson, D. L. 2008. Acinetobacter baumannii: emergence of a successful pathogen. *Clin Microbiol Rev,* 21**,** 538-82.

Penwell, W. F., Arivett, B. A. & Actis, L. A. 2012. The Acinetobacter baumannii entA gene located outside the acinetobactin cluster is critical for siderophore production, iron acquisition and virulence. *PLoS One,* 7**,** e36493.

Perez-Roth, E., Armas-Gonzalez, E., Alcoba-Florez, J. & Mendez-Alvarez, S. 2011. PCR-based amplification of heterogeneous IS257-ileS2 junctions for molecular monitoring of high-level mupirocin resistance in staphylococci. *J Antimicrob Chemother,* 66**,** 471-5.

Peter J. G. Rauch, Ronald Palmen, Burds, A. A., Leslie A. Gregg-Jolly, Zee, J. R. v. d. & Hellingwerf, K. J. 1996. The expression of the Acinetobacter calcoaceticus recA gene increases in response to DNA damage independently of RecA and of development of competence for natural transformation. *microb,* 142**,** 1025-1032.

Peters, G., Locci, R. & Pulverer, G. 1981. Microbial colonization of prosthetic devices. II. Scanning electron microscopy of naturally infected intravenous
catheters. . *Zentralbl. Bakteriol. Mikrobiol. Hyg. B,* 173**,** 293–299.

Peterson, J. & Phillips, G. J. 2008. New pSC101-derivative cloning vectors with elevated copy numbers. *Plasmid,* 59**,** 193-201.

Philippe, N., Alcaraz, J. P., Coursange, E., Geiselmann, J. & Schneider, D. 2004. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. *Plasmid,* 51**,** 246-55.

Philips, S. & Novick, P. R. 1979. Tn554-a site represser-controlled transposon in Staphylococcus aureus. *Nature,* 278**,** 476-478.

Phillips, I., Culebras, E., Moreno, F. & Baqueor, F. 1987. Induction of the SOS response by the new 4-quinolones. *Antimicrob Agents Chemother,* 20**,** 631-638.

Piddock, L. J. 2006. Multidrug-resistance efflux pumps - not just for resistance. *Nat Rev Microbiol,* 4**,** 629-36.

Pinho, M. G., Filipe, S. R., de Lencastre, H. & Tomasz, A. 2001. Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in Staphylococcus aureus. *J Bacteriol,* 183**,** 6525-31.

Plasterk, R. H. A. 1996. The Tc1/mariner transposon family. *In:* SAEDLER, H. & GIERL, A. (eds.) *Current Topics in Microbiology and Immunology.* Berlin, Heidelberg: Springer.

Poirel, L. & Nordmann, P. 2006. Carbapenem resistance in Acinetobacter baumannii: mechanisms and epidemiology. *European Society of Clinical Microbiology and Infectious Diseases*.

Post, V. & Hall, R. M. 2009. AbaR5, a large multiple-antibiotic resistance region found in Acinetobacter baumannii. *Antimicrob Agents Chemother,* 53**,** 2667-71.

Post, V., White, P. A. & Hall, R. M. 2010. Evolution of AbaR-type genomic resistance islands in multiply antibiotic-resistant Acinetobacter baumannii. *J Antimicrob Chemother,* 65**,** 1162-70.

Poteete, A. R. & Fenton, A. C. 2000. Genetic requirements of phage λ Red-mediated gene replacement in Escherichia coli K-12. *Journal of bacteriology,* 182**,** 2336-2340.

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M. & Peters, G. 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.,* 4**,** 295–305.

Projan, S. J., Moghazeh, S. & Novick, R. P. 1988. Nucleotide sequence of pS194, a streptomycin-resistance plasmid from Staphylococcus aureus. *Nucleic Acids Res,* 16 2179-2187.
Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B. & Claverys, J. P. 2006. Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae. *Science,* 313**,** 89-92.
Pujol, M., Peña, C., Pallares, R., Ariza, J., Ayats, J., Dominguez, M. A. & Gudiol, F. 1996. Nosocomial Staphylococcus aureus bacteremia among nasal carriers of methicillin-resistant and methicillin-susceptible strains. *The American Journal of Medicine,* 100**,** 509-516.
Queck, S. Y., Khan, B. A., Wang, R., Bach, T.-H. L., Kretschmer, D., Chen, L., Kreiswirth, B. N., Peschel, A., DeLeo, F. R. & Otto, M. 2009. Mobile Genetic Element-Encoded Cytolysin Connects Virulence to Methicillin Resistance in MRSA. *PLoS Pathog,* 5**,** e1000533.
Quinones, M., Kimsey, H. H. & Waldor, M. K. 2005. LexA cleavage is required for CTX prophage induction. *Mol Cell,* 17**,** 291-300.
Rajamohan, G., Srinivasan, V. B. & Gebreyes, W. A. 2009. Biocide-tolerant multidrug-resistant Acinetobacter baumannii clinical strains are associated with higher biofilm formation. *J Hospital Infection,* 73**,** 287-289.
Rameckers, J., Hummel, S. & Herrmann, B. 1997. How Many Cycles Does a PCR need? Determinations of Cycle Numbers Depending on the Number of Targets and the Reaction Efficiency Factor. *Naturwissenschaften,* 84**,** 259–262.
Ramirez, M. S., Adams, M. D., Bonomo, R. A., Centron, D. & Tolmasky, M. E. 2011. Genomic analysis of Acinetobacter baumannii A118 by comparison of optical maps: identification of structures related to its susceptibility phenotype. *Antimicrob Agents Chemother,* 55**,** 1520-6.
Ramsay, J. P., Sullivan, J. T., Stuart, G. S., Lamont, I. L. & Ronson, C. W. 2006. Excision and transfer of the Mesorhizobium loti R7A symbiosis island requires an integrase IntS, a novel recombination directionality factor RdfS, and a putative relaxase RlxS. *Mol Microbiol,* 62**,** 723-34.
Ramsey, M. A., Bradley, S. F., Kauffman, C. A. & Morton, T. M. 1996. Identification of chromosomal location of mupA gene, encoding low-level mupirocin resistance in staphylococcal isolates. *Antimicrob Agents Chemother,* 40**,** 2820-3.
Rao, B. J. & Radding, C. M. 1995. RecA protein mediates homologous recognition via non-Watson—Crick bonds in base triplets. *Royal Society Publishing,* 347**,** 5-12.
Recchia, G. D. & Hall, R. M. 1997. Origins of mobile gene cassettes found in integrons. *TRENDS IN MICROBIOLOGY* 5**,** 389-394.
Redfield, R. J. 2001a. Do bacteria have sex? *Nat Rev Microbiol,* 2**,** 634-639.
Redfield, R. J. 2001b. Do bacteria have sex? *Nat Rev Microbiol,* 2.
Reif, H. J. & Saedler, H. 1975. IS1 is Involved in Deletion Formation in the gal Region of E. coli K12. *molecular genetics and genomics,* 137**,** 17--28.
Reyrat, J.-M., Pelicic, V., Gicquel, B. & Rappuoli, R. 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. *Infection and immunity,* 66**,** 4011-4017.
Riccio, M. L., Franceschini, N., Boschi, L., Caravelli, B., Cornaglia, G., Fontana, R., Amicosante, G. & Rossolini, G. M. 2000. Characterization of the Metallo- -Lactamase Determinant of Acinetobacter baumannii AC-54/97 Reveals the Existence of blaIMP Allelic Variants Carried by Gene Cassettes of Different Phylogeny. *Antimicrob Agents Chemother,* 44**,** 1229–1235.
Rice, L. 1998. Tn916 Family Conjugative Transposons and Dissemination of Antimicrobial Resistance Determinants. *Antimicrob Agents Chemother,* 42**,** 1871–1877.
Rice, Louis B. 2008. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. *The Journal of Infectious Diseases,* 197**,** 1079-1081.

Richards, R. L., Haigh, R. D., Pascoe, B., Sheppard, S. K., Price, F., Jenkins, D., Rajakumar, K. & Morrissey, J. A. 2015. Persistent Staphylococcus aureus Isolates from Two Independent Cases of Bacteremia Display Increased Bacterial Fitness and Novel Immune Evasion Phenotypes. *Infect Immun,* 83**,** 3311–3324.

Rieck, B., Tourigny, D. S., Crosatti, M., Schmid, R., Kochar, M., Harrison, E. M., Ou, H. Y., Turton, J. F. & Rajakumar, K. 2012. Acinetobacter insertion sequence ISAba11 belongs to a novel family that encodes transposases with a signature HHEK motif. *Appl Environ Microbiol,* 78**,** 471-80.

Riley, R. L. & Edward, A. N. 1989. Cleaning the air: The theory and the application of Ultraviolet air disinfection. *ATS Journals,* 139.

Roach, D. J., Burton, J. N., Lee, C., Stackhouse, B., Butler-Wu, S. M., Cookson, B. T., Shendure, J. & Salipante, S. J. 2015. A Year of Infection in the Intensive Care Unit: Prospective Whole Genome Sequencing of Bacterial Clinical Isolates Reveals Cryptic Transmissions and Novel Microbiota. *PLoS Genet,* 11**,** e1005413.

Roberts, M. C., Sutcliffe, J., Courvalin, P., Jensen, L. B., Rood, J. & Seppala, H. 1999. Nomenclature for Macrolide and Macrolide-Lincosamide- Streptogramin B Resistance Determinants. *Antimicrob Agents Chemother,* 43**,** 2823–2830.

Rocco, F., De Gregorio, E., Colonna, B. & Di Nocera, P. P. 2009. Stenotrophomonas maltophilia genomes: a start-up comparison. *Int J Med Microbiol,* 299**,** 535-46.

Rose, A. 2010. TnAbaR1: a novel Tn7-related transposon in Acinetobacter baumannii that contributes to the accumulation and dissemination of large repertoires of resistance genes. *Bioscience Horizons,* 3**,** 40-48.

Rosen, D. A., Pinkner, J. S., Jones, J. M., Walker, J. N., Clegg, S. & Hultgren, S. J. 2008. Utilization of an intracellular bacterial community pathway in Klebsiella pneumoniae urinary tract infection and the effects of FimK on type 1 pilus expression. *Infect Immun,* 76**,** 3337-45.

Rouch, D. A., Byrne, M. E., Kongt, Y. C. & Skurray, R. A. 1987. The aacA-aphD Gentamicin and Kanamycin Resistance Determinant of Tn4001 from Staphylococcus aureus: Expression and Nucleotide Sequence Analysis. *J General Microbiology,* 133**,** 3039-3052.

Rouch, D. A. & Skurray, R. A. 1989. IS257 from Staphylococcus aureus: member of an insertion sequence superfamily prevalent among Gram-positive and Gram-negative bacteria. *Gene,* 76**,** 195-205.

Rousseau, P., ardin, C., Tolou, N., Salomé, L. & Chandler, M. 2010. A model for the molecular organisation of the IS911 transpososome. *Mobile DNA,* 1**,** 1-8.

Rowe-Magnus, D. A. & Mazel, D. 2002. The role of integrons in antibiotic resistance gene capture. *Int J Med Microbiol,* 292**,** 115-25.

Rowland, S. J. & Dyke, K. G. H. 1990. Tn552, a novel transposable element from Staphylococcus aureus *Mol Microbiol,* 4**,** 961-975.

Roy, S., Viwanathan, R., Singh, A. & Basu, S. 2010. Gut colonization by multidrug-resistant and carbapenem resistant Acinetobacter baumannii in neonates. *Eur. J. Clin. Microbiol and Infect Dis.,* 29**,** 1495-1500.

Saedler, H. & Heiss, B. 1973. Multiple Copies of the Insertion-DNA Sequences IS1 and IS2 in the Chromosome or E. coli K-12. *Molecular Genetics and Genomics,* 122**,** 267--277.

Saedler, H. & Reif, H. J. 1974. S2, A Genetic Element for Turn-off and Turn-on of Gene Activity in E. coli. *molecular genetics and genomics,* 132**,** 265--289.

Saha, B., Singh, A. K., Ghosh, A. & Bal, M. 2008. Identification and characterization of a vancomycin-resistant Staphylococcus aureus isolated from Kolkata (South Asia). *Journal of Medical Microbiology,* 57**,** 72-79.

Saitou, N. & Nie, M. 1987. The neighbor-joining method: A new method for reconstructing Phylogenetic trees. *mol biol evol.,* 4**,** 406-427.

Salyers, A. A. & Amabile-cuevas, C. F. 1997. Why Are Antibiotic Resistance Genes So Resistant to Elimination? *Antimicrob Agents Chemother,* 41**,** 2321–2325.

Samanovic, M. I., Ding, C., Thiele, D. J. & Darwin, K. H. 2012. Copper in microbial pathogenesis: meddling with the metal. *Cell Host Microbe,* 11**,** 106-15.
Sauer, B. & Henderson, N. 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proceedings of the National Academy of Sciences,* 85**,** 5166-5170.
Sauer, B. & Henderson, N. 1990. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *The New Biologist,* 2**,** 441-449.
Saylers, A. A., Shoemaker, N. B., Stevens, A. M. & LI, L. 1995. Conjugative Transposons: an Unusual and Diverse Set of Integrated Gene Transfer Elements. *microb reviews,* 59**,** 579–590.
Schaberg, D. R. & Zervos, M. J. 1986. Intergeneric and Interspecies Gene Exchange in Gram-Positive Cocci. *Antimicrob Agents Chemother,* 30**,** 817-822.
Schaefer, M. R. & Kahn, K. 1998 Cyanobacterial Transposons Tn5469 and Tn5541 Represent a Novel Noncomposite Transposon Family. *J Bacteriol,* 180**,** 6059–6063.
Schara, K., JANŠA, V., ŠUŠTAR, V., DOLINAR, D., PAVLIČ, J. I., LOKAR, M., KRALJ-IGLIČ, V., VERANIČ, P. & IGLIČ, A. 2008. MECHANISMS FOR THE FORMATION OF MEMBRANOUS NANOSTRUCTURES IN CELL-TO-CELL COMMUNICATION. *CELLULAR & MOLECULAR BIOLOGY LETTERS,* 14**,** 636-656.
Schijffelen, M. J., Boel, C. E., van Strijp, J. A. G. & Fluit, A. C. 2010. Whole genome analysis of a livestock-associated methicillin-resistant Staphylococcus aureus ST398 isolate from a case of human endocarditis. *BMC Genomics,* 11**,** 376.
Schirmer, T. 1998. General and Specific Porins from Bacterial Outer Membranes. *J Structural Biology,* 121**,** 101–109
Schleifer, K. H. & Kloos, W. E. 1975. Isolation and Characterization of Staphylococci from Human Skin. I. Amended Descriptions of Staphylococcus epidermidis and Staphylococcus saprophyticus and Descriptions of Three New Species: Staphylococcus cohnii, Staphylococcus haemolyticus, and Staphylococcus xylosus. *International J Systematic Bacteriology,* 25**,** 50-61.
Schmidt, H. & Hensel, M. 2004. Pathogenicity Islands in Bacterial Pathogenesis. *Clinical Microbiology Reviews,* 17**,** 14-56.
Schoenfelder, S. M., Lange, C., Eckart, M., Hennig, S., Kozytska, S. & Ziebuhr, W. 2010. Success through diversity - how Staphylococcus epidermidis establishes as a nosocomial pathogen. *Int J Med Microbiol,* 300**,** 380-6.
Schwarz, S., Fessler, A. T., Hauschild, T., Kehrenberg, C. & Kadlec, K. 2011. Plasmid-mediated resistance to protein biosynthesis inhibitors in staphylococci. *Ann N Y Acad Sci,* 1241**,** 82-103.
Schwarz, S., Kehrenberg, C., Doublet, B. & Cloeckaert, A. 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol Rev,* 28**,** 519-42.
Schwarz, S. & Noble, W. C. 1994. Tetracycline resistance genes in staphylococci from the skin of pigs. *J applied microbiology,* 76**,** 320-326.
Schwarz, S. & Wang, Z. 1993. Tetracycline resistance in Staphylococcus intermedius. *Lett Appl Microbiol,* 17**,** 88-91.
Scott, P., Deye, G., Sirinivasan, A., Murray, C., Moran, K., Hulten, E., Fishbain, J. & Craft, D. 2007. an Outbrek of Multi-Drug Resistant acinetobacter baumannii-calcoaceticus Complex Infection in the US Military Health Care System Associated with Military Operations in Iraq. *Clinical infectious diseases,* 44**,** 1577-1584.
Seah, C., Alexander, D. C., Louie, L., Simor, A., Low, D. E., Longtin, J. & Melano, R. G. 2012. MupB, a new high-level mupirocin resistance mechanism in Staphylococcus aureus. *Antimicrob Agents Chemother,* 56**,** 1916-20.
Seggewiss, J., Becker, K., Kotte, O., Eisenacher, M., Yazdi, M. R. K., Fischer, A., McNamara, P., Al Laham, N., Proctor, R., Peters, G., Heinemann, M. & von Eiff, C. 2006. Reporter Metabolite Analysis of Transcriptional Profiles of a Staphylococcus aureus Strain with Normal Phenotype and Its Isogenic hemB Mutant Displaying the Small-Colony-Variant Phenotype. *Journal of Bacteriology,* 188**,** 7765-7777.

Seifert, H., Dijkshoorn, L., Gerner-Smidt, P., Pelzer, N., Jernberg, I. T. & Vaneechoutte, M. 1997. Distribution of Acinetobacter Species on Human Skin: Comparison of Phenotypic and Genotypic Identification Methods. *j clinical micro,* 35**,** 2819–2825.

Sentchilo, V., Ravatn, R., Werlen, C., Zehnder, A. J. B. & van der Meer, J. R. 2003. Unusual Integrase Gene Expression on the clc Genomic Island in Pseudomonas sp. Strain B13. *Journal of Bacteriology,* 185**,** 4530-4538.

Seputiene, V., Povilonis, J. & Suziedeliene, E. 2012. Novel variants of AbaR resistance islands with a common backbone in Acinetobacter baumannii isolates of European clone II. *Antimicrob Agents Chemother,* 56**,** 1969-73.

Severin, A., Tabei, K., Tenover, F., Chung, M., Clarke, N. & Tomasz, A. 2004. High level oxacillin and vancomycin resistance and altered cell wall composition in Staphylococcus aureus carrying the staphylococcal mecA and the enterococcal vanA gene complex. *J Biol Chem,* 279**,** 3398-407.

Shaikh, F., Spence, R. P., Levi, K., Ou, H. Y., Deng, Z., Towner, K. J. & Rajakumar, K. 2009. ATPase genes of diverse multidrug-resistant Acinetobacter baumannii isolates frequently harbour integrated DNA. *J Antimicrob Chemother,* 63**,** 260-4.

Shaw, K. J., P.N., R., Hare, R. S. & Miller, G. H. 1993. Molecular Genetics of Aminoglycoside Resistance Genes and Familial Relationships of the Aminoglycoside-Modifying Enzymes. *microb reviews,* 57**,** 138-163.

Sherratt, D. J., Arciszewska, L. K., Blakely, G., Colloms, S., Grant, K., Leslie, N. & McCulloch, R. 1995. Site-specific recombination and circular chromosome segregation. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences,* 347**,** 37-42.

Shore, A., Rossney, A. S., Keane, C. T., Enright, M. C. & Coleman, D. C. 2005. Seven novel variants of the staphylococcal chromosomal cassette mec in methicillin-resistant Staphylococcus aureus isolates from Ireland. *Antimicrob Agents Chemother,* 49**,** 2070-83.

Shpanchenko, O. V., Zvereva, M. I., Ivanov, P. V., Bugaeva, E. Y., Rozov, A. S., Bogdanov, A. A., Kalkum, M., Isaksson, L. A., Nierhaus, K. H. & Dontsova, O. A. 2005. Stepping transfer messenger RNA through the ribosome. *J Biol Chem,* 280**,** 18368-74.

Sidhu, M. S., Heir, E., Leegaard, T., Wiger, K. & Holck, A. 2002. Frequency of Disinfectant Resistance Genes and Genetic Linkage with -Lactamase Transposon Tn552 among Clinical Staphylococci. *Antimicrobial Agents and Chemotherapy,* 46**,** 2797-2803.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J. D. & Higgins, D. G. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol,* 7**,** 539.

Siguier, P., Gourbeyre, E. & Chandler, M. 2014. Bacterial insertion sequences: their genomic impact and diversity. *FEMS Microbiol Rev,* 38**,** 865-91.

Siguier, P., Gourbeyre, E., Varani, A., Ton-Hoang, B. & Chandler, M. 2015. Everyman's Guide to Bacterial Insertion Sequences. *Microbiol Spectr,* 3**,** MDNA3-0030-2014.

Simon, R., Priefer, U. & Puhler, A. 1983. A broad host range mobilization system of In vivo genetic engineering: Transposon mutagenesis in Grma-negative bacteria. *Nature Biotechnology,* 1**,** 784-791.

Sjöström, J. E., Lofdhal, S. & Philipson, L. 1975. Transformation Reveals a Chromosomal Locus of the Gene(s) for Methicillin Resistance in Staphylococcus aureus. *J Bacteriol,* 123**,** 905-915.

Skulj, M., Okrslar, V., Jalen, S., Jevsevar, S., Slanc, P., Strukelj, B. & Menart, V. 2008. Improved determination of plasmid copy number using quantitative real-time PCR for monitoring fermentation processes. *Microb Cell Fact,* 7**,** 6.

Slater-Radosti, C., Van Allera, G., Greenwooda, R., Richard , N., Kellerb, P. M., DeWolf, W. E., Fana, F., Paynea, D. J. & Jaworski, D. D. 2001. Biochemical and genetic characterization of the action of triclosan on Staphylococcus aureus. *Antimicrob Agents Chemother,* 48**,** 1–6.

Smani, Y., McConnell, M. J. & Pacho´ n, J. n. 2012. Role of Fibronectin in the Adhesion of Acinetobacter baumannii to Host Cells. *PLoS One,* 7**,** e33073.
Smith, J. M., Dowson, C. G. & Spratt, B. G. 1991. Localized sex in bacteria. *Nature,* 349**,** 29-31.
Smith, M. C. & Thorpe, H. M. 2002. Diversity in the serine recombinases. *Mol Microbiol,* 44**,** 299-307.
Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M. & Snyder, M. 2007. New insights into Acinetobacter baumannii pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes Dev,* 21**,** 601-14.
Smoot, J. C., Barbian, K. D., Van Gompel, J. J., Smoot, L. M., Chaussee, M. S., Sylva, G. L., Sturdevant, D. E., Ricklefs, S. M., Porcella, S. F., Parkins, L. D., Beres, S. B., Campbell, D. S., Smith, T. M., Zhang, Q., Kapur, V., Daly, J. A., Veasy, L. G. & Musser, J. M. 2002. Genome sequence and comparative microarray analysis of serotype M18 group A Streptococcus strains associated with acute rheumatic fever outbreaks. *Proc Natl Acad Sci U S A,* 99**,** 4668-73.
Smyth, D. S., Wong, A. & Robinson, D. A. 2011. Cross-species spread of SCCmec IV subtypes in staphylococci. *Infection, Genetics and Evolution,* 11**,** 446-453.
So, M., Heffron, F. & McCarthy, B. J. 1979. The E. coli gene encoding heat stable toxin is a bacterial transposon flanked by inverted repeats of IS. *Nature,* 277**,** 453-456.
Soge, O. O., Beck, N. K., White, T. M., No, D. B. & Roberts, M. C. 2008. A novel transposon, Tn6009, composed of a Tn916 element linked with a Staphylococcus aureus mer operon. *J Antimicrob Chemother,* 62**,** 674-80.
Song, L., Pan, Y., Chen, S. & Zhang, X. 2012. Structural characteristics of genomic islands associated with GMP synthases as integration hotspot among sequenced microbial genomes. *Computational Biology and Chemistry,* 36**,** 62-70.
Sridhar, J., Sambaturu, N., Sabarinathan, R., Ou, H. Y., Deng, Z., Sekar, K., Rafi, Z. A. & Rajakumar, K. 2010. sRNAscanner: a computational tool for intergenic small RNA detection in bacterial genomes. *PLoS One,* 5**,** e11970.
Starikova, I., Harms, K., Haugen, P., Lunde, T. T., Primicerio, R., Samuelsen, O., Nielsen, K. M. & Johnsen, P. J. 2012. A trade-off between the fitness cost of functional integrases and long-term stability of integrons. *PLoS Pathog,* 8**,** e1003043.
Stark, M. W., Boocock, M. R. & Sherratt, D. J. 1992. Catalysis by site-specific recombinases. *Trends in Genetics,* 8**,** 432-439.
Stewart, P. R., Dubin, D. T., Ghikramane, S. C., Inglis, B., Mathews, P. R. & Poston, S. M. 1994. IS257 and small plasmid insertions in the mec region of the chromosome of staphylococcus aureus. *Plasmid,* 31**,** 12-20.
Stokes, H. W. & Hall, R. M. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol,* 3**,** 1669-1683.
Struve, C., Bojer, M. & Krogfelt, K. A. 2008. Characterization of Klebsiella pneumoniae type 1 fimbriae by detection of phase variation during colonization and infection and impact on virulence. *Infect Immun,* 76**,** 4055-65.
Sullivan, J. T., Trzebiatowski, J. R., Cruickshank, R. W., Gouzy, J., Brown, S. D., Elliot, R. M., Fleetwood, D. J., McCallum, N. G., Rossbach, U., Stuart, G. S., Weaver, J. E., Webby, R. J., de Bruijn, F. J. & Ronson, C. W. 2002. Comparative Sequence Analysis of the Symbiosis Island of Mesorhizobium loti Strain R7A. *Journal of Bacteriology,* 184**,** 3086-3095.
Sullivan, M. J., Petty, N. K. & Beatson, S. A. 2011. Easyfig: a genome comparison visualizer. *Bioinformatics,* 27**,** 1009-10.
Sullivan, M. J., Yasbin, R. & Young, F. 1984. New shuttle vectors for Bacillus subtilis and Escherichia coli which allow rapid detection of inserted fragments. *Gene,* 29**,** 21-26.
Sun, Y., Emolo, C., Holtfreter, S., Wiles, S., Kreiswirth, B. N., Missiakas, D. & Schneewind, O. 2018. Staphylococcal protein A contributes to persistent colonization of mice with Staphylococcus aureus. *J Bacteriol,* 200.

Sutherland, R., Boon, R. J., Griffin, K., Masters, P. J., Slocombe, B. & White, A. R. 1985. Antibacterial Activity of Mupirocin (PseudomonicAcid), a New Antibiotic for Topical Use. *Antimicrob Agents Chemother,* 27**,** 495-498.

Takeuchi, F., Watanabe, S., Baba, T., Yuzawa, H., Ito, T., Morimoto, Y., Kuroda, M., Cui, L., Takahashi, M., Ankai, A., Baba, S., Fukui, S., Lee, J. C. & Hiramatsu, K. 2005. Whole-genome sequencing of staphylococcus haemolyticus uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. *J Bacteriol,* 187**,** 7292-308.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol,* 30**,** 2725-9.

Tansirichaiya, S., Mullany, P. & Roberts, A. P. 2016. PCR-based detection of composite transposons and translocatable units from oral metagenomic DNA. *Fems Environ Microbiol,* 363**,** fnw195.

Tansirichaiya, S., Reynold, L., Cristarella, G., Wong, L., Rosendahl, K. & Roberts, A. P. 2017. Reduced susceptibility to antiseptics is conferred by heterologous housekeeping genes. *Microb Drug Resistance*.

Taylor, K. L. & Churchward, G. 1997. Specific DNA Cleavage Mediated by the Integrase of Conjugative Transposon Tn916. *J Bacteriol,* 174**,** 1117–1125.

Teitzel, G. M. & Parsek, M. R. 2003. Heavy Metal Resistance of Biofilm and Planktonic Pseudomonas aeruginosa. *Applied and Environmental Microbiology,* 69**,** 2313-2320.

Tennent, J. M., Young, H.-K., Lyon, B. R., Amye, S. G. B. & Skurray, R. A. 1988. Trimethoprim resistance determinants encoding a dihydrofolate reductase in clinical isolates of Staphy/ococcusaureus and coagulase-negative staphylococci. *J Med. Microbiol.,* 26 67-73.

Tenover, F. C., Weigel, L. M., Appelbaum, P. C., McDougal, L. K., Chaitram, J., McAllister, S., Clark, N., Killgore, G., O'Hara, C. M., Jevitt, L., Patel, J. B. & Bozdogan, B. 2003. Vancomycin-Resistant Staphylococcus aureus Isolate from a Patient in Pennsylvania. *Antimicrobial Agents and Chemotherapy,* 48**,** 275-280.

Thomas, C. M. & Nielsen, K. M. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol,* 3**,** 711-21.

Thomas, J., Sorourian, M., Ray, D., Baker, R. J. & Pritham, E. J. 2010. The limited distribution of Helitrons to vesper bats supports horizontal transfer. *Gene,* 474.

Thomson, J. M. & Bonomo, R. A. 2005. The threat of antibiotic resistance in Gram-negative pathogenic bacteria: β-lactams in peril! *Current Opinion in Microbiology,* 8**,** 518-524.

Thorpe, H. M. & Smith, M. C. 1998. In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. *Proc Natl Acad Sci U S A,* 95**,** 5505-10.

Tonouchi, N., Tsuchida, T., Yoshinaga, F., Horinouchi, S. & Beppu, T. 2014. A Host–Vector System for a Cellulose-ProducingAcetobacterStrain. *Bioscience, Biotechnology, and Biochemistry,* 58**,** 1899-1901.

Top, E. M. & Springael, D. 2003. The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. *Current Opinion in Biotechnology,* 14**,** 262-269.

Torres García, M., Tejedor Junco, M. T., González Martín, M. & González Lama, Z. 1996. Selection of subpopulations resistant to amikacin and netilmicin of gentamicin-resistant clinical strains of Staphylococcus aureus and Staphylococcus epidermidis. *Zentralblatt für Bakteriologie,* 284**,** 58-66.

Toussaint, A. & Merlin, C. 2002. Mobile elements as a combination of functional modules. *Plasmid,* 47**,** 26-35.

Toussaint, A., Merlin, C., Monchy, S., Benotmane, M. A., Leplae, R., Mergeay, M. & Springael, D. 2003. The Biphenyl- and 4-Chlorobiphenyl-Catabolic Transposon Tn4371, a Member of a New Family of Genomic Islands Related to IncP and Ti Plasmids. *Applied and Environmental Microbiology,* 69**,** 4837-4845.

Towner, K. J. 1997. Clinical importance and antibiotic resistance of Acinetobacter spp. *J Med. Microbiol.,* 46**,** 721-746.

Towner, K. J. 2009. Acinetobacter: an old friend, but a new enemy. *Journal of Hospital Infection,* 73**,** 355-363.
Trzcinskia, K., Coopera, B. S., Hryniewiczb, W. & Dowsona, C. G. 2000. Expression of resistance to tetracyclines in strains of methicillin-resistant Staphylococcus aureus. *Antimicrob Agents Chemother,* 45**,** 763–770.
Tuchscherr, L., Medina, E., Hussain, M., V¨olker, W., Heitmann, V., Niemann, S., Holzinger, D., Roth, J., Proctor, R. A., Becker, K., Peters, G. & L¨offler, B. 2011. Staphylococcus aureus phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. *EMBO journal,* 3**,** 129-141.
Turlan, C. & Chandler, M. 2000. Playing second fiddle: second-strand processing and liberation of transposable elements from donor DNA. *Trends Microbiol,* 8**,** 268-74.
Turner, A. G., Ong, C.-l. Y., Gillen, C. M., Davies, M. R., West, N. P., McEwan, A. G. & Walker, M. J. 2015. Manganese Homeostasis in Group A Streptococcus Is Critical for Resistance to Oxidative Stress and Virulence. *mBio,* 6**,** e00278-15.
Turton, J. F., Woodford, N., Glover, J., Yarde, S., Kaufmann, M. E. & Pitt, T. L. 2006. Identification of Acinetobacter baumannii by detection of the blaOXA-51-like carbapenemase gene intrinsic to this species. *J Clin Microbiol,* 44**,** 2974-6.
Tzouvelekis, L. S. & Bomono, R. A. 1999. SHV-type beta-lactamases. *Current Pharm Des,* 5**,** 847-864.
U´beda, C., Barry, P., Penade´s, J. & Novick, R. P. 2006. A pathogenicity island replicon in Staphylococcus aureus replicates as an unstable plasmid. *PNAS*.
Ubeda, C., Maiques, E., Knecht, E., Lasa, I., Novick, R. P. & Penades, J. R. 2005. Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Mol Microbiol,* 56**,** 836-44.
Ubeda, C., Olivarez, N. P., Barry, P., Wang, H., Kong, X., Matthews, A., Tallent, S. M., Christie, G. E. & Novick, R. P. 2009. Specificity of staphylococcal phage and SaPI DNA packaging as revealed by integrase and terminase mutations. *Molecular Microbiology,* 72**,** 98-108.
Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M. & Rozen, S. G. 2012. Primer3--new capabilities and interfaces. *Nucleic Acids Res,* 40**,** e115.
Valle, J., Vadillo, S., Piriz, S. & Gomez-Lucia, E. 1991. ToxicShockSyndromeToxin1 (TSST-1)Productionby Staphylococci Isolated from Goats and Presence of Specific Antibodies to TSST-1 in Serum and Milk. 57**,** 889-891.
Vallenet, D., Nordmann, P., Barbe, V., Poirel, L., Mangenot, S., Bataille, E., Dossat, C., Gas, S., Kreimeyer, A., Lenoble, P., Oztas, S., Poulain, J., Segurens, B., Robert, C., Abergel, C., Claverie, J. M., Raoult, D., Medigue, C., Weissenbach, J. & Cruveiller, S. 2008. Comparative analysis of Acinetobacters: three genomes for three lifestyles. *PLoS One,* 3**,** e1805.
van Aartsen, J. J. & Rajakumar, K. 2011. An optimized method for suicide vector-based allelic exchange in Klebsiella pneumoniae. *J Microbiol Methods,* 86**,** 313-9.
van der Meer, J. R. & Sentchilo, V. 2003. Genomic islands and the evolution of catabolic pathways in bacteria. *Current Opinion in Biotechnology,* 14**,** 248-254.
Van Dyne, G. D. 2002. A structural view of tyrosine recombinase site-specific recombination. *In:* CRAIG, N., CRAIGIE, R., GELLERT, M. & LAMBOWITZ, A. (eds.) *Mobile DNA II.* Washington, DC: ASM Press.
Vaneechoutte, M., Young, D. M., Ornston, L. N., De Baere, T., Nemec, A., Van Der Reijden, T., Carr, E., Tjernberg, I. & Dijkshoorn, L. 2006. Naturally transformable Acinetobacter sp. strain ADP1 belongs to the newly described species Acinetobacter baylyi. *Appl Environ Microbiol,* 72**,** 932-6.
Vaz-Moreira, I., Novo, A., Hantsis-Zacharov, E., Lopes, A. R., Gomila, M., Nunes, O. C., Manaia, C. & Halpern, M. 2011. Acinetobacter rudis sp. nov., isolated from raw milk and raw wastewater. *Int J systematic evolutionary microbiol,* 61**,** 2837-2843.
Vernikos, G. S. & Parkhill, J. 2008. Resolving the structural features of genomic islands: a machine learning approach. *Genome Res,* 18**,** 331-42.

Vig, K., Megharaj, M., Sethunathan, N. & Naidu, R. 2003. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. *Adv Environm Res,* 8**,** 121-135.
Vila, J., Marti, S. & Sanchez-Cespedes, J. 2007. Porins, efflux pumps and multidrug resistance in Acinetobacter baumannii. *J Antimicrob Chemother,* 59**,** 1210-5.
von Eiff, C., Becker, K. W., Metze, D., Lubritz, D., Hockmann, J., Schwarz, T. & Peters, J. 2001. intracellular Persistence of Staphylococcus aureus Small-Colony Variants within Keratinocytes: A Cause for Antibiotic Treatment Failure in a Patient with Darier's Disease. *J Clinical Infectious Diseases,* 32**,** 1643-1647.
von Eiff, C., Peters, G. & Becker, K. 2006. The small colony variant (SCV) concept -- the role of staphylococcal SCVs in persistent infections. *Injury,* 37 Suppl 2**,** S26-33.
Wan, T. W., Higuchi, W., Khokhlova, O. E., Hung, W. C., Iwao, Y., Wakayama, M., Inomata, N., Takano, T., Lin, Y. T., Peryanova, O. V., Kojima, K. K., Salmina, A. B., Teng, L. J. & Yamamoto, T. 2017. Genomic comparison between Staphylococcus aureus GN strains clinically isolated from a familial infection case: IS1272 transposition through a novel inverted repeat-replacing mechanism. *PLoS One,* 12**,** e0187288.
Wang, C., Li, M., Dong, D., Wang, J., Ren, J., Otto, M. & Gao, Q. 2007. Role of ClpP in biofilm formation and virulence of Staphylococcus epidermidis. *Microb Infect.,* 9**,** 1376–1383.
Wang, L. & Archer, G. L. 2010. Roles of CcrA and CcrB in excision and integration of staphylococcal cassette chromosome mec, a Staphylococcus aureus genomic island. *J Bacteriol,* 192**,** 3204-12.
Wang, L., Safo, M. & Archer, G. L. 2012. Characterization of DNA sequences required for the CcrAB-mediated integration of staphylococcal cassette chromosome mec, a Staphylococcus aureus genomic island. *J Bacteriol,* 194**,** 486-98.
Wang, R. F. & Kushner, S. R. 1991. Construction of versatile low-copy number vectors for cloning, sequencing and gene expression in Escherichia coli. *Gene,* 100**,** 195-199.
Wang, Y., Wu, C. M., Lu, L. M., Ren, G. W., Cao, X. Y. & Shen, J. Z. 2008. Macrolide-lincosamide-resistant phenotypes and genotypes of Staphylococcus aureus isolated from bovine clinical mastitis. *Vet Microbiol,* 130**,** 118-25.
Wang, Z. X., Jiang, C. P., Cao, Y. & Ding, Y. T. 2013. Systematic review and meta-analysis of triclosan-coated sutures for the prevention of surgical-site infection. *Br J Surg,* 100**,** 465-73.
Wanner, S., Schade, J., Keinhorster, D., Weller, N., George, S. E., Kull, L., Bauer, J., Grau, T., Winstel, V., Stoy, H., Kretschmer, D., Kolata, J., Wolz, C., Broker, B. M. & Weidenmaier, C. 2017. Wall teichoic acids mediate increased virulence in Staphylococcus aureus. *Nat Microbiol,* 2.
Watanabe, T. & Fukasawa, T. 1961. Episome-mediated transfer of drug resistance in Enterobacteriaceae *American Society for Microbiology J,* 81**,** 669-678.
Weigel, L. M., Clewell, D. B., Gill, S. R., Clark, N. C., McDougal, L. K., Flannagan, S. E., Kolonay, J. F., Shetty, J., Killgore, G. E. & Tenover, F. C. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of Staphylococcus aureus. *Science,* 302**,** 1569-71.
Weinert, T. A. & Hartwell, L. H. 1988. The RAD9 Gene Controls the Cell Cycle Response to DNA Damage in Saccharomyces Cerevisiae. *Science,* 241**,** 317-322.
Werckenthin, C., Schwards, S. & Roberts, M. R. 1996. Integration of pT181-Like Tetracycline Resistance Plasmids into Large Staphylococcal Plasmids Involves IS257. *Antimicrob Agents Chemother,* 40**,** 2542–2544.
Westh, H., Hougaard, D., Vuust, J. & Rosdahl, V. 1995. Prevalence of erm Gene Classes in Erythromycin-Resistant Staphylococcus aureus Strains Isolated between 1959 and 1988. *Antimicrob Agents Chemother,* 39**,** 369–373.
Whittle, G., Shoemaker, N. B. & Salyers, A. A. 2002. The role of Bacteroides conjugative transposons in the dissemination of antibiotic resistance genes. *Cell Mol Life Sci,* 59**,** 2044-54.

Widerstrom, M., McCullough, C. A., Coombs, G. W., Monsen, T. & Christiansen, K. J. 2012. A multidrug-resistant Staphylococcus epidermidis clone (ST2) is an ongoing cause of hospital-acquired infection in a Western Australian hospital. *J Clin Microbiol,* 50**,** 2147-51.

Williams, C. L., Neu, H. M., Gilbreath, J. J., Michel, S. L., Zurawski, D. V. & Merrell, D. S. 2016. Copper Resistance of the Emerging Pathogen Acinetobacter baumannii. *Appl Environ Microbiol,* 82**,** 6174-6188.

Williams, K. P. 2002. Integration sites for genetic elements in prokaryotic tRNA and tmRNA genes: sublocation preference of integrase subfamilies. *Nucleic Acids Res,* 30**,** 866-75.

Wisplinghoff, H., Rosato, A. E., Enright, M. C., Noto, M., Craig, W. & Archer, G. L. 2003. Related Clones Containing SCCmec Type IV Predominate among Clinically Significant Staphylococcus epidermidis Isolates. *Antimicrobial Agents and Chemotherapy,* 47**,** 3574-3579.

Wos-Oxley, M. L., Plumeier, I., von Eiff, C., Taudien, S., Platzer, M., Vilchez-Vargas, R., Becker, K. & Pieper, D. H. 2010. A poke into the diversity and associations within human anterior nare microbial communities. *ISME J,* 4**,** 839-51.

Wozniak, R. A. F. & Waldor, M. K. 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol,* 8**,** 552-563.

Wright, G. D. 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol,* 5**,** 175-86.

Wright, L. D. & Grossman, A. D. 2016. Autonomous Replication of the Conjugative Transposon Tn916. *J Bacteriol,* 198**,** 3355–3366.

Xu, F. F. & Imlay, J. A. 2012. Silver(I), mercury(II), cadmium(II), and zinc(II) target exposed enzymic iron-sulfur clusters when they toxify Escherichia coli. *Appl Environ Microbiol,* 78**,** 3614-21.

Yamaguchi, A., Nakashima, R. & Sakurai, K. 2015. Structural basis of RND-type multidrug exporters. *Front Microbiol,* 6**,** 327.

Yang, Z., Jiang, X., Wei, Q., Chen, N. & Lu, Y. 2009. A novel and rapid method for determining integration frequency catalyzed by integron integrase intI1. *J Microbiol Methods,* 76**,** 97-100.

Yanisch-Perron, C., Vierira, J. & Messing, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpI8 and pUC19 vectors. *Gene,* 33**,** 103-119.

Yaron, S., Kolling, G. L., Simon, L. & R., M. K. 2000. Vesicle-Mediated Transfer of Virulence Genes from Escherichia coli O157:H7 to Other Enteric Bacteria. *Applied and Environmental Microbiology,* 66**,** 4414–4420.

Yesilkaya, H., Meacci, F., Niemann, S., Hillemann, D., Rusch-Gerdes, S., Group, L. D. S., Barer, M. R., Andrew, P. W. & Oggioni, M. R. 2006. Evaluation of molecular-Beacon, TaqMan, and fluorescence resonance energy transfer probes for detection of antibiotic resistance-conferring single nucleotide polymorphisms in mixed Mycobacterium tuberculosis DNA extracts. *J Clin Microbiol,* 44**,** 3826-9.

Young, D. M., Parke, D. & Ornston, L. N. 2005. Opputunities for genetic investigation afforded by Acinetobacter baylyi, a nutrionally versatile bacterial species that is highly competent for natural transformation. *ann reviews,* 59**,** 519-551.

Yu, D., Ellis, H. M., Lee, E. C., Jenkins, N. A., Copeland, N. G. & Court, D. L. 2000. An efficient recombination system for chromosome engineering in Escherichia coli. *Proc Natl Acad Sci U S A,* 97**,** 5978-83.

Zaneveld, J. R., Nemergut, D. R. & Knight, R. 2008. Are all horizontal gene transfers created equal? Prospects for mechanism-based studies of HGT patterns. *Microbiology,* 154**,** 1-15.

Zarb, P., Coignard, B., Griskeviciene, J., Muller, A., Vankerckhoven, V., Weist, K., Goossens, M. M., Vaerenberg, S., Hopkins, S., Catry, B., Monnet, D. L., Goossens, H., Suetens, C., National Contact Points for the Ecd, C. & Hospital Contact Points for

the Ecd, C. 2012. The European Centre for Disease Prevention and Control (ECDC) pilot point prevalence survey of healthcare-associated infections and antimicrobial use. *Eurosurveillance,* 17.

Zhang, Y. M. & Rock, C. O. 2008. Membrane lipid homeostasis in bacteria. *Nat Rev Microbiol,* 6**,** 222-33.

Zhou, L., Mitra, R., Atkinsin, P. W., Hickman, A. B., Dyda, F. & Craig, N. 2004. Transposition of hAT element links transposable elements and V(D)J recombination. *nature,* 432**,** 995-1001.

Zhu, L., Yan, Z., Zhang, Z., Zhou, Q., Zhou, J., Wakeland, E. K., Fang, X., Xuan, Z., Shen, D. & Li, Q.-Z. 2013. Complete Genome Analysis of Three Acinetobacter baumannii Clinical Isolates in China for Insight into the Diversification of Drug Resistance Elements. *PLoS One,* 8**,** e66584.

Zhu, W., Clark, N. C., McDougal, L. K., Hageman, J., McDonald, L. C. & Patel, J. B. 2008. Vancomycin-resistant Staphylococcus aureus isolates associated with Inc18-like vanA plasmids in Michigan. *Antimicrob Agents Chemother,* 52**,** 452-7.

Zimbler, D. L., Penwell, W. F., Gaddy, J. A., Menke, S. M., Tomaras, A. P., Connerly, P. L. & Actis, L. A. 2009. Iron acquisition functions expressed by the human pathogen Acinetobacter baumannii. *Biometals,* 22**,** 23-32.

Zinder, N. D. & Lederberg, J. 1952. Genetic exchange in Salmonella. *J Bacteriol,* 64**,** 679-99.

Zuckerkandl, E. & Pauling, L. 1965. Evolutionary Divergence and Convergence, in Proteins. *J Theoretical Biology***,** 97-166.