Studies on a bacterial plasmid (pBURNS1) from a hospital Burns Unit conferring resistance to chlorite

By

Rabiat Olaronke Gambari

A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

School of Biosciences

College of Environmental and Life Sciences

University of Birmingham

AUGUST 2020

ABSTRACT

Pseudomonas aeruginosa is a widely distributed opportunistic pathogen. This research focuses on *P. aeruginosa* 943 isolated from water sampled from the Burns Unit of the Queen Elizabeth Hospital, Birmingham. The isolate has an IncP-1β plasmid which carries a *cdm* gene on a TniC-like class 1 integron. This work aims to determine the phenotype conferred by the plasmid and thus assess its significance in the hospital.

Comparison of pBURNS1 with other IncP-1β plasmids showed mostly conserved regions except for the accessory elements. Phylogenetic assessment of pBURNS1 showed that a set of ORFs identified in IncP-1β plasmids have sequence similarity to the *parABCDE* region of RK2 could act as a multimer resolution and addiction system in pBURNS1.

The phenotypes of both WT *P. aeruginosa* 943 and *E. coli* (+/- pBURNS1::Tn*1723*) was investigated. The plasmid did not promote biofilm formation in either species but confers chlorite resistance strongly in *E. coli* and only marginally in *P. aeruginosa*. Both species (+/- pBURNS1::Tn*1723*) biofilms persisted after treatment with 0.5 ppm chlorine dioxide for 3 hrs.

In conclusion, there is an increased risk of spread of antibiotic or biocide resistance when IncP-1 plasmids such as pBURNS1 are present within a biofilm.

This thesis is dedicated to my two wonderful children,

Ismail and Nabilah (Ummuhani).

ACKNOWLEDGEMENT

I would like to thank my supervisor Prof Chris Thomas for his tremendous academic support and availability throughout my studies. I am honoured to have had the opportunity to work with you sir. My co-supervisor, Dr Mark Webber, thanks for all your help and encouragement. Thank you to my sponsors, Tetfund and University of Ilorin. Thank you, Elton for being so kind and helpful, Thanks Tony for sharing your expertise in bioinformatics and Jo for being the best bench mate. Thank you to George, Jack and Yousra, Hadi and Anand for checking up with me. To my Dad, Dr Ahmed K. Gambari. Thank you for your financial support. Thanks to my Mum and siblings Khadija, Rahma, Habiba and Mohammed including my sister-in-law Halima for their encouragement. Many thanks to my inlaws Prof and Dr. (Mrs) Ambali and the entire family. My senior colleagues at the University of Ilorin, Prof Kolawole, Dr Mrs Saliu, Dr Agbabiaka, Dr Sule amongst others too many to mention. May God bless you all abundantly. Thanks to my Uncle Dr Munir Yusuf, Mr MacDonald Mopho, my friends back home- Amina, Maria and Safiya and my friends here in the UK, everyone else that has ever showed me kindness. Thank you all for being part of my journey. Lastly and most importantly thanks to God for the strength and grace to complete what I started.

Table of contents

LIST OF FIGURES

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 1

Introduction and Background

Literature review

1.1 Pseudomonadaceae

Pseudomonaceae are a family of rod-shaped gamma proteobacteria commonly found in both soil and aquatic environments. They can be characterized as being Gram negative, non-spore forming, non-fermenting and usually possess a polar flagellum. They usually exist as either free living or as opportunistic pathogens. Members of the family Pseudomonaceae are chemoorganotrophs and can survive by utilizing minimal organic compounds. Some genera belonging to this family include *Pseudomonas, Nitrosomas, Nitrobacter, Azotomonas, Azobacter* and *Gluconobacter* (Palleroni, 1981).

1.1.1 Pseudomonas aeruginosa

Pseudomonas aeruginosa is one of the most common members of the family Pseudomonadaceae. They are Gram-negative rod-shaped opportunistic pathogens found as part of the normal flora in both clinical and environmental settings (Fonseca and Sousa, 2007). *Pseudomonas aeruginosa* can carry multiple mobile genetic elements in its genome and yet exhibit stability (Lambert, 2002). In recent times, multiple outbreaks have shown that *P. aeruginosa* has been associated with the spread of multi drug resistance determinants within the clinical settings. An electron micrograph of *P. aeruginosa* is seen below in Figure 1-1.

Pseudomonas aeruginosa has the tendency to become resistant after exposure to antibiotics, easily acquiring mobile genetic elements such as integrons, plasmids and transposons from the environment, acquiring spontaneous mutation of regulatory genes and possessing high lipid content cell wall to restrict antimicrobial permeability. (Lambert, 2002). *Pseudomonas aeruginosa* has been isolated from water sources such as recreational swimming pools, surface water and drinking water, however, hospital water and plumbing materials have been the major source of nosocomial outbreaks (Mena and Gerba, 2009; Wellington et al, 2013).

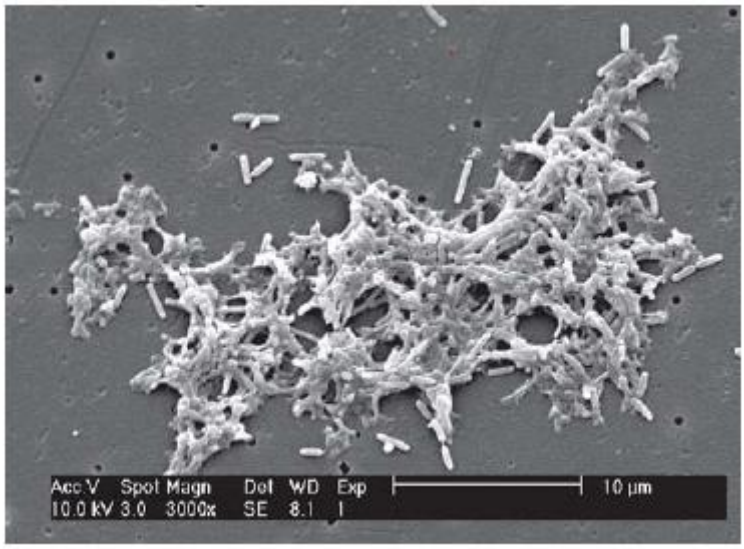


Figure 1-1 Electron micrograph of *P. aeruginosa* cells that have formed a biofilm

A 24hr P. aeruginosa biofilm whose dense exopolysaccharide matrix holds cells in place on a substratum with water channels visible as circular gaps. Figure adapted from (Tamashiro et al, 2009)

1.1.2 Nosocomial P. aeruginosa infections

Infections that patients contract while they are receiving treatment in the hospital or health facility are referred to as Nosocomial infections (Hosein et al, 2005). There are various nosocomial infections caused by a wide variety of bacteria, viruses and fungi. However, it has been noted that the acquisition of secondary bacterial infections is one of the most common. About 300,000 cases of bacterial nosocomial infections occur in the UK annually, however, measures such as hand washing regularly have begun to reduce the frequency (PHE, 2014).

Studies comparing environmental and clinical isolates have shown that most nosocomial infections due to *Pseudomonas aeruginosa* can occur either via patient to patient transmission or from environmental strains (Orsi et al, 1994)**.** Specific units in hospitals have reported outbreaks of *Pseudomonas aeruginosa* related infections which include the

Intensive Care Unit (Trautmann et al, 2001) Burns Unit (Kolmos et al, 1993), Neonatal Unit (Walker et al, 2014) and the Cystic Fibrosis Unit (Panagea et al, 2005). Many other studies have also linked nosocomial outbreaks due to *Pseudomonas aeruginosa* within the hospital water supply (Grundmann, 1993).

1.2 Antimicrobial resistance in bacteria

Antimicrobial resistance is on the rise worldwide and it has become one of the world's major health concerns in recent times (O'Neil, 2014). It has been observed geographically that the indiscriminate use of antimicrobials in both humans and animals has contributed greatly to the present high rate of multidrug resistance (Lebeaux et al, 2013). In developing countries, antibiotic usage is not well controlled in patients due to lack of awareness of antibiotic resistance among the population and engaging in self-medication of unprescribed antibiotics (Okumura et al, 2002). Another causative factor is the insufficient development of new antimicrobials by pharmaceutical companies over the years due to their focus on obtaining profit in the long run. A brief timeline of the production of antibiotics and development of antibiotic resistance is shown in Figure 1-2 below.

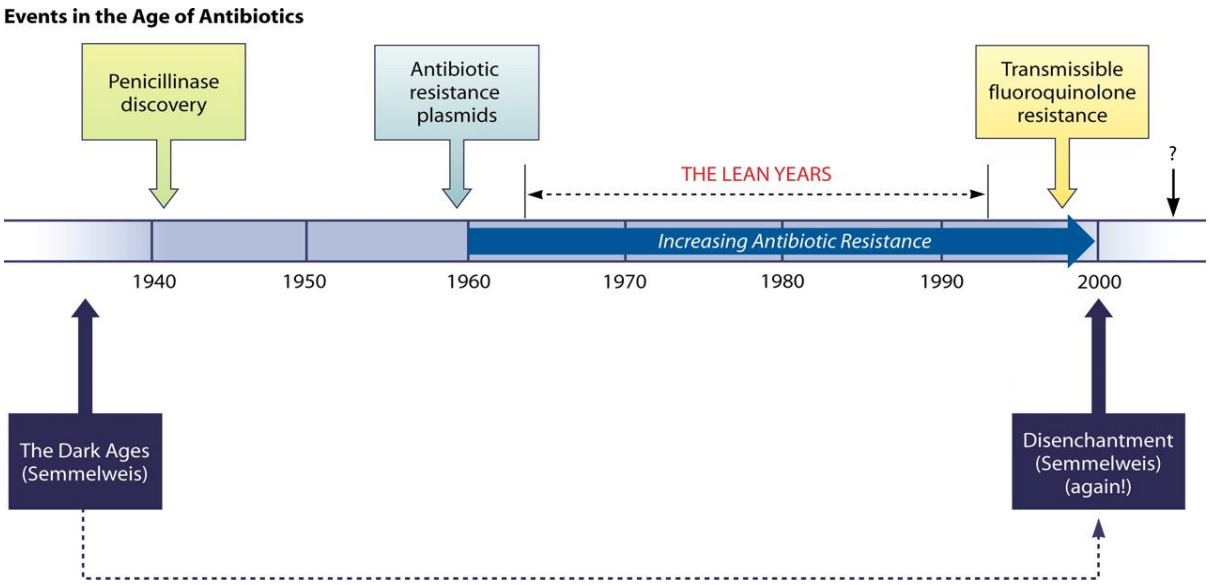


Figure 1-2. The history of antibiotic production and development of antibiotic resistance

The era was when the first antibiotic, Penicillin was discovered in the 1940s and the advent of resistance plasmids coincided with the long and steady rise in antibiotic resistance. Contributing to this was production of fewer new antibiotics by pharmaceutical companies in the beginning of the new millennium. Figure adapted from (Davies and Davies, 2012)

Mechanisms by which multi drug resistance bacteria confer resistance include development of efflux pumps, inactivation of the antibiotic mutation of target site and development of antibiotic degrading enzymes. Antibiotic resistant bacteria may also possess genes that encode the expression of one or more of these mechanisms to maintain their continuity (Levy, 1998).

1.2.1 Antibiotic versus Biocide resistance

Antimicrobials are commonly classed as either antibiotics (or antifungals or antiprotozoal depending on the kind of aetiological agent involved) or biocides. Antibiotics can be described as a chemical substance either naturally sourced or artificially synthesised that can cause bactericidal or bacteriostatic effects on bacteria (Poole, 2002). They are mainly administered for treatment in humans and animals while biocides are chemical molecules

that can cause similar effects by mitigating the growth or activity of bacteria and other microorganisms found on inanimate objects or substances. Examples of biocides are disinfectants and preservatives (SCENIHR, 2009).

Until recently, biocide resistance has not been given a lot of attention. A possible link between biocide resistance and antibiotic resistance has been established. There is a tendency of cross-resistance and co-resistance to occur and this has called for more research on the levels of biocide resistance and its effects or influence on antibiotic resistance patterns in patients (Levy, 2002). Cross-resistance can be described as the acquisition of antimicrobial resistance to several different antimicrobials simultaneously through a single resistance mechanism e.g. the mechanism of multiple drug efflux pumps while co-resistance describes the presence of multiple resistance genes on one genetic platform such as a plasmid or an integron (Chapman, 2003). An example is the RK2 plasmid which confers resistance to Ampicillin, Kanamycin and Tetracycline due to the expression of three resistance genes (Chapman, 2003). Cross-resistance could be a clinical problem if the resistance mechanism of the biocide closely matches those of the antibiotic or antimicrobial and therefore the bacteria become less susceptible to the antibiotic (Poole, 2002).

Biocides are widely used in both clinical and industrial settings to mitigate the spread of pathogenic microorganisms to susceptible individuals. The most common biocides used in the hospital setting include chlorine, chlorine dioxide, sodium hypochlorite and quaternary ammonium compounds such as benzalkonium chloride (Russel, 1995). As with antibiotics, indiscriminate use of some biocides among everyday household items such as soap could be a significant contributor to antimicrobial resistance through cross-resistance. For example, subinhibitory concentrations of triclosan in soap was found to increase the tolerance of bacteria to both triclosan and antibiotics through efflux pumps. Due to other environmental

concerns of triclosan such as issues with its biodegradability, it has been since banned from use in soaps and other products (Carey et al, 2015).

1.2.2 Mechanisms of biocide resistance

Biocides have a less specific mechanism of action to bacteria than antibiotics. Most biocides target the cell wall or cell membrane or alter the genes responsible for their synthesis. They commonly destroy cells by creating pores which then lead to cell lysis (Russel, 1995). Other biocides such as chlorine-containing compounds target the protein synthesis of important enzymes necessary for cell growth (Russel, 2003). Biocide resistance genes can be regulated by repressors when the biocide is not exerting selective pressure, or they can be constitutively expressed. Common biocide resistant bacteria of clinical significance include those encoding resistances to triclosan, chlorhexidine, quaternary ammonium compounds, chlorine and silver (Gupta et al, 2018).

Some bacteria can survive treatment if they can metabolize the biocides as their principal substrate. A study showed the degradation of benzyl benzoate by *Acinetobacter spp.* isolated from a river. The bacteria were cultured *in vitro* using the biocide as the only source of nutrient (Göttsching and Schmidt, 2007). Also, the formation of persister cells could contribute to the reduced susceptibility of biofilms to both antibiotics and biocides (Lewis, 2010). Persister cells are a group of non-replicating and non-infectious bacterial cells that continue to survive after being exposed to antibiotics while most other cells are susceptible (Stewart, 2002). Toxin-antitoxin (TA) systems present on both chromosomes and some naturally occurring plasmids have been found to be associated with the survival of persister cells. The TA consist of genes encoding a stabile toxin and a labile antitoxin present simultaneously in the genome. Once the cell loses the plasmid or chromosome, the

accumulation of the toxin in the cell stops growth and thus helps in the maintenance of the genetic element within daughter cells (Lewis, 2015). The TA systems consisting of the *relBE* locus, a protein synthesis inhibitor, have been known to control cell ribosomal activity during translation thereby leaving the cell in a stationary state. This system contributes to the ineffectiveness of several antibiotics in lysis of persister cells (Keren et al, 2004, Lewis, 2015). The *relB* gene encodes the antitoxin while *relE* is a corepressor and cleaves specific mRNA targets. Both *relB* and *E* form a complex that binds to *relO* to regulate transcription of *relBE*. Lon is a cofactor that degrades *relB* leaving *relE* the chance to act on mRNA (Overgaard et al, 2009).

1.3 Chlorite dismutase

Chlorite dismutase (*Cdm)* is a heme-based enzyme that catalyses the breakdown of chlorite to chloride and molecular oxygen (Celis et al, 2015). *Cdm* was first found and studied in perchlorate reducing bacteria such as *Dechloromonas agitate*, *Azospirra spp* and *Dechlorobacter spp*; however, in recent times it has been found present in various non-perchlorate reducing bacteria such as opportunistic nosocomial pathogens like *Pseudomonas aeruginosa* and *Klebsiella pnemoniae* (Celis et al, 2015), Cyanobacteria (Schaffner et al, 2015a), nitrifying bacteria (Maixner et al, 2008) and *Staphylococcus aureus* (Mayfield et al, 2013).

The significance of *cdm* in non-perchlorate reducing bacteria is yet to be fully understood. In a study by Celis et al (2015) it was suggested that presence of the *cdm* in *Klebsiella pneumoniae* may protect its host from the accumulation of toxic chlorite due to the breakdown of chlorate under anaerobic conditions. The biotechnological benefits and mechanisms of chlorite dismutases (Hofbauer et al, 2014) have been explored but no study

has explored the presence of *cdm* gene and the survival of clinically important bacteria in the hospital setting.

1.3.1 Classification and Structure of Chlorite dismutase

Structurally, chlorite dismutase either exists as a dimer or pentamer in most Gram-negative bacteria (Hofbauer et al, 2014). They can be either functional or non-functional based on their ability to enzymatically degrade chlorite (Schaffner et al, 2015a). The functional Cdms are further grouped into two lineages, Lineage I and II Cdms. Lineage I comprise of Cdm proteins that have a longer sequence length (Maixner et al, 2008). They also have the complete N-terminal and C-terminal folds. Examples of Lineage I Cdm include those isolated from *Dechloromonas aromatica, Geobacillus sterothermophillus,* and *Candidatus Nitrospira defluvii* while Lineage II belongs to the Cdm proteins which have a shorter sequence length (Schaffner et al, 2015a). An example of a well-studied Cdm in Lineage II is Cdm from *Nitrobacter winogradski* (Nwcdm) found to occur structurally in a dimeric form while other Lineage I have a pentameric quaternary structure (Hofbauer et al, 2014). Other dimeric Cdms are from the Cyanobacteria which also have a shortened N-terminal (Schaffner et al, 2015b). The pentameric crystal structure of *Candidatus Nitrospira defluvii,* a Lineage 1 Cdm protein has been established as seen in Figure 1-3 (Sundermann et al, 2014).

The non-functional Cdms often referred to as Cdm/Cld-like protein have a limited Cdm activity on chlorite substrate due to the replacement of the distal arginine residue with a different amino acid. Examples of Cdm-like proteins are from those found in *Staphylococcus aureus, Geobacillus stearothermophilus and Listeria monocytogenes* (Schaffner et al, 2015a). In functional Cdms, the distal arginine residue (Arg173) and proximal histidine residue to the heme is conserved as seen in Figure 1-4 below. It has been concluded that the

presence of Arg173 plays an important role in the degradation of chlorite (Liebensteiner et al, 2016; Hofbauer et al, 2014). Recently, chlorite dismutases have been linked with dye reducing bacteria by having similar phylogenetic backgrounds with the dye decolorising peroxidases (DyP) due to their similar phylogenetic background.

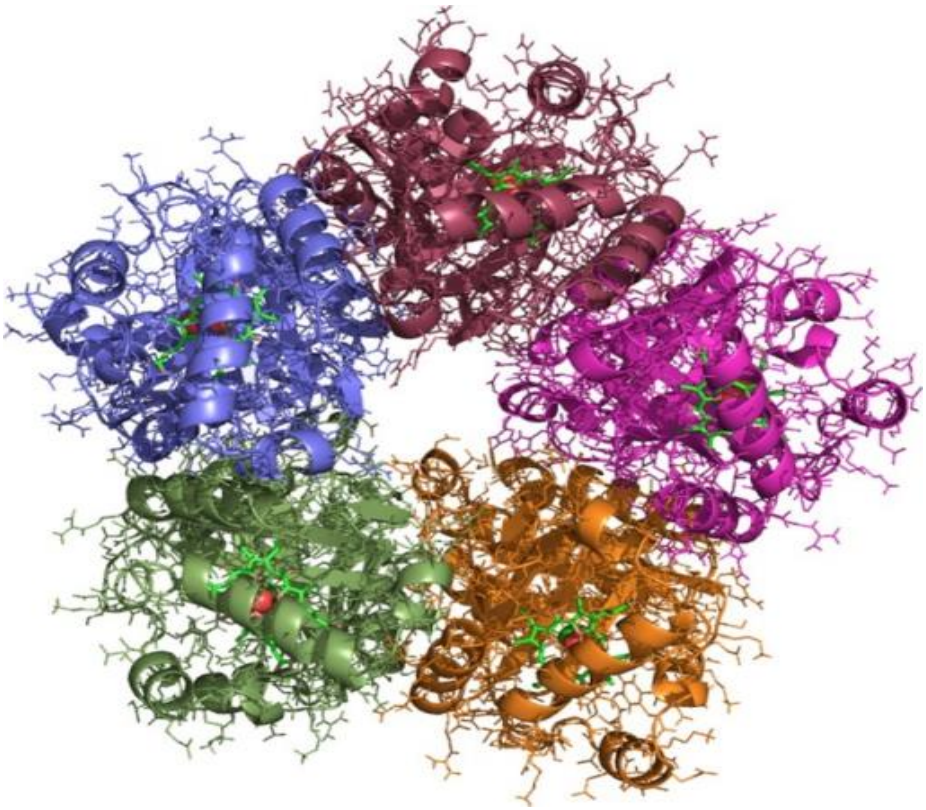


Figure 1-3. Crystal structure of chlorite dismutase isolated from Candidatus *Nitrospira defluvii* in the pentameric form.
The different colours represent the five separate monomers, the secondary structures are shown as ribbons, substrate ions are represented as bubbles while the heme group and side chains are shown as branched sticks. Figure from (Sundermann et al, 2014).

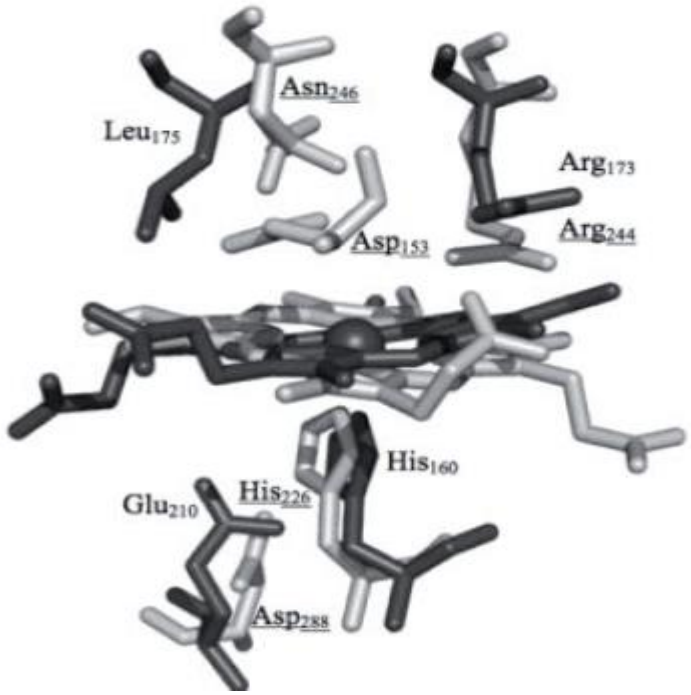


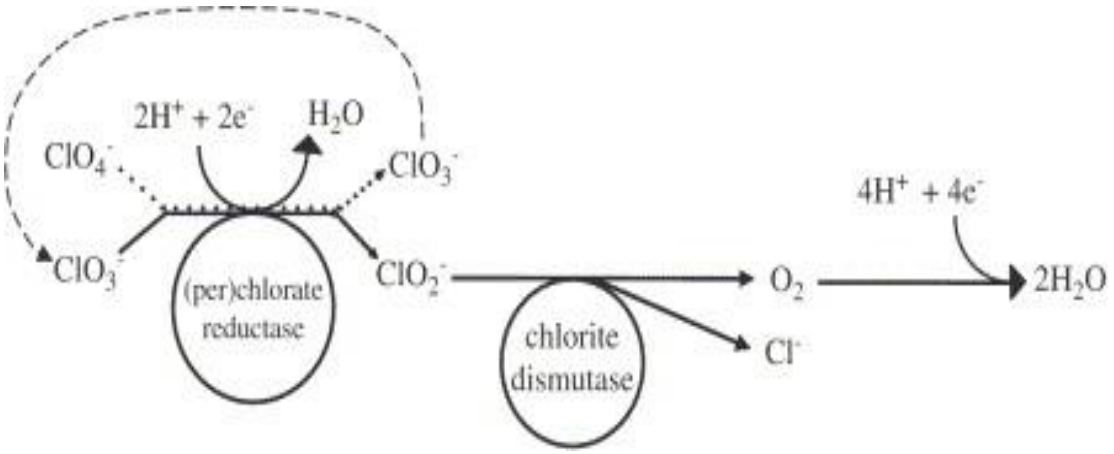
Figure 1-4. Active sites of DyP and Cdm
The figure shows the comparison of active sites in dye decolorizing peroxidases (DyPs) in light grey and Cdms in dark grey. Both enzymes have arginine at a similar distal ligand to the heme group and histidine at the proximal end. Figure adapted from (Hofbauer et al, 2014)

1.3.2 Origin of Chlorite in the environment

Chlorine oxyanions including highly oxidizing chlorate, chlorite and chloride are all products of the breakdown of disinfectant agents, chlorine dioxide and hypochlorite. Chlorate (ClO_3^-) is produced commercially for its use as a herbicide and as a precursor to chlorine dioxide-based bleaching in the paper and pulp industry (Sorlini et al, 2014). Perchlorate, on the other hand, has been determined to be the most abundant chlorine oxyanion in the environment and is also manufactured for its use in rocket propellants (Coates and Achenbach, 2004). In the past, improper disposal of effluents from production of perchlorate has been found to be the source of perchlorate contamination in aquatic environments (Liebensteiner et al, 2016).

Chlorite (ClO_2^-) is the breakdown product of chlorate by perchlorate-reducing bacteria. It can be used as a disinfectant due to its bactericidal and toxic effect on most bacteria. A reaction of chlorite and hypochlorite catalysed by hydrochloric acid produces chlorine dioxide (Sorlini et al, 2014). During anthropogenic activities like water purification, the reaction of chlorine dioxide with organic matter may cause it to break down into by products such as chlorite and chloride in low quantities. These levels substantially decrease when post-oxidation measures such as treatment with activated charcoal and aluminium sulphate are employed (Sorlini et al, 2014). Both perchlorate and chlorate reducing bacteria are strict anaerobes that use the enzymes perchlorate reductase and chlorate reductase respectively to form chlorite (Liebensteiner et al, 2016). An equation showing the breakdown from perchlorate to chloride is seen in Figure 1-5 below.

*



Key: Cl^- -Chloride ClO_2^- - Chlorite ClO_3^- - Chlorate ClO_4^-- Perchlorate

*Dotted and dashed lines show that perchlorate reductase acts on both ClO_3^- and ClO_4^- to produce ClO_2^-

Figure 1-5. Chemical equation of the degradation of Perchlorate

The breakdown of Perchlorate and subsequently chlorate begins by the loss of two hydrogen ions and the release of water by perchlorate reductase and then anaerobically followed by the dissolution of chlorite into chloride and oxygen by the enzyme chlorite dismutase. In low concentrations chloride is non-toxic to bacteria and no form of further bioremediation is necessary. Oxygen in some cases is hydrolysed back to water by gaining four hydrogen atoms. Figure adapted from (Nerenberg et al, 2006)

1.3.3 Mechanism of degradation of chlorite

Chlorite dismutase activity is initiated by binding to chlorite in its ferric form to give a Fe (III)-chlorite complex. The heme is oxidized to compound 1 (ferryl (Fe(IV)=O) porphyrin cation radical) and chlorite is reduced to hypochlorite which is transiently regulated by distal arginine residue to form a hypochlorite-oxo iron (IV) compound linked by an O-O bond (Nerenberg et al, 2006) as shown in Figure 1-6 below. When there is an increase in pH, some hypochlorite escapes and causes decomposition of the ferric complex which results in the irreversible inactivation of the Cdm enzyme and persistence of chlorite but at a lower pH of 5 or 6. Ferric III- peroxyhypochlorite is then formed which is gradually reduced to chloride

and dioxygen (Schaffner et al. 2015b). Isotopically labelled oxygen molecules were used to show that the oxygen molecule end-product is due to an intermolecular redox reaction of chlorite rather than hydrolysis (Mehboob et al, 2009). In microaerophilic environments, an oxidase enzyme is present to utilize the oxygen produced from chlorite dismutase activity as in the case of cytochrome c oxidase in *Ideoella dechloratans*. Chloride serves as a micronutrient for certain photosynthetic microorganisms and one of its functions is its involvement in cellular transport and metabolism (Raven, 2017).

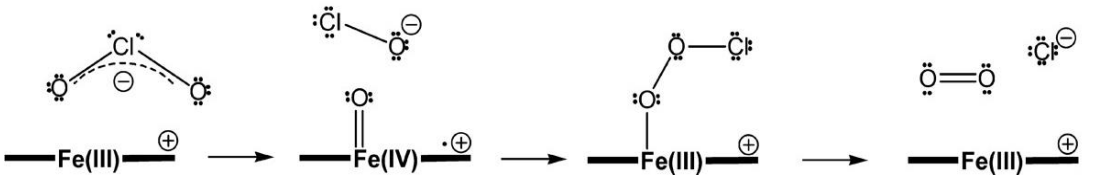


Figure 1-6. Mechanism of chlorite dismutase activity.

An Fe(III) chlorite complex is formed with the interaction of the heme and chlorite. This complex is then oxidized into Fe(IV)=O (oxoiron(IV) porphyrin cation radical and hypochlorite through the cleavage of the O-Cl bond. The hypochlorite is further reduced to ferric III-peroxyhypochlorite and finally chloride and dioxygen are released through dissociation of the Ferric oxygen bond. Figure taken from (Mylnek et al, 2011)

1.3.4 Chlorite dismutase gene

The chlorite dismutase gene (*cdm*) has been well studied and found to be present across a huge range of diverse phyla including proteobacteria, cyanobacteria and archae (Mehboob et al, 2009). From BLAST search, it was observed that most of the bacteria containing this gene have marine and aquatic origins. Although the natural source of chlorite in the environment remains unknown, some of the *cdm* gene-carrying bacteria do not produce chlorite intracellularly making the gene function in those organisms a mystery ((Hofbauer

et al, 2014). The chlorite dismutase gene from *Nitrospira defulvii*, a bacterium which uses nitrite as its sole electron acceptor, recovered from a wastewater treatment effluent suggests that the bacterium contributes to the breakdown of chlorite for its survival in the environment with competing bacteria (Maixner et al, 2008). The *cdm* gene in perchlorate reducing bacteria *Dechloromonas agitata* strain CKB showed functionality since the gene was up regulated in the presence of chlorite (Bender et al, 2002).

Little is known about the toxicity of chlorite in the environment. *In vitro,* it seems to have the same strength of potency to both Cdm^+ and Cdm^- bacteria. The MIC of a *Klebsiella pneumoniae* strain encoding Cdm, was found to be <20mM of $NaClO_2$ alongside ΔCdm bacteria (Schaffner et al, 2015b). It is thought that the toxicity of chlorite is similar to hypochlorous acid (household bleach) which acts on cells by partly unfolding proteins (Celis et al, 2015b). Also, the hypochlorous acid formed during chlorite reaction with the enzyme at a high concentration may cause destruction of the heme group. The high acidity of chlorite causes it to persist with the cell much longer and the negative charge of chlorite prevents it from getting into the cell (Celis et al, 2015). Cyanobacteria Cdms on the other hand, are less sensitive to the hypochlorous acid formed during the reaction and therefore are more stable than the pentameric Cdms.

1.4 Mobile genetic elements in bacteria

A mobile genetic element is a sequence of DNA that can disassociate or duplicate from its original position in a host and relocate to a different site within or outside its host (Zhang et al, 2011). There are various types of mobile genetic elements present but the most common in bacteria are transposons which could be either retrotransposons or DNA transposons and insertion sequences (Burrus et al, 2002).

1.4.1 Transposons

Transposons, also known as transposable elements, are mobile genetic elements that contains a transposase gene which can direct a stretch of DNA to be transported to a different region in the genome (Burrus et al, 2002). Usually they are flanked by insertion sequences which are a short sequence of inverted repeats surrounding the transposase gene. Transposons play a major role in the dissemination of antibiotic resistance genes in bacteria (Whittle and Salyers, 2002). Several clinically important transposons carry within them multiple antibiotic resistance genes and/or integrons. Composite transposons have dual insertion sequences, one at each end, which carry the transposase gene within each insertion sequence (Salyers et al, 1995). They usually carry a resistance gene and a classic example is Tn10. Non-composite transposons on the other hand do not have insertion sequences but rather they have inverted repeat sequences at their distal ends. They usually contain a resistance gene, a resolvase gene and transposase gene present at the centre of the transposon (Whittle and Salyers, 2002). A typical example is the Tn3 as shown in Figure 1-7 below.

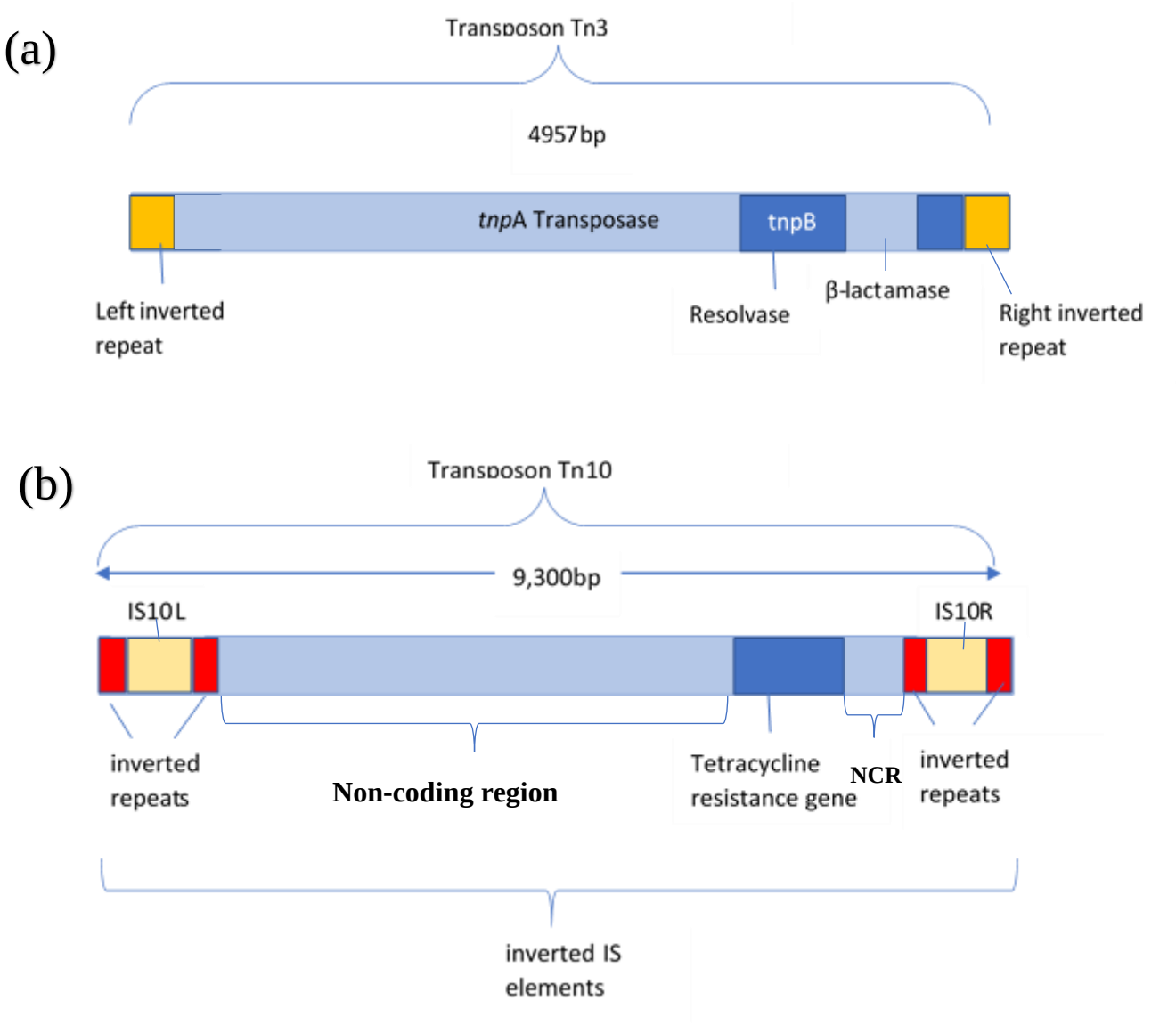


Figure 1-7. Non-composite and Composite Transposons

Above are examples of (a) a Non-composite transposon (Tn3) and (b) a composite transposon (Tn10). Composite transposons are different from non-composite transposons due to their mode of transposition owing to the location of the transposase gene. Tn 3 contains the β-lactamase gene and upstream the resolvase gene responsible for recombination events of the transposon. The Tn10 contains a tetracycline resistance gene with the transposase present within the insertion sequences at both ends. Design of Figure adapted from (Russel(iGenetics) n.d.)

Retrotransposons make DNA copies by first being transcribed by RNA polymerase to mRNA and then back to cDNA by the enzyme reverse transcriptase. The cDNA is then

allocated to a part of the bacterial genome (Whittle and Salyers, 2002). DNA transposons on the other hand are not copied but are excised by the enzyme transposase which then form sticky ends that are complementary to its new target site in the genome (Whittle and Salyers, 2002). Transposons are an effective vector for resistance gene dissemination; a single transposon can carry multiple transposons or integrons within it. An example is Tn1403 class II 19.9kb transposon, which was isolated from *Pseudomonas aeruginosa* from a clinical setting. It has within it, a class 1 integron and three transposons. In total, it encodes resistance to chloramphenicol (*cal*), ampicillin (*β-lactamase*), streptomycin (*aadA*) and Spectromycin (*aphC*) (Vezina and Levesque, 1991; Stokes et al, 2007).

1.4.2 Conjugative transposons

Conjugative transposons are mobile genetic elements that have properties closely related to both plasmids and transposons (Salyers et al, 1995). Like transposons, they can excise from a segment of DNA and relocate to a different section of the genome and like plasmids; they form circular intermediates and are able to transfer in a similar manner via the *oriT* (origin of transfer) site (Scott, 2002). Conjugative transposons can excise and transfer through conjugation with a recipient cell. After excision occurs, they then form a circular intermediate which gets nicked at the *oriT* and a strand is transferred to the recipient strain (Salyers et al, 1995). After transfer, the single strand in the recipient is replicated into double stranded DNA while replication of a complementary strand in the donor occurs during transfer. The circular strands then linearize and integrate into the bacterial genome (Salyers et al, 1995).

1.4.3 Insertion sequences

Insertion sequences can be described as short mobile DNA elements that do not carry any structural genes except those necessary for their transposition. This includes transposase and its regulators. They are usually flanked by inverted repeats enabling the element to be transposed from one location to another. They can exist in the bacterial genome on their own but are most often associated with a transposon (Mahillon and Chandler, 1998). There are several clinically significant insertion sequences in pathogenic bacteria. Some emerging subtypes of insertion sequences can carry antibiotic resistance genes as passenger genes located next to the transposase. These types of insertion sequences are referred to as transporter insertion sequences (Siguier et al, 2014).

1.5 Integrons

Integrons are a sequence of DNA that comprises an integrase gene (*int*1) responsible for a site-specific recombination of a set of gene cassettes, an attachment site *attl* and sub attachment sites in between the gene cassettes with an integrase promoter upstream of the *attl* site that assists the expression of the integrase (Mazel, 2006). A second promoter (Pc) is responsible for the transcription of all the genes contained in the gene cassettes with the first gene cassette always being more strongly expressed than the subsequent ones (Guerin et al, 2011). Figure 1-8 below shows the basic components of a classic integron. Each gene cassette is separated by a 59 bp element which varies slightly in size and sequence between species (Mazel, 2006). Integrons are found in both Gram-negative and Gram-positive bacteria and are largely considered to be spread through horizontal gene transfer.

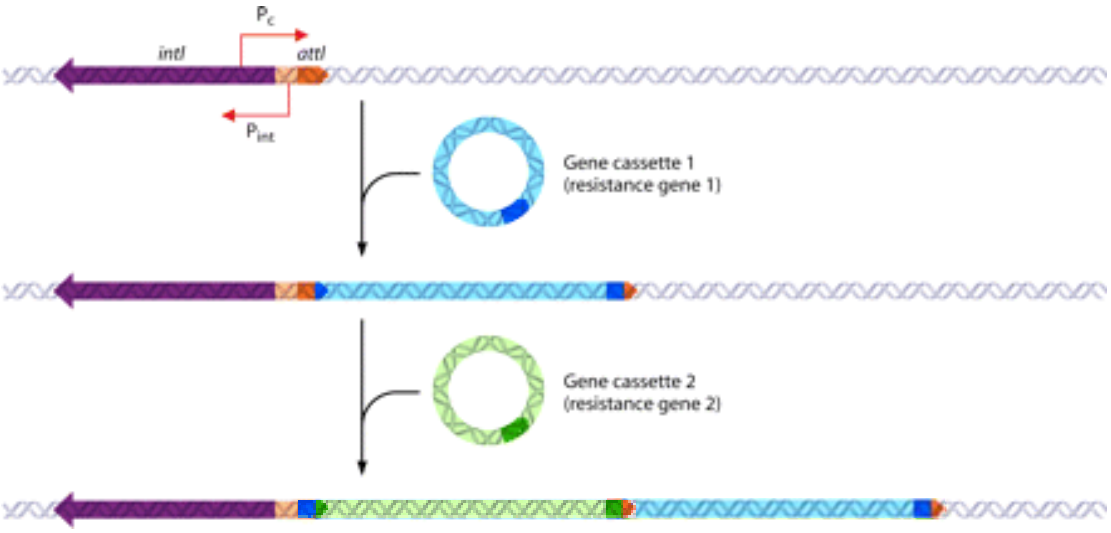


Figure 1-8. Stages of site-specific recombination in an Integron

The integrase gene is shown in purple, the promoter Pc for the gene cassettes and the promoter for the integrase gene in the opposite direction. Gene cassettes 1 and 2 in blue and green respectively are different resistance genes that can be integrated at the attachment site attI in orange through site specific recombination. Figure adapted from Davies and Davies, 2010)

Class 1 integrons are of major clinical importance due to the conserved nature of the types of genes present in the gene cassettes. Normally, they would have a conserved region at the 3' end of the gene cassettes, a *qacEΔ1* gene encoding quaternary ammonium compounds and a *sul1* gene encoding sulphonamide resistance (Gaze et al, 2011). Integrons are commonly found in pathogenic bacteria when present within a transposon. This provides the ability to disperse efficiently within the bacterial community and therefore may contribute to the spread of antibiotic or biocide resistance (Mazel, 2006).

The above mentioned mobile genetic elements have one thing in common. They all have the capacity to obtain and carry antimicrobial resistance genes. Most of the time, they provide an added advantage to their host organism individually but in some cases, they rely on each other's capabilities to carry out their functions. For example, an integron is not mobile and

thus is often found on transposons. Transposons except conjugative transposons are not able to transfer into other bacteria on their own and this is where merging into a plasmid genome will prove useful.

1.6 Plasmids

Plasmids are extra-chromosomal genetic elements present in a large diversity of Gram negative and Gram-positive bacteria. They can be mobile and sometimes un-mobilizable; the latter occurring without any genes necessary for conjugative transfer or mobilization (Røder et al, 2013). Plasmids offer the host bacterial cells advantages that are beneficial but not essential to the normal functioning of the bacterial cell (Røder et al, 2013). They are used for genetic engineering to produce large amounts of proteins such as insulin. In this case the plasmid is called a vector. Plasmid mediated transfer of resistance is one of the most efficient methods by which pathogenic and commensal bacteria can acquire resistance genes in a natural state through conjugation (Hennequin et al, 2012).

Some of the advantageous traits encoded by plasmids include antibiotic resistance, resistance to biocides, metal and hydrocarbon degrading capabilities. They serve as vehicles for the exchange of resistance genes through horizontal gene transfer. Plasmids are commonly distinguished based on their incompatibility status (Inc) (Norberg et al, 2011). Plasmid incompatibility most commonly occurs when two plasmids are unable to be maintained in the same cell line because during DNA replication, the cell cannot distinguish between the multiple regulatory mechanisms present so, related plasmids are treated as a single pool and random selection results in one or the other becoming dormant in the host cell (Norberg et al, 2011). Broad host range plasmids give the most threat to the spread of antibiotic resistance due to their ability to transfer an advantageous trait in the form of DNA to a diverse range of bacteria (Schluter et al, 2003).

Plasmids self-replicate in their host cell and possess genes responsible for encoding proteins that help to achieve this process. The first stage of plasmid replication frequently referred to as initiation, is characterized by the recruitment of plasmid encoded proteins to help DNA polymerase to bind to a free hydroxyl group at the 3' end of a plasmid-generated primer complementary to the parent strand (Del Solar, 1998). The site where this occurs is termed *oriV* (origin of vegetative replication). In plasmids, there are two common types of replication, the rolling circle replication and theta replication (Del Solar, 1998). The former involves a site-specific cleavage of the plasmid DNA strand to produce the -OH end and the latter involves the use of initiation proteins that synthesise an RNA primer at the *oriV*. In this case, replication can be unidirectional or bidirectional (Del Solar, 1998).

The second stage is elongation, where DNA polymerase holoenzyme and other host proteins are involved in the lengthening of the complementary strand. Replication only occurs from the 5' to 3' region and the continuous complementary strand is called the leading strand. The lagging strand occurs from the opposite direction of the leading strand forming short discontinuous Okazaki fragments which are later joined by DNA ligase. The final stage referred to as termination occurs when DNA polymerase reaches a stop signal on the parent strand (Del Solar, 1998).

Plasmids can be categorized into either low copy number (2-7) or high copy number plasmids based on the number of plasmid copies capable of occupying its host cell. Low copy number plasmids require a partitioning system which allows for the stable maintenance of the plasmids in each daughter cell during cell division (Baxter and Funnel, 2014). Partitioning systems are designed to position the plasmids at different sites in the cell. Elements involved in this process include two proteins known as the centromere binding protein and ATPase which generate energy needed for partitioning plus the *par* site which

acts as the site of assembly (Baxter and Funnel, 2014). All the major genetic elements are occasionally dependent on another for successful dissemination. Figure 1-9 below shows how a plasmid can accommodate a gene cassette, integron, transposon within its genome which will all be replicated as the plasmid replicates in a host organism.

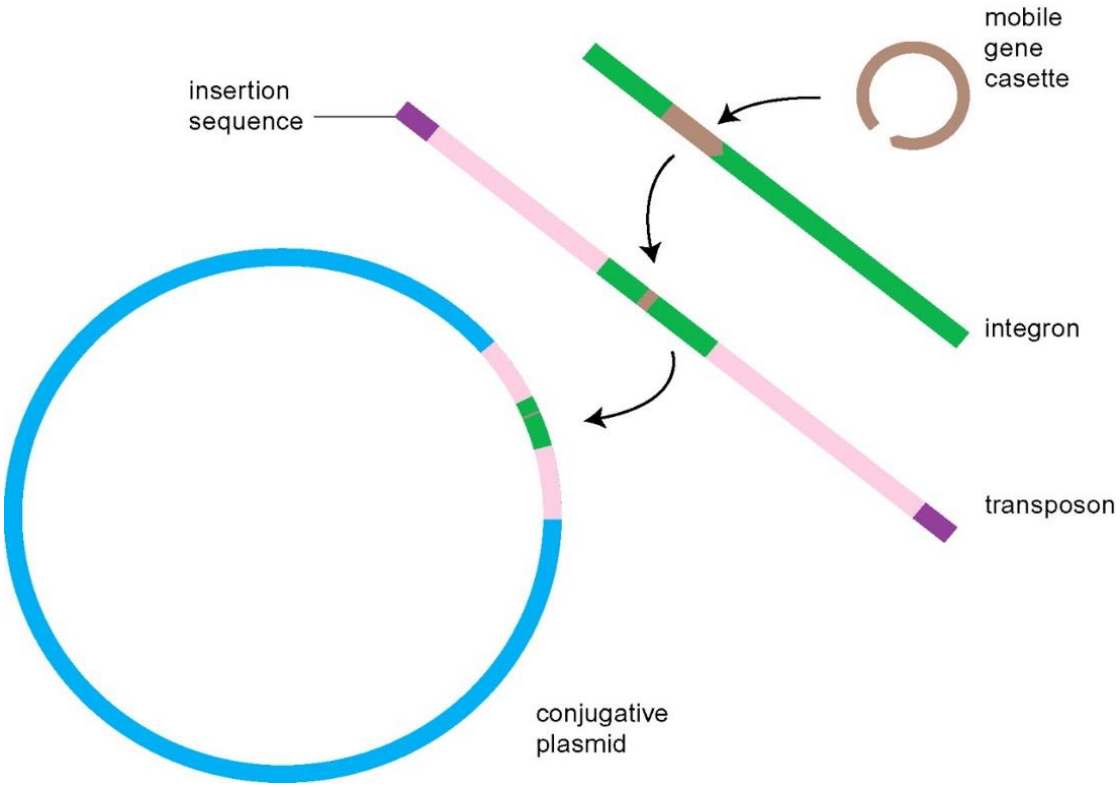


Figure 1-9. Relationship between a plasmid, transposon, insertion sequences, integron and gene cassettes

Mobile gene cassettes are resistance genes that can be integrated via site specific recombination by the integrase gene into an integron. The integron is a sequence of DNA which contains a promoter, the integrase gene, an attachment site for the integration of one or more gene cassettes. Integrons are non-mobilizable and are often present on transposons, which with the aid of the gene transposase and flanked insertion sequences, can translocate to various sections of a another larger mobilizable element such as a conjugative plasmid. A conjugative plasmid contains genes necessary for transfer to other bacteria and thus, can mobilize all these other genetic elements at once to another cell. Figure from Norman et al, (2009)

1.6.1 Plasmids and biocide resistance

Plasmids play an important role in the resistance of bacteria to biocides. Resistance to biocides can either be self-encoded by the bacteria through defensive measures such as initiating biofilm formation, mutation of targets for the biocide and acquired resistance through horizontal gene transfer by the possession of mobile genetic elements (Russel, 1997). Numerous plasmids found in both Gram-negative and Gram-positive bacteria have genes that encode mechanisms for decreased biocide uptake, inactivation or modification of cell surface serotypes and encoding the expression of multiple efflux pumps (Poole, 2002). Plasmid mediated resistance to metals have occurred with biocides such as mercury and silver, of which the most common are quaternary ammonium compounds (QACS), triclosan and parabens all of which are encoded by plasmid or transposon borne genes (Russell, 1997).

A study on *Pseudomonas aeruginosa* biofilms showed that the presence of genes encoding production of catalase and not the thickness of the biofilms was responsible for protecting biofilms from destruction by hydrogen peroxide (Mah and O'Toole, 2001). On the other hand, the thickness of the biofilm may have an impact on survival regarding the ability of the biocide to reach deeper layers in non-catalase producing strains (Mah and O'Toole, 2001).

1.6.2 Conjugation transfer of plasmids

Bacterial conjugation is a mechanism of horizontal gene transfer in prokaryotes which involves the movement of a single DNA strand from a plasmid with conjugative and transfer ability into another bacterial cell with the aid of pili (Freese et al, 2014). Plasmids can be regarded as conjugative in nature if they have a set of genes that encode processes of gene transfer and pilus production. After conjugation between plasmid-carrying (donor) and

plasmid-free bacteria (recipients), the recipients now carrying the plasmid are called transconjugants (Freese et al, 2014).

There are three major steps in conjugation: Mating pair formation, DNA synthesis and DNA transfer and maturation. The mating pair formation (mpf) system comprises of about 10 transfer proteins including the pilus subunit protein called pilin which is responsible for the production of the pili and other proteins to initiate conjugation (Strand et al, 2014). The mpf coordinates the pili to extend towards a recipient cell and when contact is made to pull the recipient cell closer to establish synthesis of a transmembrane pore which enables the transfer of a single strand of DNA from donor to recipient (Thomas, 2014). Figure 1-10 below highlights the key stages in bacterial conjugation.

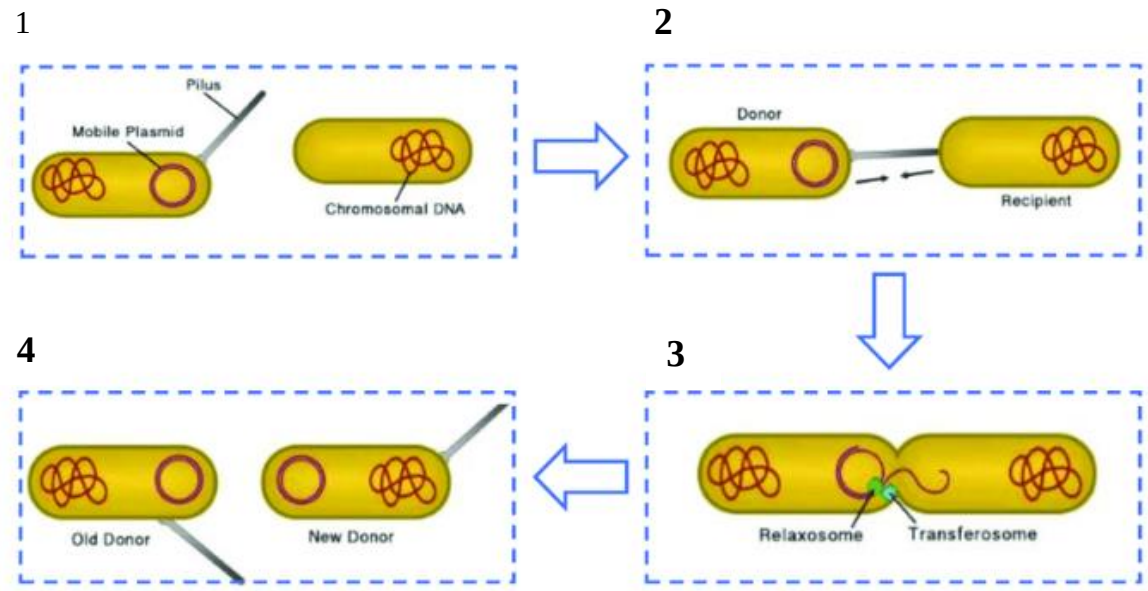


Figure 1-10. Stages of Bacterial Conjugation

Stages 1-4 show the process of conjugation between two cells. It begins with the initiation of mating pair formation and pilus extension. By rolling circle formation, one DNA strand is nicked from a single strand from the donor cell and transferred via the pili to the recipient cell. A complementary strand is then formed in both cells. Figure adapted from (Wang et al, 2018)

A mating bridge is formed between the two cells that contracts from the depolymerisation of the pilus at its end (Thomas, 2014). The second major phase involves the nicking of one strand of plasmid DNA at a region known as *oriT* leaving a free 3' end which will serve as a primer for in a rolling circle replication. The plasmid may also encode the enzyme DNA primase which in addition to other plasmid encoded genes necessary for replication, assists in the self-replication of the single strand with a complementary strand of DNA (Thomas, 2014).

1.7 IncP-1 plasmids

IncP-1 plasmids are an example of low copy number, broad host range plasmids that have several sub-groups differing in terms of evolutionary backbone, insertion sites and type of transposable elements (Popowska and Krawczyk-Balska, 2013). For differentiation, these plasmids are called IncP in *E. coli* and IncP-1 in *Pseudomonas aeruginosa*. Phylogenetic analysis has revealed subdivisions of the IncP-1 plasmids based on divergence in the *trfA* protein necessary for initiation of plasmid replication. Components of a typical IncP-1 plasmid are shown in Figure 1-11 below and a list of important genes with their respective functions are highlighted in Table 1-1. IncP-1 plasmids were classified into α, β and ϑ subtypes when their diversity was recognised (Haines et al, 2006) but more recently, further classification of these subgroups into α, β, γ, δ, ε, ζ and η has been established (Popowska and Krawczyk-Balska, 2013). IncP-1 α plasmids have a backbone closely related to RK2 and normally possess antibiotic resistance genes as accessory elements while IncP-1β plasmids have a plasmid backbone like R751 and frequently possess genes encoding mercury resistance (Norberg et al, 2011).

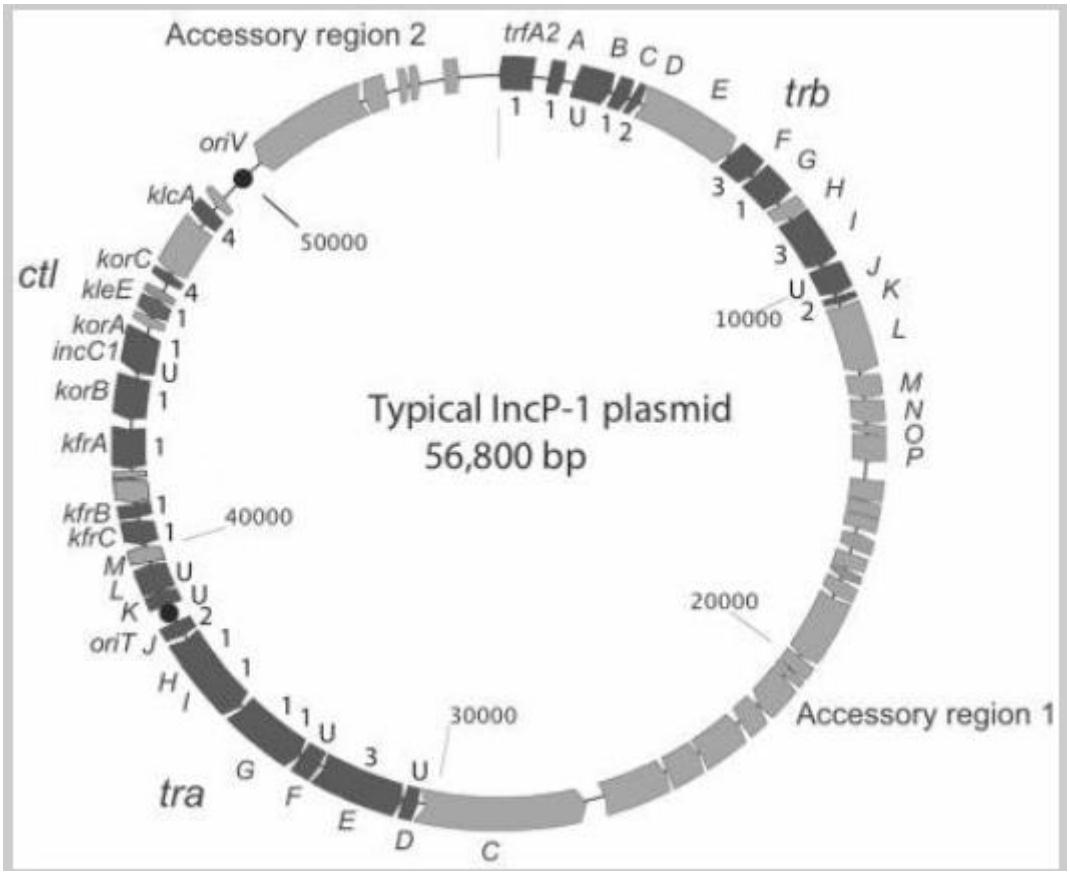


Figure 1-11. An IncP-1 Plasmid map

A plasmid map showing the conserved regions of IncP-1 plasmids, the tra and trb regions necessary for conjugation, initiation and transfer; OriV and trfA, the sites for initiation of DNA replication; genes for partitioning and plasmid stability kor, IncC, kle and klu regions and the accessory regions are all represented in the map (Figure from Sen et al, 2013).

Table 1-1. A list of some IncP-1 plasmid genes and their functions

Genes	**Encoding function**
kfrA	Regulation
korB	Partitioning and regulation
IncC	Partitioning and regulation
korA	Regulation
kleF	Plasmid Maintenance?
kleE	Plasmid Maintenance?
kleB	Plasmid Maintenance?
kleA	Plasmid Maintenance?
korC	Regulation
klcB	Plasmid Maintenance?
klcA	Antirestriction
TrfA	Replication initiation
ssb	ssDNA binding
traI	DNA relaxase
traK	*ori*T binding
traL	Conjugative transfer
oriV	Origin of replication
oriT	Origin of transfer
Tra operon	Transfer
Trb operon	Transfer
parA	Multimer resolution
parD, parE	Addiction

? represents unverified functions
Table adapted from (Aggarwal, 2015)

1.7.1 IncP-1 plasmids isolated from water sources

IncP-1 plasmids have a backbone that provides replication, stability and broad host conjugative transfer capability. Its occurrence in water or wastewater may pose a high risk of accumulation or proliferation of antibiotic resistant genes (ARG) in the environment (Bahl et al, 2009; Schluter et al, 2007). Water serves as a suitable reservoir for some pathogenic and opportunistic bacteria to thrive despite it having moderate to minimal nutrient content (Van Nevel et al, 2013). Mineral or spring water which is usually unprocessed and does not undergo biological or chemical treatment has been found to contain bacteria with antibiotic resistant genes (Falcone-Diaz et al, 2012).

Two groups of IncP-1 plasmids, namely IncP-1 α and ε plasmids have been isolated in estuarine waters (Oliveira et al, 2011). Also, effluents from downstream of wastewater treatment plants (WWTP) have been shown to contain plasmid borne antibiotic resistant bacteria which proves that plasmids could be further disseminated to other water sources such as surface and ground water (Allen et al, 2010; Zhang et al, 2007). Most of the reported IncP-1 plasmids had originated from the final effluents of wastewater treatment facilities (Schluter et al, 2007). One example is the IncP-1α plasmid pTB11 closely related to the first IncP-1α plasmid isolated from the Birmingham hospital in 1986 (Tennstedt et al, 2005).

1.7.2 Inc P-1 plasmids carrying resistance genes

Inc P, W and U plasmids have been classified as the most promiscuous plasmids because of their ability to replicate and transfer in a wide range of bacteria. IncP-1 plasmids have been isolated from both clinical and environmental bacteria and have been more commonly been affiliated with Gram negative *E. coli, Klebsiella, Pseudomonas spp* (Sen et al, 2013). Sites for integration of mobile genetic elements such as transposons in IncP-1 plasmids tend to be

either between the *tra* and *trb* operons or between the *trfA* and *oriV* regions (Thorsted et al, 1998).

Both antibiotic resistance genes and biocide resistance genes have been found in IncP-1α and IncP-1β plasmids (Droge et al, 2000). An example of an IncP-1α plasmid is pB5 which carries both resistance genes *aadA2*, *aacC1*, *tetA, tetR, aphA, sul1* which are all antibiotic resistance genes and *qacEΔ1*, a biocide resistance gene. These genes encode resistances to streptomycin (*aadA2* and *aacC1*), tetracycline, kanamycin, gentamicin and sulfonamide respectively (Droge et al, 2000). An IncP-1β plasmid carrying both types of resistance genes is plasmid pB12 which carries resistance genes *tetA, aacCA4, oxa 2, sul1* and *qacA* conferring resistances to tetracycline, streptomycin, spectinomycin, β-lactam, erythromycin and sulphonamide and quaternary ammonium compounds respectively (Droge et al, 2000).

1.8 Biofilms

In nature, bacteria can exist either as individual free-living cells otherwise termed as planktonic or they can form aggregates made up of layers of slime in a dense exopolysaccharide matrix usually adhered to a surface called a biofilm (Stewart, 2002). The two phases can be reversible since planktonic cells can join or form biofilms and vice versa depending on the present environmental conditions. The exopolysaccharide matrix serves as a binder and provides a structure and channels for both nutrients and water to reach the living bacteria within the biofilm (Walker and Moore, 2005). Some bacterial biofilms become a threat to human health when they are found in hospital settings since they are often very difficult to detect and remove efficiently. They also become a concern when strains present in them carry multi drug resistant determinants (Walker and Moore, 2005).

Most *Pseudomonas aeruginosa* strains are known to form biofilms with other microorganisms and with the help of their biofilm producing properties, enable them to exist in localized surfaces for long periods of time. The biofilm formed has been shown to protect the strains from undesirable environmental conditions and antimicrobials (Taylor et al, 2014). *Pseudomonas* biofilms are composed of surface mucoid polysaccharides or alginate and extracellular glycolipoprotein (Lagoumintzis et al, 2003).

Biofilms in hospital settings are either present within the patient or on abiotic materials. In clinical infections, planktonic cells that are associated with acute diseases can normally be treated with antibiotics but when biofilms are formed within human tissue, they can cause more chronic conditions such as cystic fibrosis, burn wound infections and complications in tissue transplants (Bjarnsholt, 2013). Some clinical infections occur when contaminated abiotic surfaces encounter human tissues such as the use of catheters, artificial bone and tissue transplants (Bjarnsholt, 2013).

Biofilms are more difficult to eradicate than planktonic cells because the biofilms are composed of layers of bacteria and exopolysaccharide matrix that can withstand antimicrobial activity (Kolmos et al, 1993). Biofilms also have the tendency to form over time in rubber and plastic tubing if left unwashed for a given period. Hospital water from contaminated tubing was suspected of being the source of a *P. aeruginosa* outbreak on 3rd degree burns patients shortly after being admitted in the hospital. Proper disinfection of the tubing used in hydrotherapy of burns patients resulted in a decrease in new cases (Kolmos et al, 1993; Tredget et al, 1992). The stages of biofilm development are illustrated in Figure 1-12 below.

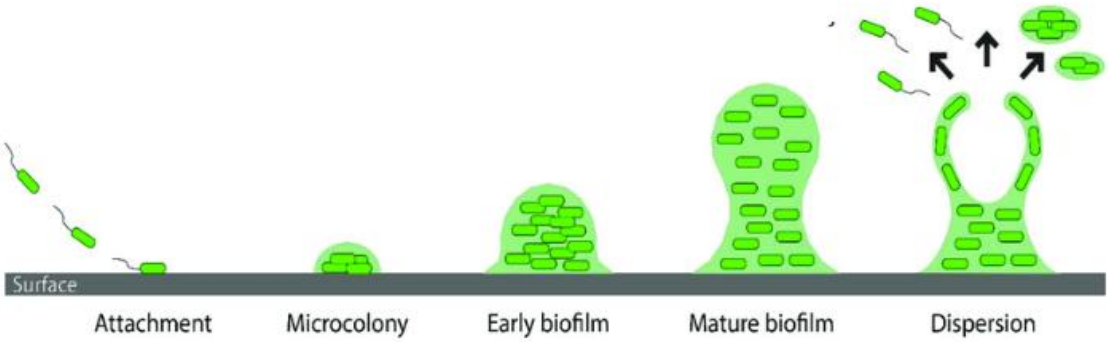


Figure 1-12. Stages of Biofilm development

(i) Initial attachment of the cells on a substratum or surface marks the first stage of biofilm formation. (ii) The second stage involves the continuous growth of the cells to form a dense structure with multiple layers. (iii) At the third stage, termed the maturation stage, the biofilm forms a dense extracellular polysaccharide matrix which is secreted by the bacteria (iv) Stage four, the formation of water channels and quorum sensing molecules begins in the mature biofilm. (v) The last and final stage, parts of the biofilm become loose and disperses to form new microcolonies. Figure adapted from (da Silver et al, 2017)

Biofilms have the tendency to survive, to a greater degree than planktonic cells, in low nutrient and oxygen deprived environments. They can also protect their inhabitants from harsh environmental conditions such as pH, temperature and desiccation as a form of adaptive mechanism (Stewart, 2002). This therefore prevents total eradication of biofilm bacteria. Cooperation between members of a biofilm community can influence an exchange of beneficial genetic traits amongst each other. Alongside mutualism and syntrophism, cheat associations may also occur whereby one strain is benefitting without contributing to the survival of the biofilm community. In vitro, it has been shown that during quorum sensing, signals are sent to *P. aeruginosa* populations to produce enzymes to degrade a certain available substrate. Other bacteria may not respond to this signalling and still make use of the available resources provided by *P. aeruginosa* (Dandekar et al, 2012; Davey and O'Toole, 2000; Popat et al, 2012). Another example is a strain that produces oxygen as a

product of metabolism may influence the growth of aerobic opportunistic pathogens that find themselves in an anaerobic biofilm environment.

1.8.1 Susceptibility of Biofilms to Biocides

Besides the high tolerance of biofilms to antibiotics, biofilms have become increasingly resistant to many biocides (Russell, 2002). The genetic composition of bacterial strains in a biofilm play a major role in determining their survival in the presence of biocides. Some factors that contribute to biofilm survival include change to the physical structure of the biofilm, expression of multidrug efflux pumps and modification of the cell membranes (Fux et al, 2005).

1.8.2 Bacterial conjugation in biofilms

Biofilms provide a niche that could promote horizontal gene transfer by conjugation since the bacterial cells are packed close to each other. The ability of a strain to form a biofilm therefore increases its plasmid transfer potential in the environment (Ghigo, 2001).

While some studies have showed that rates of conjugation are higher in planktonic cells than on a solid agar surface (Freese et al, 2014), others have observed that the transfer efficiency of plasmids in either biofilms or suspension may be specific to the class of plasmids. For example, IncP-1 plasmids such as RK2 are known to transfer better on a solid surface than in suspension (Thomas, 2014). Methods of detecting plasmid transfer efficiency have become more advanced and therefore give an accurate estimation of transfer rates. The population of transconjugants observed using fluorescence scanning microscopy superseded the numbers observed through plating on selective media (Hausner and Wuertz, 1999).

Epilithons are a type of biofilm that grows on the surface of rocks in an aquatic environment. They are made up of a vast variety of microrganisms such as bacteria fungi and algae. Plasmid transfer via conjugation has been documented in marine bacteria on epilithons found in rivers (Haines et al, 2006).

1.9 Water and wastewater treatment and the spread of resistance

The accumulation of antibiotics from both clinical settings and high cell density of bacteria from both natural (soil) and the biological treatment of wastewater in such wastewater treatment plants (WWTP) may lead to proliferation of multidrug resistance bacteria in water systems (Rizzo et al, 2013). If the tertiary stage of wastewater treatment fails due to biocide resistance, the risk of exposing antibiotic and biocide resistant genes to the environment may lead to a public health crisis. The WWTP serves as a middle channel where all forms of wastewater carrying resistance genes could be linked with domestic water supply as illustrated in Figure 1-13 below.

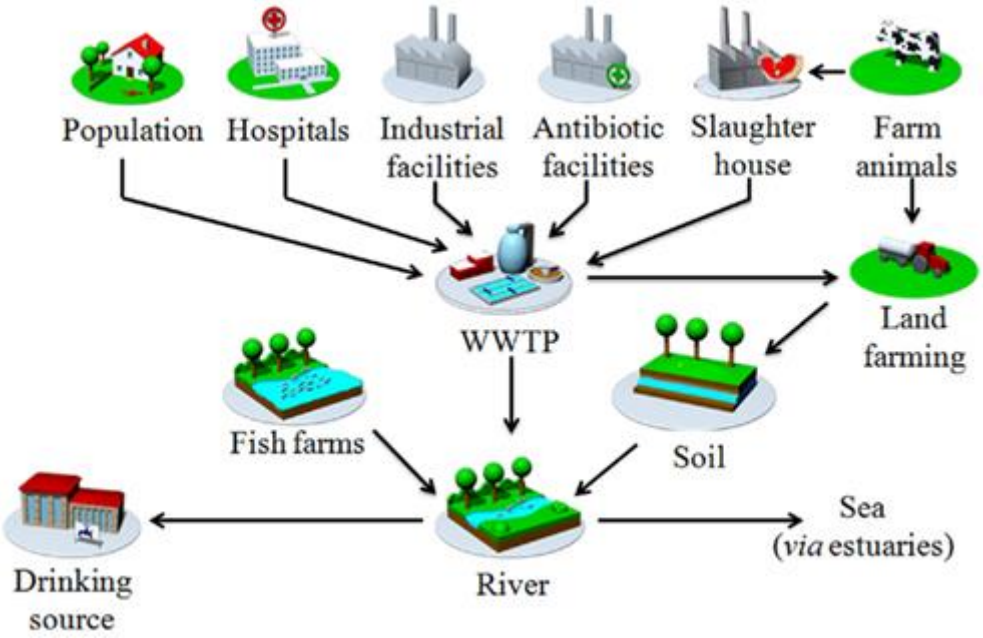


Figure 1-13. Wastewater treatment and spread of resistance genes

The wastewater treatment plant has the highest concentration of antibiotic resistant bacteria since it collects waste from hospitals to farmlands. Recycled and treated water from these plants can still harbour resistance genes which return to the natural environment. Figure adapted from (Stalder et al, 2012).

1.9.1 Water as a source of nosocomial infections

Nosocomial infections from *Legionella pneumophilla* and *Pseudomonas aeruginosa* have been commonly linked with the hospital water supply. Treated tap water in hospital units have been reported to cause *Pseudomonas aeruginosa* outbreaks in Intensive Care Units and Burns Units due to the frequent contact with hospital water to immunocompromised patients and exposed wounds respectively (Trautmann et al, 2005; Anaissie et al, 2002).

A study showing the Whole Genome Sequencing of isolates from a Burns Unit in a new hospital identified the water supply as the main source of *Pseudomonas sp* contamination and dissemination possibly via a breeding biofilm within plumbing parts (Quick et al, 2004). Figure 1-14 shows the various routes by which water could be a source of nosocomial

infection in a hospital setting. Various prevention and control measures are routinely put in place to mitigate possible causes of *Pseudomonas* contamination in the clinical setting. Such measures include monitoring shower flow rates, amendments to previous cleaning protocols and the redesign of plumbing parts and materials (Breathnach et al, 2012).

PCR assays have been used to detect *Pseudomonas aeruginosa* in hospital water (Baghal Asghari et al, 2013). Despite so many studies on prevalence of *Pseudomonas aeruginosa* in hospital water, chlorinated or treated water has not been associated with the enrichment of *P. aeruginosa* biofilms in hospital water supply in the UK (Curran, 2012).

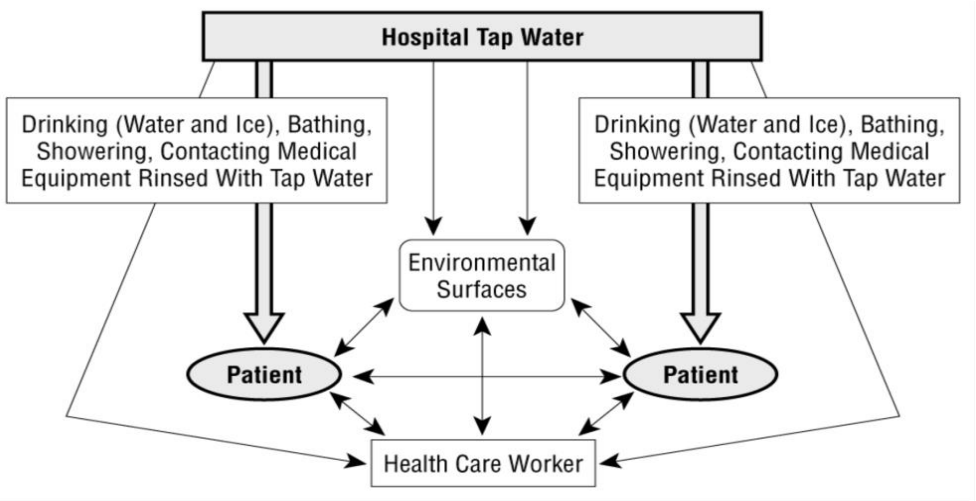


Figure 1-14. Transmission of waterborne pathogens in the hospital setting

Water borne outbreaks in health care settings are transmitted either directly from the water source itself or indirectly through contact with hospital equipment or from other patients, medical personnel, and cleaning staff. The mode of transmission is interchangeable between these links and can therefore be acquired from source to patient or from the patient to the water source. Figure adapted from (Anaissie et al, 2002)

1.9.2 Chlorine dioxide water treatment

Chlorine dioxide was first discovered in 1925 and was first used as a disinfectant in 1946 for antimicrobial purposes (Russell, 2002). Although it is commonly produced for use in paper and pulp manufacture, it is still the most used water treatment strategy at the tertiary

stage of the water purification. One of the reasons chlorine dioxide is often preferred over chlorine as a biocide is because it does not react with ammonium to form trihalomethanes and other toxic by-products as chlorine does. It also exists in its unchanged chemical state when dissolved in water (Russell, 2002).

However, the use of chlorine dioxide in these processes gives rise to the production of chlorite and chlorate in the environment. This occurs from the hydrolysis of chlorine dioxide to chlorite and chlorate ions (Kaczur and Cawlfield, 2000). The concentration of chlorite in water has been thought to contribute to premature delivery in pregnant women in Italy and chlorite from sampled drinking water consumed by a group of pregnant women showed that levels of chlorite exceeded the limit allowed by the Italian guidelines (Aggazzotti et al, 2004).

The efficacy of 100 ppm chlorine dioxide for 15 seconds on multi drug resistant strains of commonly encountered nosocomial bacteria such as *Pseudomonas aeruginosa*, *Acetinobacter baumanni* and *Staphylococcus aureus (*MSRA) was far greater than the use of hypochlorite with the same concentration (Hinenoya et al, 2015). Its efficacy may vary based on the material or bacteria to be disinfected. For instance, in one study, hypochlorite was found to be more effective than chlorine dioxide against Enteric bacteria isolated from settled hospital water sludge (Tsai and Lin, 1999).

1.9.3 Contamination of treated hospital water supply

The discovery of antibiotic resistance genes in chlorinated water supply has been widely reported (Shi et al, 2013, Schwering et al, 2013, Sorlini et al, 2014, Murray et al, 1984). It has also been documented that chlorination decreased the population of bacteria in sewage effluents while increasing the proportion of surviving bacteria carrying antibiotic resistance

genes (Murray et al, 1984). Metagenomic analysis of chlorinated drinking water and tap water showed that chlorine disinfection greatly contributed to the plasmid-mediated concentration of antibiotic resistance genes in water (Shi et al, 2013). Another study showed that antibiotic resistance genes from waste-water effluents are activated in the presence of chlorine stress over a period (Huang et al, 2011). These findings greatly encourage a modified method of tertiary disinfection of water especially in clinical settings.

A 17-month study investigating the effectiveness of chlorine dioxide treatment of hospital water on Legionella showed that only the ward farthest from the chlorine dioxide source still showed survival of the bacteria after chlorine dioxide treatment. Chlorine dioxide levels in the hospital water were no higher than 0.8ppm. On initial treatment, chlorine dioxide and chlorite levels were relatively equal, and their concentration decreased with increase in the distance from the source, but over time this stabilized in the water sourced from both upper and lower levels of the hospital building (Srinivasan, 2003).

1.9.4 Mechanisms of action of chlorine dioxide as a biocide

One of the reasons why chlorine has been largely replaced by chlorine dioxide in water treatment is due to the development of chlorine resistant bacteria. Some levels of chlorine resistance have been seen in species of *Pseudomonas, Serratia* and other Gram-negative bacteria such as some strains of *E. coli* (Charlton, 1933). Chlorine dioxide has been found to be effective against a wide range of microorganisms including bacteria (Knapp and Battisti, 2001), viruses (Li et al, 2004) and protozoa (Korich et al, 1990).

In bacteria, the enzymes necessary for cell membrane synthesis are disrupted by the action of chlorine dioxide leading to a defective membrane formation (Knapp and Battisti, 2001). In spore forming bacteria such as *Bacillus subtilis*, chlorine dioxide only causes damage to

the cell membrane rather than DNA as with vegetative bacteria. Nevertheless, spore formers such as *Clostridium perfringens* are usually less resistant to chlorine dioxide than other non-spore formers. It has also been found that chlorine dioxide is less effective on bacterial biofilms that have been detached from their substratum than planktonic cells (Behnke and Camper, 2012). Slow growing planktonic strains in their stationary phase of growth tend to be less susceptible to chlorine dioxide than bacteria in logarithmic phase of growth (Taylor et al, 2000).

1.10 Background of study

A whole genome sequence of environmental and clinical isolates was investigated to determine the origin of *Pseudomonas* infections of the Queen Elizabeth hospital in Birmingham. Two different IncP-1 plasmids designated pBURNS1 and pBURNS2, were identified from *Pseudomonas aeruginosa* strains from genetic sequences and assembled contigs from the Burns unit. Thirteen strains carrying pBURNS1 were isolated from room 8 whereas only two strains were found carrying pBURNS2 and these came from room 9 of the Burns unit (Quick et al, 2014). The pBURNS1 plasmid further analysed and was found to have a putative *cdm* gene that encoded resistance to chlorite (Aggarwal, 2014).

Figure 1-15 shows the distribution of pBURNS1 plasmid based on the phylogenetic reconstruction of the clade E isolates in room 8. Although none of the patients sampled carried the same strain with the plasmids, the IncP-1 plasmid exhibits high stability and has a broad host range, therefore it is necessary to determine if this plasmid can spread to other existing bacteria through horizontal gene transfer in the biofilm and to what frequency it achieves this.

Several isolates from room 8 carried pBURNS1, an IncP-1β plasmid which had a chlorite dismutase gene (*cdm*). The *cdm* gene confers resistance to chlorite, which is toxic to the host cell, by breaking down chlorite into chloride and oxygen. It is believed that the immediate source of these isolates could be from a biofilm in the shower plumbing because the plasmid carrying strain was found also isolated from the water source and on surfaces around the room.

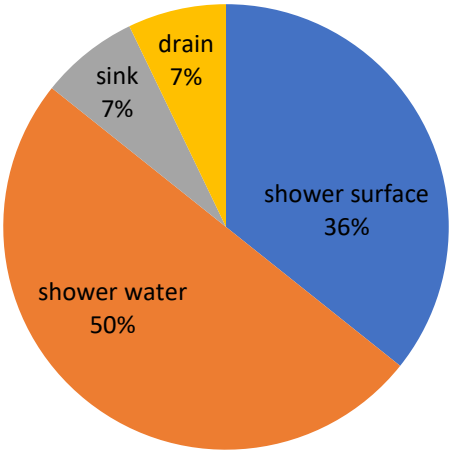


Figure 1-15. A pie chart showing the distribution of different environments where pBURNS1 was isolated in room 8 of the Burns unit, Queen Elizabeth Hospital, Birmingham. *Data for chart adapted from (Quick et al, 2014)*

1.11 The Aims of this Research

Research has shown that presence of biofilms in hospital plumbing and water distribution systems may play a role in prevalence of nosocomial Pseudomonal infections among patients (Walker et al, 2014). Treatment of water by chlorination has been known to concentrate bacteria in water supply and thus some bacteria carrying resistance plasmids may thrive better than bacteria without resistance determinants. Plasmid borne resistance in

hospital settings is a threat to human health since it could lead to outbreaks of antibiotic and biocide resistance infections among susceptible patients. The biofilm-forming potential of *Pseudomonas aeruginosa* may make the bacterium less susceptible to antibiotics and biocides such as chlorine dioxide treatment. Such biofilm environment may select for the biocide and transfer genetic traits amongst existing members of the biofilm thereby complicating antimicrobial therapy (Schwering et al, 2013).

This research is aimed at understanding the significance of a chlorite dismutase gene carrying plasmid in the hospital plumbing system especially in the water supply seem to harbour surviving bacteria containing this IncP-1β plasmid named after the unit it was found, pBURNS1.

It is hypothesized that the chlorite dismutase gene present in pBURNS1 plasmid would be functional by conferring resistance to chlorite in its host strain and may enable survival of other bacteria carrying other resistance genes when present in a biofilm. Secondly, the investigation of phylogenetic traits of pBURNS1, the class 1 integron and the chlorite dismutase protein will determine the epidemiological origin and relative significance of its occurrence in clinical settings.

Thirdly, the chlorine dioxide treatment when in contact with the biofilm containing pBURNS1, will break down to chlorite and may allow the proliferation of bacteria carrying this plasmid in the system. Thus, the presence of pBURNS1 may be explained by the resistance it confers to chlorite and the selective pressure created by water treatment in the QE Hospital Birmingham. Other bacteria that do not have the plasmid are expected not to survive treatment.

Lastly, I hypothesize that chlorite will select for the products of pBURNS1 transfer. Hence, in a biofilm containing pBURNS1 there will be an increased survival of donors and transconjugants in the presence of chlorite and eventual loss of plasmid-free recipients without the plasmid.

Specific Objectives of this research:

1. To determine if chlorite can select for the transconjugants obtained from pBURNS1 transfer in both biofilm and planktonic environments.
2. To investigate the Cdm phenotype in both the original strain *P. aeruginosa* 943 and in *E. coli* and phylogenetic origin of *cdm* in *P. aeruginosa* 943.
3. To investigate the advantage the *cdm* gene in pBURNS1 gives its host in a hospital setting
4. To investigate the level of survival of plasmid-carrying biofilms in hospital biocides such as chlorite and chlorine dioxide
5. To determine if the pBURNS1 plasmid promotes biofilm production

Chapter 2

Material and methods

2.1 Bacterial strains, growth conditions and plasmids

The work described in this thesis used both *E. coli* and *P. aeruginosa*. The specific bacterial strains used are listed in Table 2-1 below. All antibiotics and chemicals used otherwise stated were obtained from Sigma Aldrich, UK. A list of plasmids used in this study are listed in Table 2-2.

Table 2-1. Bacterial strains used in this study

Species	Strain	Genotype	Selective marker	Reference
E. coli	C600	F- *ton*A21 *thi*-1 *thr*-1 *leu*B6 *lac*Y1 *gln*V44 *rfb*C1 *fhu*A1 λ-	-	Appleyard, 1953
E. coli	C600(pBURNS1::Tn*1723*)	(pBURNS1::Tn*1723*) transformants	Kan^R	Aggarwal, 2015
E. coli	MV10 Nal^R	C600 *trp*E5 Nal^R	Nal^R	Hershfield et al, 1974
E. coli	MV10Nal^R (pBURNS1::Tn*1723*)	(pBURNS1::Tn*1723*) transconjugants	Kan^R	Aggarwal, 2014
E. coli	S17-1	*pro* Δ*hsd*R *hsd*M^+ *rec*A Tp^R Sm^R ΩRP4-Tc::MuKn::Tn*7*	Tp^R Sm^R	Simon et al, 1984
E. coli	DH5α	*sup*E44Δ*lac*U*(ϕ80lacZ*ΔM*15) hsdR17 rec*A*1 end*A*1 gyrA96 thi-1 relA1*	Nal^R	Invitrogen
E. coli	DH5α (pBURNS1::Tn*1723*)	(pBURNS1::Tn*1723*) transformants	Kan^R	This study
P. aeruginosa	943	Wild type, clinical naturally occurring strain carrying pBURNS1	-	Quick et al, 2014
P. aeruginosa	943 Cured strain	Isogenic original strain cured of pBURNS1 by a pCURE plasmid	-	Aggarwal, 2015
P. aeruginosa	PAO11061 Rif^R	*leu* r^- Rif^R	Rif^R	Lasocki et al, 2007
P. aeruginosa	PAO11061 Rif^R (pBURNS1)	*leu* r^- Rif^R carrying pBURNS1 transconjugants	Rif^R	This study

Because pBURNS1 does not encode any resistance genes, Nikhil Aggarwal (2015) previously tagged it with a Kanamycin resistance gene in transposon Tn*1723* to generate pBURNS1::Tn*1723* allowing easy selection after transfer by transformation or conjugation (Table 2-2). *P. aeruginosa* 943 is the original strain with pBURNS1 isolated from room 8

of the Burns Unit of the Queen Elizabeth Hospital, Birmingham and was cured using a pCURE 12 plasmid (Aggarwal, 2015). This renders the strain plasmid-free and was used as a comparison to the original strain in this work. This cured strain is referred to as *P. aeruginosa* 943 (cured). The name pBURNS1::Tn*1723* is used in describing the tagged plasmid in specific experiments, pBURNS1 refers to the untagged version used in the Bioinformatics work, however, where it is clear that conclusions will also apply to the parent plasmid, pBURNS1 is used in the rest of the report.

Table 2-2. List of plasmids used in the study

Plasmid	Description	Source/Reference
pBURNS1	IncP1β plasmid with cdm gene on an integron.	Quick et al, 2014
pBURNS1::Tn*1723*	pBURNS1 tagged with Tn*1723* to provide Kanamycin resistance	Aggarwal, 2015
pCDM1	cdm1 promoter cloned into pJH10 derivative pMUP9 upstream of *lacZ* gene	This study
pCDM2	cdm2 promoter cloned into pJH10 derivative pMUP9 upstream of *lacZ* gene	This study
pMUP9	*mupZ* promoter cloned into pJH10 derivative pMUP9 upstream of *lacZ* gene	Connolly et al, 2019

2.2 Growth media

Both *E. coli* and *P. aeruginosa* strains were grown in either Luria Broth (LB) or Luria agar (LA) which is Luria broth added to agar (1.5% wt/vol) (Sigma-Aldrich Ltd, UK, Cat. no. L2897) prepared and autoclaved from the Central services (School of Biosciences, University of Birmingham) or M9 medium with the essential amino acids needed. All strains were grown at 37ºC with or without shaking at 200rpm. The recipes for Luria Broth and M9 broth (400ml) are shown below.

Luria Broth

Prepared in sterile glass bottles, Luria broth is made up of yeast extract 10g/l, tryptone 5g/l and NaCl 5g/l (Bertani, 1951).

M9 Broth (For *E. coli* strains)

M9 broth is made up of 200ml sterile distilled water, 200ml M9 salts which comprises of Na_2HPO_4-$2H_2O$ (75.2 g/L), KH_2PO_4 (30 g/L), NaCl (5 g/L) and NH_4Cl (5 g/L). To prepare a stock of 10x M9 salt solution, 2ml sterile 40% Glucose, 1ml Thiamine 4% (w/v), 400 µl 50 mg/ml Leucine (50 µg/ml), 400 µl 50 mg/ml Threonine (50 µg/ml), 400 µl $MgSO_4$ (1M), 400 µl 50 mg/ml Tryptophan (50 µg/ml) (for MV10 Nal^R only) and 400 µl 100 mM $CaCl_2$ (1.0 mM) is required (final concentrations in brackets).

Nalidixic acid (Nal) of final concentration 25 µg/ml and Kanamycin (Kan) final concentration of 50 µg/ml were added to either L-broth or L-agar to select for *E. coli* MV10 Nal^R and *E. coli* C600 (pBURNS1::Tn*1723*). 100 µg/ml final concentration of Kan was used to select for *P. aeruginosa* 943(pBURNS1::Tn*1723*).

2.3 Plasmid DNA isolation

2.3.1 Maxi prep plasmid isolation method

2.3.1.1 Preparation of solutions

This method is based on the original alkaline-SDS lysis method of Birnboim and Doly (1979). Preparation of solutions that were needed for the maxi-prep was done before starting the experiment. These include Resuspension buffer, NaOH- SDS (4M NaOH/ 2% SDS), 3M Sodium Acetate pH 5.0, 1x TNE solution (above mentioned all autoclaved before use), Ethidium Bromide solution and Water/CsCl saturated isopropanol.

Lysis buffer

Lysis buffer is prepared by adding 50 mM glucose, 10 mM EDTA and 25 mM Tris HCl pH 8.0. 3M sodium acetate was pH corrected with 11.5% v/v acetic acid and sterilized by autoclaving.

TNE buffer

TNE buffer was made by preparing 10x TNE stock and then diluted to 1 x TNE (working solution) by adding 9 parts of SDW to 1-part 10x TNE. 1X TNE was prepared by adding 100 mM Tris HCl pH 7.5, 50 mM NaCl and 5 mM EDTA.

NaOH-SDS mix

NaOH-SDS mix (0.2M NaOH, 1% w/v SDS) was prepared by adding 1 part 0.4M NaOH and 1 part 2% w/v SDS.

Ethidium bromide solution

Ethidium bromide solution was prepared by adding 10mg ethidium bromide pellets to 1ml sterile distilled water. The container this is prepared in is labelled as toxic and wrapped in foil due to its light sensitivity.

Water/CsCl saturated isopropanol

This solution was prepared by adding 0.6 volumes of Isopropanol (54 ml), 1.06 g of CsCl per 1 ml of sterile distilled water

2.3.1.2 Maxi Plasmid DNA preparation

DNA can be obtained in a large scale by using maxi-prep plasmid purification. It is based on the alkaline lysis method (Smith and Thomas, 1983) producing large quality and quantity plasmid DNA. It involves growing bacterial cells in liquid media, and by DNA replication, the plasmids are produced in a large quantity, harvested and then subjected to alkaline lysis. This is followed by salt neutralization, debris separation from purified plasmid by centrifugation. Caesium ions creates a gradient which allows slightly different densities of chromosomal and plasmid DNA molecules to accumulate each form a band at a density region. Ethidium bromide binds to DNA making it visible under UV light but also decreasing its buoyant density. Covalently closed circular plasmid DNA cannot bind as much ethidium bromide as linear (chromosomal fragments) so the two types of DNA form bands at different densities.

2.3.1.3 Alkaline SDS method

A 400 ml overnight culture of a plasmid carrying strain was grown with selection with antibiotics and was centrifuged at 6,000 *g* for 10 mins at 4ºC. The pellet was resuspended in

25 ml lysis buffer. To this, 50 ml of freshly made NaOH-SDS was added and mixed vigorously. It was then left on ice for 10 mins. To the solution, 37.5 ml of 3M sodium acetate was added and inverted 5 times. It was left on ice for 15 mins after which it was centrifuged at 10,000 *g* for 20 mins at 20ºC to remove proteins and cell debris which contains chromosomal DNA. The supernatant was filtered through a 1MM Whatman filter into an empty centrifuge pot containing 100 ml of isopropanol, mixed properly and stored at -20ºC.

The pots were further centrifuged at 10,000 *g* for 15 mins at 4ºC, the supernatant discarded, and the pellet dried off.

2.3.1.4 Caesium chloride gradient method

The pellet from the alkaline-SDS method above (2.3.1.3) was resuspended in 3 ml 1x TNE solution (100 mM Tris pH 8.0, 50 mM NaCl, 5 mM EDTA pH 8.0) and transferred to a tube containing 4.62g caesium chloride. This was dissolved by shaking gently and topped up to 4.5 ml with additional TNE. To the same tube 0.5 ml of ethidium bromide solution was added and mixed. After this had dissolved, the contents were transferred to a 2X Beckman ultracentrifuge tubes. The tubes were heat sealed and centrifuged at about 100,000rpm (550,000 *g*) overnight at 20ºC on a Beckman TL 100 centrifuge. Once spun, the tubes were visualized by UV illumination and the plasmid DNA was collected using a syringe and needle.

Purification of the DNA sample began with the removal of ethidium bromide from the samples using isopropanol saturated with water and caesium chloride (CsCl) until all the ethidium bromide had been removed from the top pink layer. The remaining DNA was transferred to a 1.5 µl centrifuge tube and 50 µl SDW, 100 µl sodium acetate and 530 µl isopropanol was added to it. It was centrifuged at 11,000 *g* for 5 mins and the supernatant

discarded. The resulting pellet was dissolved in 200 µl 1X TNE (100 mM Tris pH 8.0, 50 mM NaCl, 5 mM EDTA pH 8.0), 25 µl 3M sodium acetate and 500 µl 70% ethanol (v/v) and mixed. Finally, the tube was centrifuged for 10 mins at 11,000 *g* and the supernatant discarded. The pellet was dried and resuspended in 1:10 1X TNE. The resulting plasmid DNA was stored at -20ºC for further use.

2.3.2 Mini prep isolation method

The mini prep plasmid isolation method is based on the same principles as the maxi prep isolation method differing mainly by using proprietary solutions. Usually preps obtained using this method are smaller in quantity and of less quality than maxi preps. However, it is a faster and easier way of isolating plasmid DNA for genetic manipulations if the method is properly followed.

The kit used for all plasmid preps in this study was the Isolate II Plasmid Mini Kit (Bioline, UK). The protocol for low copy number plasmid isolation was followed for isolating pBURNS1::Tn*1723* (5-7 copy number) according to the manufacturer's instructions. The bacterial cells were harvested by centrifuging 5-10 ml of overnight *E. coli* suspension for 30 seconds at 11,000 *g*. The supernatant was discarded and 500 µl of resuspension buffer was added and vortexed. The bacterial cells were lysed by adding 500 µl of lysis buffer and mixed gently. Afterwards, a neutralization buffer was added and mixed thoroughly.

The lysate was clarified by centrifuging for 10 mins at 11,000 *g* at room temperature. DNA was bound by decanting 750 µl of clarified solution into a spin column, followed by the washing of the silica membrane by adding 500 µl of wash buffer (which was previously preheated to 50ºC) and centrifuged for 1 min. The wash process was repeated using 600 µl wash buffer with ethanol and centrifuged for 1 min. The flow-through was discarded and

the silica membrane dried by centrifuging for two mins. The mini column was placed in a micro centrifuge tube and 50 µl of P buffer preheated to 70ºC was added. It was then centrifuged for 1 min at 11000xg. The resulting plasmid DNA was split into aliquots and stored at -20ºC.

2.4 Conjugation

Conjugation experiments were conducted in order to transfer plasmid from *E. coli* C600(pBURNS1::Tn*1723*) into *E. coli* MV10nal^{R}. The pBURNS1 plasmid was previously tagged with a kanamycin resistance gene present in the Tn*1723* transposon for selection purposes and was designated as pBURNS1::Tn*1723* (Aggarwal, 2015). The pBURNS1::Tn*1723* plasmid was transformed into *E. coli* C600 to be used as the donor strain. The recipient strain, *E. coli* MV10nal^{R} has a chromosomally-encoded resistance to nalidixic acid. After conjugation, the transconjugants were plated on LA plates containing both KAN (50 µg/ml) and NAL (25 µg/ml).

Both a filter-based conjugation method and spot-based method was used to transfer pBURNS1::Tn*1723* plasmid from a donor strain to a recipient strain. Positive and negative controls were also conducted by screening donor and recipients on each selective agar used.

2.4.1 Filter-based method of conjugation

For the filter-based method, a 0.2 µm Millipore filter was used. Aliquots of donor and recipient overnight culture were added to a single universal bottle in the ratio of 1:10. The bottle was gently swirled, and the mixture transferred into a sterile syringe. The Millipore filter was uncapped and placed above an empty universal bottle to capture the supernatant. The liquid was then injected through the filter with the bacteria being collected on the top of the filter. The supernatant was discarded and using sterile forceps, the filter was carefully

placed on an L-agar plate without any antibiotics and incubated at 37ºC for 3-4 hrs. After incubation, the bacteria remaining on the filter were resuspended in normal saline, serially diluted and then plated on antibiotic selective plates to determine the number of Colony Forming Units (CFU).

2.4.2 The spot-based conjugation

The spot-based conjugation method was carried out by spinning down 1 ml of overnight cultures and resuspending in 1 ml of normal saline then placing 10 µl of donor and recipient strain on top of each other on a properly dried LA plate (48ºC in the oven for 15 mins) either overnight at or incubated for about 3 hrs at 37ºC. The spot was then re-streaked on an LA plate containing the appropriate antibiotic to select for the transconjugants.

2.5 Preparation of Competent cells and Transformation

Transformation is a method of horizontal gene transfer where DNA is taken up by competent bacteria through their permeable membrane. For efficiency, the cells need to be competent to improve the chances of obtaining successful transformants.

2.5.1 Preparation of competent cells

The preparation of competent cells begins with a 5ml starter overnight culture which was diluted 1:100 e.g. 250 µl in 25 ml broth in a 250 ml flask and grown at 37ºC at 200rpm until an OD_{600} of 0.4-0.6 was reached. A bottle of 100mM Calcium chloride was placed on ice to cool. The cells were pelleted in a 4ºC centrifuge for 7 mins at 5,000 g using a swing out rotor. The supernatant was discarded, and the pellet was resuspended in pre-chilled $CaCl_2$ of a volume of 2 ml per 5 ml culture or 10 ml for a 25 ml culture. The tubes were vortexed to resuspend the pellet and the cells were incubated on ice for 20 mins. After incubation, the

cells were placed back into the centrifuge and set to pellet for 7 mins at 4ºC at 5000 g. The supernatant was again discarded, and the pellet resuspended by shaking gently in pre-chilled 100mM $CaCl_2$ at a volume of 0.5 ml per 5 ml bacteria culture (Nakata et al, 1997). The competent cells were stored at 4ºC and used within a period of two weeks.

2.5.2 Transformation

Transformation of pBURNS1::Tn*1723* plasmid into either *E. coli* or *Pseudomonas* strains was conducted during this project. To a microcentrifuge tube, 5 µl of plasmid DNA and 100 µl of competent cells was added. This mixture was left on ice for 30 mins and then heat shocked to allow movement of the DNA into the competent bacteria by placing in a 42ºC water bath for 45 secs. The microcentrifuge tube was then returned to the ice for 5 mins and then 1 ml of L-broth was added to the cells. It was placed on a shaker for 1 hour at 37ºC in a water bath and removed to be plated out on selective plates. The plates were incubated overnight at 37ºC.

2.6 Restriction digest and Gel Electrophoresis

2.6.1 Restriction digests

Restriction enzymes (class 2 restriction endonucleases) are used to cut DNA strands at a specific site for constructing new sequences and checking for correct DNA sequences.

Restriction enzymes were purchased from New England Biolabs (NEB) and the high-fidelity enzymes were preferably used if available to avoid use of two non-compatible buffers. The units of enzyme and amount of buffer were based on the manufacturer's instructions.

About 100 ng of plasmid DNA was pipetted into a microcentrifuge tube. For a 25 µl digest, a mix of 0.5 µl (or 5 units of DNA), 2.5 µl of 10x buffer and SDW up to 25 µl was made

and incubated for 1 hr in a 37ºC water bath. For cloning purposes, double the amount of constituents was used for a 50 µl restriction digest and incubated for longer 1.5-2 hrs in a water bath at 37ºC.

For the digest of pBURNS1::Tn*1723* DNA, a 20 µl total mix was made generically using 10 µl of DNA, 2 µl of Cutsmart Buffer, 1 µl each of SacI and AatII and 6 µl of SDW.

Afterwards, 4 µl of loading buffer was added, mixed briefly, centrifuged for a few seconds and then loaded on a 1% agarose gel to run for about 40 mins at about 120V in an electrophoresis tank. The gel was viewed under UV light using a Bio-rad Gel Doc™ XR+ system to detect the fragmented bands which should correspond to the lengths of DNA cut by the restriction enzymes. A negative control which differed from the final mix by replacement of DNA with SDW was included.

2.6.2 Agarose gel electrophoresis

Plasmid DNA isolation and PCR fragments were analysed using Agarose gel electrophoresis. To prepare a 1% w/v agarose gel, 100ml of 1x TAE buffer prepared from a stock solution of 50x TAE was added to a clean flask, 1g of agarose was added to the 1x TAE buffer and swirled gently. The mixture was heated and cooled before adding 2 µl of ethidium bromide (final concentration of 0.5 µg/ml). The flask was gently swirled again and poured into a sealed gel tray. The appropriate-sized combs were inserted into the gel. After the gel solidified, 1 x TAE buffer was poured into a gel electrophoresis tank and the gel tray carefully placed in the tank.

2.6.3 Preparation of DNA sample for gel electrophoresis

To visualize DNA (either after restriction digests, pure plasmid DNA or PCR fragments) through gel electrophoresis, a mix of DNA and loading dye was made for each of the samples placed in a microcentrifuge tube. Sterile distilled water (SDW) was added to the DNA made up to 20 µl. To each 20 µl tube, 4 µl of 6x purple loading dye loading dye (NEB) was added. Each tube was mixed and centrifuged briefly to ensure proper mixing. A 1kb DNA ladder O' Gene Ruler (Invitrogen) which serves as a DNA sizing ladder, was added to a well on the gel at a volume of 5 µl. In the other adjacent wells, depending on the amount needed, the DNA mix was individually added to a well. Electrophoresis was set up to run at 120V and the gel bands were visualized and photographed under UV light. The intensity of the bands may suggest the amounts of DNA present. The bands of interest were spotted by using the DNA ladder to access the correct size.

2.6.4 Purification of DNA from an agarose gel

After the gel electrophoresis has been completed, further cloning processes may require extraction of pure DNA either PCR products or fragments from restriction digests from the gel through Gel Purification. Under UV light, and using a protective face gear, the appropriate bands were identified based on size, and carefully cut out using a sharp blade and put in a 1.5 ml microcentrifuge tube. A gel purification kit, GFX™ PCR DNA and Gel Band Purification Kit (Sigma-Aldrich, UK) was used to recover the DNA. The gel slices were weighed and based on the weight, 30 µl capture buffer type 3 added for each 30 mg of gel slice was placed into the tube and incubated for 15-30 mins at 60ºC inverting after every 5 mins.

Once completely dissolved, the solution was transferred into a GFX MicroSpin column in a collection tube. and incubated at room temperature for one min then spun at 16,000 *g* for 30 seconds. The flow-through was discarded and 500 µl of Wash Buffer Type 1 was added to the column and spun again at 16,000 *g* for 30 seconds. The collection tube was discarded, and the column placed into a DNase free 1.5 ml microcentrifuge tube and then 20 µl of Elution Buffer Type 4 was added and incubated at room temperature for 1 min. The assembled column was spun at 16,000 g to recover the purified DNA. The DNA was stored at -20ºC for further applications.

2.7 Polymerase Chain reaction

Polymerase Chain reaction is an important tool in molecular biology that uses changes in temperature to amplify short sections of DNA into many identical copies. It involves denaturing at a high temperature of up to 98ºC to initially separate the double stranded DNA. This is followed by the annealing of two primers, a forward and a reverse primer at both ends of the defined segments on the template strands. The annealing temperature is ideally about 55ºC but determined by 5ºC below the calculated melting temperature of the primer from its complementary sequence). The next step is the elongation phase by DNA polymerase from the primers to generate the complementary strands in opposite directions using free deoxynucleotide triphosphates (dNTPs) present in the reaction mix. The cycle is repeated for 20-30 rounds with the same process which causes a doubling in number after each completed round (Figure 2-1). Velocity DNA polymerase (Bioline, London, UK), a thermostable DNA polymerase was used for all the PCR in this work.

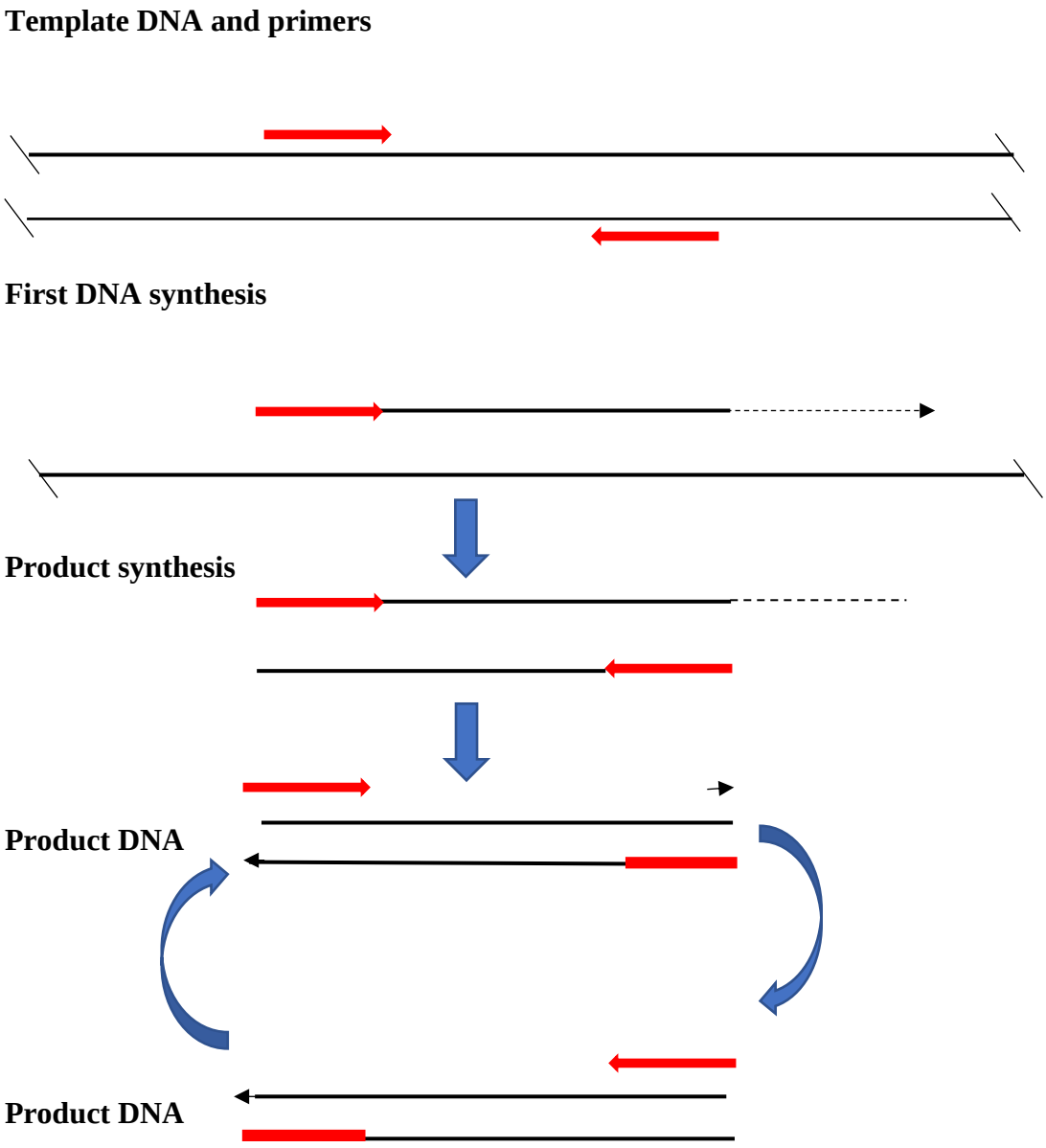


Figure 2-1. PCR amplification of DNA

For the first two cycles, the top and bottom strands are amplified to an unknown length. Subsequent cycles however will be of a definite shorter length since the newly synthesized strand is primed by the reverse primer up till the forward primer region. The complementary strands become templates for the generation of further copies and there is an exponential increment in the amount of DNA produced.

2.7.1 Primer Design

The primers were designed using the online Premier Biosoft International software, NetPrimer which automatically computes the melting temperature of primer sequences inputted. It also recognises potential problems with the intended primer sequence such as hairpins and low GC content and calculates the stability based on the sequence. Ideally, qualities of a good primer will include a length of about 17 bases, stable binding GC clamp at the 5' end, avoidance of more than 4 repeats of a base such as AAAA in the primer and at least 60% GC content. SnapGene (SnapGene Chicago, USA), a software used to analyse plasmid DNA sequences, was used in the creation of primer sequences and helped in avoiding multiple binding sites of the primer. The designed oligonucleotides were then synthesized by Alta Bioscience (Birmingham, UK). The primers used for this study are listed in Table 2-3 below.

2.7.2 PCR Reaction

The Thermocycler used was the Senso Quest Thermocycler (Gottingen, Germany). PCR reactions were made up from a master mix of 50 µl. Typical reactions consisted of 1 µl of Velocity DNA Polymerase, 1 µl of template DNA solution, 5 µl of stock dNTP solution (2.5 mM each A, G, C, T), 2 µl forward primer (10pmol) and reverse primer (10 pmol), 10 µl of 5x Buffer (including Mg^{2+}) 1.5 µl of DMSO and sterile distilled water to make a total of 50 µl reaction mix.

Table 2-3. List of Primers used in this study

Name	Sequence 5'-3'	Tm (ºC)
forward	ATTGAATTCGGCAGATCCACACACAGCAC	59.01
reverse	TCCGGATCCGTGTTCGGTATTTTTGCCGC	60.68
pCDMForward1	*gcacaattGCACGAACCCAGTGGACATAC	60.04
pCDM Reverse1	gctaagcttTGCTCTAAATGTCTAACTTTGTTTTCG	61.35
pCDM Reverse2	tcacccgggCTCGGAGCCGCCAATAAATG	63.02
Rabiat lacZ Reverse	CGTAGGTAGTCACGCAACTCGC	62.52

*lower case letters represent restriction sites that have been added at the 5' end of the primer while Upper case letters are the annealed primer sequences

2.7.2.1 Template DNA

The template DNA was either plasmid from a boiled prep from whole bacteria carrying the plasmid or from a purified plasmid prep. For the boiled prep, a grown colony or patch of bacteria containing the plasmid was taken from an agar plate using a sterile toothpick and was suspended in 50 µl sterile distilled water in a 0.2 ml PCR tube (Fisher Scientific, Loughborough, UK). It was then boiled for 10 mins at 98ºC and afterwards centrifuged at 11,000x g for 1 min in a MiniSpin benchtop centrifuge (Eppendorf, Hamburg, Germany) and 1 µl of the supernatant used as a template for PCR. If purified plasmid DNA was used, it was diluted to 1:1000 because it was very concentrated and 1 µl of the diluted solution used as template. The conditions for PCR are shown in Table 2-4.

Table 2-4. PCR conditions

Steps	Velocity Polymerase		
Initial Denaturation	98ºC	2 min	
Denaturation	98ºC	30 sec	20-30x
Primer Annealing	Tm- 5 ºC	30 sec	
Elongation	72ºC	15 sec/Kb	
Final Elongation	72ºC	7 mins	

2.8 Ligations

Ligations were performed to create new plasmids using the enzyme DNA ligase to join the insert and vector together. Prior to ligation, restriction digests were performed both on the plasmid of interest (Vector) and the fragment to be inserted with the same enzymes. A mixture of 50 ng of vector with a molar ratio of 3:1 (insert to vector), 1 µl of T4 DNA ligase (Promega, Madison, USA), 2 µl of 10x ligase buffer and SDW up to a total volume of 20 µl was found to be favourable for ligation to take place. The mixture was left overnight at room temperature or 4ºC and 5-10 µl of the ligate transformed into competent bacterial cells and plated on L-Agar containing the appropriate selective antibiotics.

2.8.1 pGEM-T ligation

Occasionally, during failure of direct ligation of PCR product to vector, the fragment was first was cloned into a commercial vector, pGEM-T which carries an ampicillin resistance gene and *lacZ* gene and then later digested with specific digestive enzymes found on the pGEM-T plasmid alongside the A-tailed fragment to be cloned. A-tailing was achieved by following the standard Taq polymerase procedure using the insert fragment as template but

only adding a single nucleotide, deoxyadenosine triphosphate to the mix. The PCR reaction was run at 72ºC for 20 mins in a thermocycler. Once the cycles have been completed, the product is run on a gel and purified. The pGEM-T mix includes, 4 µl of the purified A-tailed insert, 1 µl of linearized pGEM-T easy ligase, and 6 µl of ligase buffer provided by the manufacturer. The mix was left overnight at room temperature then transformed into competent DH5α cells and plated on Ampicillin and X-gal IPTG L- agar plates. The blue-white screening was used to detect for positive ligations. The white or pale blue colonies indicate that there has been a disruption of the *lacZ* gene due to the disruption of the insert while the dark blue colonies indicate there has not been disruption of the *lacZ* gene by the insert and therefore expression of the β-galactosidase will occur giving off a blue coloured end product of the substrate.

2.9 Growth curve experiments

To determine the effect of chlorine dioxide or chlorite on planktonic bacteria carrying pBURNS1 or pBURNS1::Tn*1723* as compared to their plasmid-free counterpart, the growth rate of the strains showing the lag, log and stationery phases of growth was carried out using a SPECTROSTAR nano plate reader (BMG Labtech, Ayesbury, UK). This was achieved by measuring the OD_{600} of the planktonic strains in a range of concentrations of the biocide while incubating at 37ºC for 13-14.5 hrs.

2.9.1 Preparation of Biocide Concentration

A stock of chlorine dioxide (ClO_2) was made to 12 ppm by dissolving one ClO_2 tablet (Lifesystems, UK) in 125 ml of sterile distilled water (SDW). From the manufacturer's instructions, one tablet of ClO_2 dissolved in 1 litre of water will yield about 1.5ppm ClO_2 which should be effective in killing water-borne bacteria. Where necessary, the glassware

used for chlorine dioxide experiments were pre-treated with 1.25ppm ClO_2 for an hour and rinsed with deionised water before use. Fresh stock was made for every experiment due to the degradative nature of ClO_2.

The 12ppm stock solution allowed investigation of the effects of a range of concentrations of chlorine dioxide above and below 1.5 ppm (from 6ppm to 0.0375ppm). For ClO_2, using a multi-channel pipette, 200 µl of the 12ppm stock was placed in the first column of a round bottomed 96 well microtiter plate (COSTAR) and the rest of the wells were filled with 100 µl of SDW. The rest of the wells excluding the positive control were serially diluted from the first column.

For chlorite, a stock of 100mM sodium chlorite (6750ppm chlorite) (Sigma Aldrich, UK) was prepared and diluted to make 8mM in 200 µl of SDW in the first column. Test concentrations of chlorite ranged from 8 mM to 0.5 mM and for both biocides, a two-fold or four-fold dilution was made from the first column down to the last concentration excluding the positive control and the negative control (blank) column. Pipette tips were changed after each serial dilution.

2.9.2 Preparation of Bacterial Suspension

Bacterial species of interest were obtained from a cryogenic stock at -80ºC and from a second subculture on an agar plate, a single colony was inoculated into 5 ml lysogeny broth (LB) (yeast extract (10 g/l), tryptone (5 g/l) and 5 g/l NaCl) (Bertani, 1951) in a sterile universal bottle at 37ºC for 24hrs while shaking at 200rpm. From the overnight culture, 50 µl of bacterial suspension was diluted 100-fold to make 5 ml of bacterial suspension.

Using a multi-channel pipette, 100 µl of the bacterial suspension was added to the 100 µl biocide dilutions in all the wells including the positive control in column 11 and excluding the negative control in column 12.

After adding the bacterial suspension thereby reducing the previous concentration by half, the microtiter plate was placed in the plate reader, and the settings were incubation at 37ºC for 13-14.4 hrs, shaking for 15 secs before optical density reading, 30 cycles at 30min for each cycle.

2.9.3 Generation time estimates

The generation time for each of the strains at different concentrations of biocides was established from the growth curves obtained after about 13 hrs of incubation. This was evaluated by observing the difference in the start and end of the logarithmic phase of growth of each sample and dividing this by the time interval. The equation used to calculate the generation time is $G = t / (3.3 \log b/B)$

G (generation time) = (time, in mins or hours)/ n (number of generations)

t = time interval in hours or mins

B = number of bacteria at the beginning of a time interval

b = number of bacteria at the end of the time interval

Since the number of bacteria was measured by optical density using a spectrophotometer, this was converted to number of bacterial cells by multiplying the OD by the dilution factor before calculation (Beal et al, 2019). A plot of generation time versus concentration of biocide was produced.

2.10 Biofilm formation assay

2.10.1 Bead system of biofilm formation

To estimate the effect of chlorine dioxide and chlorite on pre-formed biofilms, the bead system of biofilm formation was employed. This method was used in two major experiments,

1. The survival of products of transfer of pBURNS1::Tn*1723* plasmid in a biofilm with the selection of chlorite

2. Survival of plasmid carrying strains versus non-plasmid carrying strains in a range of concentrations of biocide

The bead system adopted from Popat et al, (2007) is known to form efficient biofilms within 24 hrs. The beads measure about 6mm by 4mm in size with a hollow in the middle which forms most of the biofilm since this part is not exposed to shear forces during incubation by shaking.

2.10.2 The transfer of pBURNS1::Tn1723 plasmid in a biofilm while selecting with chlorite

Four sterile (autoclaved) plastic beads were placed in 5 ml L-broth media and inoculated by adding 50 µl of a 5 ml overnight culture of the recipient strain *E. coli* MV10 Nal^R for 24hrs. The beads were transferred to fresh medium and incubated for a further 24hrs to make a 48hr biofilm. 50 µl of a 5ml overnight culture of the donor strain (*E. coli* C600 (pBURNS1:: Tn*1723*) was added to the 48hr biofilm in 5 ml fresh L-broth. This was grown without any chlorite for 24hrs to allow survival of both donors and recipients. The resulting biofilm was expected to be comprised of donors, recipients and transconjugants.

A single bead was removed from each set and was subjected to scraping for 30 seconds and vortexing for two mins to resuspend the biofilm formed and this was plated for counts on selective media. Serial dilutions of the resuspended biofilm were plated on L-Agar containing KAN (50 µg/ml) to select for the donors, NAL (25 µg/ml) to select for the recipients and both KAN and NAL antibiotics to select for transconjugants. The other set of beads that have not been resuspended were aseptically placed in fresh medium and the control set re-incubated. For the 'test' set, 5 mM and 0.2 mM $NaClO_2$ was added to L-broth and M9 media respectively before re-incubation for 24hrs at 37ºC. After this treatment stage, the procedure of resuspension and plating was repeated, and the procedure of treatment carried out as previously till the last bead is resuspended.

2.10.3 Level of biofilm survival in chlorine dioxide

The bead system was employed using single species of bacteria to test the level of survival of bacterial biofilms carrying the pBURNS1::Tn*1723* plasmid in chlorine dioxide compared to biofilms that do not carry the plasmid. In this method, single specie *E. coli* biofilms and *Pseudomonas aeruginosa* 943 biofilm with and without the plasmid were grown using the beads and then subjected to varying concentrations of the biocide for a specific contact time. Single colonies were picked from subcultures made from a cryogenic stock and inoculated into 5 ml of LB or M9 media and grown for 24 hrs. From the overnight culture, 50 µl of the suspension was added to 5 ml M9 or LB containing two sets of experiments, controls and test sets, each set having three replicates. The beads were then incubated for 24 hrs and 48 hrs at 37ºC. Figure 2-2 below shows a description of the procedure.

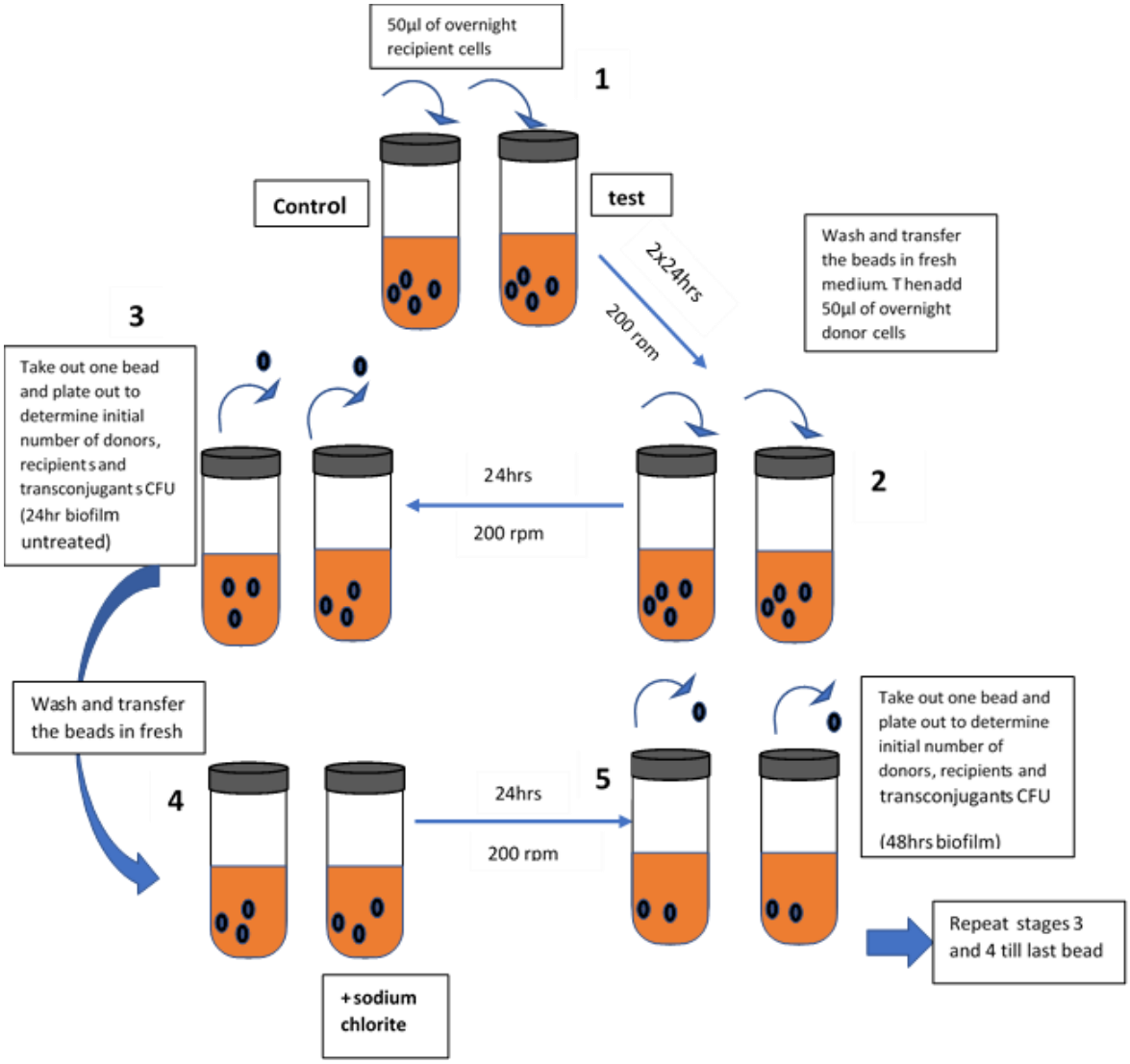


Figure 2-2. Biofilm bead experiment for the transfer of pBURNS1::Tn*1723* while selecting with chlorite.

After a 24 or 48 hr of growth, the biofilms on the bead were rinsed twice with 5 ml of SDW to remove any planktonic cells from the surface of the biofilm and then placed individually in sterile bijou bottles containing the range of concentrations of biocide to be investigated for a period of 3 hrs. After treatment, the beads were neutralized by transferring aseptically into 1 ml of phosphate buffered saline (PBS) and then resuspended by scraping and vortexing for 2 mins. The resuspended detached biofilm was serially diluted and plated to

estimate the number of surviving bacteria on the biofilm which was measured in CFU/ml per bead.

An alternative bead biofilm method was employed which involved growing the beads all together in 1 litre conical flask rather than in separate batches of 4 beads per universal bottle. This method, when employed gave better reproducibility of replicates especially for the *Pseudomonas* biofilms which gave very variable results with the universal bottle growth. In this method, 12 beads were aseptically placed in a 1 L conical flask and 150 ml of L broth was added. To grow a 24-hr biofilm, 150 µl of an overnight culture was added to the flask and incubated at 37ºC for 24 hrs while shaking at 200rpm.

Chlorine dioxide Treatment of Biofilms

For a 48-hr biofilm, after 24 hrs, the beads were rinsed by twice adding 150 ml of SDW to the flask, swirling gently and decanting to remove planktonic cells. Fresh L-broth was added to the beads and re-incubated for a further 24 hrs. Once a 24hr or 48hr biofilm was formed, each bead in triplicate was placed in a bijou bottle containing 5 ml of chlorine dioxide solution made up to a specific concentration or a range of concentrations normally 0.125 ppm to 0.5 ppm. Treatments of biofilms were set at a contact time of 3hrs. After treatment, each bead from each concentration in triplicate and negative controls were taken out using sterile forceps and placed in a microcentrifuge tube containing 1ml of PBS. The biofilm was resuspended by scraping for 30 secs using a sterile tweezer and vortexing for 2 mins using a whirlimixer. Once resuspended, 100 µl of the suspension was taken out with a micropipette and serially diluted for plating on selective agar and colonies were counted after 24 hr incubation at 37ºC. Figure 2-3 below shows a cartoon version of the procedure.

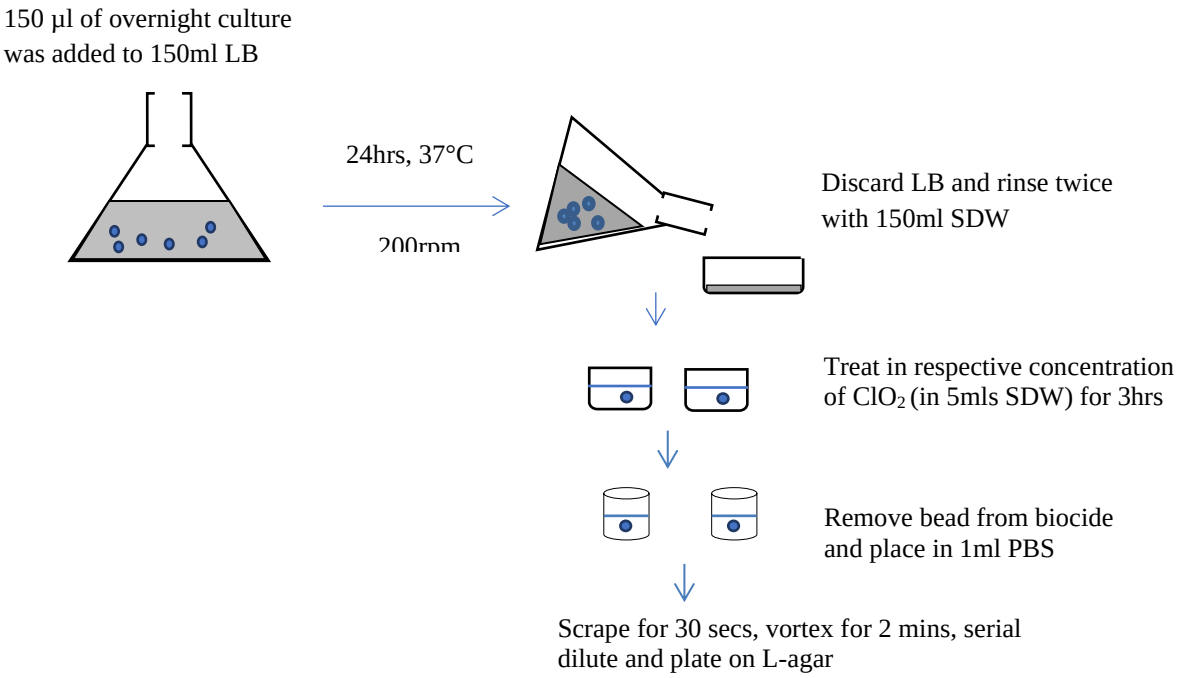


Figure 2-3. Procedure to test for the survival of +/- pBURNS1::Tn*1723* bacteria in the presence of Chlorine dioxide

2.10.4 Survival of planktonic strains in chlorine dioxide

To determine survival after treatment with chlorine dioxide, planktonic bacteria were subjected to 1 hr in the presence of the biocide. A sample was then serially diluted in normal saline and spread on L-agar for colony counts.

2.10.4.1 Preparation of bacterial suspension

Bacterial strains from a cryogenic stock frozen at -80ºC were streaked into subcultures on L agar where single colonies were picked and inoculated in 5 ml LB or M9 broth used to make overnight cultures. Biological replicates of colonies from both plasmid positive and negative strains were incubated at 37ºC for 24 hrs while shaking at 200rpm. A 1:100 serial dilution of each overnight was made and incubated for about two hours at 37ºC for 24 hrs while shaking at 200rpm until the OD_{600} was between 0.08 and 0.10. At this point, bacteria from

1 ml of culture were harvested, the pellet washed twice, resuspended in 1 ml of saline and diluted further to 1:100 in normal saline to obtain approximately 1 x10^5 CFU/ml cells.

2.10.4.2 Preparation of challenge plate

To determine the survival of planktonic bacteria in ClO_2, a range of concentrations of ClO_2 from 0.0625ppm to 0.50ppm were used. A sterile 96-well microtiter round bottom plate (COSTAR, Sigma-Aldrich) was used for this experiment. The first well column was made up to 200 µl of 1 ppm from a 6 ppm ClO_2 stock. Other wells were filled with 100 µl SDW. A two-fold serial dilution was made to provide concentrations of 0.125 ppm to 1 ppm and the tips were changed after each dilution. Since an equal volume of bacterial suspension was added, the final ClO_2 concentration was reduced by half making the range of treatment to be 0.0625 ppm to 0.50 ppm. A column was used as a positive control with no biocide added.

Once the challenge plate has been set up, 100 µl of the bacterial suspension previously prepared was added to each of the wells except the negative controls. After 1 hr, 100 µl of each concentration for each strain/species was serially diluted separately and plated into LA plates with appropriate antibiotic selection.

2.10.4.3 Preparation of Biocide stock

For the stock solutions, chlorine dioxide solution was made up to a concentration of 12 ppm by diluting with 125 ml of SDW in chlorine demand free glassware. The glassware was covered completely with foil to prevent break down of chlorine dioxide by sunlight. The glassware was prepared as mentioned previously in chapter 2.9.1.

The source of chlorite on the other hand, was the chemical compound Sodium Chlorite ($NaClO_2$) (CAS number: 7758-19-2) which will dissolve easily in water to release chlorite

ions. Properties of sodium chlorite are listed in Table 2-5 below. A stock solution of 100mM sodium chlorite dissolved in SDW was estimated and used for further experiments. Unlike chlorine dioxide, the shelf life of sodium chlorite is far longer with the same effectiveness as the first day of production provided it was protected from light sources. The nature of chlorite which causes it to be very stable in water suggests that in the industrial setting and during the production of chlorine dioxide, high levels of chlorite present in effluents can remain stable for a long period if protected from sunlight for example in closed plumbing systems. This could contribute to selection of chlorite dismutase gene in the environment.

Table 2-5. Physical and chemical properties of sodium chlorite and chlorine dioxide

Property	**Chlorite**	**Chlorine dioxide**
Molecular weight (g/mol)	90.45	67.46
Colour	White	Yellow/ reddish yellow
Physical state	Solid	Gas
Melting point	180-200°C	-59ºC
Boiling point	Decomposes	11ºC
Density	2.468 g/ml	1.640g/ml (0ºC; liquid) 1.614g/ml (10ºC; liquid)
Solubility		
Water	390 g/L at 30 ºC	3.01 g/L at 25°C and 34.5 mm Hg
Organic solvents	No data	
Incompatibilities	Organic matter, sulphur, powdered coal; a powerful oxidizer	Organic materials, heat, phosphorus, potassium hydroxide, sulphur, mercury, carbon monoxide; unstable in light; a powerful oxidizer

(EPA, 2004)

2.11 Determination of Minimum Inhibitory Concentration, Minimum Bacteriocidal Concentration and Minimum Biofilm Eradication Concentration in sodium chlorite and/or chlorine dioxide

2.11.1 Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) can be defined as the lowest concentration of an antibiotic or a biocide that can inhibit the growth of an organism.

2.11.1.1 Preparation of Bacterial Suspension

A 5 ml overnight culture in either L-broth or M9 media was set up by inoculating a single colony from a subculture plate. The overnight was diluted 1:100 by adding 50 µl to 5 ml LB and incubated for 1.5 hours to capture the exponential phase of growth. After incubation, the optical density was measured using a spectrophotometer to OD_{600} = 0.08-0.10 which should have approximately 10^8 CFU/ml of bacterial cells. The bacterial culture was further diluted 1:100 (10^6 CFU/ml) which was the final bacterial concentration to be used. Some of the bacterial culture was also diluted 1:10 (10^7 CFU/ml) (Weigand et al, 2008)

2.11.1.2 Preparation of biocide concentration

For chlorite, a stock of 150mM $NaClO_2$ was prepared by weighing 0.27132g of sodium chlorite and dissolving it in 20 ml of SDW. In the second column of a 96 well microtiter plate, 171 µl of 150 mM $NaClO_2$ and 29 µl of LB or M9 broth was added down each column to make up 128 mM. To the rest of the wells excluding column 1, 100 µl of LB or M9 was added. A 2-fold serial dilution from column 2 was conducted horizontally while changing tips at each dilution. The range of concentrations were 128 mM to 1 mM. After 100 µl of the bacterial suspension was added the concentrations were reduced by half. In the first

column, 29 µl of L broth, 171 µl of 150 mM $NaClO_2$ and 10 µl of 10^7 CFU/ml bacterial suspension to give a final concentration of 128 mM $NaClO_2$.

Once the challenge plate was set up, 100 µl of 10^6 CFU/ml bacterial suspension was added to each well apart from the negative controls and blank columns. The microtiter plate was sealed with cling film to prevent contamination and evaporation and incubated at 37ºC for 24 hrs. A preview of the plates is shown in Figure 2-4 below. The MIC was read using a plate reader or by the unaided eye. The concentration present in the first well which shows no turbidity from bacterial growth is termed as the MIC (Weigand et al, 2008). The experiment was done in triplicates.

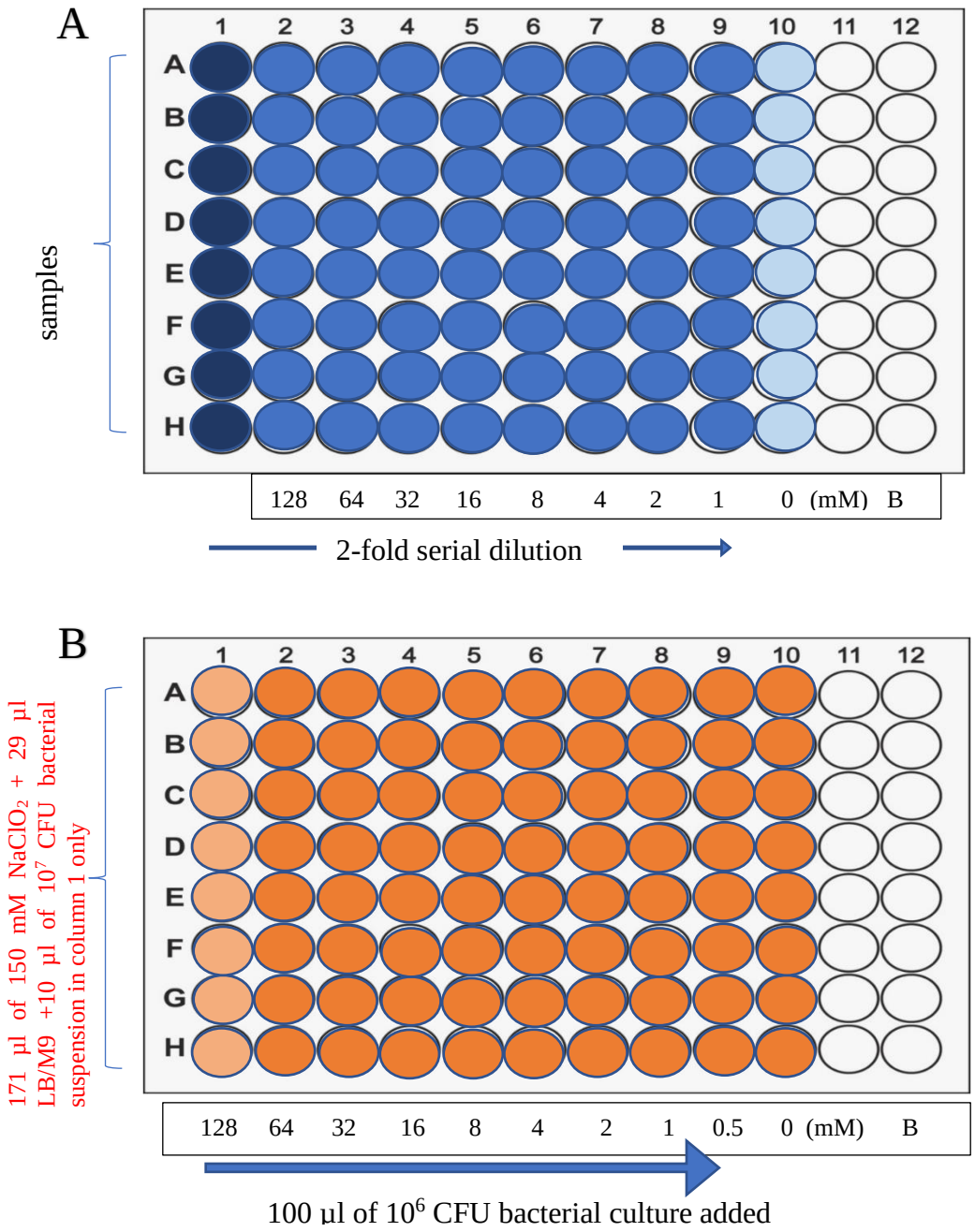


Figure 2-4. Schematic representation of the MIC assay plates.

A. Represents the preparation of biocide concentration. The dark blue wells indicate biocide dilutions in L Broth or M9 media, the wells in light blue have no biocide added. Blank/grey wells indicate blank samples. All wells except well 1(deep blue), which was separately prepared with 171 µl of 150 mM $NaClO_2$ + 29 µl LB/M9 were to be serially diluted.
B. shows the plate after the bacterial suspension has been added reducing the biocide concentrations by half. The dark orange wells indicate all the wells that included 100 µl of 10^6 CFU/ml bacterial suspension while light orange indicate the addition of 10^7 CFU/ml bacterial suspension. Blank/grey wells indicate blank samples.

2.11.2 Minimum Bacteriocidal Concentration (MBC)

The MBC refers to the lowest concentration of a biocide or antibiotic that will completely kill an organism in planktonic phase. The MBC was estimated by spot plating aliquots of the MIC well and other adjacent wells that showed a clear broth on L-agar. This was incubated for 24 hrs at 37ºC. The concentration which results in no surviving bacteria that could give rise to colonies was taken as the MBC.

2.11.3 Minimum Biofilm Eradication Concentration (MBEC)

To determine the minimum concentration of chlorine dioxide that would successfully eradicate a 24hr biofilm in a monoculture state, sterile 96-well microtiter plates were filled with 150 µl of a 1:100 diluted overnight culture of desired strain in all wells except the blanks and negative control columns. Sterile PCR non-skirted plates were used as pegs and inserted into the 96 well plates containing the bacterial suspension (Figure 2-5). The microtiter plate was incubated at 37ºC without shaking for 24hrs.

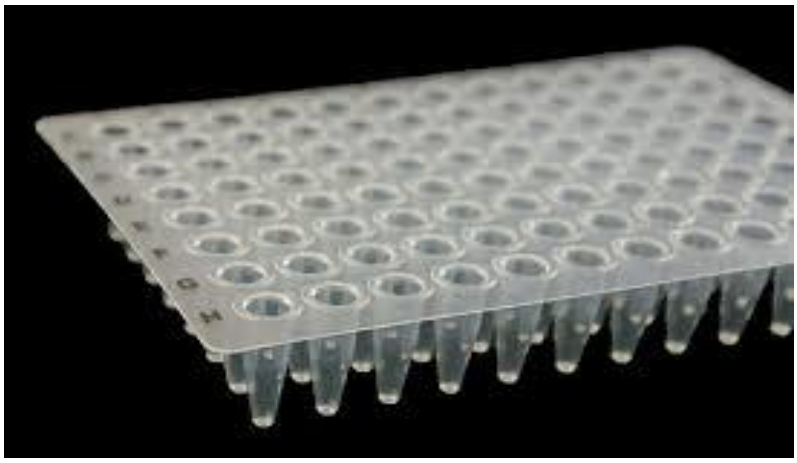


Figure 2-5. Photo of non-skirted PCR plates used as the pegs for the MBEC assay

Photo adapted from Elkay Laboratory products

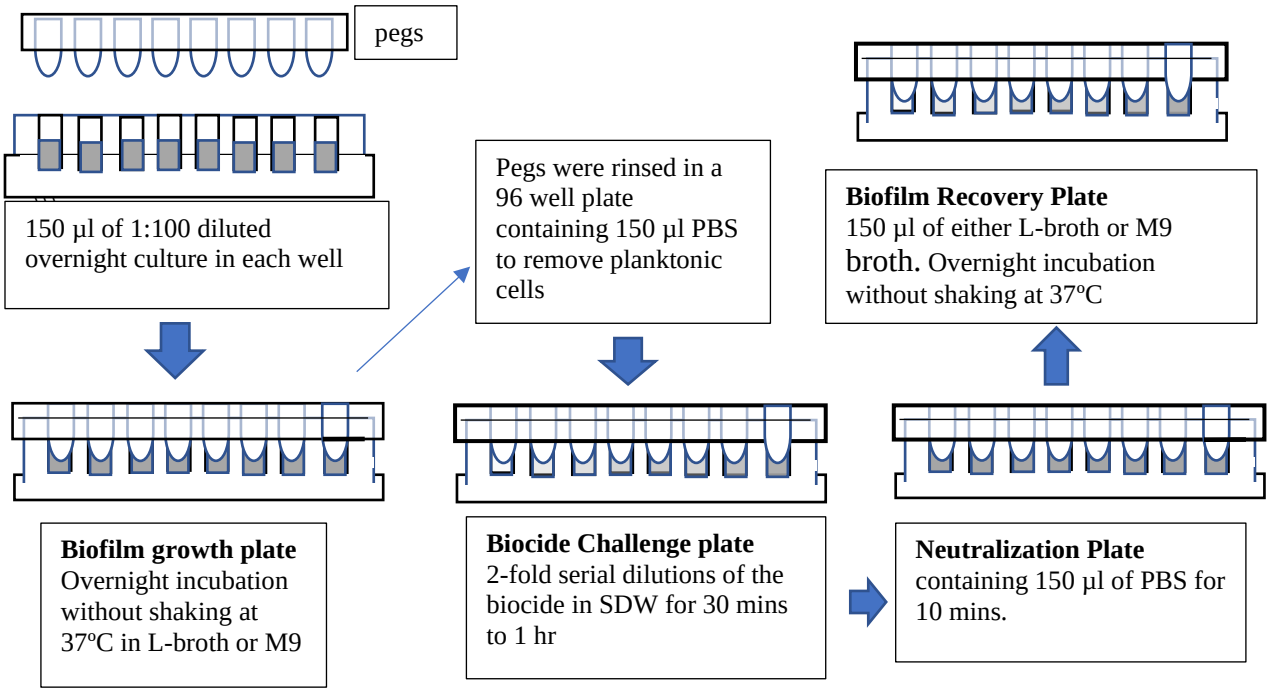


Figure 2-6. Method for determining MBEC of bacteria in chlorine dioxide

Schematic representation of how the bacterial MBEC was carried out using chlorine dioxide as a biocide. At the final stage the wells that did not show any turbidity in the biofilm recovery medium after a 20- 24hr incubation was referred to as the MBEC. Figure adapted from (Hengzhuang and Ciofu, 2014)

After incubation, the pegs were removed and placed in a 96 well plate filled with 150 µl PBS to rinse off planktonic cells from the biofilm. The pegs were then transferred to the challenge plate containing 2-fold serial dilutions of the biocide in SDW as shown in Figure 2-6 above. Contact time for 24 hr biofilms grown in M9 was 30 mins while 24hr biofilms grown in L-broth were left for 1 hour in chlorine dioxide solutions.

After treatment, the pegs were transferred to a 96 well plate that contained 150 µl of PBS in each well and left for 10 mins to neutralize the biocide. Finally, the pegs were placed in a recovery plate filled with 150 µl of either L-broth or M9 broth, covered with a lid and incubated for 24hrs at 37ºC without shaking. The highest concentration of biocide that

resulted in wells that did not go turbid was said to be the MBEC for the species in chlorine dioxide.

2.12 Determination of gene expression activity using β-galactosidase assay

To study the promoters upstream of the chlorite dismutase gene, they were inserted into a broad host range IncQ plasmid carrying *lac*Z as both transcriptional and translational fusions replacing the *mup*Z promoter in pMUP9 (see Figure 2-7 below). After the two promoters 1 and 2 were cloned into the pMUP9 vector by restriction digest and ligation, the new plasmids pCDM1 and pCDM2 were then transformed into *E. coli* S17-1 which was then mated with *E. coli* C600 followed by selection of transconjugants using NAL 25 µg/ml, TET 15 µg/ml and KAN 50 µg/ml on LA plates. The plasmids were transformed directly into *P. aeruginosa* 943 rather than transfer through conjugation. This process is further explained below.

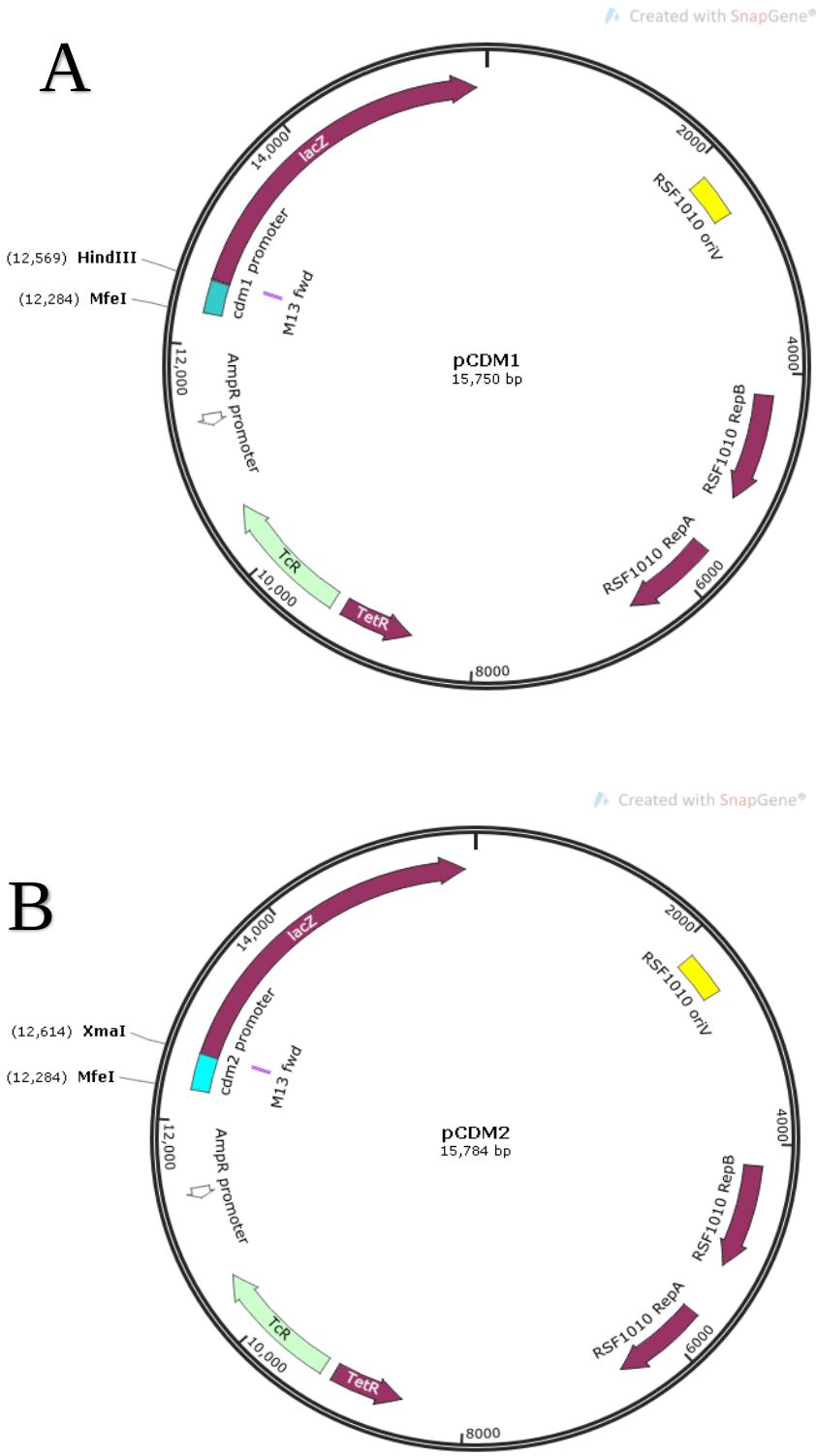


Figure 2-7. Map of A. pCDM1 and B. pCDM2 which were constructed by cloning cdm1 and cdm2 promoter respectively upstream of the *lacZ* gene replacing *mupZ* in pMUP9.

Cdm 1 promoter was cloned into the pMUP9 plasmid using the restriction sites MfeI and HindIII while cdm2 was cloned via the MfeI and Xma I sites. The insert fragments were first obtained from the pBURNS1:Tn1723 plasmid via PCR before cloning into the vector.

2.12.1 Cell growth for β-galactosidase assay

Using a sterile tip, 2.2 ml 96-well polypropylene blocks (Marsh Biomedical Products, Fisher) containing 1 ml of L-broth was inoculated with a single colony of the isolate to be assayed. Antibiotics were added to the L-broth for selection. The blocks were then covered with a sterile mat cap (Marsh Biomedical Products, Fisher) and incubated at 37ºC with shaking at 200 rpm for 24 hours. A minimum of three replicates for each strain including controls were made. After 24 hrs, 10 µl of each grown culture was used to inoculate fresh L-broth in a second block also containing 1 ml of L-broth to make a ratio of 1:100. Antibiotics were again added at a standard concentration.

2.12.2 Determination of OD_{600} of the bacterial culture and preparation of ONPG (O' nitrophenyl-β-D -galactopyranoside)

First, 4mg/ml O' nitrophenyl-β-D -galactopyranoside (ONPG) was prepared fresh each time in Z-buffer (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4.2H_2O$, 10 mM KCl) without β-mecarptoethanol. This solution in addition to PBS was pre-chilled on ice for 20 mins. Using a multi-channel pipette, 50 µl of each culture was added to a standard flat bottomed 96 well plate (COSTAR) containing 150 µl of the pre-chilled PBS. Blanks containing 200 µl PBS were included at this point. The OD_{600} reading was taken and necessary dilutions were made until each sample reached a value between 0.4-0.6. The OD_{600} was measured using a plate reader.

2.13 Permeabilization of cells

Fresh β-mercaptoethanol was added to an aliquot of Z-buffer inside a fume hood. A polypropylene block with 450 µl of Z-buffer was set up in each well using a multi-channel pipette in the fume hood. Freshly prepared stock of 10% SDS was diluted to 0.1% and 8µl

of 0.1 % SDS and 15 µl chloroform was further added to each well/sample. The diluted culture plate was taken from ice and 50 µl was added to the permeabilization plate using a multi-channel pipette. The cell mixture was aspirated 10-15 times using a pipette. Blank reactions with PBS replacing the cells were included in the wells.

2.13.1 Assay of β-galactosidase Activity

The chloroform was left to settle at the bottom of the wells of the blocks for 10 mins in the fume hood. Using a multi-channel pipette, 100 µl of the permeabilized cells were transferred into duplicate flat-bottomed microtiter plates in the fume hood. The assay was initiated at time zero by adding 48 µl of ONPG to each well using a multichannel pipette. For an end point assay, the plates were mixed thoroughly and incubated at room temperature until a faint yellow colour developed for a maximum of 1 hr. The assay was terminated by adding 48 µl of 1M Na_2CO_3 with the start and stop time noted. The plate reader was used to measure A_{420} and A_{550} values.

Miller unit = measurement of β-galactosidase activity

$1000 \times [(OD_{420} - 1.75 \times OD_{550})] \times [(t \times v \times OD_{600})]$

2.13.2 Transformation of pcdm1 and pcdm2 into P. aeruginosa 943

For the β-galactosidase assay, the pCDM1 and pCDM2 plasmids were transformed into *P. aeruginosa* 943 +/- pBURNS1. The process was adapted from the method used by Thomas et al, (1982). The *P. aeruginosa* strains were made competent by pelleting 10mls of overnight cultures in L broth grown at 37ºC in L-broth. The OD was standardized to 1.0 at 625 nm. The pellet formed was washed with ice cold 100 mM $MgSO_4$ and incubated in 4 ml pre-chilled $CaCl_2$ for 20 mins. The suspension was centrifuged, and the pellet resuspended in 1 ml of pre-chilled 100mM $CaCl_2$. 1 µl each of pCDM1 and pCDM2 plasmid

prep was mixed with 100 µl of the competent cells and incubated on ice for 1 hr. After incubation, it was heat shocked for 2 mins on a heat block at 42ºC. 1 ml of L-broth was added and incubated at 30ºC for 2 hrs. After this, 9 ml of L-broth containing 10 µg/ml was added and incubated overnight at 30ºC. 200 µl of samples were plated on L-agar containing 100 µl/ml Tetracycline to select for the transformants and incubated overnight at 30ºC.

2.14 Statistical analysis

Statistical analysis was carried out using Graphpad prism 7. Statistical significance of data was determined using either students t-test or 2-way ANOVA followed by either Tukey's multiple comparisons test or Sidak's multiple comparison test as a post hoc to determine which of the variable data sets were statistically different with a p-value of <0.05. When P-values are higher, the null hypothesis is accepted meaning the two data sets are not significantly different from each other.

Chapter 3

Characterization of pBURNS1 plasmid

3.1 Introduction

Plasmids are carriers of genetic information. They can carry resistance genes within their genome and may carry other genetic determinants such as transposons and integrons which have the capacity to also carry resistance genes and to promote movement to new locations (Bennet, 2008). One of the plasmid's main function is to promote multiplication of the plasmid DNA and this is strengthened if the plasmid provides an advantage to the bacterial hosts in the environments where they are found. This gives a higher level of adaptation and survival to the host cell compared to bacteria that do not have the plasmid (Bennett, 2008).

IncP plasmids are known to be promiscuous since they are transmissible between diverse bacteria via conjugation and highly stable, so it is clinically important when they are found to be present in the hospital environment (Bahl et al, 2009). Although bacterial strains carrying IncP-1α plasmids serve as a greater threat since they often carry mainly multiple antibiotic resistance genes, those carrying IncP-1β have a similar capability of acquiring resistance genes through the uptake of mobile genetic elements from the clinical environment (Popowska & Krawczyk-Balska, 2013).

IncP-1β plasmids are known to be found in environmentally-occurring bacteria whose habitats include sewage, effluents and soil (Bahl et al, 2009), therefore their presence in hospital isolates calls for concern because little is known of the significance of plasmids from environmental sources in hospital systems: whether they are harmless and simply coincidental or if the hospital environment may be contributing to the proliferation and survival of other pathogenic or opportunistic bacteria carrying the plasmid. In this case, the properties and characteristics of such plasmids must be investigated and linked with previous findings in literature.

The phenotypic analysis of IncP-1 plasmids is important to understand the risks involved in their presence in clinical environments, by comparing the advantageous properties they give a bacterial cell. Studying the sequence of the plasmids, their physiological and biological functional in relation to their common ancestors allow for this comparison. The chlorite dismutase gene has been found in a wide range of archaea, bacteria such as proteobacteria and cyanobacteria (Celis et al, 2014). Deciding on the appropriate action when faced with plasmid-borne transmission of antibiotic/biocide resistance in the hospital environment depends on investigating the cause, spread, origin and level of threat to human health of these plasmids and their host organisms.

The genome of pBURNS1 has an average G+C content of 65% and a total of 77 open reading frames were identified during annotation. The chlorite dismutase gene which we have designated *cdm* was found to be the only gene conferring any resistance in the plasmid. It is found within an integron enclosed in a transposon and therefore contains genes such as integrase and transposase among others which encode for their functions.

3.2 Aims

This chapter aims to determine the phylogenetic relationship between pBURNS1 and the class 1 integron it carries with elements reported in other bacterial species. Therefore, the specific aims of the work in this chapter are to:

- Extract pBURNS1::Tn*1723* plasmid from *E. coli* C600 (pBURNS1::Tn*1723*) using maxi prep plasmid isolation method.
- Determine the possible origin of the pBURNS1 plasmid and/or genes using phylogenetic data and bioinformatics.
- Confirm the presence of the *cdm* gene pBURNS1 using PCR.

3.3 Maxi prep isolation of plasmid DNA from *E. coli* C600 (pBURNS1::Tn*1723*)

Although Nikhil Aggarwal (2015) had tagged pBURNS1 DNA with an antibiotic resistance marker by the insertion of Tn*1723*, no one had isolated the plasmid DNA to prove it was replicating independently. To do this and in order to obtain large amounts of plasmid DNA with high quality, a large scale isolation using the alkaline SDS method followed by a caesium chloride density gradient (see Chapter 2.3.2.1 and 2.3.1.4) was conducted using *E. coli* C600 (pBURNS1::Tn*1723*) which carries the tagged plasmid generated by Aggarwal (2015). A small quantity (1 µl) of plasmid DNA was run on an agarose gel to determine the presence of the plasmid in the final solution. A single band showed the presence of the plasmid as seen in Figure 3-1 below but the yield of DNA was observed to be quite low.

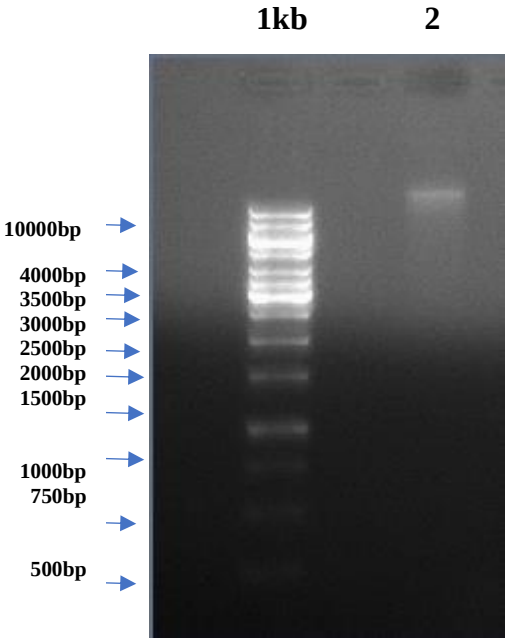


Figure 3-1. Plasmid DNA isolated from (*E. coli* C600 pBURNS1::Tn*1723*) via Maxi prep

Lane 1 shows the 1kb Gene O ruler ladder and in lane 2, a single band can be seen which represents pBURNS1::Tn1723 isolated from E. coli C600 (pBURNS1::Tn1723)

3.4 Restriction digest of pBURNS1::Tn*1723*

The sequence of pBURNS1::Tn*1723* was initially analysed to determine palindromes to help determine what enzymes to cleave pBURNS1 with to give a characteristic set of bands. NEBcutter V2.0 software was used to predict the expected fragment sizes. AatII and SacI were chosen because together they should give four fragments, three sites for AatII and one site for SacI (Table 3-1 and Figure 3-2).

Table 3-1 Coordinates for AatII and Sac1 restriction digests of pBURNS1::Tn*1723*

#	Ends	Coordinates	Length (bp)
l	AatII-AatII	7844-37772	29929
2	AatII-SacI	37773-50981	13209
3	AatII-AatII	58871-7843	9280
4	SacI- AatII	50982-58870	7889

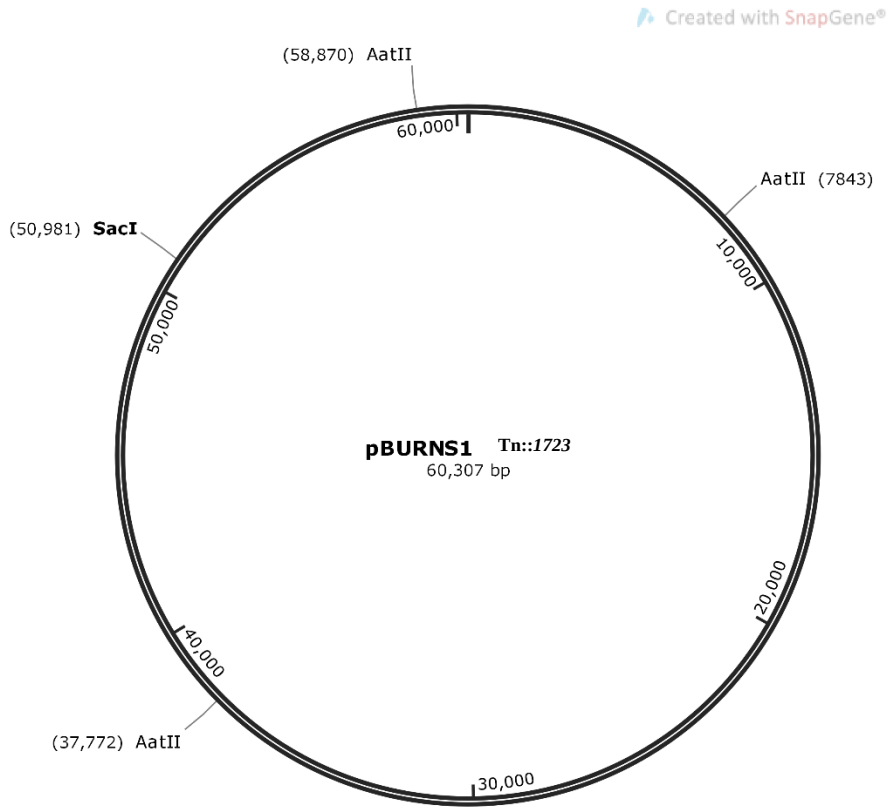


Figure 3-2. Map of the tagged pBURNS1::Tn*1723* plasmid showing the restriction sites AatII and SacI

As a test to confirm the presence of the pBURNS1::Tn1723 plasmid, restriction digests were carried out using restriction enzymes AatII and SacI which cuts the plasmid DNA thrice and once respectively at coordinates as shown above.

It could be seen that the maxi prep DNA gives four different clear bands of the expected sizes (Figure 3-3). Two of the mini prep plasmid DNA preparations did not appear to digest well. The third seems to have been given enough digestion to confirm the presence of the two smaller bands. It can therefore be concluded that the DNA extracted is pBURNS1::Tn*1723*.

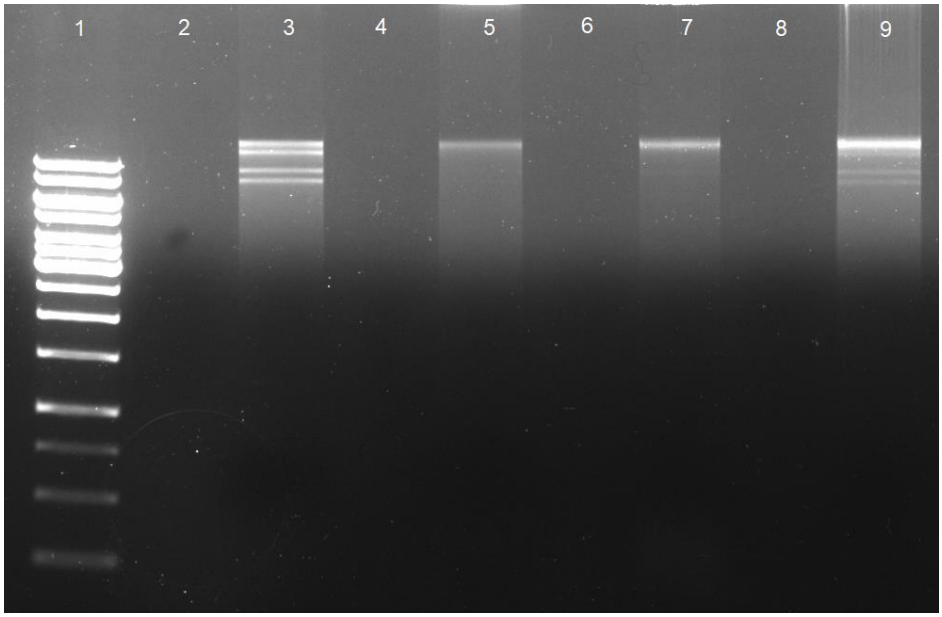


Figure 3-3. Restriction digest of pBURNS1::Tn*1723* plasmid using SacI and AatII restriction enzymes.

Lane	Enzymes	Isolate	Description
1			Gene O Ruler 1kb ladder
3	SacI, AatII	C600 (pBURNS1::Tn*1723*)	pBURNS1::Tn*1723* (maxi prep)
5,7,9	SacI, AatII	C600 (pBURNS1::Tn*1723*)	pBURNS1::Tn*1723* (various mini preps)

3.5 Preparation of bacterial strains containing pBURNS1::Tn*1723*

3.5.1 Transformation of pBURNS1::Tn1723 into E. coli

To study the plasmid in a different host, pBURNS1::Tn*1723* plasmid DNA was transferred into both *E. coli* DH5α and *E. coli* C600 competent cells via transformation. Depending on the quality of competent cells and efficiency of the transformation protocol, single small

cream coloured colonies were observed on the selective plates. The antibiotic(s) to be used in the media would select for the plasmid and the host strain. Confirmation of the plasmid in the transformants was done through selection of a minimum of six colonies from each selective plate and doing a boiled prep PCR using primers followed by PCR amplification of a known region of the plasmid such as the *trbB* gene and *cdm* gene.

3.5.2 Conjugation of E. coli C600 (pBURNS1::Tn1723) with E. coli MV10nal^R

A selection of six or more small cream coloured transconjugant colonies were then further screened by extracting plasmid DNA as well as doing a boil prep and performing PCR to detect a unique segment in the plasmid. Both spot-based and filter-based conjugation techniques as described in Chapter 2.4.1 and 2.4.2 respectively produced numerous colonies for all the *E. coli* strains used and needed to be diluted up to 10^{-3} to be counted individually.

3.6 PCR amplification of *cdm* gene from pBURNS1::Tn*1723*

To check that the *cdm* gene is present in pBURNS1::Tn*1723* plasmid, forward and reverse primers were designed to amplify the whole of the *cdm* gene using PCR. PCR was carried out as described in Chapter 2.7.2 and the PCR product was then analysed on a 1% agarose gel using gel electrophoresis. Figure 3-4 below shows the band of 552bp chlorite dismutase gene when viewed under UV light. The presence of the *cdm* gene was checked via PCR in *P. aeruginosa* 943 and *E. coli* strains/samples carrying pBURNS1::Tn*1723* through transformation and conjugation before conducting experiments.

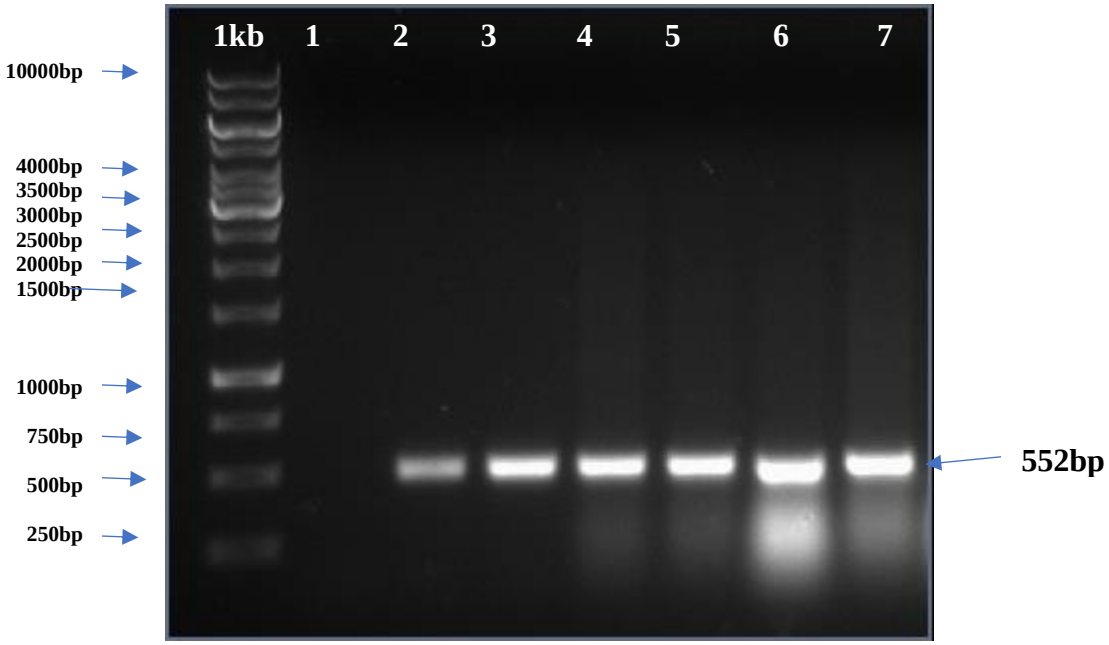


Figure 3-4. PCR product of *cdm* in pBURNS1::Tn*1723* using *cdm* forward and reverse primers

Lane	Isolate	Species	Description
1kb			Gene O Ruler 1kb ladder
1	C600	*E. coli*	Negative control
2	C600 (pBURNS1::Tn*1723*)	*E. coli*	sample 1
3	MV10Nal^{R} (pBURNS1::Tn*1723*)	*E. coli*	sample 2
4	943	*P. aeruginosa*	WT
5	pBURNS1::Tn*1723* (plasmid DNA prepared from *E. coli*)	*E. coli*	Positive control
6	Boil prep from C600 (pBURNS1::Tn*1723*)	*E. coli*	Positive control
7	DH5α (pBURNS1::Tn*1723*)	*E. coli*	sample 3

3.7 Bioinformatics of pBURNS1 plasmid

Bioinformatics is a term given to a series of tools that can analyse and interpret a vast amount of biological data using computational biology. The relationship between DNA sequences from various sources, phylogenetic origins and epidemiological backgrounds can easily be demonstrated using these tools. Examples of these kind of computerized software are BLAST, ARTEMIS, FASTA and CLUSTALW to mention a few (Huang et al, 2009). The benefits of using the above-mentioned software include analysing large amounts of DNA sequences at a time and performing sequence alignments and annotations.

3.7.1 Annotation of pBURNS1 plasmid

The first bioinformatic analysis of pBURNS1 was carried out by an MSc student Nikhil Aggarwal who worked in the Thomas lab prior to the start of my PhD. However, this work has not yet been published and the plan is that the work that Nikhil carried out will be incorporated into publications arising from my PhD work. The annotation carried out by Nikhil was incomplete, so I have checked what he did and completed the annotation process to provide a solid foundation for my work. The annotation has been completed and submitted to Genbank (Accession number MN386974.1). I used the Artemis Comparison Tool (ACT) to compare the pBURNS1 plasmid genome with a selected number of IncP-1 plasmids from BLAST and annotated the core sites, 59 base elements and attachment sites of the integron, the *klu*A and *klu*B genes not previously annotated in pBURNS1.

The pBURNS1 plasmid is 50,465bp (Figure 3-5) that belongs to the IncP-1β incompatibility group as explained in Chapter 1. The archetype of this subgroup is the R751 plasmid backbone (Thorsted et al, 1998) and so pBURNS1 is expected to be a conjugative plasmid because the DNA sequence shows that it carries both *tra* and *trb* genes necessary for plasmid

transfer. Other essential genes include the genes responsible for replication - *trfA* and *oriV* (Popowska and Krawczyk, 2013).

In many IncP-1 plasmids there are insertions of transposable elements and/or phenotypic determinants between *oriV* and *trfA* but close inspection shows that this is not the case in pBURNS1. The *oriV* region is defined by the binding sites for *trfA* and in parallel work carried out in the lab by PhD student Alessandro Lazdins and then PDRA Anand Maurya it has been established that all 10 *trfA* binding sites (called iterons) are necessary either for *oriV* activation or for the normal control of the replication process. Iterons 1 to 9 run in the same direction and iteron 10 is inverted (Lazdins et al, 2020). At the Iteron 1 and *trfA* end there is a group of inverted repeats that have been proposed to be hotspots for insertions and that seem to be characteristic of IncP-1β plasmids, not being found in other subgroups (Thorsted et al, 1998).

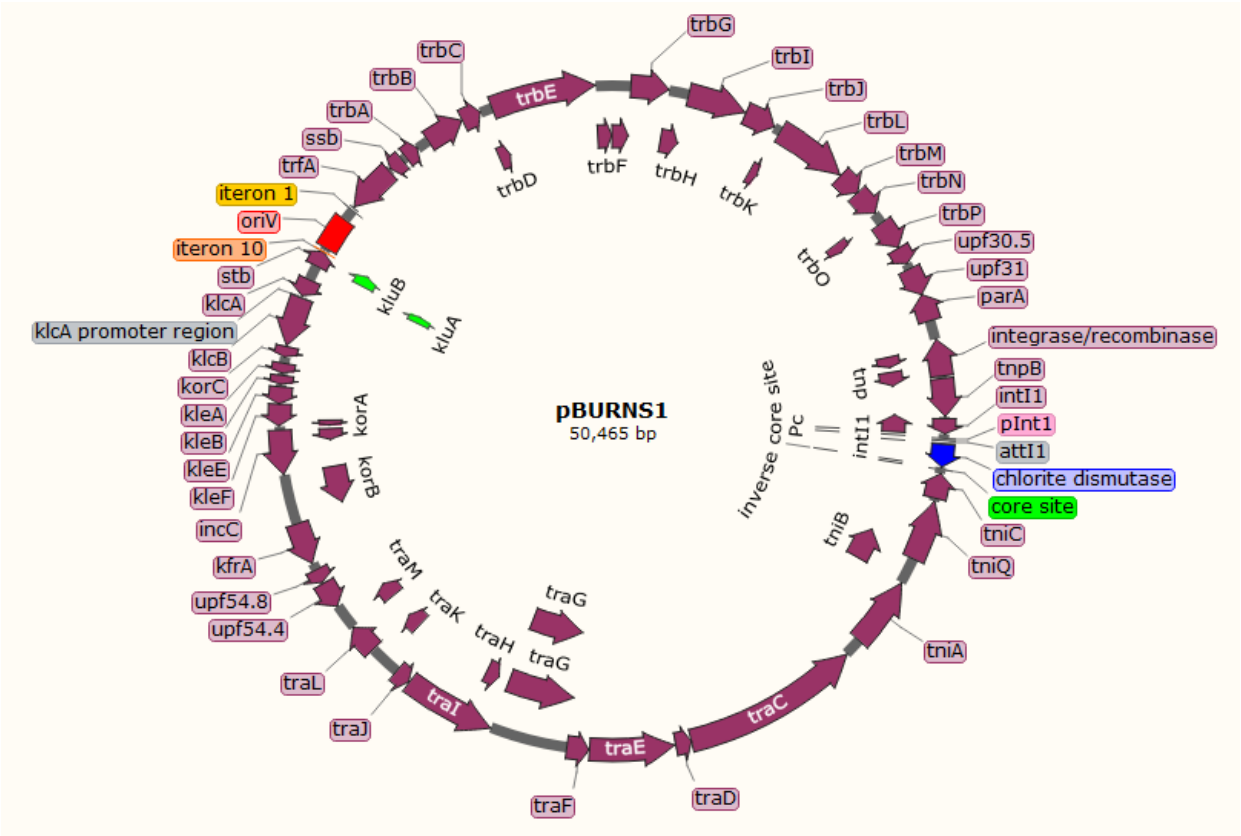


Figure 3-5. Complete annotated map of pBURNS1 plasmid (untagged)

A fully annotated map of pBURNS1 plasmid which shows all the functional open reading frames which includes the tra and trb genes responsible for conjugation, the replication initiation site trfA, the integron and associated genes the transposon associated genes and regulatory genes.

At the other end in pBURNS1, adjacent to Iteron 10 are two short ORFs that are called *kluA* and *kluB* in R751, with unknown function. A BLAST search with the product of the *kluA* ORF showed similarity to ParD (of RK2), a regulator and antidote for the addiction toxin RelE/ParE in RK2. A second BLAST search, with the product of the second ORF, *kluB* showed similarity to ParE (RK2) (See Figure 3-6). This suggests that these two ORFs are part of the set of stabilisation functions encoded by R751. In the IncP-1α plasmids such as RK2, the *parABCDE* gene block encodes the *parDE* addiction system (Schluter et al, 2007). (See Figure 3-7).

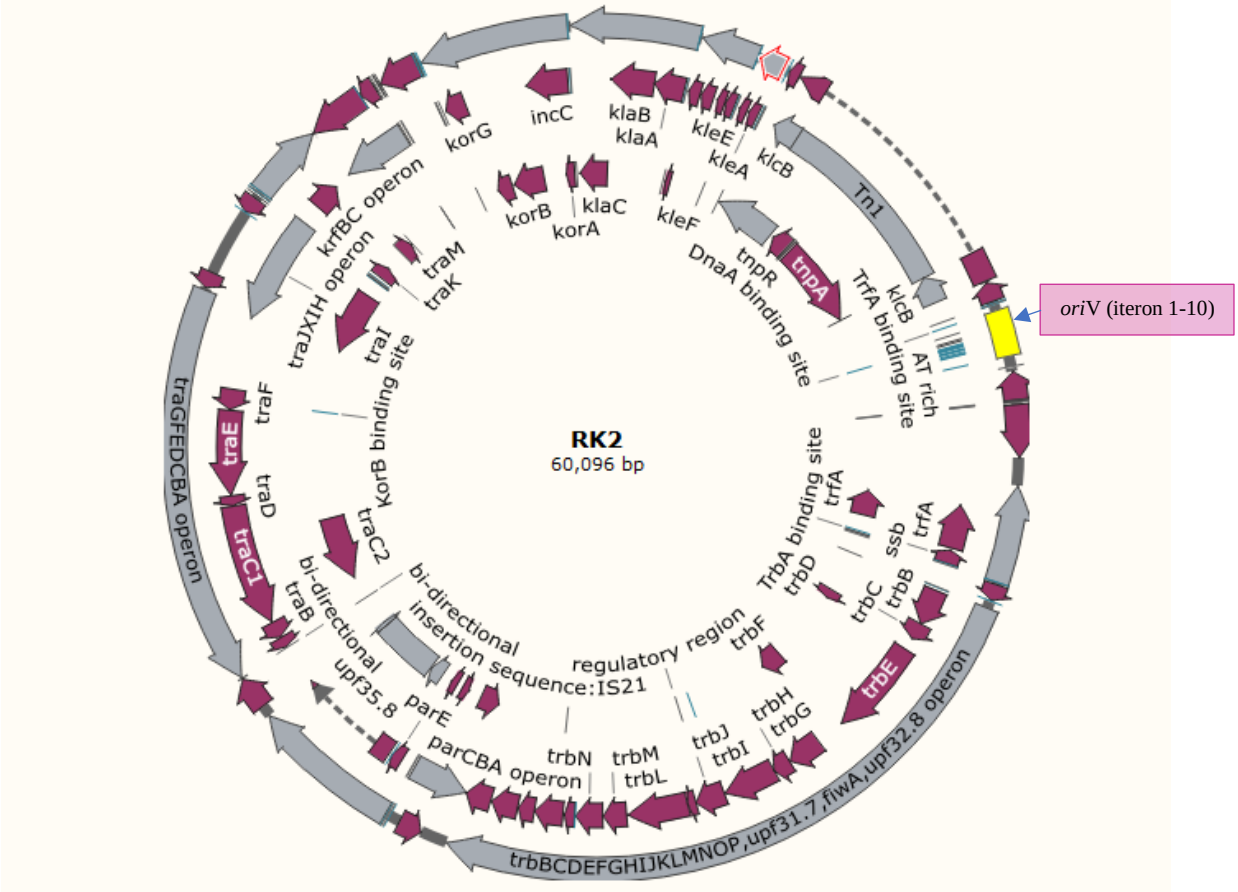


Figure 3-6. The complete annotated sequence of RK2

RK2 is the archetype of IncP-1α plasmids and was first isolated from a hospital in Birmingham (Pansegrau et al, 1994). It has three antibiotic resistance genes conferring resistance to Ampicillin, Tetracycline and Kanamycin. It also has a functioning set of tra and trb genes responsible for conjugative transfer alongside other genes for plasmid replication. The par A-E operon is only present in IncP-1α plasmids.

The only known phenotype associated with the *parABC* block is multimer resolution and pBURNS1 has *parA* encoded at the same location, at the end of the *trb* gene block (Easter et al, 1998) (Figure 3-7). Thus, it seems likely that pBURNS1 has both multimer resolution and addiction systems encoded in the *oriV-trb* sector of the plasmid. The plasmid is therefore likely to be highly stable from the combination of these plus the active partitioning

proteins IncC and KorB encoded in the replication, stable inheritance and regulation region, the hub of the regulatory circuits typical of IncP-1 plasmids (Thorsted et al, 1998).

At the *oriV* region in pBURNS1, the genes which were designated *kluA* and *kluB* were put into BLAST search to determine if they were present in all IncP-1 plasmids. A BLAST search with the *parDE* and *trfA* DNA sequence from RK2 (Figure 3-6) identified a list of plasmids. To determine how plasmids in the *trfA* BLAST search list but not in the *parDE* list differed from pBURNS1, the sequence in this region for the plasmid of this sort that was highest on the list, pRWC72a (Rahube et al, 2014), was inspected (see Figure 3-8).

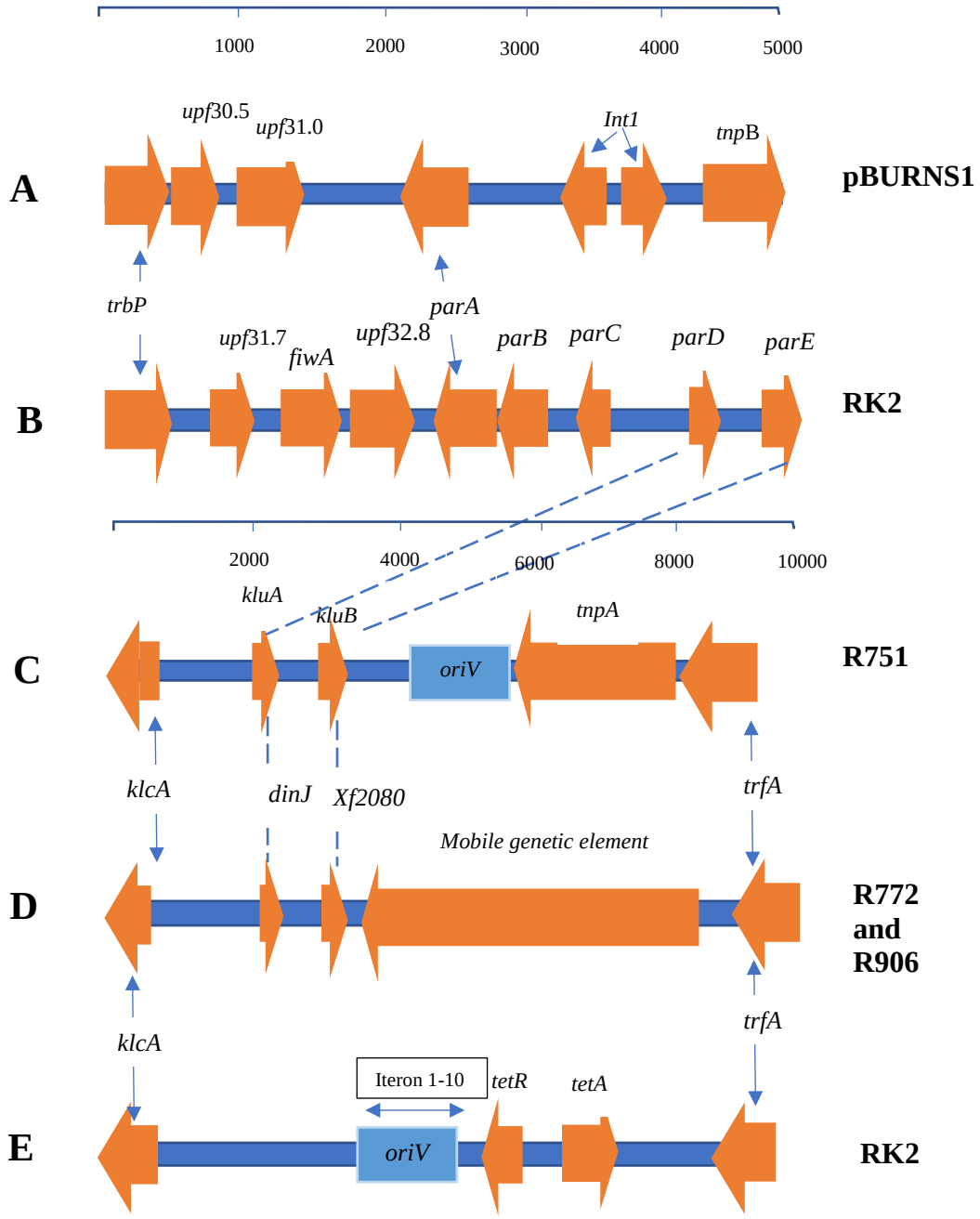


Figure 3-7. Schematic diagram comparing homologous ORFs in IncP-1β plasmids

parA is located upstream of upf31.0 in pBURNS1 ***(A)*** *and upf 32.8 in RK2* ***(B)*** *both at a similar position at the end of the trb genes. In RK2, parD encodes the antitoxin to plasmid addiction protein encoded by parE. Blast search of the proteins showed similar sequences to kluA and kluB found in IncP-1ϐ plasmids like R751* ***(C)*** *In some other IncP-1ϐ plasmids R772 and R906 two ORF dinJ and xf2080* ***(D)*** *are in a similar position as kluA and kluB between klcA and iteron 10 but are dissimilar in sequence. In RK2, an IncP-1α plasmid* ***(E)*** *and some IncP-1ϐ plasmids (Figure 3-8), none of these two ORFS are present.*

A BLAST search with the *klcA*-i10 *oriV* region from pRWC72a gave a set of results showing two additional highly similar plasmid sequence with no insertions in this region but then many others that lined up but had extra sequence interrupting this region (Figure 3-8). Some of these plasmids were on the list with *parDE* genes like pBURNS1 but others were not. Inspection of these latter revealed two short ORFS whose DNA sequences was quite different from the pBURNS1 *parDE* genes. However, on further analysis the first ORF was found to encode a protein with similarity to a potential regulator RelB and DinJ while the second ORF encodes a protein that also has similarly to RelE/ParE. The phylogeny of the IncP-1β plasmids can therefore be refined by the structure of this region.

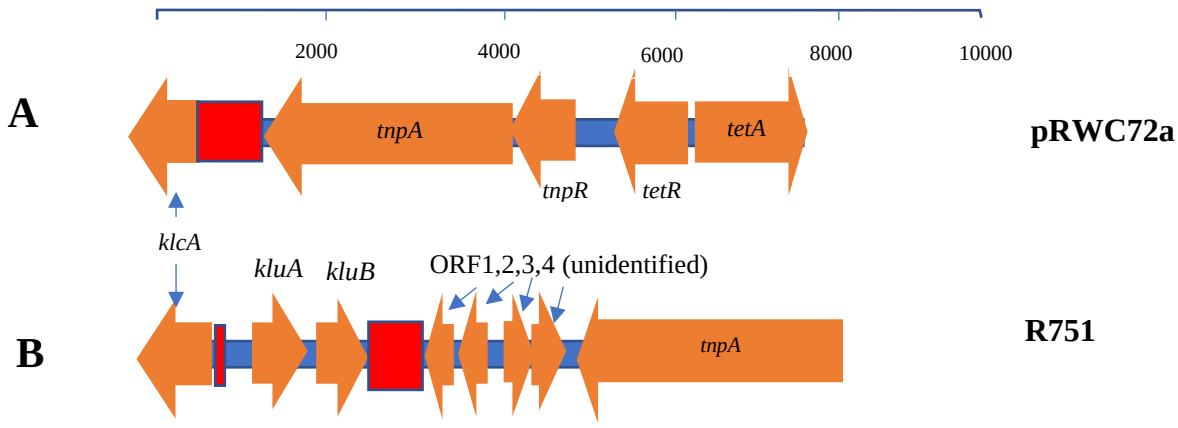


Figure 3-8. Comparison between two IncP-1β plasmids based on the *klcA*-i10 *oriV* region.

The plasmid pRWC72a was one of three plasmids identified via BLAST that did not have any annotated genes resembling parDE between klcA and i10 of oriV region but had the trfA genes. Regions in red show the matches of the1090bp segment between the klcA and tnpA in pRWC72a where kluA and kluB absent and R751 where kluA and kluB are present and split the region into segments of 80bp and 851bp.

3.8 Comparison of pBURNS1 with other closely related IncP-1β plasmids

Based on the annotations previously made, a closer look at some of the closely related IncP-1β plasmids was deemed necessary to make further comparisons. The previous analysis

focused mainly on the *oriV* region and not the entire genome. Here, the pBURNS1 plasmid (untagged) was compared with other IncP-1β plasmids by obtaining their sequences through BLASTn which showed a cross section of closely aligned sequences. Plasmids whose backbone had both closest identity and highest percentage with pBURNS1 were chosen for comparison. Knowledge of the relationship between the pBURNS1 plasmid and the other closely related plasmids can give an insight to its origin.

Plasmid pDB1 was originally isolated from *Variovorax* sp DB1 from the soil environment (Kim et al, 2013). It has the entire *tra* region (*traC-N* genes) and *trb* operon almost identical to that of pBURNS1. Regions that differ between the two plasmids include the sites of insertion between *trfA* and *oriV*. The transposon and integron which includes the *cdm* gene in pBURNS1 is absent in pDB1. Absent in pBURNS1 but found in pDB1 is a transposon carrying genes for 2,4-dicholorophenoxy-acetic acid degradation. The *trfA* gene, ssb, *trbA, B, C, D*, and *trbE* have 98% identity with pBURNS1 (Figure 3-9).

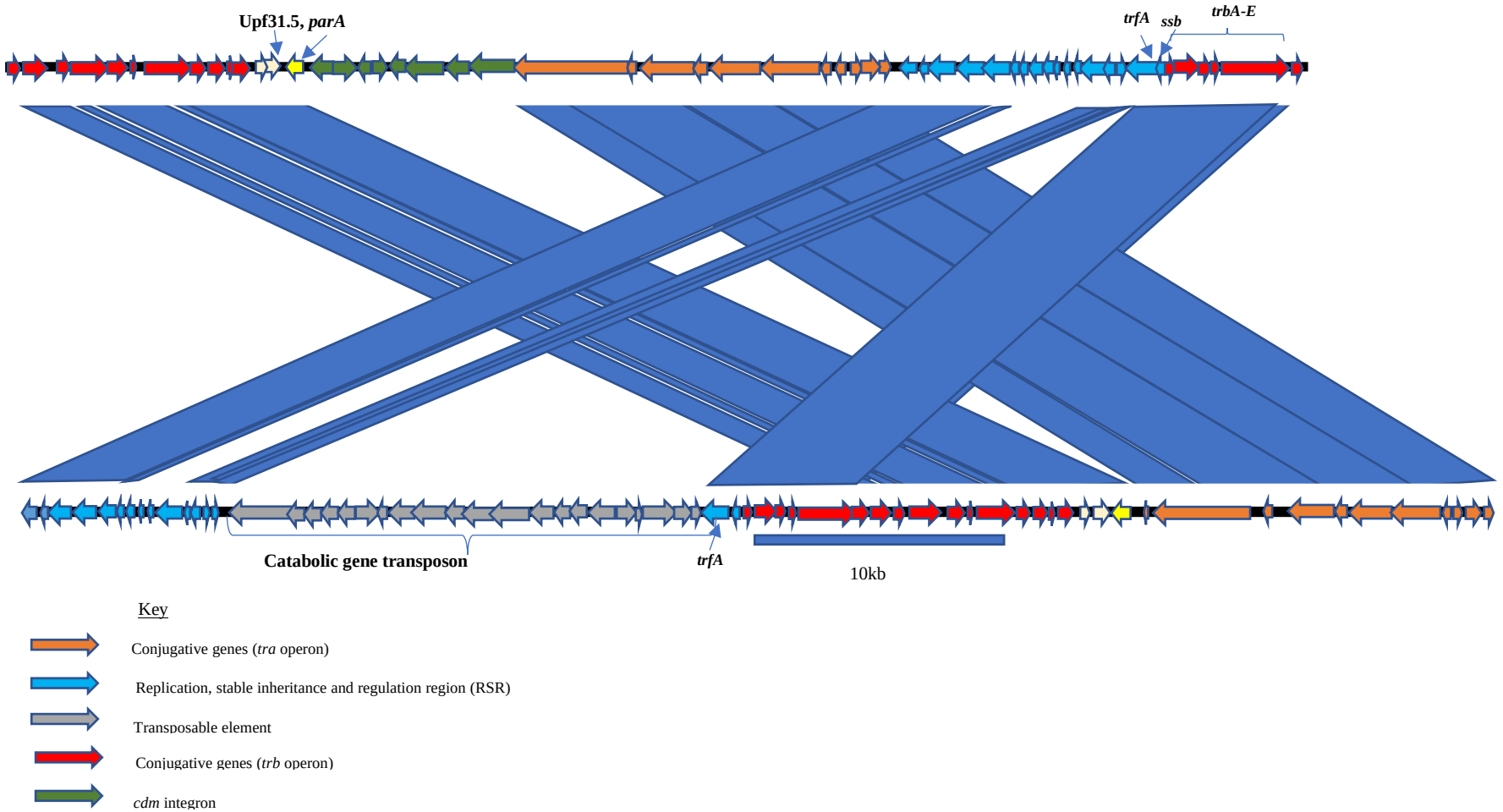


Figure 3-9. Plasmid sequence comparison between pBURNS1 (top) and pDB1, JQ436721 (bottom)

The major differences between both plasmids are the presence of the cdm integron in pBURNS1 and its absence in pDB1 and the absence of the mer genes (catabolic gene transposon) in pBURNS1 and their presence in pDB1 between trfA and the tra operon.

Plasmid pKSP212 is an IncP-1β plasmid isolated from a hospital waste-water treatment plant (Sen et al, 2013). It has the whole of *trb* and *tra* operon at 99% identity with those of pBURNS1. Replacing the transposon in pBURNS1 is an integron containing the *int*1 gene, *aac*, *sul* and a hypothetical protein in pKSP212. Some components of the replication, stable inheritance and regulation region (RSR) *kor*, *kle* and *klc* genes are present in both plasmids (Figure 3-10)

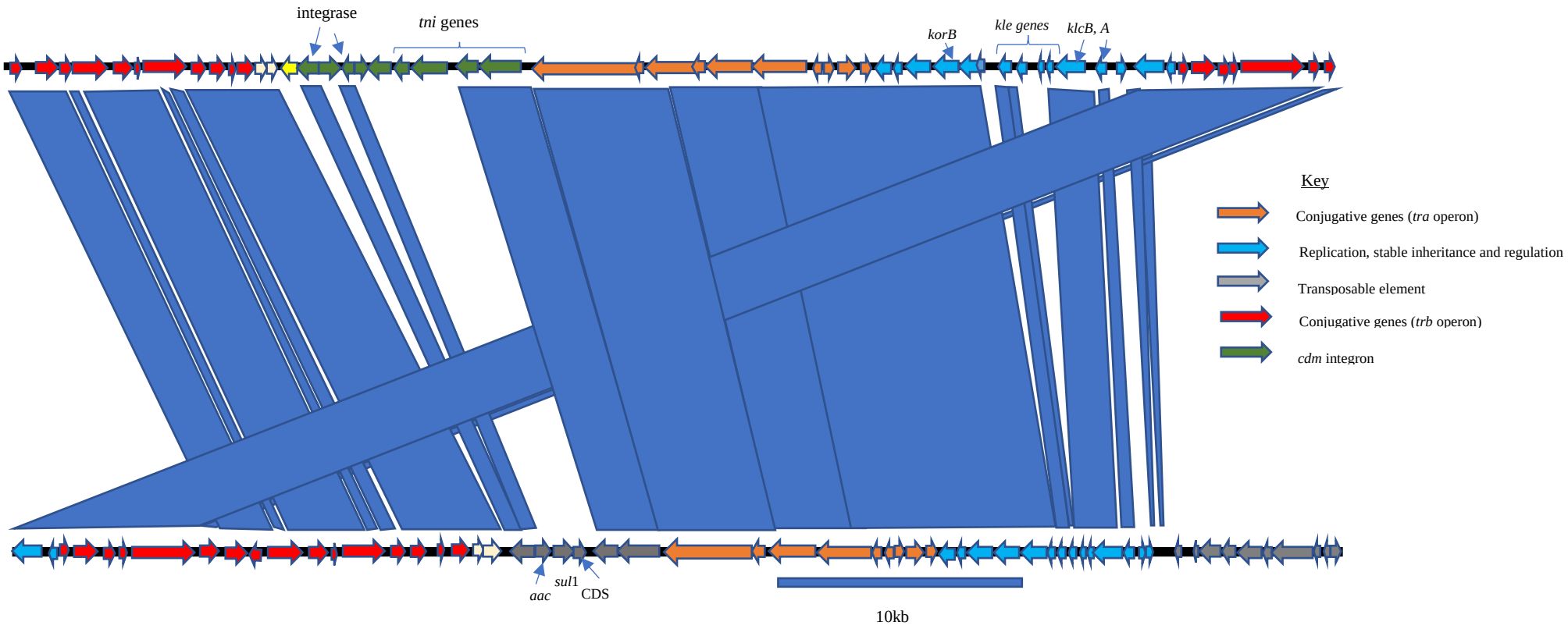


Figure 3-10. Plasmid sequence comparison between pBURNS1 (top) and pKSP212, JX469831.1 (bottom)

In both plasmids, the tra and trb operon show a very similar percentage identity. The main difference lies in the presence of antibiotic resistance genes and mercury resistance genes in pKSP212 and their absence in pBURNS1. The tni genes and integrase in pBURNS1 show identity with those in pKSP212.

The plasmid pTP6 was isolated from a mercury-polluted river sediment in Kazakhstan (Smalla et al, 2006). In pTP6, part of the transposon, *cdm* integron of pBURNS1 are replaced by the *mer* operon carrying the genes necessary for mercury resistance within Tn*5058*. *tniQ*, *tniB* and *tniA* of pBURNS1 have 89% identity with those of pTP6. The only resistance genes present in pTP6 are the *mer* genes (Figure 3-11).

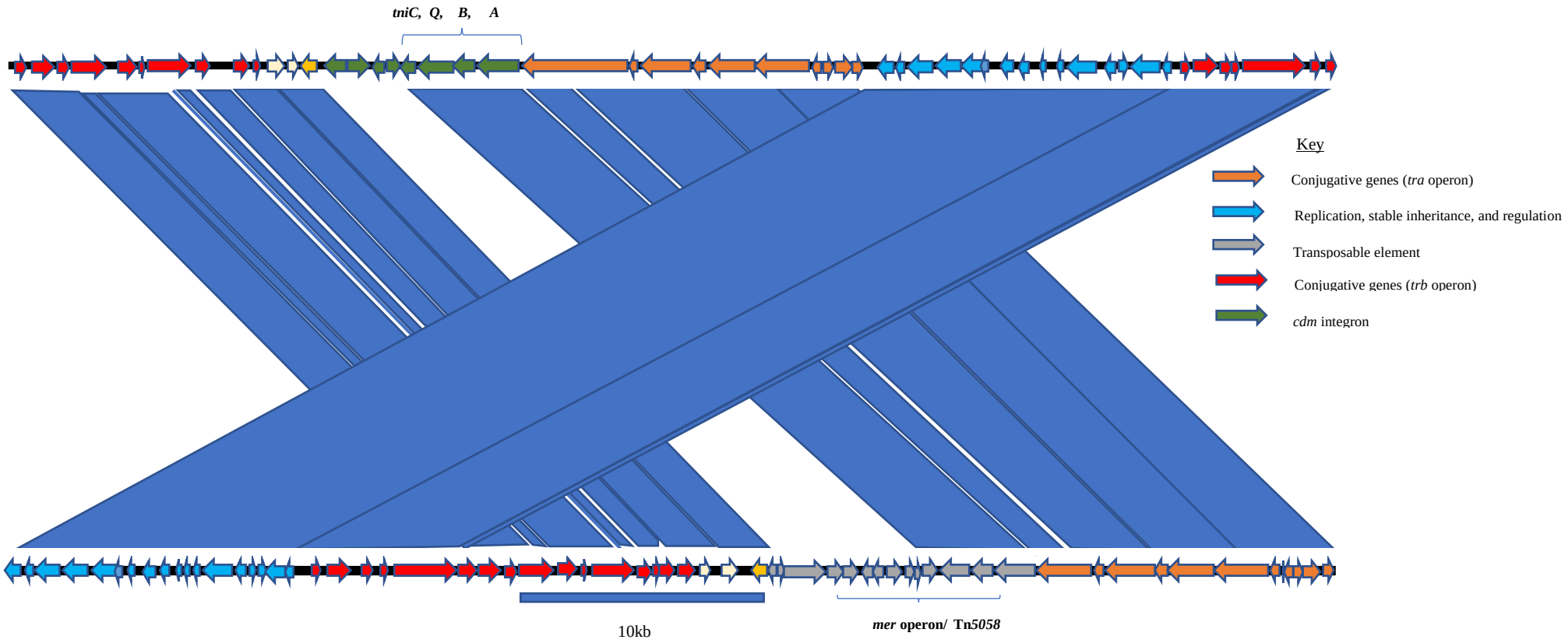


Figure 3-11. Plasmid sequence comparison between pBURNS1 (top) and pTP6, AM048832.1 (bottom)

In this comparison, part of the cdm integron in pBURNS1 is replaced by the mer operon in the pTP6, the complete tni genes are homologous in both plasmids, as are the trb, tra operon and replication, stable inheritance and regulation region (RSR).

R751, first isolated from *Enterobacter aerogenes* (Thorsted et al, 1998) is referred to as the prototype backbone for IncP-1β plasmids as RK2 is for IncP-1α plasmids because they were the first IncP-1β sequenced (Thorsted et al, 1998) and first plasmid placed in a different subgroup (Pansegrau et al, 1994) respectively. As with the previously mentioned plasmids, pBURNS1 is highly similar to R751 with the main difference being the accessory genes they carry. Transposon Tn*4321* is present in R751 but absent in pBURNS1. The *tnpA* and *tnpB* in pBURNS1 is absent in R751 but the *tniC, Q, B* and *A* in both are homologous (Figure 3-12). The class1 integron genes have 95% identity.

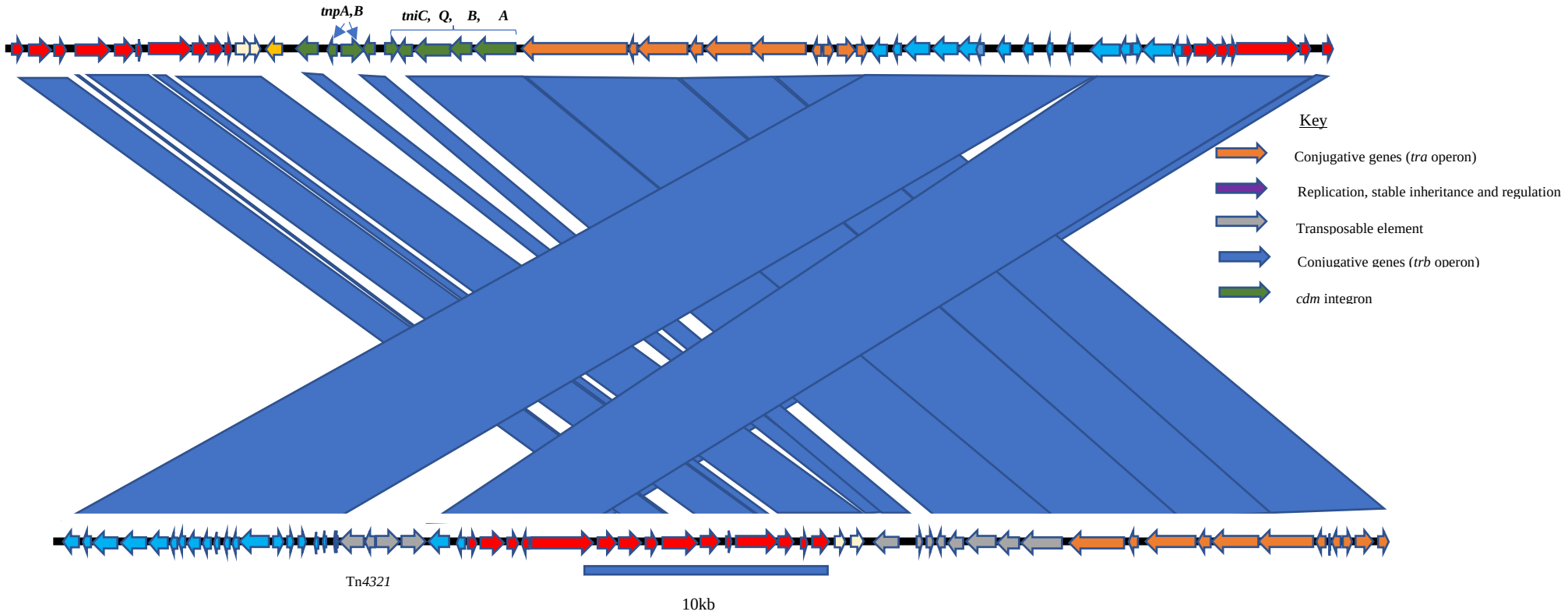


Figure 3-12. Plasmid sequence comparison between pBURNS1 (top) and R751, U67194.4 (bottom)

In this plasmid comparison, there is high identity (homology) between the trb and tra genes, the replication, stable inheritance and regulation region (RSR) and integron. The only major differences are the absence of cdm gene in R751 and the transposon between RSR and trfA in pBURNS1

The plasmid R772 was first isolated from *Proteus mirabilis* from clinical sources (Petroviski and Stanisich, 2011). R772 differs mainly from pBURNS1 with the presence of a transposon comprising of a complete set of mercury encoding genes upstream of *trfA*. Also present in the transposon, Tn*511-3* are *socD* (santhopine degradation), *aphA* (aminoglycoside resistance), transposase (*tnpA*) and resolvase (*tnpR*)genes. The entire transposon in pBURNS1 is absent in R772. A second transposon Tn*514* between the *tra* and *trb* genes is also absent in pBURNS1 (Figure 3-13).

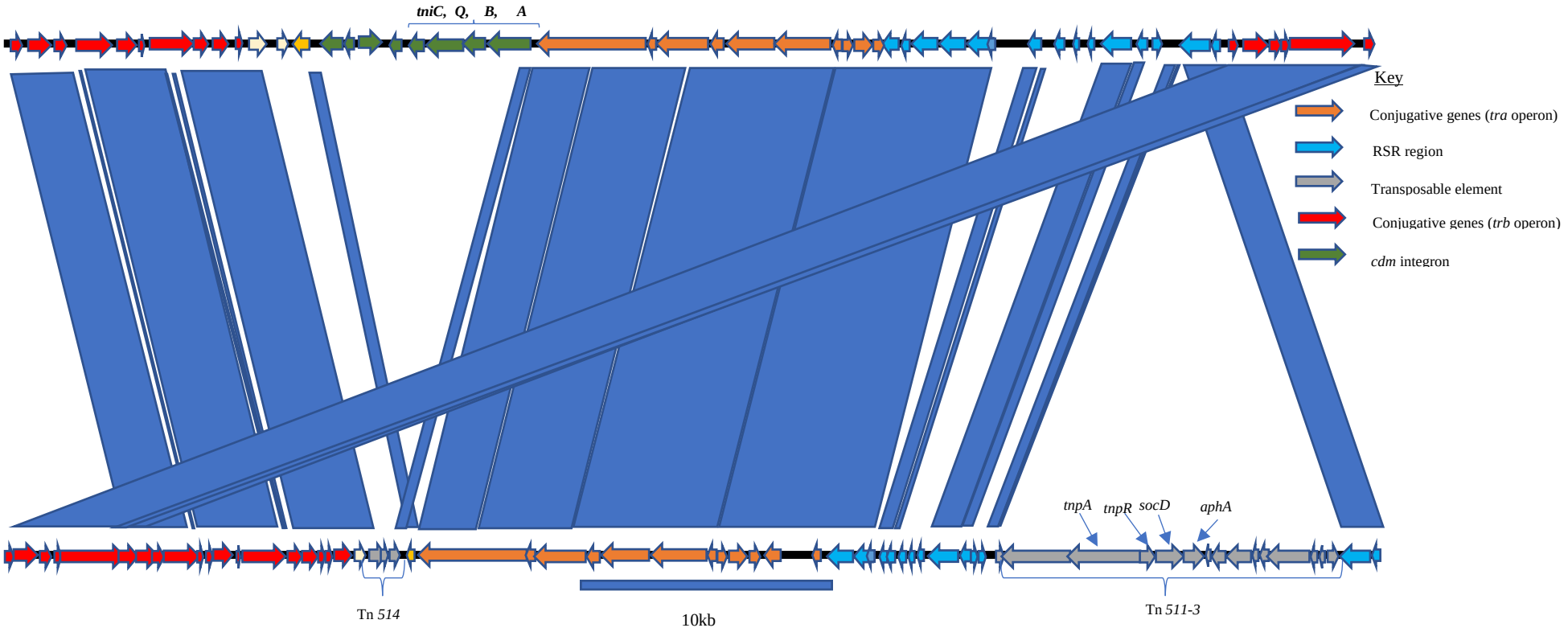


Figure 3-13. Plasmid sequence comparison between pBURNS1 (top) and R772, KF743817.1 (bottom)

The main difference between pBURNS1 and R772 is the presence of two transposons Tn514 and Tn511-3 in R772 and their absence in pBURNS1. The entire cdm integron and tni genes are absent in R772. There are also differences in the replication, stable inheritance and regulation region

R906 has 99-100% identity with the entire *trb* and *tra* regions and the *trfA* and ssb region have 96%identity with pBURNS1. A transposon Tn*510*/Tn*5393* located between *trfA* and *oriV*, in R906, in addition to the *mer* operon, carries *strA* (aminoglycoside-3-phosphotransferase) and *strB* (aminoglycoside-6-phosphotransferase) resistance genes (Figure 3-14). All complete nucleotide sequences of these plasmids where downloaded from Genbank and the Artemis and ACT software were used to make double alignments and compare the differences between pBURNS1 and six other IncP-1β plasmid genomes as seen in Table 3-2.

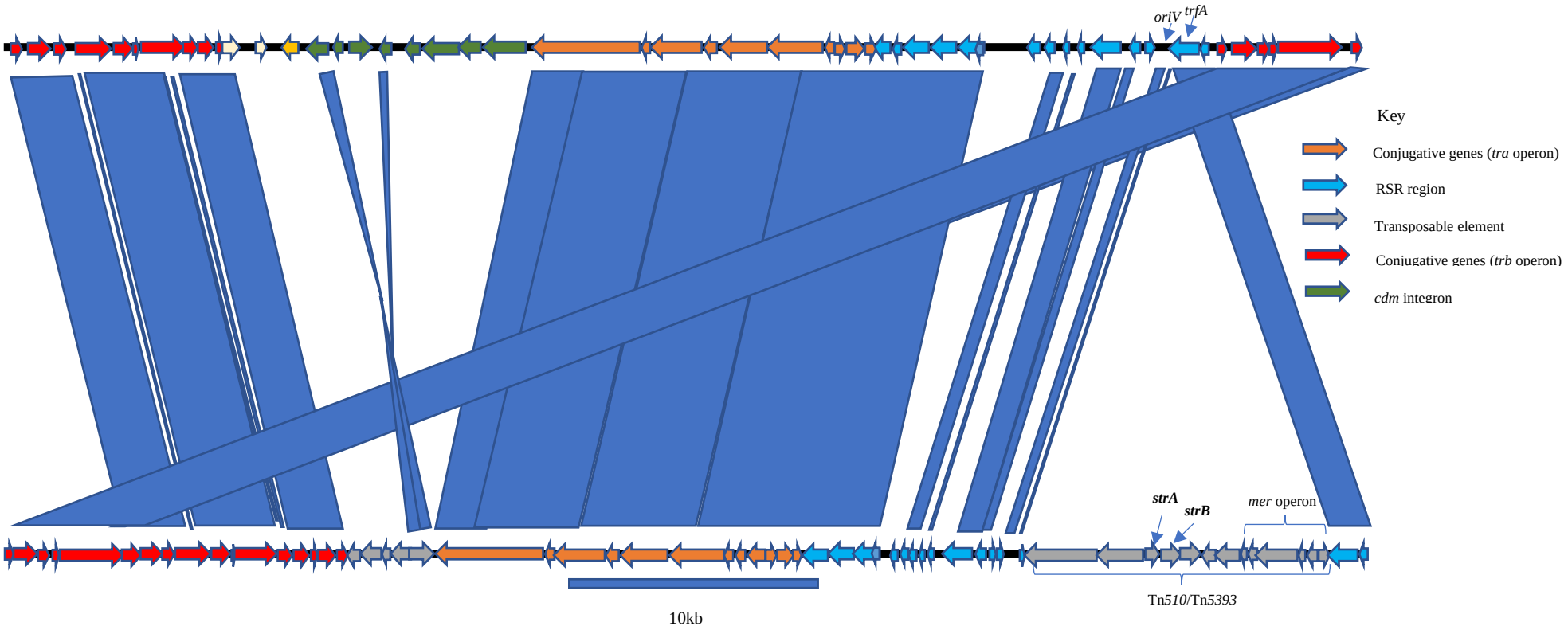


Figure 3-14. Plasmid sequence comparison between pBURNS1 (top) and R906, KF743818.1 (bottom)

R906 like R772 have two insertions between trfA and oriV and between the trb and tra operons. The integron between tra and trb in R906 shares no similarity with the integron in pBURNS1 except with the int1 genes. Regions of close identity in the two plasmids include the transfer genes and the replication, stable inheritance and regulation region (RSR).

Table 3-2. The similarity of pBURNS1 sequence to closely related IncP-1β plasmids using BLAST

Plasmid	Inc type	Organism	Source	*Coverage of pBURNS1 (%)	%identity *	Accession number	Reference/ Date deposited
pDB1	IncP-1β	*Variovorax sp.* DB1	soil	80	99.64	JQ436721	Kim et al, 2013
pTP6	IncP-1β	Uncultured bacterium	Mercury-polluted river sediment	92	99.5	AM048832.1	Smalla et al, 2006
R751	IncP-1β	*Klebsiella aerogenes*	Peritoneal-dialysis catheter	93	99.4	U67194	Thorsted et al, 1998
pKSP212	IncP-1β	uncultured bacterium	Hospital waste-water treatment plant	87	98.8	JX469831	Sen et al, 2013
R906	IncP-1β	*Bordetella bronchiseptica*	Porcine	81	98.5	KF743818	Petrovski and Stanisich, 2011
R772	IncP-1β	*Proteus mirabilis*	Clinical specimen	79	98.5	KF743817	Petrovski and Stanisich, 2011

*

Identity	*The percentage degree of similarity between the two plasmid sequences being compared*
Coverage	*The area within both sequence which have the most accurate matches. This may vary with sequence length*

3.8.1.1 Genetic context of chlorite dismutase gene within the class 1 integron and transposon

Analysis of the integron content showed that alongside the *cdm* gene, the Class 1 integron itself is flanked by a TniC- like non-composite transposon. The transposon also contains the *tnpA* and *tnpB* genes which code for transposase and resolvase, respectively. The *tnpA* is closely related to the IS3/IS 911 family protein while the *tnpB* is similar to the IS2 family protein sequence (Post et al, 2007). These type of Class 1 integrons differ from the typical class 1 integrons which have a conserved segment 3' region containing *qacEΔ1* and *sul1*genes. Instead, they tend to end with the transpositional protein TniC. The reason was suggested to be based on the environmental rather than clinical origin of the integron (Samuelsen et al, 2008). A map showing the *cdm* gene within a class 1 integron and TniC transposon can be seen in Figure 3-15. Several TniC transposons have been found in *Pseudomonas sp* isolates and are also frequently associated with multidrug resistant metallo-β-lactamases (Samuelsen et al, 2008).

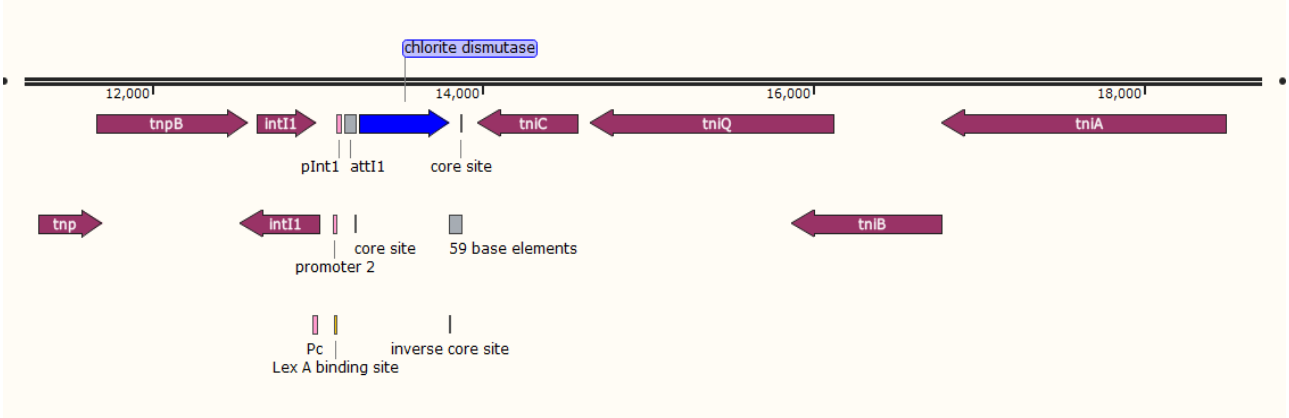


Figure 3-15. Map of the transposon region present in pBURNS1 showing the chlorite dismutase gene within an integron.

A screenshot of the annotated regions of the pBURNS1 plasmid showing the cdm gene within an integron and next to a transposon. The 59 base elements, promoter regions and core sites are also highlighted.

The entire integron showed 100% identity to an element in the chromosome of *Pseudomonas rhodesiae* strain BS2777 (Accession LT629801). This includes the integrase, chlorite dismutase gene, *tniC* and *tniQ* but without the *tnpB* ruling out it being part of a transposon. *P. rhodesiae* is a naturally occurring Pseudomonad, a gamma protobacteria closely related to *P. flourescens* and was first identified in mineral water/ springs in France (Coroler et al, 1996). Little is known about its clinical significance (Coroler et al, 1996). Another similar match of the *cdm* gene and integron was in *Pseudomonas putida* isolate from a hospital toilet facility (Santos et al, 2010). Along with the *cdm* gene which was represented as an open reading frame of unknown function, the class 1 integron carried a metallo-β-lactamase gene conferring resistance to β-lactams including carbapenems (Santos et al, 2010). A cartoon representation of various integrons carrying *cdm* is presented in Figure 3-16.

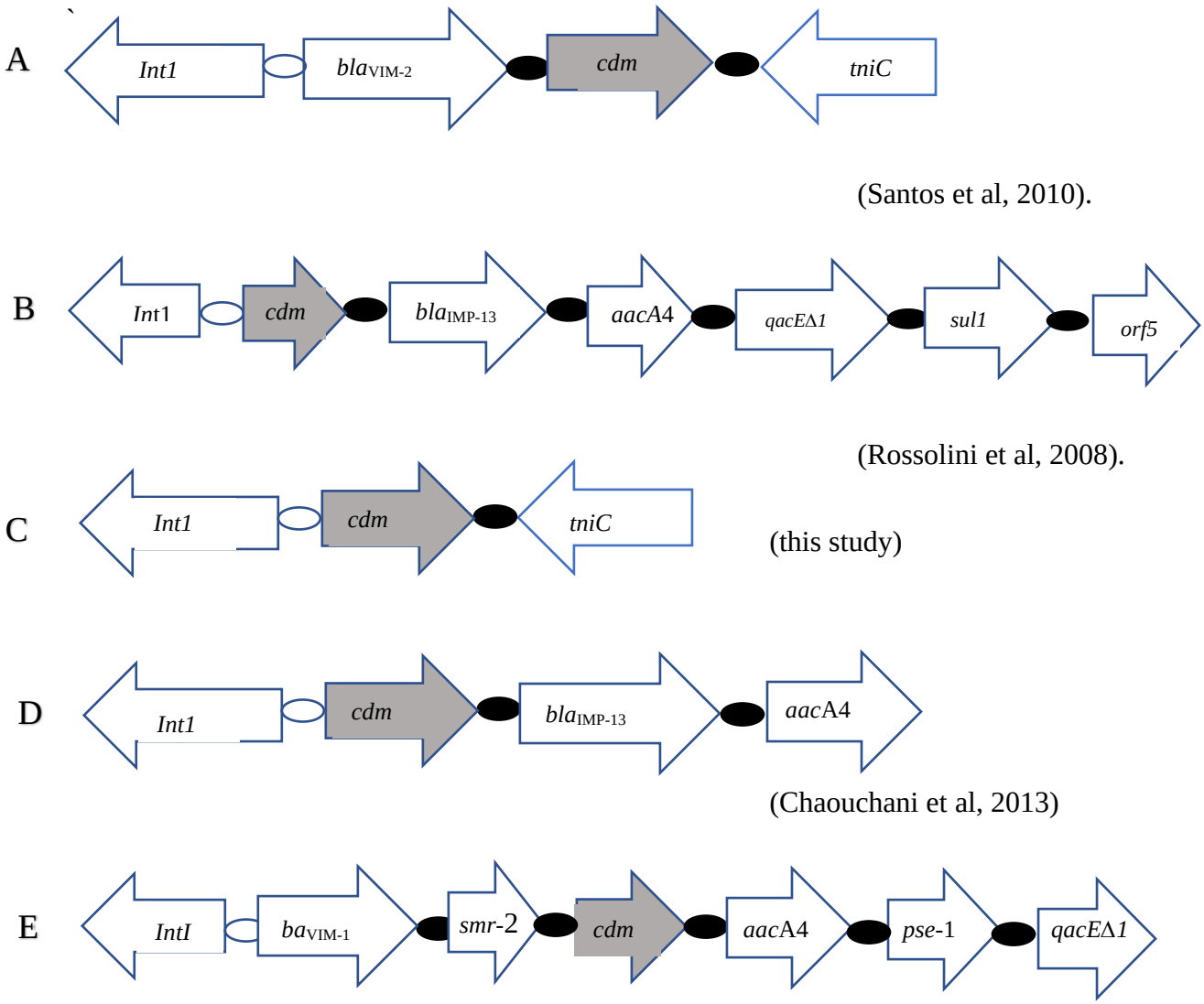


Figure 3-16. Other integrons showing BLAST matches to the *cdm* gene in pBURNS1

A. The P. putida PFi integron carries the cdm gene in one of its gene cassettes along with a blaVIM-1 gene. This integron like pBURNS1 has the TniC gene rather than the conserved 3' end of class 1 integrons. ***B.*** *The P. aeruginosa integron carries the cdm gene, aacA4 (aminoglycoside 6'N acetyltransferase type 1) which confers resistance to kanamycin. bla_{IMP-13} a metallo β lactamase gene conferring resistance to beta lactam antibiotics and the genes contained in the 3' conserved region (qacEΔ1, sul1, ORF5).* ***C.*** *P. aeruginosa integron contained in the pBURNS1 plasmid. It consists of the cdm gene as the only resistance gene in the integron.* ***D.*** *Klebsiella pneumoniae integron carrying cdm and identical to B except without the 3' conserved region.* ***E.*** *P. aeruginosa integron carrying cdm and a host of other resistance genes including smr-2, aacA4, bla_{VIM-1} and pse-1.*

The *P. aeruginosa* strain which contains In89 in its chromosome was isolated from a urine specimen in Italy in a survey to identify metallo-β-lactamases in Gram negative bacteria. About 93% of the isolates that carried these metallo-β-lactamase genes were from *P. aeruginosa* strains (Rossolini et al, 2008). A second integron discovered during a survey/metallo-β-lactamase screening of *P. aeruginosa* collected over four years from hospitals in Italy along with the *cdm* gene, had a host of other resistance genes including a metallo β- lactamase gene, *aacA4* gene, *smr-2* gene and *pse-1* gene (Toleman et al, 2005).

Klebsiella pneumoniae also carried a pBURNS1 *cdm* match on an integron and alongside, a $bla_{IMP\text{-}13}$ and an *aacA4* gene conferring resistance to beta-lactamases and kanamycin respectively. The strain was isolated from a polluted river in Tunisia (Chaouchani et al, 2013). From the above *cdm* blast matches, it can be observed that the *cdm* gene in Gram negative bacteria within the clinical setting is most likely to be present on mobile genetic elements especially class 1 integrons with or without accompanying antibiotic resistance genes. From Table 3-3 below it is also observed that when *cdm* is found in environmental isolates, this gene is likely to be part of the chromosome. The environmental habitats of the strains in which the *cdm* gene is found suggests that it originated from an aquatic environment which managed to by-pass the disinfection process in clinical settings and could eventually get into the human system with or without being involved in the cause of nosocomial infections.

Table 3-3. List of the top BLAST matches of the *cdm* gene found on class 1 integrons

Accession number	Strain	Location of strain	*Identity with pBURNS1 gene	Location of *cdm*	Year of isolation	Reference
LT629801.1	*P. rhodesiae*	Water	100%	Chromosome	2016	unpublished
FJ172676.1	*P. aeruginosa*	Urine sample	100%	Unspecified/genomic	2004	Rossolini et al, 2008
FJ237530.1	*P. putida*	Hospital toilet	100%	Unspecified/genomic	2002	Santos et al, 2010
AJ784256.1	*P. aeruginosa*	Nosocomial	100%	Unspecified/genomic	2002	Toleman et al, 2005
HE605041.1	*Klebsiella pneumoniae*	Polluted river	99.28%	Unspecified/genomic	2010	Chouchani et al, 2013
CP021382.1	*Cellvibrio sp*	Fresh water pond	83.39%	Chromosome	2014	unpublished

The matches provided a mixture of both clinical and environmental bacterial strains contained mostly on class 1 integrons most of which had metallo-β-lactamase genes alongside the cdm gene. It also suggests a link between the chromosome bound cdm gene found in the purely environmental bacteria and opportunistic pathogens found within the hospital environment. The identity refers to the degree of homology with the other plasmid.

**Definition of Identity:*

Identity	*The percentage degree of similarity between the two plasmid sequences being compared*
Coverage	*The area within both sequence which have the most accurate matches. This may vary with sequence length*

3.9 Phylogenetic analysis of the Cdm protein

The chlorite dismutase protein sequence was run through BLAST to establish matches in order to determine the phylogenetic background to the protein encoded pBURNS1. A large database of matches was found which included mostly Gram-negative bacteria from the phyla α, β and γ Proteobacteria, Cyanobacteria, Enterobacteria and Firmicutes. Common species found to have the closest matches and their phylogenetic relationship are shown in Figure 3-17 below. The Cdm protein from *P. aeruginosa* isolate fell closest to other *Pseudomonas sp* groups such as *P. putida* and *P. rhodesiae.* The enteric bacteria of the family Enterobacteriaceae were clustered closer together as sister taxa, so also a group of Gram-negative opportunistic pathogens. As expected, the Cdm protein from the Cyanobacteria group were also clustered as a clade and have a nearer ancestral background with each other than other bacterial groups. The phylogenetic tree indicates that the Cdm protein in *Pseudomonas* spp and Enterobacteriaceae have had the most recent mutations in contrast to the Cyanobacteria group. However, the *Pseudomonas spp* clade has a more closely shared ancestor with the Cyanobacteria than with the Enterobacteriaceae clade. The complete Cdm protein sequence is found in Appendix 1 of this thesis.

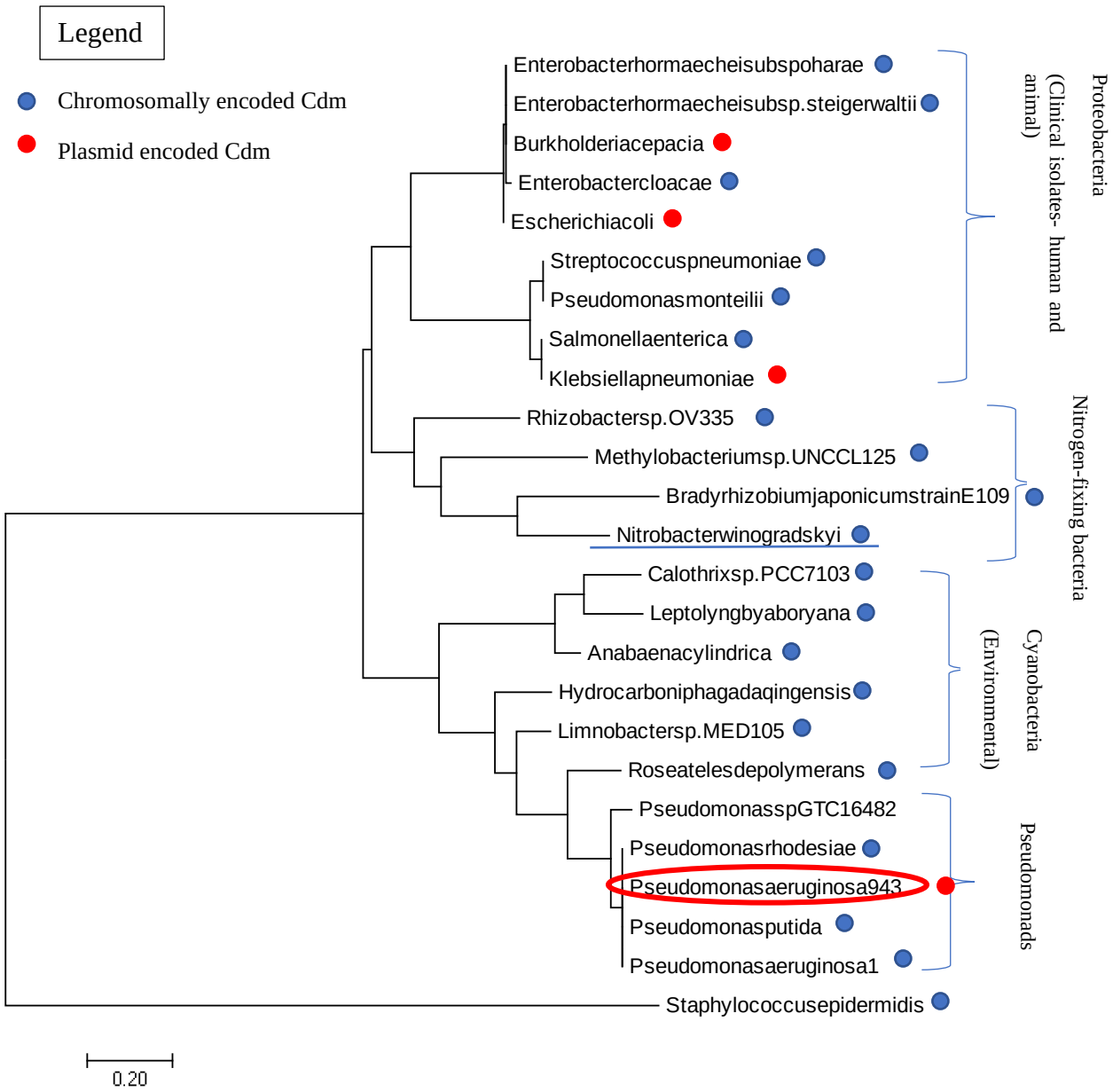


Figure 3-17. A phylogenetic tree which represents the ancestral relationship between the Cdm protein found in the *P. aeruginosa* 943 isolate and other bacteria through BLAST

The Cdm protein from P. aeruginosa 943 pBURNS1 plasmid compared with other bacteria in the database showed a closer ancestral lineage relationship with cyanobacteria than the proteins present in the clinical bacteria. Circled in red is the pBURNS1 strain showing where it fits in the tree. Underlined in blue is Nitrobacter winogradsky, the archetype of lineage II chlorite dismutases. Multiple sequence alignments were carried out using Clustal Omega and the tree was assembled using MEGA v7.0.

3.10 Discussion

The first bacteria where chlorite dismutases (*cdm*) were found were perchlorate reducing bacteria (PCRB) (Xu et al, 2003). The presence of the enzyme was justified because chlorite is produced intracellularly in PCRB and being toxic to the host cell, there was a need to convert it to chloride which is non-toxic. Phylogenetically, *cdm* has been found in a wide range of bacteria and archaea suggesting an early ancestral presence (Hofbauer et al, 2014). However, its significance remains unclear since some of its host organisms excluding PCRB do not produce chlorite intracellularly (Maixner et al, 2008). *P. aeruginosa* 943 Cdm (Pacdm) is present on a class 1 integron without the conserved 3' end gene cassettes, instead having the *tni*C gene. Sequence identity through protein BLAST showed 100% match with *P. rhodesiae* which had the *cdm* within its chromosome. The host of other matches in *Pseudomonas* strains and *Klebsiella* had their *cdm* within class 1 integrons with a peculiar presence of metallo-β-lactamase among the gene cassettes. Cdms, Cdm-like proteins and Dye decolorizing peroxidases (Dyps) belong to the same super family and have a common ancestral lineage. It is suggested that the clustering of Cdm proteins from diverse families on a phylogenetic tree possess a common physiological function such as the ability to degrade chlorite (Hofbauer et al, 2014).

From the phylogenetic tree, Proteobacteria and Cyanobacteria are two distinct phyla whose Cdm protein belong to the same group as the *Nitrobacter winogradski* (Nwcdm) collectively called the Clade/ Lineage II Cdm proteins (Mlynek et al, 2011). Although most of the cyanobacteria have an active *cdm*, they do not necessarily have the capacity to degrade chlorite and can be limited in catalytic efficiency (Hofbauer et al, 2014). For Cdm to be catalytically efficient it must possess five key residues. Ile88, Trp97, Leu122, Arg127 and Glu167. Cdms can be differentiated based on the ability of the protein to bind to the heme.

Those that bind weakly are moderately or non-functional while those that bind strongly are usually highly functional (Mlynek et al, 2011).

It can be assumed that very early on there was a distinction between the opportunistic pathogenic organism's Cdm and those found in free-living Cyanobacteria and *Pseudomonas sp*. The clade where the pBURNS1 Cdm was found in the phylogenetic tree (as shown in a red circle in above) falls within the non-pathogenic protobacteria clade. The location of the pBURNS1 Cdm may give an insight to the origin of the *P. aeruginosa* 943 strain. It is more likely that the natural habitats of *P. putida, P. rhodesiae* and *Rhoseateles depolymerans* are the immediate environmental source of *P. aeruginosa 943* rather than from the hospital environment/patients. This is in line with the outcome of the whole genome sequencing of *Pseudomonas* isolates from the QE hospital, Birmingham which suggests that the source of *P. aeruginosa* was most likely from the hospital water supply (Quick et al, 2014) and the likelihood that bacteria carrying IncP-1 plasmids and class 1 integrons can be enriched in aquatic environments that have suspended matter or objects in them such as plumbing parts (Barron et al, 2018).

Nitrogen fixing- Cyanobacteria containing *cdm* do not produce chlorite intracellularly and can contain either *cdm* or *dyp* genes within their genome. Phenotypically, they resemble the Nwcdm by belonging to the same Lineage II of functional *cdm*, but the physiological role of *cdm* cannot be affiliated to Cyanobacteria as a phylum since Schaffner et al, (2015) showed that only 4% of all sequenced genomes carried the *cdm* gene and 13% carried the *dyp* gene. Therefore, the presence and purpose of *cdm* in Cyanobacteria remains unclear.

The normal habitat of most of the strains found on the phylogenetic tree are aquatic/ environmental therefore the Cdm protein has a definite link to environmental aquatic

isolates. Members of the family Enterobacteriaceae and Pseudomonadaceae carrying *cdm* according to the phylogenetic tree have had recent mutations. It could be observed that the opportunistic pathogens cluster closely together in the same clade. Cyanobacteria carrying *cdm* also belong to clade II and its role in the environment has also not been fully verified nor understood. While the information provided by the phylogenetic tree can suggest an ancestral relationship between different protein sequences among various phyla, more evidence is needed to draw effective conclusions on the origin of the Cdm protein. However, based on the phylogenetic analysis, the origin of Pacdm is most likely to be from other environmental Pseudomonad isolates but since it is carried by a broad host range plasmid it could originate by horizontal gene transfer from other host bacteria.

However, close relationships within diverse phyla may suggest a common physiological function of *cdm* within different groups and the habitat of the harbouring bacteria may suggest the presence of a common substrate needed to be degraded by *cdm*.

Class 1 integrons have been around long before the introduction of antibiotics (Petrova et al, 2011). *tniC* was first detected in Tn*402* transposon of R751. *tniQ*, *tniA*, *tniB* genes provide transposase functions while *tniR* provides resolvase activity (Brown et al, 1996). The pBURNS1 integron has all *tni* genes and thus may be able to transpose the integron within the plasmid and chromosome of host strain. Most Tn*402*-like transposons have a truncated *tniR* which could render the integron non-transposable (Stokes et al, 2006). Upstream of the integrase gene in pBURNS1 are *tnpA* and *tnpB* genes that should be able to mobilize the integron through transposition.

It is often difficult to classify integrons into clinical or environmental due to a constant cycle of integration of waste from the hospital into the environment and vice versa (Gillings et al,

2008). The *tniC* integrons were initially found on chromosomes in non-clinical bacteria (Brown et al, 1996). This could explain our finding of the entire pBURNS1 integron on the chromosome of *P. rhodesiae*. Since IncP1 plasmids have hotspots on specific areas of its genome, acquisition of lateral gene transfer elements such as integrons and transposons may take place. The success of integrons therefore lies in their transposition abilities from chromosomes to other mobile genetic elements such as plasmids (Stokes et al, 2006). Although class 1 integrons have mostly been associated with hospital settings, class 1 integrons with and without resistance genes have been isolated from water sources that have little or no links with hospital effluents (Rosser and Young, 1999).Therefore, pBURNS1 may be a vector for the dissemination of the integron from a possible environmental origin.

Comparing the *klcA*-i10 (iteron 10) of pRWC72a with R751 and pBURNS1 revealed that the *kluA* and *kluB* genes found in the latter two are absent in the former. When the sequence *klu*A and *kluB* was run through BLAST, there were only a limited number of plasmids that showed identity (25 hits) compared to the much larger number of hits obtained when the region replacing *kluA* and *kluB* was run. Since the pRWC72a plasmid does not have the *parD* or *parE* genes or any homologues to these genes, it will be worth determining how related these set of plasmids are phylogenetically to pBURNS1. IncP-1β plasmids closely related to pBURNS1 along with IncP-1β plasmids with and without *parDE* homologues were analysed using the Geneious prime software to create multiple sequence alignments and MEGA 7.0 to generate a phylogenetic tree.

Since pBURNS1 now has a *parDE* homologue, it could mean an increased level of plasmid stability which was missed in earlier studies (Thorsted et al, 1998). This may also give a hint that other gene pairs with similar protein sequence homologies may likely have the same

functions in an IncP-1 plasmid such as the KlcB and KorA and TrbA proteins (Thomas et al, 1995).

3.11 Conclusion

The pBURNS1 plasmid is a circular, self-replicating and self-transmissible plasmid and this was confirmed by performing restriction digests, gel electrophoresis, conjugation and transformation tests using hosts' carrying the plasmid. Bioinformatics was used to identify the potential origin of the Cdm protein by identifying the hosts ancestral phylogeny.

The pBURNS1 Cdm protein can be classified as belonging to the Lineage II group of Cdm/clds similar to Cdm isolated from *Nitrobacter winogradsky* with amino acid sequence length of about 182aa. This group is structurally and biochemically different from the Lineage I group (eg. *Dechloromonas agitata* and other perchlorate reducing bacteria Cdms) but still highly active/functional *cdm* genes. The pBURNS1 plasmid was found in a biofilm in the Burns unit and it is likely that other strains of bacteria and other phyla would also be present within the biofilm. The phylogenetic analysis gives an insight to the probability of the plasmid being in *Pseudomonas aeruginosa* through horizontal or lateral gene transfer. From the results, the likelihood that the former was the case is low because the clade the pBURNS1 Cdm protein belonged to was that of Pseudomonads specifically. Therefore, if other organisms in the mixed species biofilm had acquired pBURNS1, it would be from *P. aeruginosa* and not vice versa. Further study of the annotation of pBURNS1 showed a newly discovered variation within the site of replication initiation (*oriV*) and *klcA*. The two genes in R751 and pBURNS1 designated as *kluA* and *kluB,* responsible for cell maintenance through toxin-antitoxin post segregational systems and multimer resolution respectively, are either absent, present or have a different sequence but similar function within IncP-1β plasmids. These functions are coordinated and encoded by the *par* operon in IncP-1α

plasmids. Comparison of pBURNS1 to a selection of IncP-1β plasmids showed high identity between most parts of the genome excluding the *oriV-trfA* sites and between *trbA* and *trbB* which are common hotspots for insertions of mobile genetic elements such as transposons.

Chapter 4

Investigating the phenotypic properties of the pBURNS1 plasmid

4.1 Introduction

The genomes of several *cdm* carrying strains have been sequenced and their chlorite degrading activity checked (Bender et al, 2002; Maixer et al, 2018; Mylnek et al, 2011). Some of these bacteria can degrade chlorite while some of them do not. Of all the literature found, this is the first recorded *cdm* to be found in a *Pseudomonas aeruginosa* plasmid rather than on the chromosome. It's presence on an IncP-1 plasmid may further increase the spread of resistance to other bacteria through horizontal gene transfer.

Following the discovery of pBURNS1 it is important to determine what the significance of the spread of *cdm* within the hospital setting might be. For example, how important is this spread with respect to survival of other bacteria within the hospital setting and hospital water supply? Recent screening of hospitals including the QE hospital (Quick et al, 2014) have shown that the basic chlorine dioxide treatment of hospital water supply may be insufficient from keeping environmental opportunistic pathogens at bay. The presence of *cdm* in hospital isolates is becoming more common but is frequently overlooked, being identified as a putative open reading frame without questioning its occurrence (Rossolini et al, 2008; Santos et al, 2010).

4.2 Aims

In this work, the consequences of the *cdm* gene on an IncP-1 plasmid was investigated, its functionality (chlorite degrading ability) in both the original host (*P. aeruginosa* 943) and laboratory *E. coli* strains, its transfer potential to other surrounding bacteria while using similar hospital water conditions with and without selection of chlorite.

Based on available information, it was proposed that chlorine dioxide could break down to chlorite after contact with organic matter such as a biofilm and that the chlorite produced

may be enough to give selective pressure on *cdm*-carrying pBURNS1 strains in a biofilm and therefore, leading to increased bacterial survival in chlorine dioxide treated water (Figure 4-1). Therefore, it was hypothesised that the pBURNS1 plasmid, when present in a biofilm, can transfer chlorite resistance to surrounding bacteria producing *cdm* functional transconjugants which will have a selective advantage over bacteria not carrying the plasmid.

The specific aims of this section are

- To test the transfer pBURNS1::Tn*1723* from one strain to another in a growing biofilm
- To test for the survival of plasmid-carrying and non-plasmid-carrying strains in various concentrations of chlorine dioxide
- To test for the survival of plasmid-carrying and non-plasmid-carrying strains in various concentrations of chlorite.
- Determine the Cdm+ phenotype in *P. aeruginosa* 943 and *E. coli* (pBURNS1::Tn*1723*)

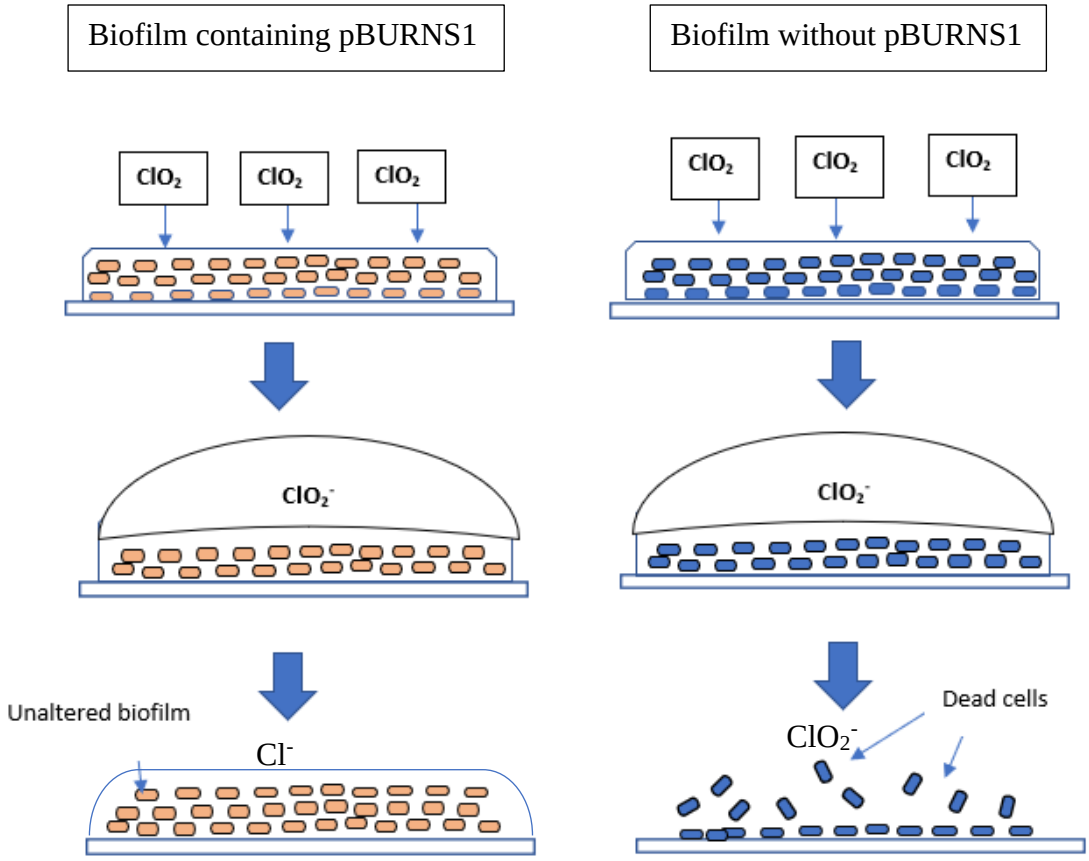


Figure 4-1. Diagram describing the hypothesis of the action of chlorine dioxide on a biofilm containing pBURNS1

The brown cells contain pBURNS1 which carries the cdm gene responsible for the breakdown of toxic chlorite to chloride. The dark blue cells represent a grown biofilm which does not contain within its cells the pBURNS1 plasmid. It is hypothesized that the cdm gene will be protective of a pBURNS1 carrying biofilm

4.3 Determination of inhibitory conditions for strains carrying pBURNS1::Tn*1723* and their corresponding plasmid free-strains

As part of the initial protocol to investigate the Cdm phenotype, it was necessary to test the survival of each plasmid carrying *E. coli* and *P. aeruginosa* strain along with the plasmid free versions. This was done to have an initial idea of the benchmark biocide concentrations

to use in future experiments. The lowest concentrations that would prevent planktonic and biofilm growth where therefore tested.

4.3.1 Minimum inhibitory concentration and Minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in sodium chlorite were determined for both *E. coli* and *P. aeruginosa* strains with and without pBURNS1::Tn*1723* plasmid. The sodium chlorite solution was prepared using both LB (Luria Broth) and SDW as solvent/ stock solution. It was observed that there was no difference between the MIC and MBC results of the two and therefore it was concluded that unlike chlorine dioxide, the sodium chlorite does not lose biocidal effect through contact with constituents of LB (no chlorine demand). The MIC and MBC was the same for both *E. coli* C600 (pBURNS1::Tn*1723*) and MV10 Nal^R (pBURNS1::Tn*1723*) (Table 4-1). Although *E. coli* C600 had a higher MIC and MBC than their mutant counterpart MV10 Nal^R, there was no detectable difference between the MIC and MBC of *P. aeruginosa* 943 WT and cured. This means that the Cdm phenotype seems to be absent from the original *P. aeruginosa* 943 WT strain, but we seem to see it when the plasmid is present in *E. coli.*

Table 4-1 MIC and MBC of planktonic plasmid positive and negative strains in chlorite

Strain	Sodium chlorite concentration			
	MIC (mM)		**MBC** (mM)	
	M9	**LB**	**M9**	**LB**
***E. coli* C600**	0.25	4	1	8
***E. coli* C600 (pBURNS1::Tn*1723*)**	1	16	2	>32
***E. coli* MV10nal ^{R}**	0.25	2	1	4
***E. coli* MV10nal ^{R} (pBURNS1::Tn*1723*)**	1	16	2	>32
***P. aeruginosa* 943 (cured)**	1	4	2	4
***P. aeruginosa* 943**	1	4	2	4

4.3.2 Minimum biofilm eradication concentration (MBEC) in Chlorine dioxide

It was also hypothesised that pBURNS1or pBURNS1::Tn*1723* may enhance the production of biofilm in the host strain when exposed to chlorite or chlorine dioxide as many plasmids have been observed to promote biofilm formation (Ghigo, 2001). This was determined by comparing the biofilm formation in the plasmid carrying strain to that of the plasmid free strain. Twenty-four and 48hr biofilms grown on separate beads without any selection were used to determine biofilm formation of pBURNS1 plasmid positive and negative strains. After 24 hrs, the results for *P. aeruginosa* showed no statistical difference between plasmid carrying and plasmid free, however after 48hrs, there is a slight decrease in the *P. aeruginosa* 943 biofilm (Figure 4-2).

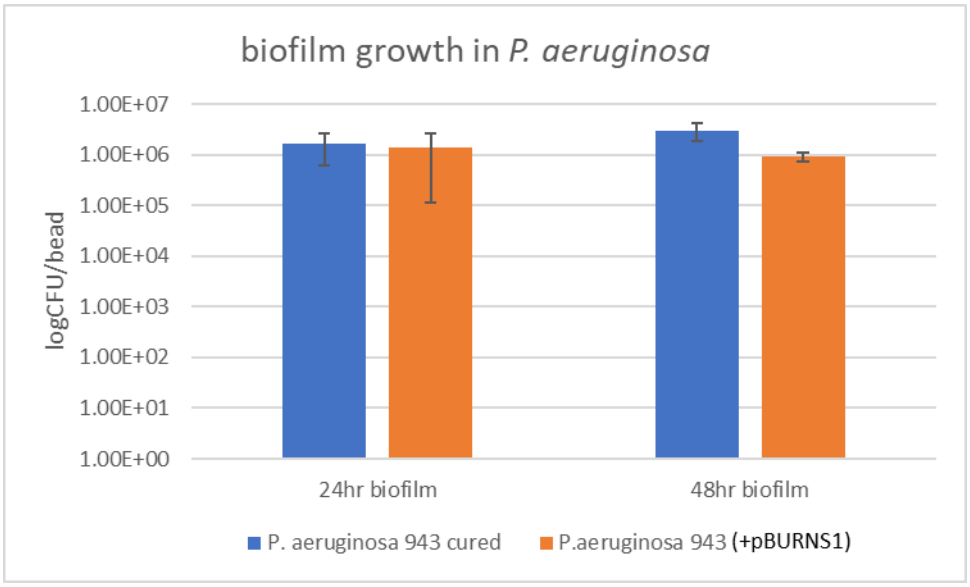


Figure 4-2. Resuspended *P. aeruginosa* biofilm counts grown on separate beads in LB after 24 and 48hrs without selection

The bead system was used to determine if the presence of pBURNS1 encourages biofilm formation ability in the host. Biofilms with and without pBURNS1 were grown separately under the same conditions for either 24 or 48 hrs. There was no statistical difference between the means of the two strains either at 24hr s and 48 hrs. The above chart is based on three replicates.

E. coli 48hr biofilms were also tested and a similar result but no statistical significance between the means of plasmid positive and negative was observed (Figure 4-3). The data therefore provides no evidence for pBURNS1::Tn*1723* promoting biofilm formation.

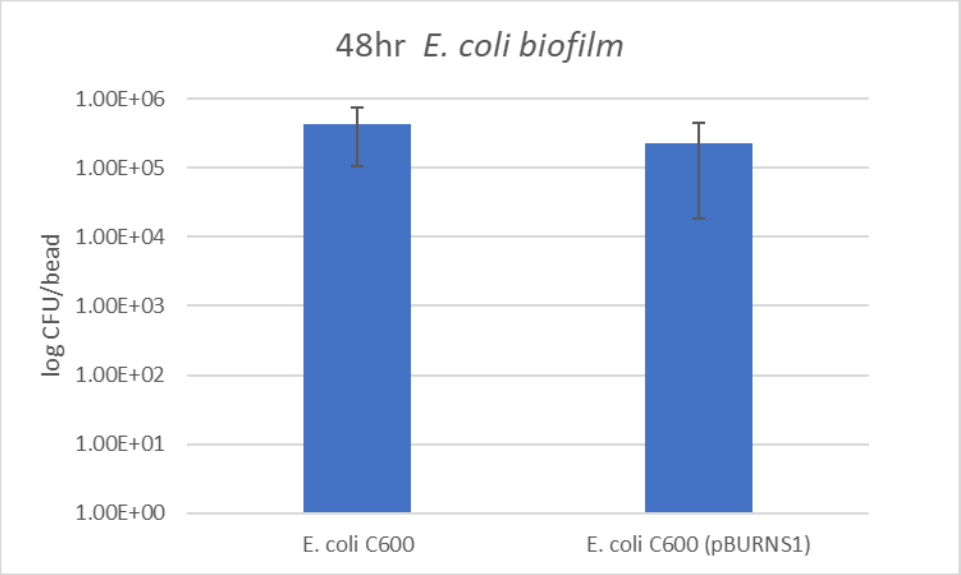


Figure 4-3. Resuspended *E. coli* biofilm counts grown on separate beads in LB after 24 and 48hrs without selection

The bead system was used to determine if the presence of pBURNS1::Tn1723 encourages biofilm formation ability in E. coli. Biofilms with and without pBURNS1::Tn1723 were grown separately under the same conditions for either 24 or 48 hrs. There was no statistical difference between the means of the two strains either at 24hrs and 48 hrs.

However, it is possible that pBURNS1 might change the nature of the biofilm formed such that it is more robust and more resistant to harmful chemicals. We therefore decided to determine the minimum biofilm eradication concentration (MBEC) of both *E. coli* C600 (pBURNS1::Tn*1723*) and C600. The MBEC was only conducted for Chlorine dioxide and not for chlorite since the question we are only interested in the events occurring in the hospital water supply. We hypothesise that chlorite should only be relevant if it is generated by chlorine dioxide interacting with the biofilm. The results after a number of repeats showed that there was no apparent difference in the MBEC of C600 pBURNS1::Tn*1723* and C600. The MBEC of both was 0.76 ppm after 24 hr biofilms of both were formed on pegs and treated with Chlorine dioxide solutions from 0.6 ppm to 1.5 ppm for 30 mins. The biofilms were grown using M9 minimal media. Positive control well was from the pegs that

were untreated with chlorine dioxide. A total of four replicates for each species were made as seen in Figure 4-4 below.

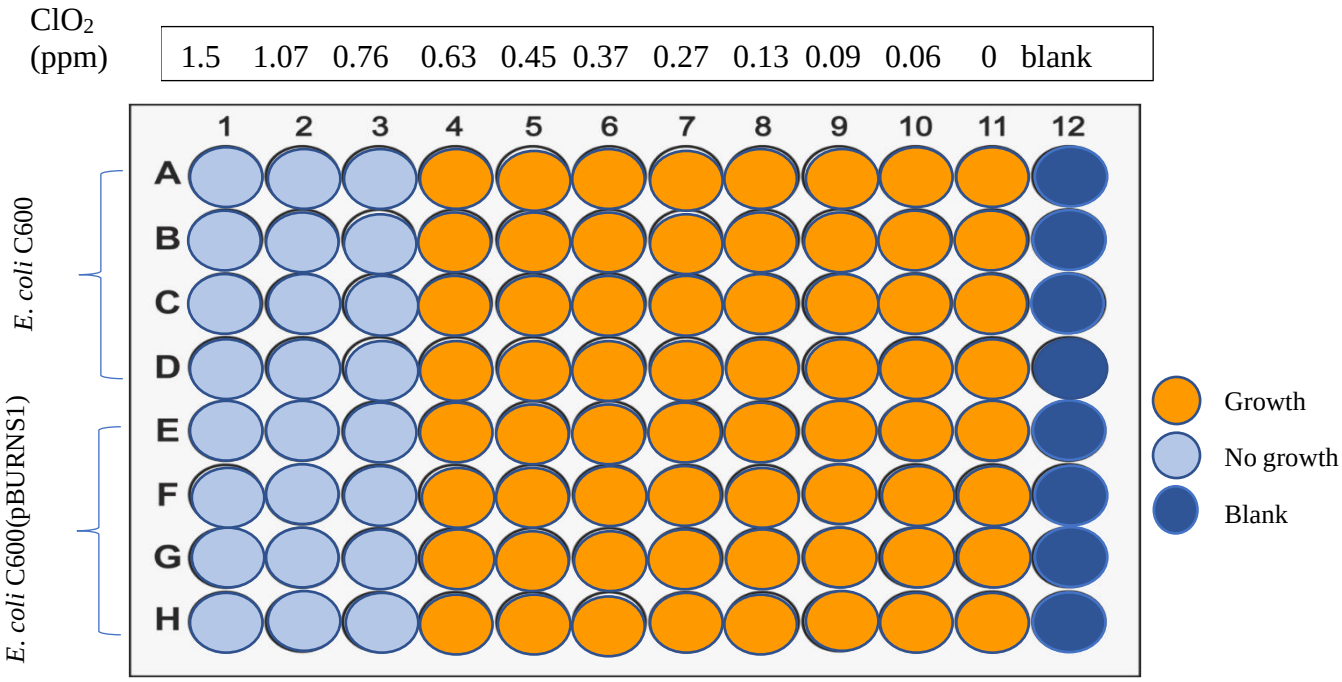


Figure 4-4. Results of the MBEC of 24hr *E. coli* biofilms in chlorine dioxide solutions

The MBEC shows no difference in a 24hr E. coli biofilm survival in chlorine dioxide irrespective of the presence or absence of the pBURNS1::Tn1723 plasmid. A total of four replicates were conducted in each experiment and the concentrations in wells without any sign of turbidity were chosen as the MBEC (0.76ppm ClO_2). Wells in orange show turbidity due to bacterial growth. The blank wells (dark blue) did not have any bacteria added before incubation while wells A11- H11 did not have any biocide added 0ppm ClO_2.

4.4 Biofilm bead system

The pBURNS1 plasmid isolated from *Pseudomonas aeruginosa* 943 carries a putative chlorite dismutase gene (*cdm*) located on an integron which encodes the production of an enzyme chlorite dismutase. When the *cdm* gene is expressed, the degradation of chlorite to chloride and oxygen should take place. From the previous work done by (Quick et al, 2014)

the pBURNS1 plasmid found in *Pseudomonas* strains was present both in the shower water and the environments around the shower in room 8 of the Burns unit. The plastic shower head rosette is thought to be capable of breeding biofilms and the water force exerted from the shower head can act as a possible a source of spread to other areas in the room. Also, when a thermo-mixer valve in room 9 was dismantled, a biofilm was found to be present and contained the same *Pseudomonas aeruginosa* strains as seen in the water and shower surfaces in the same room and thus there are chances of those strains carrying a transmissible plasmid within them. Therefore, we investigated the consequence of pBURNS1 plasmid being present in a *Pseudomonas* mixed biofilm using an *in vitro* bead biofilm model.

In this study, to mimic the possible biofilm reservoir in the affected room, a surface area in which a biofilm can be formed without a lot of shearing was needed and thus biofilms were grown on plastic beads rather than glass beads. The use of the bead system to generate biofilm has been successfully attempted previously (Popat et al, 2012, O'Rourke et al, 2015, Poltak and Cooper, 2011). A simple protocol was designed for biofilm formation using the plastic beads as a substratum where the colonized bead could be agitated and resuspended to give approximate counts of bacteria before and after selection with chlorite. Using this protocol also helped in monitoring the rate of biofilm growth/biomass and survival of bacteria over time.

The biofilm bead system is one of the ways in which cells from a growing biofilm can be enumerated. In experimenting with different biofilm model systems, glass and plastic beads were used to grow biofilm, but plastic beads were found to give a more reproducible and larger amount of biofilm per bead than the glass beads (see Figure 4-5). The plastic beads used had a hollow in the centre and white and black beads where obtained to create

distinction during use. The hollow was not subjected to shear forces at all during shaking and it was physically observed to contain most of the biofilm. Biofilm formed was resuspended by applying force through vortexing and by scraping the hollow of the bead for 20 seconds. At first, a 200 µl pipette tip was used for scraping the hollow but this was later changed and the edge of sterile forceps was used due to fact that the smooth surface area of the pipette tip may not be effective at removing most of the biofilm from the hollow substratum. The design of biofilm growth and survival experiments was made to determine how many donors, recipients and transconjugants survived after being exposed to a fixed level of biocide treatment and after a fixed length of time. The biofilm bead system was used in two major experiments:

1. The transfer of pBURNS1::Tn*1723* plasmid and with the selection of chlorite.

2. Survival of plasmid-carrying strains versus non plasmid carrying strains in a range of concentrations of chlorite/chlorine dioxide.

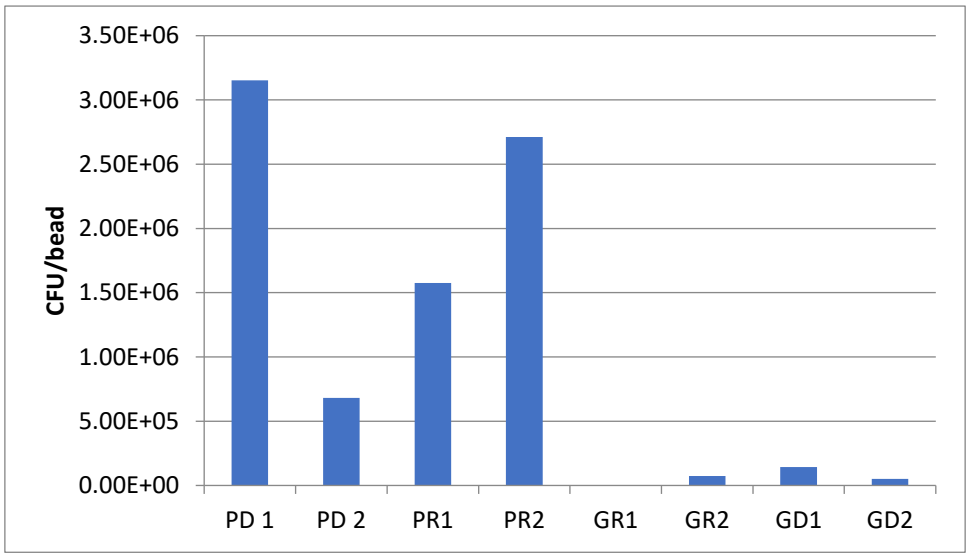


Figure 4-5. Determination of appropriate bead to grow robust biofilm between glass and plastic beads

PD1= Plastic + Donor with 5mM sodium chlorite treatment

PD2= Plastic +Donor without treatment

PR1= Plastic + Recipient with treatment

PR2= Plastic +Recipient without treatment

GD1=Glass +Donor with treatment

GD2=Glass +Donor without treatment

GR1= Glass + recipient with treatment

GR2= Glass +recipient without treatment

Donor strain referred to here is E. coli C600 (pBURNS1::Tn1723), recipient strain is E. coli MV10 NAL^R. 24hr biofilms of each were grown on both glass and plastic beads and treated for an hour with 5mM $NaClO_2$, the plastic beads grew generally better biofilms than the glass, so they were chosen for further use.

4.5 Level of survival of pBURNS1 positive and negative *P. aeruginosa* and *E. coli* strains in chlorine dioxide

In order to determine if there is a difference in the survival of plasmid-carrying strain against a plasmid free strain in chlorine dioxide and to what concentration a difference can be seen, *E. coli* C600 (pBURNS1::Tn*1723*), *E. coli* MV10 Nal^R (pBURNS1::Tn*1723*) and *P. aeruginosa* 943 along with their corresponding plasmid free strains were grown as biofilms and also in a planktonic state and treated with a range of concentrations of chlorine dioxide that can be found in treated hospital water taken from the final outlet (0.125 ppm-0.50 ppm)

for 1 hr or 3 hrs. The plasmid positive and negative *E. coli* and *P. aeruginosa* species were first tested in their planktonic state to determine if there is an immediate difference in chlorine dioxide survival between the two species. The presence of *Pseudomonas aeruginosa* 943 in other sections in room 8 of the Burns unit shows that the plasmid-carrying strain has been disseminated from one point to another within a short distance. This could have been either through surface cleaning where the bacteria survive the cleaning agent and get transmitted via the cleaning vessel, or from aerosolization of water droplets from the shower to the sink and drain. Most importantly, the fact that the plasmid-carrying strain was found in the water samples implies that the bacteria have the capacity to survive in a planktonic state in the ClO_2-treated hospital water.

Planktonic survival

When the susceptibility of the planktonic cultures of *P. aeruginosa* 943 and its plasmid cured derivative was tested, it was observed that for both strains, there was approximately a 5-fold drop in CFU count after treatment for 1 hr at 0.0625 ppm chlorine dioxide (Figure 4-6). At concentrations of 0.125 ppm and higher, neither strains survived 1 hr of treatment. Two-way ANOVA showed no statistical difference in the survival rate of the two strains. However, there was a difference between the means of the control and 0.0625 ppm in *P. aeruginosa* 943 WT.

Also, with *E. coli* C600, when the planktonic plasmid positive and negative strains were treated for an hour, while there was a significant difference seen between 0.0625 ppm and 0.125 ppm ClO_2, there was also a 100-fold reduction in CFU with the wild type C600 but only a 10-fold reduction with C600(pBURNS1::Tn*1723*) (Figure 4-7).

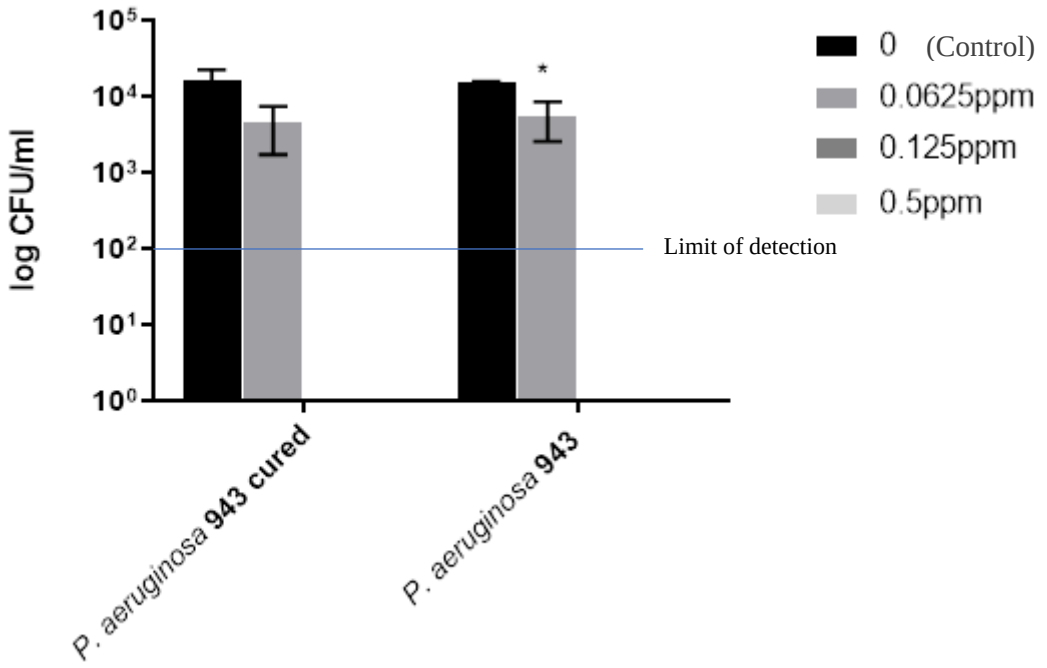


Figure 4-6. Planktonic survival of *P. aeruginosa* 943 and *P. aeruginosa* 943 (cured) in the presence of Chlorine dioxide

A comparison between the plasmid carrying P. aeruginosa 943 and cured strain in a planktonic state. The controls of each strain were not treated. This experiment shows results from a biological triplicate (n=3) and mean value of counts of surviving bacteria on growth media. Data with asterix show statistical significance p-value <0.05. 0.125ppm and above was enough to completely eradicate all the P. aeruginosa cells in planktonic state after a one-hour contact time. There was no significant difference between the means of either strain however there was a difference between the means of the control and 0.0625ppm in P. aeruginosa 943 WT.

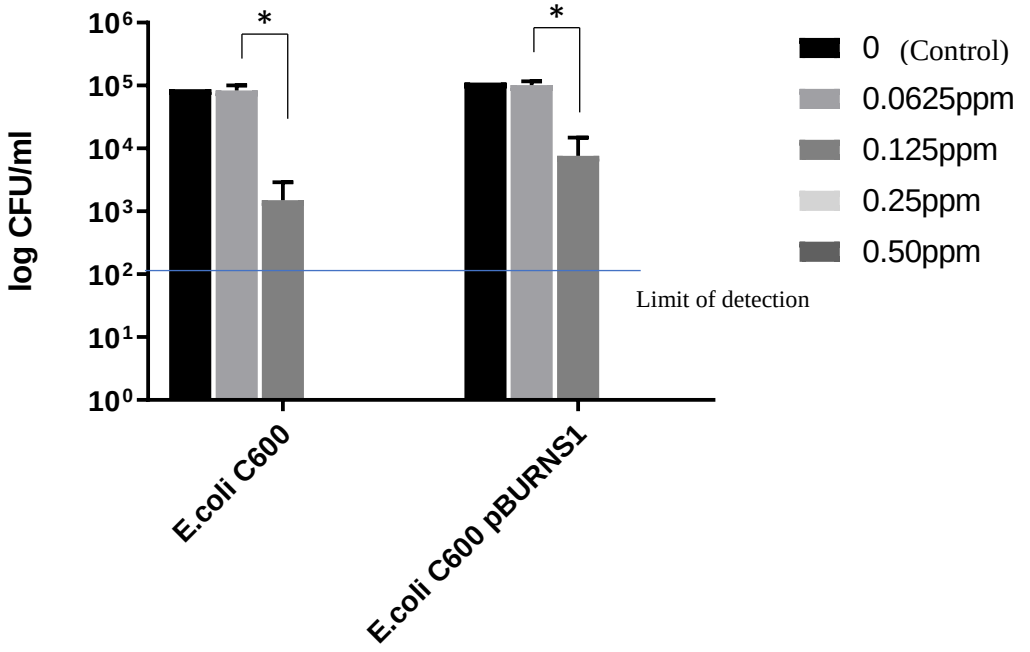


Figure 4-7. Planktonic survival of *E. coli* C600 and *E. coli* C600 (pBURNS1::Tn*1723*) in ClO_2

The data represent the level of surviving E. coli cells remaining when planktonic plasmid-carrying and plasmid-free bacteria were treated with different concentrations of ClO_2. In both cases, the cells did not survive at 0.25 ppm and above for one hour under laboratory conditions. This experiment shows results from a biological triplicate (n=3). Asterisk labelled bars show statistical significance between the means at 0.0625ppm and 0.125ppm of E. coli C600 and C600 (pBURNS1::Tn1723)

From the results so far, planktonic strains of *P. aeruginosa* seems to survive in ClO_2 one-fold lower than *E. coli* strains with or without the plasmid (0.125 ppm). This shows that generally, planktonic *E. coli* may survive better than *P. aeruginosa* in chlorine dioxide treated water. Although the reason is not clear, the important factor is that they both do not survive one hour in 0.25 ppm which is used in the hospital water system. *E. coli* (pBURNS1::Tn*1723*) plasmid-carrying planktonic cultures seem to survive to a greater capacity than *E. coli* without the plasmid.

Biofilm survival

Pseudomonas aeruginosa strains produce pyocyanin and slime when grown as a biofilm culture which makes an uneven distribution of biocide on the biofilm. The consequence of this was the development of non-reproducible replicates using the bead system for biofilm survival. This issue was not observed in *E. coli* biofilms. Slime and pyocyanin production are both products of phase variation and could occur randomly during biofilm growth. Results seen in Figure 4-8 show that there is no evident increase in 24 hr biofilm survival of *P. aeruginosa* 943 over the cured *P. aeruginosa* 943 at the range of concentrations of chlorine dioxide used. At the highest concentration of 0.5 ppm, there was minimal effect of the biocide on the biofilms formed on both plasmid positive and negative strains. A paired t-test showed that the survival of the two strains with ClO_2 was statistically different from each other (p-value =0.0081).

The possibility that the lack of adequate killing of the plasmid positive and negative biofilms was due to the short contact time and age of biofilm led to the determination of survival of plasmid positive and plasmid free *P. aeruginosa* 943 in chlorine dioxide for 3 hrs contact time as seen in Figure 4-9**.** A two tailed t-test showed that there was no significant difference between the survivals of the two strains.

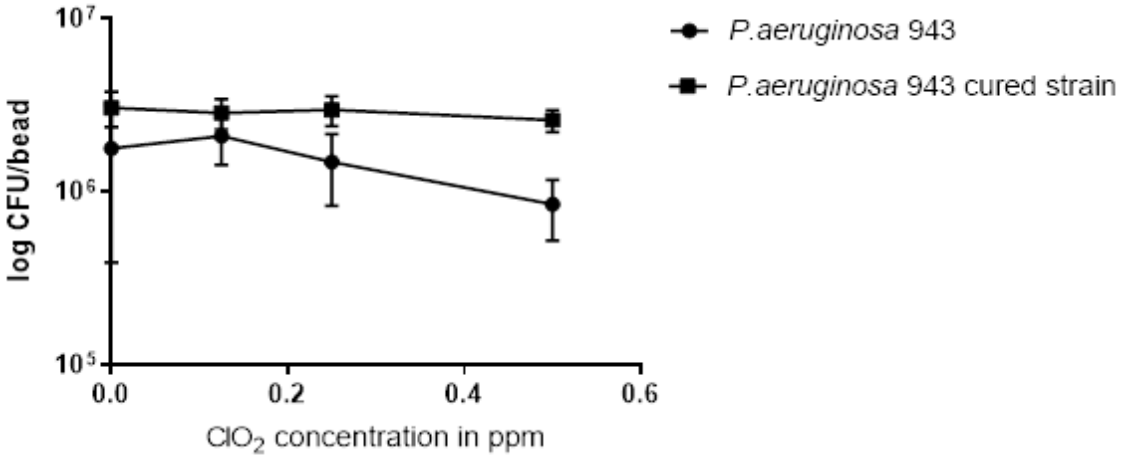


Figure 4-8. Survival of a 24hr *P. aeruginosa* biofilm after ClO_2 exposure for 1hour

Comparison of WT P. aeruginosa 943 and the cured P. aeruginosa 943 after a 24hr biofilm of each formed on a bead was treated. Concentrations of 0.125, 0.25 and 0.50 were used. There is more evidence of cell number reduction in the plasmid positive than plasmid negative strains. The results were compiled from six replicate counts.

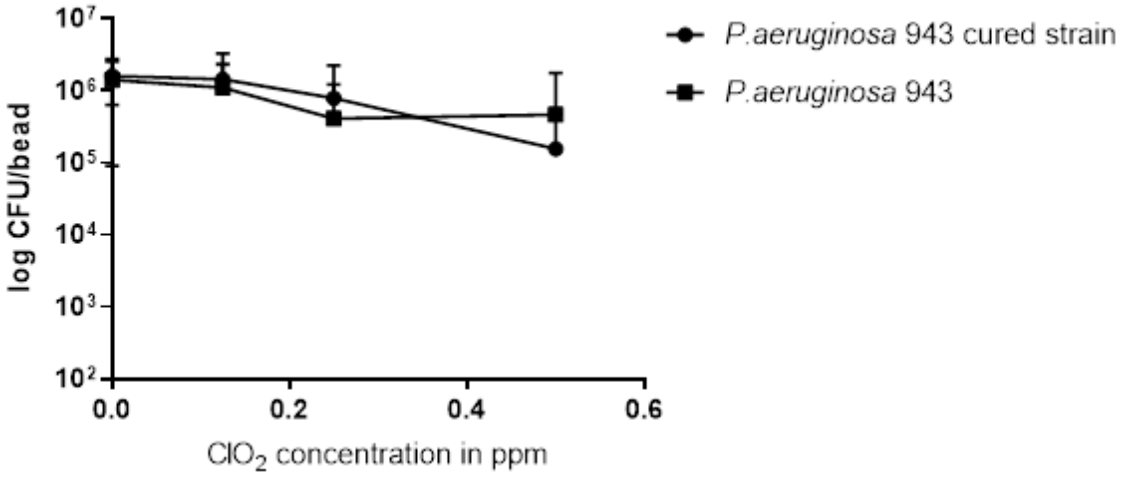


Figure 4-9. Survival of a 24hr *P. aeruginosa* biofilm after ClO_2 exposure for 3hours

Comparison between WT P. aeruginosa 943 and the cured P. aeruginosa 943 after a 24hr biofilm of each formed on a bead was treated for 3hrs. There is a minimal difference between the two strains at the 0.125ppm and 0.25ppm but at the highest concentration of 0.5ppm, the plasmid-carrying strain survives better than the plasmid free strain with less than 10-fold increase. The results were compiled from six replicate counts.

After a series of experiments using a range of ClO_2 concentrations, it was observed that the concentration 0.25 ppm seemed to make a difference between the plasmid positive and plasmid negative strains in *P. aeruginosa* 943 and using a more robust biofilm of 48 hrs growth gave more consistency when treating for 3 hrs as shown in Figure 4-9.

With no evident difference between *P. aeruginosa* 943 biofilm and its plasmid free (cured) counterpart in the presence of chlorine dioxide attention was focused on experiments with *E. coli*. Therefore *E. coli* C600 and *E. coli* C600 (pBURNS1::Tn*1723*) were used to grow a 24 hr biofilm and treated with the same range of concentrations of chlorine dioxide that were used for the *Pseudomonas* strains. Figure 4-10 shows the treatment of a 48 hr *E. coli* C600 and C600(pBURNS1::Tn*1723*) biofilm for 3 hrs. At 3 hrs it was observed that a killing effect of chlorine dioxide is evident but occurs at a similar level in both plasmid positive and negative strains. A two-way ANOVA test showed no significant difference between the means of the surviving strains. In the treatment of *E. coli* 48 hr biofilms, 0.50 ppm was not enough to kill off all the biofilms in both plasmid positive and negative strains.

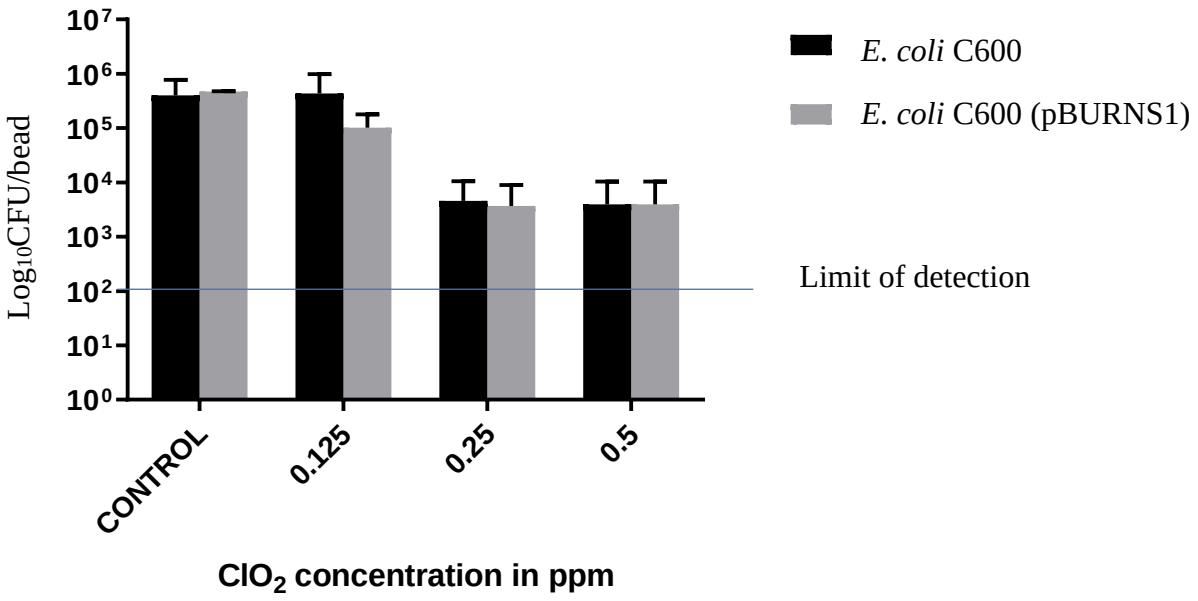


Figure 4-10. Survival of a 48 hr *E. coli* biofilm after ClO_2 exposure for 3 hrs

Above represents the surviving E. coli with and without pBURNS1::Tn1723 after contact treatment with a range of concentrations of ClO_2. The results show that the E. coli biofilms of both strains where equally robust but the treatment with 0.25 ppm and 0.50 ppm resulted in approximately 100-fold reduction in CFU. 48 hr biofilms survive 0.50 ppm almost as well as the 0.25 ppm treatment irrespective of the presence of the plasmid.

There were issues with poor reproducibility in *P. aeruginosa*, so a slight modification to the biofilm survival experiment was made where multiple (12) beads were placed in a 1 L conical flask for each of the strains to produce more uniformity in replicates to determine if there was a difference in survival due to the carriage of the plasmid in *P. aeruginosa* at 0.25 ppm ClO_2 (Figure 4-11). Results show that there was a significant difference between the means of the untreated and treated of each individual species, however no significant difference was found between the means of the two strains either before or after treatment.

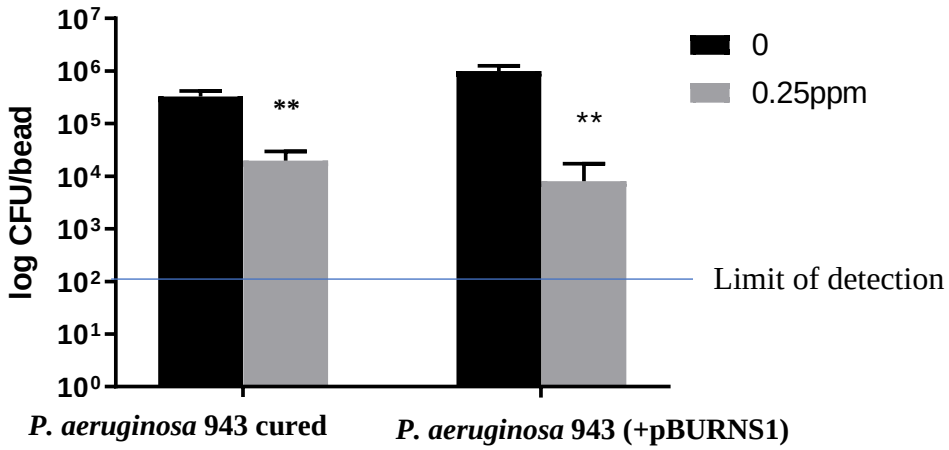


Figure 4-11. Survival of 48 hr *P. aeruginosa* biofilm in 0.25 ppm ClO_2 for 3 hrs (using a 1 L conical flask)

*Bar charts showing the survival of plasmid positive and negative strains of P. aeruginosa 943 in 0.25 ppm ClO_2. Seven replicates of biofilm formed on beads were grown in a single 1 L conical flask to reduce variability obtained from growing them in different universal bottles. (SD/mean= +/-9.29E+03/ 8.04E+03 and 9.80E+03/ 1.99E+04 for P. aeruginosa 943 and P. aeruginosa 943 cured respectively). ClO_2 concentration of 0.25 ppm was used and about 100-fold decrease in cell count occurred in both strains after treatment. Data with an asterisk show statistical significance. P-value ** represents p<0.001.*

4.6 Estimate of transconjugants in 24, 48- and 72-hr *E. coli* mixed biofilms with chlorite selection

Horizontal gene transfer via conjugation is an important factor that determines the spread of significant genetic determinants from a single bacterium to multiple bacteria within a length of time depending on their transfer frequency (Hausner et al, 1999). Although in IncP plasmids, plasmid transfer was more efficient on solid surfaces than in liquids (Zhong et al, 2011), It has also been observed that bacterial conjugation is likely to have higher mating success rates in liquids rather than solid media due to random mating events (Hausner and Wuertz, 1999). However, in biofilms, less random events occur (Davey and O'Toole, 2000). The dense bacterial population within extracellular matrix will only allow mating events to

occur in a step wise manner with only the closest recipients to incoming donors becoming transconjugants initially (Hausner and Wuertz, 1999).

Based on work carried out so far on pBURNS1 (Aggarwal, 2015), the plasmid's ability to act as a selective advantage to drive transfer from a biofilm, and the ability of the products of transfer to express the *cdm* gene and survive in the presence of chlorite has not yet been established. Therefore, the role of pBURNS1 in the transfer of chlorite resistance to a recipient bacterium in a biofilm and subsequent expression of chlorite degrading ability of the transconjugants was investigated.

To determine if the products of transfer of pBURNS1 plasmid in a biofilm will be selected by chlorite, an experiment for biofilm formation using the bead system was set up. Plasmid transfer by conjugation was measured when the donor strain C600 (pBURNS1::Tn*1723*) was added to the 48 hr recipient biofilm (MV10 Nal ^{R}) and allowed to grow for a further 24 hrs allowing for conjugation to be established on the bead before treatment. The transconjugants, donors and remaining recipients were treated together (at the same time) by adding a fixed concentration of sodium chlorite in either Luria broth (LB) or M9 minimal media during incubation at 37ºC overnight. The experimental design is shown in Figure 2-2. The experiment was done in triplicate and duplicate experiments were conducted as negative controls (without addition of chlorite to liquid media) which have been referred to as donor, recipient and transconjugant controls.

The mixed biofilm survival experiment was established to determine the survival of products of plasmid transfer on the biofilm under selective pressure from chlorite. Based on the protocol mentioned in chapter 2.10.2, the amount of biofilm remaining on the bead was resuspended before and after exposure to chlorite and the ratio of donors, recipients and

transconjugants was estimated from the bacterial counts. Each bar represents counts of all three species within a biofilm obtained from a single bead. The experiments were conducted in triplicates with a control group and a test group to show the untreated and treated results respectively with increasing age of biofilm.

The initial 24-hr biofilm consists of a 48hr recipient biofilm and a 24-hr donor planktonic cells mixed within 24 hrs without any treatment. The 48-hr biofilm was a second passage of the 24-hr biofilm only this time, treated with chlorite for 24 hrs and the 72-hr biofilm was the surviving 48hr biofilm treated for a further 24 hrs.

The recipient biofilm was formed initially because it was expected that these were the cells that would die off due to the biocide based on the hypothesis, therefore to remove any bias that the loss of recipients were as a result of unequal cell numbers between donors and recipients. This method of generating recipient biofilms first before adding planktonic donors was also utilized by Angles et al. (1993). Another rational for growing a recipient biofilm first is to observe transfer rates between incoming pBURNS1 carrying strains from the possible water source (donors) and the resident recipient bacteria present in form of a biofilm.

The experiment was conducted using either L-broth or M9 minimal media as liquid media to grow the biofilm and using 5 mM and 0.2 mM $NaClO_2$ respectively. These two concentrations were used to determine the effect of high and low concentrations on the biofilm. The remaining cells in the biofilm before and after treatment was determined by resuspending the viable bacterial cells and plating them out on solid selective agar plates. The suspension was also plated to determine if the constituents would differ significantly from the biofilm population. This was achieved by serial diluting 1 ml of the spent medium

and plating out 100 µl of the lower concentrations. The dislodged biofilm cells in PBS were spot plated using the Miles and Misra method (Miles and Misra, 1938) and colonies counted by eye, the average recorded as colony forming units per bead. In Figure 4-12 A, the donors, transconjugants and recipients obtained from the biofilm and suspension respectively were grown without treatment across a span of 72hrs.

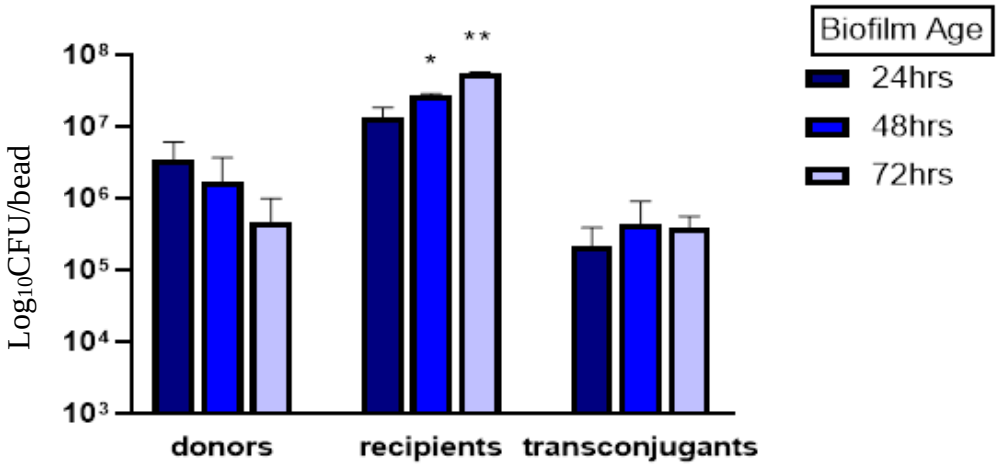
Results showed that the control donors declined with increase in biofilm age at 48 hrs and 72 hrs in Figure 4-12A while the recipients increased slightly over time. This could be a result of the recipient biofilm being well established on the bead and fewer microcolonies of donors left with each rinse and re-incubation. Also, In Figure 4-12A (the controls) it can be observed that the recipient biofilm counts were at least 10-fold higher than the donors. This was expected since the recipient biofilm is a few days older than the donor biofilm to begin with. The term 'recipients' refers to the number of plasmid free *E. coli* MV10 Nal ^{R} while the transconjugants are recipients that have received the plasmid and thus the chlorite degrading phenotype. Each bar represents the average colony count obtained from three 20 µl spots from each bead on three different plates, Kanamycin (Kan), Nalidixic acid (Nal) and KanNal plates. To obtain an approximate total number of recipients and donors, the mean colony counts that grew on KanNal were subtracted from the Nal and Kan plates respectively. Multiple t-tests showed that there was a significant difference between the means of recipients before and after treatment at 48 hrs and 72 hrs. The P-values were determined using the Holm-Sidak method as 0.043 and 0.001 respectively.

In Figure 4-12B (test group), the recipients based on average counts did not seem to survive the initial treatment at 48 hrs but appears at 72 hrs. However, the recipients seen here were found to be transconjugants after screening. This was investigated by replica-plating the colonies that grew (few) on Nal plates (recipients) on KanNal plates to determine if the *E.*

coli MV10 Nal^R had obtained the plasmid or not. Results showed that, all the colonies that grew on Nal were also Kan resistant making them transconjugants rather than recipients.

Control biofilm LB (0mM)

A



5mM $NaClO_2$ biofilm LB

B

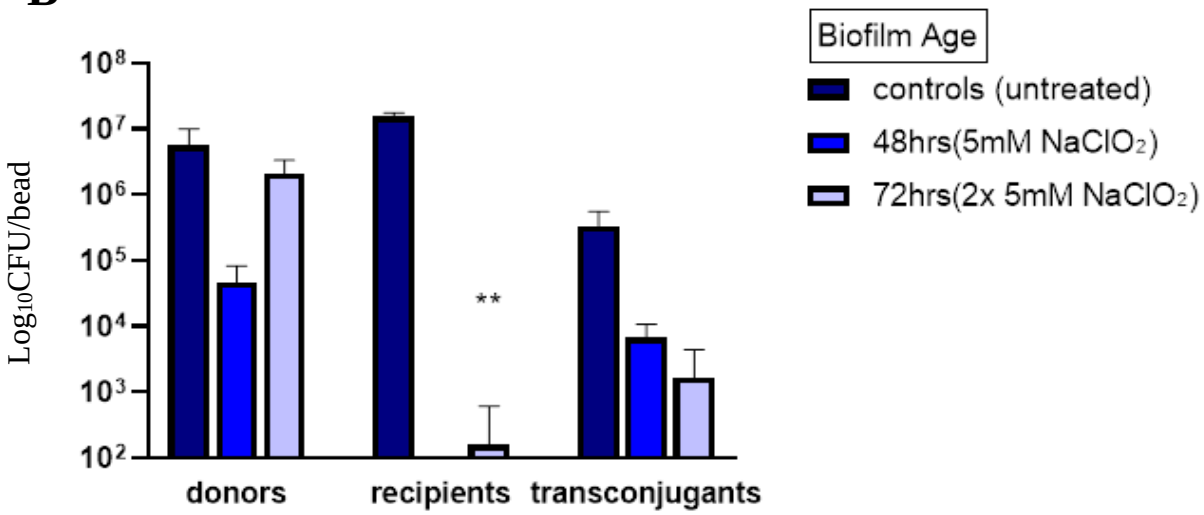


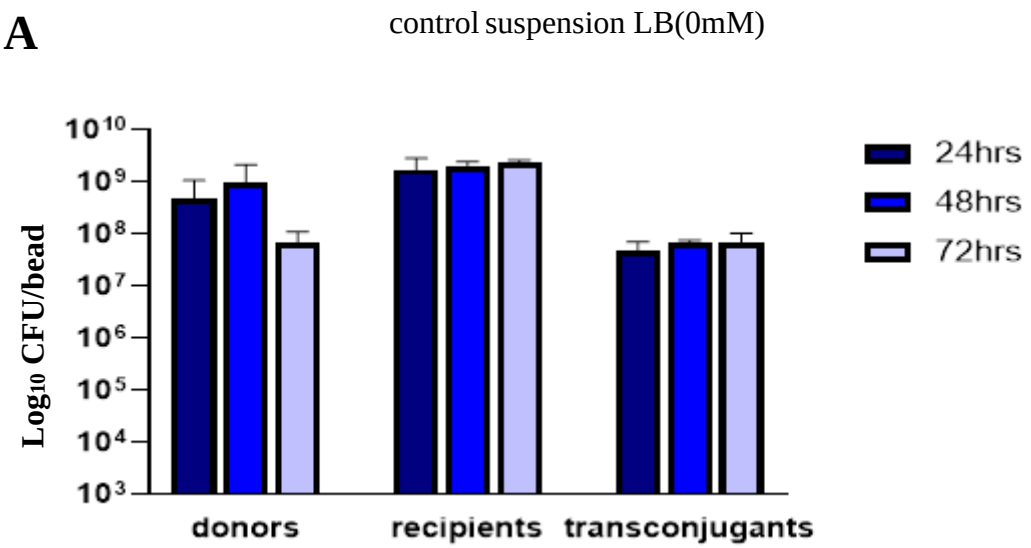
Figure 4-12. Presence of pBURNS1::Tn*1723*-carrying *E. coli* in a mixed biofilm while selecting for chlorite (5mM $NaClO_2$ in LB)

*Above are the resuspended biofilm counts with **(A)** representing the control group (-$NaClO_2$) **(B)** the treated group using L-broth as growth media (+$NaClO_2$). A. shows the growth of the products of transfer over a 72-hr period (The amount of biofilm recovered over 3 days of rinsing off planktonics and resuspending for plate counts). It shows that the effect of chlorite on plasmid free recipients is greater than donors and transconjugants. Data with Asterix show statistical significance (* for p<0.05 and ** for p<0.001).*

As expected, the donors after the initial 5 mM $NaClO_2$ treatment seemed to regain chlorite resistance when treated twice at 72 hrs as they did without treatment (24 hrs). However, there was a general 100-fold or more decline in all species including the plasmid carrying strains after the initial 5 mM treatment. In general, counts from the untreated biofilm were less than the counts obtained from the untreated suspension because the bead has a small surface area available to the biofilm compared to the liquid suspension. A similar result was seen with the suspension in Figure 4-13. By comparing the controls and tests, in A and B respectively, the recipients in the liquid suspension are absent which means they are being killed off by the biocide and not just escaping the biofilm.

There was a significant difference between the means of the recipients before and after treatment in initial treated biofilm at 72 hrs p-value <0.001 and there was a significant difference determined by Holm-Sidak method in the donors before and after treatment at 48 and 72 hrs with p-values of 0.025 and 0.036 respectively.

A



B

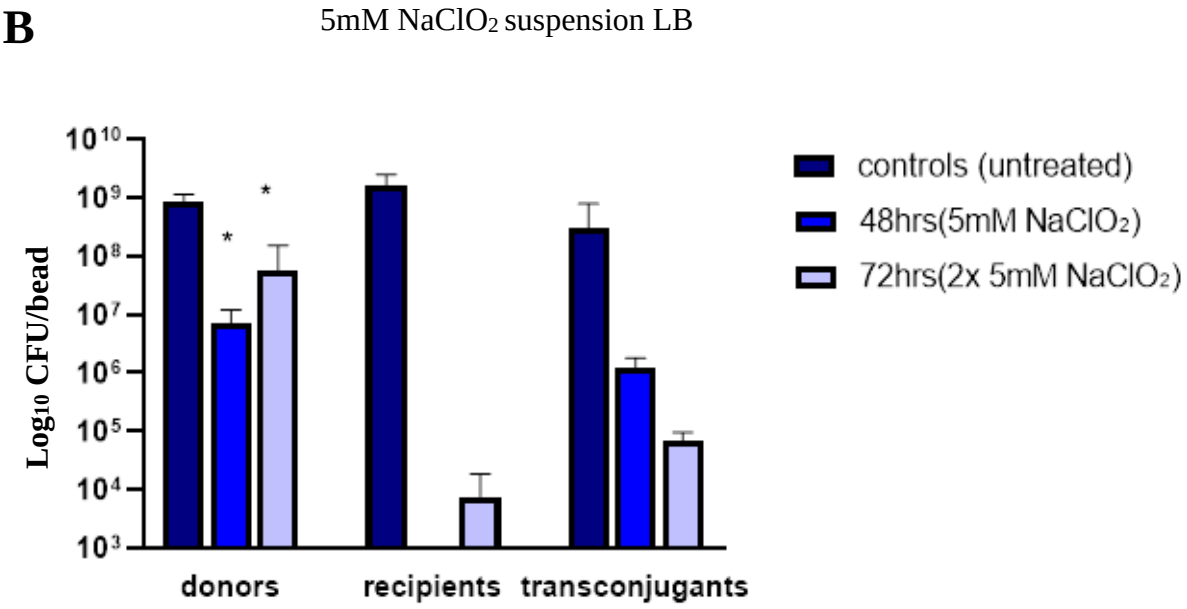
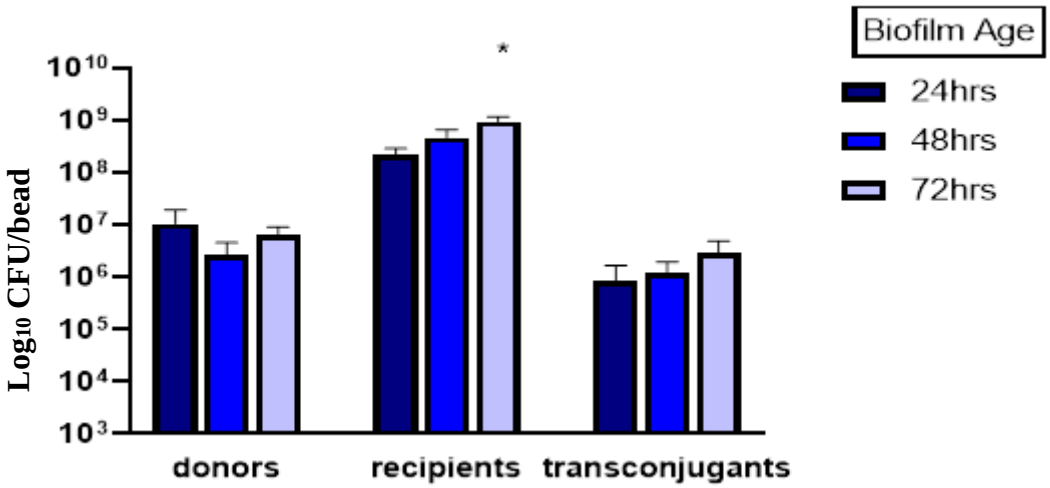


Figure 4-13. Presence of pBURNS1::Tn*1723*-carrying *E. coli* in a mixed biofilm suspension while selecting for chlorite (5mM $NaClO_2$ in LB)

The CFU/ml of E. coli present in the liquid suspension media where the biofilm was formed ***(A)*** *representing the control group(-$NaClO_2$)* ***(B)*** *the treated group using L-broth as growth media (+$NaClO_2$). As in the biofilm, it shows that the effect of chlorite on plasmid free recipients is greater than donors and transconjugants. Data with Asterix show statistical significance (* for p<0.05 and ** for p<0.001).*

Each bar shown in all the figures represents the average counts obtained from 3 individual beads. The beads were submerged in liquid media constantly in motion (200 rpm) during incubation to discourage conjugation in the liquid. Figure 4-14 (**A)** and **(B)** are the results of the same experiments as in Figure 4-12 but differing in the medium used (M9 in place of LB) and the concentration of sodium chlorite used (0.2 mM rather than 5 mM for LB). Minimal medium was used to mimic the low to negligible nutritional content of hospital water. This is important since part of our aim is to determine the fate of pBURNS1 within a biofilm in the hospital chlorine dioxide treated water. M9 minimal media as its name implies, in contrast to other rich media, has only the essential minerals and nutrients which the organism needs to survive with the key constituents mentioned in Chapter 2.2. In the untreated control biofilm set grown in M9 (Figure 4-14**A)**, there was a statistically significant difference between the recipients before and after treatment at 72 hrs with p-value = 0.019. In the treated biofilm set (Figure 4-14**B**) at a concentration of 0.2 mM, the donors, recipients and transconjugants all showed statistical significance between the means of untreated and 72 hrs (treated for 2 x 24hr), however, only the recipients showed a significant difference between the control and 48 hrs (initial treatment).

A



B

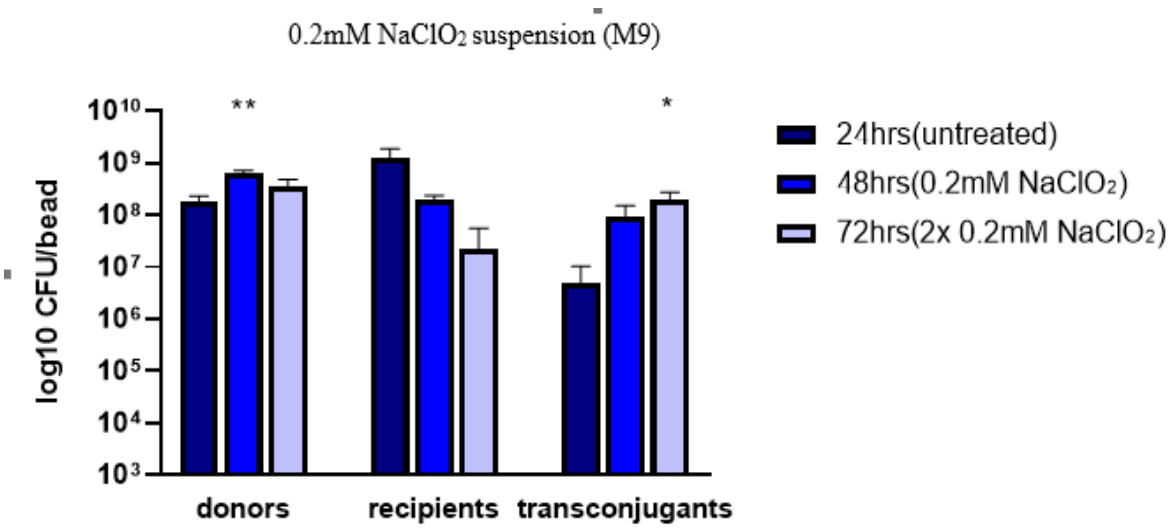


Figure 4-14. Presence of pBURNS1::Tn*1723*-carrying *E. coli* in a mixed biofilm while selecting for chlorite (0.2mM $NaClO_2$ in M9)

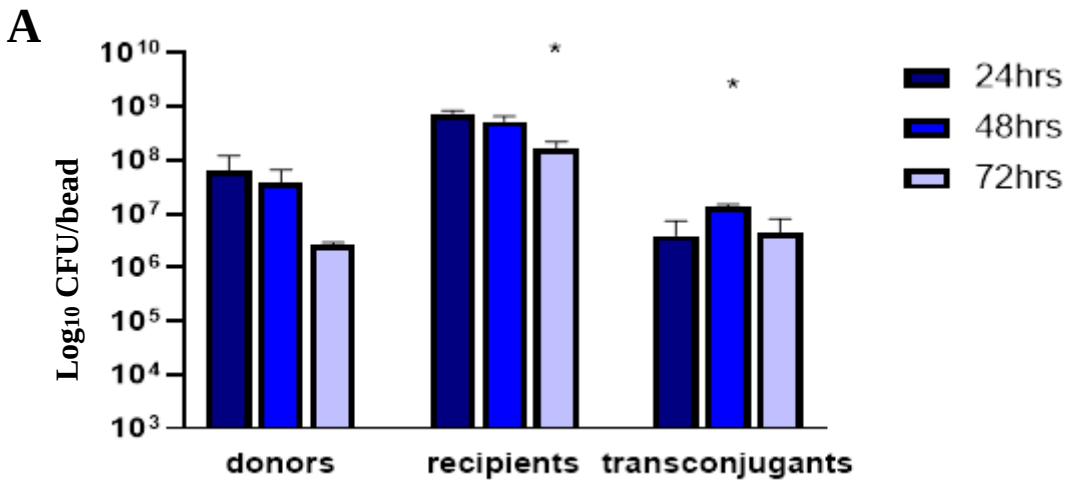
*Above are the resuspended biofilm counts with **(A)** representing the control group(-$NaClO_2$) **(B)** the treated group using M9 as growth media (+$NaClO_2$). A. shows the growth of the products of transfer over a 72hr period (The amount of biofilm recovered over 3 days of rinsing off planktonics and resuspending for plate counts). It shows a gradual increase in survival of plasmid positives over plasmid negatives with time after treatment. Data with Asterix show statistical significance (* for p<0.05 and ** for p<0.001).*

Although in contrast to the LB results, all the recipients were not totally killed off by the 0.2 mM sodium chlorite, there was a 10-fold or less decrease in recipients and an opposing 10-fold increase in total number of donors and transconjugants on the bead in the biofilm, a very similar survival pattern found in the treated suspension (Figure 4-15**B**). In the untreated M9 biofilm results, (Figure 4-14A) there was a significant difference between the means of the 24 hrs and 48 hrs biofilm for recipients and between the 24 hr and 72 hrs biofilm for the transconjugants. p-values were 0.017 and 0.032 respectively. In the treated suspension (Figure 4-15**B**), t-tests showed statistical significance in the donors at 48 hrs and transconjugants at 72 hrs. P-values were 0.006 and 0.035 respectively. Table 4-3 shows an estimate of CFU/bead obtained by the control species both in M9 and LB after 24 hrs of growth. Due to the complexity of the figures, Table 4-2 gives a breakdown of the age of each of the constituents of the biofilm (donors, recipients and transconjugants) for that experiment.

Table 4-2. Description of the legend in Figure 4-12 to Figure 4-15

		Total treatment time	
	untreated	M9	LB
24 hrs	48 hrs recipient biofilm + donors for 24 hrs	-	-
48 hrs	72 hrs recipient biofilm + donors for 48 hrs	0.2 mM for 24 hrs	5 mM for 24 hrs
72 hrs	96 hrs recipient biofilm + donors for 72 hrs	0.2 mM for 48 hrs	5 mM for 48 hrs

Control suspension M9 (0mM)



0.2mM $NaClO_2$ suspension (M9)

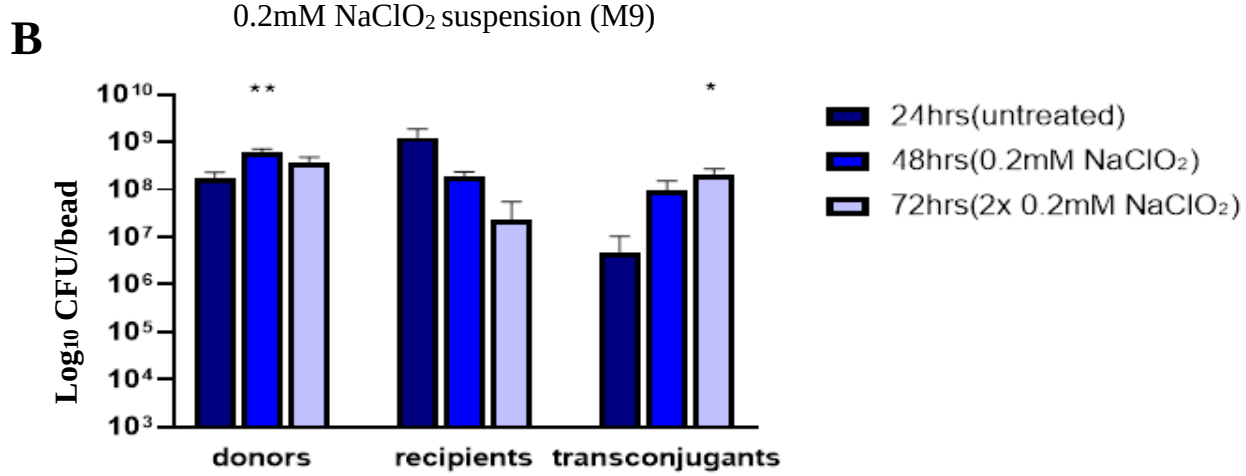


Figure 4-15. Presence of pBURNS1::Tn*1723*-carrying *E. coli* in biofilm liquid suspension while selecting for chlorite (0.2mM $NaClO_2$ in M9)

The CFU/ml of bacteria present in the liquid suspension media where the biofilm was formed ***(A)*** *representing the control group(-$NaClO_2$)* ***(B)*** *the treated group using M9 as growth media (+$NaClO_2$). As with the results in the biofilm, it shows a gradual increase in transconjugants and decrease in plasmid free recipients with treatment over time. Data with Asterix show statistical significance (* for p<0.05 and ** for p<0.001).*

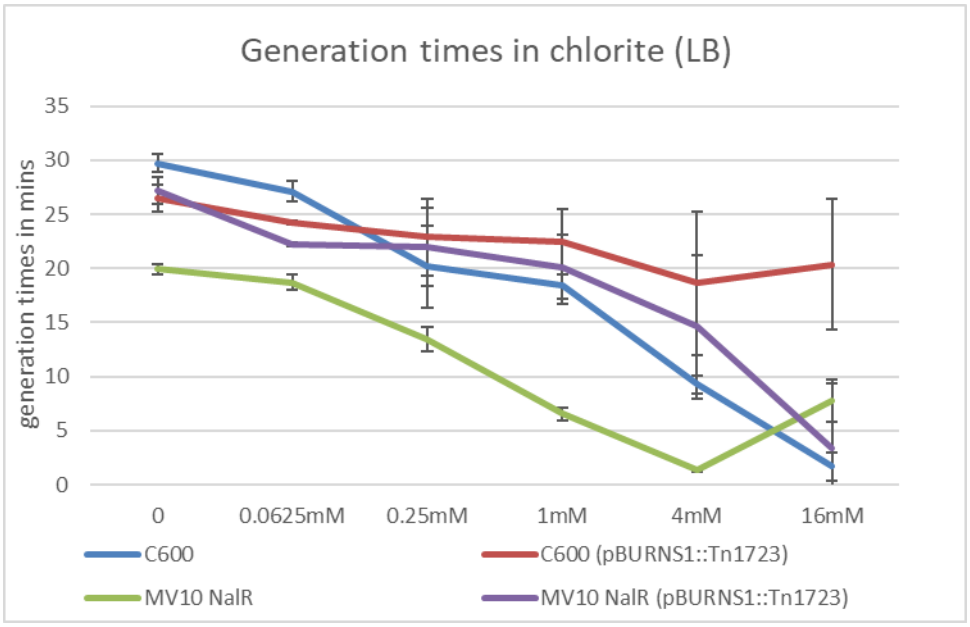
Table 4-3 Comparison between control biofilm biomass in LB and M9 media

	Controls (average CFU/bead)	
	M9 biofilm	LB biofilm
Donors	10^6 -10^7	10^5-10^6
Recipients	10^8-10^9	10^7-10^8
Transconjugants	10^6-10^7	10^5-10^6

4.6.1 Generation times of E. coli +/- pBURNS1::Tn1723 in the presence of sodium chlorite

A phenotype can be described as the observable characteristics determined by the expression of the genome in a living cell (Orgogozo et al, 2015). It allows us to differentiate between bacterial cells based on their differing genotypes and can give an insight to which genes are actively being expressed and those that are recessive. The growth curves were generated as described in Chapter 2.9 using a 96 well plate spectrophotometer. Following the analysis of growth curves of strains carrying pBURNS1::Tn*1723* against plasmid-free counterparts, the generation times were calculated in the presence of chlorite. Figure 4-16A and B show the generation times obtained from the growth curves of plasmid positive and negative *E. coli* strains in sodium chlorite. Concentrations of sodium chlorite used were 0.25, 1, 4 and 16mM in LB; 0.015, 0.0625, 0.25, 1 and 4mM in M9 minimal media. It can be observed that the plasmid negative strains have a shorter generation time than the plasmid positives with increase in chlorite concentration in both LB and M9 experiments. This shows that Cdm is highly active at the exponential phase of growth and peaks between 0.0625 and 0.25 in M9; 1 and 4mM in LB.

A



B

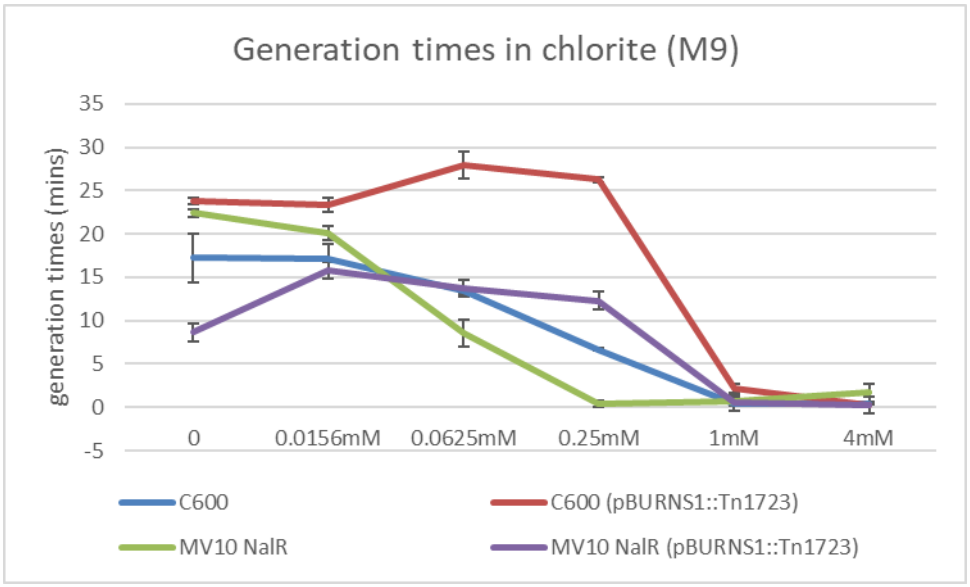


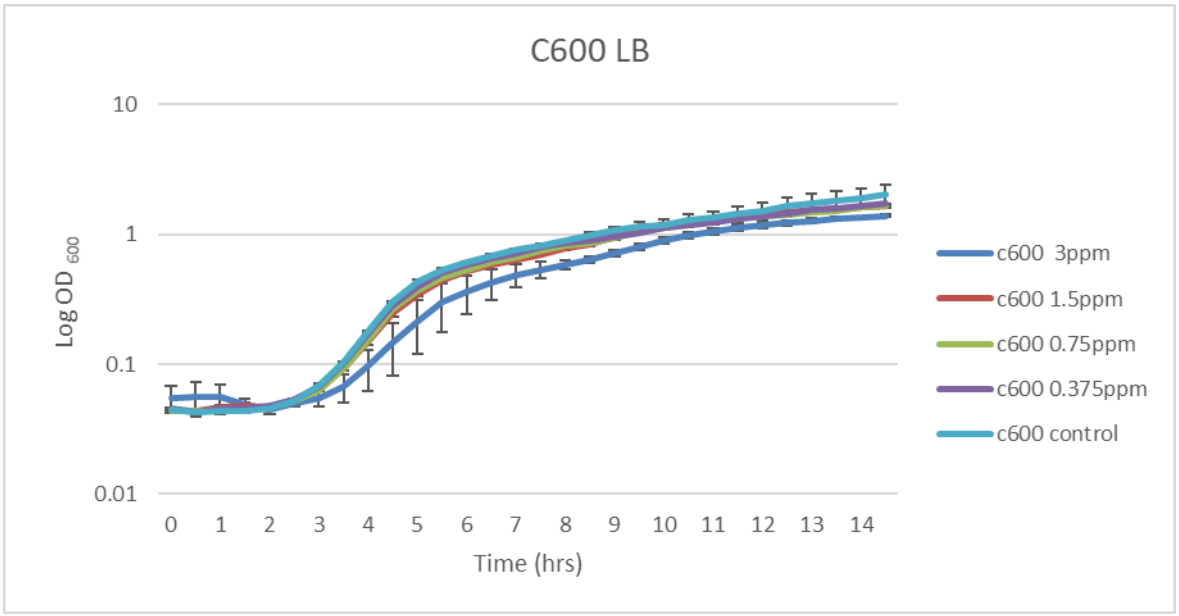
Figure 4-16. The generation time estimated for the *E. coli* strains with and without the pBURNS1::Tn*1723* plasmid.

The results were obtained by estimating the start and end of the exponential phases of bacterial growth and the time interval between them in (A)LB and (B)M9. The generation times least affected were those strains with the plasmid present. This shows that the toxic effect of chlorite is less in plasmid positive strains. In M9, a similar pattern is seen with increase in chlorite concentration.

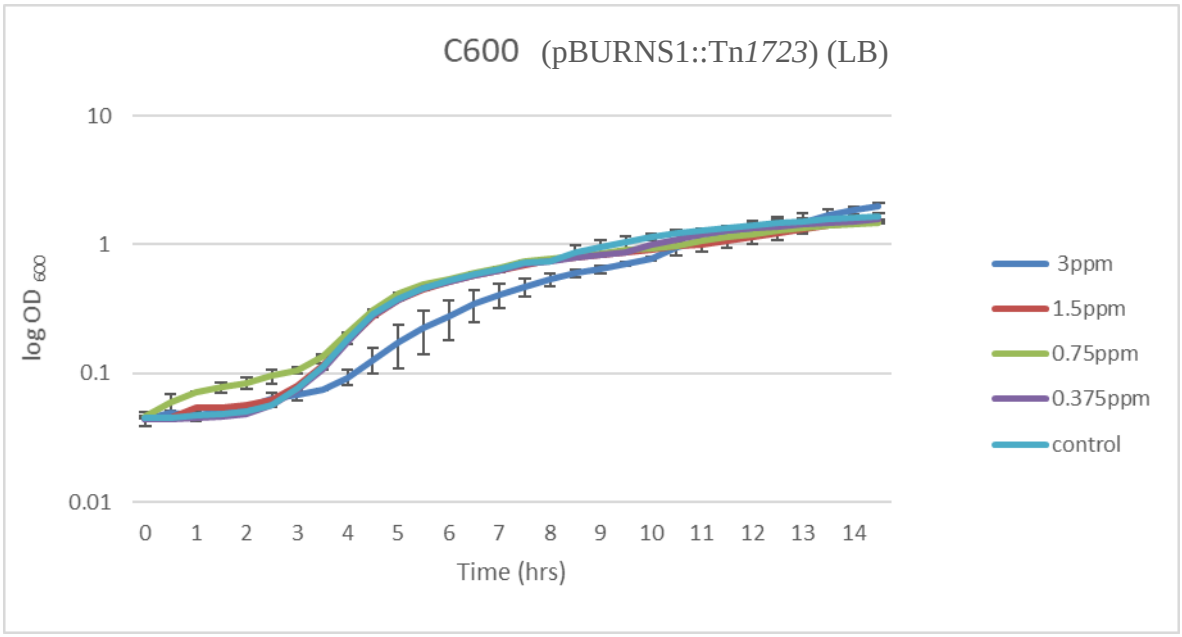
4.6.2 Determination of a Cdm phenotype in pBURNS1::Tn1723 positive and negative strains in Chlorine dioxide solutions.

E. coli strains with and without pBURNS1::Tn*1723* were grown in a range of chlorine dioxide concentrations to determine if there will be an increased phenotypic survival of plasmid positive strains as compared to their plasmid negative counterparts. Both LB and M9 media were used to grow the planktonic species in 96 well microtiter plates. In LB, it was observed that there was little or no effect of the biocide (ClO_2) on any of the strains after incubation. This could be due to the interaction of chlorine dioxide with the rich media and therefore a reduction in biocidal activity. The components of rich medium such as tryptone and yeast extract may interact with the biocide (De Leersnyder et al, 2018) and cause a decreased chlorine content. Figure 4-17 below shows minimal negative effect of chlorine dioxide on *E. coli* C600 and MV10nal ^{R} with and without pBURNS1::Tn*1723*.

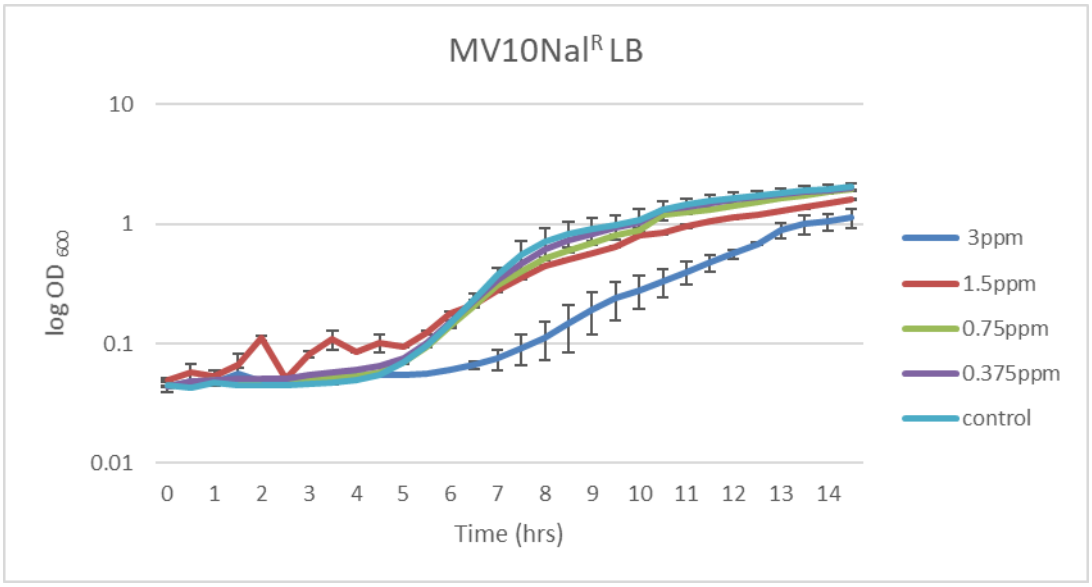
A


C600 LB
10
1
0.1
0.01
Log OD 600
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (hrs)
c600 3ppm
c600 1.5ppm
c600 0.75ppm
c600 0.375ppm
c600 control

B


C600 (pBURNS1::Tn1723) (LB)
10
1
0.1
0.01
log OD 600
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (hrs)
3ppm
1.5ppm
0.75ppm
0.375ppm
control

C



D

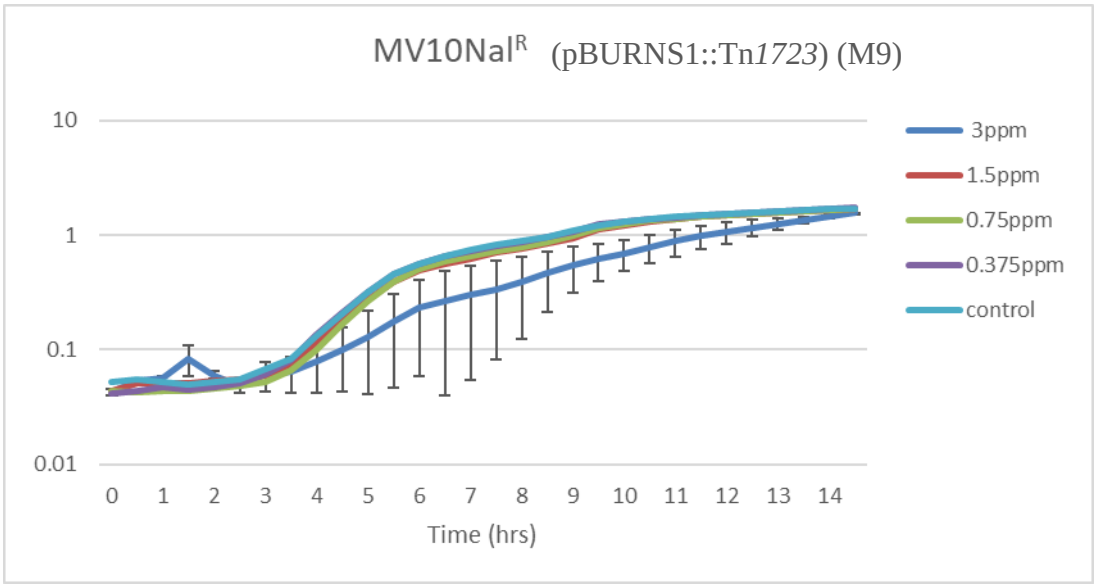
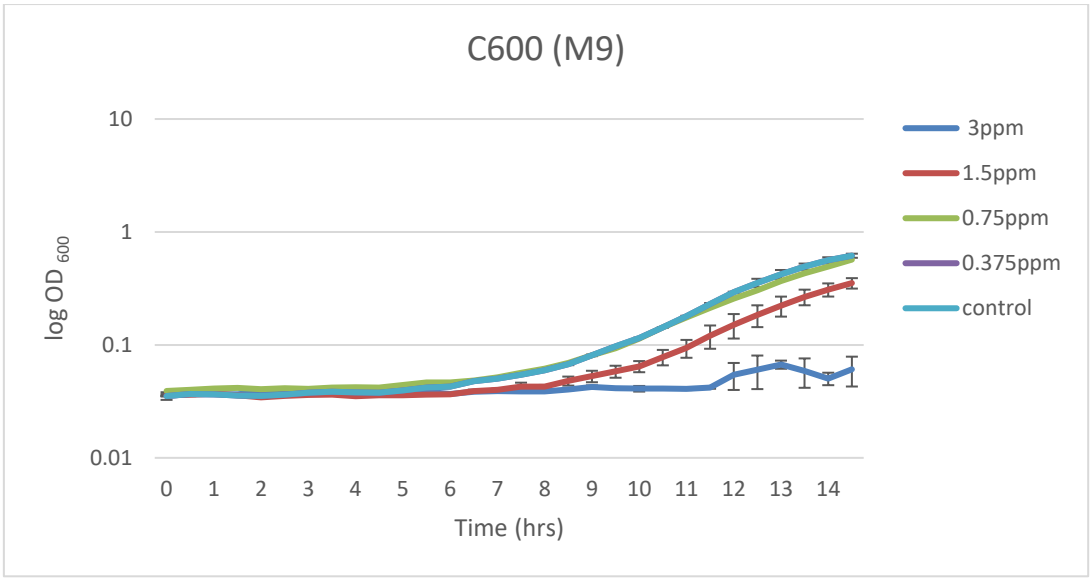


Figure 4-17. Growth curves of *E. coli* with and without pBURNS1::Tn*1723* in chlorine dioxide (LB)

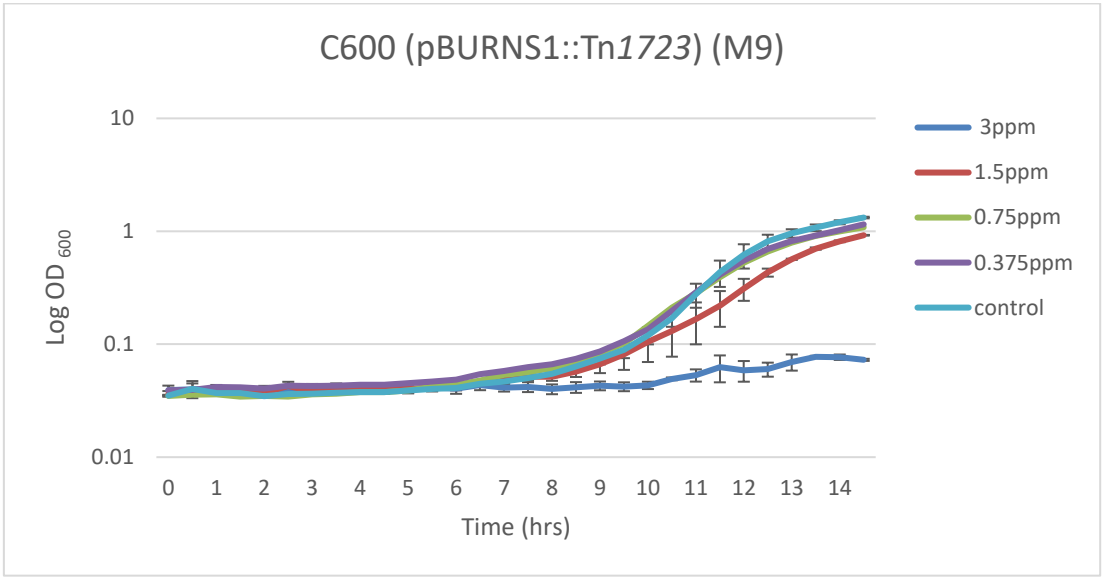
E. coli C600 and C600 pBURNS1::Tn1723 (A and B) and E. coli MV10 nal ^{R} and MV10 nal ^{R} pBURNS1::Tn1723 (C and D) grown in LB with 0.375-3ppm ClO_2 for up to 14.5 hrs in a plate reader. Four concentrations were tested, however, there is no significant difference between the plasmid carriers and plasmid-free E. coli. It is hypothesized that the chlorine dioxide interacts with the constituents of the LB growth medium to a large extent and is reduced before its activity on the cells takes place.

In M9 medium, however, the *E. coli* species with and without the plasmid exhibited a very long lag phase followed by a late exponential phase starting at about 8- 10hrs of incubation. (Figure 4-18**)**. The *E. coli* C600 strains had a faster exponential phase starting at about 7hrs while the MV10nal^{R} strains had a slower exponential phase starting after 10 hours of incubation. In conclusion, the *E. coli* growth curves in chlorine dioxide and M9 did not show a Cdm phenotype as evident as when grown in chlorite. Therefore, it is possible that the chlorine dioxide used in the hospital water systems under the conditions used in the lab do not produce enough chlorite to make a difference in survival of strains carrying pBURNS1::Tn*1723* over plasmid-free ones.

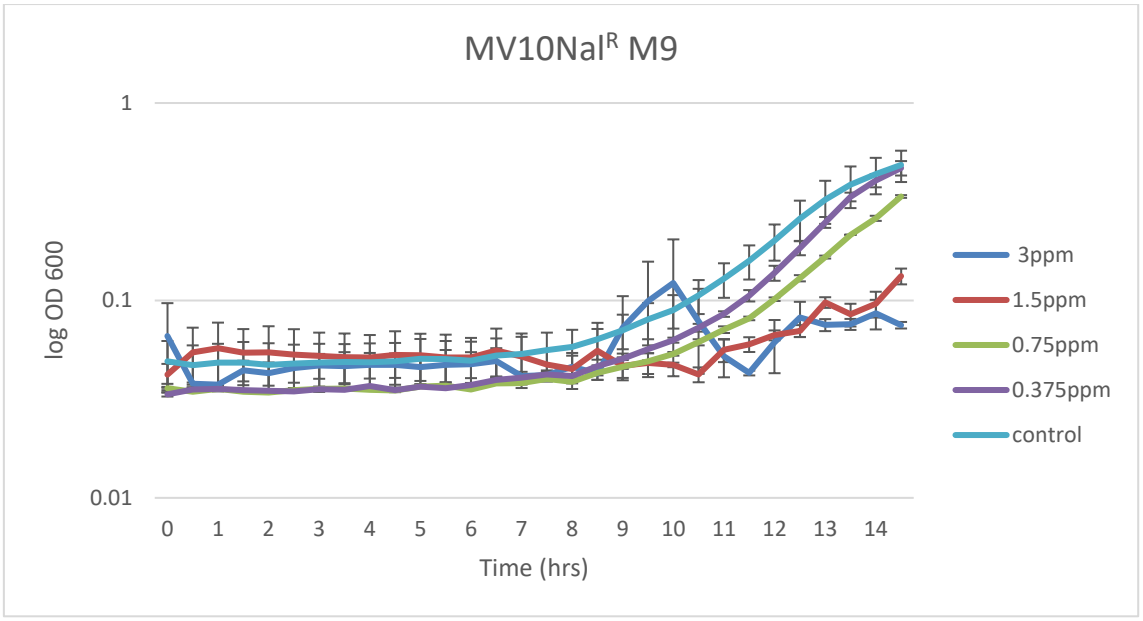
A


C600 (M9)
log OD 600
10
1
0.1
0.01
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (hrs)
3ppm
1.5ppm
0.75ppm
0.375ppm
control

B


C600 (pBURNS1::Tn1723) (M9)
Log OD 600
10
1
0.1
0.01
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (hrs)
3ppm
1.5ppm
0.75ppm
0.375ppm
control

C



D

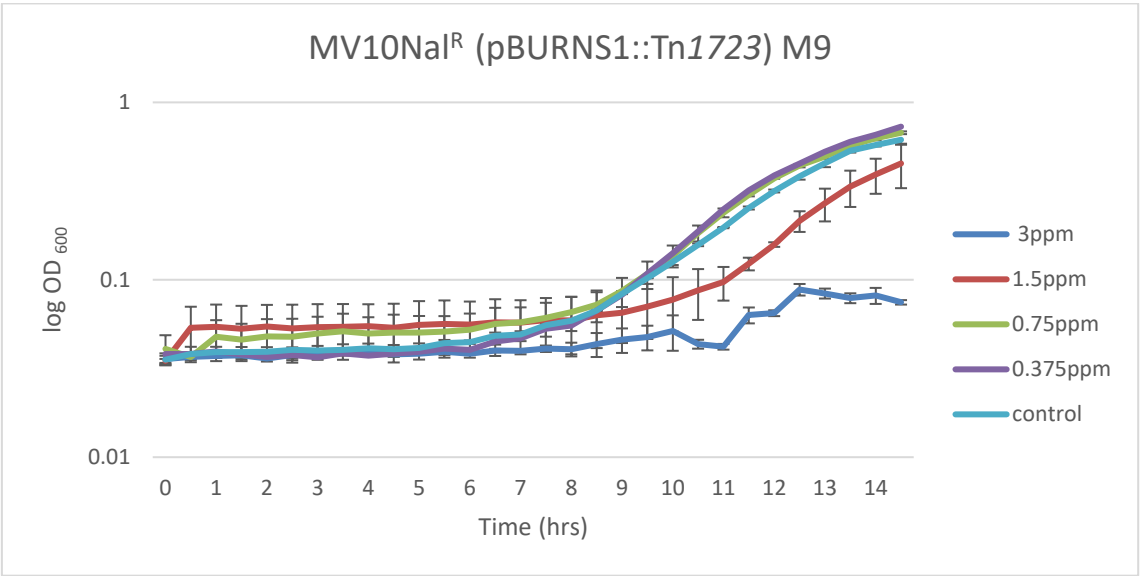


Figure 4-18. Growth curves of *E. coli* C600 with and without pBURNS1::Tn*1723* in chlorine dioxide (M9)

Growth curves of E. coli C600 and C600 pBURNS1::Tn1723 (A and B) and E. coli MV10Nal^R and MV10 Nal^R pBURNS1::Tn1723 (C and D) grown in M9 media with 0.375-3ppm ClO_2 for up to 14.5 hrs. There is evidence of a late log phase which starts after 10hrs.There seems to be a slight advantage of pBURNS1::Tn1723 in ClO_2. In all cases, 3ppm completely kills off the planktonic strains after 14.5hrs of growth.

In some cases, when comparing growth rates of plasmid-carrying strains with their plasmid free counterparts, strains carrying the plasmid grow slower than strains without the plasmid due to fitness cost of the former. In IncP plasmids the highly stable characteristic makes it rare to exhibit fitness costs, however, it majorly depends on the host strains (De Gelder et al, 2007). From the results, *E. coli* C600 (pBURNS1::Tn*1723*) and MV10Nal^{R} (pBURNS1::Tn*1723*) did not show any lag due to fitness cost due to the presence of the plasmid in both LB and M9 media but rather a slightly better growth yield more notably shown in M9.

4.7 Analysis of the Cdm phenotype in WT *P. aeruginosa* 943 and *E. coli* (pBURNS1::Tn*1723*)

In this research, it was necessary to identify the Cdm phenotype in both a laboratory *E. coli* strain and the WT *P. aeruginosa* 943 isolate to determine the significance of the pBURNS1::Tn*1723* plasmid in a hospital environment. Growth curves of the WT *P. aeruginosa* 943 and *E. coli* (pBURNS1::Tn*1723*) and their plasmid free counterparts were conducted and analysed in the presence of chlorite, and the generation times calculated.

The procedure used to determine this was explained in section 2.9. The kanamycin tagged pBURNS1 plasmid- pBURNS1::Tn*1723* was transformed into *E. coli* C600 competent cells and the transformants selected for the presence of the plasmid by plating them on Kanamycin (50 µg/ml) plates. A boil prep PCR was conducted to confirm the presence of *cdm* gene in both plasmid positive *E. coli* and *P. aeruginosa* isolates. *E. coli* C600 and *E. coli* C600 (pBURNS1::Tn*1723*) were grown adjacent to each other in a 96 well plate and placed in the plate-reader overnight at 37ºC grown using LB and minimal media. As seen in Figure 4-19**,** In *E. coli* (M9), with increase in time and concentration of chlorite, the plasmid free *E. coli*

(dotted lines), can be seen to gradually decline in cell density while plasmid carrying *E. coli* C600(smooth lines) grew much better, up to a chlorite concentration of 1mM (red bar).

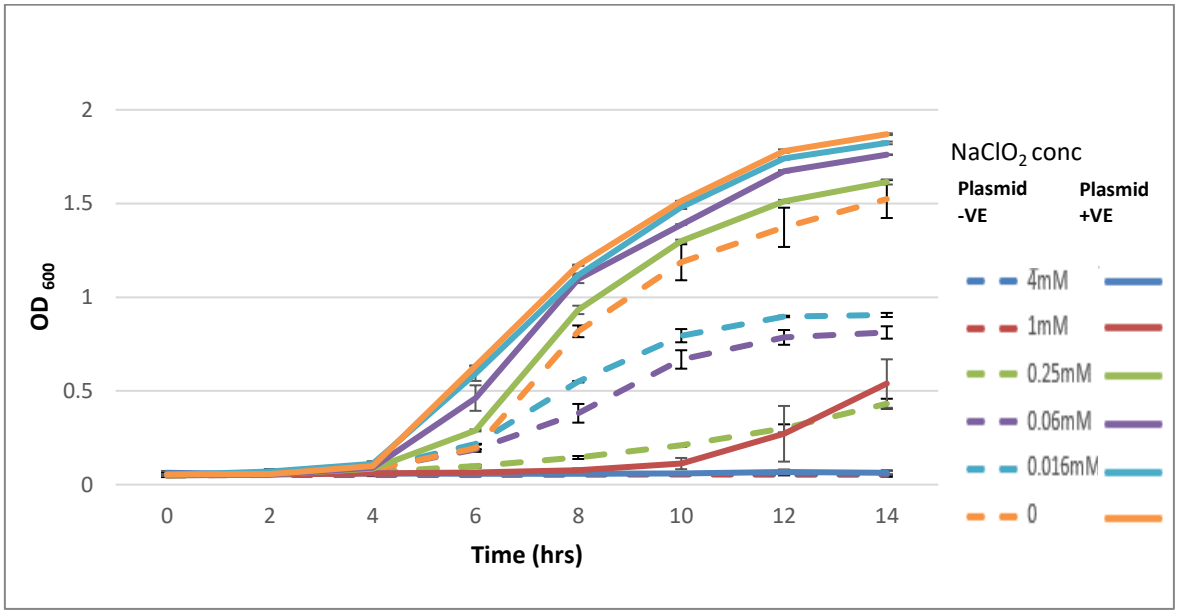


Figure 4-19. Growth curves of *E. coli* C600 +/-pBURNS1::Tn*1723* in M9 minimal media at 37°C with a range of concentrations of sodium chlorite.

Growth curves of E. coli (+/- pBURNS1::Tn1723) in M9 and a range of concentrations of sodium chlorite over a 14hr period. The different colors represent the concentrations of $NaClO_2$ tested. The pBURNS1::Tn1723-carrying strains are in plain lines while plasmid free strains are dashed lines. The graphs show a difference between growth patterns in plasmid positive and negative E. coli with the plasmid positives growing at a higher chlorite concentration than plasmid negatives. It has been observed that sub lethal concentrations of chlorite make cells divide faster than the negative control (without chlorite).

When the same experiment was conducted with *P. aeruginosa* 943 and *P. aeruginosa* (cured), (Figure 4-20) with M9, there was not much difference in lag phase of the two strains before and after the addition of chlorite as observed previously in *E. coli*.

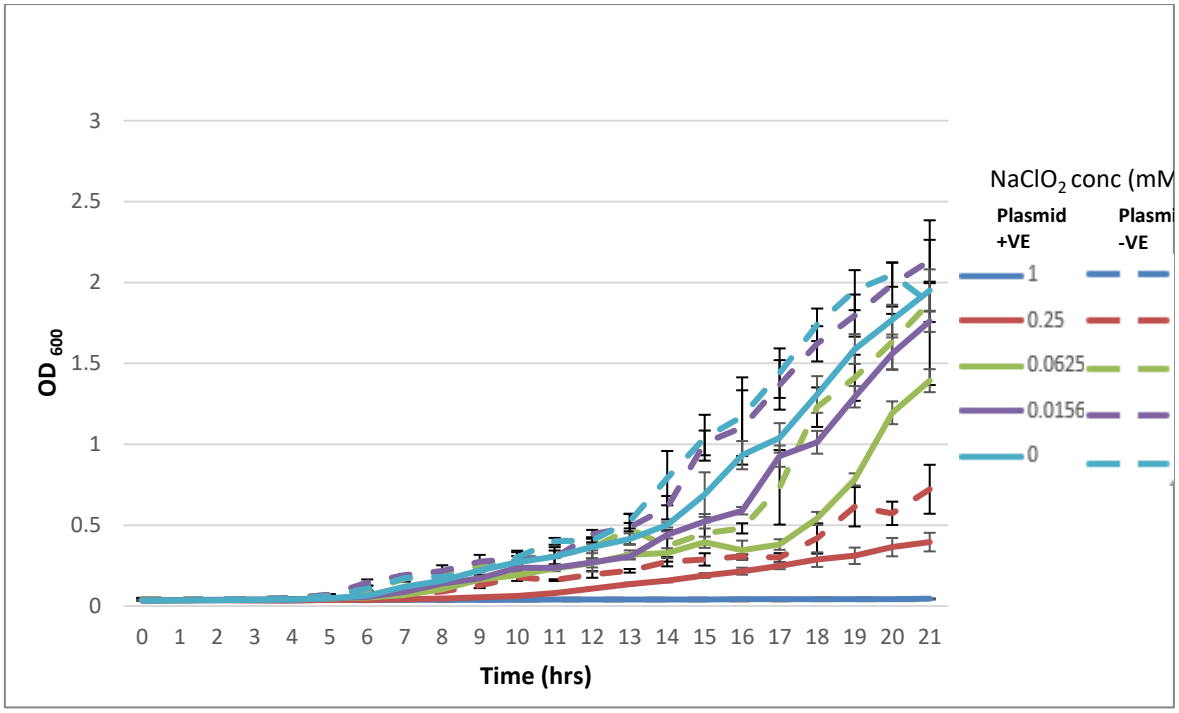


Figure 4-20. Growth curves of *P. aeruginosa* 943 and *P. aeruginosa* 943 *(cured)* in M9 minimal media at 37°C with a range of concentrations of sodium chlorite.

Growth curves of P. aeruginosa 943 (+/- pBURNS1) in M9 and a range of concentrations of sodium chlorite. The different colors represent the concentrations of $NaClO_2$ tested. The pBURNS1-carrying strains are in plain lines while plasmid free strains are dashed lines. The graph does not show a clear difference between the WT P. aeruginosa 943 and P. aeruginosa (cured) in growth patterns in chlorite.

It was observed that there was a difference between the effects of chlorite on the plasmid positive and negative *E. coli* and *P. aeruginosa* 943. Since the MICs of *P. aeruginosa* also showed no difference in chlorite, it was proposed that there is little or no Cdm phenotype in the original host strain, *P. aeruginosa* 943. For both *P. aeruginosa* 943 strains, they both showed a higher sensitivity to chlorite than the plasmid free *E. coli* strain with the lowest concentration of chlorite to permit survival at 0.25 mM.

did. Meanwhile, *E. coli* C600 did not grow at 4 mM $NaClO_2$ (Figure 4-21) Growth curves using the same parameters as above but with rich medium for the same set of strains showed

a similar pattern although with the *E. coli* strains survived much higher concentrations in LB than in M9 while *E. coli* C600(pBURNS1::Tn*1723*) did. When *P. aeruginosa* (+/-pBURNS1) was grown in LB and sodium chlorite, growth curves showed that there was minimal difference between the two strains. (Figure 4-22).

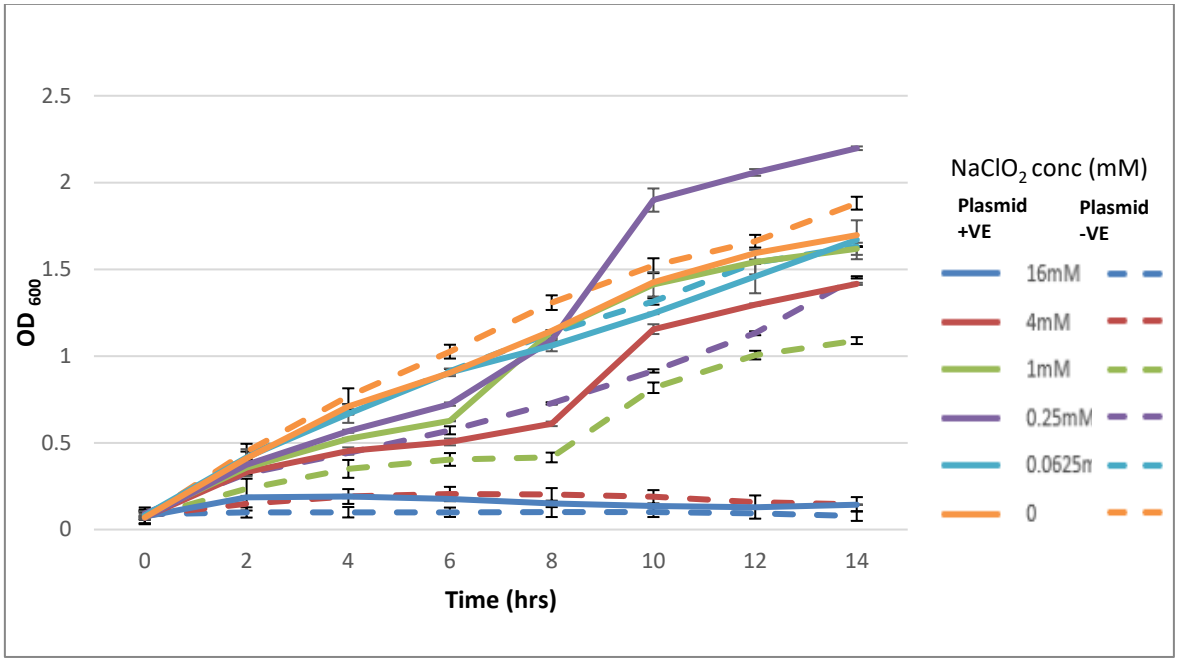


Figure 4-21. Growth curves of *E. coli* C600 +/-pBURNS1::Tn*1723* in L-Broth at 37ºC with a range of concentrations of sodium chlorite.

The E. coli strains with and without pBURNS1::Tn1723 growth curves in rich media. A similar result with the growth curves in M9 with the pBURNS1::Tn1723-carrying strains growing better than the plasmid-free strains with increase in chlorite concentration. At 4mM the pBURNS1 free E. coli does not survive the effects of chlorite.

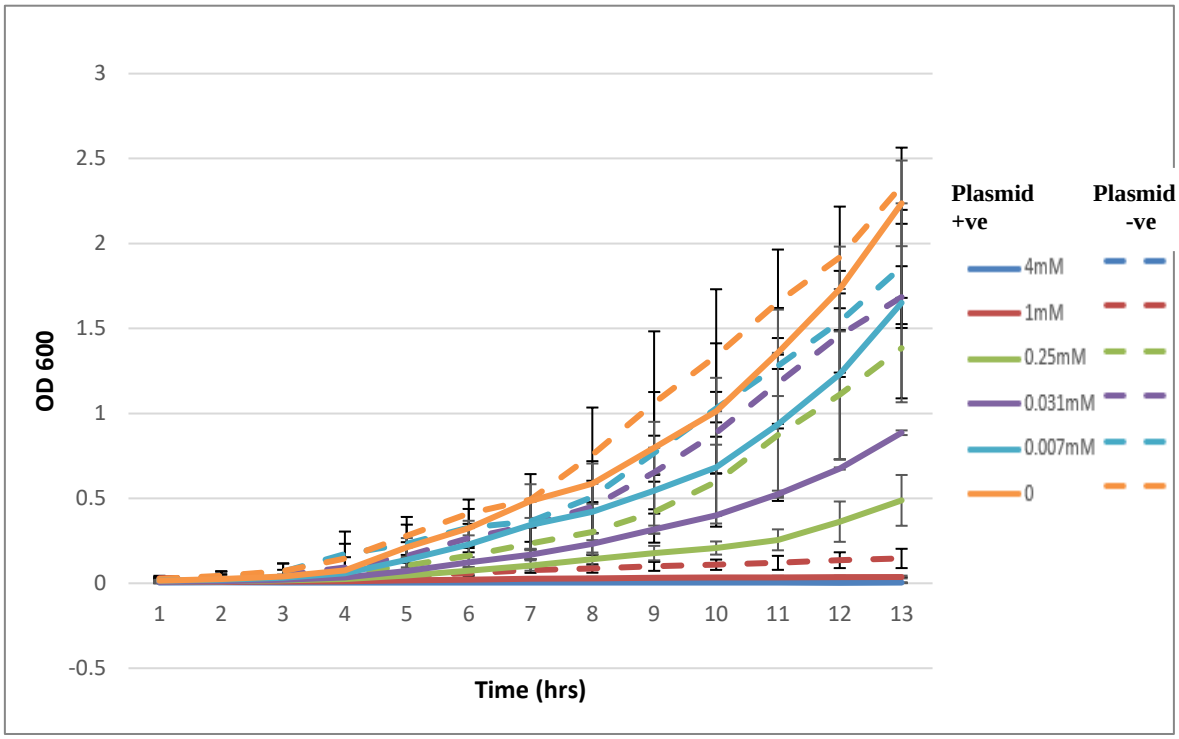


Figure 4-22 Growth curves of *P. aeruginosa* 943 and *P. aeruginosa* 943 *(cured)* in L-Broth at 37°C with a range of concentrations of sodium chlorite.

In L-broth, P. aeruginosa does not show the same level of chlorite resistance as observed in E. coli and there is minimal difference between pBURNS1-carrying and pBURNS1-free strains as seen using minimal media. It appears that a sub-optimal concentration of chlorite may promote cell division.

4.8 Discussion

Although initially used to study the evolutionary changes and morphological growth patterns of bacterial biofilm populations (Poltak and Cooper, 2011), the bead system of generating biofilm in the laboratory was found to be very efficient in monitoring plasmid transfer in a biofilm and survival of plasmid +/- strains during continuous growth since it consistently gave reproducible data. Apart from being a low expense equipment for growing biofilms, it has been used successfully to test the effectiveness of biocides against *P. aeruginosa* biofilms (Konrat et al, 2016). The bead system was also successful in accessing the single

species survival which was achieved by placing an already grown biofilm on the bead for a specific amount of time. However, this method is also thought to be advantageous with some species and not others. It was difficult to get reproducible results with the *P. aeruginosa* biofilms due to the issues mentioned earlier on the high variability within replicates. This was however not the case with the *E. coli* biofilms. In the study of Smith and Hunter (2008), *P. aeruginosa* isolates were found to vary greatly in their ability to form biofilms on polyethylene and it was later found that the lower biofilm producers had the non-mucoid phenotype while others that produced efficient biofilms had mucoid properties. Workentile et al (2013) observed in their study that several identical clones of *P. aeruginosa* isolated from a single sample gave variable phenotypic properties similar to what was observed in this study.

According to the results, there was no observable difference in survival between plasmid+/- *P. aeruginosa* biofilms in chlorine dioxide. This could mean that the *cdm* gene is specific to only chlorite therefore chlorine dioxide would have to be broken down first to chlorite for an effect to be seen between the two strains. This would explain the lack of difference in the planktonic survival of both *E. coli* and *P. aeruginosa* +/- pBURNS1. A study by Behnke and Camper (2012) showed that *P. aeruginosa* biofilms had little or no effect from low doses of chlorine dioxide (0.5-2.0ppm) but completely biocidal effects occurred at 10 ppm ClO_2 within 30 minutes. This concentration far exceeds the limits allowed for water treatment for human use.

We were told that the concentration of chlorine dioxide is maintained in the QE hospital water plumbing system is about 0.25 ppm. The results from Figure 4-6 and Figure 4-7 show that the 0.25 ppm concentration of ClO_2 is enough to remove planktonic *E. coli* and *P. aeruginosa* irrespective of the presence or absence of pBURNS1.Chlorine dioxide has been

found to be very effective against bacteria in a planktonic state. At a concentration of 1ppm, chlorine dioxide was able to kill all *P. aeruginosa* planktonic cells of an initial cell concentration of 10^7 CFU/ml at a contact time of 30mins (Behnke and Camper, 2012). A 5-log reduction in original cell concentration occurred when 0.9ppm chlorine dioxide was used to treat *E. coli* cells for 5 mins only (Foschino et al, 1998). The results showed that the major cut-off concentration between complete survival was about 0.25 ppm for planktonic cells as rightly used in the hospital. However, fluctuations that may occur in a normal setting would be crucial if the cut-off is very close to what is being used to prevent planktonic bacteria surviving long enough to form biofilms in the plumbing system. Based on the results presented here, the presence of *P. aeruginosa* 943 in various locations in room 8 of the Burns unit shows that the isolate did not seem to occur in a planktonic state from the water supply but more likely from a more resistant biofilm seeding the planktonic versions in the water and immediate surroundings.

This led on to further experiments to determine if we would see a chlorite resistance phenotype when a strain carrying pBURNS1 is exposed to the range of concentrations of ClO_2 found in the hospital water system. The *P. aeruginosa* biofilms +/- pBURNS1 treated for 1 hr and 3 hrs in ClO_2 as seen in Figure 4-8 and Figure 4-9 respectively did not completely eradicate the 24-hr single species biofilm at 0.5ppm ClO_2. The same was the case for a 24-hr *E. coli* biofilm +/- pBURNS1::Tn*1723*. In both cases, the biofilms were being affected by chlorine dioxide but were not completely eradicated within the contact times used. Therefore, young or newly formed biofilms may be expected to survive the ClO_2 treatment of the hospital water at 0.5 ppm. It is also possible that a longer contact time may have completely eradicated the biofilms.

The plasmid transfer experiment was only carried out using *E. coli* strains/ biofilms. The results determined that the transconjugant biofilm would survive repeated exposure to a high concentration of chlorite due to the presence of the newly acquired plasmid along with the initial donors, although the donors have a greater capacity to survive than the transconjugants. The reason for this could be because the donors at this stage may not have formed robust biofilms because the recipient 48 hr biofilm may have occupied most of the surface area of the bead. It has been shown that when donors are added to a pre-existing recipient biofilm, they stay on top of the recipients rather than form their own microcolonies (Ma and Bryers, 2013). Therefore, the absence of recipients after treatment means there will be enough surface area for donors to establish which was demonstrated in the results (Figure 4-12). Non- pathogenic Listeria were able to acquire and transfer benzalkonium resistance to both pathogenic and non-pathogenic Listeriae efficiently via conjugation (Katharios-Lanwermeyer et al, 2012). Low concentrations of biocides such as triclosan and chlorhexidine was also found to stimulate plasmid transfer in *E. coli* (Jutkina et al, 2018).

In M9, there was an increase in the number of transconjugants even after treatment and this may be due to the limited nutrients, increased porosity of biofilm or the reduced chlorite concentration stimulating biofilm formation (Figure 4-14). Although during their study Molin and Tolker-Niellsen (2003) did not observe an increased number of transconjugants with richer growth medium, Ma and Bryers (2013) observed that poor nutrient availability increases the porosity of biofilm and this allows donors to reach further into the biofilm to the recipients. Water environments have minimal nutritional provision for bacterial cells and so therefore we can expect more survival of transconjugants in water systems such as where pBURNS1 was originally found (Quick et al, 2014).

However, when grown in rich medium and using high concentrations of chlorite, the donors thrive better than the transconjugants (Figure 4-12). At the end of 48hrs of treatment, only transconjugants and recipients are found to remain on the biofilm and in the suspension in which the biofilm was formed in L-broth. Biofilm formation is a natural defense mechanism for bacteria such as *P. aeruginosa*. It plays a vital role in the ineffectiveness of biocides and antibiotics since the exopolysaccharide matrix helps to reduce the cells direct exposure.

The transconjugants whether grown in LB and M9 survived better than the plasmid free recipients in terms of biofilm viable cell counts. It can be deduced that this survival is due to the presence of pBURNS1::Tn*1723* which contains the *cdm* gene from the results shown in Figure 4-9. It was also hypothesised that the lower number of transconjugants formed was due to the rapid killing of the recipient biofilm and since the transconjugants have been notably found to form microcolonies on top of recipient cells, may fall off the biofilm while still being viable when the recipients are being killed off. This was concluded to be the case since higher transconjugant counts was seen when the second cell wash (to remove planktonic cells) of the bead was plated on KanNal L-Agar. The counts of transconjugants obtained from the wash were higher than those gotten when the biofilm from the beads was resuspended post-wash (data not shown).

In LB, the transconjugant biofilm population with newly acquired plasmids was notably less robust than its surviving population in M9 minimal media. In the former there was a decline with further treatment while in the latter there was an increase with increase in treatment time. It could be because the recipients were not completely eradicated as in 5mM LB biofilm. Thus, survival of recipients may play a role in the presence and survival of transconjugants. In M9, the amount of biofilm biomass produced was generally at least 10-fold higher than in LB therefore it can be suggested that the lack of nutrients may increase

the overall biofilm biomass as seen in Table 4-3. Naves et al (2008) in their study also observed a distinctive increase in biofilm formation in *E. coli* strains grown in M9 minimal media compared to those grown in LB. It was also observed that avian pathogenic *E. coli* formed more robust biofilms in nutrient deficient media than in nutrient rich and suggested the increase in proliferation of biofilms in avian water systems may be contributed to this factor (Skyberg et al, 2007).

Although there was not a complete eradication of the recipients after treatment in M9, the gradual reduction shows the effect of chlorite on the recipient biofilm. Since there wasn't a lot of loss of recipients, this gives the transconjugants more time to form and express the chlorite resistance gene. There was a higher survival of zinc resistant transconjugants in the presence of heavy metal polluted soil than when there was when no selective pressure was present. A similar result was seen when zinc sulphate was added to uncontaminated soil (De Rore et al, 1994). This could mean a sub-optimal concentration of chlorite may be what is needed for the transconjugants to thrive and remain in the biofilm.

The hypothesis of the *cdm* gene providing an advantage to pBURNS1-carrying strains in chlorine dioxide treated hospital water will be rejected. Based on the results, under laboratory conditions, there is not enough evidence that suggest that the hypothesis is valid. In *E. coli* the resistance to chlorite seems to be specific and according to the results of the growth curves, it takes a minimum concentration of chlorite (0.25mM sodium chlorite in M9) to show a difference between survival of a plasmid carrying *E. coli* strain and a plasmid free one. This concentration of chlorite equivalent to 90.4mg/L $NaClO_2$ far exceeds the level of chlorite that could be found in chlorine dioxide water systems the plumbing systems (roughly 0.8-1.0mg/L chlorite levels) (Srinivasan et al, 2003). Therefore, it is not realistic to equate this survival to what happens in the hospital plumbing system. An exception to this

is if a low/suboptimal concentration of 0.8mg/ml can influence survival of plasmid carrying biofilms over a long-term occurrence which is unknown. When chlorine was used to treat *P. aeruginosa*, considerable cell damage was perceived, however, when the dosage reduced to below the threshold 0.3mg/L for 12 hours, the surviving cells regenerated back to the same amounts observed before any treatment (Mao et al, 2018). Therefore, sub-optimal concentrations of most biocides may turn out to be more detrimental than useful.

Phenotypic variations are quite common in bacteria and several factors can contribute to the absence of a phenotype where a related gene is present in a genome. These include environmental and physiological factors, genetic factors such as mutations and alternations of the gene within the host. *E. coli* strains are genetically different from *P. aeruginosa* strains based on their genome size (Stover et al, 2000). It is hypothesized that regulatory systems expressed chromosomally may be involved in suppressing the chlorite dismutase enzyme being produced. Research showed that the degradation ability of IncP-1 plasmids is dependent on both plasmid and chromosomally encoded regulatory systems and therefore when the hosts or plasmids are interchanged, there could be phenotypic changes involved in the host cell (Shintani et al, 2010). In this study, similar results on survival of pBURNS1 or pBURNS1::Tn*1723* carrying strains were seen within *E. coli* species and *Pseudomonas* species. For example, pBURNS1::Tn*1723* carried by *E. coli* C600; MV10 Nal^R and pBURNS1 in *P. aeruginosa* 943; *P. aeruginosa* PAO1161 showed similar different survival patterns in chlorite, therefore the changes we see in expression could be an organism-specific issue.

The presence or absence of other environmental /external factors could also result in a change in phenotype in a host cell. *P. aeruginosa* can be both aerobic and facultative anaerobic, however during biofilm formation, they tend to proliferate better during

anaerobic conditions (Bollinger et al, 2001). It was also observed that unlike *E. coli*, *P. aeruginosa* ATPase production is highly dependent on oxygen levels within the biofilms and expression of this enzyme increased with increase in oxygen levels to the biofilm (Xu et al, 1998). The Cdm enzyme produces considerable amounts of oxygen during break down of chlorite and thus may signal a transcriptional regulator to control expression of *cdm*.

From the results, it can be observed that transconjugants that have recently acquired the pBURNS1::Tn*1723* can be selected by chlorite in a biofilm. In higher concentrations of sodium chlorite however, more of the transconjugants are present in the supernatant rather than on the biofilm as compared to the donors. One possible reason why this occurs is due to the presence of surface exclusion proteins (Figure 4-23) which are present in IncP1 plasmids where transconjugants will try to prevent transfer from occurring in a closely related plasmid such as those within the donor strain. In IncP1α, RK2 plasmid contains Tra1 and Tra2 regions with *tra* and *trb* genes encoding for mating pair formation except *trbK*, an entry exclusion lipoprotein is predominantly present in the cytoplasmic membrane (Haase et al, 1996). *trbJ* is the second gene in IncP plasmids that is involved in surface exclusion. It enhances the exclusion process when *trbK* is present but based on tests, its entry exclusion activity is not as efficient as *trbK* (Pansegrau et al,1994).The TrbJ protein is found in the cellular periplasm IncP and F plasmids, other incompatibity groups that have been shown to exhibit surface inclusion include Inc I, N, W and H. Integrative conjugative elements and mobilizable plasmids such as ColE1 have also been found to possess surface exclusion genes (De la Cruz and Garcillan-Barcia, 2008). This could mean that when the recipient biofilm is formed, the donors are introduced and contact with the recipients in the lower layers of the biofilm form the transconjugants. After treatment, the newly formed transconjugants will

either form their own microcolonies or may attach to the donor strains whose surface exclusion proteins may cause them to fall off.

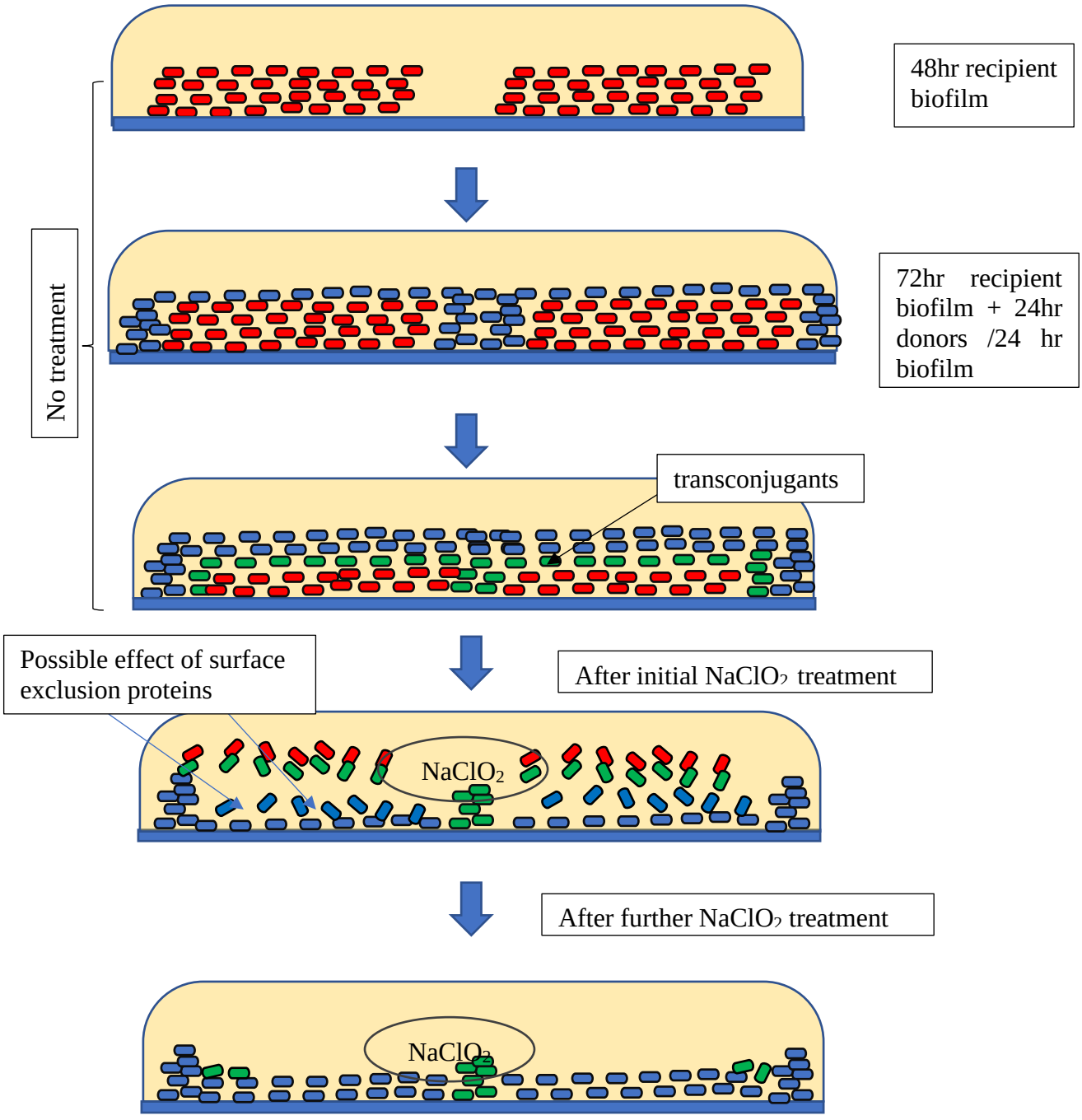


Figure 4-23. Cartoon depicting the survival of products of plasmid transfer in a biofilm before and after selection with chlorite

This illustration above shows the hypothesis of what occurs after the donor and recipient strains in a biofilm are formed and subjected to sodium chlorite. The red cells represent the

recipients; the blue represents the donors; and the green represents the transconjugants. The recipients initially formed larger microcolonies on the bead but were killed off after treatment. What is left after treatment are mostly donors and a small number of transconjugants.

Conjugative plasmids possess one or more surface exclusion genes which prevent the re-entry of a closely related plasmid into a cell that already has a plasmid by preventing recipients that have obtained a plasmid to be available for conjugation.

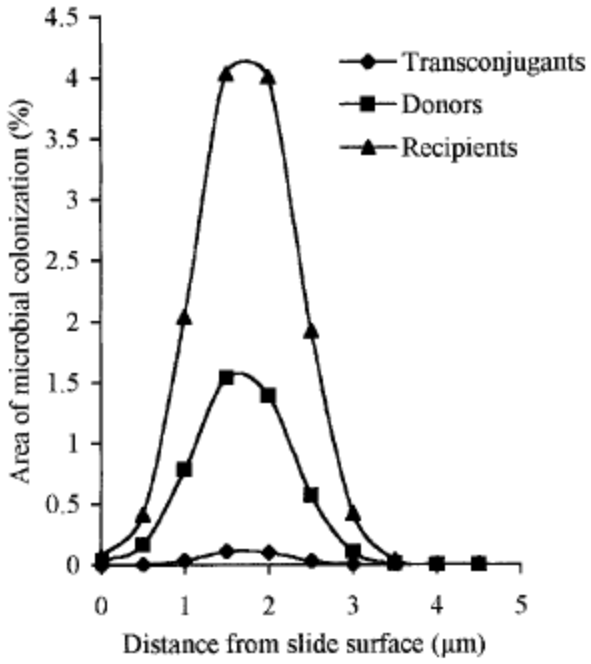


Figure 4-24. Spatial distribution of donors, transconjugants and recipients on a slide surface in a 24hr biofilm (volume $7.3x10^{4}\mu m^{-3}$) grown in rich medium

Figure from (Hausner and Wuertz, 1999)

Transconjugants are not also evenly distributed on a biofilm as donors or recipients in termsof biomass (Figure 4-24). Christensen et al, (1998) showed that the transconjugants are often associated with recipient biofilm and rarely form separate microcolonies. In plasmid transfer experiments, after treating the biofilm population, the transconjugants

survive treatment but not at the same rate as the donors on the biofilm. Some transconjugants may be washed off as viable cells with the dead recipient cells if they remain associated after treatment.

Competition between members of the biofilm could be another factor that determines the presence of transconjugants in the presence of chlorite. Bacteria in a mixed biofilm often outcompete each other depending on relative fitness and ability to form a biofilm. It can therefore be deduced that the presence of transconjugants in a biofilm grown in 5mM LB and 0.2mM M9 is dependent on the survival of the parent recipient strain.

4.9 Summary

Pseudomonas aeruginosa found in hospital settings have almost always been associated with the presence of a biofilm within the vicinity. Chlorine dioxide is used to treat the hospital water before it is passed through tanks and the water works and plumbing. Planktonic *P. aeruginosa* and *E. coli* + or - pBURNS1 will not survive 0.25 ppm chlorine dioxide (the concentration expected to be in the water at the taps in the QE hospital) when exposed for one hour. Lower concentrations however did not have a bactericidal effect on the strains, therefore it is critical that the concentrations of ClO_2 used in the hospital water systems do not decline or fluctuate for up to an hour. Biofilms have been known to be up to 1000 times more resistant to both antibiotics and biocides than the planktonic organisms (Behnke and Camper, 2012). From this research, there is little or no evidence to show that the carriage of pBURNS1::*Tn1723* containing *cdm* by a host has a higher survival rate than the same strains without the plasmid in the planktonic state. They both survived and were killed off at the same concentration of chlorine dioxide used.

When the biofilms were treated with chlorine dioxide, a similar result showing no significant difference between the plasmid positive and negative strains was seen. A limitation to the biofilm experiments was that biocide treatment was not extended (either based on contact time length or level of concentration) until all the strains were killed off before comparison between plasmid positive and plasmid negative strains were made. However, the rationale behind the design was to use the range of concentrations of ClO_2 used in the hospital and to see how effective it would be against *P. aeruginosa* (pBURNS1) biofilms if formed within 24 or 48 hrs within the plumbing system. The advantage of carrying the plasmid was therefore not seen in terms of survival of plasmid carrying strains as either planktonic or single species biofilm in the presence of chlorine dioxide.

In plasmid transfer and chlorite selection experiments, results showed that pBURNS1::Tn*1723* is highly transmissible from one *E. coli* strain to another with and without selection (controls) with chlorite and within a biofilm, transconjugants are formed after 24 hrs with the donors and recipients present. Treatment with 5 mM and 0.2 mM in LB and M9 respectively showed that the hypothesis was true, in both cases there was killing of recipients (plasmid free) and survival of donors and transconjugants, however, the donors survived at a higher rate. The lower survival of transconjugants may be due to several factors such as surface inclusion proteins, competition and spatial distribution of the species. The concentration of chlorite used in this experiment is far higher than what would be found in the hospital treated water and despite there being an advantage of pBURNS1, this can only occur in areas of significantly higher concentrations of chlorite.

The growth curves of *E. coli* and *P. aeruginosa* strains with and without pBURNS1::Tn*1723* and pBURNS1 plasmid gave a first observation of the absence of Cdm+ phenotype in the latter. We do know however, that the phenotype can be seen in all tested strains of *E. coli*.

We also did not see a distinct phenotype in *P. aeruginosa* PAO1161 with the plasmid (not shown). Growth curves in chlorine dioxide showed little or no difference in growth rate or survival between plasmid carrying and plasmid free strains. However, at the late log phase/stationary phase a small difference can be seen between *E. coli* +/- pBURNS1::Tn*1723*. It was discovered that only a certain high concentration of chlorite (1 mM or 90 mg/L) would give an advantage to the plasmid carrying strains. Such an environment with high concentrations may not be present in the hospital water plumbing.

Chapter 5

Determination of *cdm* promoter activity through β-galactosidase assay

5.1 Introduction

While chapter 4 focused on the phenotypic significance of the pBURNS1 plasmid in the hospital environment, this chapter solely focuses of the transcriptional and translational expression of the *cdm* promoter to investigate the poor levels of *P. aeruginosa* Cdm+ phenotype. Reporter plasmids are useful tools in determining the strength of regulatory functions such as those of promoters in a way that can be measured and quantified. The activity is based on the activity of β-galactosidase on either lactose or in this case O-nitrophenyl-beta-D-galactopyranoside (ONPG) which changes it from colourless to yellow (Honeyman et al, 2002). With a transcriptional fusion, the product will only include promoter activity based on the reporter gene alone (*lacZ*) while with the translational fusion, the products will include part of the Cdm protein (gene of interest) merged with the LacZ protein as end products (Honeyman et al, 2002).

5.2 Aim

This section aims to investigate the large difference in phenotype previously observed between the chlorite resistance phenotype in the WT *P. aeruginosa 943* strain and *E. coli* C600 (pBURNS1::Tn*1723*) in the presence of chlorite. It was hypothesized that both transcriptional and translational fusions may produce large amounts of LacZ in *E. coli* rather than in *P. aeruginosa* to explain the low expression earlier observed. The procedure in chapter 2.12 was followed accordingly.

Specifically, this chapter aims to

- Determine the promoter activity of the *cdm* gene in *P. aeruginosa* 943 and *E. coli* DH5α with and without (pBURNS1::Tn*1723*).

5.3 β- galactosidase assays using pMUP9 *lacZ* expression vector to determine *cdm* promoter activity

The results obtained from the growth curves in Figure 4-19 to 4-22 showed that there is a Cdm phenotype when pBURNS1::Tn*1723* is present in *E. coli* but none when it is present in the original *P. aeruginosa* WT isolate. One possible reason for the inability to observe a phenotype for the *cdm* gene in *P. aeruginosa* may be due to the difference in transcriptional or translational expression of the gene by the *cdm* promoter in pBURNS1 in the two different bacteria. To test this hypothesis, the *cdm* promoter was cloned from pBURNS1 and inserted upstream of the *lacZ* gene in a reporter plasmid (pMUP9). Once cloned, the recombinant plasmids designated pCDM1 (transcriptional fusion) and pCDM2 (translational fusion) were conjugated and transformed into *E. coli* DH5α and *P. aeruginosa* respectively (with and without pBURNS1). The next steps involved β-gal assays using the new clones to detect the levels of *lacZ* expression.

5.3.1 PCR Amplification of Pcdm1 and Pcdm2 from pBURNS1

The first step to the construction of pCDM1 and pCDM2-*lacZ* fusions was to amplify the promoter fragments from pBURNS1 using the primers mentioned above. The PCR reaction settings and mix are laid out below. Four of each fragment was amplified to get enough DNA for the cloning ahead. The results showed products of 280bp and 385bp for Pcdm1 and Pcdm2 respectively (Figure 5-1).

PCR reaction for Pcdm1 and Pcdm2 includes 5 µl of plasmid/ boil prep DNA template, 0.5 µl of Velocity Polymerase, 0.75 µl of DMSO, 1 µl each of cdm1 forward primer and cdm1 reverse primer, 2.5 µl of dNTPs, 5 µl of 5x Buffer and 34.25 µl of SDW. PCR settings are shown in Table 5-1.

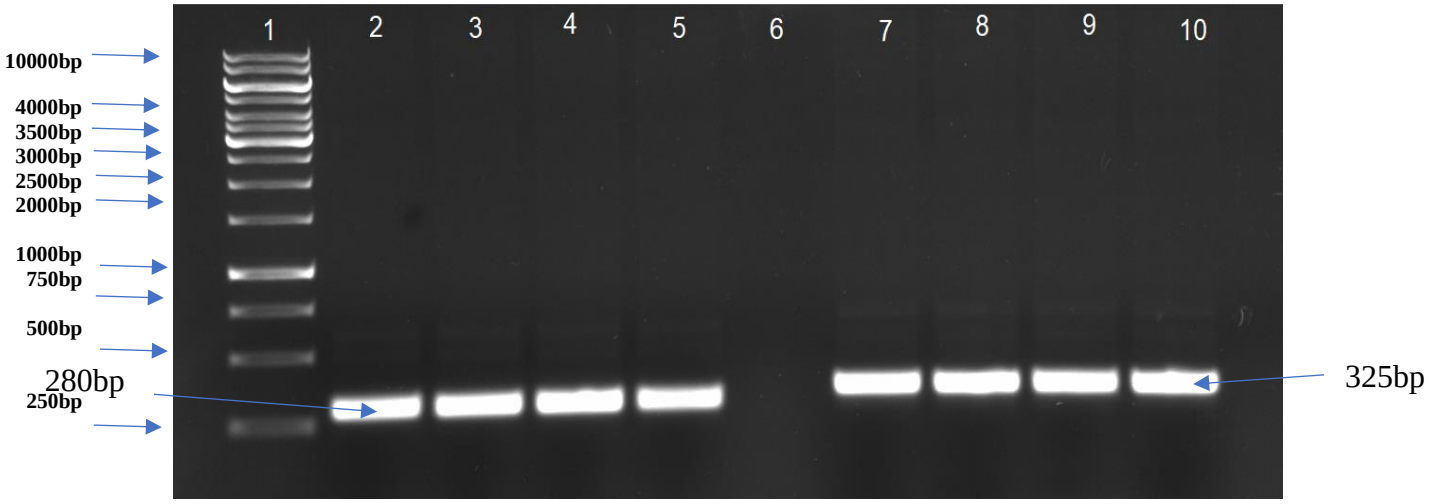


Figure 0-1. PCR amplification of the two *cdm* promoter regions from pBURNS1: Pcdm1 and Pcdm2

The 280bp bands represents the amplified Pcdm1 fragment while the 325bp bands are the second promoter region Pcdm2. These fragments were cut out and gel purified as inserts for further cloning.

Lane	Description
1	1kb Gene O ruler ladder
2-5	Pcdm1 PCR product (10 µl)
6	Negative control (H_2O in place of DNA)
7-10	Pcdm2 PCR product (10 µl)

Table 0-1 PCR settings for Pcdm1 and Pcdm2 amplification

Temperature (ºC)	time	Description
98	2 secs	Denaturation
98	30secs	Denaturation
56.5	30 secs	Annealing
72	15secs	Elongation
72	7 mins	Elongation

5.3.1.1 PCR amplification and cloning of pCDM1 and pCDM2 promoter fragments into pMUP9 (pJH10 backbone)

Primers were designed to anneal the *cdm* promoter region. This was followed by PCR amplification of two *cdm* promoter regions designated as (Pcdm1 and Pcdm2) from pBURNS1. The forward primer was designed to anneal 12 bp before the -35 region of the Pc promoter (see Figure 5-2A) while two separate reverse primers were used to anneal the two promoter fragments before the transcriptional start site and before the ribosome binding site of the *cdm* gene. (see Figure 5-2B). The reason for constructing a second promoter region, Pcdm2 was to provide the *cdm* Shine-Dalgarno (ribosomal binding site) sequence to enable *cdm* translation to take place in the reporter plasmid. These two promoter fragments were cloned in pMUP9 replacing the *mup*Z promoter upstream of *lacZ* as shown in Figure 2-7 and renamed pCDM1 and pCDM2 plasmids.

Figure 5-3 shows a snapshot highlighting the positions on the pBURNS1 sequence where the promoters are located as well as the primers.

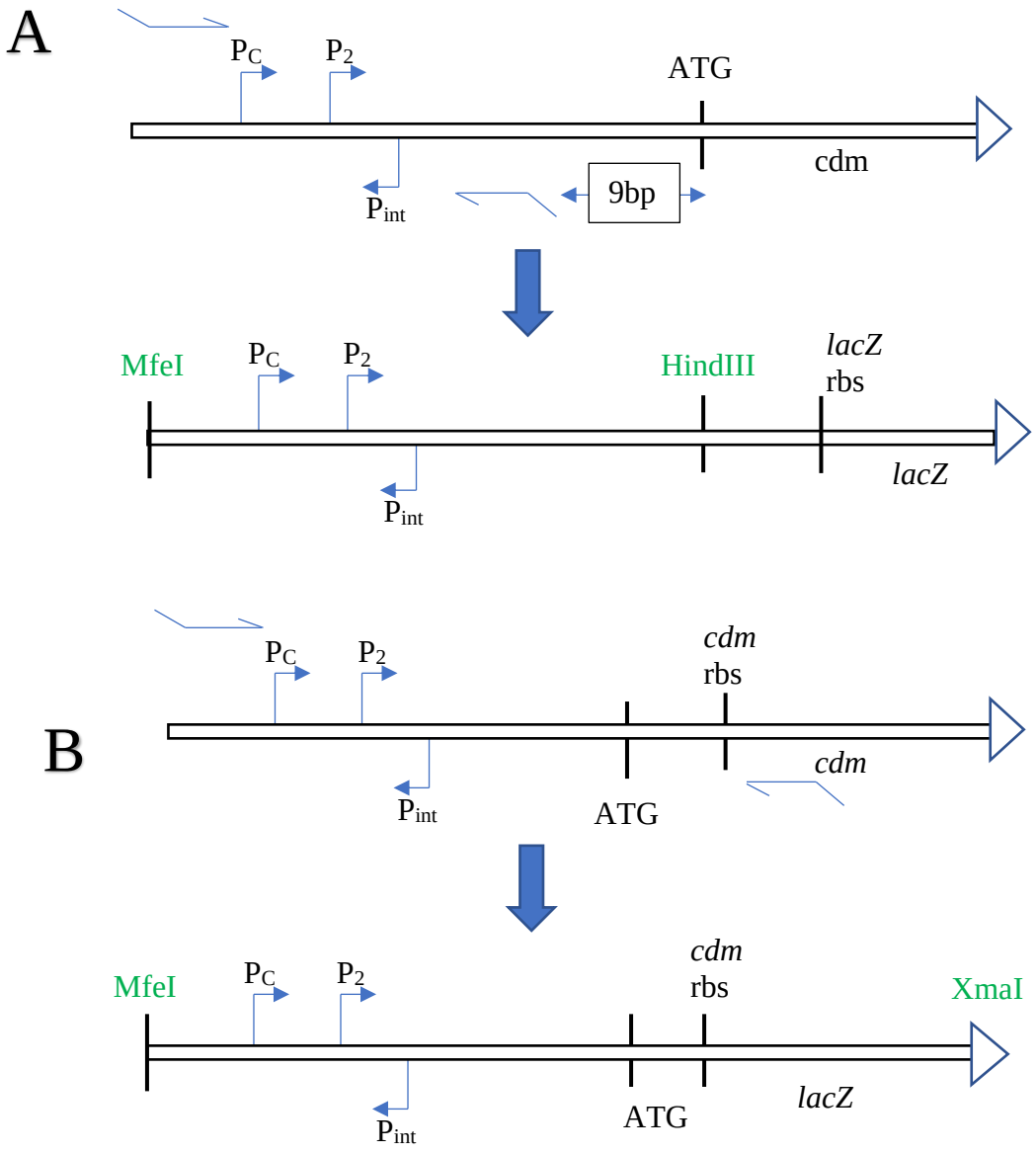
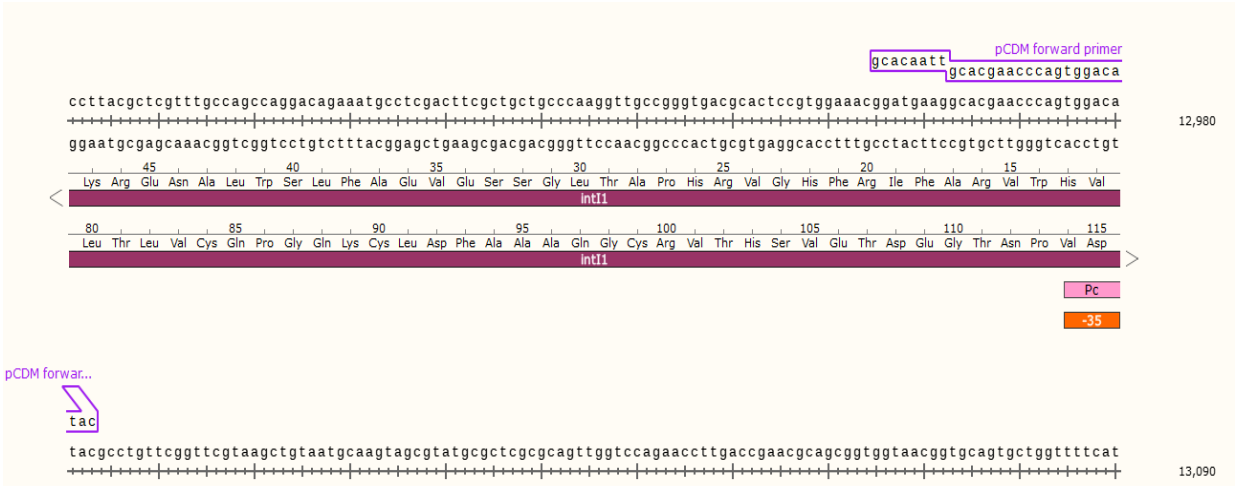


Figure 5-1. Schematic representation of the A. Pcdm1 and B. Pcdm2 amplification and fusion with the *lacZ* gene

The PCR amplification of cdm1 and cdm2 from pBURNS1 for the fusion upstream a promoter-less lacZ gene. The β-galactosidase assay was aimed at determining the level of transcriptional and translational activity respectively. The cdm ribosome binding site in B is included in the formation of pCDM2. Pc and P_2 are the two cdm promoters while P_{int} is the integrase promoter. Highlighted in green are the restriction sites. rbs stands for ribosome binding site while ATG represents the transcriptional start site.

A



B

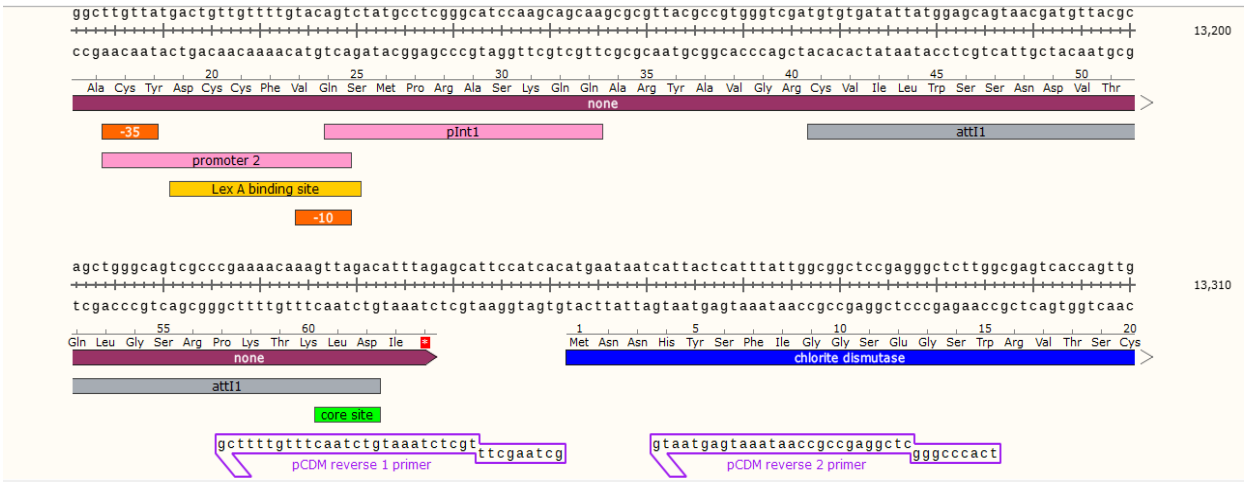


Figure 5-2. Snapshots of A. cdm1 and cdm2 forward promoter primer and B. cdm1 and cdm2 reverse primers as annealed sequences

The cdm gene is highlighted in blue, the primers are represented as arrows, promoters are in pink, lexA binding site in yellow, -35 and -10 promoter consensus in orange, the attachment site (attI) of the integron is highlighted in grey and core site in green.

5.3.1.2 Cloning of Pcdm1 and Pcdm2 inserts into pGEMT through restriction digest

The PCR products Pcdm1 and Pcdm2 were designed to have 5' restriction enzyme overhangs of MfeI, HindIII and MfeI, XmaI respectively. Once they were cut out and gel purified, Pcdm1 and Pcdm2 insert were initially cloned into a commercial pGEMT vector due to difficulty in direct ligation with the vector. This was done by A-tailing. After A-tailing as described in section 2.8.1, the fragments were ligated with pGEMT and transformed into DH5α competent cells. The transformants formed were screened for the presence of the insert by sequencing extracted plasmid DNA via mini prep before ligation to the pMUP9 vector. The pMUP9 when digested was expected to release the *mup*Z promoter that was to be replaced with the *cdm* promoters (see Chapter 2.8.1). To prepare the insert and vector for ligation, sticky ends of the fragments were needed and therefore, restriction digests were conducted using their corresponding restriction enzymes. The digest was run for 1hr 30 mins at 37ºC for both inserts and vector. The restriction digest mix prepared for Pcdm1 and pMUP9 is shown in Table 5-2

Table 0-2 Restriction digest mix for Pcdm1 and pMUP9 vector

	pMUP9 (vector) digest	**pGEMT (Pcdm1)**
DNA	20 µl	20 µl
HindIII	2 µl	2 µl
MfeI	2 µl	2 µl
Cutsmart	5 µl	5 µl
Sterile Distilled Water	21 µl	21 µl

Table 0-3 Restriction digest mix for Pcdm2 and pMUP9 vector

	pMUP9 (vector)	pGEMT (Pcdm2)
DNA	20µl	20 µl
XmaI	2 µl	2 µl
MfeI	2 µl	2 µl
Cutsmart	5 µl	5 µl
Sterile Distilled Water	21 µl	21 µl

The normal restriction digest protocol was followed as stated in chapter 2.6.1. Alongside the inserts, the 15 µl of the vector plasmid prep (pMUP9) was digested using the same enzymes with the insert. A mix of reagents was prepared as shown in Table 5-3. After digest, the products were purified without using running a gel. 1 µl of both inserts and vectors were run on a gel to estimate the quantities ahead of ligation as shown in Figure 5-4 below.

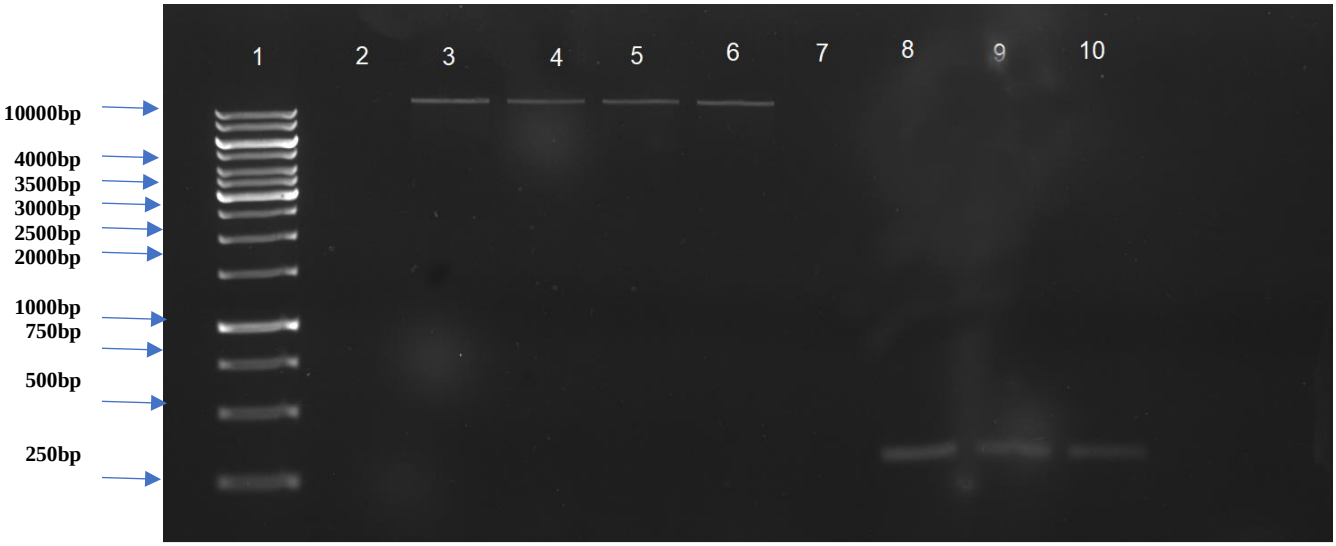


Figure 0-4 Gel electrophoresis results showing 1 µl of pMUP9 vectors and Pcdm 1 promoter insert digests.

To provide an estimate of the amounts of DNA in both insert and vector for the ligation, 1 µl of each was run on a gel side by side for comparison. The vector was shown to be more intense in brightness than the inserts.

Lane	Description
1	1kb gene o ruler ladder
3-6	MfeI and HindIII digested vector pMUP9
8-10	MfeI and HindIII digested cdm1 insert

5.3.1.3 Ligation of Pcdm1 and Pcdm2 into pMUP9 reporter plasmid

After the inserts were successfully digested from pGEMT and the vector was also digested and then gel purified. 10 µl of the ligated product was then transformed into *E. coli* DH5α.

Ligation of Pcdm1 and pMUP9

The favourable ligation ratio in this case was 1:2 and the following ligation mix was used. 10 µl pMUP9 vector, 5 µl Pcdm1 insert, 1 µl DNA ligase, 2 µl ligase buffer (x10) and 3 µl

Sterile Distilled Water. This ligation mix, when transformed into *E. coli* DH5α competent cells, produced 4 colonies. Boil prep PCR was conducted on these colonies and from the negative control to confirm the successful pCDM1 clones using the Pcdm1 forward and reverse primers. See Figure 5-5 below.

PCR mix for both pCDM1 and pCDM2 screening comprised of 5 µl of DNA, 1 µl of Velocity DNA polymerase, 10 µl of Cutsmart buffer and 1.5 of DMSO.

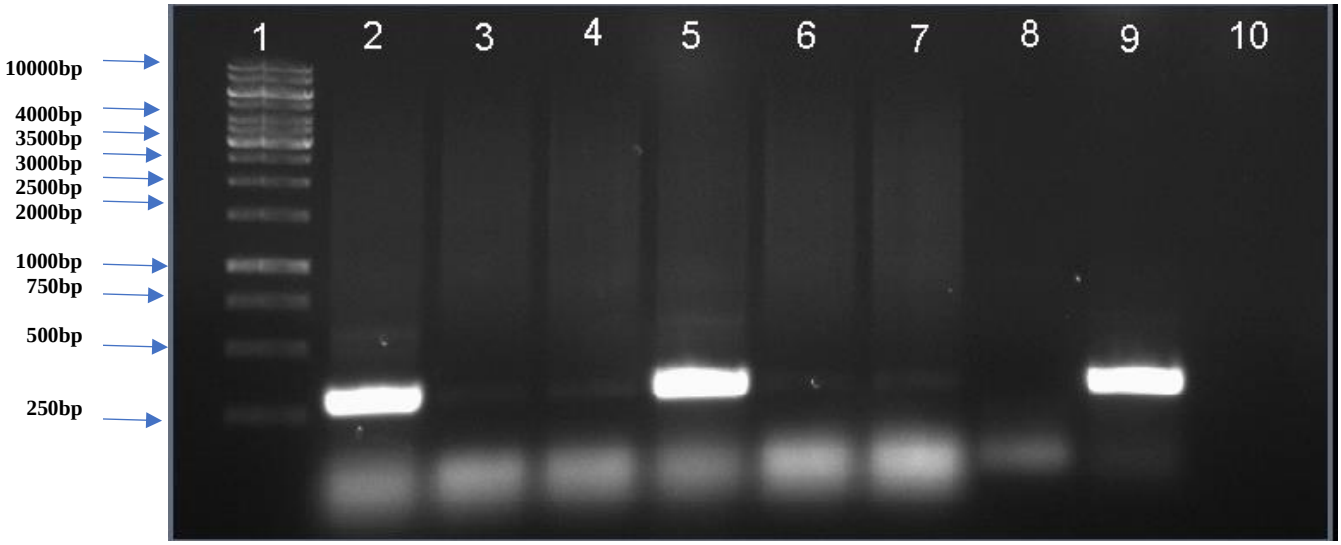


Figure 0-5. PCR screening of the pCDM1 transformants using the cdm1 promoter forward and reverse primers.

Lane	Description
1	1kb ladder Gene o ruler
2 and 5	Successful ligation clones (pcdm1)
3 and 4	Negative control pMup9 transformants
6 and 7	Ligation boil preps with no insert
8	Negative control (H_20)
9	Positive control (pBURNS1::Tn*1723*)

Ligation of Pcdm2 and pMUP9

The ligation ratio attempted was 1:1 of vector to insert. The ligation mix consisted of 8 µl pMUP9 vector, 8 µl Pcdm2 insert, 1 µl DNA ligase and 2 µl ligase buffer (x10).

This ligation resulted in 15 colonies on a tetracycline 15 µg/ml plate (LA). The colonies were screened by PCR amplifying the promoter regions (see Figure 5-6).

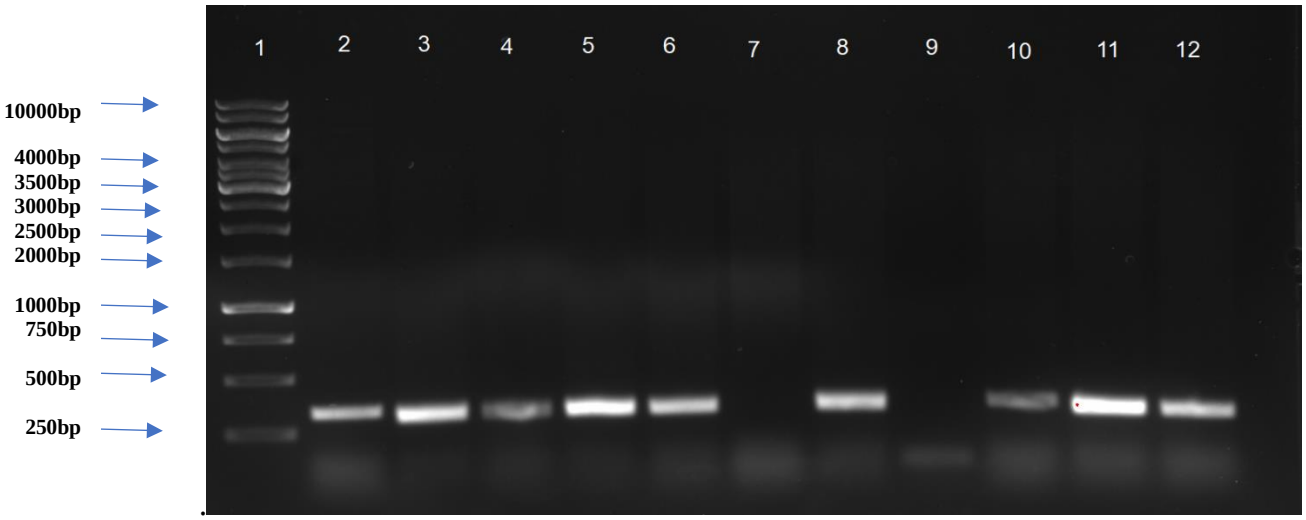


Figure 0-3. PCR screening of the pCDM2 transformants using the cdm2 promoter forward and reverse primers.

On initial screening of 12 random transformants, not all the colonies contained the newly cloned pCDM2 plasmid. Thus, positive recombinants were recorded while negative ones were repeatedly screened.

Lane	Description
1	Gene O ladder 1kb ladder
2-6, 8, 10-12	Successful pCDM2 clones
7	Unsuccessful ligations
9	Negative control (H_2O)

5.3.1.4 Preparation of strains for β-galactosidase assays

Two successful clones designated T1 and T2 for each of pCDM1 and pCDM2 were used as the test variants. The negative control was the empty pMup9 vector as observed after sequencing. The positive control was pMUP9 plasmid with *lacZ* controlled by the *mup*Z promoter. Colonies of these variants were grown into 5 ml overnight cultures in LB and mini prep plasmid isolation was carried out for each strain. The plasmids were then transformed into *E. coli* S17-1 competent cells. This was done because the pMUP9 (pJH10 derivative) is non- conjugative but mobilizable and the S17-1 has the IncP genes for conjugative transfer within its genome (Strand et al, 2014). The transformants were grown on L-Agar supplemented with tetracycline (15 µg/ml) before being mated with *E. coli* DH5α and DH5α (pBURNS1::Tn*1723). P. aeruginosa* 943 and 943cured. The transconjugants, each carrying all four variants were selected using the antibiotic selections as seen in Table 5-4.

Table 0-4 Antibiotics used for selection after conjugation of pCDM1 and pCDM2 into test strains

S17.1 (pCDM1 variants) and S17.1 (pCDM2 variants)	Mated with	Transconjugants selected with
	DH5α	Tet + Nal
	DH5α (pBURNS1::Tn*1723*)	Tet + Nal+ Kan
	P. aeruginosa 943	Tet + Kan /M9-pro Tet
	P. aeruginosa 943 cured	Tet +Kan /M9-pro Tet

To confirm whether the *cdm* promoters have been integrated in the correct orientation and have replaced the *mup*Z promoter and upstream *lacZ* in pMUP9, the forward primer used to amplify the promoters and a second constructed primer *lacZ* reverse were used. Boiled prep DNA was used as the template DNA for the PCR reaction because the initial screening gave

some negative results (Figure 5-7). More colonies from the transformants or transconjugants carrying the new plasmids were screened once this occurred (Figure 5-8).

The conjugation transfer of pCDM1 and pCDM2 from S17.1 to DH5α and DH5α(pBURNS1) was successful and up to 12 colonies from the transconjugants were screened for the presence of Pcdm1 and Pcdm2 using PCR. On the other hand, the conjugations to the *P. aeruginosa* strains produced colonies after selection but on screening with PCR, *cdm* could not be identified. After many unsuccessful attempts, I opted to transform the plasmids directly into *P. aeruginosa* 943 and 943 cured using the transformation method described in section 2.13.2.

The following PCR mix was used: 1 µl of DNA, 1.5 µl of DMSO, 5 µl of dNTPs, 10 µl of Buffer, 2 µl each of Pcdm1 forward primer and *lacZ* reverse primer and lastly, 27.5 µl of sterile distilled water to prepare a 50 µl total mix. The PCR setting is shown in Table 5-5.

Table 0-5 PCR setting for cdm1 or cdm2 and *lacZ* reverse amplification

Temperature (ºC)	Time		Description
98	2secs		Denaturation
98	30secs		“
56	30 secs	x30 cycles	Annealing
72	15secs		Elongation
72	7 mins		“

The PCR products were analyzed by gel-electrophoresis (Figure 5-7 and Figure 5-8)

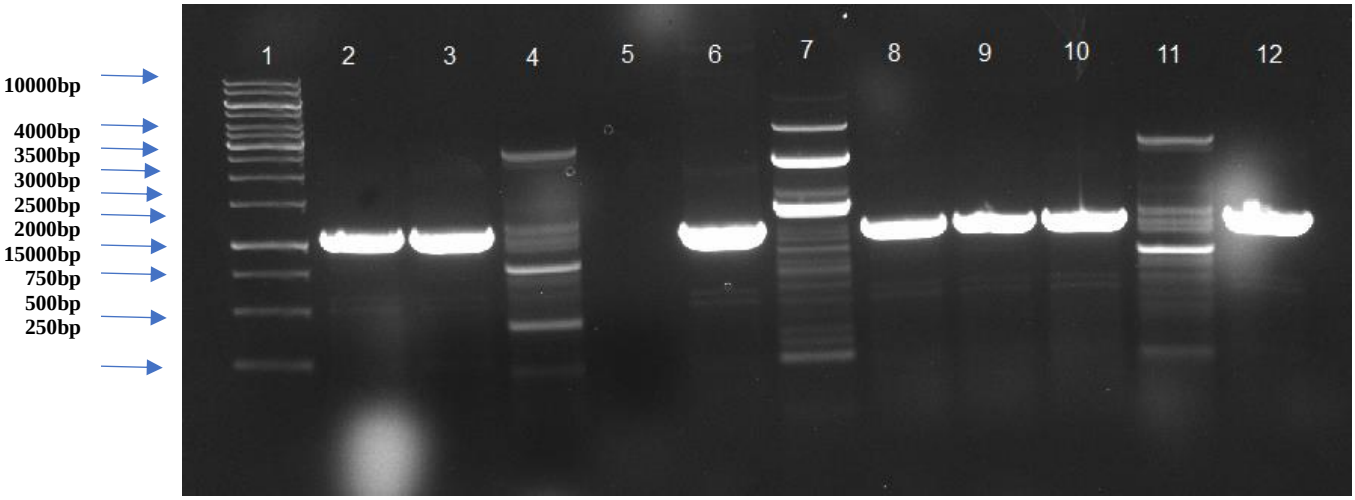


Figure 0-4. Screening for presence of pCDM1 in *P. aeruginosa* 943 transformants using cdm1 forward and *lacZ* reverse primers

Positive bands are single bands identical with the positive control on lane 6 at the 1500bp mark. The variants tested were listed as 2-1 to 2-4. 2-1 and 2-2 are two positive test strains carrying pcdm2, 2-3 is negative control that contain no promoter and 2-4 is pMUP9 (the pCDM1 backbone but under the mupZ promoter). All negative results were repeated by screening more colonies.

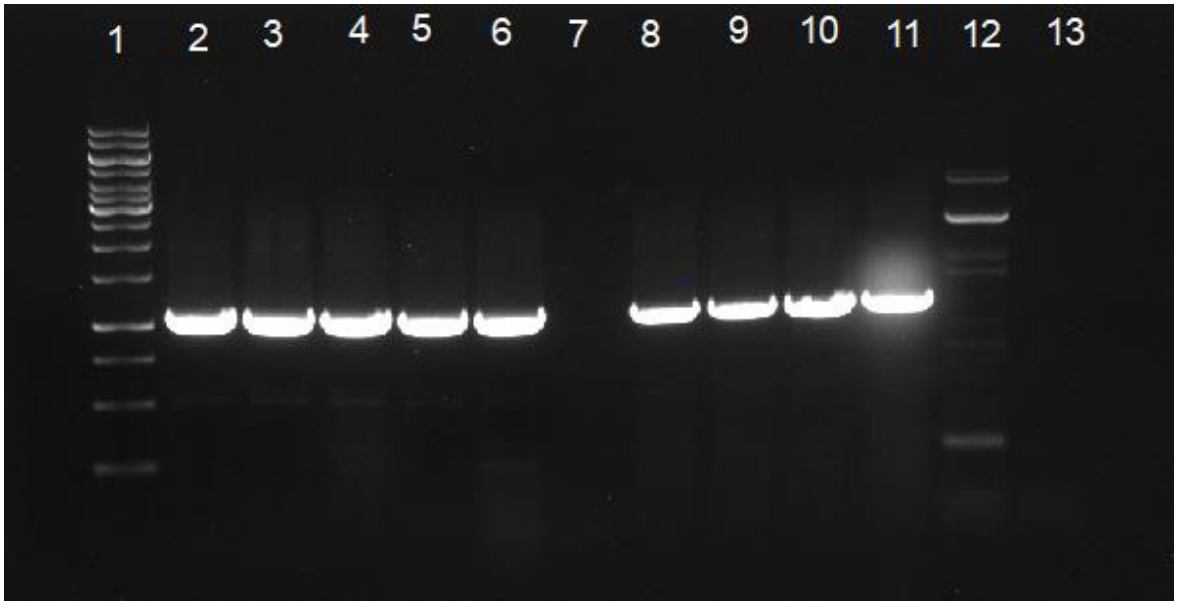


Figure 0-5 Further PCR screening for transformants and transconjugants with the pCDM1 and pCDM2 plasmids using cdm1 forward and *lacZ* reverse primers

Positive bands are single bands identical with the positive control on lane 6 at the 1500bp mark. The variants tested were listed as 1-1 to 1-4. 1-1 and 1-2 are two positive test strains carrying pcdm1, 1-3 is negative control that contain no promoter and 2-4 is pMUP9 (the pCDM1 backbone but under the mupZ promoter).

Lane	Description	results
1	1kb gene o ruler ladder	
2	*P.aeruginosa* 943 pcdm1-1	positive
3	*P.aeruginosa* 943 pcdm1-2	positive
4	*P. aeruginosa* 943 cured pcdm1-1	positive
5	*E. coli* DH5α pcdm1 positive control	positive
6	*E. coli* DH5α pcdm1	positive
7	Negative control (H_20)	negative
8	*P. aeruginosa* 943 pcdm1-3	positive
9	*P. aeruginosa* 943 pcdm2-2	positive
10	*P. aeruginosa* 943 pcdm2-1	positive
11	*P. aeruginosa* 943 cured pcdm2-3	positive
12	*E. coli* DH5α pcdm1-3	negative

The positive results were noted, strains grown in L- broth, with appropriate antibiotics, recorded and stored in the -80º freezer.

5.3.2 Comparison between the β-galactosidase assay of E. coli DH5α pCDM1 and pCDM2 (+/- pBURNS1::Tn1723) and P. aeruginosa 943 pCDM1 and pCDM2 (+/- pBURNS1::Tn1723)

The β-galactosidase assay was carried out for *E. coli* DH5α with and without pBURNS1 to determine if there is a difference with the carriage of the IncP-1 plasmid in the level of *lacZ* expression by the promoters. The main aim, however, was to determine whether there is a difference in the activity detectable from the transcriptional and translation signals responsible for *cdm* expression in *P. aeruginosa* and *E. coli*.

The newly formed pCDM1 and pCDM2 plasmids were transformed and mated into the pBURNS1 carrying and pBURNS1 free strains to determine the efficacy of the pCDM1 (transcriptional) and pCDM2 (translational) fusion expression. In *E. coli,* with emphasis on the test strains T1 and T2, (clones of the successful promoter *lacZ* fusions) there seemed to be a general decrease in promoter activity in the pBURNS1 carriers as compared with the pBURNS1-free strains. This occurred during both transcriptional and translational expression. Students t-test conducted showed a statistically significant difference between the means of pBURNS1 plasmid carriers and pBURNS1 free strains in all the variants tested except the negative control (Figure 5-9). On the other hand, there was a stronger promoter activity for transcriptional expression than in translation. However, there is a decrease in promoter activity from the translational fusion (pCDM2) which may suggest that there are regulatory mechanisms in place at the start of translation. Regulation of gene expression can occur either in the transcriptional phase or during translation (post-transcriptional).

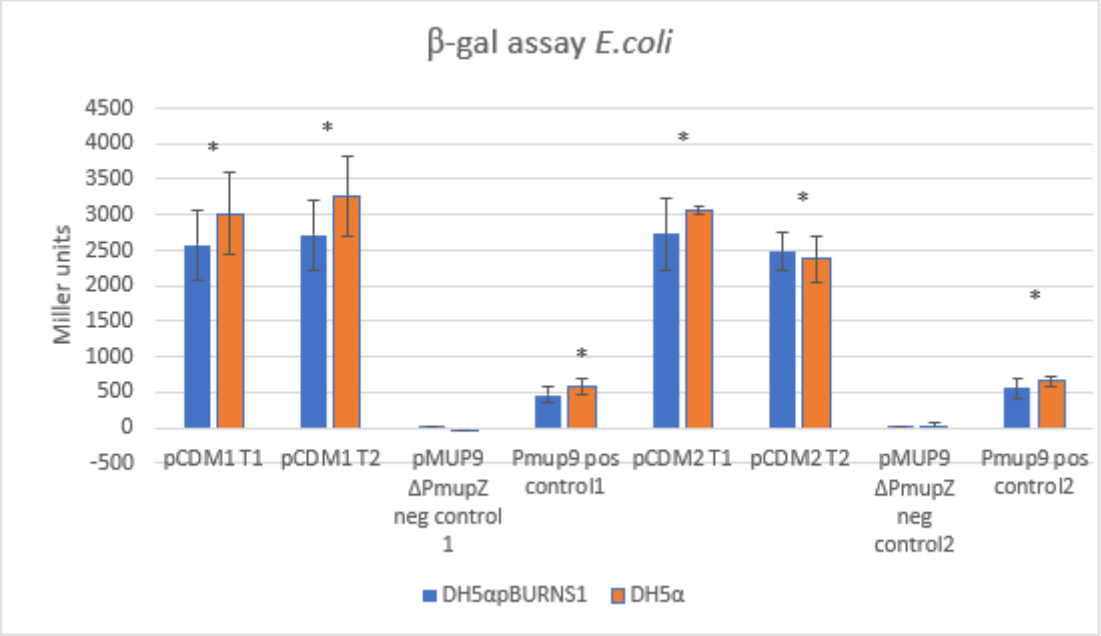


Figure 0-6 β-gal assay of Pcdm1 &2 promoter *lacZ* fusion in *E. coli* DH5α and DH5α pBURNS1::Tn*1723*

The blue bars represent E. coli DH5α pBURNS1::Tn1723 while the orange represent E. coli DH5α. T1 and T2 are identical clones carrying pCDM1 or pCDM2 as indicated, pCDM1/2ΔT1 and Pmup9ΔmupZ are both negative controls with no promoter therefore no expression of lacZ while pMUP9(the pCDM1 backbone but with the mupZ promoter) is the positive control. The test pcdm1 and 2 (T1 and T2) show strong promoter activity compared to the positive controls. The T1 and T2 translational fusions on the left (pCDM2) gave a similar but lower level of promoter activity as the transcriptional fusions on the right (pCDM1), the presence of pBURNS1::Tn1723 (in blue) shows a decrease in expression levels of both pCDM1 and pCDM2. Data in Asterix represent statistical significance (p<0.05) between the means of the pBURNS1- carrying and pBURNS1::Tn1723-free counterparts.

In *P. aeruginosa*, a similar result as seen in *E. coli* DH5α was observed with expression of *lacZ.* Unexpectedly the promoter activity for transcriptional and translational expression was 5 x higher than in *E. coli*. A second difference was that the promoter activity in WT *P. aeruginosa* 943 was higher than its cured counterpart with translational expression (Figure 5-10). During the assay, the colour change (from colourless to yellow) occurred within 3 mins in *P. aeruginosa* compared to >10mins in *E. coli* which indicated at first instance a

possible higher *lacZ* expression in *P. aeruginosa*. The positive controls used had a *tac* promoter which is weaker hence lower activity, although additional controls such as the test of *E. coli* pCDM plasmids during the *P. aeruginosa* assays would have been beneficial in clarifying the results. The negative controls show that the high levels of *lacZ* activity is not likely due to technical error.

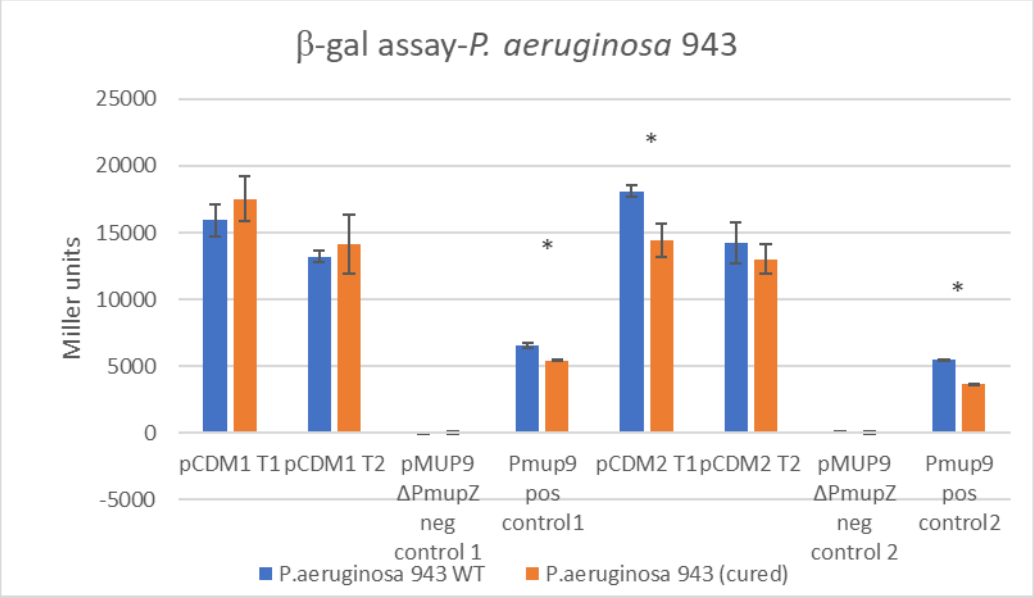


Figure 0-7 β-gal assay of pCDM1 &2 promoter-*lac*Z fusions in *P. aeruginosa 943* and *P. aeruginosa 943*(cured)

The blue bars represent P. aeruginosa 943WT (pBURNS1) strains while the orange represent the P. aeruginosa 943 (cured)strains. T1 and T2 are two positive test strains carrying pcdm1 or pcdm2, pcdm1/2ΔT1 are negative controls that contain no promoter and pcdm1/2 pMUP9 (the pCDM1 backbone but under the mupZ promoter). The test pcdm1 and 2 (T1 and T2) show very strong promoter activity compared to the positive controls. The T1 and T2 translational fusions on the left (pCDM2) gave a similar but lower level of promoter activity than the transcriptional fusions on the right (pCDM1), the presence of pBURNS1 plasmid (in blue) shows a decrease expression levels in pCDM1 but an increase in pCDM2. Data in Asterix represent statistical significance (p<0.05) between the means of the pBURNS1- carrying and pBURNS1-free counterparts.

Integron promoters have been grouped into strong, weak and other variants of this according to the strength of integron and the sequence (Barne et al, 2007). The integron Pc promoter upstream *cdm* was found to be a weak promoter PcW since it has the consensus sequence of a weak promoter however, with very strong activity due to a single base mutation of G to C allowing a strong affinity for the σ^{70} subunit of RNA polymerase, it was therefore designated to be PcW_{TGN-10} (Barne et al, 1997; Guerin et al, 2011). In this promoter, the second and third bases before the -10 hexamer were a 'TC' rather than a 'TG' (Barne et al, 1997; Jove et al, 2010) as seen in Figure 5-11. The experiments were all done in triplicates and the standard deviation plotted as error bars. Statistical significance shows the difference between the mean of the pBURNS1-carrying strains and pBURNS1-free strains.

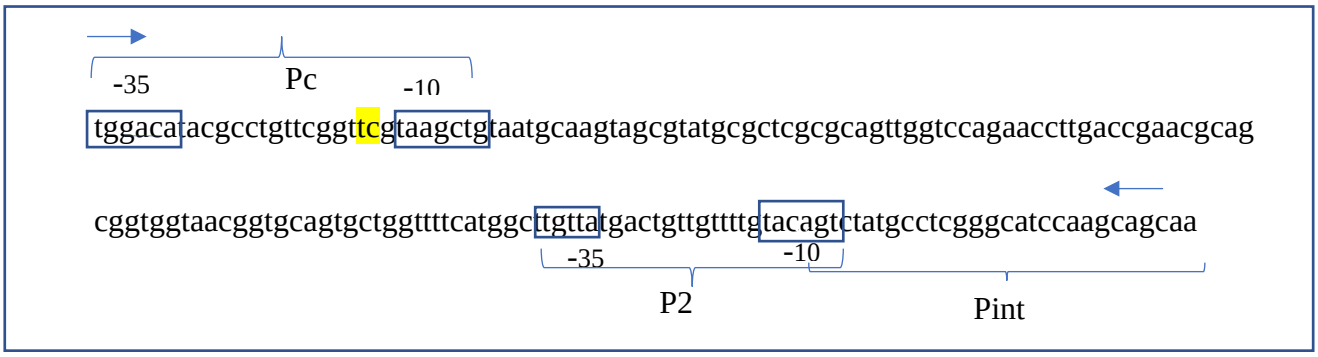


Figure 0-8 Promoter region showing the Pc, P_2 and P_{int} upstream of the *cdm* in pBURNS1

The bases highlighted in yellow are necessary to cause an increase in the rate of transcription in E. coli. The -35 and -10 regions are shown in boxes and the sequences in brackets denote Pc, P_2 and P_{int}

In general, the β-gal assay did not explain the reason for the poor Cdm phenotype in *P. aeruginosa* 943 as compared to *E. coli* and it is assumed from the results that it is not due to poor expression of the *cdm* promoter in *P. aeruginosa* 943.

5.4 Discussion

The overexpression of *lacZ* in *P. aeruginosa* was unexpected based on our previous findings in Chapter 4, however high levels of *lacZ* expression in *P. aeruginosa* could have arisen based on several reasons, one of which could be quorum sensing. Quorum sensing was involved in oxidative stress response in *P. aeruginosa* when transcription of *sod*A (encoding manganese-co factored superoxide dismutase [Mn-SOD]) greatly increased when there was an absence of iron in the cells (Bollinger et al, 2001).

The results show that *P. aeruginosa* is actively expressed based on the pCDM-*lacZ* fusion β-gal assay. However, this does not explain the difference in Cdm phenotype in the WT strain when in contact with chlorite. Most *cdm* carriers are either, perchlorate or chlorate reducers, this is the first reported *cdm* that is present on a highly transmissible plasmid within a non-PRB strain.

Some genes present in plasmids can be dormant or inactive which can be determined by the inability of the gene present to produce the end-product protein necessary for a phenotype. Transcription of these genes into mRNA can be regarded as a determining factor to whether a gene is active or not (Price et al, 2016). Genes that are not being transcribed are there for said to be inactive or non-functional (Price et al, 2016).

Another possible reason for the level of expression of *cdm* in *Pseudomonas aeruginosa* could be the fact that the *cdm* gene is not needed. It has been said that some genes that are redundant or not being used tend to be overexpressed (Price et al, 2016). The results showing that chlorine dioxide cannot be a possible source of selective pressure under the given concentrations show that in the hospital environment, there is little or no use for the *cdm* gene to be expressed. There have been reported that highly expressed genes do not

necessarily have a phenotype but may be useful at other growth conditions. The more highly expressed a gene is, the more the fitness cost to the cell (Price et al, 2016).

When pBURNS1::Tn*1723* was transformed into *E. coli* Nissle 1917, it was observed that the plasmid-carrying strain did not grow at all at 24ºC (room temperature) while the plasmid free strain grew normally at that temperature or 37ºC. This shows that physiological conditions can play a role in both expression and survival of plasmid carrying strains. It is possible that in *P. aeruginosa* 943, rather than cell loss at that temperature or condition provided, the plasmid functions could be shut off temporarily therefore the observations of no phenotype. This does not mean the gene and promoter regions are not active and functional.

5.5 Summary

The issue of why *P. aeruginosa* had a smaller Cdm phenotype was further investigated by determining the *cdm* promoter expression levels in both *E. coli* and *P. aeruginosa 943* strains with and without the pBURNS1 plasmid. Therefore, a β-galactosidase assay was conducted using two promoter regions Pcdm1 and Pcdm2 which would provide transcriptional and translational expressions respectively. To do this, pcdm1-*lacZ* and pcdm2-*lacZ* fusions were made and cloned into a reporter plasmid. Results showed that promoter activity in *E. coli* was high and activity in *P. aeruginosa* was even 5x higher based on *lacZ* expression. This was not expected, however there are instances where a gene is functional but due to environmental and physiological interference, may not produce the expected phenotype under those conditions. The oxygen concentration in cells, the presence of transcriptional regulators and other chromosomally encoded regulators could be a reason for this. Research has also shown that usually highly expressed but un-useful genes do not show a phenotype due to the fitness cost to the host organism (Price et al, 2016).

Chapter 6

Overall Discussion

6.1 Overall Discussion and future work

Hospital acquired infections caused by plasmid mediated resistance are becoming more prevalent in recent times (Baghal Asghari et al, 2013). Among all forms of horizontal gene transfer of resistance determinants, transfer by plasmid conjugation within a biofilm seems to be the most observed process by which bacteria exchange beneficial traits in water/plumbing systems (Anaissie et al, 2002). *Pseudomonas aeruginosa* biofilms are inherently the cause of major nosocomial outbreaks in Europe (Baghal-Asghari et al, 2013). IncP-1 plasmids have the mechanism for self- replication and conjugation with the presence of genes that encode production of proteins to carry out these activities (Haines et al, 2006). They also have broad host range therefore the presence of a resistance gene in an isolated strain is likely to be present in other species within proximity, for example, in a biofilm (Ghigo et al, 2001). It is also well known that waste-water treatment plants may be a reservoir for the proliferation and dissemination of antibiotic resistance genes obtained from the environment (Wellington et al, 2013).

IncP-1 plasmids belong to the highly stable and conjugative plasmids with IncI, W and F. They are commonly found in the soil and water environments and carry both heavy metal and antibiotic resistance genes (Droge et al, 2000). Plasmids also carry other genetic determinants such as integrons and transposons that could each separately carry resistance genes. More often these mobile and non-mobile genetic elements work simultaneously in the dissemination of resistance (Burrus et al, 2002).

The pBURNS1 plasmid containing a putative *cdm* gene was found in room 8 of the Burns unit of the Queen Elizabeth hospital, Birmingham (Quick et al, 2014). Co-incidentally the RK2 plasmid isolated from a Burns unit in 1969 during a *Pseudomonas aeruginosa* outbreak

in Birmingham was also an IncP-1 plasmid (Pansegrau et al, 1994). The pBURNS1 IncP-1β plasmid was the principal focus of study of the rooms for this work.

The *cdm* gene also referred to as *cld* in other papers, is the only functional resistance gene present within an integron and transposon in the pBURNS1 plasmid. The integron has a class 1 integrase gene and therefore is regarded as a class1 integron. They were first isolated from environmental strains and are also found in chromosomal DNA. Downstream of the integron are a set of *tni* genes, *tniQ*, *tniA*, *tniB* which are characteristic of Tn*402*-like transposons which have been around since before the beginning of the antibiotic era (Stokes et al, 2006; Post et al, 2007).

Bacteria such as *Pseudomonas aeruginosa* have been successful in maintaining their growth in clinical environments due to their biofilm-forming abilities and their ability to acquire useful resistance determinants via horizontal gene transfer (Fonseca and Sousa, 2007). From this work, I analyzed the situation in the hospital environment where pBURNS1 was first found and tried to develop laboratory experiments based on those conditions and the possible reasons for the presence of *cdm* in *P. aeruginosa* strain in the hospital setting were investigated. Antibiotic and biocide resistance genes on plasmids aid in the survival of their host strains in the environments that threaten their proliferation and existence (Shintani et al, 2010).

Chlorite itself is not naturally found in the environment and where present, is linked to anthropogenic sources (Goblirsch et al, 2011). Therefore, this research investigated the *cdm* gene and the possible ways it relates within the hospital setting. Chlorine dioxide is thought to break down to chlorite and ultimately chloride during disinfection and therefore the levels are occasionally monitored due to its toxicity in high concentrations (Hicks et al, 2011). In

northern Italy, chlorite was found in drinking water at levels considered high (about 500 µg/L) enough to contribute to congenital abnormalities in pregnant women (Righi et al, 2002). The presence of high concentrations of chlorite in water systems could enrich the proliferation of Cdm+ bacteria in the environment. Blast matches also showed 100% identity with part of a class1 integron in a *Klebsiella pneumonia* isolate from polluted river water in Tunis (Chouchani et al, 2013). From previous research, the presence of *cdm* has almost always been associated with water-dwelling host organisms or in the toilet/plumbing units of hospitals (Santos et al, 2010; Toleman et al, 2005; Rossolini et al, 2008).

The 24 and 48-hr *P. aeruginosa* and *E. coli* biofilms, as expected, survived 0.5 ppm ClO_2 for 3 hrs while their planktonic counterparts did not survive 0.25 ppm for 1 hr. In a study by Behnke et al, (2011), *P. aeruginosa* single species biofilms exhibited less than 10-fold reduction at from 0.1-2.5 ppm chlorine dioxide treatment with contact time of 30 mins. It was observed that *P. aeruginosa* biofilms and a combination of a commercial sanitizer and chlorine dioxide was able to further increase log reductions of the biofilm compared with using chlorine dioxide alone (Kreske et al, 2006). The reason for the low level of disinfection could be due to the biofilm structure and increase in chlorine demand which alters the final concentration of the biocide. This experiment focused on comparing the plasmid positive with plasmid-negative strains to decipher if the carriage of the plasmid increases survival of the host in chlorine dioxide. The results for both biofilm and planktonic cultures did not significantly differ in terms of presence or absence of the pBURNS1 plasmid (Figure 4-6 to Figure 4-11).

Comparison with other IncP-1β plasmids based on close identity and coverage showed that the plasmids are widely disseminated across various genera in the β-protobacteriaceae. pBURNS1 shares a common backbone with R751, the IncP-1β backbone that has

mechanisms for conjugation and transfer, replication, active partitioning and stability (Thorsted et al, 1998). The major differences between pBURNS1 and R751 is the presence of the class1 integron located between the *tra* and *trb* genes which carries the *cdm* gene (Aggarwal, 2015). When pBURNS1 was compared to other closely related plasmids the major difference was presence and location of accessory genes. *P. aeruginosa* has been found to be a suitable and common host for IncP-1 plasmids. This occurrence has been linked with the G+C content of IncP-1 plasmids closely correlating with that of the *P. aeruginosa* (Chiu and Thomas, 2004). The phylogenetic tree in Figure 3-17 shows that the Cdm protein is found within the Pseudomonads clade and share the closest common ancestor with *Roseateles depolymerans* of class betaproteobacteria which was isolated from river water (Suyama et al, 1999). This evidence shows that the Cdm protein has associated with Pseudomonad strains for a long time.

The pBURNS1 class1 integron matches in BLAST showed 100% identity with *P. rhodesiae* and was the only strain in which the *cdm* integron was found in the chromosome. It has been observed that integrons probably originated from the chromosomes of bacteria and transfer to plasmids only occurred due to the introduction of transposable elements such as insertion sequences and transposons (Guerin et al, 2011). The presence of class1 integrons in strains isolated from hospital environments is of utmost importance to public health. They can confer multiple resistances to antibiotics and biocides to a wide range of organisms within a short time through lateral gene transfer and their association with mobile genetic elements (Rosser and Young, 1999).

It was discovered that pBURNS1/ R751 may have two regions homologous to *parDE* of RK2 plasmid designated as *kluA* and *kluB*. This equivalent region to the *parABCDE* locus in IncP-1α plasmids was regarded absent in R751 (Thorsted et al, 1998). The *kle* and *klc*

regions represented the only systems conferring stability to the plasmids until this discovery (Thorsted et al, 1998). Presence of plasmid stability and maintenance in host strains has been found to be useful in adverse environmental conditions such as one with depleted nutrient supply and little or no selective pressure (Panseqrau et al, 1994). This factor could explain the reoccurrence of pBURNS1 from water sources with little nutrient availability in the Burns unit at many days' interval (Quick et al, 2014). Fifty years ago, the RK2 plasmid was isolated from a Burns unit in Birmingham (Panseqrau et al, 1994). Presence of a closely related plasmid in the same type of unit only recently means that resistance plasmid proliferation is still not under control which signifies the importance of this work.

Changes to the plumbing design of Burn units and other sections such as the intensive care units notorious for plasmid mediated outbreaks may need to be made. These include the need to put risk of biofilm formation into consideration by installing materials that are easily disassembled for proper routine disinfection (Hoisen et al, 2005), piping materials must have smooth surfaces and if possible, biofilm resistant coatings used (Mahapatra et al, 2015). The flow rate of treated water going into the taps needs to be put into consideration as well. An even distribution of flow may go a long way in preventing opportunistic pathogens forming biofilms. It was observed that the rooms 8 and 9 of the Burns Unit were at the distal end farthest away from the main pipeline and therefore where the flow rate is slowest (Figure 6-1). In this case, multiple inlets from the main source may be useful rather than from just one or two. The lesser the flow rate the more opportunities for biofilms to form and the longer a biofilm accumulates, the more likely it becomes an avenue for plasmid dissemination (Donlan, 2001).

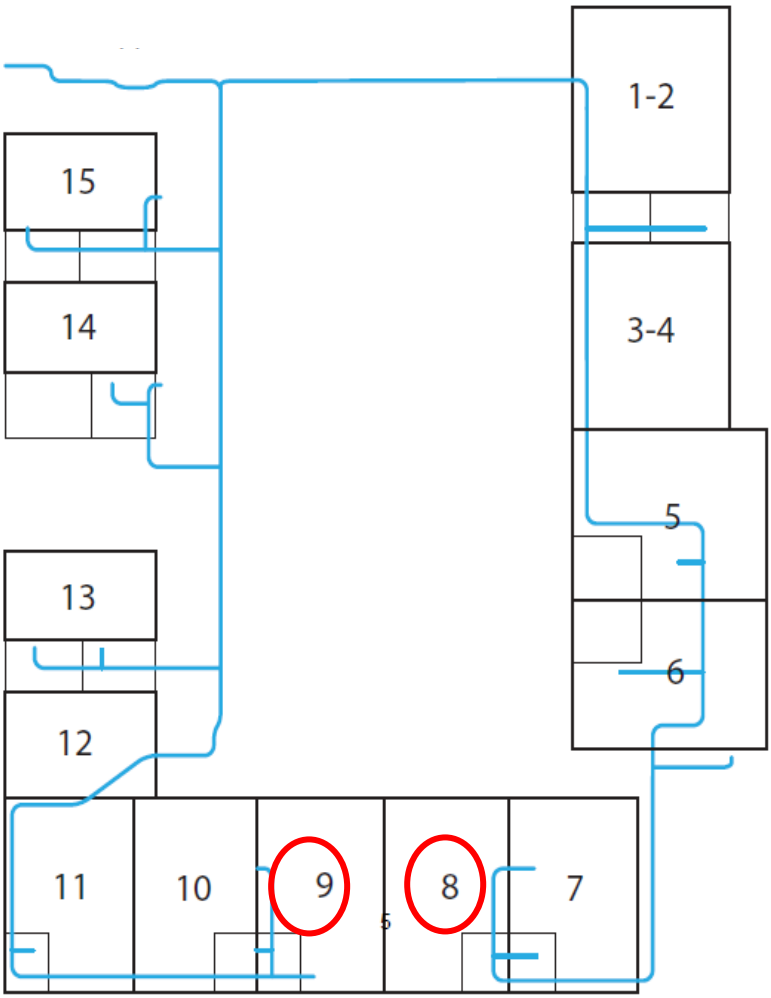


Figure 6-1. Diagram showing the water distribution frame-work and rooms of the Burns unit, QE Hospital

Highlighted in red circles are the rooms where pBURNS1 (room 8) and pBURNS2 (room 9) were isolated, the initial stating water pressure from the source may not be equivalent to the endpoints. Figure adapted from (Quick et al, 2014).

Plasmids are useful in their host strains only when they provide an advantage to their host especially when present in adverse environmental conditions. This is because carrying a plasmid usually has a fitness cost to the host and therefore more resources will be needed to maintain the plasmid (Carrol and Wong, 2018). However, it has been shown that plasmids that have both mechanisms for partitioning and toxin antitoxin systems have reduced fitness

cost to the host cell (Sayeed et al, 2005) and the fitness cost of a plasmid depends greatly on the genotypic traits of the host organism (Humphrey et al, 2012).

Toxin-antitoxin systems help to ensure stability of plasmids in host strains. However, it was discovered in this study that some incP-1β plasmids don't have any *parDE* homologues such as the pRWC2a plasmid. This plasmid was isolated from a waste-water treatment plant and was found to be closely related to other IncP-1β plasmids isolated from the same source (Rahube et al, 2014). However, on inspection of the *klcA*-i10 region of these plasmids, they had variable regions of this segment. pB10 and pB4 had the *din* and *xf*2080 orfs, pB8 had *kluA* and *kluB* like R751 and pBURNS1 while pB3 had no insertions or orfs in this segment like pRWC2a. The phylogenetic tree in Figure 6-2 coincidentally reflects this relationship based on the *klc*A-i10 of *oriV* region. In pRWC2a, the region is empty and *yacA* and *yacB* were said to be responsible for provision of a toxin-antitoxin system (Rahube et al, 2014).

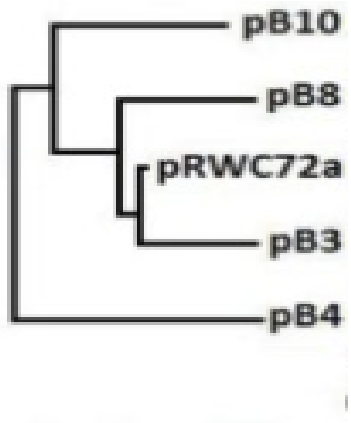


Figure 6-2. Phylogenetic relationship between IncP-1 plasmids isolated from wastewater treatment plants. Figure adapted from (Rahube et al, 2014)

A second phylogenetic tree showing the plasmids mentioned above with different *klcA*-i10 region including pBURNS1 and with RK2 representing IncP-1α was assembled to compare the whole genome relationship between their common ancestor. The tree was assembled using Genious prime software (Figure 6-3).

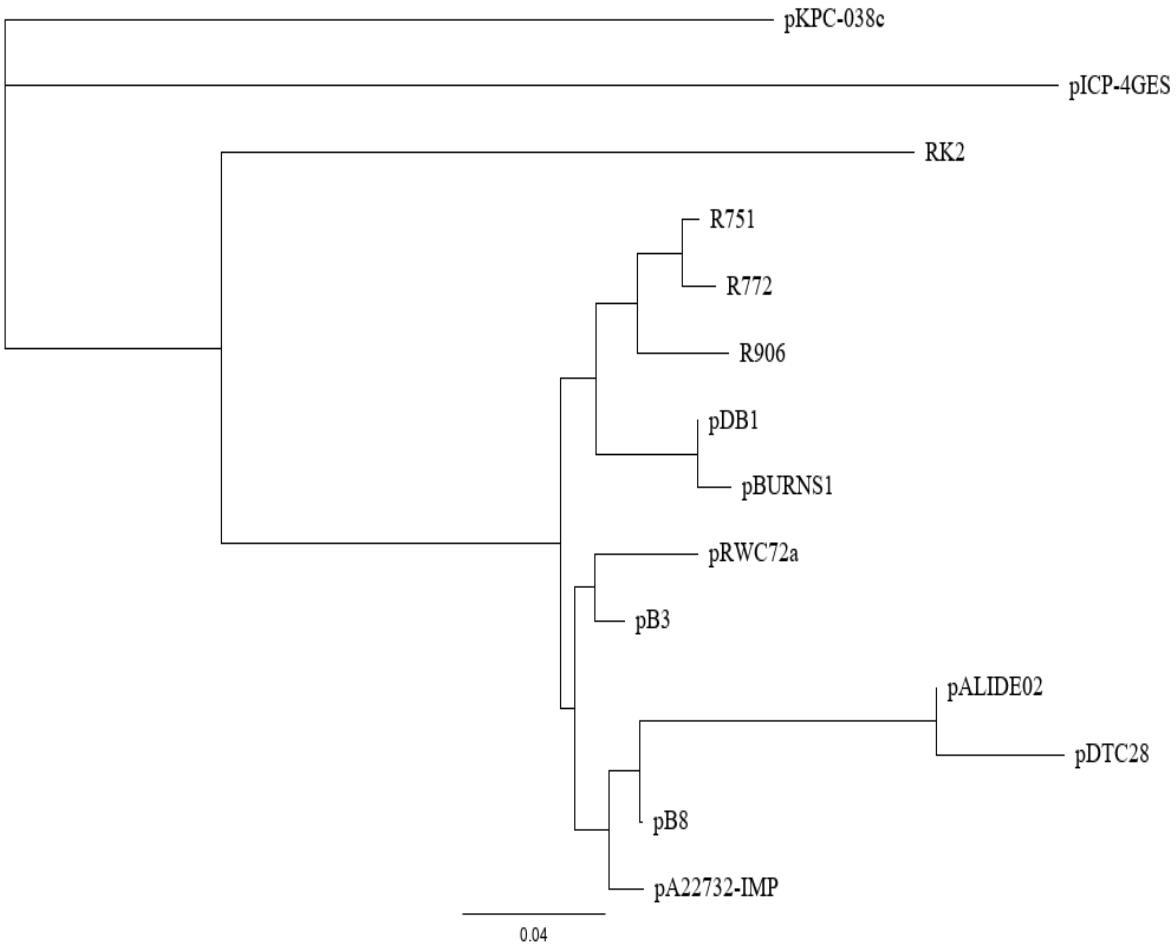


Figure 6-3. Phylogenetic tree comparing whole genomes of IncP-1 plasmids that have a variable *klcA*- i10 region with RK2 representing IncP-1α

The tree was generated using Geneious prime using whole genome alignments of the 14 plasmids. The plasmid sequences were obtained from BLAST and consists of plasmids with a variable klcA-i10 oriV region. RK2 was added to compare with an IncP-1α plasmid. According to the tree, pBURNS1 showed closest ancestral relationship with pDB1.

The transferability of genetic determinants into multiple hosts makes IncP-1 plasmids of great importance. The pBURNS1 plasmid is highly conjugative between *E. coli* strains in

both biofilm and planktonic states so it was of interest. To determine whether the Cdm phenotype is preserved after conjugation to a new host. The experimental hypothesis suggested that a growing *E. coli* biofilm selected by high concentrations of chlorite will eliminate plasmid-free biofilms and enable the survival of donors and to a lesser degree transconjugants. The recipient biofilm was designed to be older than the donors to ensure the target (recipient) strains were more numerous than the ones expected to survive. The initial decrease in biofilm cell numbers for all the strains tested showed that chlorite activity on the cells is fast, occurring before the chlorite-degrading mechanism is fully expressed. After further treatment however, the donors regained enough biofilm growth on the bead.

In this study, we wanted to test if the presence of the *cdm* gene or pBURNS1 would influence the increase in biofilm formation in their host strain. Results using the beads showed that within a 24 hr and 48 hr period of growing biofilms of the same strain +/-pBURNS1::Tn*1723* without selection for chlorite, there was no significant difference resulting from plasmid carriage as indicated by the resuspended biofilm counts (Figure 4-2) and (Figure 4-3).

I also determined the survival of +/- pBURNS1::Tn*1723* and pBURNS1 in *E. coli* and *P. aeruginosa* biofilms respectively with the presence and absence of chlorine dioxide to test if there would be increased biofilm formation in the plasmid positive bacteria. However, the bead system gave variable results with the normal procedure which prompted amendments to give reproducible results. A 24-hr and 48-hr biofilm of *E. coli* and *P. aeruginosa* (+/- pBURNS1) was almost 100% resistant to 0.5 ppm (maximum concentration of ClO_2 in water tanks in the QE hospital) of chlorine dioxide left for a maximum of 3 hrs. There was no difference between strains with and without the pBURNS1 plasmid (Figure 4-8 to Figure 4-10). The variation in estimating the surviving *P. aeruginosa* biofilm after being exposed to chlorine dioxide was largely due to random formation of slime and pyocyanin amongst

some replicates. The concentration that influenced a reduction in cell count in both (+/-pBURNS1) strains was 0.25ppm and this was solely used to test the difference between the strains. Results in Figure 4-11 showed the plasmid free *P. aeruginosa* biofilm did not have an advantage in terms of surviving biofilm over the plasmid free counterpart. Planktonic strains (+/-pBURNS1) exposed to chlorine dioxide did not also show increased survival in pBURNS1-carrying strains. This may point to the fact that the *cdm* gene may be specific to chlorite degradation and without giving enough time for chlorine dioxide to degrade to chlorite, we may not see a difference. The concentration of chlorite formed also matters. A very low concentration of chlorite may not show an effect in plasmid positive strains except over a long period of time. Unfortunately, a long-term experiment planned could not be carried out after extensive preparations were made so it is described here as potential future work. The reason it was not carried out was because the strain to be used in the experiment-*E. coli* Nissle 1917 (one without nutrient requirements i.e prototrophic) did not grow at room temperature (25ºC) when the pBURNS1::Tn*1723* was present which was quite intriguing. The long-term experiment involved growing plasmid-free and plasmid carrying strains separately under the same conditions. Two different coloured beads (black and white) would be used to differentiate between seeding and older biofilms since one bead would be taken out after a few days and the donors, recipients and transconjugant counts enumerated. The preformed biofilms on the beads would subsequently be monitored for survival in only water as growth medium and this was the reason why we required a strain without growth requirements such as *E. coli* Nissle. The two strains would be subjected to a much lower concentration of chlorite such as the concentration found in hospital water system. The aim would be to determine if lower concentrations of chlorite could make pBURNS1::Tn*1723* -carrying strains survive better over time compared to plasmid free strains.

In chlorine dioxide however, there is a probability that *E. coli* (pBURNS1::Tn*1723*) may have a long-term advantage in ClO_2 treated water. The growth curves showed that *E. coli* carrying pBURNS1::Tn*1723* had a slightly increased survival rate at 1.5 ppm over a 14 hr period in chlorine dioxide (Figure 4-18) but when the MBEC was conducted there was no difference in biofilm survival between (+/-pBURNS1::Tn*1723*). Therefore, it seems we are only able to see a difference (+/-pBURNS1::Tn*1723*) during the growth phase when the planktonic strains are transitioning to biofilms in the 96 well plates.

From the results, it can be deduced generally that planktonic strains are not expected to find their way past the main storage tanks treated with 0.5 ppm ClO_2. If this happens, three reasons maybe responsible are fluctuation of ClO_2 levels in the tanks, presence of biofilms already in the tank; and lastly, contamination of the water ways from within the hospital from the patients or health care workers. Once planktonic *P. aeruginosa* escape the tanks it may take 48 hrs only to form biofilms resistant enough to withstand the 0.25 ppm ClO_2 within the plumbing. Once planktonic bacteria can be controlled, biofilm formation will be greatly reduced. Although there was no significant difference between plasmid positive and negative planktonic cells, results showed that there was a narrow range between survival and almost no effect of chlorine dioxide on them (0.125 and 0.25ppm) in *P. aeruginosa* and *E. coli* respectively. Since 0.25ppm of ClO_2 is the concentration found in the hospital plumbing, care needs to be taken to avoid fluctuation for a maximum of 48hrs.

From the results of the growth curves *E. coli* (+/-pBURNS1::Tn*1723*) in LB and M9 while selecting with chlorite, a greater negative effect can be seen on the plasmid free strains compared to the plasmid carrying strain (Figure 4-17 to Figure 4-18). This level of survival could not be seen in *P. aeruginosa* 943 (+/-pBURNS1), therefore it is only evident that there is a difference between expression of the Cdm enzyme in *E. coli* but not *P. aeruginosa* under

the same conditions but it is not known if this is due to the fact that the *cdm* gene in *P. aeruginosa* is not being expressed. Therefore, at much lower concentrations of chlorite, there may be a Cdm^+ phenotype in *P. aeruginosa* 943. Multi drug resistant *P. aeruginosa* have been demonstrated to have an increased level of survival when treated with suboptimal concentrations of chlorine (Shrivastava et al, 2004). Another observation was the fact that lower concentrations of chlorite seemed to give rise to an increase in optical density of the *P. aeruginosa* cells which grew better than the negative controls with time. *P. aeruginosa* biofilms have been found to produce more biofilm biomass when subjected to antibiotic concentrations below the MIC compared to strains that were not subjected to antibiotic exposure (control biofilms) (Bagge et al, 2004). During the degradation of chlorite by chlorite dismutase, it was observed that high levels of the intermediary hypochlorite produced can cause an irreversible deactivation of the Cdm enzyme (Hofbauer et al, 2014) this may be what is happening in *P. aeruginosa* at higher chlorite concentrations. For some reason, it does not apply to *E. coli* which could withstand up to >16mM chlorite. The level of chlorite commonly found in hospital water supply is far below what was used in the experiment and therefore the results of *P. aeruginosa* cannot be completely dismissed if we compare with real life settings. Therefore, it could be that the increased growth may be in preparation for biofilm formation at that concentration.

The β-galactosidase assay showed that the *cdm* transcriptional and translational signals are not the reason for the difference in chlorite resistance between *P. aeruginosa* and *E. coli* (+/- pBURNS1). The results showed higher β-gal activity when present in *P. aeruginosa* than when in *E. coli*. In *E. coli*, both transcriptional and translational expression were high with very similar levels between pCDM1 and pCDM2. In *P. aeruginosa* however, despite generally very high levels of expression, the translational expression was notably higher

than the translational levels. The positive control showed a much lower level of expression since the *mup*Z promoter may be weaker *tac* promoter normally used with IPTG (Connolly et al, 2019). Therefore, it was an expected result. However, the main point to be taken from this is to focus on the other possible reasons *P. aeruginosa* phenotype is not as evident as seen in *E. coli*. The promoter type was found to be a mutant to the PcW_{-10TGN} which is different from the normal weak promoters in terms of strength. Therefore, the high level of expression is not entirely unexpected in *P. aeruginosa*.

In conclusion, the pBURNS1 IncP-1β plasmid had a sole chlorite dismutase gene commonly found in both perchlorate and non-perchlorate reducing bacteria in hospital water supply. The *cdm* gene is active and functional with the ability to degrade chlorite to less harmful chloride (Mlynek et al, 2011). The epidemiological and phylogenetic reasons for its occurrence were investigated in two hosts *E. coli* and *P. aeruginosa.* Evidence shows that the pBURNS1 plasmid exhibits conjugative and high stability functions in its host. The products of plasmid transfer can be selected with chlorite and plasmid free cells will be outcompeted in the presence of certain levels of chlorite over time. Chlorine dioxide may act as a long-term advantage to the planktonic cells carrying the plasmid in the water supply low concentrations but do not have a positive effect on 48 hr *P. aeruginosa* biofilms with pBURNS1. The Cdm+ phenotype is more obvious in *E. coli* (pBURNS1::Tn*1723*) than in WT *P. aeruginosa* 943 and we know the reason is not due to the *cdm* promoter activity in both species. To relate the possible biological role to chlorine dioxide, future studies should employ more long-term treatment of both planktonic and biofilms containing pBURNS1 with low amounts of the biocide (competition assays). The bead system of biofilm formation will be an efficient way of measuring this because a different colored bead can be used for each evolutionary passage to estimate the bacterial population (Popat et al, 2012). Also,

while bead biofilm cell counting techniques gave reproducible data for most of this work, it was difficult to measure a uniform biocide survival for slime forming *P. aeruginosa*. A technique that would have been useful at this stage is the LIVE/DEAD® *Bac*Light™ Bacterial Viability for microscopy (Robertson et al, 2019) a staining technique that could be used to identify and differentiate between lysed and viable bacteria after biocide (chlorine dioxide and chlorite) treatment using fluorescence.

Further work involves fluorescent gene tagging of the donors, recipients and transconjugants to visualize their distribution as a mixed biofilm after exposure to both high and low concentrations of chlorite. The pBURNS1 plasmid would be tagged with gfp, which will give a green color when transformed to the donor strain under a fluorescent microscope (green fluorescent protein), the recipient would have the rfp (red fluorescent protein) tagged in its chromosome. The bead on which the biofilm has been formed will be visualized to access the spatial distribution of the biofilm using fluorescence microscopy. The reason behind the room temperature sensitivity of *E. coli* Nissle (pBURNS1) which was earlier observed in this study was not clear and would have been investigated if time had permitted. Also, the transfer rate of pBURNS1 from *P. aeruginosa* to *E. coli* and vice versa would have been done except for time constraints and lastly detection and isolation of IncP-1 plasmids from treated tap water along with identification of plasmid hosts using 16S rRNA are other areas for future work. It would be useful to test other strains of bacteria for the transferability of pBURNS1 especially those normally present in hospital plumbing such as *Mycobacterium spp* and *Listeria spp.* The effects of low concentrations of chlorine dioxide (0.1-0.25ppm) on a mixed biofilm structure and population containing pBURNS1 would be another area to explore.

Appendix

Appendix 1

Amino acid sequence and translation to peptide of *P. aeruginosa* 943 chlorite dismutase

>similar to chlorite dismutase - 13252: 13803 MW: 21364.44

```
         10        20        30        40        50        60        70        80        90
         |         |         |         |         |         |         |         |         |
ATGAATAATCATTACTCATTTATTGGCGGCTCCGAGGGCTCTTGGCGAGTCACCAGTTGTGAAACACTCATTGGCATACCTCTTGAGATTGTGGAGAGGG  100
M  N  N  H  Y  S  F  I  G  G  S  E  G  S  W  R  V  T  S  C  E  T  L  I  G  I  P  L  E  I  V  E  R  V   34

         |         |         |         |         |         |         |         |         |
TCAATGTAGTTAATATGCCCTCAACAAATTTGATCGAACGTGGCACCTGGGTGCTTCAAGGCTTTACAAGCAATGTTCGGTACGCCGAACGGCATGAGAT  200
  N  V  V  N  M  P  S  T  N  L  I  E  R  G  T  W  V  L  Q  G  F  T  S  N  V  R  Y  A  E  R  H  E  I   67

         |         |         |         |         |         |         |         |         |
CAATCAACTTCGCGCAAAACAGGAGGAATTAAACCGTCCTACGTCATCATGCGCTGCACTCATTCCCATAAAGAAAAGTCCAGAATGGTGGGCAATGTCA  300
 N  Q  L  R  A  K  Q  E  E  L  N  R  P  T  S  S  C  A  A  L  I  P  I  K  K  S  P  E  W  W  A  M  S   100

         |         |         |         |         |         |         |         |         |
CAAGAGGAACGGCGCGAAATTTTTGAAGCAAAGTCCCACCACACAGAAATTGGACTGGCTTATCTTCCAGAAATCGCAAGACAGCTACATCATTCTCGCG  400
Q  E  E  R  R  E  I  F  E  A  K  S  H  H  T  E  I  G  L  A  Y  L  P  E  I  A  R  Q  L  H  H  S  R  D  134

         |         |         |         |         |         |         |         |         |
ACCTCGGAGAGCCGTTTGACTTCTTAACCTGGTTCGAGTTTGCTCCGGAGCATACTGCAATATTCAACAAACTGTTGGCTCAGTTACGCTCATCAAAAGA  500
  L  G  E  P  F  D  F  L  T  W  F  E  F  A  P  E  H  T  A  I  F  N  K  L  L  A  Q  L  R  S  S  K  E  167

         |         |         |         |         |         |         |         |         |
GTGGGAATATGTCGAACGCGAGATAGATATCCGACTGGTGAAAATGTCTAA                                                    551
 W  E  Y  V  E  R  E  I  D  I  R  L  V  K  M  S  *                                                    183
```

Appendix 2 Comparison of translated sequence of ORFs in pBURNS1 with other IncP-1β plasmids. * represents the comparison of pKV29, pAKD-1 and pBURNS1 only. ? represents unconfirmed function

Protein	Function	Length of protein in pBURNS1 (aa)	Number of mutations unique to pBURNS1	Conserved aa (%)	Number of mutations in other plasmids
upf54.4	Unknown	217	2	99.1	0
upf54.8	Unknown	115	1	99.1	0
KfrA	Regulation	343	10	96.8	1
KorB	Partitioning and regulation	349	5	98	2
IncC	Partitioning and regulation	358	5	98.6	0
KorA	Regulation	100	1	99	0
KleF	Plasmid Maintenance?	176	10	93.7	1
KleE	Plasmid Maintenance?	137	6	95.6	0
KleB	Plasmid Maintenance?	71	6	91.5	0
KleA	Plasmid Maintenance?	78	4	94.9	0
KorC	Regulation	85	1	97.6	1
KlcB*	Plasmid Maintenance?	404	28	93	0
KlcA	Plasmid Maintenance?	142	3	97.9	0
TrfA	Replication initiation	406	7	97.8	2
ssb	ssDNA binding	113	2	98.2	0
TraI	DNA relaxase	746	2	98.5	9
TraK	*ori*T binding	133	10	92.5	0
TraL	Conjugative transfer	241	1	99.6	0

Figure based on data from (Aggarwal, 2015; Gambari et al, n.d)

List of references

Aggarwal, N. (2015). An IncP-1 plasmid in *Pseudomonas aeruginosa* from the QEHB Burns Unit (Unpublished M.Sc thesis). University of Birmingham, Birmingham.

Aggazzotti, G, Righi, E, Fantuzzi, G, Biasotti, B, Ravera, G, Kanitz, S, Sciacca, S. (2004). Chlorination by-products (CBPs) in drinking water and adverse pregnancy outcomes in Italy. *Journal of Water and Health*, *2*(4), 233–247.

Akimitsu, N, Hamamoto, H, Inoue, R.-I, Shoji, M, Akamine, A, Takemori, K.-I, Hamasaki, N. & Sekimizu, K. (1999). Increase in resistance of methicillin-resistant *Staphylococcus aureus* to β-lactams caused by mutations conferring resistance to benzalkonium chloride, a disinfectant widely used in hospitals. *Antimicrobial agents and chemotherapy*, 43**,** 3042-3043.

Allen, H. K, Donato, J, Wang, H. H, Cloud-Hansen, K. A, Davies, J, & Handelsman, J. (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nature Reviews Microbiology*, 8, 251–259

Angles, M. L, Marshall, K. C, & Goodman, A. E. (1993). Plasmid transfer between marine bacteria in the aqueous phase and biofilms in reactor microcosms. *Applied and Environmental Microbiology*, *59*(3), 843–850

Anaissie, E. J, Penzak, S. R, & Dignani, M. C. (2002). The hospital water supply as a source of nosocomial infections: A plea for action. *Archives of Internal Medicine*, *162*(13), 1483–1492.

Anzai, Y, Kim, H, Park, J. Y, Wakabayashi, H, & Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *International Journal of Systematic and Evolutionary Microbiology*, *50*(4), 1563–1589.

Appleyard, R. K. (1953). Segregation of lambda lysogenicity during bacterial recombination in *E. coli* K-12. *Cold spring harbor symposia on quantitative biology*, *18*, 95–97.

Bagge, N, Schuster, M, Hentzer, M, Ciofu, O, Givskov, M, Greenberg, E. P, & Høiby, N. (2004). *Pseudomonas aeruginosa* Biofilms Exposed to Imipenem Exhibit Changes in Global Gene Expression and β-Lactamase and Alginate Production. *Antimicrobial Agents and Chemotherapy*, *48*(4), 1175–1187.

Baghal Asghari, F, Nikaeen, M, & Mirhendi, H. (2013). Rapid monitoring of *Pseudomonas aeruginosa* in hospital water systems: A key priority in prevention of nosocomial infection. *FEMS Microbiology Letters*, 343, 77–81.

Bahl, M. I, Burmølle, M, Meisner, A, Hansen, L. H, & Sørensen, S. J. (2009). All IncP-1 plasmid subgroups, including the novel ε subgroup, are prevalent in the influent of a Danish wastewater treatment plant. *Plasmid*, *62*(2), 134–139.

Barne, K. A, Bown, J. A, Busby, S. J. W, & Minchin, S. D. (1997). Region 2.5 of the *Escherichia coli* RNA polymerase σ70 subunit is responsible for the recognition of the "extended -10" motif at promoters. *EMBO Journal*. *16*(13), 4034–4040.

Barrón, M. D. la C, Merlin, C, Guilloteau, H, Montargès-Pelletier, E, & Bellanger, X. (2018). Suspended materials in river waters differentially enrich class 1 integron- and IncP-1 plasmid-carrying bacteria in sediments. *Frontiers in Microbiology*, 9, 1443.

Baxter, J. C, & Funnell, B. E. (2014). Plasmid Partition Mechanisms. *Microbiology Spectrum*, *2*(6)876.

Beal, J, Farny, N. G, Haddock-Angelli, T, Selvarajah, V, Baldwin, G. S, Buckley-Taylor, R. (2019). Robust Estimation of Bacterial Cell Count from Optical Density. *BioRxiv*, 803239.

Behnke, S, & Camper, A. K. (2012). Chlorine dioxide disinfection of single and dual species biofilms, detached biofilm and planktonic cells. *Biofouling*. 28(6), 635-647

Bellanger, X, Guilloteau, H, Bonot, S, & Merlin, C. (2014). Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. *Science of the Total Environment*, *493*, 872–882.

Bender, K. S, O'Connor, S. M, Chakraborty, R, Coates, J. D, & Achenbach, L. A. (2002). Sequencing and transcriptional analysis of the chlorite dismutase gene of *Dechloromonas agitata* and its use as a metabolic probe. *Applied and Environmental Microbiology*, *68*(10), 4820–4826.

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: Acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, *153*(SUPPL. 1).

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli. Journal of Bacteriology*, *62*(3), 293–300.

Birnboim, H. C, & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids research*, *7*(6), 1513–1523.

Bjarnsholt, T. (2013). The role of bacterial biofilms in chronic infections. *APMIS*. (136), 1-51

Bollinger, N, Hassett, D. J, Iglewski, B. H, Costerton, J. W, & McDermott, T. R. (2001). Gene expression in *Pseudomonas aeruginosa*: Evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. *Journal of Bacteriology*, *183*(6), 1990–1996.

Breathnach, A. S, Cubbon, M. D, Karunaharan, R. N, Pope, C. F, & Planche, T. D. (2012). Multidrug-resistant *Pseudomonas aeruginosa* outbreaks in two hospitals: association with contaminated hospital waste-water systems. *The Journal of Hospital Infection*, *82*(1), 19–24.

Brown, H. J, Stokes, H. W, & Hall, R. M. (1996). The integrons In0, In2, and In5 are defective transposon derivatives. *Journal of Bacteriology*, *178*(15), 4429–4437.

Burrus, V, Pavlovic, G, Decaris, B, & Guédon, G. (2002). Conjugative transposons: The tip of the iceberg. *Molecular Microbiology*, Vol. 46, pp. 601–610.

Caldwell, B. A, Ye, C, Griffiths, R. P, Moyer, C. L, & Morita, R. Y. (1989). Plasmid

expression and maintenance during long-term starvation-survival of bacteria in well water. *Applied and Environmental Microbiology*, *55*(8), 1860–1864.

Carey, D. E, & McNamara, P. J. (2015). The impact of triclosan on the spread of antibiotic resistance in the environment. *Frontiers in microbiology*, *5*, 780.

Carroll, A. C, & Wong, A. (2018). Plasmid Persistence: Costs, Benefits and the Plasmid Paradox. *Canadian Journal of Microbiology*. *64*(5), 293-304.

Celis, A. I, Geeraerts, Z, Ngmenterebo, D, Machovina, M. M, Kurker, R. C, Rajakumar, K, Dubois, J. L. (2015). A dimeric chlorite dismutase exhibits O2-generating activity and acts as a chlorite antioxidant in *Klebsiella pneumoniae* MGH 78578. *Biochemistry*, *54*(2), 434–446.

Chandler, M, & Siguier, P. (2013). Insertion Sequences. In *Brenner's Encyclopedia of Genetics: Second Edition*.

Chapman, J. S. (2003). Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *International Biodeterioration and Biodegradation*, *51*(4), 271–276.

Charron, D, Bédard, E, Lalancette, C, Laferrière, C, & Prévost, M. (2015). Impact of electronic faucets and water quality on the occurrence of *Pseudomonas aeruginosa* in water: A multi-hospital study. *Infection Control and Hospital Epidemiology*. *36*(3), 311–319.

Charlton, D. (1933). Chlorine tolerant bacteria in water supplies. *Journal (American Water Works Association), 25*(6), 851-854.

Chiu, C. M, & Thomas, C. M. (2004). Evidence for past integration of IncP-1 plasmids into bacterial chromosomes. *FEMS Microbiology Letters*. *241*(2), 163–169.

Chouchani, C, Marrakchi, R, Henriques, I, & Correia, A. (2013). Occurrence of IMP-8, IMP-10, and IMP-13 metallo-β-lactamases located on class 1 integrons and other extended-spectrum β-lactamases in bacterial isolates from Tunisian rivers. *Scandinavian Journal of Infectious Diseases*. 64(5), 293-304.

Christensen, B. B, Sternberg, C, Andersen, J. B, Eberl, L, Møller, S, Givskov, M, & Molin, S. (1998). Establishment of new genetic traits in a microbial biofilm community. *Applied and Environmental Microbiology*, *64*(6), 2247–2255.

Coates, J. D, & Achenbach, L. A. (2004). Microbial perchlorate reduction: Rocket-fuelled metabolism. *Nature Reviews Microbiology*, 2, 569–580.

Conlan, S, Mijares, L. A, Becker, J, Blakesley, R. W, Bouffard, G. G, Brooks, S, … Segre, J. A. (2012). *Staphylococcus epidermidis* pan-genome sequence analysis reveals diversity of skin commensal and hospital infection-associated isolates. *Genome Biology*, *13*(7).

Connolly, J. A, Wilson, A, Macioszek, M, Song, Z, Wang, L, Mohammad, H. H, … Thomas, C. M. (2019). Defining the genes for the final steps in biosynthesis of the complex polyketide antibiotic mupirocin by *Pseudomonas fluorescens* NCIMB10586. *Scientific reports*, *9*(1), 1542.

Coroler, L, Elomari, M, Hoste, B, Gillis, M, Izard, D, & Leclerc, H. (1996). *Pseudomonas*

rhodesiae sp. nov, a new species isolated from natural mineral waters. *Systematic and Applied Microbiology*, *19*(4), 600–607.

Curran, E. T. (2012). Outbreak column 3: Outbreaks of *Pseudomonas spp* from hospital water. *Journal of Infection Prevention*. *13*(4), 125–127.

da Silva, D, C Schofield, M, Parsek, M, & Tseng, B. S. (2017). An Update on the Sociomicrobiology of Quorum Sensing in Gram-Negative Biofilm Development. *Pathogens*, *6*, 51.

Dandekar, A.A.; Chugani, S.; Greenberg, E.P. (2012). "Bacterial quorum sensing and metabolic incentives to cooperate". *Science*. 338 (6104): 264–266

Davey, M. E, & O'toole, G. A. (2000). Microbial Biofilms: from Ecology to Molecular Genetics. *Microbiology and Molecular Biology Reviews*. *64*(4), 847–867.

Davies, J, & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews : MMBR*, *74*(3), 417–433.

De Gelder L, Ponciano J,M, Joyce P, Top E,M.(2007). Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship. Microbiology. ;153:452–63.

de Geus, D. C, Thomassen, E. A. J, Hagedoorn, P. L, Pannu, N. S, van Duijn, E, & Abrahams, J. P. (2009). Crystal Structure of Chlorite Dismutase, a Detoxifying Enzyme Producing Molecular Oxygen. *Journal of Molecular Biology*, *387*(1), 192–206.

De Leersnyder, I, De Gelder, L, Van Driessche, I, & Vermeir, P. (2018). Influence of growth media components on the antibacterial effect of silver ions on *Bacillus subtilis* in a liquid growth medium. *Scientific Reports*. *8*(1).

De Rore, H, Top, E, Houwen, F, Mergeay, M, & Verstraete, W. (1994). Evolution of heavy metal resistant transconjugants in a soil environment with a concomitant selective pressure. *FEMS Microbiology Ecology*. *14*(3), 263–273

Del Solar, G, Giraldo, R, Ruiz-Echevarria, M. J, Espinosa, M, & Diaz-Orejas, R. (1998). Replication and control of circular bacterial plasmids. *Microbiology and Molecular Biology Reviews : MMBR*, *62*(2), 434–464.

Donlan, R. M. (2001). Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases*, *33*(8), 1387–1392.

Dröge, M, Pühler, A, & Selbitschka, W. (2000). Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Molecular and General Genetics*, *263*(3), 471–482.

Easter C.L, Schwab, H. and Helinski, D.R. (1998) Role of the *parCBA* operon of the broad-host-range plasmid RK2 in stable plasmid maintenance Journal of Bacteriology 180, 6023-6030

Falcone-Dias, M. F, Vaz-Moreira, I, & Manaia, C. M. (2012). Bottled mineral water as a potential source of antibiotic resistant bacteria. *Water Research*. *33*(8), 1387–1392.

Fonseca, A. P, & Sousa, J. C. (2007). Effect of shear stress on growth, adhesion and biofilm

formation of *Pseudomonas aeruginosa* with antibiotic-induced morphological changes. *International Journal of Antimicrobial Agents*. *30*(3), 236–241.

Foschino, R, Nervegna, I, Motta, A, & Galli, A. (2016). Bactericidal Activity of Chlorine Dioxide against *Escherichia coli* in Water and on Hard Surfaces. *Journal of Food Protection*. *61*(6), 668–672.

Freese, P. D, Korolev, K. S, Jiménez, J. I, & Chen, I. A. (2014). Genetic drift suppresses bacterial conjugation in spatially structured populations. *Biophysical Journal*. *106*(4), 944–954

Fux, C. A, Costerton, J. W, Stewart, P. S, & Stoodley, P. (2005). Survival strategies of infectious biofilms. *Trends in Microbiology*, (13) 34–40.

Gambari, R. O,Aggarwal, N, Quick, J,Perez-soto, N, Wearn C. M, Webber, M. A, Loman, N. J, Thomas,(n.d.) C. M. A bacterial plasmid from a hospital Burns Unit conferring resistance to chlorite. *Unpublished.*

Garcillán-Barcia, M. P, & de la Cruz, F. (2008). Why is entry exclusion an essential feature of conjugative plasmids? *Plasmid*, 60(1), 1-16.

Gaze, W. H, Zhang, L, Abdouslam, N. A, Hawkey, P. M, Calvo-Bado, L, Royle, J, … Wellington, E. M. H. (2011). Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME Journal*. *5*(8), 1253–1261.

Ghigo, J. M. (2001). Natural conjugative plasmids induce bacterial biofilm development. *Nature*, *412*(6845), 442–445.

Gillings, M. R, Krishnan, S, Worden, P. J, & Hardwick, S. A. (2008). Recovery of diverse genes for class 1 integron-integrases from environmental DNA samples. *FEMS Microbiology Letters*. *287*(1), 56–62.

Goblirsch, B, Kurker, R. C, Streit, B. R, Wilmot, C. M, & Dubois, J. L. (2011). Chlorite dismutases, DyPs, and EfeB: 3 microbial heme enzyme families comprise the CDE structural superfamily. *Journal of Molecular Biology*, Vol. 408, pp. 379–398.

Göttsching, A, & Schmidt, S. (2007). Productive degradation of the biocide benzylbenzoate by *Acinetobacter sp*. strain AG1 isolated from the River Elbe. *Research in Microbiology*, *158*(3), 251–257.

Guérin, E, Jové, T, Tabesse, A, Mazel, D, & Ploy, M. C. (2011). High-level gene cassette transcription prevents integrase expression in class 1 integrons. *Journal of Bacteriology*, *193*(20), 5675–5682.

Gupta, P, Bhatia, M, Gupta, P, & Omar, B. J. (2018). Emerging Biocide Resistance among Multidrug-Resistant Bacteria: Myth or Reality? A Pilot Study. *Journal of pharmacy & bioallied sciences*, *10*(2), 96–101.

Haase, J, Kalkum, M, & Lanka, E. (1996). TrbK, a small cytoplasmic membrane lipoprotein, functions in entry exclusion of the IncPα plasmid RP4. *Journal of Bacteriology*, *178*(23), 6720–6729.

Haines, A. S, Akhtar, P, Stephens, E. R, Jones, K, Thomas, C. M, Perkins, C. D, Fry, J. C.

(2006). Plasmids from freshwater environments capable of IncQ retrotransfer are diverse and include pQKH54, a new IncP-1 subgroup archetype. *Microbiology*, *152*(9), 2689–2701.

Hassett, D. J, Ma, J. F, Elkins, J. G, McDermott, T. R, Ochsner, U. A, West, S. E. H, … Iglewski, B. H. (1999). Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Molecular Microbiology*. *34*(5), 1082–1093.

Hausner, M, & Wuertz, S. (1999). High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Applied and Environmental Microbiology*. *65*(8), 3710–3713.

Helena Rebouças, R, Viana de Sousa, O, Sousa Lima, A, Roger Vasconcelos, F, de Carvalho, P. B, & dos Fernandes Vieira, R. H. S. (2011). Antimicrobial resistance profile of *Vibrio* species isolated from marine shrimp farming environments (*Litopenaeus vannamei*) at Ceará, Brazil. *Environmental Research*, *111*(1), 21–24.

Hengzhuang W, Høiby N, Ciofu O. (2014) Pharmacokinetics and Pharmacodynamics of Antibiotics in Biofilm Infections of *Pseudomonas aeruginosa* In Vitro and In Vivo. In: Donelli G. (eds) Microbial Biofilms. Methods in Molecular Biology (Methods and Protocols), vol 1147. Humana Press, New York, NY

Hennequin, C, Aumeran, C, Robin, F, Traore, O, & Forestier, C. (2012). Antibiotic resistance and plasmid transfer capacity in biofilm formed with a CTX-M-15-producing *Klebsiella pneumoniae* isolate. *Journal of Antimicrobial Chemotherapy*, *67*(9), 2123–2130.

Hershfield, V, Boyer, H. W, Yanofsky, C, Lovett, M. A, & Helinski, D. R. (1974). Plasmid ColEl as a molecular vehicle for cloning and amplification of DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *71*(9), 3455–3459.

Heurlier, K, Dénervaud, V, Haenni, M, Guy, L, Krishnapillai, V, & Haas, D. (2005). Quorum-sensing-negative (lasR) mutants of *Pseudomonas aeruginosa* avoid cell lysis and death. *Journal of Bacteriology*, *187*(14), 4875–4883.

Hicks, S. D, Petersen, J. L, Bougher, C. J. and Abu-Omar, M. M. (2011), Chlorite Dismutation to Chlorine Dioxide Catalyzed by a Water-Soluble Manganese Porphyrin. *Angew. Chem. Int. Ed*, *50,* 699-702

Hinenoya, A, Awasthi, S. P, Yasuda, N, Shima, A, Morino, H, Koizumi, T, Yamasaki, S. (2015). Chlorine dioxide is a better disinfectant than sodium hypochlorite against multi-drug resistant *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Acinetobacter baumannii*. *Japanese Journal of Infectious Diseases*. *68*(4), 276–279.

Hofbauer, S, Hagmüller, A, Schaffner, I, Mlynek, G, Krutzler, M, Stadlmayr, G, Furtmüller, P. G. (2015). Structure and heme-binding properties of HemQ (chlorite dismutase-like protein) from *Listeria monocytogenes*. *Archives of Biochemistry and Biophysics*, *574*, 36–48.

Hofbauer, S, Schaffner, I, Furtmüller, P. G, & Obinger, C. (2014). Chlorite dismutases - a heme enzyme family for use in bioremediation and generation of molecular oxygen. *Biotechnology Journal*, *9*(4), 461–473.

Honeyman, A. L, Cote, C. K, & Curtiss, R. (2002). Construction of transcriptional and translational lacZ gene reporter plasmids for use in *Streptococcus mutans*. *Journal of Microbiological Methods*. *49*(2), 163–171.

Hosein, I. K, Hill, D. W, Tan, T. Y, Butchart, E. G, Wilson, K, Finlay, G, Ribeiro, C. D. (2005). Point-of-care controls for nosocomial legionellosis combined with chlorine dioxide potable water decontamination: A two-year survey at a Welsh teaching hospital. *Journal of Hospital Infection*. *61*(2), 100–106.

Huang, D. W, Sherman, B. T, & Lempicki, R. A. (2009). Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research*, *37*(1), 1–13.

Huang, J. J, Hu, H. Y, Tang, F, Li, Y, Lu, S. Q, & Lu, Y. (2011). Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. *Water Research*. *45*(9), 2775–2781.

Huang, J, Wang, L, Ren, N, Ma, F, & Juli. (2002). Disinfection effect of chlorine dioxide on bacteria in water. *Water Research*, *31*(3), 607–613.

Humphrey, B, Thomson, N. R, Thomas, C. M, Brooks, K, Sanders, M, Delsol, A. A, Enne, V. I. (2012). Fitness of *Escherichia coli* strains carrying expressed and partially silent IncN and IncP1 plasmids. *BMC Microbiology*, *12*(1), 53.

Jafar, E. (2013). Isolation of a Novel Antibiotic Resistance Plasmid DNA from Hospital Isolates of *Pseudomonas aeruginosa*. *Journal of Clinical & Experimental Pathology*, *3*(2).

Jové, T, Da Re, S, Denis, F, Mazel, D, & Ploy, M.-C. (2010). Inverse Correlation between Promoter Strength and Excision Activity in Class 1 Integrons. *PLOS Genetics*, *6*(1), 1–10.

Jutkina, J, Marathe, N. P, Flach, C.-F, & Larsson, D. G. J. (2018). Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. *Science of The Total Environment*, *616–617*, 172–178.

Kaczur, J. J, & Cawlfield, D. W. (2004). Chlorine Oxygen Acids and Salts, Chlorous Acid, Chlorites, and Chlorine Dioxide. In *Kirk-Othmer Encyclopedia of Chemical Technology*.

Katharios-Lanwermeyer, S, Rakic-Martinez, M, Elhanafi, D, Ratani, S, Tiedje, J. M, & Kathariou, S. (2012). Coselection of cadmium and benzalkonium chloride resistance in conjugative transfers from nonpathogenic *Listeria spp*. to other listeriae. *Applied and Environmental Microbiology*, *78*(21), 7549–7556.

Keren, I, Shah, D, Spoering, A, Kaldalu, N, & Lewis, K. (2004). Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *Journal of Bacteriology*, *186*(24), 8172–8180.

Kim, D. U, Kim, M. S, Lim, J. S, & Ka, J. O. (2013). Widespread occurrence of the tfd-II genes in soil bacteria revealed by nucleotide sequence analysis of 2,4-dichlorophenoxyacetic acid degradative plasmids pDB1 and p712. *Plasmid*, *69*(3), 243–248.

Knapp,J. E, and Battsiti, D. L, (2001). Chlorine dioxide. In: Block SS, ed. In *Disinfection, sterilization, and preservation* (pp. 215-225). Lippincott Williams & Wilkins

Kolmos, H. J, Thuesen, B, Nielsen, S. V, Lohmann, M, Kristoffersen, K, & Rosdahl, V. T. (1993). Outbreak of infection in a burns unit due to *Pseudomonas aeruginosa* originating from contaminated tubing used for irrigation of patients. *Journal of Hospital Infection*, *24*(1), 11–21.

Konrat, K, Schwebke, I, Laue, M, Dittmann, C, Levin, K, Andrich, R, Schaudinn, C. (2016). The bead assay for biofilms: A quick, easy and robust method for testing disinfectants. *PLoS ONE*, *11*(6).

Korich, D. G, Mead, J. R, Madore, M. S, Sinclair, N. A, & Sterling, C. R. (1990). Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. *Applied and Environmental Microbiology*. *56(5) 1423-1428*

Kostan, J, Sjöblom, B, Maixner, F, Mlynek, G, Furtmüller, P. G, Obinger, C, … Djinović-Carugo, K. (2010). Structural and functional characterisation of the chlorite dismutase from the nitrite-oxidizing bacterium " *Candidatus Nitrospira defluvii"*: Identification of a catalytically important amino acid residue. *Journal of Structural Biology*, *172*(3), 331–342.

Kreske, A. C, Ryu, J.-H, Pettigrew, C. A, & Beuchat, L. R. (2016). Lethality of Chlorine, Chlorine Dioxide, and a Commercial Produce Sanitizer to *Bacillus cereus* and *Pseudomonas* in a Liquid Detergent, on Stainless Steel, and in Biofilm. *Journal of Food Protection*, *69*(11), 2621–2634.

Król, J. E, Nguyen, H. D, Rogers, L. M, Beyenal, H, Krone, S. M, & Top, E. M. (2011). Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air-liquid interface. *Applied and Environmental Microbiology*, *77*(15), 5079–5088.

Król, J. E, Wojtowicz, A. J, Rogers, L. M, Heuer, H, Smalla, K, Krone, S. M, & Top, E. M. (2013). Invasion of *E. coli* biofilms by antibiotic resistance plasmids. *Plasmid*, *70*(1), 110–119.

Lagoumintzis, G, Christofidou, M, Dimitracopoulos, G, & Paliogianni, F. (2003). *Pseudomonas aeruginosa* Slime Glycolipoprotein Is a Potent Stimulant of Tumor Necrosis Factor Alpha Gene Expression and Activation of Transcription Activators Nuclear Factor κB and Activator Protein 1 in Human Monocytes. *Infection and Immunity*, *71*(8), 4614–4622.

Lambert, P. A. (2002). Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa. Journal of the Royal Society of Medicine*, *95 Suppl* 4, 22–26.

Lasocki, K, Bartosik, A. A, Mierzejewska, J, Thomas, C. M, & Jagura-Burdzy, G. (2007). Deletion of the parA (soj) homologue in *Pseudomonas aeruginosa* causes ParB instability and affects growth rate, chromosome segregation, and motility. *Journal of Bacteriology*, *189*(15), 5762–5772.

Lautenschlager, K, Boon, N, Wang, Y, Egli, T, & Hammes, F. (2010). Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition. *Water Research*, *44*(17), 4868–4877.

Lazdins A, Maurya AP, Miller CE, Kamruzzaman M, Liu S, et al. (2020) Potentiation of curing by a broad-host-range self-transmissible vector for displacing resistance plasmids to tackle AMR. PLOS ONE *15*(1)

Lebeaux, D, Chauhan, A, Rendueles, O, & Beloin, C. (2013). From in vitro to in vivo Models of Bacterial Biofilm-Related Infections. *Pathogens*. 2(2) 288-356.

Leungtongkam, U, Thummeepak, R, Tasanapak, K, & Sitthisak, S. (2018). Acquisition and transfer of antibiotic resistance genes in association with conjugative plasmid or class 1 integrons of *Acinetobacter baumannii*. *PLOS ONE*, *13*(12), 1–12.

Levy, S. B. (1998). The challenge of antibiotic resistance. *Scientific American*, *278*(3), 46–53.

Levy, S. B. (2002). Active efflux, a common mechanism for biocide and antibiotic resistance. *Journal of Applied Microbiology*, *92*(s1), 65S-71S.

Lewis, K. (2008). Multidrug tolerance of biofilms and persister cells. *Current Topics in Microbiology and Immunology*, (322) 107–131.

Lewis, Kim. (2010). Persister Cells. *Annual Review of Microbiology*.

Li, J. W, Xin, Z. T, Wang, X. W, Zheng, J. L, & Chao, F. H. (2004). Mechanisms of inactivation of hepatitis a virus in water by chlorine dioxide. *Water Research*, *38*(6), 1514–1519.

Liebensteiner, M. G, Oosterkamp, M. J, & Stams, A. J. M. (2016). Microbial respiration with chlorine oxyanions: Diversity and physiological and biochemical properties of chlorate- and perchlorate-reducing microorganisms. *Annals of the New York Academy of Sciences*, *1365*(1), 59–72.

Lindqvist, M. H, Johansson, N, Nilsson, T, & Rova, M. (2012). Expression of chlorite dismutase and chlorate reductase in the presence of oxygen and/or chlorate as the terminal electron acceptor in *Ideonella dechloratans*. *Applied and Environmental Microbiology*, *78*(12), 4380–4385.

Livermore, D. M. (2002). Multiple Mechanisms of Antimicrobial Resistance in *Pseudomonas aeruginosa*: Our Worst Nightmare? *Clinical Infectious Diseases*. (2002) 34(5) 634-640.

Loveday, H. P, Wilson, J. A, Kerr, K, Pitchers, R, Walker, J. T, & Browne, J. (2014). Association between healthcare water systems and *Pseudomonas aeruginosa* infections: A rapid systematic review. *Journal of Hospital Infection*, (86), 7–15.

Lyras, D, Chan, A. W. S, McFarlane, J, & Stanisich, V. A. (1994). The surface exclusion system of RP1: Investigation of the roles of trbJ and trbK in the surface exclusion, transfer, and slow-growth phenotypes. *Plasmid*, *32*(3), 254–261.

Ma, H, & Bryers, J. D. (2013). Non-invasive determination of conjugative transfer of plasmids bearing antibiotic-resistance genes in biofilm-bound bacteria: Effects of substrate loading and antibiotic selection. *Applied Microbiology and Biotechnology*, *97*(1), 317–328.

Mah, T. F. C, & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial

agents. *Trends in Microbiology*, *9*(1), 34–39.

Mahillon, J. & Chandler, M. 1998. Insertion sequences. *Microbiology and Molecular Biology Reviews*, 62**,** 725-774.

Maixner, F, Wagner, M, Lücker, S, Pelletier, E, Schmitz-Esser, S, Hace, K, Daims, H. (2008). Environmental genomics reveals a functional chlorite dismutase in the nitrite-oxidizing bacterium "*Candidatus Nitrospira defluvii*." *Environmental Microbiology*, *10*(11), 3043–3056.

Mao, G, Song, Y, Bartlam, M, & Wang, Y. (2018). Long-term effects of residual chlorine on *Pseudomonas aeruginosa* in simulated drinking water fed with low AOC medium. *Frontiers in Microbiology*, *9*(5), 879.

May, T, & Okabe, S. (2008). *Escherichia coli* harboring a natural IncF conjugative F plasmid develops complex mature biofilms by stimulating synthesis of colanic acid and curli. *Journal of Bacteriology*, *190*(22), 7479–7490.

Mayfield, J. A, Hammer, N. D, Kurker, R. C, Chen, T. K, Ojha, S, Skaar, E. P, & DuBois, J. L. (2013). The chlorite dismutase (HemQ) from *Staphylococcus aureus* has a redox-sensitive heme and is associated with the small colony variant phenotype. *Journal of Biological Chemistry*, *288*(32), 23488–23504.

Mazel, D. (2006). Integrons: Agents of bacterial evolution. *Nature Reviews Microbiology*, Vol. 4, pp. 608–620.

Mehboob, F, Junca, H, Schraa, G, & Stams, A. J. M. (2009). Growth of *Pseudomonas chloritidismutans* AW-1T on n-alkanes with chlorate as electron acceptor. *Applied Microbiology and Biotechnology*, *83*(4), 739–747.

Mena, K. D, & Gerba, C. P. (2009). Risk assessment of *Pseudomonas aeruginosa* in water. *Reviews of Environmental Contamination and Toxicology*, *201*, 71–115.

Merkey, B. V, Lardon, L. A, Seoane, J. M, Kreft, J. U, & Smets, B. F. (2011). Growth dependence of conjugation explains limited plasmid invasion in biofilms: An individual-based modelling study. *Environmental Microbiology*. 13(9) 2435-2452

Miles, A. A, Misra, S. S, & Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene*, *38*(6), 732–749.

Mlynek, G, Sjöblom, B, Kostan, J, Füreder, S, Maixner, F, Gysel, K, Djinović-Carugo, K. (2011). Unexpected diversity of chlorite dismutases: A catalytically efficient dimeric enzyme from *Nitrobacter winogradskyi*. *Journal of Bacteriology*, *193*(10), 2408–2417.

Molin, S, & Tolker-Nielsen, T. (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Current Opinion in Biotechnology*, 14, 255–261.

Murray, G. E, Robin, R. S, Junkins, B, & Kushner, D. J. (1984) Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria. (1984). *Applied and Environmental Microbiology*, *48*(1), 73–77.

Nakata Y, Tang X, Yokoyama K.K. (1997) Preparation of Competent Cells for High-Efficiency Plasmid Transformation of *Escherichia coli*. In: Cowell I.G, Austin C.A.

(eds) cDNA Library Protocols. Methods in Molecular Biology™, vol 69. Humana Press

Naves, P, Del Prado, G, Huelves, L, Gracia, M, Ruiz, V, Blanco, J, Soriano, F. (2008). Measurement of biofilm formation by clinical isolates of *Escherichia coli* is method-dependent. *Journal of Applied Microbiology*, *105*(2), 585–590.

Nerenberg, R, Kawagoshi, Y, & Rittmann, B. E. (2006). Kinetics of a hydrogen-oxidizing, perchlorate-reducing bacterium. *Water Research*, *40*(17), 3290–3296.

Nilsson, T, Rova, M, & Smedja Bäcklund, A. (2013). Microbial metabolism of oxochlorates: A bioenergetic perspective. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1827*(2), 189–197.

Norberg, P, Bergström, M, Jethava, V, Dubhashi, D, & Hermansson, M. (2011). The IncP-1 plasmid backbone adapts to different host bacterial species and evolves through homologous recombination. *Nature Communications*, *2*(1) 268.

Norman, A, Hansen, L. H, & Sørensen, S. J. (2009). Conjugative plasmids: vessels of the communal gene pool. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *364*(1527), 2275–2289.

O'Rourke, D, Fitz Gerald, C. E, Traverse, C. C, & Cooper, V. S. (2015). There and back again: Consequences of biofilm specialization under selection for dispersal. *Frontiers in Genetics*, 6, 18.

Okumura J, Wakai S, Umenai T. (2002) Drug utilisation and self-medication in rural communities in Vietnam. *Soc Sci Med* 54: 1875-86

Oliveira, C. S, Lázaro, B, Azevedo, J. S. N, Henriques, I, Almeida, A, & Correia, A. (2012). New molecular variants of epsilon and beta IncP-1 plasmids are present in estuarine waters. *Plasmid*, *67*(3), 252–258.

Orgogozo, V, Morizot, B, & Martin, A. (2015). The differential view of genotype-phenotype relationships. *Frontiers in Genetics*, *6*(MAY).

Orsi, G. B, Mansi, A, Tomao, P, Chiarini, F, & Visca, P. (1994). Lack of association between clinical and environmental isolates of *Pseudomonas aeruginosa* in hospital wards. *Journal of Hospital Infection*, *27*(1), 49–60.

Otter, J. A, Vickery, K, Walker, J. T, deLancey Pulcini, E, Stoodley, P, Goldenberg, S. D, Edgeworth, J. D. (2015). Surface-attached cells, biofilms and biocide susceptibility: Implications for hospital cleaning and disinfection. *Journal of Hospital Infection*. 89(1), 16-27

Overgaard, Martin & Borch, Jonas & Gerdes, Kenn. (2009). RelB and RelE of *Escherichia coli* Form a Tight Complex That Represses Transcription via the Ribbon–Helix–Helix Motif in RelB. Journal of molecular biology. 394. 183-96.

Padilla, C, & Vásquez, C. (1993). Plasmid-mediated antibiotic resistance in *Pseudomonas aeruginosa* from well water sediments and their transformation into *Escherichia coli*. *Letters in Applied Microbiology*. 16(1) 17-20.

Palleroni, N. J, & Holmes, B. (1981). *Pseudomonas cepacia* Sp. Nov, Nom. Rev.

International Journal Of Systematic And Evolutionary Microbiology, *31*(4), 479–481.

Panagea, S, Winstanley, C, Walshaw, M. J, Ledson, M. J, & Hart, C. A. (2005). Environmental contamination with an epidemic strain of *Pseudomonas aeruginosa* in a Liverpool cystic fibrosis centre, and study of its survival on dry surfaces. *Journal of Hospital Infection*. 59(2) 102-107.

Pansegrau, W, Lanka, E, Barth, P. T, Figurski, D. H, Guiney, D. G, Haas, D, … Thomas, C. M. (1994). Complete nucleotide sequence of birmingham IncPα plasmids. Compilation and comparative analysis. *Journal of Molecular Biology*, *239*(5), 623–663.

Petrova, M, Gorlenko, Z, & Mindlin, S. (2011). Tn5045, a novel integron-containing antibiotic and chromate resistance transposon isolated from a permafrost bacterium. *Research in Microbiology*, *162*(3), 337–345.

Poltak, S. R, & Cooper, V. S. (2011). Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. *ISME Journal*, *5*(3), 369–378.

Popat, R, Crusz, S. A, Messina, M, Williams, P, West, S. A, & Diggle, S. P. (2012). Quorum-sensing and cheating in bacterial biofilms. *Proceedings of the Royal Society B: Biological Sciences*. 279(1748) 4765-4771

Popowska, M, & Krawczyk-Balska, A. (2013). Broad-host-range IncP-1 plasmids and their resistance potential. *Frontiers in Microbiology*, Vol. 4.

Post, V, Recchia, G. D, & Hall, R. M. (2007). Detection of gene cassettes in Tn402-like class 1 integrons [7]. *Antimicrobial Agents and Chemotherapy*, *51*(9), 3467–3468.

Price, M. N, Wetmore, K. M, Deutschbauer, A. M, & Arkin, A. P. (2016). A comparison of the costs and benefits of bacterial gene expression. *PLoS ONE*, *11*(10).

PUBLIC HEALTH ENGLAND (2014). Healthcare associated infections (HCAI): guidance, data and analysis

Quick, J, Cumley, N, Wearn, C. M, Niebel, M, Constantinidou, C, Thomas, C. M, Loman, N. J. (2014). Seeking the source of *Pseudomonas aeruginosa* infections in a recently opened hospital: An observational study using whole-genome sequencing. *BMJ Open*. 4(11)

Rahube, T. O, Viana, L. S, Koraimann, G, & Yost, C. K. (2014). Characterization and comparative analysis of antibiotic resistance plasmids isolated from a wastewater treatment plant. *Frontiers in Microbiology*. *5*(10)

Raja, C. E, & Selvam, G. S. (2009). Plasmid profile and curing analysis of *Pseudomonas aeruginosa* as metal resistant. *International Journal of Environmental Science and Technology*, *6*, 259–266.

Raven, J. A, (2017). Chloride: essential micronutrient and multifunctional beneficial ion, *Journal of Experimental Botany*, 3(68), 359–367.

Righi, E, Bechtold, P, Tortorici, D, Lauriola, P, Calzolari, E, Astolfi, G, Aggazzotti, G. (2012). Trihalomethanes, chlorite, chlorate in drinking water and risk of congenital

anomalies: A population-based case-control study in Northern Italy. *Environmental Research*, *116*, 66–73.

Rizzo, L, Manaia, C, Merlin, C, Schwartz, T, Dagot, C, Ploy, M. C, Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of the Total Environment*. *1*,447, 345-60

Robertson, J, McGoverin, C, Vanholsbeeck, F, Swift, S, (2019). Optimisation of the Protocol for the LIVE/DEAD® BacLightTM Bacterial Viability Kit for Rapid Determination of Bacterial Load. *Frontiers in Microbiology*.10, 801.

Røder, H. L, Hansen, L. H, Sørensen, S. J, & Burmølle, M. (2013). The impact of the conjugative IncP-1 plasmid pKJK5 on multispecies biofilm formation is dependent on the plasmid host. *FEMS Microbiology Letters*. *344*(2) 186-192

Rogues, A. M, Boulestreau, H, Lashéras, A, Boyer, A, Gruson, D, Merle, C, Gachie, J. P. (2007). Contribution of tap water to patient colonisation with Pseudomonas aeruginosa in a medical intensive care unit. *Journal of Hospital Infection*, *67*(1), 72–78.

Rosser, S. J, & Young, H. K. (1999). Identification and characterization of class 1 integrons in bacteria from an aquatic environment. *Journal of Antimicrobial Chemotherapy*, *44*(1), 11–18.

Rossolini, G. M, D'Andrea, M. M. and Mugnaioli, C. (2008), The spread of CTX-M-type extended-spectrum β-lactamases. *Clinical Microbiology and Infection*, 14, 33-41.

Russell, A. D. (1995). Mechanisms of bacterial resistance to biocides. *International Biodeterioration and Biodegradation*, *36*(3–4), 247–265.

Russell, A. D. (1997). Plasmids and bacterial resistance to biocides. *Journal of Applied Microbiology*, 83, 155–165.

Russell, A. D. (2002). Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. *Journal of Applied Microbiology*. *92* Suppl:121S-35S

Saito, K, Yoneyama, H, & Nakae, T. (1999). nalB-type mutations causing the overexpression of the MexAB-OprM efflux pump are located in the mexR gene of the *Pseudomonas aeruginosa* chromosome. *FEMS Microbiology Letters*, *179*(1), 67–72.

Salyers, A. A, Shoemaker, N. B, Stevens, A. M, & Li, L. Y. (1995). Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiological Reviews*, *59*(4), 579–590.

Samuelsen, Ø, Buarø, L, Toleman, M. A, Giske, C. G, Hermansen, N. O, Walsh, T. R, & Sundsfjord, A. (2009). The first metallo-β-lactamase identified in Norway is associated with a TniC-like transposon in a *Pseudomonas aeruginosa* isolate of sequence type 233 imported from Ghana. *Antimicrobial Agents and Chemotherapy*, *53*, 331–332.

Santos, C, Caetano, T, Ferreira, S, & Mendo, S. (2010). Tn5090-like class 1 integron carrying blaVIM-2 in a *Pseudomonas putida* strain from Portugal. *Clinical Microbiology and Infection*, *16*(10), 1558–1561.

Sayeed, S, Brendler, T, Davis, M, Reaves, L, & Austin, S. (2005). Surprising dependence

on postsegregational killing of host cells for maintenance of the large virulence plasmid of *Shigella flexneri*. *Journal of Bacteriology*, *187*(8), 2768–2773.

SCENIHR. (2012). Health Effects of Artificial Light Risks. *Environment*, p. 118.

Schaffner, I, Hofbauer, S, Krutzler, M, Pirker, K. F, Bellei, M, Stadlmayr, G, Obinger, C. (2015a). Dimeric chlorite dismutase from the nitrogen-fixing cyanobacterium *Cyanothece sp*. PCC7425. *Molecular Microbiology*, *96*(5), 1053–1068.

Schaffner, I, Hofbauer, S, Krutzler, M, Pirker, K. F, Furtmüller, P. G, & Obinger, C. (2015b). Mechanism of chlorite degradation to chloride and dioxygen by the enzyme chlorite dismutase. *Archives of Biochemistry and Biophysics*, *574*, 18–26.

Schlüter, A, Szczepanowski, R, Pühler, A, & Top, E. M. (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiology Reviews*, 31, 449–477.

Schröder, G, & Lanka, E. (2005). The mating pair formation system of conjugative plasmids - A versatile secretion machinery for transfer of proteins and DNA. *Plasmid*. *54*(1):1-25

Schwering, M, Song, J, Louie, M, Turner, R. J, & Ceri, H. (2013). Multi-species biofilms defined from drinking water microorganisms provide increased protection against chlorine disinfection. *Biofouling*. *29*(8):917-28.

Sen, D, Brown, C. J, Top, E. M, & Sullivan, J. (2013). Inferring the evolutionary history of IncP-1 plasmids despite incongruence among backbone gene trees. *Molecular biology and evolution*, *30*(1), 154–166.

Shahcheraghi, F, Feizabadi, M. M, Yamin, V, Abiri, R, & Abedian, Z. (2003). Serovar determination, drug resistance patterns and plasmid profiles of *Pseudomonas aeruginosa* isolated from burn patients at two hospitals of Tehran (IRAN). *Burns*, *29*(6), 547–551.

Shahid, M, Malik, A, & Sheeba. (2003). Multidrug-resistant *Pseudomonas aeruginosa* strains harbouring R-plasmids and AmpC β-lactamases isolated from hospitalised burn patients in a tertiary care hospital of North India. *FEMS Microbiology Letters*.228 (2), 181-186.

Shi, P, Jia, S, Zhang, X. X, Zhang, T, Cheng, S, & Li, A. (2013). Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water. *Water Research*, *47*(1), 111–120.

Shintani, M, Takahashi, Y, Yamane, H, & Nojiri, H. (2010). The Behavior and Significance of Degradative Plasmids Belonging to Inc Groups in *Pseudomonas* within Natural Environments and Microcosms. *Microbes and Environments*, *25*(4), 253–265.

Shrivastava, R, Upreti, R. K, Jain, S. R, Prasad, K. N, Seth, P. K, & Chaturvedi, U. C. (2004b). Suboptimal chlorine treatment of drinking water leads to selection of multidrug-resistant *Pseudomonas aeruginosa*. *Ecotoxicology and Environmental Safety*, *58*(2), 277–283.

Sia, E. A, Roberts, R. C, Easter, C, Helinski, D. R, & Figurski, D. H. (1995). Different

relative importances of the par operons and the effect of conjugal transfer on the maintenance of intact promiscuous plasmid RK2. *Journal of Bacteriology*, *177*(10), 2789–2797.

Siarkou, V. I, Vitti, D, Protonotariou, E, Ikonomidis, A, & Sofianou, D. (2009). Molecular epidemiology of outbreak-related *Pseudomonas aeruginosa* strains carrying the novel variant blaVIM-17 metallo-β-lactamase gene. *Antimicrobial Agents and Chemotherapy*, *53*(4), 1325–1330.

Siguier, P, Gourbeyre, E, & Chandler, M. (2014). Bacterial insertion sequences: Their genomic impact and diversity. *FEMS Microbiology Reviews*, *38*(5), 865–891.

Simon, R, Priefer, U, & Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in Gram negative bacteria. *Bio/Technology*, *1*(9), 784–791.

Skyberg, J. A, Siek, K. E, Doetkott, C, & Nolan, L. K. (2007). Biofilm formation by avian *Escherichia coli* in relation to media, source and phylogeny. *Journal of Applied Microbiology*. *102*(2) 548-554.

Smalla, K, Haines, A. S, Jones, K, Krögerrecklenfort, E, Heuer, H, Schloter, M, & Thomas, C. M. (2006). Increased abundance of IncP-1β plasmids and mercury resistance genes in mercury-polluted river sediments: First discovery of IncP-1β plasmids with a complex mer transposon as the sole accessory element. *Applied and Environmental Microbiology*. *72*(11) 7253-7259

Smith, K, & Hunter, I. S. (2008). Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. *Journal of Medical Microbiology*, *57*(8), 966–973.

Smith, C. A, & Thomas, C. M. (1983). Deletion mapping of kil and kor functions in the *trfA* and trfB regions of broad host range plasmid RK2. *Molecular & General Genetics : MGG*, *190*(2), 245–254.

Sørensen, S. J, Sørensen, A. H, Hansen, L. H, Oregaard, G, & Veal, D. (2003). Direct Detection and Quantification of Horizontal Gene Transfer by Using Flow Cytometry and gfp as a Reporter Gene. *47*(2):129-33.

Sorlini, S, Gialdini, F, Biasibetti, M, & Collivignarelli, C. (2014). Influence of drinking water treatments on chlorine dioxide consumption and chlorite/chlorate formation. *Water Research*, *54*, 44–52.

Srinivasan, A, Bova, G, Ross, T, Mackie, K, Paquette, N, Merz, W, & Perl, T. M. (2003). A 17-Month Evaluation of a Chlorine Dioxide Water Treatment System to Control *Legionella* Species in a Hospital Water Supply . *Infection Control & Hospital Epidemiology*. (8), 575-9

Stalder, T, Barraud, O, Casellas, M, Dagot, C, & Ploy, M.C. (2012). Integron Involvement in Environmental Spread of Antibiotic Resistance. *Frontiers in Microbiology*, *3*, 119.

Stewart, P. S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology*. *292*(2),107-13.

Stokes, H. W, Elbourne, L. D. H, & Hall, R. M. (2007). Tn1403, a multiple-antibiotic resistance transposon made up of three distinct transposons. *Antimicrobial Agents and*

Chemotherapy, *51*(5), 1827–1829.

Stokes, H. W, Nesbø, C. L, Holley, M, Bahl, M. I, Gillings, M. R, & Boucher, Y. (2006). Class 1 integrons potentially predating the association with tn402-like transposition genes are present in a sediment microbial community. *Journal of Bacteriology*, *188*(16), 5722–5730.

Stover, C. K, Pham, X. Q, Erwin, A. L, Mizoguchi, S. D, Warrener, P, Hickey, M. J, … Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, *406*(6799), 959–964.

Strand, T. A, Lale, R, Degnes, K. F, Lando, M, & Valla, S. (2014). A new and improved host-independent plasmid system for RK2-based conjugal transfer. *PLoS ONE*. 9(3), e90372.

Sündermann, A, Reif, M. M, Hofbauer, S, Obinger, C, & Oostenbrink, C. (2014). Investigation of Ion Binding in Chlorite Dismutases by Means of Molecular Dynamics Simulations. *Biochemistry*, *53*(29), 4869–4879.

Suyama, T, Shigematsu, T, Takaichi, S, Nodasaka, Y, Fujikawa, S, Hosoya, H, … Hanada, S. (1999). *Roseateles depolymerans* gen. nov, sp. nov, a new bacteriochlorophyll a-containing obligate aerobe belonging to the β-subclass of the Proteobacteria. *International Journal of Systematic and Evolutionary Microbiology*, *49*(2), 449–457.

Tamashiro, E, Antunes, M. B, Palmer, J. N, Cohen, N. A, & Anselmo-Lima, W. T. (2009). Implications of bacterial biofilms in chronic rhinosinusitis. *Brazilian Journal of Infectious Diseases*. *13(3) 232-235*

Tao, H .E, Xue-li, E, Wang Hong-wei, et al. (2008). Development of Chlorite and Influencing Factors in Drinking Water Treatment with Chlorine Dioxide, *Journal of Environment and Health*, *2*(25), 101–103

Taylor, P. K, Yeung, A. T. Y, & Hancock, R. E. W. (2014). Antibiotic resistance in *Pseudomonas aeruginosa* biofilms: Towards the development of novel anti-biofilm therapies. *Journal of Biotechnology*. 191, 121-130.

Taylor, R. H, Falkinham, J. O, Norton, C. D, & LeChevallier, M. W. (2000). Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. *Applied and Environmental Microbiology*, *66*(4), 1702–1705.

Tennstedt, T, Szczepanowski, R, Krahn, I, Pühler, A, & Schlüter, A. (2005). Sequence of the 68,869 bp IncP-1α plasmid pTB11 from a waste-water treatment plant reveals a highly conserved backbone, a Tn402-like integron and other transposable elements. *Plasmid*, *53*(3), 218–238.

Thomas, C. M. (2014). Acquisition And Spread Of Antibiotic Resistance Genes By Promiscuous Plasmids. *Encyclopedia of Genetics***,** 119.

Thorsted, P. B, MacArtney, D. P, Akhtar, P, Haines, A. S, Ali, N, Davidson, P, Thomas, C. M. (1998). Complete sequence of the IncPβ plasmid R751: Implications for evolution and organisation of the IncP backbone. *Journal of Molecular Biology*. *282*(5), 969-990.

Trautmann, M, Lepper, P. M, & Haller, M. (2005). Ecology of *Pseudomonas aeruginosa* in the intensive care unit and the evolving role of water outlets as a reservoir of the

organism. *American Journal of Infection Control*, 33 (5 Suppl 1), S41-9.

Trautmann, M, Michalsky, T, Wiedeck, H, Radosavljevic, V, & Ruhnke, M. (2001). Tap Water Colonization With *Pseudomonas aeruginosa* in a Surgical Intensive Care Unit (ICU) and Relation to *Pseudomonas* Infections of ICU Patients . *Infection Control & Hospital Epidemiology*, *22*(1), 49–52.

Tredget, E. E, Shankowsky, H. A, Mark Jofie, A, Inkson, T. I, Volpel, K, Paranchych, W, Burke, J. F. (1992). Epidemiology of infections with *Pseudomonas aeruginosa* in burn patients: The role of hydrotherapy. *Clinical Infectious Diseases*, *15*(6), 941–949.

Tsai, C. T, & Lin, S. T. (1999). Disinfection of hospital waste sludge using hypochlorite and chlorine dioxide. *Journal of Applied Microbiology*, *86*, 827-833.

Umile, T. P, & Groves, J. T. (2011). Catalytic generation of chlorine dioxide from chlorite using a water-soluble manganese porphyrin. *Angewandte Chemie - International Edition*. 5, 695-698.

Van Nevel, S, De Roy, K, Boon,.N.(2013). Bacterial invasion potential in water is determined by nutrient availability and the indigenous community, *FEMS Microbiology Ecology*, 85:3, 593–603

Vezina, G, & Levesque, R. C. (1991). Molecular characterization of the class II multiresistance transposable element Tn1403 from *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, *35*(2), 313–321.

Walker, J, & Moore, G. (2015). *Pseudomonas aeruginosa* in hospital water systems: Biofilms, guidelines, and practicalities. *Journal of Hospital Infection*, 89, 324–327.

Walker, J. T, Jhutty, A, Parks, S, Willis, C, Copley, V, Turton, J. F, Bennett, A. M. (2014). Investigation of healthcare-acquired infections associated with *Pseudomonas aeruginosa* biofilms in taps in neonatal units in Northern Ireland. *Journal of Hospital Infection*, *86*(1), 16–23.

Walker, J. T, Mackerness, C. W, Mallon, D, Makin, T, Williets, T, & Keevil, C. W. (1995). Control of *Legionella pneumophila* in a hospital water system by chlorine dioxide. *Journal of Industrial Microbiology*, *15*(4), 384–390.

Wang, X, Zheng, P, Ma, T, & Song, T. (2018). Small Universal Bacteria and Plasmid Computing Systems. *Molecules*, *23*, 1307.

Wellington, E. M. H, Boxall, A. B. A, Cross, P, Feil, E. J, Gaze, W. H, Hawkey, P. M, Williams, A. P. (2013). The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infectious Diseases*, 13, 155–165.

Whittle, G, & Salyers, A. A. (2003). Bacterial Transposons—An Increasingly Diverse Group of Elements. In *Modern Microbial Genetics* (385–427).

Workentine, M. L, Sibley, C. D, Glezerson, B, Purighalla, S, Norgaard-Gron, J. C, Parkins, M. D, Surette, M. G. (2013). Phenotypic Heterogeneity of *Pseudomonas aeruginosa* Populations in a Cystic Fibrosis Patient. *PLoS ONE*. *8*(4), e60225

Xu, J, & Logan, B. E. (2003). Measurement of chlorite dismutase activities in perchlorate

respiring bacteria. *Journal of Microbiological Methods*, *54*(2), 239–247.

Young, S. B, & Setlow, P. (2003). Mechanisms of killing of *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. *Journal of Applied Microbiology*, *95*(1), 54–67.

Zhang, T, Zhang, X. X, & Ye, L. (2011). Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *PLoS ONE*.

Zhang, Y, Lu, J, Guo, J, Wang, Q, Lian, J, Wang, Y,Yang, J. (2016). Isolation and characterization of a perchlorate-reducing *Acinetobacter bereziniae* strain GWF. *Biotechnology and Biotechnological Equipment*, *30*(5), 935–941.

Zhong, X, Droesch, J, Fox, R, Top, E. M, & Krone, S. M. (2012). On the meaning and estimation of plasmid transfer rates for surface-associated and well-mixed bacterial populations. *Journal of Theoretical Biology*, *294*, 144–152.