The prevalence and potential mobility of Tn*21*-like mercury resistance transposons in *E. coli* isolated from dairy cattle slurry

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Abstract

The risk of antimicrobial resistance as well as antimicrobial resistant infections arising from agricultural environments is widely debated. Through the movement and acquisition of plasmids, which carry complex and varying resistance gene cassettes, there is an undeniable rise in resistances occurring across the world though the impact of this movement needs to be evaluated.

A total of 926 *E. coli* isolates that had been cultured from slurry samples and from the slurry tank on the Sutton Bonington dairy unit using Tryptone-Bile X-glucuronide (TBX) growth media with or without cefotaxime, or isolated using CHROMagar ESBL media, were tested for mercury and antibiotic resistance. Seven *E. coli* isolates were phenotypically mercury resistant and confirmed by PCR to carry *merA, merC, merR* and *intI*, suggesting they carried a Tn21-like transposon. All isolates were tested for phenotypic resistance to 21 antibiotics representing 15 antibiotic classes. All the isolates found to be mercury resistant were found to be resistant to at least 4 antibiotics, with 2 isolates taken from the slurry tank being resistant to 6 antibiotics and those taken from the muck heap being resistant to 4.

Potential mobility of the Tn*21* like transposons was determined using transposon trapping using RP4-8 using gentamicin resistance as a phenotypic marker. Only two of the isolates out of seven showed transposons which were able to move into RP4-8 and then into *E. coli* K12 MG6155 strains.

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1. Introduction

The word antibiotic simply describes a molecule produced by an organism which inhibits the growth of other microbes, which can happen in a variety of different ways (Clardy et al, 2009). Antimicrobial however is another term which is frequently used but differs in that it refers to any substance that kills or inhibits the growth of bacteria. Antibiotic or antimicrobial resistance occurs when the antimicrobial in question loses its ability to inhibit bacterial cell growth. In the context of bacterial resistance, and their ability to survive, both terms have been used interchangeably however there is a very large difference between the two. Antimicrobials cover antibiotics such as penicillin, biocides, heavy metals and a wide range of other substances whereas antibiotic only covers those substances produced by bacteria.

Antibiotics have been used therapeutically for decades in humans, being used to treat, or prevent bacterial infections, in surgery to prevent post-surgical infections. Antibiotics are also used widely in agriculture to treat or prevent infections, as growth promoters in livestock, in fish farming and in fruit production. Antibiotics kill or inhibit the growth of bacteria, the two most common methods of this are by interrupting cell wall synthesis (e.g. β-lactams) or protein synthesis (e.g. streptomycin), however membrane disruption (Aztreonam), DNA replication interruption (e.g. quinolones) and Folic acid metabolism (trimethoprim) are other ways in which bacterial growth can be tackled (Kohanski et al, 2010).

Antimicrobial resistance (AMR) is a widespread and well-known threat with the World Health Organisation (WHO) classing it as a threat to global health, however resistance genes have been within bacteria for thousands if not millions of years (Filippova et al, 2014). The presence of antimicrobial resistant microorganisms is not an unnatural phenomenon; however, the increasing prevalence of antimicrobial resistant bacteria, particularly multi-drug resistance is being driven by antimicrobial exposure in health care, agriculture, and the environment. This problem is then made worse by poor standards of infection control, sanitation, access to clean water, and access to quality assured antimicrobials and diagnostics coupled with increased travel as well as migration (Holmes et al, 2016). The

increasing levels of antimicrobial resistance being seen in soil, water, animals and in clinical settings could be blamed on the overuse of antibiotics in agriculture, as well as human use. There is substantial evidence to suggest that with over 70% of all antibiotics in the US being used in agriculture and in other countries approximately 50% of total consumption is for agricultural use, that localised resistances are occurring in farms and spreading outward (O ’Neill, 2015).

A key area of interest and research is the co-selection of antimicrobial resistance genes in bacteria. These genes can confer resistance to antibiotics, biocides commonly used in hospitals, agriculture and industry for cleaning and even to toxic metals which have been used in medicine, dentistry, or agriculture (Ventola, 2015). These metals include silver, copper, zinc, arsenic, and the focus of this research, mercury. Mercury is no longer used as an antimicrobial, due to its toxicity. It is unclear whether the wide dissemination of mercury resistance operons is due to the human use of mercury and its persistence in the environment, the use of other antimicrobial metals, such as silver and copper in clinical and agricultural settings co-selecting for mobile genetic elements carrying mercury resistance, co-selection of mercury resistance operons that also carry antibiotic and biocide resistance, or from the natural persistence of mobile genetic elements that carry mercury resistance operons (Yazdankhah et al, 2014).

In agricultural environments such as slurry tanks, antibiotics at sub inhibitory levels can be found, depending on how the farm is being run and the treatment of the animals. It is thought that the constant presence of even sub optimal levels of antibiotics can lead to the selective pressure to keep and maintain resistance genes which confer mercury and Sulphonamide resistance (Pal et al, 2015). Mathematical models of slurry tanks, which can fill to 3 million litres, have predicted that if these waste reservoirs are not emptied then this could cause a selective pressure for resistant bacteria (Baker et al, 2016). This research also postulates that these environmental changes would increase the amount of horizontal gene transfer occurring in the slurry tank which would then lead to greater resistance. Despite this

research being mathematical and based on theoretical models it does show the potential effects that antimicrobial resistance could have on an isolated microbial population.

Antimicrobial resistance has become an increasing threat to public health and is a global priority recognised by World Health Organisation (Prestinaci, et al, 2015). Despite this, and the wealth of literature on resistance from clinical settings leading to a possible “post antibiotic” era, there are still gaps in our knowledge. These gaps include what potential threat to human health could arise from agriculture due to the co-selection of toxic metal resistance (mercury) and antibiotic resistances (Seiler and Berendonk, 2012).

1.0.1 Antibiotic resistance

Antibiotic resistance has been seen in a large number of different bacterial species and through a variety of different mechanisms including: efflux of the active antibiotic out of the cell, changes in permeability of the cell wall, modification of the antibiotic through the use of enzymes, antimicrobial degradation, the use and acquisition of different metabolic pathways to the one that the antibiotic affects, antibiotic target modification and the overproduction of the target enzyme (Magnet and Blanchard, 2005). One method of resistance and a major contributor to the growing resistance to antibiotics comes from intrinsic resistance in the form of the LPS in Gram-negatives. There are also several other broad-spectrum efflux pumps, including major facilitator families, multidrug and toxic efflux, resistance nodulation and division family, small multidrug resistance family pumps and efflux systems such as the TolC and MerC which appear to contribute greatly to resistance in Gram-negative bacteria and a few Gram-positives (Webber & Piddock, 2003). The variety and sheer number of these pumps indicates their importance in the environment and in bacteria in protecting against toxic molecules. Their wide distribution is consistent with these efflux pumps ancient nature (Alcalde-Rico et al, 2016).

Penicillin was the first natural antibiotic discovered and used for treatment. Since its discovery numerous other β- lactams have been discovered such as the cephalosporins and carbapenems (van Hoek et al, 2011). Semi synthetic penicillin’s (e.g amoxicillin) were

developed to be a broad-spectrum antibiotic that could withstand the stomach acid in the gastrointestinal tract. This ability produces higher levels of antibiotic in the blood and urine than does its counterpart, ampicillin. Carbapenems such as imipenem and meropenem can bind to penicillin binding proteins preventing synthesis of the bacterial cell wall. Carbapenems affect both Gram-negatives and Gram-positives and so are known as broad spectrum antibiotics (Papp-Wallace et al, 2011). The β-lactam class of antibiotics works specifically by binding to penicillin-binding proteins and inhibiting cell wall synthesis. This in turn interferes with the interlinking of peptidoglycan and leads to a decrease in structural integrity finally leading to a cell death (Andes and Craig, 2005).

Other examples of antibiotics with a wide occurrence of bacterial resistance are the sulphonamides, the oldest synthetic drugs, being implemented in Germany by Domagk in 1933 (Brownlee, 1949). Sulfamethoxazole is commonly used, however trimethoprim, a pyrimidine inhibitor of dihydrofolate reductase, is an antibacterial related to pyrimethamine which is potentiated by sulphonamides and the trimethoprim. Sulfamethoxazole drug combination including trimethoprim is the form most often used. Due to the complementary effects each has this combination reduces the occurrence of resistance to each antibiotic (Grape, 2006). Sulphonamide competitively inhibits the enzyme dihydropteroate synthase (DHPS) which is part of the folate biosynthetic pathway, which is essential to produce thymine and consequently in bacterial cell growth (Roberts, 2002). Resistance to this synthetic antibiotic was seen soon after its implementation. As it is a synthetic compound modification or degradation by natural means were not to be expected, however chromosomal sulphonamide resistance occurs at low levels through the *folP* gene which encodes DHPS. This is through point mutations such as a 2-aa insertion in DHPS of both *Neisseria meningitidis* and *Streptococcus pneumoniae* which was found to confer sulphonamide resistance (Lopez et al, 1987). Acquired resistance to sulphonamides was found in the 1960's but plasmid mediated resistance through the genes, *sul1* and *sul2*, was later found in the 1980's (Sundström et al, 1988). Resistance has now been reported for

almost all antibiotics in a wide variety of bacterial species. Penicillin was first prescribed in the 1940's and by the 1950's bacteria had already become resistant, leading to the loss of the progress that pharmaceuticals had taken previously (Sengupta et al, 2013). Vancomycin was introduced in 1972 and by 1979 vancomycin resistance was reported in coagulase-negative staphylococci (Sengupta et al,2013).

During the 1950's and 1960's tetracycline was widely used as it was effective at treating intracellular and extracellular pathogens. Tetracycline was particularly useful to treat outpatients as it was cheap, could be taken orally and had few side effects. Despite this, currently tetracycline and oxytetracycline have limited use but are used in the treatment of nongonococcal urethritis and other chlamydial infections (Toomey and Barnes, 1990). Apart from medicinal uses oxytetracycline has also been used as an additive to livestock feed as it stimulates weight gain in some domestic animals such as pigs (Chopra et al, 1981). Bacteria can be resistant to tetracyclines in one of three ways: limiting the access of tetracycline to the ribosomes, altering the ribosome to prevent the binding of tetracycline, and producing tetracycline-inactivating enzymes (Speer et al, 1992). In the 1950's chloramphenicol was also widely in use in treating conjunctivitis. This broad-spectrum antibiotic produced by *Streptomyces* has been in limited use since (Rose et al, 2005). Resistance to chloramphenicol can occur by enzymatic inactivation by acetylation mainly by acetyltransferases or, in some cases, by chloramphenicol phosphotransferases. Resistance is also available through target site mutation/modification, decreased outer membrane permeability, and the presence of efflux pumps (Fernández et al, 2012).

In 1959 methicillin was used as a substitute to benzylpenicillin, as it has the phenol group of benzylpenicillin disubstituted with methoxy groups to tackle *S. aureus* that had become resistant (Chambers, 1997). In 1961 however, there were reports from the United Kingdom of *S. aureus* isolates that had acquired resistance to the newly introduced methicillin which are now referred to as MRSA. This form of resistance comes from the *mecA* gene which is thought to have been acquired from a distantly related species and encodes a methicillin-

resistant penicillin-binding protein. The *mecA* gene is carried on a mobile genetic element known as the staphylococcal cassette chromosome mec (SCCmec), of which four forms varying in genetic size have been found and contribute to multidrug resistance in MRSA (Saber et al, 2017).

Aminoglycosides are highly potent broad-spectrum antibiotics that include gentamicin, streptomycin, kanamycin and tobramycin. Gentamicin and other aminoglycosides are known to exert both a bacteriostatic and bactericidal effect to prevent bacterial growth. Aminoglycosides act by preventing bacterial protein synthesis by binding to prokaryotic ribosomes (Mingeot-Leclercq et al, 1999). The first of the aminoglycosides was streptomycin in 1944 and subsequently led to the discovery of the others. Within six years of aminoglycoside production, resistant strains of *S. aureus* were discovered (Zaman et al, 2017).

The WHO has selected 12 priority bacterial species which are seen to be a threat to world health and need new antibiotics to combat them. These species are separated into three categories: Critical, High and Medium. In the critical category is *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* all of which have been observed to be carbapenem resistant. Next in the high-risk category are *Enterococcus faecium, Staphylococcus aureus, Helicobacter pylori, Campylobacter, Salmonella spp.* and *Neisseria gonorrhoeae*, which have been seen to carry vancomycin, clarithromycin and fluoroquinolone resistance. Finally, in the medium risk category are *Streptococcus pneumoniae, Haemophilus influenzae* and *Shigella spp.* which are ampicillin and fluoroquinolone resistant. These priority pathogens were selected based on several factors including a ten-year trend of resistance and transmissibility (Govindaraj and Vanitha, 2018).

The societal cost of antimicrobial resistance is a key concern in combating the rising tide of resistance (Michaelidis et al, 2016). For each case of resistance that occurs the cost of treating the infection will increase and over the years as reviews have been written to ascertain the effects of resistance on society and economics it is becoming ever clearer that

antimicrobial resistance is a burden to society. This is especially shocking when it has been estimated that over $20 billion could be lost in America alone (Diazgranados et al, 2005). By 2050 this could reach a global loss of $100 trillion (O'neil, 2014).

1.0.2 Biocides

Biocide resistance is also thought to contribute to the AMR problem. Biocides have been used for centuries with accounts of their use in wound treatment being recorded since 1867 (Lister, 1867). These powerful antibacterial products include chemicals such as Chlorine and Ethanol as well as many others that provide a wide range of uses such as antifouling agents and disinfectants. The 20th century saw a plethora of new chemicals being used for disinfection, sterilization, and preservation with the development of quaternary ammonium compounds and cationic biocides (Russell, 1999). The concentration of the biocide in a formula is of the utmost importance, particularly finding a balance between its efficacy and its toxicity. This is clear in the health care environment where three levels of disinfection are required depending on the risk of transmission to other patients and the rate of microbial survival, these three levels are low, intermediate and high (Rutala and Weber, 1999).

The increased usage of biocides such as phenolics at low concentrations has raised concerns about the efficiency of the products in use. Concerns are also raised about the potential for these sub lethal doses of biocides to allow for biocide resistant bacteria to emerge. In fact, several studies have been published relating to this concern and the emergence of biocide and antibiotic resistant bacteria due to the presence of sub lethal doses within the environment (Moken et al, 1997) (Thomas et al, 2000). As such the consensus is that biocides should be considered carefully in relation to their use in any health care environment (Maillard, 2005). Understanding of the mechanisms of how biocides act is lacking and thus research on the effects of bacterial resistance towards biocides is disappointing (Russel, 2002). This gap in understanding of the effects comes in part from the fact that several factors affect how the biocide works. Some of these factors are inherent to the biocide and others to the microorganism being affected, which can include: pH,

temperature, Organic load, type of organism and presence of a biofilm (Jean-Yves Maillard, 2005). Additionally, most biocides, unlike antibiotics, do not have a specific cell target and so resistance does not emerge from target gene mutations. When resistance does occur, it's mediated by mechanisms that are poorly characterized such as efflux pump up regulation and changes to the structure of the cell wall (Poole, 2002).

Cross resistance between biocides and antibiotics is thought to occur in four ways. The first of these being when the biocide and antimicrobial compound work on the same target such as the cell wall. The second, when both have the same transport mechanism such as through cellular porins. The third, where both biocide and antibiotic can be accommodated by the same mechanism of resistance and the fourth being where antibiotic and biocide resistance genes are carried on the same mobile genetic element (Poole, 2004). Though there are many studies and reports looking at this naturally occurring co-selection one large scale study conducted by Pal *et al* (2015) provided a comprehensive visualisation for the patterns of resistance occurring between biocides, metals and antibiotics (Seiler and Berendonk, 2012). This look into carriage and linkage between resistance genes on plasmids, however, yielded little novel information and insight into previously unknown patterns of transmission. This potentially shows that there is little opportunity for biocides and metals to promote horizontal gene transfer of antibiotic resistance but conversely the study showed ample opportunities for the chemicals involved to promote the selection of antibiotic resistant bacteria through chromosomal biocide/metal resistance genes (Pal et al, 2015). Despite this however the evidence still shows that there is the possibility for biocides to establish relationships with other resistance genes. These associations can be made through linkage and co-carriage of antimicrobial resistance genes on mobile genetic elements which helps in their propagation.

1.0.3 Metal ion Resistance

Metals have been used in human therapy for centuries, with examples of their use including Victorians using mercury to treat syphilis and the Romans using zinc tablets for eye

treatments, dating back to 140 BC (Giachi et al, 2013). Despite their toxicity to humans some of these metals are still used today, such as zinc, copper, silver, and arsenic (Jaishankar et al, 2014). Metals have been used by humans for thousands of years but were released into the biosphere millions of years ago, when the Earth became oxygenic. The ability of bacteria to resist the effects of these metals has gone on for much longer than human usage, with permafrost samples showing resistance genes dating back to between five thousand to one million years ago (Kashuba et al, 2017). Copper has been used as an antifungal and in agriculture as an antimicrobial as well as for the reduction in microbial populations found in hospitals by lining surfaces with the metal (Marais et al, 2010). Copper is essential in many cellular processes and acts as an electron donor or acceptor in enzymes, however it can also take part in Fenton-like reactions in the generation of hydrogen peroxide and superoxide (Grass et al, 2011). Silver has no known beneficial effects in biology and is the second most toxic metal to *E. coli.* In the 17th to 19th centuries silver nitrate was used to treat ulcers. However due to the introduction and popularity of sulphonamides in the 1930's and antibiotics in the 1940's silver has become redundant and is very rarely used in medical treatment apart from burn treatments and in silver impregnated dressings (Klasen, 2000). Mercury, like silver, has no known positive biological effect and despite being used to treat syphilis and its use in diuretics, its toxicity was its most notable feature and so lead to its almost complete reduction in use, apart from in dental amalgam, which typically contains 43–54 % elemental mercury (Franke, 2007). In relation to agriculture farms are very unlikely to come across any form of mercury in the environment though mercury was used commonly from the late 19th century to the 1970's to treat plant disease with aryl, aloxyl and alkyl organomercurials being used as antifungals.

The above list is but a small group of the wide variety of metals still being used and should be considered a very small insight into the previous and present uses of metals in medical therapy. Despite the historical use of metals, the gaps in our knowledge of how they kill bacterial cells are yet to be filled. However, it is understood that the chemistry of the metal in

question drives its function and thus gives it its effects on biological compounds. The mechanisms of how these metals work is generally considered to come from the affinity these metals have for the biological components within the cell. An example of these toxic effects is through displacing essential metals in enzymes key for cellular function, by participating in chemical reactions in the cell that are harmful and by binding to the functional groups contained in biological molecules. These all contribute to damage to the DNA, proteins, cell wall, enzyme function and cellular processes within the cell (Nies, 1999). Despite the discontinuation in the use of mercury as an antimicrobial, it is not known why mercury resistance is still being found in agriculture after decades of it not being used. It could simply be a matter of that it is naturally occurring within bacterial species or potentially localised pockets of selection and evolution.

1.0.4 Mobile Genetic Elements

Of the mobile genetic elements that are carriers of resistance genes, plasmids represent a huge pool of diversity that bacteria can draw from and recombination is a key and essential driver of evolution in these genetic elements allowing for the adaption of these resistances (Rankin et al, 2011). As an example of genetic co-selection, the plasmid *R100*, was not only found to encode mercury resistant but also conferred resistance to streptomycin, sulphonamides, tetracycline and chloramphenicol. What we could begin to see a lot more of however is the variance in antibiotic resistant genes being carried on the same mobile genetic element such as in animal manure and water contaminated with antibiotics (Sun et al, 2016). We know that Tn*21* commonly carries resistance to sulphonamides and quaternary ammonium compounds. As Tn*21* is disseminated however, there is a high probability that it will pick up resistance genes to other antibiotics that it would not usually carry. This would produce unique resistomes within the slurry tanks as shown by genetic analysis in a study by Mindlin (2002) which showed that many transposons are recombinants originating from interactions between related and unrelated mobile elements.

1.0.5 Co-selection

Co-selection of antibiotic and metal resistance genes is primarily important due to co-selection promoting and maintaining resistance in bacterial populations found in the environment in the absence of antibiotics (Pal et al, 2017). It may be due to co-selection that we are seeing in the mercury resistant isolates found in agricultural settings. Mercury resistance has been found linked with resistance genes to colistin as well as tetracycline, chloramphenicol, ampicillin and sulphonamide, with colistin resistance being found in pig farms from China (Campos et al, 2016). The ability for bacteria to pick up genes from mobile genetic elements and for them to be selected for on transposable elements alongside the specific genes being specifically selected for may be very important in selecting for multiple resistance.

Multi drug resistant *Shigella spp.* isolated from Japan in the 1950's was an omen for the MDR *Enterobacteriaceae* and were found less than 7 years after the first use of antibiotics (Fair and Tor, 2014). It is now our understanding that this co-occurrence of resistance is not just happening by chance but is being specifically selected for by mobile genetic elements carrying these genes (Gaze et al., 2011). One study looking at the effects of copper on the prevalence of co-selective resistance in water found that after just 6 hours of exposure the bacterial levels of resistance genes increased by at least one-fold. 117 antimicrobial-resistance genes were detected from 12 types of genetic elements of which most markedly were integrons and transposons, leading to significantly increased bacterial resistance to the antibiotics tested, including rifampin, erythromycin, kanamycin and others (Zhang et al, 2018).

Mobile Genetic elements such as plasmids, transposons and integrons are the cause of movement between bacterial species however, again, there are still questions left unanswered by recent developments and findings in antimicrobial resistance. Agents with the potential for co-selection of antibiotic resistance include biocides, heavy metals and antibiotics. Differences between these agents and their target of action would initially lead to

an assumption that there is very little in common between the different agents and the altered susceptibility of microorganisms. Point mutations in bacteria can lead to them becoming resistant to antibiotics, however similar mechanisms are seen only rarely against biocides with the mutation and upregulation of *fabI* in relation to triclosan being an example (Rensch et al, 2013). Experimental evidence in *S. aureus* which holds a variety of chromosomal and plasmid-borne efflux pumps, and laboratory chromosomal efflux mutants to single-agent biocides do present co-selection for reduced sensitivity to fluoroquinolone (Huet et al, 2008). However, these findings in respect of *S. aureus* biocide-antibiotic co-resistance have been highly variable between different studies, between strains and between the antimicrobial agents used (Sheldon, 2005).

Mechanisms of co-selection for heavy metal resistance have been seen that are like those of biocides such as extracellular adaptations: cell envelope changes, efflux and global regulatory responses, plus co-resistance via gene linkage (Seiler and Berendonk, 2012). Other differences include metal use in agriculture which is usually over a long period of time and at sub lethal levels. Pathogenic bacteria and copper in waste can mix and cause antimicrobial resistance gene acquisition but this waste when mixed with the soil microbiota can cause a particularly advantageous scenario for the development and transfer of multiple resistance determinants (Seiler and Berendonk, 2012). To contrast the assumption that the presence of metals induces co-selection, some metals, lead and mercury, have been associated with counter-selection leading to reduced antibiotic resistance in bacterial populations (Hölzel et al, 2012). Despite a lot of data describing correlations between the presence of metals and antibiotic resistance evidence for causal links are hard to find. An exception to this is provided by Stepanauskas *et al* (2006) who reported that microorganisms from river environments which were subjected to heavy metal stresses such as cadmium, showed significantly increased frequencies of resistance to antibiotics, compared to microorganisms from water that did not contain metals.

1.1 Mercury Toxicity

Mercury resistance was the first toxic metal resistance to be found and is arguably one of the most well understood. Mercury is one of the most toxic metals and is particularly known for its potency, with no known beneficial effects to organic life. Despite this toxicity it has not prevented humans from using it, in medicine as a treatment for infections (e.g. mercury (II) chloride for syphilis) or as a prophylactic (eyedrops for new born children to prevent syphilitic eye infections) or in agriculture to control plant diseases from the late nineteenth century until the 1970's. Aryl-aloxyl-, and alkyl- organomercurials became widely used in the 1950's, used as antifungal seed dressings, and as pesticides and fungicidal sprays (Huisingh, 1974). Mercury and its salts are also used such as Mercury (I) chloride which is used in medicine in acousto-optical filters and in electrochemistry (Rustagi and Singh, 2010). Despite the wide array of uses in previous decades mercury is now very rarely used as its toxicity and impact on the environment has become more widely understood.

Mercury, especially organomercury and mercuric salts, are highly toxic and can be fatal if inhaled and harmful if absorbed through the skin (Cincinnati, OH: US Environmental Protection Agency Region; 2004). An example of the potential harm of mercury once released into the environment and the toxic nature of this heavy metal comes from the Chisso corporation, which dumped methylmercury contaminated waste into Minamata bay between 1932 and 1968. This caused the suffering of over 3000 people from serious deformities, severe mercury poisoning symptoms, or death from what became known as Minamata disease, which is a neurological condition (Ministry of the Environment, Government of Japan. Minamata disease: The History and Measures. 2002).

Minamata demonstrates the toxicity of mercury to multicellular life but its strength is not lost on prokaryotic life. The only difference between the two however is that bacteria have adapted to this stress by the evolution of resistance genes. The environment and the animal microbiome present a huge pool for antimicrobial resistance, however human activity is changing the resistome present in both through contamination of water systems and soil

contamination and as this has happened the accumulation of resistance genes from use of antimicrobial agents and heavy metals compounds as therapeutics, metaphylactic, prophylactics and growth promoters has increased (Woolhouse et al, 2015). In agriculture other toxic metals are used as growth promoters to increase yields, such as copper and silver however mercury is not one of these being used yet mercury resistance genes are still present in faecal matter from pigs, chickens and cows (Pal et al., 2017). Despite its toxicity elemental mercury is still used in select practices such as in dental amalgams.

Bacteria with mercury resistance have been found in a wide variety of environments including sediment, soil, water, permafrost and from humans and animals (Robinson and Tuovinen, 1984). Mercury resistant bacteria which reduce Hg^{2+} to Hg^{0} frequently carry *mer* genes on plasmids of the isolates. However, variation in the methods of reduction may be greater than we currently know (Liebert et al, 1997). Despite a lack of understanding of the variations of mercury reduction due to differences in mercury genes in different isolates the basic method of resistance is known.

Hg^{2+} has a high affinity for proteins containing thiol groups which makes it particularly toxic to biological systems (Liebert et al., 1999). Resistance to this comes simply in the reduction of Hg^{2+} to Hg^{0} in the cytoplasm of bacteria by MerA (Mercuric reductase). This volatilisation of mercury by mercury resistant *E. coli* was first identified in 1964 when resistant strains were found to produce volatile mercury under physiological conditions (Magos et al, 1964). Shortly after this, the mechanisms of resistance to organic and inorganic mercury were elucidated leading to a clearer understanding of mercury resistance (Komura et al, 1971). The volatile mercury moves out of the cytoplasm and out of the bacteria in a vaporous form once it has been reduced to Hg^{0} and so the cell has removed the toxin by utilizing the chemistry of mercury. It is the transport into the cell of the toxic mercury, its conversion to mercury metal which is volatile at physiological conditions and subsequent volatilization out of the cell that is key to resistance. Once the cell becomes overloaded with soluble mercury ions and is

unable to reduce soluble mercury ions to mercury metal faster than the mercury is being imported in is when resistance begins to fail.

1.1.1 Gram-negative mercury resistance

The detoxification of mercury and organo-mercury compounds involves the use of a positively regulated transposon system containing essential genes. The essential genes of the generic mercury operon are taken from Gram-negative examples as these are the most widely studied. Mercury gene regulation in the mercury operon carried in Tn*21* and Tn*501* is both positively and negatively regulated by MerR which acts as a DNA binding protein, binding to a region of dyad symmetry within the mercury operator promoter region (Lund and Brown, 1987). When Hg^{2+} is present it binds to MerR causing an allosteric change in the protein linked to the operator region of the operon. This leads to an under winding of the DNA and subsequently allows improved access of RNA polymerase to the transcription start site (Ansari et al, 1995).

MerP is a small periplasmic mercury binding protein that has homology to the flexible amino terminal domain of MerA (Barkay et al, 2003). MerP next transfers Hg^{2+} to MerT, an inner membrane protein. MerT transfers Hg^{2+} into the cytosol by an inner membrane spanning protein which passes the Hg^{2+} to MerA (Boyd and Barkay, 2012). The reduction of the ionic version of mercury into its reduced form is catalysed by MerA (the mercury reductase) that uses NADPH as a reductant. After being transported inside the cytosol the Hg^{2+} is reduced by MerA and converted into volatile and insoluble Hg^{0} (Smith et al, 1998).

Both Gram-negative and Gram-positive bacterial mercury operons may encode additional mercury related genes. These commonly are MerB which functions as an organomercurial lyase, MerC and MerF which function in mercury uptake and MerE which is assumed to be a broad mercury transporter of Hg^{2+} and CH_3Hg_1 (Kiyono et al, 2009).

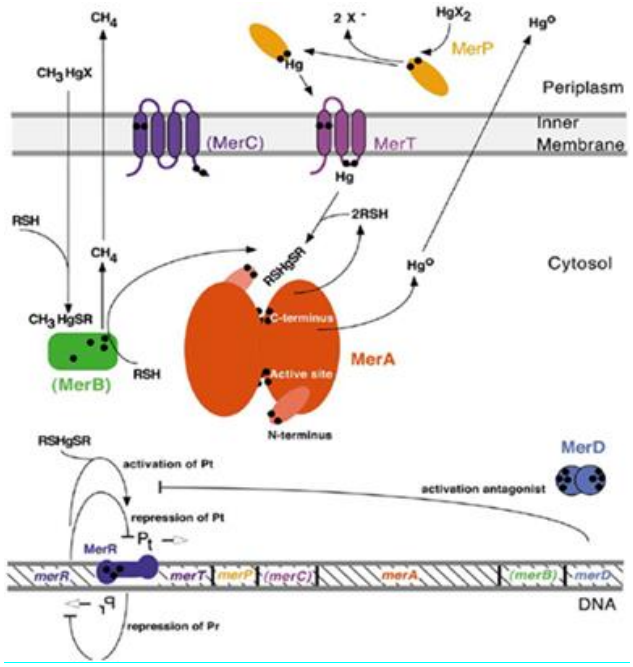


Figure 1.1. Model of Gram-negative mercury operon. Parenthesise around protein or gene names indicates the genes or proteins that are not commonly part of a standard mercury operon (Barkay et al, 2003). Permission to reproduce this Figure has been granted by Oxford University Press.

Other Gram-negative mercuric ion resistance operons also encode additional mercuric ion import proteins such as MerC in the Tn*21* mercury operon and MerF in Tn*5053* (Wilson et al, 2000) (Sahlman et al, 1997). Most research and most of our understanding of bacterial mercury resistance comes from Gram-negative models and Tn*21* and Tn*501*. Studies indicate a wide variety in diversity in mercury operons carried by Gram-negative bacteria at the DNA sequence level (Kholodii et al, 1993). The genes for mercury resistance have been in existence for thousands of years, and potentially more, as shown by permafrost samples ranging from five thousand to one million years old (Kashuba et al, 2017). This evolution and further adaptation of the mercury operon could have been from early exposure to sources of mercury and other toxic metals.

1.1.2 Gram-positive mercury resistance

Mercury resistance is arguably one of the best understood heavy metal resistances carried by bacteria due to its early discovery (Barkay et al, 2003). The first description of mercury resistance came in the 1960's, from *S. aureus* isolated from wounds (Novick and Roth, 1968). Shortly after this discovery, in 1964 Magos and co-authors showed the linkage of mercury resistance to penicillin resistance plasmids, which was followed by the finding that metal ion resistances were being carried on plasmids that carried penicillinase genes in 1967 (Novick, 1967). In this same year plasmids isolated from *Escherichia coli* and *Staphylococcus aureus* were shown to carry antibiotic resistance genes and metal ion resistance genes within the same plasmids (Hobman and Crossman, 2015).

With a few exceptions' mercury resistance operons from Gram-negative bacteria conferring the resistance genes are generally found on transposons carried on plasmids and conversely there is little evidence for plasmid-borne mercury operons in Gram-positive bacteria isolated from the environment (Shiratori et al,1989) (Belliveau & Trevors, 1990). Research also indicates that the mechanism of mercuric ion resistance in Gram-positives is mostly the same as that in Gram-negatives (Hobman and Crossman 2015). Differences can be seen between Gram-positive and negative *mer* operons in organisation of the operon structure. One such example of difference comes from comparing the mercury operon structure in *S. aureus* and *Bacillus* strain RC607 (Laddaga et al 1987). The sequences for the broad-spectrum mercury resistance from both have been identified with *S. aureus* found to contain a resistance region which contains seven open reading frames. Only two of which were readily identified by comparison with sequenced Gram-negative mercury genes. The *Bacillus* sequence however, predicts five contiguous open reading frames, one of which clearly corresponds to mercuric ion reductase (*merA*) and a sixth which is a distally located reading frame that corresponds to organomercurial lyase (*merB*).

Other examples of differences come from the Gram-positive plasmid *pI258* which is also found on *S. aureus* and on various *Bacillus* strains. The mercury resistance carried by *S.*

aureus with this plasmid contains *merR, merA, merB and merT* homologues, and some additional open reading frames, as do the *Bacillus* mercury resistance operons, both of which confer broad spectrum resistance to mercury (Chu et al, 1992). Research from Malachowa and DeLeo (2010) has shown that mercury resistance in some *S. aureus* strains is carried on the *SCC* mercury element. This *SCC/mec* element carries antibiotic resistance genes, beta-lactam resistance genes *mecA/mecC*, recombinase genes and a variety of accessory genes. Resistance in the methicillin resistant *S. aureus* is in part caused by the modified penicillin binding protein, PBP2a, which is encoded by the gene *mecA*. A second gene, *mecC* also causes methicillin/beta-lactam resistance. Both genes are notable for the fact that they cause resistance and are situated on what is potentially mobile genetic elements, the Staphylococcal Cassette Chromosome *mec* (SCCmec) (Shore et al, 2011). Toxic metal resistance markers were commonly found with these elements such as *czrC* which confers cadmium and zinc resistance. From this element found in *S. aureus* it would only take geographical dissemination for them to acquire other heavy metal resistance genes (Monecke et al, 2016).

1.1.3 Tn*21*

Tn*21* is one of the most heavily studied mercury resistances and contains the class I Integron In2, which encodes resistance to sulphonamides and streptomycin-spectinomycin (Essa et al, 2003). Tn*21* was found on the conjugative plasmid NR*1* also known as R*100,* which was isolated in Japan in 1950. In more recent years Tn*21* and similar or related plasmids have been found and isolated from *E. coli* from agricultural environments such as slurry tanks, non-clinical sources as well as mercury exposed Gram-negative gut bacteria (Sunde et al, 2009). Tn*21* is a Tn*3* subfamily transposable element which also carries the integron In*2*, located between the mercury resistance operon and the genes responsible for transposition. This integron also carries a gene encoding a protein responsible for efflux of quaternary ammonium compounds, and other small molecules, which will be discussed further in later sections.

It is Tn*21* and the closely related group of mobile genetic elements referred to as the Tn*21* family that are thought to be responsible for the increase of mercury resistance in bacteria and its dissemination worldwide (Liebert et al, 1999). The carriage is due to the integron which encodes a system of site-specific acquisition for antibiotic resistance genes. It is as such that as Tn*21* moves it accumulates an ever-increasing arsenal of resistance genes, which has the potential to contribute to the global threat of antibiotic resistance. Tn*21* also carries genes *tnpA, tnpR and tnpM* which are used for its own transposition for independent movement (De et al, 1982). It is because of this that these mobile elements have become so wide spread as the integrons being carried by Tn*21* and its relatives due to the expression systems within the integron as well as the ready mobility associated with these genetic elements. Not only are the integrons able to move when incorporated in other mobile genetic elements but the cassettes are also able to readily move which also contributes to the wide distribution of these genetic catalysts for antimicrobial resistance.

It is now thought that Tn*21* (figure 1.2.) evolved from an insertion of an In2 ancestor into an *urf2M* gene of a mercury resistance transposon as shown in figure 1.3. Deletions and gene insertions could lead to the formation of Tn*21* variations which could carry variations in antibiotic resistance. The original Tn*21* isolated in Japan from the 1950's was found to include a 1.6kb region which corresponds to IS1353 which has not been found in German isolates (Grinsted et al, 1990). This suggests that the IS1353 region found in Tn*21* a member of the IS3 family, appears to have moved into In*2* which has then moved into an early form of Tn*21* after the integron was inserted into and was acquired due to the spread of an ancestor Tn*2411* and its close relatives which does not contain the IS1353 (Brown et al, 1996). This means that In*2* is identical to In*0* apart from the fact it includes an *aadA1* gene cassette which determines resistance to streptomycin and spectinomycin which points towards variations in resistances (Clennel, 1995) (Radstrom et al, 1994).

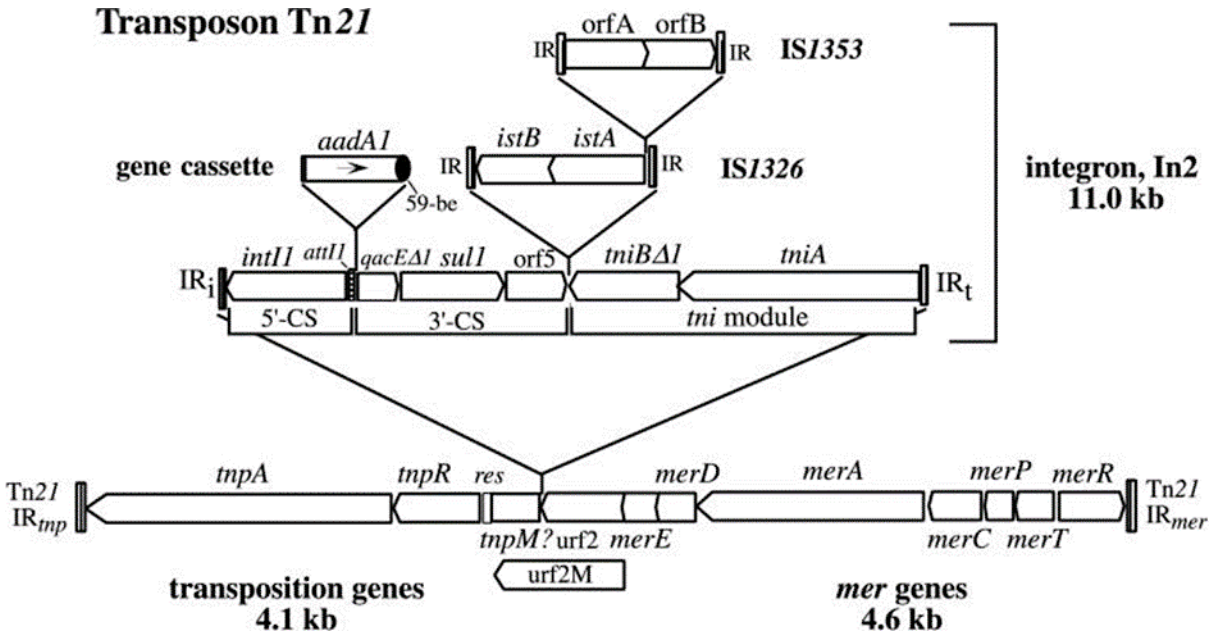


Figure 1.2. Structure of Transposon Tn*21* with symbols depicting the transposition region (Tnp) the *mer* operon and the Integron. The vertical bars in the image depict insertion sequences and flanking inverted repeats of transposons. The Tnp region consists of the transposase (*tnpA*), the resolvase (*tnpR*), the resolution site (*res*) as well as the transposition regulator (*tnpM*). The 5'-CS region contains the genes necessary for the integrase which can be found in figure1.1. The 3'-CS contains the genes for resistance to quaternary ammonium compounds, sulphonamides and an ORF of unknown function as well as the two insertion sequences, IS1353 and IS1326. The mercury operon consists of the necessary genes to confer mercury resistance, the structural genes *merD* and *merR* as well as the structural genes *merT, merP, merC* and *merA*. Sul indicates genes conferring resistance to sulphonamides and aad*A1* is shown to give resistance to spectinomycin and streptomycin (Liebert et al, 1999). Permission to reproduce this Figure has been granted by American Society for Microbiology.

The mobility of Tn*21* has been clearly demonstrated. In one study looking at variations in *mer* loci, antibiotic resistance phenotypes were found in unequal distributions. Resistances to ampicillin, streptomycin, and tetracycline were found to be more frequent than the

resistances to other antibiotics such as chloramphenicol, kanamycin, and sulfadiazine (Fang et al., 2016). This leads to the question, if Tn*21* and similar plasmids are responsible for the spread of resistances worldwide are they truly responsible for as much of the mercury and antibiotic co-selection being seen in soils, farms and clinical settings.

Several studies have shown that certain antibiotic resistance genes are more likely to be isolated, due to their current use, or presence in the environment being studied (Davies and Davies, 2010). However, certain resistances are more likely to be found together and being co-selected for, one example is tetracycline and streptomycin resistance (Tadesse et al, 2012). Regardless of variations in antibiotic resistance, resistance to antibiotics has generally been found to be higher in those bacterial isolates that are resistant to mercury compared to those that are sensitive to the metal (Liebert et al, 1997).

A contradiction to this finding however is that other studies have indicated a reduction in the variation of antibiotic resistance when co-selected with mercury and with only a sharp increase in antibiotic resistance gene carriage linked with other toxic metals such as copper (Hölzel et al, 2012). These contrasting findings show that co-selection of mercury and antibiotic resistance still requires further research especially into the variations and patterns of resistance being selected for.

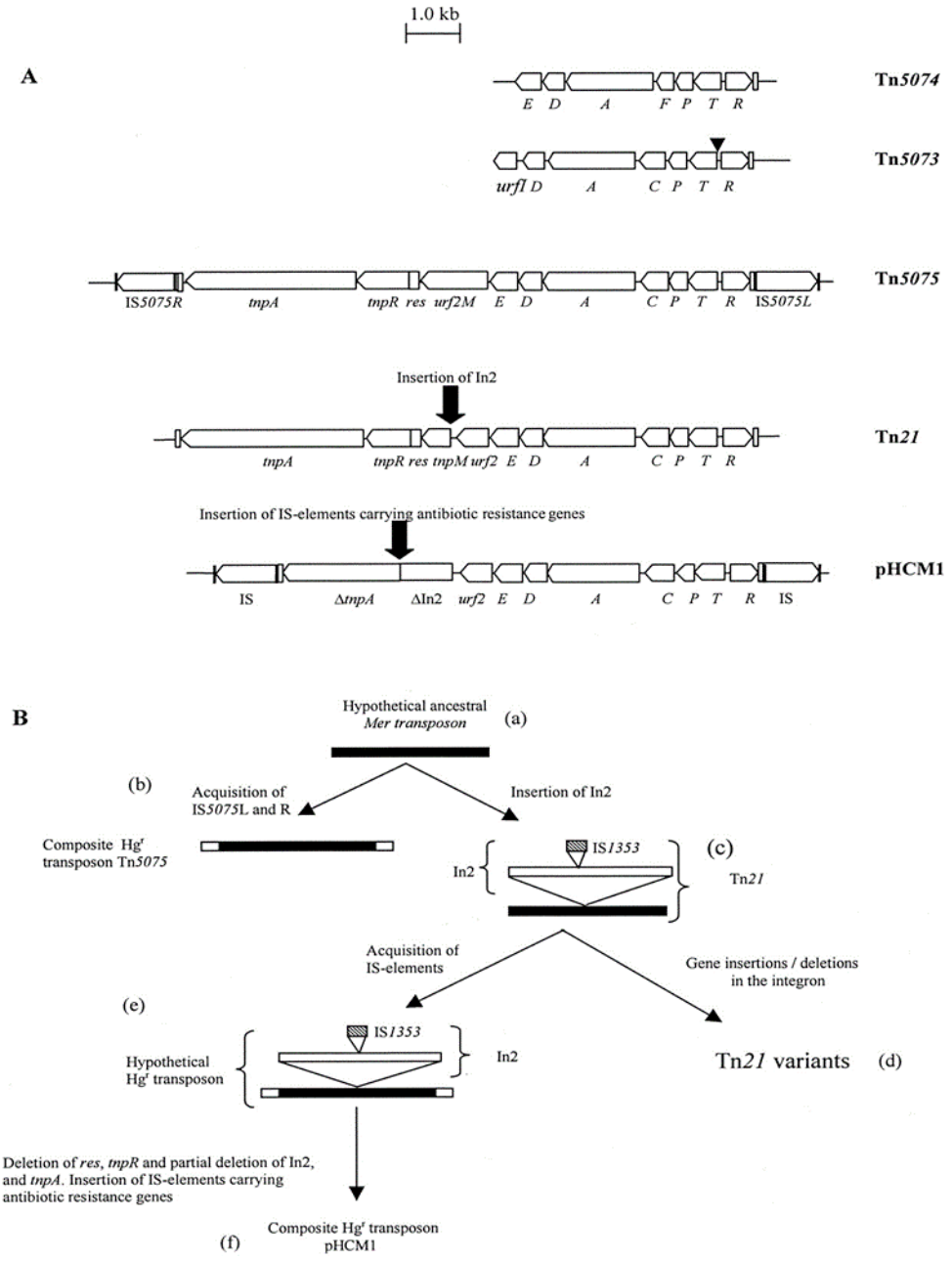


Figure 1.3 (A) Showing genetic maps for Tn*5074*, Tn*5073*, Tn*5075*, Tn*21* and pHCM1. Vertical black bars are indicative of 10-12 bp inverted repeat sequences surrounding the IS elements. The transposition regions marked as Tnp consist of *tnpA, tnpR* and *tnpM* as well as the resolution site res. Points where antibiotic resistance genes or In2 have inserted in Tn*21* and Phcm1 are indicated by black arrows. The mercury resistance encoding regions consist of *merR, merD, merA, merC, merF, merP* and *merT*. (B) Shows the hypothesised evolutionary pathway for Tn*5075*, Tn*21*, and the Tn*21*-like transposon in pHCM1. (a) An

ancestral *mer* transposon could have acquired either IS5075L and IS5075R to become Tn*5075* indicated as (b). Conversely an integron related to In*2* could have been integrated resulting in the formation of Tn*21* indicated as (c). (d) presents gene insertions and deletions in In*2* which would lead to Tn*21* variants. Tn*21* could have also acquired IS elements, which would lead to the formation of a precursor to the Tn21-like transposon in pHCM1 (e). Deletions of transposition genes and partial deletion of In*2*, which would then be followed by insertion of antibiotic resistance gene-carrying IS elements, would result in the formation of the Tn*21*-like transposon in pHCM1 (f). (Essa et al, 2003). Permission to reproduce this Figure has been granted by American Society for Microbiology.

1.2 Integron structure

Integrons are versatile and adaptive genetic acquisition systems found in bacterial genomes, transposons and plasmids. These genetic systems are ancient elements capable of creating genetic diversity and complexity with the potential to shape how an organism adapts and responds to its environment (Gillings et al, 2015). To further summarise Integrons, these elements can move between different species and are a common component of bacterial genomes, with at least 15% of all bacteria sequenced found to contain an integron (Rowe-Magnus et al, 2002).

Integrons are organised genetic elements which conventionally share three common elements. The first of these elements is the *intI1* gene which is the gene encoding the integrase protein from the tyrosine recombinase family (Riccio et al, 2001). This integrase gene removes the selected gene and integrates it into the element through site specific recombination. The Integrase catalyses the recombination of a gene cassette at the second feature of the integron, the integron associated recombination site, designated att1 which is the primary site of attachment of the gene cassettes (Anuradha Ravi et al, 2014). Finally, the last essential piece of the Integron is the Integron associated promoter which will then express the integrated gene cassettes, such as the mercury resistance genes (*merEDACPTR*), designated Pc, as shown in figure 1.4 (Liebert et al,1999). This gene

capture system allows for the quick and complex generation of genetic variation and diversity of adaptive phenotypes which, includes resistance to almost all known antibiotics (Ghaly et al, 2017). In0 and In2 contain an IS1326 which has caused deletions of adjacent 3'-conserved segments and sequences for transposition modules, they both retain a complete copy of only one of four genes required for transposition of related transposons and so are defective derivatives of transposons (Brown et al, 1996).

Figure 1.4 The most common configuration of an integron. *intI1* the gene for the integrase, Pc the intron associated promoter and att1, the integron associated recombination site, with the arrow showing a single Open reading frame (ORF). In some cases, the Pc (Promoter) can be found between the integrase gene and the recombinase site (Gillings, 2014). Permission to reproduce this Figure has been granted by American Society of Microbiology.

Integrons have been associated with the spread of the adaptive ability to resist the activity of antibiotics and heavy metals. Many hospital outbreaks caused by extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* have been reported, and most of these were found to carry the blaTEM-1, blaTEM-2, and blaSHV-1 gene derivatives (de Champs et al, 1991). These genes are found on large conjugative plasmids of the incompatibility types IncC, IncFI and IncH12 (Eisen et al, 1995). IncH12 a close relative of Inh1 which is one of the most common incompatibility groups of plasmids in *Enterobacteriaceae* has been found to carry a wide range of resistance genes including resistances to quinolones, amphenicols and β-lactams with reports of co-occurrence of ESBL's (Liangxing et al, 2016).

In addition to genes conferring resistance to antibiotics the IncH12 plasmids also harbour the resistance genes to heavy metals including resistances to copper (*pcoABCDRSE*), silver (*silESRCBAP*), arsenic (*arsCBRH*), as well as the Tn*1696*-related mercury operon (*merEDACPTR)* (García-Fernández & Carattoli, 2010). As an example of this *R478*, the

prototypical *ST1-IncHI2* plasmid encodes the resistance cassettes to the toxic metals described above.

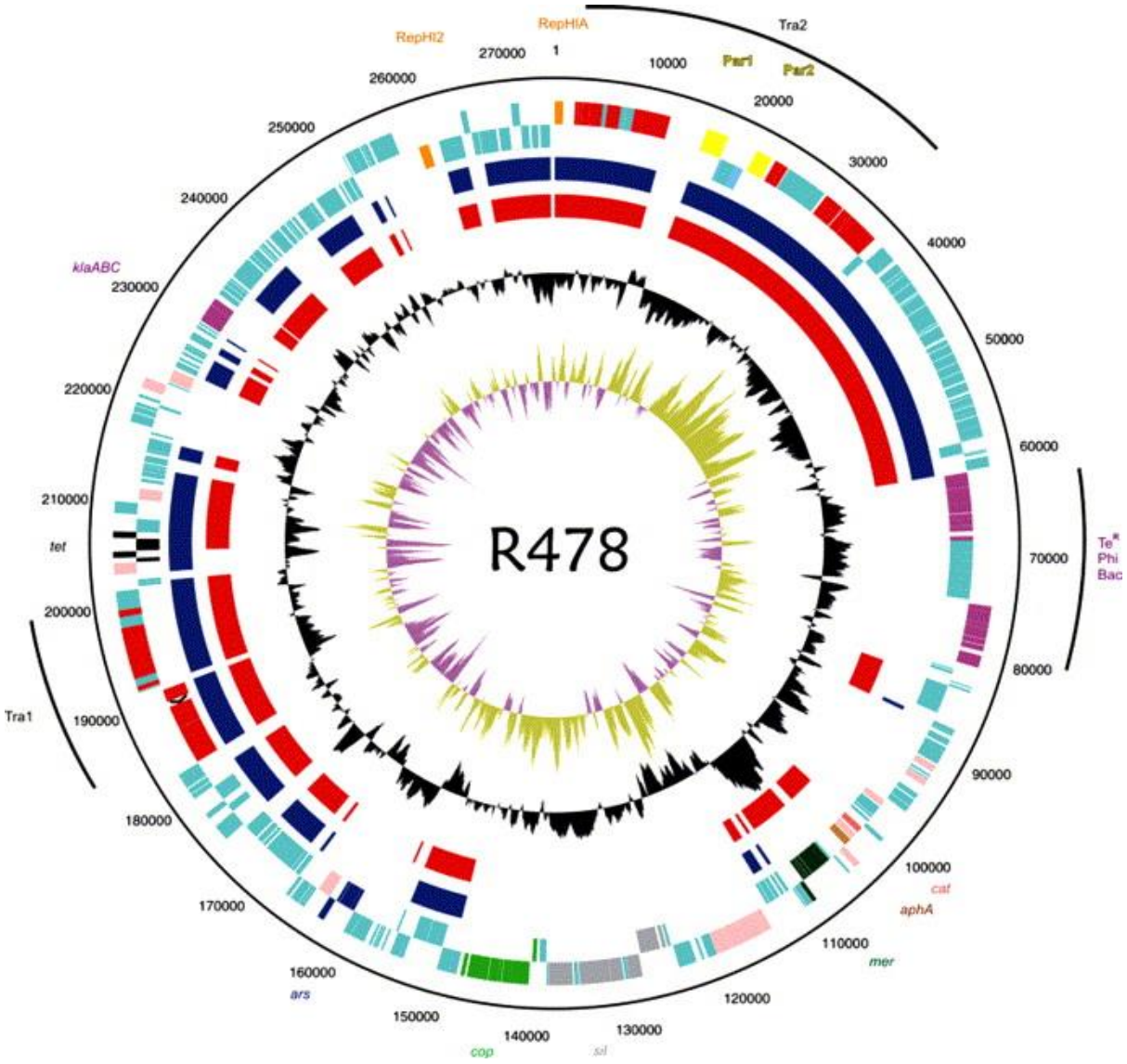


Figure 1.5 Plasmid map of R478. Resistance genes include: organic mercury resistance (*mer*), silver resistance (*sil*), copper resistance (cop), arsenic resistance (*ars*), tetracycline resistance (*tet*), chloramphenicol resistance (*cat*) and kanamycin resistance (*aphA*) (Gilmour et al, 2004). Permission to reproduce this Figure has been granted by Elsevier

The first Integron found on the chromosome of a bacteria was found in *Vibrio cholerae* and carried hundreds of gene cassettes with largely unknown function (Rowe-Magnus et al, 2002). The function of these genes has for the most part been elucidated however there are

still large gaps in the knowledge of what genes integrons carry and what their functions are. Chromosomally carried integrons are a normal part of environmental bacteria however it is now thought that integrons carried on plasmids are a more recent phenomenon driven mainly by human antibiotic selection (Gillings, 2014). Mobile Integrons are split in to 5 classes, of which class 1, 2 and 3 are associated with antibiotic resistance found in clinical environments. Class 4 integrons are found on SXT element of *Vibrio* cholerae and class 5 found on the pRSV1 plasmid of *Alivibrio salmonicida.* The differences between the 5 integron classes is divergent integron integrase sequences (Gillings, 2014).

Class 1 integrons are the focus of this research due to their ability to not only reside within chromosomes but also to be contained within other mobile genetic elements such as plasmids and transposons, these are known as clinical class 1 integrons. Due to the lateral transfer of these MGE contained integrons shown in Figure 1.5, the class 1 integrons have spread to nearly all species of Gram-negative bacteria (Ghaly et al, 2017). Due to transfer of the early ancestors of this gene into human commensal microflora and the ensuing selection pressures, these integrons have acquired over 130 resistance genes (Partridge, 2009). Most gene cassettes carried by class 1 integrons of known function, confer resistance to antibiotics or quaternary ammonium compounds (QAC) however there is increasing evidence of additional genes with additional functions (Partridge et al, 2009). Classes 4 and 5 however are mainly associated with *V. cholerae* and *A. salmonicida* (Partridge, 2011). These Integrons, as mentioned before, share a pool of around 130 gene cassettes which in the majority confer antibiotic resistance. These genes have heterogeneous *attC* sites and codon usage show strong evidence for the acquired accumulation of the genes, incrementally over time from a range of phylogenetic backgrounds (Partridge et al, 2009). It is not uncommon for integrons to be carrying a large variation of gene cassettes with unknown function.

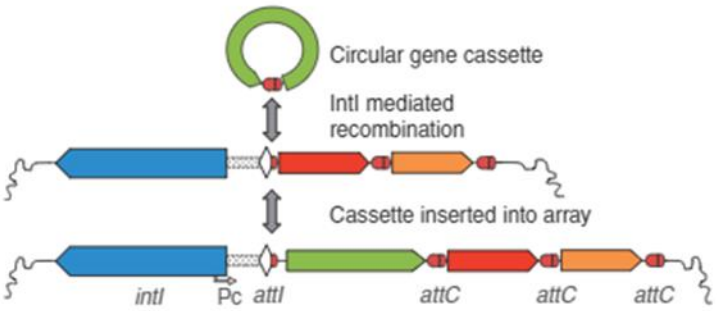


Figure 1.6 The *intI* gene which is responsible for the recombination of the *attC* site on the pictured circular gene cassettes and the integron recombination site, *attI*. This allows for the insertion of multiple different gene cassettes in a sequential manner giving host to what is known as a tandem cassette within the integron which can potentially exceed hundreds of genes (Gilling et al, 2015). Permission to reproduce this Figure has been granted by Springer Nature.

1.2.1 Resistance gene cassettes and variations

Due to the role of the gene *intI1* in acquisition of gene cassettes, it is possible for an integron to potentially contain hundreds of gene cassettes in sequence. It is this potential and ability that is leading to the increase of antimicrobial resistance and the use of the *intI1* as a marker for anthropogenic pollution. Though several of the genes are of an unknown function and are yet to name, a clear majority belong to those conferring resistance to antibiotics, biocides and heavy metals such as mercury (Gilling et al, 2015).

Tn*21* and several elements closely related to Tn*21* (de la Cruz and Grinsted, 1982) contain one or more genes for resistance to various antibiotics, such as *sul1* conferring resistance to sulphonamides and *qacEΔ1* which gives resistance to quaternary ammonium compounds. These are located at or near the *aadA* gene of Tn*21* which confers resistance to streptomycin (Schmidt, 1984). As one example gene cassettes carrying known antibiotic resistance genes such as *aadA1* have several variants including: *aadA1a, aadA1a-ant (3")-1a, aadA2* and *aadA4*. Each cassette may also have several minor variants. Some gene cassettes identified in mobile resistance integrons (MRI) include ORFs potentially encoding proteins of yet unknown function (Partridge et al, 2009).

So far more than 40 gene cassettes have been identified, and it is a reasonable expectation that many more will be discovered in the future. All but five of the known cassettes encode resistance determinants but it is not thought that this is a true reflection of the gene variety carried on these elements; rather it is likely to reflect experimental selection bias. (Recchia and Hall, 1995). There is obviously the potential for many more to be found in the environment as selection for antimicrobials is ever increasing and the way that bacteria interact with each other and share their genetic information in a vast community of species is still yet to be fully elucidated. Chromosomal integrons are most commonly found in various classes of Proteobacteria (beta through epsilon) but have also been reported in *Chlorobi, Cyanobacteria, Spirochaetes*, and *Planctomycetes* (Boucher et al, 2007). As more genomes are sequenced, the range of species and phyla that contain integrons are likely to expand.

The integrons of *Vibrio* have been studied extensively where cassette arrays within *Vibrio* integrons are large, with the reported arrays containing between 36 and 219 cassettes which accounts for between 0.7% and 3.1% of the *Vibrio* genome (Boucher et al, 2006). These Integron cassettes are also found in xanthomonads however the xanthomonad arrays are considerably shorter than those contained in *Vibrio*, which have between 1 and 22 cassettes. The cassettes and the arrays in the xanthomonads are associated with specific strains or pathovars of the species, suggesting a role for the gene cassettes in providing pathogenicity to specific plant species in which they colonise (Gillings et al, 2005). In the integrons described above, the integron integrase gene and the gene cassettes are transcribed in opposite directions (Boucher et al, 2007). Promoters within the integron promote integrase gene driven expression of gene cassettes within the associated array, but the strength of expression drops off as cassettes become more distal to the promoter (Collis and Hall, 1995). This may be why the cassette arrays in clinical class 1 integrons rarely contain more than six cassettes, as any additional cassettes may not be expressed because of their distance from the promoter.

Most comparative studies have been conducted on the afore mentioned *Vibrio*, where acquisition, loss, and rearrangement of the gene cassettes generate a wide diversity within serotypes, strains, and species (Rowe-Magnus et al, 2003). There also appears to be frequent movement of cassettes between the *Vibrio* as well, with high rates of loss or gain of individual genetic elements. These rearrangements generate significant differences in cassette content and order. The speed in which diversity is generated in the *V. cholerae* integron cassette array allows for the repeated rearrangement and cross-species sharing of genes. This activity generates diverse genotypes in *V. cholerae* that can then be acted upon by natural selection, allowing for rapid adaptation to local conditions and survival of the genetic resistances (Boucher et al, 2011). In-depth analyses of 12 *Vibrio spp* isolated from coral mucus showed that only 1 to 10% of the cassettes in their arrays were common.

Gene cassettes are a huge reservoir of genetic novelty with combined studies of metagenomic and chromosomal gene cassettes showing that around 65% of cassettes and the polypeptides that they encode have no known homologues in known DNA or protein databases. Furthermore, an additional 15% exhibit homology to a variety of conserved hypothetical proteins, while another 20% have enough homology to characterized and known proteins that the function of these peptides might be predicted (Holmes et al, 2003).

Clearly as shown from the research above, integrons and the gene cassettes they carry are an important resource for bacterial adaptation and survival. It is not surprising that integrons have a major role in the survival and adaptation of bacteria to antibiotics and antimicrobial therapy. Because integrons can assimilate such a vast pool of gene cassettes with a variety of functions, they are preadapted and ready for the acquisition and expression of resistance determinants. This allowed for integron containing cells to quickly fix resistance genes and become part of the bacterial survival strategies under the strong selection pressures imposed by antimicrobial use. Environmental gene cassettes that have been incorporated as resistance determinants have been described, such as efflux pumps encoded by the *qac* gene family (Gillings et al, 2009). The use of antibiotics in human medicine and agriculture

then allowed for the selective pressures to fix rare occurrences where integrons had acquired gene cassettes of importance to antibacterial resistance such as the mercury resistance genes. This sampling and incorporation of genes from the environmental resistome by integrons has resulted in the accumulation of gene cassettes with diverse functions and ways of dealing with a similarly diverse number of antibiotics and antimicrobials (Allen et al, 2010)

1.2.2 *qac* Genes – the missing pieces

Chromosomal integrons carry a variety of gene cassettes, most with unknown function. These also have diversity between their *IntI1* genes (Gillings et al, 2008). Evidence to prove that the class 1 integron is comparatively mobile comes from conserved left-hand breakpoints occurring exactly 107bp beyond the *IntI1* stop codon at the same position where ISPa7 is inserted into class 1 integrons, which are carried by some *P. aeruginosa* isolates (Riccio et al, 2005). In addition to this there is a conserved right-hand break point which is found 43bp after *attC*. The presence of such precisely conserved break points from a wide range of chromosomal landscapes and across different species is strong evidence that the chromosomal class 1 integron is mobile and highly conserved (Gillings et al, 2008). As further evidence, the study of biofilm and bacteria from soil and water suggest that as much as 1-5% of bacteria present may carry a class 1 integron (Riccio et al, 2005). The chromosomal class 1 integrons appear to have been in the right place at the right time and were well equipped to deal with the ever increasing human need to control the bacteria in their environment. From looking at clinical class 1 integrons we can see that the integrase genes are essentially identical across isolates suggesting a recent event leading to the dissemination of a single form of the integrase into the environment. The clinical class 1 integron that we know now is thought to have been incorporated into a Tn*402* like transposon or close relative (Gillings et al, 2008). This new mobile element then contained the integrase as well as cassettes already present with the machinery capable of moving the Tn*402* transposon out of the cell. The exact path leading to the capture of this transposon

with clinical isolates is unknown however the present evidence points towards the *qac* genes.

The *qac* genes confer versatile efflux pumps capable of transporting a variety of molecules including toxic cationic particles (Paulsen et al, 1996). These genes are found in approximately half of all cassette arrays carried by integrons. Quaternary ammonium compounds were used in clinical settings in the 1930's along with sulphonamides, which started being used later in the 1930's. From here antibiotic selection and acquisition occurred. Next *sul* genes were selected for and were next on the growing list of resistances being acquired by this element (Rowe-Magnus et al, 2002). It is then over time that the Tn*402* like element lost its ability to transpose and became incorporated with the human microbiota. It was able to acquire gene cassettes incorporated in chromosomes and resistances to the over 130 antibiotics, along with other resistance genes such as mercury Tn*402* transposon. The exact path leading to the capture of this transposon with clinical isolates is unknown. However present evidence points towards the *qac* genes (Partridge et al, 2009).

The ability for integrons to sample gene cassettes, along with the linkage of a class 1 integron and a plasmid hunting element is what has made the relatives and descendants of this Tn*402* like transposon so successful at spreading and disseminating into the environment (Lupo et al, 2012). This same element has been found in a wide range of isolates from clinical settings as well as from animals from farms. In *E. coli* isolated from farms, integrons can be found in up to 80% of isolates (Sepp et al, 2009). This could be due to the use of quaternary ammonium compounds in agriculture and the successive leaps from one environment into the next. The story of the Tn*402* transposon and the evidence behind it is very simple and clean cut, the successive evolution of the transposon and integron is portrayed to be a neat occurrence of events, however this is unlikely, evolution is not quite so simple and there is potentially a lot more to this story; however this would explain why

even to the present day there are resistances to antibiotics appearing that haven't been used in decades.

Current research has quite finely pointed out the importance of antibiotic resistance with the O'Neill reports suggesting a mass of preventable deaths to come within the next 50 years if the problem not tackled and better understood (O'neill, 2014). Research has pointed out, in several instances, the dissemination of mobile genetic elements and their arsenal of resistance genes due to addiction systems. Resistance to colistin, a last resort antibiotic has been found in bacteria isolated from pigs from China and with all this we are moving ever closer to the post antibiotic era (Paulsen et al., 1996).

1.2.3 Sulphonamide (*sul*) gene cassettes

Today sulphonamides are infrequently used due to widespread resistance (Sköld et al,2000). Due to the limited current use of sulphonamides, the selective process for resistance has all but ceased and so there is little warranting study on these genes. However, the precise definition of the target for sulphonamides in bacteria and how it evolves to resist the actions of sulphonamides could throw light on general mechanisms for occurrence and spread of resistance in bacteria.

Sulphonamides target the enzyme dihydropteroate synthase (DHPS) which catalyses the condensation of p-aminobenzoic (PABA) acid and 7,8- dihydro-6-hydroxymethylpterin-pyrophosphate (DHPPP) to form dihydropteroic acid. Mammalian cells are not dependent on the synthesis of folic acid, and as such generally lack DHPS. Higher eukaryotic cells however can utilize dietary folates by uptake through transport systems, which most prokaryotes and some lower eukaryotes lack. These prokaryotes must therefore synthesize folates from small molecules, including the formation of dihydropteroic acid catalysed by DHPS. This is the basis for the effect of sulphonamides on bacteria and not on eukaryotic cells and for their broad spectrum of antibacterial activity (Domagk and Hegler, 1942).

In the case of *E. coli*, resistance to tetracyclines, sulphonamides, and streptomycin or spectinomycin are generally the most prevalent (Guerra et al, 2003). Despite the research previously conducted there is still a lot left to uncover about the pattern of distribution of antimicrobial resistance genes. For example, previous studies showed that the distribution of antimicrobial resistance genes from pathogenic *E. coli* isolates taken from pigs may differ significantly from those found in other animal species (Lanz et al, 2005).

The simultaneous presence of more than one resistance gene was significantly higher for sulphonamides (15%) and streptomycin (34%) than with tetracycline which showed simultaneous presence in only 4% of over 300 samples (Boerlin et al, 2005). Similar results have also been found in other studies giving evidence to the frequent co-carriage of these genes on transposable elements. In one study looking at *E. coli* from avian hosts sixty-three percent of the clinical isolates were positive for the class 1 integron markers *intI1* and *qacE*Δ*1*, furthermore fifty percent of the clinical isolates positive for the integron marker gene *intI1* as well as for the *qacE*Δ*1* and *aadA1* cassettes also contained the mercury reductase gene *merA* (Essa et al, 2003).

1.2.4 *aadA1* (spectinomycin and streptomycin) gene cassettes

Resistance to aminoglycosides such as spectinomycin and streptomycin can be mediated by plasmid specified genes that encode aminoglycoside-modifying enzymes such as the *aadA1* gene carried by Tn*21* (Leung et al, 1992). The *aadA1* gene itself encodes the protein known as streptomycin Adenylytransferase and can adenylate either the 3-hydroxylgroup on the amino-hexose III ring of streptomycin or the 9-hydroxyl group on the actinamine ring of spectinomycin (Davies and Benveniste, 1974).

The *aadA1* gene is commonly found in both Gram-positive and Gram-negative bacteria likely due to its ability to be carried and found readily on plasmids such as the R plasmid and is also linked with transposons such as Tn*1331 and* Tn*7* in *E. coli* (Fling et al, 1985). The fact that this gene is so highly distributed among bacteria shows how mobile genes can potentially be and how widely they can be disseminated. However, the fact that its identity is

so highly conserved in some species like *E. coli,* where it can be close to 100% identical at the amino acid level (Fling et al, 1985), and due to this prevalence and generally good conservation it could be assumed that this gene was acquired in an early ancestor on the Tn*21* transposon.

Environments high in antibiotics should lead to a higher presence of Tn*21* like transposons in *E. coli*. It's the mobile genetic elements, such as the transposons, that are of interest to modern research into this field. The ability to predict the movement of antimicrobial resistances could help us understand where the next outbreak of the next superbug could occur and if we could predict what it would be resistant to we could be better prepared to fight it whenever and wherever it appears. However, what is not understood is the mechanisms by which mercury resistance is being transported into agricultural environments such as slurry tanks and in fields when mercury has not been used for decades.

Many studies report the positive correlation between resistance to antibiotics and metals in a wide variety of environments, from swine waste lagoons, to soil and waste from dairy farms (Pan et al, 2006). One study found that along a gradient of metal exposure the bacteria were more tolerant to the metals, as well as antibiotics, compared to bacteria from a control site (Perry and Wright, 2013). However, despite this correlation, causality is not ensured as many other factors besides metal exposure differ between studies. Research into the antimicrobial resistance of bacteria found to be resistant to mercury, isolated from slurry tanks, would provide an insight to why mercury resistance genes are still being found in these isolates when they have had no interaction with mercury. This research builds on the foundation that integrons are transposable genetic elements and can be used as an indicator of anthropogenic pollution. In the case with this project, the pollution mentioned would be antimicrobials contained in the animal faeces accumulating in slurry tanks and in the waste matter, with mercury resistance being a flag indicating the presence of acquired antimicrobial resistance in *E. coli.* It is also the use of the *IntI1* gene as a marker for anthropogenic pollution that will elucidate the current situation of acquired antimicrobial

resistance in these bacteria, due to the fact it has a highly conserved sequence and is usually found in high abundance in areas of high anthropological impact and in low abundance where there is little impact (Gilling et al, 2015).

1.3 Aims and Objectives

The research being conducted in this project will be used to:

1. Observe the current resistance genes in *E. coli* isolated from cow slurry from Sutton Bonington slurry tank and if there is variation in resistance held by different isolates. This will show how prevalent they are on the farm and what other resistances are being carried.

2. investigate the mobility and potential of the transposable elements to move into environmental samples with acquired genes using transposon trapping, thus giving an insight to how easily and frequently movement could be occurring in the slurry tank.

3. Elucidate whether novel resistances found during the transposon trapping experiment are being carried on the same mobile elements along with mercury resistance. As well as this, are more resistance genes carried with mercury resistance than usually found in isolates without the mercury operon?

2. Materials and Methods

2.1 Media & Solutions

All the media used was prepared according to manufacturer's instructions, dispensed into glass containers, and autoclaved at 121˚C for 15 minutes unless otherwise stated. The media was then cooled to 55˚C in a water bath before approximately 25ml was poured in each petri dish.

2.1.1 Maximum Recovery Diluent (MRD)

4.75g of MRD powder (Oxoid, CM0733) was dissolved in 500ml of Reverse Osmosis (RO) water. The medium was then autoclaved and stored at ambient temperature.

2.1.2 Mueller-Hinton broth & Mueller-Hinton agar

11.5g of MH broth powder (Sigma-Aldrich, 70192) was dissolved in 500ml of RO water and autoclaved. The prepared medium was stored at ambient temperature. For MH agar, 19g of MH agar powder (Oxoid, CM0337) was added to 500ml of distilled water which was then autoclaved and stored at 55˚C.

2.1.3 Luria-Bertani (LB) broth & agar

10g of LB broth powder (L3022, Sigma-Aldrich) was dissolved in 500ml of RO water and autoclaved. For the agar 7.5g of Bacteriological Agar (Oxoid, LP0011) was added to the above composition before autoclaving.

2.1.4 NA agar

14g of Nutrient agar powder (Oxoid, CM0003) was dissolved in 500ml of RO water. The medium was then autoclaved, and prepared plates were stored at 2-8˚C.

2.1.5 TE buffer (pH 8)

0.0372g of disodium EDTA (ethylenediamine tetra-acetic acid) (Sigma-Aldrich) and 0.12g of Tris Base (Sigma-Aldrich) were dissolved into 80ml of RO water to prepare 10mM Tris-HCl and 1mM EDTA buffer. The pH of the solution was then adjusted to pH 8 using concentrated stock of HCl by slowly adding the HCl into the solution while measuring with a pH meter. The solution was then made up to 100ml of final volume and autoclaved at 121˚C for 15 minutes. The prepared solution was stored at room temperature.

2.1.6 $HgCl_2$ solution preparation

A stock solution of 50µg/ml of $HgCl^2$ was produced by adding 0.5g of $HgCl^2$ powder to 10ml of MilliQ water, which was then vortexed to dissolve. The solution was stored in sterile glass bottles.

2.2 Bacterial isolates

Bacterial isolates were isolated from slurry samples collected between 16th May 2017 and the 25th January 2018. These strains had been isolated on TBX media, with or without cefotaxime, or on CHROMagar ESBL, and confirmed as *E. coli* by oxidase and indole tests.

2.2.1 Storage of the isolates

All isolates were stored on Microbank beads containing specially formulated broth (Pro-Lab Diagnostics, UK) and kept in -80˚C. A loopful from pure NA cultures was swirled into the beads and left for about 2-3 minutes. Using a pipette, the broth was removed from the tubes and discarded. The microbanks were kept at -80˚C.

2.2.2 Bacterial control strains and RP4-8 plasmid characteristics

Bacterial strains	**Characteristsics**	**References**
pMG101	Resistance to chloramphenicol, ampicillin, tetracycline, streptomycin, sulphonamide, mercury, tellurium and silver	McHugh et al, 1975
ATCC25922	Sensitive to neomycin, colistin, kanamycin, cephalexin, gentamicins, cefamandole, cephalothin, tetracycline, cephaloglycin, cephaloridine, nalidixic acid, and chloramphenicol.	Public Health England. Culture Collections
NCTC13353	CTX-M-15 (cefotaxime). Resistant to chloramphenicol, ampicillin, sulphonamide, azithromycin, trimethoprim-sulfamethoxazole, enrofloxacin, ceftiofur,cefotaxime, ciprofloxacin, ceftazidime, aztreonam,cefpodoxime	Public Health England. Culture Collections
NCTC13476	IMP-type	Public Health England. Culture Collections
NCTC13846	Resistant to colistin, cefoxitin, nalidixic acid,ciprofloxacin and chloramphenicol. MCR-1 positive.	Public Health England. Culture Collections
K12-MG1655	F-lambda-, rph-1. Wild type strain, no resistance to gentamycin, or mercury.	Blattner et al, 1997
BCC2	Resistant to chloramphenicol, ampicillin, streptomycin, sulphonamide, naladixic acid, azithromycin, trimethoprim-sulfamethoxazole, enrofloxacin, ceftiofur,cefotaxime, ciprofloxacin. blaCTX-M and blaTEM	Ibrahim et al, 2016
plasmid		
RP4-8	Gentamycin resistance	Quandt et al, 2004

Table 1.1 Characteristics of the bacterial control strains used with relevant details to antibiotic resistances/antimicrobials and key genes.

2.3 Bacterial phenotypic mercury resistance testing

2.3.1 Metal resistance

All *E. coli* were subjected to phenotypic and genotypic testing for mercury resistance. Control strains used are summarised in Table 1.1.

2.3.2 Phenotypic Resistance

LB agar plates were prepared (Section 2.1.23) and mercury solution was added to them. Plates with two different mercury concentrations were prepared. Isolates were tested for the ability to grow in LB agar containing 50µg/ml of $HgCl_2$. 50µg/ml plates were prepared by adding 25µl of 50mg/ml $HgCl_2$ stock solution into 25ml of molten agar, which was then thoroughly mixed. Plates were poured, allowed to set and air dried for 1 hour. Fresh bacterial cultures were then streaked onto the LB agar plates and incubated for 24h at 37˚C for *E. coli*. The plates were then observed for growth.

2.3.3 Genotypic Resistance

PCR was performed for the detection of *merA, merC, merR* and *intI1* genes. DNA was extracted as described in Section 2.7.1 The PCR mixture was prepared by adding 12.5µl of DreamTag Green (2X), 1µl of 10µM stock of the forward and reverse primer for *merA, merC, merR* and *intI1* to separate 0.2 mL microtubes so each isolate would be tested for each gene separately (Table 2.2), 8.5µl of nuclease-free water and 2µl of DNA. The PCR cycle was performed at 95˚C for 5 min followed by 30 cycles of 94˚C for 1 minute, 64˚C for 1 minute and 72˚C for 2 min. Final extension step was performed at 72˚C for 10 min. 5µl of each PCR product was run on 1.5% (w/v) TAE agarose gel at 85V for 90min for each PCR experiment.

Primer	Sequence	Band size (bp)	test to observe the presence of	references
Mercury resistance				
merA-F	ACCATCGGCGGCACCTGCGT (20)		mercuric reductase	Liebert et al 1997
merA-R	ACCATCGTCAGGTAGGGGAACAA (23)	1238	mercuric reductase	Liebert et al 1997
merC-F	CATCGGGCTGGGCTTCTTGAG(21)		mercury transport protein	Liebert et al 1997
merC-R	CATCGTTCCTTATTCGTGTGG (21)	422	mercury transport protein	Liebert et al 1997
merR-F	ATCCGBTTCTATCAGCGCAAG(21)		transcription activator	Pérez-Valdespino et al, 2013
merR-R	ACGTTCCTTNRGCTTGTGCTCG(21)	433	transcription activator	Pérez-Valdespino et al, 2013
intI-F	CCTCCCGCACGATGATC(17)		Mercury resistance operon of transposon Tn21	Kashif et al, 2013
inti-R	TCCACGCATCGTCAGGC(17)	280	Mercury resistance operon of transposon Tn21	Kashif et al, 2013
Inverse PCR				
sul1invFWa	AAGAACCGCACAATCTCGTC(20)		Detection of surrounding AMR genes	Pei et al, 2006
sul1invRVa	GGCTTCCGCTATTTGGTCTC(19)	163	Detection of surrounding AMR genes	Pei et al, 2006
tetMinvFWa	CAGAATTGTTAGAGCCATATC(21)		Detection of surrounding AMR genes	Tamminen et al, 2011
tetMinvRva	GCAGAAATCAGTAGAATTGC(20)	186	Detection of surrounding AMR genes	Tamminen et al, 2011

Table 1.2 Oligonucleotides used in the PCR reactions to amplify *merA, merC, merR and intI* as well as for the inverse PCR reactions which were designed to amplify surrounding antimicrobial resistance genes neighbouring *sul* and *tet.*

2.4 Mercury kill assay concentrations

Varying amounts of $HgCl_2$ were added to 50ml of molten LB agar contained in a 50ml Falcon Tube (BD Biosciences) which was then inverted ten times to allow for adequate mixing of the $HgCl_2$. 25ml of the contents of each Falcon tube was then poured into a 9mm wide Petri dish and allowed to set. Kill assay plates had $HgCl_2$ added to make a final concentration of 50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml, 250µg/ml and 300 µg/ml.

2.4.1 Mercury kill assay isolate preparations

Single colonies from each of the isolates were placed into separate 10ml Falcon tubes containing 8ml of LB broth. These were then incubated overnight 35˚c in a spinning incubator (Gallenkamp Orbi-safe TS netwise) and left for no more than 16 hours. The Falcon tubes were then removed from the incubator and 200µl of culture added to a 50ml Falcon tube containing 19.8ml of LB broth. These Falcon tubes were then placed into the spinning incubator for 2-3 hours. After 2-3 hours had passed 1ml of the LB broth was removed and placed into a Cuvette. This was then placed into a spectrophotometer (CECIL CE2021) with an optical density set at 0.6OD. Once isolates reached an OD reading of 0.5 100µl was transferred into an Eppendorf containing 900µl of MRD.

2.4.2 Kill assay isolate dilutions

1:10 dilutions were prepared in MRD were prepared for each isolate and each isolate was diluted down to 10^{-6} by taking 100µl from the previous Eppendorf and adding it to the 900µl in the next Eppendorf. Thorough mixing was achieved by pipetting the mixture with a Gilson pipette. The dilutions were spotted onto the MRD agar plate using the Miles and Misra technique where 20µl of the appropriate dilution was dropped onto the surface of the agar and left to dry.

2.5 Antibiotic susceptibility testing (AST)

Testing was carried out according to the Clinical and Laboratory Standards Institute method (CLSI, 2018). Antibiotic susceptibility testing was carried out on the isolates with 21 antibiotics.

Antibiotic disc name and abbreviation	**Manafacturer**	**Disc concentration (µg)**
Ampicillin (AMP)	SirScan Disc	10
amoxicillin- clavulanic acid (AMC)	Oxoid	10
aztreonam (ATM)	Oxoid	30
chloramphenicol (C)	Oxoid	30
ceftazidime (CAZ)	SirScan Disc	30
cefpodoxime (CPD)	SirScan Disc	10
ciprofloxacin (CIP)	Oxoid	5
colistin (CT)	Oxoid	10
cefotaxime (CTX)	Oxoid	30
ceftiofur (EFT)	Oxoid	30
enrofloxacin (ENR)	Oxoid	5
nitrofurantoin (F)	Oxoid	300
cefoxitin (FOX)	Oxoid	30
imipenem (IPM)	Sensi Discs	10
nalidixic acid (NA)	SirScan Disc	30
sulfonamide (S3)	Oxoid	300
streptomycin (S10)	SirScan Discs	10
azithromycin (AZM)	Oxoid	15
tetracycline (TET)	Oxoid	30
trimethoprim-sulfamethoxazole (SXT)	Oxoid	1.25+23.75
meropenem (MEM)	Oxoid	10

Table 1.3 List of the antibiotics used in the antibiotic susceptibility tests with the abbreviated names, the manufacturer of the discs used and the concentration of the antibiotic in the discs used.

2.5.1 Inoculum preparation for AST

A fresh bacterial culture was prepared by streaking single isolates on a NA agar plate and incubating overnight at 37˚C.The inoculum was prepared by adding 10ml of Mueller-Hinton broth (Section 2.1.2) into a sterile tube and emulsifying 1-2 distinct colonies from the fresh bacterial culture. The suspension was adjusted to 0.5 McFarland standard to achieve equivalent turbidity (approximately 1 to 2 x 10^8 colony-forming units per ml). The turbidity was checked visually using a Wickerham card.

2.5.2 Inoculation of test plates

Within 15 min of the inoculum preparation, a sterile cotton swab was used to inoculate the 120mm Length x width square test plates, containing 50ml of LB agar (Pro-Lab Diagnostics, REF: PL.800256). The swab was dipped into the adjusted suspension and the excess fluid was removed by firmly pressing on the side of the tube. Then the dried surface of a fresh Mueller-Hinton agar plate (Section 2.1.2) was inoculated by streaking the swab over the entire surface of the agar. To ensure even distribution of the inoculum, the plate was rotated approximately 60˚ and each time the swab was dipped in the adjusted inoculum. Lastly, the edge of the agar was swabbed to provide a complete lawn of growth on the plate. The inoculated agar was left to rest for 5 min to allow for any excess moisture to be absorbed.

2.5.3 Application of Drug-impregnated Discs

The antibiotic discs (Table 3) were either dispensed aseptically onto the inoculated agar surface using sterile tweezers or via the use of an Antibiotic Dispenser for 120mm length x width square plates. Each disc was pressed down to ensure complete contact with the agar. The discs were applied evenly over the surface with no less than 24mm distance from centre to centre. Discs which produce small diameter inhibition zones, e.g. vancomycin, were placed next to discs which give larger inhibition zones, such as the cephalosporins, to avoid

overlapping. Once all discs were dispensed, the plates were inverted and incubated at 37˚C for 24h.

2.5.4 Interpretation of the results

The diameter of each zone of clearing around each disc was measured and referred to as resistant, intermediate or susceptible according to the referencing standards for each pathogen from CLSI and EUCAST breakpoints (CLSI, 2014; EUCAST, 2017).

2.6 PCR for mercury resistance genes

2.6.1 Crude DNA extraction by boiling method

100µl of sterile RO water was added into a sterile Eppendorf tube. Two to three distinct colonies from a fresh pure culture were picked and resuspended in sterile RO water. The tube was then heated in a dry heating block (QBT2, Grant Instruments, UK) at 100˚C for 30 minutes. The tube was then transferred into a centrifuge (Sigma 1-16K) and spun at 16000 x g for 5 minutes. The supernatant was then transferred into a fresh sterile Eppendorf tube and stored at -20˚C. The NanoDrop®ND-1000 Spectrophotometer (ThermoFisher Scientific, UK) was used to measure the DNA concentration and purity (in ng/µl). The spectrophotometer was cleaned using lens tissue and 2µl of RO water was then loaded onto the machine to initiate BLANK measurement. Then 2µl of extracted DNA was loaded and measurement initiated. Samples with a DNA yield below 30ng/µl were repeated.

2.6.2 Mercury PCR ladders

Phusion High fidelity PCR master mix with GC buffer (New England Biolabs (NEB)) was used in the mercury resistance gene detection PCR reactions looking for genotypic mercury resistance. A 100bp DNA ladder (New England Biolabs (NEB)) was used for size comparison of the resulting PCR products and was added to the first lane on the agarose gel.

2.6.3 Mercury PCR controls

Controls for the PCR were *E. coli* PMG101 as the positive control and water added to the control reagents as a negative control.

2.6.4 PCR protocol, temperatures and times

2µl of crude DNA from each isolate was added to 8µl of Nuclease free water along with 1.25µl of the forward primers for *merA, merC, merR and intI* and 1.25µl of the reverse primers for *merA, merC*, *merR and intI*. Finally, 12.5µl of Phusion High fidelity PCR master mix with GC buffer (New England Biolabs (NEB)) was added to mixture. Each isolate was tested for each gene separately.

0.2 ml PCR tubes containing the solution (ThermoFisher scientific 10401203) were then placed into a C100 Thermocycler. The initial denaturation stage for the PCR was 95˚C for 5 minutes. After these 30 cycles of 94˚C for 1 minute, 1 minute at 62˚C and 2 minutes at 72˚C. After this there was a final extension of 10 minutes at 72˚C.

2.7 Inverse PCR

2.7.1 Crude DNA preparation

100µl of sterile RO water was added into a sterile microtube tube. Two to three distinct colonies from a fresh pure culture were picked and resuspended in sterile RO water. The tube was then heated in a dry heating block (QBT2, Grant Instruments, UK) at 100˚C for 30 minutes. The tube was then transferred into a centrifuge (Sigma 1-16K) and spun at 15,493g for 5 minutes. The supernatant was then transferred into a fresh sterile Eppendorf tube and stored at -20˚C. The NanoDrop®ND-1000 Spectrophotometer (ThermoFisher Scientific, UK) was used to measure the DNA concentration and purity (in ng/µl). The spectrophotometer was cleaned using lens tissue and 2µl of RO water was then loaded onto the machine to initiate BLANK measurement. Then 2µl of extracted DNA was loaded and measurement initiated. Samples with a DNA yield below 30ng/µl were repeated.

2.7.2 Restriction enzyme

EcoR1 (New England Biolabs (NEB)) was used as the restriction enzyme to digest DNA for inverse PCR. 1 µg of DNA was added to 5 µl (1X) 10X NEBuffer with 4µl of EcoR1 added to ensure adequate cutting of the genome. Nuclease free water was added to make a total Reaction Volume of 50 µl. This was then left to incubate in a 35˚C incubator for 1 hour. This was then inactivated by heating to 65˚C for a total of 20 minutes.

2.7.3 Ligation step

2µl of T4 DNA Ligase Buffer (10X) (New England Biolabs (NEB)) was firstly added to an microtube. Then 2µl of the product from the previous restriction step. 1µl of T4 DNA Ligase was added lastly after adding 15µl of Nuclease free water to make the total reaction volume 20µl. This was then kept at room temperature for 10 minutes and heat inactivated at 65˚C for 10 minutes.

2.7.4 Inverse PCR protocol, temperatures and times

1.25µl of the forward inverse primer for tetMinvFWA or sulinvFWa and 1.25µl of the reverse inverse primer for tetMinvRVa or sulinvRVa (Table 1.2) were added to 2µl of the product from the ligation step along with 8µl of Nuclease free water and 12.5µl of Phusion High fidelity PCR master mix with GC buffer to make a total volume of 25µl. This was done separately for each isolate with the tetracycline primers and the sulphonamide primers.

The PCR protocols for the sulphonamide IPCR reaction were as follows. 98˚C for 30 seconds, then 30 cycles of: 98˚C for ten seconds, 64˚C for 30 seconds, 72˚C for 5 minutes. The final extension was at 72˚C for 2 minutes. The PCR protocols for the tetracycline reaction is as follows. 98˚C for 30 seconds, then 30 cycles of: 98˚C for ten seconds, 55˚C for 30 seconds, 72˚C for 5 minutes. The final extension was at 72˚C for 2 minutes.

2.7.5 Agarose Gels

To make 1% agarose gel, 1g of agarose powder (A9539, Sigma-Aldrich) was dissolved in 100ml of TAE buffer (prepared by dissolving 242g of Tris base in water, adding 57.1ml of

glacial acetic acid and 100ml of 500mM EDTA pH 8) by microwaving for about 3 minutes at 600watts. Once cooled, 2µl of ethidium bromide (10mg/ml stock) were carefully added and the gel was poured into a gel tray with a comb to form wells and left to set.

5µl of Blue/Orange Loading dye (New England Biolabs (NEB)) was added to each microtube PCR tubes (Thermofisher scientific 10401203) and mixed by pipetting up and down. 5µl of this subsequent 30µl volume was then added to the wells of the agar. With 5µl of a 1Kb marker DNA ladder (New England Biolabs (NEB)) added to the first well and the subsequent samples from the isolates for the *tet* and *sul* reactions thereafter. The gels were electrophoresed at 85V for 90 minutes.

2.7.6 Gel visualization

Gels were removed from the gel trays and placed into a Bio rad Gel document imaging system (BioRad Gel Doc™ XR+ Gel Documentation System) where UV light was applied to visualise the bands in the agarose gel.

2.8 DNA extraction methods used for the Gram-negative isolates to be sent for gene sequencing

2.8.1 Cell preparation and lysis

A Gen Elute DNA Purification kit (New England Biolab (NEB)) was used for the extraction of DNA to be sent for sequencing. 1.5ml of an overnight LB broth was pelleted by centrifuging (Sigma 1-16K centrifuge) for 2 minutes at 16000xg. The remaining culture was then removed. The pellet was resuspended in 180µl of lysis solution T/Buffer STL for GenElute Mammalian Genomic DNA Kit (B6678). 20µl of RNase A Solution (R6148) was then added to remove RNase, this was then mixed, and incubated for 2 minutes at room temperature.

The cells were next prepared for cell lysis by adding 20µl of the Proteinase K solution to the sample. This was then mixed and incubated for 30 minutes at 55 °C.

Cells were lysed by adding 200µl of Lysis Solution C (B8803), which was then vortexed thoroughly (15 seconds), and incubated at 55 °C for 10 minutes. A homogeneous mixture was essential for efficient lysis of the cells.

2.8.2 DNA isolation

Columns were prepared by adding 500µl of the Column Preparation Solution to each pre-assembled GenElute Miniprep Binding Column seated in a 2ml collection tube. This was then centrifuged (Sigma 1-16K) at 12000g for 1 minute with the eluate being discarded. 200µl of ethanol (95–100%) was next added to the lysate and mixed thoroughly by vortexing for 5–10 seconds to prepare for binding.

The entire contents of the tube were transferred into the binding column. The contents were then centrifuged (Sigma 1-16K) at 6500 x g for 1 minute. Discard the collection tube containing the eluate and place the column in a new 2ml collection tube.

For the first wash 500µl of Wash Solution 1 (W0263) was added to the column and centrifuged for 1 minute at 6500 x g. The collection tube containing the eluate was discarded and the column placed in a new 2ml collection tube. A second wash was next performed by adding 500µl of Wash Solution to the column and centrifuged for 3 minutes at 16000 x g to dry the column.

Finally, to elute the DNA 200µl of the Elution Solution (B6803) was pipetted directly onto the centre of the column and centrifuged for 1 minute at 6500g to elute the DNA.

2.9 Transposon trapping

2.9.1 Preparation of small-scale Electro Competent Cells

A single colony from each isolate was inoculated into 10ml LB broth and incubated at 37°C for 16-18 hours at 250rpm in a shaking incubator. The overnight culture was diluted 1/100 into 4ml of LB which was incubated at 37°C in a shaking incubator at 150 rpm for 2-4hr, until partially turbid, OD600 0.2-0.6. This was then immediately placed on ice.

For each transformation, 1ml of the culture was centrifuged at 16000xg (Sigma 1-16K) (4°C) for 30 seconds. The cell pellet was resuspended in 1ml of ice cold 10% glycerol. The pelleting stage was repeated and re-suspended in 1ml of 10% glycerol. This was then centrifuged a third time and the pellet was re-suspended in 40µl of 10% glycerol, at this point the cells could be stored at -80°C for long term storage.

Roughly 40-60ng/µl of DNA from RP4-8 taken from a crude cell preparation as previously mentioned in Section 2.6.1 was added separately to 40µl of competent isolate cells which was then added to a pre-chilled 2mm Electroporation cuvette (ThermoFisher Scientific). The electroporator (Eppendorf Electroporator 220V 940000017) was set to: 2.5 V, 200 Ω, 25µF (time constant ≥4msec). Once electroporation had occurred 960µl of LB was added immediately and incubated at 37°C with shaking for 1 hour. After incubation the samples were plated out onto LB plates containing gentamycin (7µg/ml). The plates were incubated at 37°C for 16 hours.

2.9.2 Plasmid DNA from isolates containing RP4-8 preparation

Single colonies taken from the gentamicin plates were placed into separate 10ml Falcon tubes containing 8ml of LB broth. These were then incubated overnight at 35˚c in a spinning incubator (Gallenkamp Orbi-safe TS netwise) and left for no more than 16 hours. 1ml of the culture was then centrifuged in a microtube for 30 seconds at 16000 x g. The supernatant was then discarded, and the pellet was resuspended in 200µl Plasmid Resuspension Buffer (Monarch Plasmid miniprep kit NEB #T1010S/L).

The cells were lysed by adding 200µl Plasmid Lysis Buffer to the solution which was then inverted several times until a colour change to pink was observed. This was then incubated for one minute. The lysate was next neutralised by adding 400µl of Plasmid Neutralization Buffer which was inverted again until a colour change to yellow was observed and a precipitate could be seen. This was then incubated for a further 2 minutes. The lysate was next clarified by spinning for 2–5 minutes at 16000 x g. Next the supernatant was added to a spin column and centrifuged for 1 minute with the flow-through being discarded after.

The column was re-inserted into the collection tube and 200µl of Plasmid Wash Buffer was added to remove RNA, protein and endotoxin. This was centrifuged for 1 minute. Next 400µl of Plasmid Wash Buffer was added to the column and centrifuged for 1 minute. After this the column was transferred into a clean 1.5ml microfuge tube. Finally, 30µl of DNA Elution Buffer was added to the centre of the matrix and spun for 1 minute to elute DNA.

2.9.3 *E. coli* containing RP4-8 gentamicin and mercury resistance

Electroporation for the new plasmids was carried out as in section 2.9.1. Plasmids were transformed into *E. coli* MG6155 which was not gentamicin or mercury resistant before transformation. The *E. coli* MG6155 cells had a separate plasmid solution from each of the isolates taken from the slurry tank and muck heap transformed into them and were then selectively grown on 25µg/ml mercury plates and 7µg/ml gentamicin plates separately.

3. Results

The primary aim of the project was to observe the variance in antibiotic resistance in *E. coli* isolates which were found to be mercury resistant. Bacteria were isolated from animal faeces and the main slurry tanks at the University of Nottingham, Sutton Bonington farm. These isolates were then tested for phenotypic and genotypic mercury resistance as well as antibiotic resistances they carried and whether they carried any antibiotic resistance genes within the presumptive Tn*21* transposon.

3.1Phenotypic mercury resistant isolates

Isolates taken from the slurry tank and muck heap were tested for mercury resistance to allow for a collection of isolates to be made which would form the group on which the further molecular and genetic tests would be carried out.

From the 943 isolates taken from the slurry samples only three (isolates EcoSL 2205-55, EcoSL 2205-56 and EcoSL 1608-397) were phenotypically resistant to 25µg/ml of Mercury.

Four isolates from a separate collection (isolates 51, 67, 68 and 113) taken from the muck heap, situated close to the slurry tank, were also found to be phenotypically mercury resistant on the plates inoculated with 25µg/ml of Mercury.

3.2 Mercury resistance kill assay

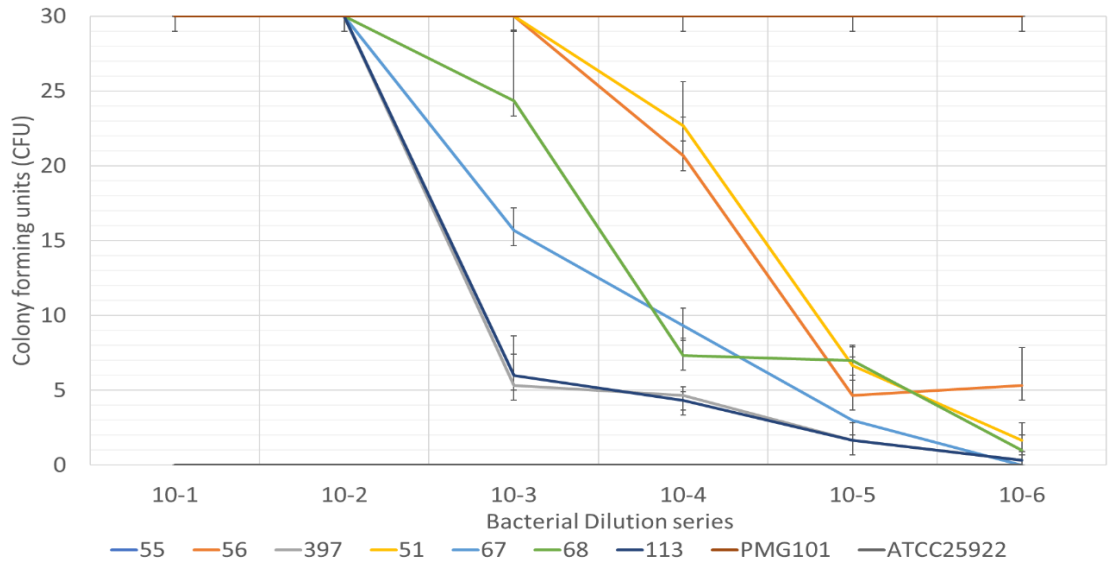
The isolates taken from the previous experiment were tested to observe how resistant they were to the effects of mercury at varying concentrations. This was to observe if similar levels of resistance were carried by all the isolates and if this could be linked to their antibiotic resistance profiles in later experiments.

All isolates were originally selected for their ability to grow on 25 µg/ml $HgCl_2$ LB plate and were also all able to grow on LB plates that were not inoculated with $HgCl_2$. These all grew above the countable range of colony forming units (>30) for all dilutions. All isolates were resistant to and survived up to 100 µg/ml of $HgCl_2$ LB plate, however a significant drop in colony forming units can be seen in isolates 51, 67, 68 and EcoSL 1608-397, with each only showing growth at the 10^{-1} dilution (figure 2.1B). No growth was seen beyond 200µg/ml of $HgCl_2$ for all isolates including PMG101, whilst the positive control and PMG101 showed no growth on 150µg/ml of $HgCl_2$ past the 10^{-1} dilution.

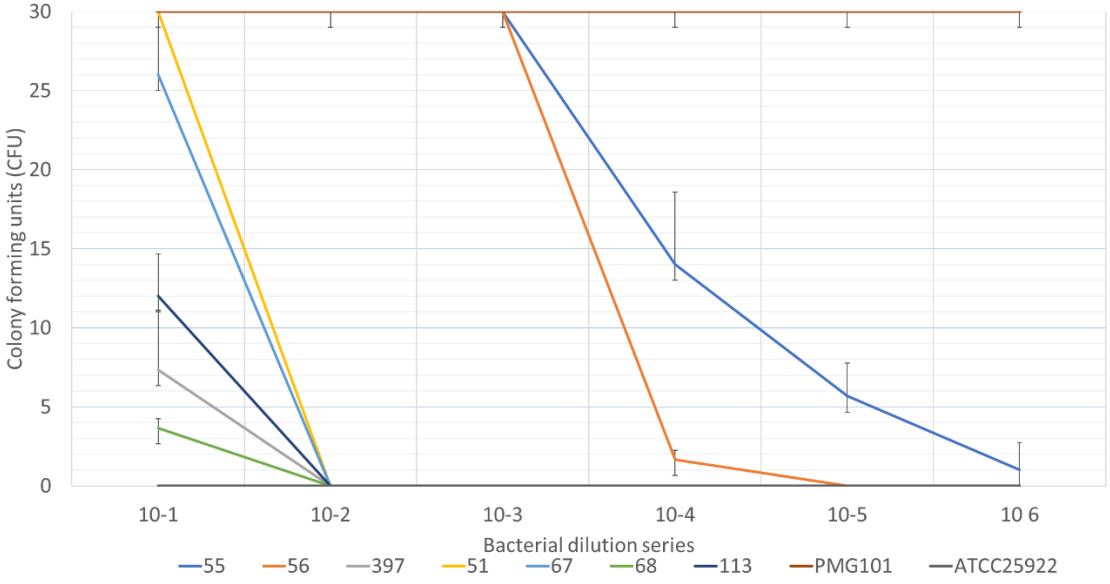
Variations in the level of resistance were seen. Isolates 55 and 56 showed >30 colonies from dilution 10^{-1} to 10^{-3} however colonies were no longer seen to grow from dilutions 10^{-5} and 10-6 for isolate 56 whereas isolate 55 showed <30 colonies from dilutions 10^{-4} to 10^{-6} (figure 2.1.A). As the levels of $HgCl_2$ increased in the plates, a drop in the colony forming units (CFU) can be observed in all isolates.

Variations can be seen in the level of resistance each isolate has, with isolates EcoSL 2205-55 and EcoSL 2205-56 showing the most consistent levels of resistance between dilutions (figure 2.1.B) however isolate 113, despite showing one of the quickest reductions in CFU did show growth (8 CFU) on plates containing 150 µg/ml of HgCl2 (Figure 2.1 C).

(Figure 2.1A)
Kill assay for bacterial dilution series placed on agar plates containing 50µg/ml of $HgCl_2$



(Figure 2.1B)
Kill assay for bacterial dilution series placed on agar plates containing 100µg/ml of $HgCl_2$



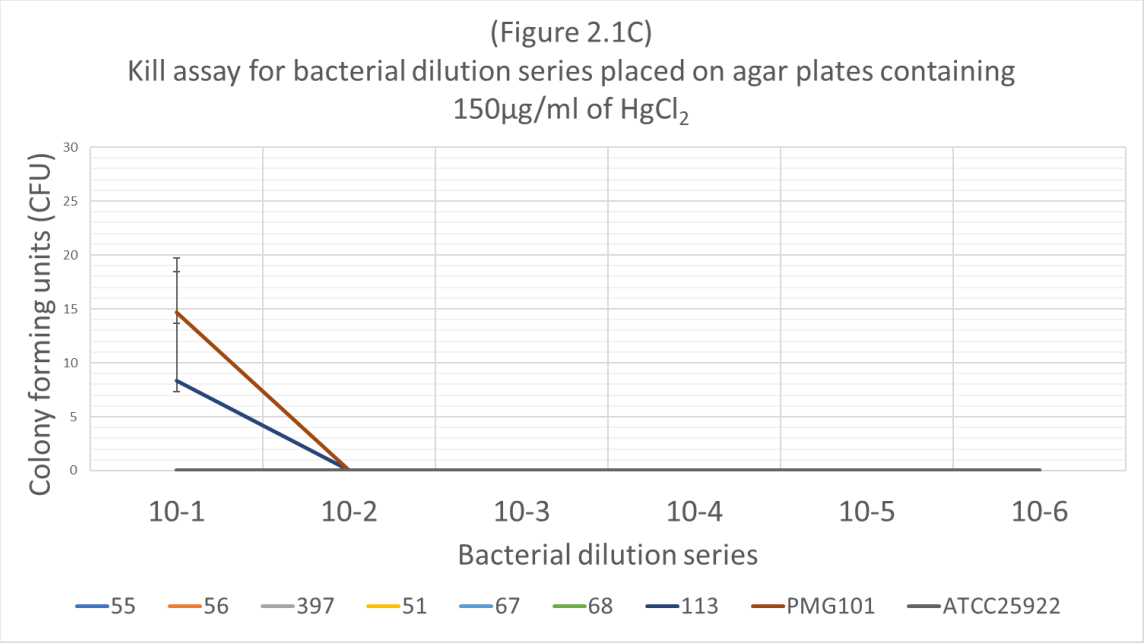


Figure 2.1 Kill assay results in colony forming units per dilution for each plate containing varying amounts of $HgCl_2$. Figures show the CFU for each isolate at each dilution stage on LB plates. Figure 2.1 A shows results for each isolate at each dilution on LB plates containing 50 µg/ml of $HgCl_2$. B shows the CFU for each isolate at each dilution stage on LB plates containing 100 µg/ml of $HgCl_2$ and figure C shows the CFU for each isolate at each dilution stage on LB plates containing 150µl of $HgCl_2$. Each isolates CFU results are indicated by a coloured line which also include the results for the positive control *E. coli* K-12 J53 PMG101 and the negative control *E. coli* ATCC25922.

3.3 Antibiotic sensitivity tests

Antibiotic sensitivity was tested to observe which antibiotics isolates were resistant to. 21 antibiotics were used including important antibiotics used in human health care such as nitrofurantoin. These tests were done to observe patterns and relationships in antibiotic sensitivities across the isolates that could have arisen from the transfer of genetic elements between bacteria. These experiments were also done to observe whether the carriage of multiple antibiotic resistances was linked to higher mercury resistance.

All isolates were completely resistant to sulphonamides and streptomycin with all but EcoSL 1608- 397 being resistant to tetracycline. Similarly, all isolates apart from EcoSL 1608- 397

were resistant to ampicillin with EcoSL 1608- 397 showing intermediate resistance. Four of the isolates were also resistant to chloramphenicol (51, 67, EcoSL 2205-55 and EcoSL 2205-56) with isolate 113 also being resistant to azithromycin, totalling in a resistance to five antibiotics.

All isolates were sensitive to cefoxitin, ceftazidime, nalidixic acid, enrofloxacin, cefpodoxime, aztreonam, ciprofloxacin, nitrofurantoin, cefotaxime and colistin. All isolates were intermediately resistant to meropenem. All isolates apart from isolate EcoSL 2205-56 were sensitive to amoxacillin-clavulanic acid with EcoSL 2205-56 being only intermediately resistant. Isolates EcoSL 2205-55, EcoSL 2205-56 and 68 were intermediately resistant to ceftiofur with the other isolates (EcoSL 1608-397, 67, 113 and 51) being completely sensitive. Isolate 113 was intermediately resistant to imipenem with the other six isolates were sensitive to this antibiotic. The sensitivities to the antibiotics tested are shown in Table 2.1.

Isolates EcoSL 2205-55 and EcoSL 2205-56 were resistant to trimethroprim-sulfamethoxazole (TP/SXT), with isolates EcoSL 1608-397, 67, 68 and 51 being sensitive to it. Isolate 113 was intermediately resistant to this. Isolates 51, 67, EcoSL 2205-55 and EcoSL 2205-56 were all resistant to chloramphenicol with the other isolates: EcoSL 1608-397, 113 and 68 sensitive. Finally, isolates EcoSL 2205-55, EcoSL 2205-56, EcoSL 1608-397 and 51 were resistant to azithromycin while isolate 68 was intermediately resistant and isolate 113 was sensitive. Table 2.1 below, shows clustering of resistances (generally sulphonamide (S3), streptomycin (S10), tetracycline (TET) and ampicillin (AMP)) and sensitivities (cefoxitin (FOX), ceftazidime (CAZ), cefotaxime (CTX), cefpodoxime (CPD), aztreonam (ATM), ciprofloxacin (CIP), enrofloxacin (ENR), nalidixic acid (NA), nitrofurantoin (F) and colistin (CT)). Despite this, variations in resistances are in all isolates against antibiotics: ceftiofur, trimethoprim-sulphamethoxazole, chloramphenicol, and azithromycin.

Isolates EcoSL 2205-55, EcoSL 2205-56, 51, 67, 68 and 113 all show resistance to at least four antibiotics from separate antibiotic classes, therefore 85.7% can be considered MDR.

Isolate EcoSL 1608-397 however only shows true resistance to two antibiotics: S3 and S10. Isolates EcoSL 2205- 55 and 113 both have the highest occurrence of intermediate resistance to 3 antibiotics (EcoSL 2205- 55 having resistances to amoxacillin-clavulanic acid (AMC), EFT and meropenem (MEM) and isolate 113 having resistance to imipenem (IPM), SXT and MEM respectively). Five out of the seven isolates show intermediate resistance to at least two antibiotics with only isolates 51 and 67 being intermediately resistant to one antibiotic, which was Meropenem.

Antibiotic Class		Penicillins	B-Lactam/ase Inhibitor comb	Second Gen Ceph	Third Gen Ceph	Third Gen Ceph	Third Gen Ceph	Third Gen Ceph	Monobactam	Carbapenem	Amino-glycosides	Fluoro-quinolones	Fluoro-quinolones	Quinolones	Folate Pathway Inhibitors	Folate Pathway Inhibitors	Phenicols	Nitrofurans	Polymyxin	tetracycline	meropenem	azithromycin
Antibiotic Abbreviations		**AMP**	**AMC**	**FOX**	**CAZ**	**CTX**	**EFT**	**CPD**	**ATM**	**IPM**	**S10**	**CIP**	**ENR**	**NA**	**S3**	**SXT**	**C**	**F**	**CT**	**TET**	**MEM**	**AZM**
Disc Concentration (µg)		10	20 & 10	30	30	30	30	10	30	10	10	5	5	30	300	1.25 & 23.7	30	300	10	30	10	30
Zone Size		++	++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	++	++	+	++	+++	+	++	++	++
55		0	20	30	34	40	20	30	44	40	0	44	34	32	0	0	4	30	18	0	21	24
56		0	16	30	36	40	20	30	46	40	0	46	32	36	0	0	4	30	18	0	21	20
397		16	24	32	32	38	24	32	42	40	4	48	34	40	0	34	32	30	16	30	20	20
51		0	20	28	30	36	24	26	34	34	0	40	30	26	0	20	0	26	16	0	20	20
67		0	20	24	28	32	23	24	30	30	10	40	30	28	0	20	0	20	17	0	22	20
68		0	20	24	30	32	20	24	30	28	10	34	26	26	0	20	30	20	17	0	20	14
113		0	20	26	28	30	24	26	38	20	10	30	30	28	0	14	28	24	17	0	20	10
+	BCC2	0	20	26	40	18	0	0	22	36	0	10	0	0	0	0	3	20	13	20	21	10
+	NCTC13476	nt	nt	0	0	0	23	0	23	20	20	0	nt	0	nt	0	0	16	14	5	25	20
+	NCTC13846	nt	nt	0	nt	nt	nt	nt	22	24	18	0	nt	0	nt	19	0	18	4	22	22	18
+	NCTC13353	0	16	36	16	18	0	10	6	36	17	0	0	20	0	0	20	19	13	6	20	6
-	ATCC25922	25	23	26	24	30	28	25	22	27	26	25	33	23	36	25	25	25	21	25	25	25

Zone Sizes Expected (this aids in appropriate disc placement)

Small <16 +	Medium 17-20 ++	Large >21 +++
Zones of Inhibition		
Sus	Int	Res
>18	14-17	<13
>17	14-16	<13
>21	18-20	<17
>18	13-17	<12
>21	18-20	<17
>23	22-20	<19
>21	18-20	<17
>21	16-20	<15
>14	12-13	<11
>26	23-25	<22
>23	22-20	<19
>26	25-19	<18
>17	15-16	<14
>18	15-17	<14
>23	20-22	<19
>19	14-18	<13
>15	12-14	<11
>17	13-16	<12
>15	12-14	<11
>23	20-22	<19
>18	14-17	<13
>19	15-18	<14
>16	11-15	<10

Amoxicillin-clavulanic acid (AMC)
Ampicillin (AMP)
Aztreonam (ATM)
Chloramphenicol (C)
Ceftazidime (CAZ)
Cefquinome (CFQ)
Cefpodoxime (CPD)
Ciprofloxacin (CIP)
Colistin (CT)
Cefotaxime (CTX)
Ceftiofur (EFT)
Enrofloxacin (ENR)
Nitrofurantoin (F)
Cefoxitin (FOX)
Imipenem (IPM)
Nalidixic Acid (NA)
Oxytetracycline (OT)
Sulfonomide (S3)
Streptomycin (S10)
Meropenem (MEM)
azithromycin (AZM)
Tetracycline (TET)
Trimethoprim-sulfamethoxazole (SXT)

Table 2.1 The resistances of each of the 7 isolates to each of the 21 antibiotics with inhibition zones measured in millimetres. Resistances are coloured red; intermediate resistances are coloured yellow, and sensitivities are coloured green. Antibiotic abbreviations are given at the top of the table and their full names can be found in the smaller table attached. Inhibition zone sizes are also given for the control strains BCC2, NCTC13353, NCTC13476, NCTC13846 and ATCC25922. Nt indicates an antibiotic that is not usually tested with a control strain so has not been tested for those control strains.

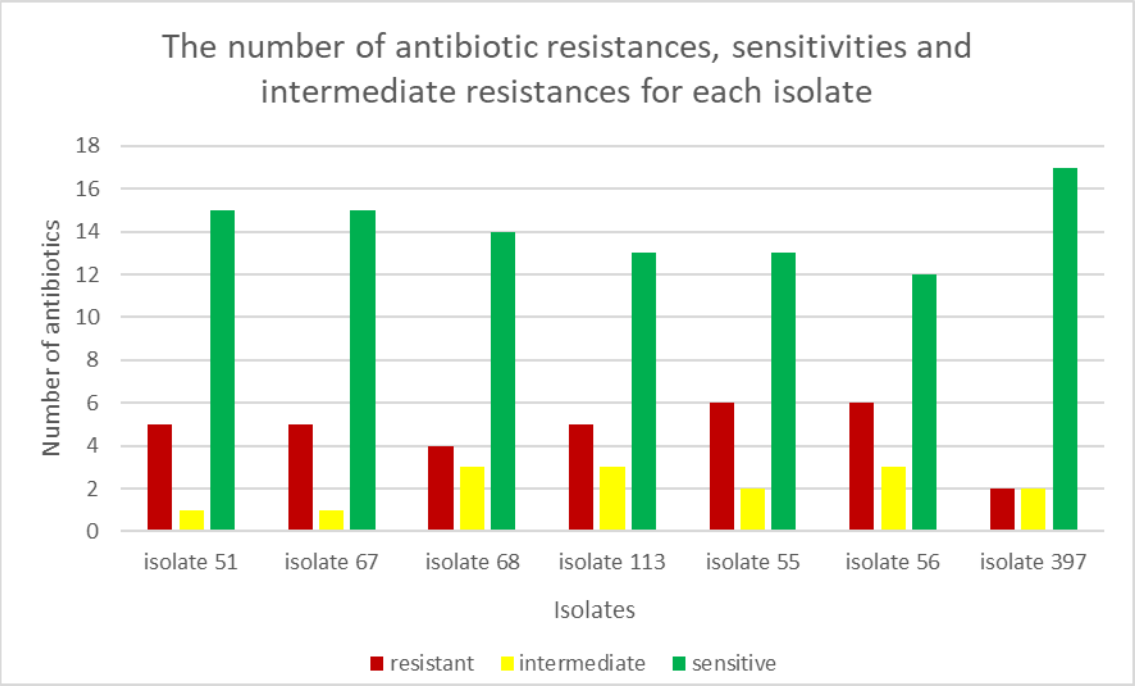


Figure 2.2 Showing the number of resistances, intermediate resistances and sensitivities displayed by each isolate that was tested. Resistance is marked by a red colour; intermediate resistances are the bars with a yellow colour and sensitivities are bars coloured green. Each isolate was tested against 21 antibiotics.

3.4 Genotypic mercury resistance PCR results

Genotypic testing of the isolates for the genes *merA, merC, merR* and *intI*1 was done to show that the phenotypic mercury resistance was due to carriage of the mer operon. The

presence of intl1 was used as an indicator of genetic transfer and a mobile genetic element which could be the cause of the antibiotic resistances shown by the isolates.

As shown in Figure 2.3, Isolates 51, 67, 68 and 113 were all positive for the presence of *merC, merR*, and the integrase *intI1* with bands present at around 422 bp's for *merC,* 433 for *merR* and 280bp for *intI1*. In Figure 2.3B, the clarity of the *intI1* genes are weak however due to trouble increasing the stringency of these tests by manipulating the MgCl content, the clarity did not improve.

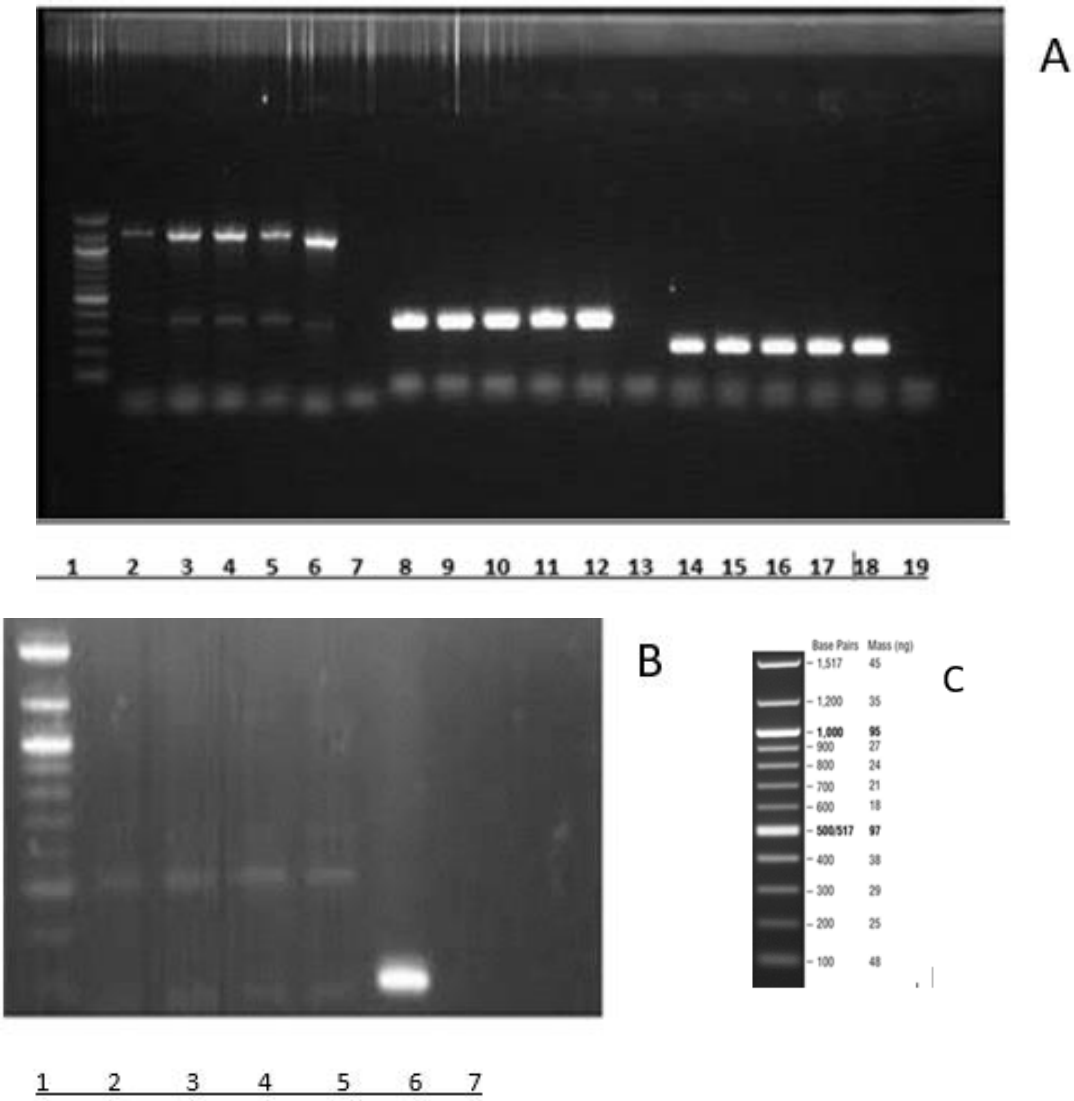
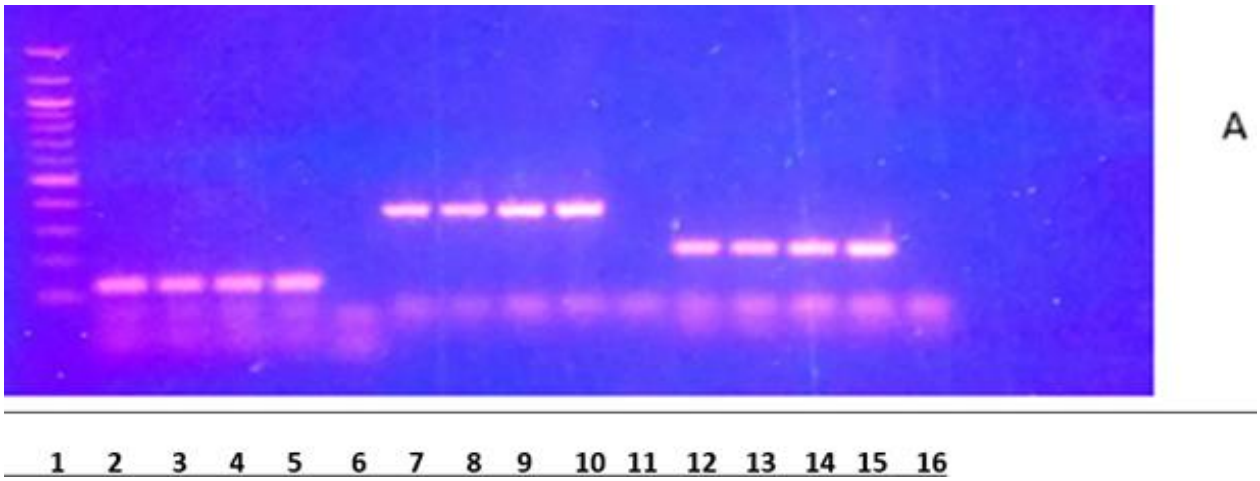


Figure 2.3 (A). PCR results for isolates tested for the presence of *merA, merC and merR*. Lane 1: 100 base pair ladder as seen in Figure 2.3 C. Lanes 2, 8 and 14: PCR products from isolate 51, lanes 3,9 and 15 contain results for isolate 67, lanes 4,10 and 16 PCR products

for isolate 68, lanes 5, 11 and 17 PCR products for isolate 113, lanes 6,12 and 18 contain the positive control strain PMG101 and lanes 7,13 and 19 contain the negative control, H_2O along with the primers and enzymes. Lanes 2-7 identify *merA*, lanes 8-13 identify *merC* and lanes 14-19 identify *merR*. (B) Isolates 51,67,68 and 113 tested for the integrase gene *int1*. Lane 1: 100bp ladder, lane 2 PCR products for isolate 51, lane 3-67, lane 4-68, lane 5-113, lane 6- PMG101 and lane 7 the negative control H_2O. (C) 100bp Quick load DNA ladder (New England Biolab (NEB)) and molecular mass of the bands once the ladder had been run which was used as a size comparison against the bands seen in the PCR gel images.

For isolate EcoSL 2205-55, EcoSL 2205-56 and EcoSL 1608- 397 it can be seen in Figure 2.4 that again *merC, merR* and *intI1*, the PCR products obtained are all present and of the expected sizes (1238bp for *merA*, 422bp for *merC*, 434bp for *merR* and 280bp for *intI1*) as indicated by comparison with the 100bp ladder in lane 1 of the images and shown in table 1.2. Bands can be seen for *merA* in isolates EcoSL 2205-55, EcoSL 2205-56 and EcoSL 1608- 397. All isolates show presence on the integrase gene as well as the majority carrying key genes of the mercury operon.



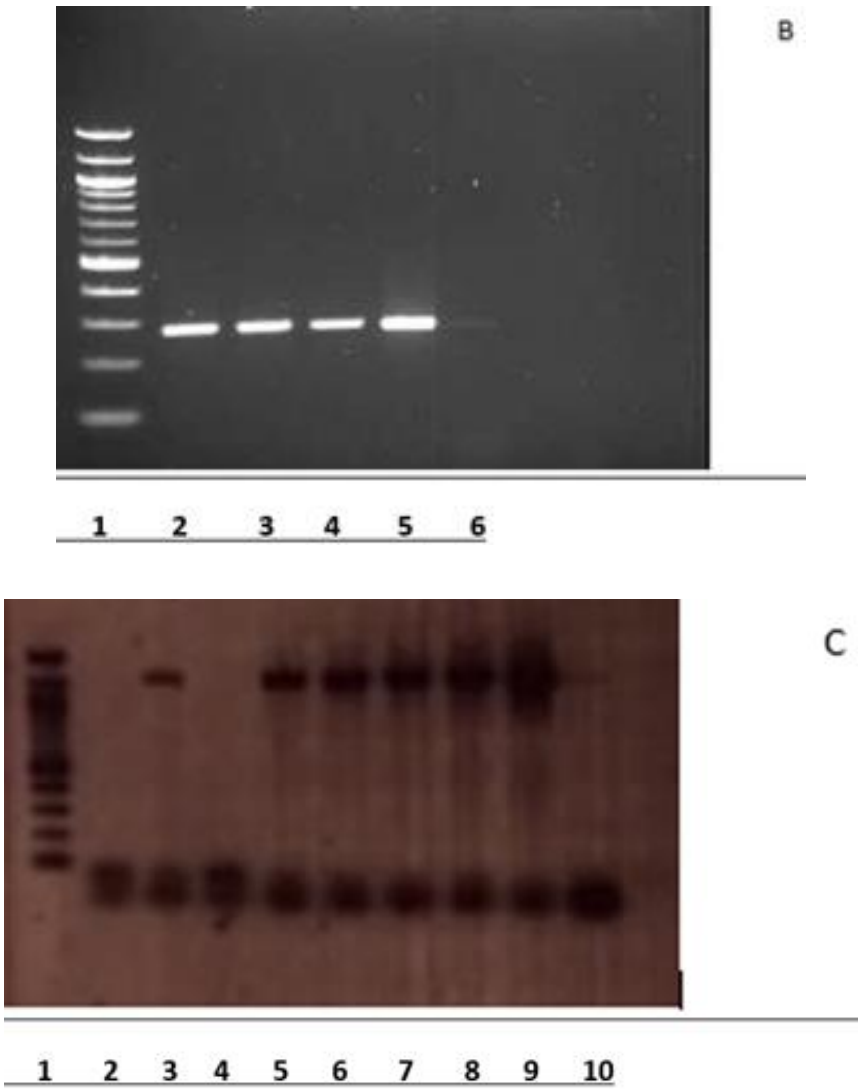


Figure 2.4 (A). Results for testing for *merA, merC and merR* in isolates EcoSL 2205-55, EcoSL 2205-56 and EcoSL 1608- 397. Lane 1 holds a 100 base pair ladder. Lane 2,7 and 12 contain PCR products from EcoSL 2205-55, lanes 3,8 and 13 contain PCR products from isolate EcoSL 2205-56, lanes 4,9 and 14 PCR products from isolate EcoSL 1608- 397, lanes 6,11 and 15 hold the positive control PMG101 and lanes 6,12 and 16 contain the negative control H_20. Lanes 2-6 tested for *merA*, lanes 7-11 tested for *merC* and lanes 12-16 tested for *merR*. It should be noted that the test for *merA* failed and results for identification of isolates containing *merA* can be found in figure 2.4C. (B) shows results for isolates containing *int1*. Lane 1 is filled with a 100 base pair ladder, lane 2- isolate EcoSL 2205-55, lane 3- EcoSL 2205-56, lane 4- EcoSL 1608- 397, lane 5 the positive control PMG101 and lane 6 the negative control H_2O. Figure 2.4C shows the results for *merA* in isolates EcoSL

2205-55, EcoSL 2205-56 and EcoSL 1608- 397. A 100 base pair ladder is in lane 1, failed tests for *merA* testing in isolates 51,67,68 and 113 are in lanes 2-5, isolate EcoSL 2205-55 is in lane 6, EcoSL 2205-56 is in lane 7, EcoSL 1608- 397 is in lane 8, the positive control PMG101 is in lane 9 and the negative control, H_2O is in lane 10.

3.5 DNA sequencing

Strains isolated from this study were to be sent to MicrobesNG for whole genome sequencing which would generate Illumina next-generation sequencing data. This would allow for observation of any additional antimicrobial resistance genes surrounding the *tet* and *sul* primers as well as any other antibiotic resistance genes that hadn't been tested for by PCR. These results would have allowed for a broader observation of the carriage and co-selection of antimicrobial resistance. This would have given a detailed image of the resistome of the isolates taken from the slurry tank as well as elucidating the structure of the genome and the mobile genetic element the antimicrobial genes are being carried on. Results were not back in time for thesis submission.

3.6 Tet and Sul IPCR

Inverse PCR is a method utilising the same technologies and ideas as PCR however it uses reverse primers to amplify genetic material that flank the region selected for by the primers. Using this method genetic material surrounding the *tet* and *sul* genes, staple genes commonly found in Tn*21* could be amplified and further sequenced to observe if any novel resistance genes which are not commonly carried on Tn*21* could be seen. This would indicate whether the Tn*21*- like transposons carried by the isolates have acquired resistance genes providing variation to the normal structure of the transposon.

When all isolates were tested using the sul1invRVa and sul1invFWa primers (Table 1.2) only isolate EcoSL 1608- 397 was seen to produce a product at around 500bp long (Figure 2.5B). When all isolates were tested using the tetMinvFWa and tetMinvRva primers (Table 1.2), isolates 67, 68 and EcoSL 2205-55 showed clear bands at around 6000bp (Figure 2.5B).

Isolate EcoSL 2205-55 also showed a larger band at around 8000bp long and a smaller one at around 2000bp long (Figure 2.5B).

Isolates 51, 67, 68, 113, EcoSL 2205-55 and EcoSL 2205-56 showed no bands of any size when tested against the *sul* primers. Isolates 51, 113, EcoSL 2205-56, EcoSL 1608-397 showed no products of any size for the *tet* primers. Table 2.2 shows the collection of genes present within all isolates tested which were prior found to resistant to mercury.

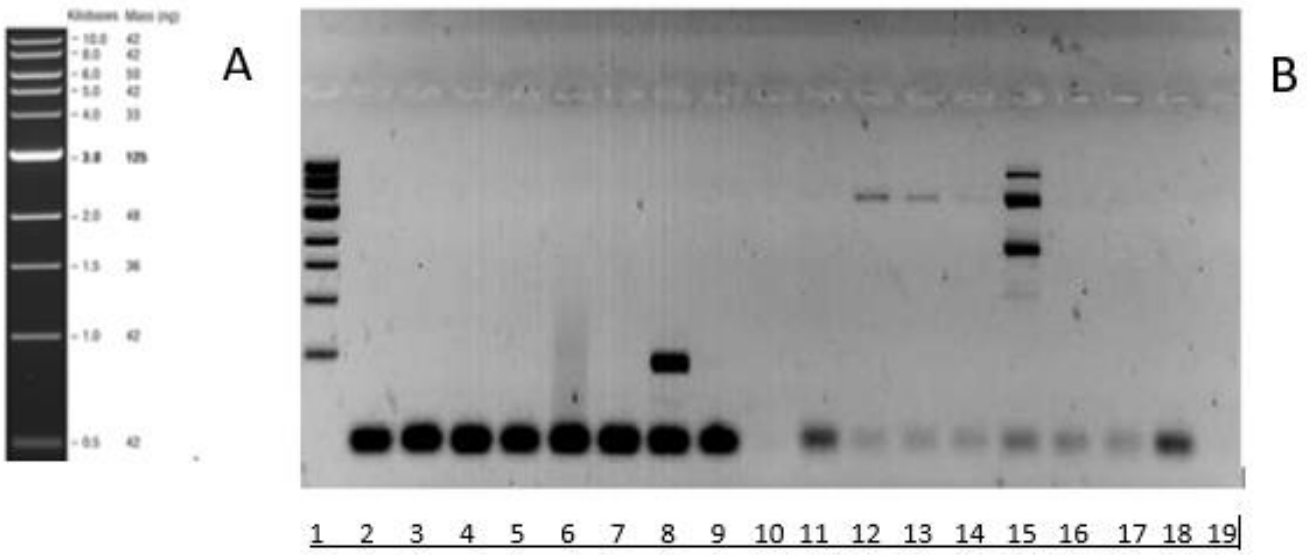


Figure 2.5 (A) shows a 1kb DNA marker ladder with each band labelled with n approximate amount of product in μg and the size in base pairs. (B) shows the inverse PCR results with lane 1 containing the 1kb ladder, lane 2 isolate 51, lane 3- 67, lane 4-68, lane 5-113, lane 6 Eco SL 2205-55, lane 7-EcoSL 2205-56, lane 8 EcoSL 1608-397, lane 9-PMG101, lane 10 the negative control, lane 11-51, lane 12-67, lane 13-68, lane 14-113, lane 15-EcoSL 2205-55, lane 16-EcoSL 2205-56, lane 17-EcoSL 1608-397, lane 18-PMG101 and lane 19-the negative control ATCC25922. Lanes 2-10 tested for *sul* and lanes 11-19 tested for the presence of *tet.*

Isolates	merA	merC	merR	intI1	tet	sul
51	√	√	√	√		
67	√	√	√	√	√	
68	√	√	√	√	√	
113	√	√	√	√	√	
55	√	√	√	√		
56	√	√	√	√		
397	√	√	√	√	√	√

Table 2.2 List of isolates and results of observed genes present in each isolate including results from the mercury gene PCR tests and inverse PCR as well as the results from the tetracycline and sulphonamide resistance inverse PCR. Ticks denote the presence of the gene corresponding to the primers used and thus its presence.

3.7 Transposon trapping

The transposon trapping experiment was used show the potential for movement of mobile genetic elements carrying resistance genes from one bacterium to another. This was to prove that mercury resistance and Tn*21* like transposons could be acquired by previously mercury and antibiotic sensitive bacteria.

For the first electroporation of the original RP4-8 plasmid all isolates taken from the slurry tank showed successful uptake of the RP4-8 plasmid after electroporation and showed growth on the gentamicin plates inoculated with 100µl, 200µl and 700µl of the LB and isolate solution (Table 2.3). The transformation efficacy was calculated for each isolate to observe the success of the integration of the RP4-8 plasmid. Due to the high number of bacteria in the plates inoculated with 700µl of the bacteria/LB solution it was impossible to count individual bacterial colonies, so the colony counts for the 200µl were used to attain a transformation efficacy.

	Amount of bacterial solution added (µl)			
Isolates	100µl	200µl	700µl	Transformation efficacy
51	120	246	>300	3.0x10-4
67	91	214	>300	2.7x10-4
68	92	183	>300	2.3x10-4
113	69	158	>300	2.0x10-4
55	88	160	>300	2.0x10-4
56	83	201	>300	2.5x10-4
397	54	107	>300	1.3x10-4

Table 2.3 Number of bacteria transformed with the RP4-8 plasmid and transformation efficacy. Each isolate was tested in 100µl, 200µl, and 700µl amounts.

The second electroporation experiment used the RP4-8 plasmid which had been transformed into the bacterial isolates taken from the slurry tank previously which were assumed to have taken up the RP4-8 plasmid, now containing gentamicin and mercury resistance. Only the *E. coli* MG6155 isolates which took up the RP4-8 from EcoSL 2205-55 and EcoSL 2205-56 showed growth of colonies on both the gentamicin (7µg/ml) and mercury (25µg/ml) LB plates. This indicates the presence of the RP4-8 plasmid containing gentamicin resistance as well as mercury resistance genes, showing successful acquisition of the marker resistance genes and RP4-8 plasmid.

4. Discussion

4.1 Antimicrobial resistance

The increased presence of antimicrobial resistance in agriculture is usually caused and amplified by the presence of antimicrobial agents such as biocides and metals being present in the environment causing a co-selection for antibiotic resistance (Gaze et al, 2013). It's not only therapeutic doses but sub inhibitory doses that can lead to the presence and prevalence of antimicrobial resistance (Andersson et al, 2012). This situation is made only more complex when considering the movement of antimicrobial resistance genes carried on mobile genetic elements such as integrons and plasmids (Singer and Williams-Nguyen,

2014). One factor potentially leading towards us seeing mercury resistant *E. coli* taken from the slurry tanks could be due to a re-emergence of the plasmids carried in the tank for several years leading to the occasional re-emergence of isolates carrying the mercury resistance operon, as changes in *E. coli* populations have been reported in just months (Duriez and Topp, 2007).

All the isolates found to be mercury resistant, except EcoSL 1608- 397, which was only resistant to two antibiotics, were found be resistant to several different antibiotic classes including sulphonamides, streptomycin and ampicillin, with the remaining six isolates being classified as multidrug resistant (Table 2.1). All isolates were completely resistant to sulphonamides and streptomycin and with all but EcoSL 1608- 397 being resistant to tetracycline. With the presence of the integrase *(intI1)* being found in all these isolates it was predicted that resistances to sulphonamides (*sul1*) should be carried among the integrons, which are well known for frequently containing gene cassettes that mediate resistance to antibiotics and are frequently found in habitats contaminated with antimicrobials (Figure 1.2). Especially as Tn*21*, known for carrying the mercury operon, also carries sulphonamide resistance as well as resistances to quaternary ammonium compounds (*qac*Δ*1*) and aminoglycoside adenyltransferases (*aadA1*) (Liebert et al, 1999). This idea is supported by studies finding elevated levels of class 1 integrons in aquatic environments contaminated with heavy metals (Wright et al, 2008).

One study looking at the diversity of *E. coli* in manure found that out of the 475 samples 80% were resistant to sulphonamides, 77% were resistant to streptomycin and 50% resistant to ampicillin (Marchant and Moreno, 2013). Four of the isolates (56.9%) taken from the slurry tank for this project were also resistant to chloramphenicol which is more than triple that of the paper which was 17.5%. These show very similar results between this study and the one conducted by Marchant and Moreno (2013)

Variations in antibiotic resistances can be seen between the isolates taken from the slurry tank and those taken from the muck heap, although they all carry very similar patterns of

resistance to ampicillin, streptomycin, sulphonamides and tetracycline (Figure 2.2). This could be due to these isolates carrying the same mobile genetic element. Much of the variation can be seen in the intermediate resistances shown by the isolates which seem to, mostly, have no consistency between isolates, apart from the antibiotic meropenem.

Findings for this part of the project were only phenotypic and did not confirm presence of the specific resistance genes which could elucidate what is being carried in these isolates. This would be done to ensure that there is no cross resistance or co- resistance being applied by one specific gene for two or more antibiotics.

4.2 Phenotypic mercury resistance and Kill assays

Bacteria have evolved many mechanisms to resist mercury's negative effects. Transposon encoded resistance to mercury can be commonly found on plasmids as is also the case for antibiotic resistance (Summers, 1986). The prevalence of mercury resistant bacteria, such as *E. coli* is shown to be higher in those environments which are polluted with mercury, mainly through the acquisition of mercury resistance genes (Barkay and Olson, 1986). In environments not containing mercury however, it would be thought that the prevalence of mercury resistant bacteria would fall as selective pressures to maintain the genes for resistance would no longer be needed. Despite the presumed lack of mercury within the slurry tank 3 isolates were found out of the 926 tested to be phenotypically and genotypically mercury resistant. As well as this, 4 isolates were found to be mercury resistant from the muck heap. This low number of mercury resistant strains could be due to the lack of mercury in the slurry tank environment providing no selective pressure, or whilst the presence of some mercury resistant isolates could be caused by some other factor. Potentially the presence of other selective pressures such as the presence of metals like copper, which is found in foot baths and antibiotics could cause the co-selection of the mercury operon and thus explain its continued presence in the isolates (Wilson-Welder et al, 2015).

Many studies have reported the positive correlation between resistance to antibiotics and metals in a wide variety of environments, from swine waste lagoons to soil and waste from

dairy farms (Pan et al., 2006). One study found that along a gradient of metal exposure the bacteria were more tolerant to the metals as well as antibiotics compared to bacteria from a control site (Perry and Wright, 2013). However, despite this correlation, causality is not ensured as many other factors besides metal exposure differ between studies, so the true cause remains unclear and yet again there is still a lot left to define. Community based studies are well known for over-exaggerating the taxonomic proportions of bacterial communities so the reported changes in resistance genes or resistant bacteria would also be overemphasised. The metal present in the environment may only be selecting for bacteria that also happen to be carrying antibiotic resistance genes, regardless of abundance or whether these genes are active. This scenario is more likely than the specific selection of strains within a species for having a wide range of resistance genes. To study antibiotic and metal resistance co-selection effectively these changes should be measured in cells that are in a complex community, measuring these two events occurring within cells in parallel doesn't give a sense of the broader scale of events that are happening. One study looking at mercury amalgam fillings in apes found that the oral and gut microflora showed an increase in antibiotic and mercury resistance in the 5 weeks after the amalgam fillings had been installed (Summers et al, 1993).

The existing research available does point to co-selection of mercury resistance genes with those for antibiotics and even other antimicrobials such as quaternary ammonium compounds, however there is a lack of research that looks at the full scale of the problem. Some studies will look at which microbes are resistant to a certain substance, whilst other studies, which are becoming more common, will look at the full range of resistance genes available in a specific isolate. Both types are only looking at one side of the problem which prevents a clear view of the situation: to gain an idea of the full scope of the problem there should be more research looking into the full resistome of the bacteria isolated from these studies. This, as mentioned previously, would elucidate the transmission and dissemination patterns of antimicrobial resistance genes as well as others which may be of interest but are

not looked at specifically because we either are not aware of them now or aren't specifically looking for them.

The research being currently conducted will elucidate the current resistance genes from *E. coli* isolated from cow slurry from the Sutton Bonington slurry tank. This will show the metal and antibiotic resistances being carried, how prevalent they are on the farm and what other resistance genes are being carried. The hope is that with this work novel resistances, unknown or unexpected will be found and with the use of transposon trapping their function will be elucidated.

Out of the 926 isolates taken from the slurry tank only three were found to be mercury resistant. This seems like a comparatively small proportion when compared to the results of other studies looking at mercury resistance presence such as the Nakahara et al (1977) study which found 58.6% of the 338 isolates were resistant to mercury. This study was conducted on clinical isolates in Japan but still shows a very high proportion of mercury resistance carriage by *E. coli* isolates. Another study looking at mercury being a driving factor for antibiotic resistance found that 713 strains out of 849 taken from a gold mining area which used mercury to make gold amalgams, had an MIC to mercury of 32 mM and 136 strains with an MIC of 64 mM (Skurnik et al, 2010). The low number found in this project could be due to initially looking for mercury resistance before looking at each isolate's antibiotic resistances during testing. It could also possibly be due to a limited re-emergence of mercury resistance carried by mobile genetic elements, as no mercury is used on the farm the mercury resistance genes carried by these elements have not proved useful and so their dissemination hasn't readily occurred due to not providing enough of a survival benefit.

All isolates initially identified as mercury-resistant, when further tested for their resistance to $HgCl_2$ did survive and show growth on LB agar plates containing 100µg/ml of $HgCl_2$, however variations were observed between isolates. Isolates 51, 67, 68, 113 and EcoSL 1608-397 only showed growth at 10^{-1} dilution at 100µg/ml whereas isolates EcoSL 2205-55 and EcoSL 2205-56 showed >30 CFU until 10^{-3}, after which isolate EcoSL 2205-55 showed

a steady decline in CFU until the final dilution 10^{-6} and isolate EcoSL 2205-56 only showed growth at 10^{-4}. This indicates that the levels of resistance in the slurry tank and muck heap differ as well as showing that the levels of resistance in the slurry tank differ between isolates. This suggests that isolates EcoSL 2205-55 and EcoSL 2205-56 could express varying levels of the proteins required for mercury resistance due to copy number or other regulatory effects. This difference in expression could be due to the structure of the plasmid that the mercury operon is being carried on and thus the promoters that could lead to an increase in expression. Looking at the isolates taken from the muck heap (51, 67, 68 and 113) these isolates all have a lower tolerance to mercury ($HgCl_2$). This comparatively low resistance could be due to the fact the muck heap does not have a system of mixing when new faecal matter from the animals is added. The sub inhibitory levels of antibiotics then contained within the faecal matter would not mix, reducing the survival advantage of having the mercury operon, due to a lack of antibiotic resistance co-selection, which is carried alongside the genes for mercury resistance.

In the slurry tank, there is a mixture of liquid and solid matter along with waste from the footbaths which are known to contain copper. This then results in for an environment rich in antibiotics and metals, which promotes the expression of antimicrobial resistance genes and results in the higher levels of resistance seen in Section 3.2. It has been hypothesised that the length of time the slurry is stored can also increase the spread of resistance. If storage time is reduced however, then the potential of spread is significantly reduced (Baker et al, 2016). Due to this there is likely to be a persistence of antimicrobial resistance that's occurring. Isolates taken from the slurry heap are resistant to mercury, albeit not as highly resistant as the isolates taken from the slurry tank, thus there could be a continuous and isolated population of mercury resistant bacteria within the muck heap.

4.3 Genotypic mercury resistance

All the mercury resistant isolates tested during this experiment proved to have all the mercury resistance genes tested for: *merA, merC and merR*, as well as the integrase gene

intI1 indicating the presence of an integron. This indicates that the whole of the mercury operon has been moved into these isolates presumably using a mobile genetic element and that this element may have the potential to move again. Further confirmation by PCR should be used to elucidate whether this is what is happening. Despite showing the presence of the key genes for mercury resistance it was not investigated as to whether the isolates contained the other genes of the operon such as *merT and merP*. For further research it would be interesting to see whether these mercury resistance genes are being carried together and next to each other in an operon structure or whether they are being split between the chromosome and the plasmid. This could be done by using whole genome sequencing which would allow for this to be determined.

There is the potential that the isolates in this project could all be carrying the same mobile genetic element as all have streptomycin and sulphonamide resistance which are carried on Tn*21* (Figure 1.2). To confirm this, the isolates should be tested for quaternary ammonium compounds as well which is also carried on Tn*21*. Ideally however sequencing of the isolates should be conducted to identify what is being carried on the integron and what antibiotic resistance genes are also present to clarify if all isolates are carrying the same integron and if the variations seen in antibiotic resistance are due to difference in the chromosomally encoded resistances between isolates.

4.4 Sequenced Inverse PCR products

Inverse PCR amplification of EcoRI cut and re-ligated genomic DNA from all isolates was attempted using the sul1invRVa and sul1invFWa primers (Table 1.2). DNA extracted from EcoSL 1608- 397 was the only sample where an inverse PCR product was produced. This product was approximately 500bp long which is comparatively small compared to the full size of Tn*21* which is around (de la Cruz and Grinsted, 1982). This could potentially mean that the extension time used in the PCR protocol wasn't long enough for this and that not much else around the *sul1* gene was amplified. In the study by Pärnänen and co-authors (2016) no contigs were produced for *sul* however several contigs were formed for *tet*, with

one 4596bp long contig and three contigs between 1800bp and 2800bp. Another reason that the products obtained were smaller than expected could be due to the location of EcoRI sites surrounding the primer annealing sites. The sul1invRVa and sul1invFWa primers anneal at around 8000bp and 10000bp along Tn*21* where the *sul* gene is located. This area is surrounded by 4 closely positioned EcoRI sites as seen in Figure 2.6(A) (Zühlsdorf and Wiedemann, 1993) and Figure 2.6 (B) (de la Cruz and Grinsted,1982). With the products that were obtained being so short it could be assumed that this is due to the elongation step of the PCR process terminating at one of these sites.

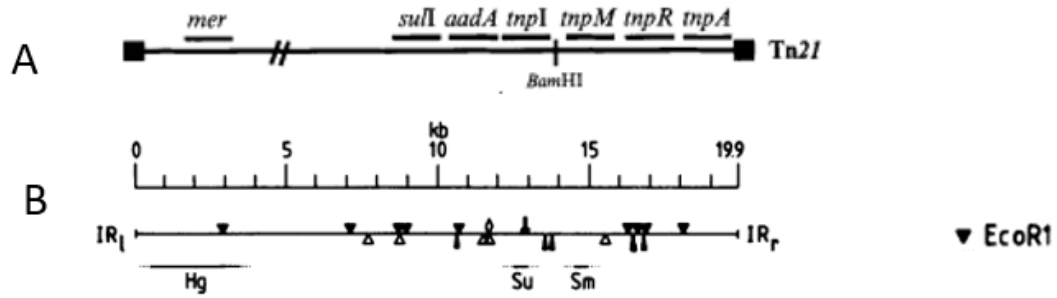


Figure 2.6 (A) shows a rough schematic of major genes in Tn*21* including the mercury operon indicated by *mer*; sulphonamide resistance, *sulI*; Aminoglycoside resistance, *aadA*, and transposase genes indicated by *tnpI*, *tnpM*, *tnpR* and *tnpA*. The solid boxes indicate inverted repeats. Location of the primer binding sites is indicated by *sulI.* (B) shows a restriction enzyme map of Tn*21* showing positions of EcoRI restriction sites with a key for the EcoRI restriction enzyme site. Permissions to reproduce these figures has been granted by the Journal of General Microbiology (A) and by the American Society of Microbiology

When all isolates were tested using the tetMinvFWa and tetMinvRva primers (Table 1.2), isolates 67, 68 and EcoSL 2205-55 showed clear bands at around 6000bp. Isolate EcoSL 2205-55 also showed a larger band at around 8000bp long and a smaller one at around 2000bp long. The 8000bp and 6000bp product are considerably larger than those obtained by Pärnänen and could hold an abundance of resistance genes. Pärnänen also found two mobile genetic elements, Tn*916* and Tn*5397*, as well as tetracycline or aminoglycoside

resistance associated with the mobile elements. Intriguingly the other isolates did not show any products from the inverse PCR. This is unsurprising for isolate EcoSL 1608- 397 as when it was tested using *tet* primers it was shown to be sensitive to tetracycline, however the others all showed resistance. The findings above indicate that the *tet* gene is not being carried on Tn*21* and that there is enough space between the primer annealing point and the next EcoRI site to obtain products of 6000bp in length. In comparison to the results from Tamminen et al (2011) observed resulting amplicons of around 1000bp. However, in their experiments DNA from environments involved in aqua culture were used without looking specifically at any organism. This means the difference in length of amplicons could be due to a difference in bacterial species the DNA came from. It would have been interesting to sequence the products from the IPCR experiment to observe what other antimicrobial resistance genes were surrounding the tet gene, and if this too was being carried on a transposable element that wasn't being looked at in this project.

The motive behind using Inverse PCR was to observe whether antibiotic resistance genes had moved into the Tn*21*-like transposons thought to be in the isolates. By using primers designed to use *sul* and *tet* as start points and using inverse PCR to circularise and amplify the regions around these genes, it would have been possible to observe any new genes, that are not commonly found in the Tn*21* structure, showing variation in resistance genes carried by the transposons and thus responsible for an increase in the potential for co-selection of mercury resistance.

To fully understand the potential for mercury resistance to be disseminated and spread it would have been interesting to see whether any contigs formed between the *tet* and *sul* primers used and the mercury genes contained within Tn*21*. By amplifying *tet* and *sul* and observing the results, it could provide an insight into neighbouring genes and whether genes allowing for transposition and movement close by could move these resistance genes. Observing these amplicons would also have allowed us to see what other antibiotic resistance genes are being carried along with mercury and *sul* or *tet*, and thus gain an idea

of the mobility potential. For the isolates that showed products from being tested with *tet* primers it would have been interesting to see what was contained within the 10kb band taken from isolate EcoSL 2205-55. Tn*21* does not contain a gene for tetracycline resistance as can be seen in Figure 1.2 and this result could potentially indicate the presence of another transposon such as Tn916 (Pärnänen et al, 2016). The original R100 plasmid does carry the *tet* gene on Tn*10* (See Figure 2.7, Liebert et al, 1999). The PCR amplicon produced from isolate EcoSL 1608-397 DNA was potentially too small at 250bp to hold any useful information on what other genes are being carried and their position in relation to other resistance genes. Unfortunately, due to time constraints It was not possible to sequence the inverse PCR amplicons.

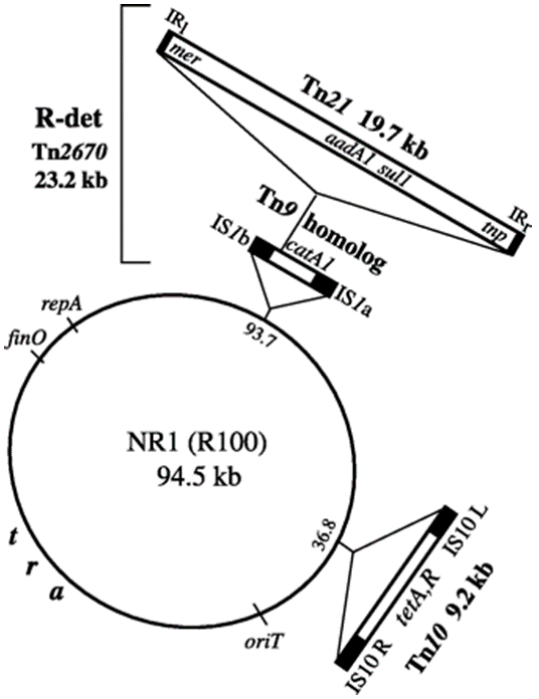


Figure 2.7 Genetic map of the multiple antibiotic resistant R100 plasmid. Inserts surrounding R100 indicate mobile genetic elements and the resistance genes they carry (Liebert et al, 1999). Permission to reproduce this Figure has been granted by American Society of Microbiology.

The reason inverse PCR was used was because inverse PCR has been found to be more sensitive than other tests such as metagenomics. A study using the same primers as the ones used in this project found the sensitivity limit of IPCR was determined to be approximately 10^{-8} copies of target gene whereas metagenomics the abundance limit for obtaining assembled contigs with target genes was approximately 10^{-3} target genes per 16S rRNA gene (Pärnänen et al, 2016).

4.5 Transposon trapping

The detection of active transposons that can directly influence a bacteria's phenotype is relatively simple to determine as can be seen with this project. RP4-8 was used and gave gentamicin resistance to the bacteria which it was inserted into (Sołyga and Bartosik, 2004). It is widely known that transposon and insertion sequences are the most viable factors in recombination within the bacterial genome (Zaratiegui, 2017). It is these elements which allow for mutations: inactivation or even overactivation of specific genes (Hubner and Henderson, 1997). Both transposons and insertion sequences are commonly found in the genomes of bacteria and within other mobile genetic elements which can then allow for the further dissemination of these elements into the bacterial population.

The results from the transposon trapping experiment proved that it is possible to transpose a large genetic element containing antimicrobial resistance genes out of the isolates taken from the slurry tank into other *E. coli* strains. RP4-8 which contained gentamicin resistance was electroporated into bacterial isolates taken from the slurry tank containing mercury resistance and subsequently electroporated into *E. coli* MG6155. RP4-8 was electroporated initially to gain mercury resistance from the Tn*21* like transposon, which would become one of two markers. *E. coli* MG6155 was grown on mercury and gentamicin agar plates to show that genetic acquisition and movement of large genetic elements such as Tn*21* does occur.

However, this was only observed in the *E. coli* K12 MG6155 strains which had taken up the plasmids from EcoSL 2205-55 and EcoSL 2205-56. Each showed growth on gentamicin plates due to resistance provided by the RP4-8 plasmid which was used in the transposon

trapping experiment as it contains resistance genes to gentamicin that could be used to determine the uptake of it into the wild type bacteria taken from the slurry tank which were not found to be originally gentamicin resistant (Figure 2.8) (Quandt et al, 2004). As well as gentamicin, mercury resistance was an identifier of transposon trapping as it showed that the mercury operon or even Tn*21*-like transposons had integrated into RP4-8.

It is unexpected that the transposon trap did not work for any of the other isolates in the project. With the size of RP4-8 being roughly 60kb and such a large plasmid being acquired by two of the isolates from the slurry tank it is unexpected that the other isolates haven't acquired the plasmid. Transposon insertion efficiency can vary depending on the size of the genes being carried. Transposons like Tc*1*/mariner are negatively affected by this as shown by a decrease in cultured cells (Muñoz-López and García-Pérez, 2010). However, in Tol*2* the efficiency begins to drop off when the genes being carried are above 10kb in size (Balciunas et al, 2006). This could potentially be a reason for not seeing more isolates with the resistance marker genes, as the insertion sequence conferring gentamicin resistance is only 800bp and the sequence of Tn*21* is 19671bp (Liebert et al, 1999). However, this assumes that Tn*21* has been acquired by RP4-8 and not smaller Tn*21*-like transposons. It is more likely that experimental errors occurred whilst electroporating the cells if only a couple of the isolates gained RP4-8. However, this does still show that it is possible for these elements to move between bacteria despite the procedure only working for two of the isolates.

It is thought that transposon trap experiments are random in that the element selected will randomly integrate, with an optimal transposon showing no sequence bias and integrate randomly at any genomic point to which the transposon has been exposed (Seringhaus et al, 2006). Despite this though it has been found that they do have preferential sequence sites into which they will integrate. If this is again true, then what we should see from these isolates once they were sequenced is integration at similar sites and therefore disruption of the genes surrounding these sites. It would have been interesting to investigate whether

from the results we achieved if the transposon traps integrated into the wild type isolates taken from the slurry tank in a random, non-bias way or whether due to the isolates all theoretically containing the same Tn*21* plasmid, whether it would have inserted here. It is the identification of the transposable elements in a specific environment or population that can lead to a greater understanding of the population's diversity as well as the direction and frequency of lateral transfer (Sołyga and Bartosik D, 2004).

The results from transposon trapping experiment proved that it is possible to trap a genetic element containing antimicrobial resistance genes into a large mobile genetic element which can then be acquired by the isolates taken from the slurry tank. However, this should be researched further as not all isolates took up RP4-8 containing mercury resistance genes.

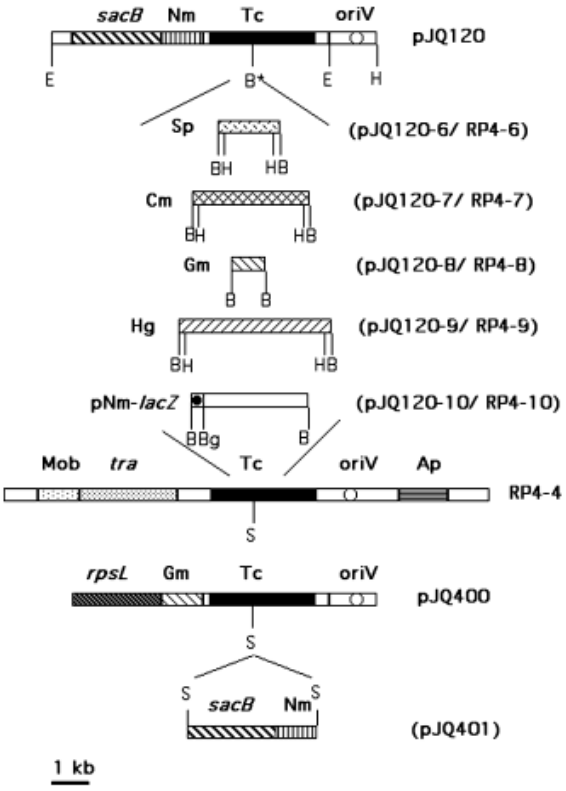


Figure 2.8 Construction of the RP4-8 plasmid containing the gentamicin resistance noted by Gm. RP4-4 is not drawn in complete detail or to exact scale and only relevant genes of RP4-4 are shown. Diagram shows the structure of RP4-4 along with insertions of relevant genes

to produce the RP4 derivatives (Quandt et al, 2004). Permission to reproduce this Figure has been granted by Elsevier.

4.6 Sequenced Isolate DNA

Strains have been sent for sequencing however results have not returned in time for submission.

5. Conclusion

Tn*21*- like transposons, indicated by their ability to resist mercury chlorides toxic effects had a low abundance in the 926 *E. coli* samples taken from the slurry tank. Variance in resistance profiles between isolates taken from the slurry tank and muck heap could be seen, indicating a potential localised evolution or acquisition of resistance genes. Transposon trapping proved that co-selective resistances, as represented by using Tn*21*-like transposons (mercury and antibiotic resistances), can move between *E. coli* through mobile genetic elements (RP4-8), and furthermore acquire large genetic elements like Tn*21*.

It is clear from the results obtained in this project that there is still a lot of work left to elucidate the prevalence and potential mobility of Tn*21*-like transposons in *E. coli* from the slurry tank and muck heap. The project shows that there is a potential threat that could arise from these agricultural environments through co-selection and the mobility of transferable genetic elements.

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