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Detection and characterization of antibiotic resistance plasmids in Cheney biosolids

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DETECTION AND CHARACTERIZATION OF ANTIBIOTIC RESISTANCE PLASMIDS IN CHENEY BIOSOLIDS

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ABSTRACT

Biosolids are organic matter produced at the end of sewage treatment process. It has been shown that during sewage treatment, resistant bacteria are selected because of the presence of antibiotics and their byproducts. These resistant bacteria are more likely to transfer resistance genes to other bacteria. In the current study, we examined Cheney biosolids for the presence of drug resistant bacteria and the role of these bacteria in transfer of resistance genes to others. We screened 100 bacteria for drug resistance and found that 68% of the isolates were resistant to two or more drugs tested. Plasmids were separated from the resistant bacteria and 13.2% of them showed the presence of plasmids. These resistance plasmids were introduced into *E. coli* MM294 to screen for the presence of antibiotic resistance genes. Plasmids were isolated from the transformants and 77.7% of the transformants showed the presence of plasmids with similar size and mobility on an agarose gel. The plasmids extracted from the transformants were digested with a restriction enzyme, *Eco*RI to verify the presence of multiple plasmids in the samples. The resistant bacteria (13.2%) that showed the presence of plasmid were tested whether they were conjugative or mobilizable type. Unfortunately, none of the isolates were conjugative or mobilizable plasmid. In short, Cheney biosolids do contain drug resistant bacteria, so there is a chance that these resistant bacteria will transfer their resistance genes to other bacteria present in biosolids.

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1.1 Significance of Antibiotics:

Antibiotics are chemicals that restrict the growth of bacteria. They are divided into two major categories based on their effect on a target cell: bacteriostatic (growth inhibitor) and bactericidal (killing agent) (Davies, 2010; Walsh, 2003). Antibiotics affect bacterial growth by many different mechanisms including: 1. disruption of bacterial cell wall (e.g., Penicillin), 2. inhibition of protein synthesis (e.g., Aminoglycosides and Tetracyclines), and 3. inhibition of DNA replication and transcription (e.g., Quinolones and Rifampin). The most commonly used antibiotics and those used in this study are listed in Table 1. Antibiotics are used everywhere - in agriculture, aquaculture, livestock, veterinary and human treatment. From the 1950s, antibiotics such as oxytetracycline and streptomycin have been used as pesticides when growing fruit, vegetable, and ornamental plants. Streptomycin is primarily used for control of fire blight in apples, pears and plants of the Rosaceae family. In the USA, approximately 10,000 tons of antibiotics are used on plants (McManus *et al.,* 2002). Animal farms and aquaculture are also using antibiotics to promote growth and prevent diseases (Cabello, 2006). For example, in swine breeding farms, antibiotics are detected in dust from the air ventilators (Hamscher *et al.,* 2003). It has been shown that antibiotic misuse or overuse has resulted in the development of drug resistant bacteria in the environment (Rhodes *et al.,* 2000). Such resistant bacteria pass

on their resistance traits to successive generations (vertical transmission) and to other members of the bacterial community (horizontal gene transfer) (Davies, 2010; Freifelder, 1987).

Table 1: Antibiotics: classes, targets and resistance mechanisms

Class / Generic name	Common uses	Target	Resistance Mechanism
Aminoglycosides Kanamycin Streptomycin	*E. coli, Klebsiella, Pseudomonas* infections Anti-tuberculosis	Binds to 30S subunit of bacterial ribosome, thereby inhibits protein synthesis	Phosphorylation, acetylation, nucleotidylation, drug efflux and target alteration
Penicillin Ampicillin	Streptococcal infections, Syphilis, and Lyme disease	Disrupts the peptidoglycan layer of bacterial cell wall	Hydrolysis, drug efflux and target alteration
Quinolones Nalidixic acid Ciprofloxacin	Urinary tract infections, pneumonia, bacterial diarrhea, and mycoplasmal infections	Bind to DNA gyrase A, thereby inhibits DNA replication and transcription	Gyrase mutations, reduced uptake and drug efflux
Tetracyclines Tetracycline	Syphilis, Lyme disease and mycoplasmal infections (also used to treat infections in plants and animals)	Binds to 30S subunit of bacterial ribosome, thereby inhibits protein synthesis	Monooxygenation, drug efflux and target alteration
Others Rifampin	Gram positive and mycobacteria	Binds to the β subunit of RNA polymerase and inhibits transcription	ADP-ribosylation, drug efflux and target alteration
Chloramphenicol (rarely used in the U.S)	Meningitis, MRSA, topical use, or for low cost internal treatment.	Inhibits bacterial protein synthesis by binding to the 50S subunit of the ribosome	Acetylation, drug efflux and target alteration

1.2 Issues with Drug Resistant Bacteria:

In 1928, Alexander Fleming discovered the first naturally occuring antibiotic, penicillin. In the 1940s, even before the introduction of penicillin into clinical practice, a bacterial enzyme penicillinase was discovered. Penicillinase breaks down penicillin by targeting the β-lactam ring present in its structure (Abraham and Chain, 1940). Antibiotic producing bacteria and fungi developed natural resistance to protect themselves from the antibiotics that they produce. This shows the prevalance of antibiotic resistance in natural settings (Walsh, 2003). However, introduction of antibiotics in clinical practice has created enormous pressure on bacteria and selected them for drug resistance. A strong correlation between the use of antibiotics and a development of resistance has been observed over the past half-century (Shlaes *et al.,* 1997). This is especially true for β-lactam class of antibiotics and their corresponding inactivating enzyme, β-lactamases (Damoa-Siakwan, 2005; Shlaes *et al.,* 1997). At this time, several classes of β-lactamases have been identified, comprising up to 1000 different β-lactamases (Bush and Jacoby, 2010). The use of antibiotics worldwide in clinical practice has led to multidrug resistant (MDR) and extensively drug resistant (XDR) strains of bacteria e.g., MDR and XDR *Mycobacterium tuberculosis* (Gandhi *et al.,* 2006 and Sekiguchi *et al.,* 2007). The Centers for Disease Control and Prevention (CDC, 2008) estimate the annual cost of treating infections caused by drug resistant bacteria to be 5.7 to 6.8 billion U.S. dollars. This shows the persistence of drug resistance in bacterial populations.

The resistance genes that confer antibiotic resistance are prevalent and widespread among bacterial communities because of horizontal gene transfer (HGT) (Shlaes *et al.,*

1997). The HGT mechanisms include: 1. transformation (cell free DNA uptake), 2. conjugation (bacterial mating) and 3. transduction (viral infection). These mechanisms have played a significant role in the evolution and transmission of β-lactam resistance among bacteria. For instance, *Pseudomonas aeruginosa* became resistant to β-lactams and aminoglycosides (another class of antibiotics), thus evolved into a life threating pathogen. Patients with cystic fibrosis are most susceptible to *P. aeruginosa* as this pathogen is highly opportunistic and infect damaged tissues by avoiding human immune defenses (Curran *et al.*, 2004; Horrevorts *et al.,* 1990).

Similarly, a gram-negative pathogen, *Acinetobacter baumnanni* is primarily hospital acquired (nosocomial) causing serious infections and death (Peleg *et al.,* 2008). These organisms persist in soil and water. Several *Acinetobacters* are resistant to various classes of drugs such as β-lactams, aminoglycosides, quinolones and tetracyclines. In addition, many *Acinetobacters* are naturally competent for DNA uptake and have high rates of natural transformation. This supports the idea that these organisms are capable of exchanging resistance genes with others (Peleg *et al.,* 2008). The toxin producing anaerobe, *Clostridium difficile* is also hospital acquired and found to cause severe intestinal infections. This gram-positive spore former can be readily transmitted by hospital personnel and equipments (Gifford and Kirkland, 2006). Once ingested, these organisms reside in the human gut but their numbers are kept low by normal gut flora. If the gut flora are eliminated by antibiotics, these *Clostridia* colonize the entire gut and release toxins which can result in the serious intestinal infection, pseudomembranous

colitis. Recently, hypervirulent toxin producing strains have also been recognized (Vernaz *et al.,* 2009).

The most famous superbug is the gram-positive bacterium, *Staphylococcus aureus.* It is a nasal commensal in 30% of the population and causes common skin infections such as impetigo and boils (Enright *et al.,* 2002 and Tenover *et al.,* 2001). In 1959, the antibiotic methicillin was discovered to suppress penicillin resistant *S. aureus,* but within 3 years methicillin-resistant *S. aureus* (MRSA) had arisen (Barber, 1961; DeLeo and Chambers, 2009). The MRSA is a well-known hospital pathogen. More than 10% of bloodstream infections in hospitals are due to MRSA and patients with MRSA have worse outcomes than those with methicillin-sensitive *S. aureus* (Selvey *et al.,* 2000). In recent years, identification of MRSA in healthy individuals in a community has become increasingly common (Delaney *et al.,* 2008; Klevens *et al.,* 2007). Thus, an increase in the development of drug resistance among many opportunistic pathogens presents a major health and economic concern.

1.3 Development of Antibiotic Resistance:

Bacteria develop antibiotic resistance primarily by: point mutations and horizontal gene transfer (HGT). The development of resistance by point mutation occurs only by chance i.e., bacteria develop mutations at a rate faster than other organisms, as they have shorter generation times and the rapid rate of DNA replication may produce errors leading to mutations (Walsh, 2003). Some of these mutations give selective advantage for their survival, such as protection against antibiotics in their surroundings. Such

resistance mutation can remain in the bacterial population and it can be passed on to successive generations through vertical transmission (from parent to offspring) (Walsh, 2003). For instance, *M. tuberculosis* developed multidrug resistance exclusively by spontaneous mutation. Streptomycin is the antibiotic most commonly used against *M. tuberculosis* but streptomycin resistant strains are creating problems in patients with compromised immune system and resulting in high morbidity and mortality rates (Shah *et al.,* 2007; Sotgiu *et al.,* 2009; Velayati *et al.,* 2009). Mutations in the ribosomal protein S12 or within the 530 loop of 16S rRNA are responsible for streptomycin resistance in *M. tuberculosis.* A single amino acid change from lysine to either arginine or threonine on S12 and 16S rRNA is enough for *M. tuberculosis* to become resistant to streptomycin (Finken *et al.,* 1993). Thus, mutation plays an essential role in the development of drug resistance in bacteria. However, bacteria can also gain drug resistance by other means such as horizontal gene transfer (Figure 1) (Fajardo, *et al.,* 2009; Freifelder, 1987; Phornphisutthimas *et al.,* 2007).

There are three types of mechanisms involved in horizontal gene transfer (HGT): 1. Transformation, 2. Conjugation, and 3. Transduction (Figure 1). Transformation is a process by which bacteria take a small amount of cell free DNA from the surroundings which is released from other dead bacteria. Conjugation is a process of gene transfer that needs direct contact between a donor and a recipient cell. The donor cell carries a special type of plasmid or fertility (F) factor which promotes gene transfer. Transduction is a process of gene transfer that occurs when viruses carrying bacterial genes infect bacteria. These mechanisms are illustrated in Figure 1. The genetic elements that are

transferred by HGT can be any of the following: small fragment of bacterial genomic DNA, a plasmid, transposon, integron and bacteriophages (Freifelder, 1987; Heuer *et al.,* 2002). Hence, it is important to analyze the presence of drug resistant bacteria and plasmids in biosolids and also for their role in transferring resistance genes to others in the bacterial community.

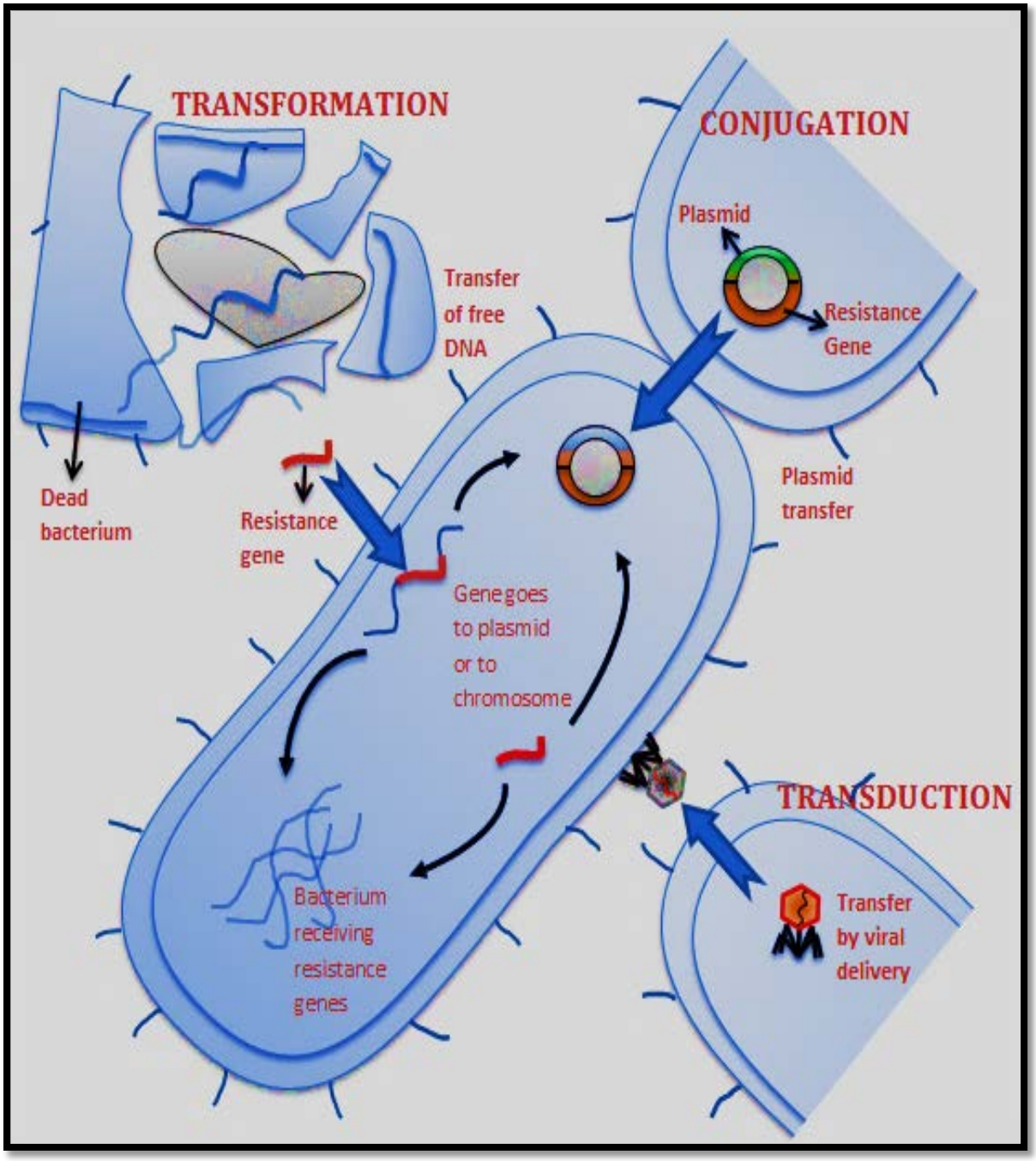


Figure 1: Mechanism of horizontal gene transfer (HGT) in bacteria. The three types of mechanisms involved in HGT: 1. Transformation (uptake of cell-free DNA), 2. Conjugation (bacterial mating), and 3. Transduction (gene transfer by viruses)

1.4 Plasmids and Antibiotic Resistance:

A plasmid is a circular, autonomously replicating DNA molecule. It may carry genes that can be beneficial to their host (e.g., genes which code for drug resistance, virulence factors etc.) (Carattoli, 2009). The resistance genes within the plasmids may be responsible for inactivation of antibiotics and the mechanisms involved are: 1. drug modification, 2. drug degradation, 3. drug efflux and 4. drug target alteration (Figure 2) (Walsh, 2003). The drug modification and degradation processes involve specific enzymes encoded by the resistance genes carried on plasmids. These enzymes recognize and bind to the incoming antibiotic molecules and either cleave or modify them chemically to prevent antibiotics from binding to their targets. Another mechanism called drug efflux involves efficient transport of antibiotics from the inside to the outside of the bacterial cell. In the target alteration mechanism, a specific cellular target for a drug is altered through mutation to prevent the drug from binding and making them ineffective. These four mechanisms are illustrated in Figure 2. All these mechanisms can be regulated by the antibiotic resistance genes present on plasmids (Walsh, 2003).

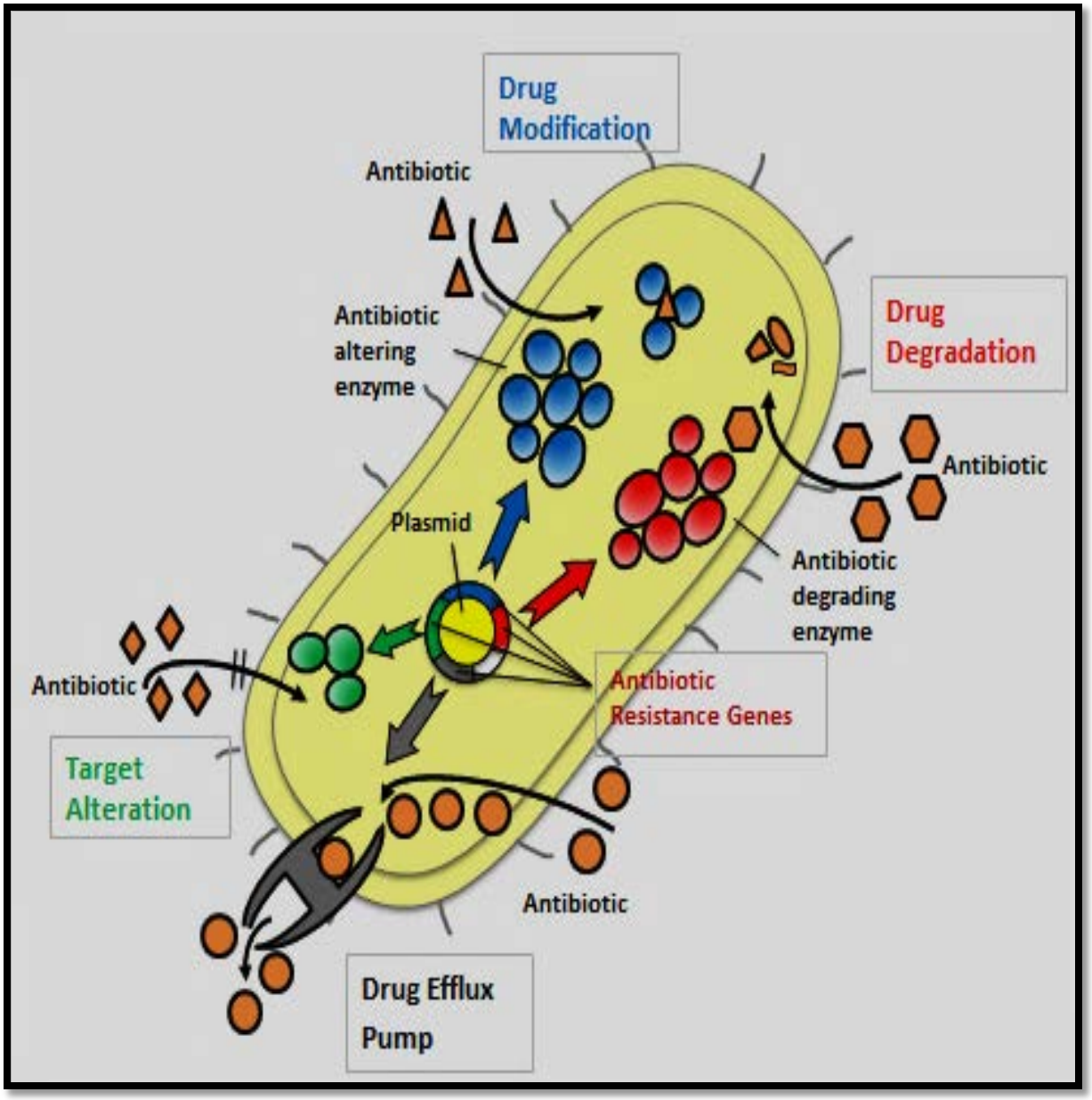


Figure 2: Mechanism of antibiotic resistance. The four mechanisms involved in antibiotic resistance are: 1. drug inactivation, 2. drug modification, 3. drug efflux, and 4. target alteration.

Again, the resistance (R) plasmids can be a threat to humans and animals because it can be transferred laterally among bacteria of different genera by HGT (Figure 1) (Couturier *et al.,* 1988). Based on their ability to move between different bacteria,

plasmids are categorized into: 1. self-transmissible or conjugative, and 2. nonconjugative or mobilizable plasmids (Salyers and Amabile-Cuevas, 1997). Conjugative plasmids carry *tra* genes needed for conjugation and they do not need any external support for their gene transfer. By contrast, nonconjugative plasmids lack *tra* gene functions, so they depend on the *tra* genes of other conjugative plasmids for their gene transfer (Freifelder, 1987). For instance, when planktonic bacteria carrying plasmids come into contact with a biofilm population composed of recipient cells with no plasmids, planktonic bacteria will form biofilms and transfer infectious plasmids to the recipient. Plasmids can transfer antibiotic resistance and virulence factors, threofore plasmids involved in biofilm formation can produce infecions that are hard to treat (Ghigo, 2001). Since, R plasmids play a significant role in gene exchange between organisms, it is important to analyze the presence of R plasmids in environmental samples such as biosolids.

1.5 Accumulation of Antibiotics in the Environment:

As antibiotics are poorly absorbed in the gut of animals and humans, a large amount of them are excreted unchanged in feces and urine (Sarmah *et al.,* 2006). For example, 40-90% of sulphonamides and tetracyclines are excreted unchanged in feces after passage through the gastro-intestinal tract of swines (Winckler and Grafe, 2001). Antibiotics are not easily degraded in soils, especially when they are spread onto soils as contaminants in sludge (Halling-Sorensen, 1998; Thiele-Bruhn, 2003). They can persist in the environment for a long time (2-6 months) and can reach ground water or aquatic

sediments through application of liquid manure or sewage sludge as fertilizers (Kuhne *et al*., 2001; Kummerer, 2001; Richardson and Bowron, 1985). In the USA, a nationwide survey was conducted regarding the presence of pharmaceuticals in river water samples. The data revealed a number of veterinary and human antibiotics in concentrations of 0.7% in 27% of 139 river water samples (Kolpin et al., 2002). Application of dairy farm manure to a garden soil caused a 70% increase in bacterial resistance against ampicillin, penicillin, tetracycline, vancomycin and streptomycin (Esiobu *et al.,* 2002). Therefore, application of manure as a fertilizer increases the chance of drug resistance among bacterial communities (Sarmah *et al.,* 2006).

1.6 Production of Biosolids:

Biosolids are nutrient-rich organic materials produced during treatment of wastewater. Once wastewater from households and industry reach a sewage treatment plant, it undergoes several physical, chemical and biological processes to reduce organic matter, eliminate heavy metals and disease causing pathogens. First, the raw sewage is filtered to remove large solid materials and the liquid undergoes aerobic and anaerobic digestion. In the aerobic treatment process, wastewater is mechanically agitated with the supply of air and the temperature is maintained at 15-20°C for 40 days to activate aerobic microorganisms which reduce biological oxygen demand and stabilize suspended solids in the wastewater (U.S. EPA, 1994). This is followed by an anaerobic digestion process in which the temperature is maintained at 20-55°C for 40 days in the absence of oxygen to transform organic materials in the sludge to gases such as methane and carbon dioxide. By the end of aerobic and anaerobic digestion processes,

there will be a reduction in the quantity of solids and pathogenic organisms. The sludge is then air dried for 2 months and lime is added to raise the pH and eliminate odors (U.S. EPA, 1994). Later, composting materials like saw dust and yard waste are added to the sludge and the final product is called biosolids. The processes involved in the production of biosolids are illustrated in Figure 3.

The biosolids are recycled and applied as fertilizer to promote plant growth as they are rich in nutrients like nitrogen, phosphorous, potassium and trace amounts of calcium, copper, iron, magnesium, manganese, sulfur and zinc (U.S. EPA, 2001). In the United States, approximately 5.6 million dry tons of biosolids are generated annually and 60% of them are used as fertilizer whereas the rest is incinerated or dumped in landfills (National Research Council, 2002). Biosolids are used as a fertilizer in public parks, agricultural lands, forest lands, reclamation sites, golf courses, road sides, plant nurseries, lawns, and home gardens (U.S. EPA, 1994).

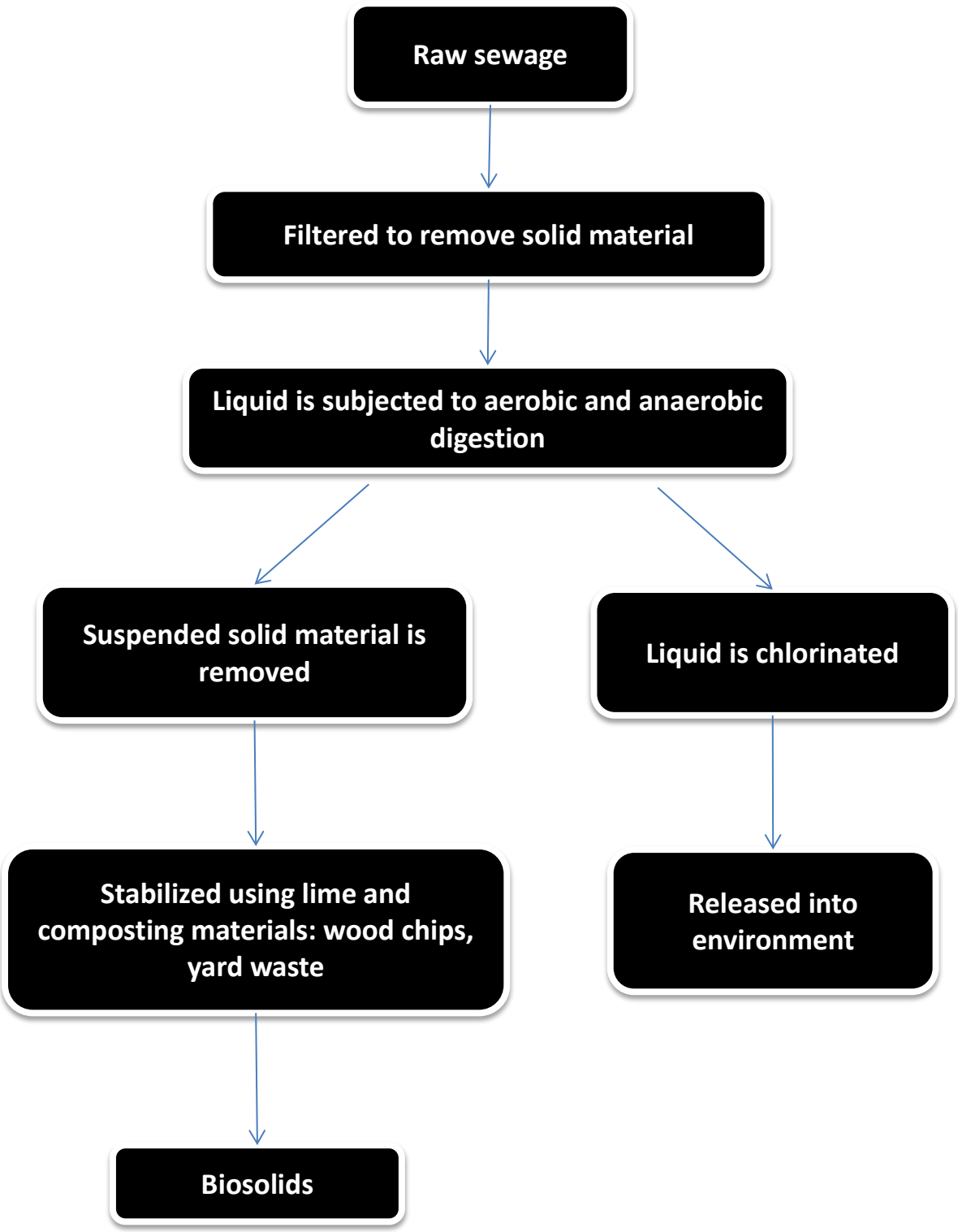


Figure 3: Production of biosolids. The biosolids undergo several processes before they are applied to lands. These include 1. aerobic and anaerobic digestion, 2. lime stabilization, and 3. composting.

1.7 Regulations for Biosolids:

As biosolids are being applied to lands as a fertilizer, it must meet some regulations and quality standards before its application. The U.S Environmental Protection Agency (EPA) established a set criterion for the concentration of heavy metals and pathogens (e.g., bacteria, viruses and parasites) permitted in biosolids for the safety of humans and animals. The EPA classified biosolids into two types based on their pathogen content - class 'A' and class 'B' (EPA, 1994). The class 'A' type undergoes some additional steps like heat drying, pasteurization, β-ray irradiation and gamma ray irradiation to further reduce the number of pathogens - but those treatment methods vary among treatment plants. In general, class 'A' is tested for the following indicator organisms and pathogens: *Salmonella*, fecal coliforms, enteric viruses and helminthes ova, whereas class 'B' is tested only for fecal coliforms such as *E.coli, Citrobacter, Enterobacter* and *Klebsiella*. The number of pathogens and their concentration allowed in class 'A' and 'B' are shown in Table 2. The class 'A' type is recommended by the U.S EPA for application to home gardens and public parks, and the class 'B' for crop lands and forestry. The potential risks associated with application of biosolids are discussed in the next section.

Table 2: The U.S EPA - Pathogen Concentration Limits

Pathogen / indicator and class	Standard concentration limits (dry wt.)
Class A	
Salmonella	**< 3 MPN/4 g total solids**
Fecal coliforms	**< 1000 MPN/g total solids**
Enteric viruses	**< 1 PFU/4g total solids**
Viable helminthes ova	**< 1 PFU/4g total solids**
Class B	
Fecal coliform density	**< 2 million MPN/g total solids**

Note: MPN – most probable numbers; PFU – plaque forming unit. Source: The U.S. EPA, 2001

1.8 Potential Hazards of Biosolids:

Although the EPA regulates biosolids use, there are risks associated with such application. Studies show that biosolids are unsafe for land application due to: 1. dispersion of bioaerosols from application sites (Baerisch *et al.,* 2007; Pillai *et al.,* 1996), 2. contamination of food and groundwater (Edmonds, 1976; Sidhu and Toze, 2009; Tamminga *et al.,* 1978; Wei *et al.,* 2010), and 3. prevalence of viruses and resistant strains of bacteria (Binh *et al.,* 2008; Ewert and Paynter, 1980; Ward *et al.,* 1981; Wong *et al.,* 2010). It has been shown that gene transfer occurs between different strains of *Enterococcus faecalis* under natural conditions in wastewater treatment plants (Marcinek *et al*., 1998). Several pharmaceutical compounds (ibuprofen, acetaminophen, gemfibrozil), and antibiotics (ciprofloxacin, ofloxacin, azithromycin) persist in biosolids despite of all the treatment processes (Radjenovic *et al.,* 2009; Spongberg and Witter, 2008). Presence of antibiotics in biosolids put selective pressure on bacteria and select

for drug resistant determinants. These resistant organisms can act as a reservoir for drug resistance plasmids (Binh *et al.,* 2008; Viau and Peccia, 2009). In addition, human and bacterial viruses present in sewage can be maintained because they are more resistant to ammonia and heat treatments (Burge *et al.,* 1983; Lund *et al.,* 1996; Viau and Peccia, 2009). These studies support an idea that resistant bacteria transfer resistance genes to other organisms through vertical and horizontal transmission. Therefore, it is necessary to examine these possibilities in biosolids before their land application.

1.9 Significance of Bacteria in *Enterobacteriaceae* Family:

The *Enterobacteriaceae* is a large family of gram negative bacteria and some members are part of a normal gut flora of humans and animals. However, members of this family such as *E.coli, Salmonella* and *Proteus* are known pathogens while others like *Enterobacter*, *Klebsiella* and *Serratia* cause secondary wound infections, respiratory and urinary tract infections (Table 3). As these members are found in human and animal intestine, they are present in sewage in large quantity and in biosolids in spite of the wastewater treatment processes. It has been shown that members of *Enterobacteriaceae* are resistant to multiple drugs and infections caused by these resistant organisms are hard to treat (Paterson, 2006; Salyers and Amabile-Cuevas, 1997). Studies show that members of this family (e.g., *Salmonella* and other fecal coliforms) are able to regrow and colonize after biosolids are applied to lands (Zaleski *et al.*, 2005). As members of the *Enterobacteriaceae* family are enteric pathogens, it is crucial to study these organisms in biosolids.

Table 3: Clinical syndromes caused by members of the *Enterobacteriaceae* family

Bacteria	Habitat / Source	Disease
***Citrobacter* spp.**	**Soil, water and wastewater**	**Urinary tract infection, infant meningitis and sepsis**
***Enterobacter* spp.**	**Soil, water and human intestine**	**Urinary and respiratory tract infections**
***E.coli* 0157:H7**	**Human intestine**	**Diarrhea and severe kidney failure**
Klebsiella pneumonia	**Nosocomial; Spread by hospital personnel and equipment**	**Pneumonia, diarrhea bacteremia and sepsis**
***Proteus* spp.**	**Soil, water, wastewater and human intestine**	**Wound infections, pneumonia and septicemia**
Serratia marcescens	**Nosocomial; Spread by hospital personnel and equipment**	**Urinary tract, wound infection and septicemia**
***Salmonella* spp.** ***Shigella* spp.**	**Contaminated food and water, animal and human intestine**	**Diarrhea, enteric fever, septicemia and reactive arthritis**

1.10 Plasmid Extraction and Transformation:

The presence of resistance genes on plasmids can be examined by transformation. For such an analysis, plasmids can be extracted from antibiotic resistant bacteria and these cell-free plasmids can be mixed with an antibiotic sensitive laboratory strains of *E. coli*.

Through the transformation process, bacteria take a small amount of cell free DNA from their surroundings and keep them in their cytoplasm as a plasmid or incorporate them into their chromosome (Andersson and Hughes, 2010). The bacteria that are susceptible to transformation in both natural and artificial environments are called competent cells. In a lab, competence can be achieved by treating the cells with chemicals (e.g., $CaCl_2$) and using high voltage electric pulse (electroporation) (Froger and Hall, 2007; Hill *et al.,* 1992). The process of transformation is illustrated in Figure 4. Using transformation process, drug resistance genes (R) present on plasmids can be identified.

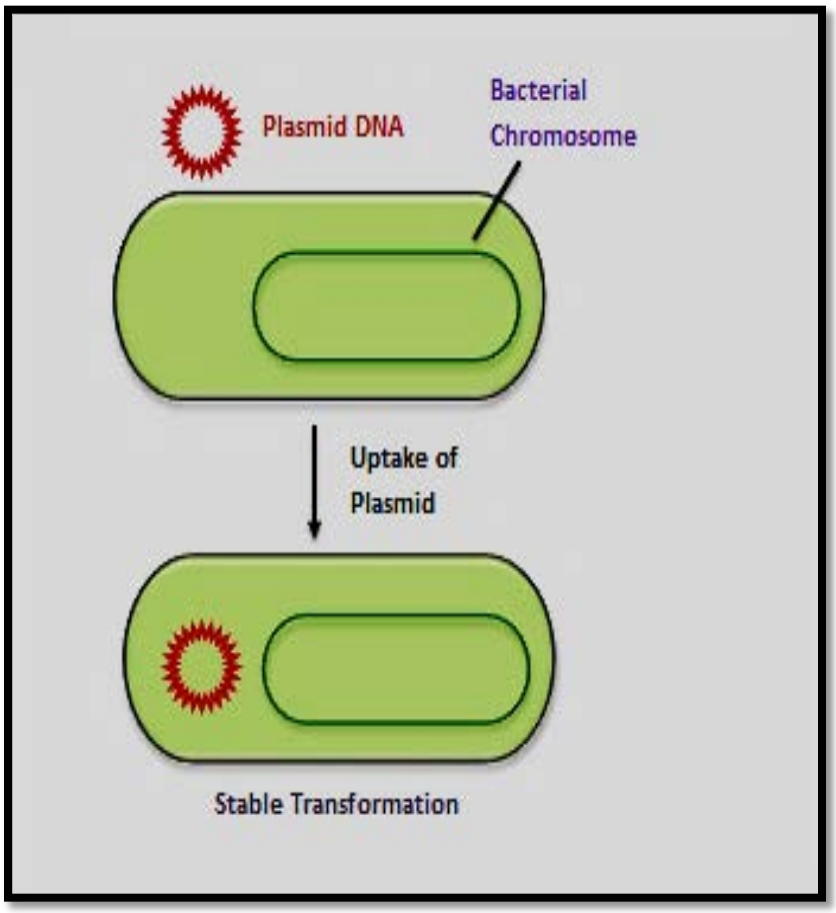


Figure 4: Mechanism of transformation in bacteria. Transformation is a process by which bacteria take a small amount of cell free DNA from their surroundings. Once the DNA enters a

cell, it will be maintained stably as a plasmid or may be integrated into their chromosome. In either case, the new DNA is passed on to successive generations.

1.11 Phage Sensitivity Assay:

To determine whether plasmids are of the conjugative or non-conjugative type, a phage sensitivity assay can be used. Viruses can replicate only in specific host. Based on the host they live in, they are classified into three types: 1. viruses that infect bacteria are called bacteriophages or phages, 2. viruses that infect plants are referred as plant viruses, and 3. viruses that infect animals and humans. For the identification of conjugative and non-conjugative plasmids, bacteriophages or phages can be used.

Bacteriophages basically consist of a nucleic acid molecule (DNA or RNA) surrounded by a protein coat called capsid (Lipton and Weissbach, 1969). After infection, phages use the bacterial ribosomes, protein-synthesizing factors and energy-generating systems for their replication, hence phages can only multiply in metabolizing bacterial cells (Sobsey *et al.,* 1995). Phages that infect *E.coli* bacteria are called coliphages. Attachment or receptor sites for coliphages are located on different parts of bacteria. Based on the receptor sites, coliphages are classified into two types: somatic and male-specific (F+) coliphages. Some receptors for coliphages are located on the bacterial cell wall and are present all the time. These receptors are recognized by phages known as somatic phages. This implies that somatic phages can infect host bacteria at any time, and these phages will attach to even dead bacteria. Some examples of somatic phages are T4, T5, λ and P22 phages (Furuse *et al.,* 1981; Long *et al.,* 2005). Receptor sites for other

coliphages are located on the fertility (sex) fimbriae of host bacteria. These fimbriae are produced only by bacteria carrying F-factor in the log growth phase under optimal conditions. Coliphages which utilize these receptor sites are known as male-specific (F+) phages. Some examples of male-specific phages are Qβ, M13, MS2 and f1 (Furuse *et al.,* 1981; Long *et al.,* 2005; Sobsey *et al.,* 1995).

Bacteriophages share many fundamental properties with human viruses. For example, F+ RNA coliphages and polioviruses, both have an icosahedral capsid with a diameter of about 25nm and a single stranded (ss) RNA genome (Havelaar, 1993; Hsu *et al.,* 1995). Both F+ RNA coliphages and enteroviruses are excreted by humans and animals. Coliphages are excreted at all times by many humans and warm-blooded animals, whereas enteric viruses of human health concern are excreted only by humans during infection which may last for few days to few weeks (Sobsey *et al.,* 1995). For these reasons, coliphages are valuable models for the study of human and animal enteric viruses (Gantzer *et al.,* 1998). In addition, coliphages are easily and rapidly cultivated in labs which makes them good indicators for the presence of enteric viruses in water, food, shellfish, and wastewater (Furuse, 1981; Sobsey *et al.,* 1995).

To identify conjugative plasmids present in biosolids, Qβ phages can be used. Qβ phage belongs to the family Leviviridae. The phage genome is surrounded by a cubic or icosahedral protein capsid without a tail. Qβ has a single-stranded RNA genome of 4,217 bases that encodes four genes for A2, A1, coat protein and the RNA replicase β subunit (Duin, 1988; Klovins *et al.,* 1998). The host for Qβ is male specific (F+) *E.coli* cells.

Phage infection starts by attachment of Qβ to the *E.coli* F pilus followed by the release of phage RNA into the host cell (Figure 5). After the release, phage directs the host machinery to make copies of the phage RNA and to form a coat protein (Takeshita *et al.,* 2012; Tsukada *et al.,* 2009). The phage heads are then assembled with the newly synthesized RNA followed by the release of phages by rupture or lysis of the bacterial cell within as little as 1 hour after infection (Figure 5). The newly released phages go and attach to other bacterial cells carrying F-factor and the process continues. These phages typically produce clear plaques on a lawn of susceptible host bacteria (Davis *et al.,* 1990; Takeshita *et al.,* 2012; Tsukada *et al.,* 2009). Thus, this technique can be used to distinguish between cells carrying conjugative and non-conjugative plasmids (Miller, 1972).

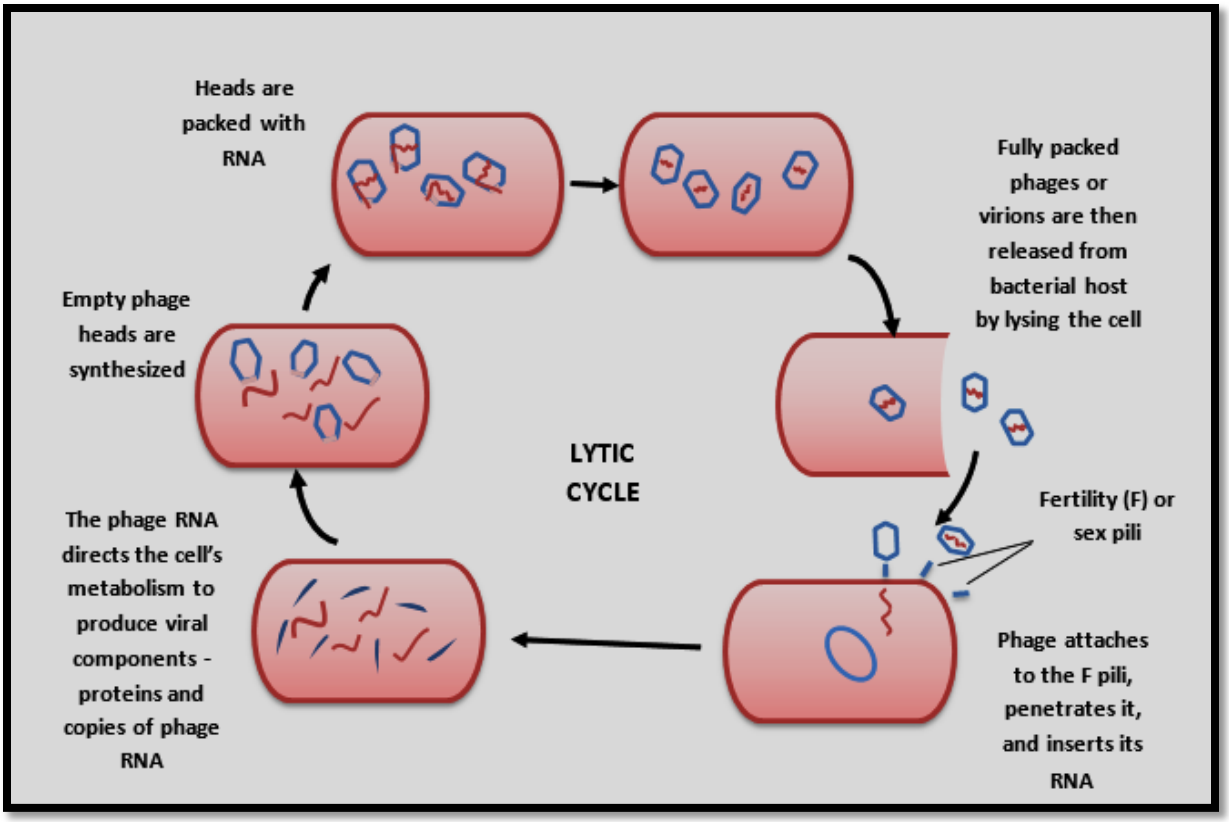


Figure 5: Infection of RNA phage and cell lysis. RNA phages such as Qβ attaches to the host bacteria through F pili and inserts their genome into the host. The phage RNA then uses the host machinery for making copies of their genome and protein coat. The newly synthesized RNAs are then packed into empty phage heads and the fully packed heads or virions are released by lysing the host cell. The released phages go and attach to other bacterial cells and the process continues.

1.12 Mechanism of Conjugation:

Once again, the plasmids involved in HGT are of two types: (i) self-transmissible or conjugative, and (ii) nonconjugative or mobilizable plasmids (Salyers and Amabile-Cuevas, 1997). The self-transmissible plasmids carry necessary genes (*tra* or transfer) to

promote their gene transfer. However, the mobilizable plasmids lack transfer function (*tra* genes) but contain an *ori* site (a specific sequence that allows for their transfer), so in the presence of conjugative plasmids they are able to transfer their genes to a recipient (Freifelder, 1987).

The mobility of plasmids can be examined by the conjugation process which requires a physical contact contact between donor and recipient cells. The donor cell is referred to as F^+ because it carries a conjugative plasmid for conjugation, whereas the recipient cell lacking a conjugative plasmid is called the F^-cell (Moat *et al*., 2002). The F plasmid encodes a sex pilus, a protein appendage which recognizes and binds to receptors on a recipient bacterial cell wall. The cell membrane of donor and recipient fuses together and creates a passage between bacteria for DNA transfer. Then, a plasmid encoded endonuclease cleaves the plasmid at a specific site called the origin of transfer (*ori T*). After that, the 5' end of a single stranded DNA starting from the *ori T* enters a recipient F^-cell (Phornphisutthimas *et al.,* 2007). Later, complementary strands are synthesized in both ($F^+\text{and}\ F^-$) bacteria by a rolling circle mechanism. It has been shown when F^+ and F^-cells are mixed together that eventually all the cells will become F^+ (Moat *et al*., 2002). The processes involved in conjugation are depicted in Figure 6. Hence, conjugative plasmids can be identified by conjugative transfer or by their susceptibility to male-specific coliphages such as Qβ and M13 phages (see section 1.11). If the plasmids are non-conjugative, they can be identified by a tripartite mating process which is described below.

Tripartite mating is similar to conjugation and can be used to identify non-conjugative or mobilizable plasmids. As mentioned earlier, non-conjugative plasmids are not able to make direct gene transfer because they lack transfer function (*tra* genes) but these plasmids carry an *ori* site (a specific sequence that allows for their transfer), so in the presence of conjugative plasmids they are able to transfer their genes to a recipient cell (Freifelder, 1987). In tripartite mating, there will be three strains involved: donor, helper and recipient. The helper strain carry *tra* genes for transfer function, so it will help the donor to transfer the plasmid to the recipient cell. It has been shown that in the presence of bacteria carrying *tra* genes, non-conjugative plasmids are able to transfer their genes to recipient bacteria (Droge *et al.,* 2000; Schluter *et al.,* 2007). Thus, non-conjugative plasmids can be identified by the tripartite mating process.

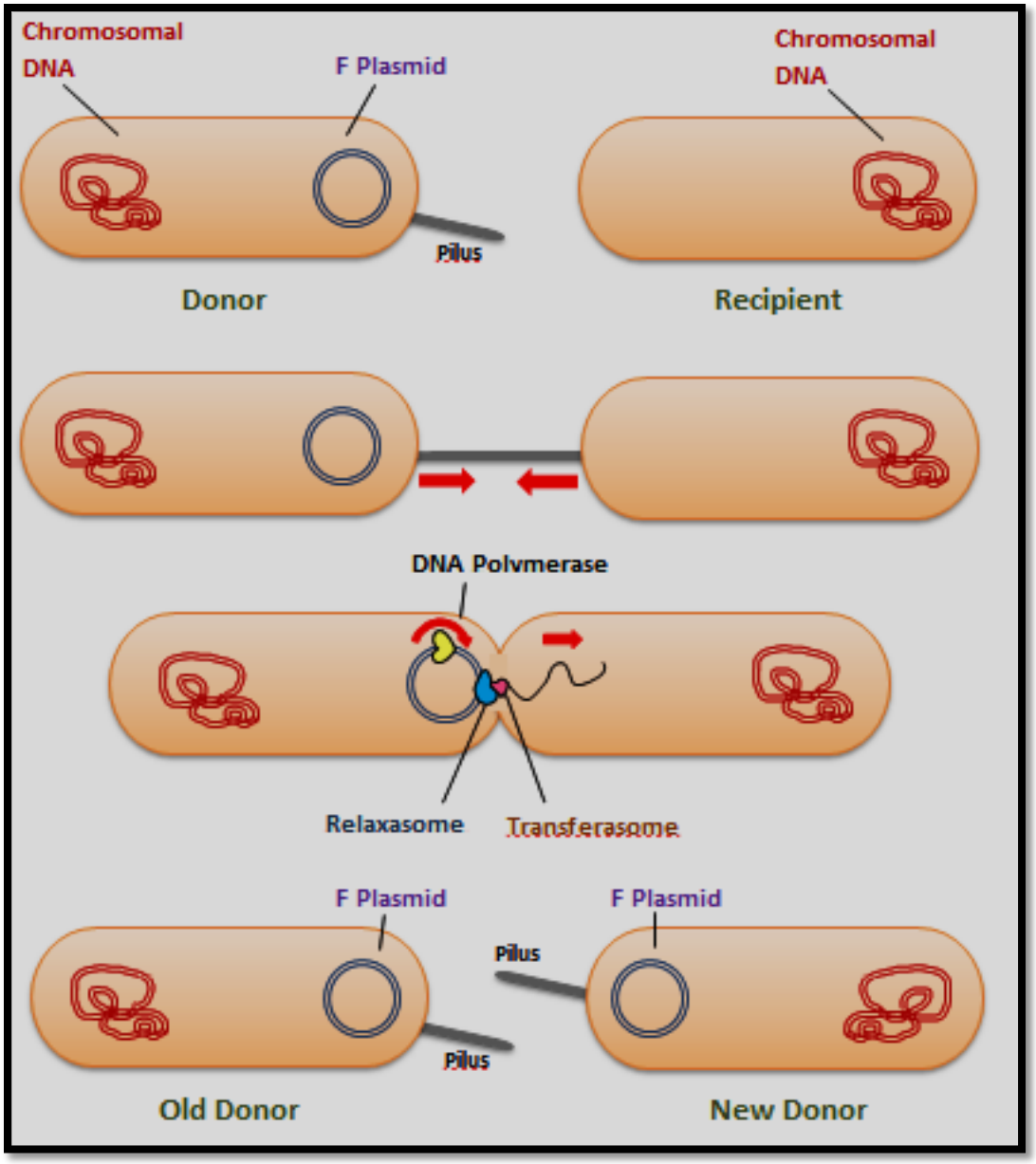


Figure 6: Mechanism of conjugation in bacteria. The donor cell contains an F plasmid which encodes an F pilus for conjugation. The F pilus is a protein appendage which recognizes and binds to receptors on a recipient cell wall, and then the cell wall fuses together and creates a passage between the two bacteria for plasmid DNA transfer.

1.13 Objectives:

The goal of this study was to (1) examine the presence of drug resistant bacteria in Cheney biosolids, (2) to screen them for the presence of resistance (R) plasmids, (3) to verify the location of resistance genes on R-plasmids, and (4) to identify whether the R-plasmids are of a conjugative or a non-conjugative type.

2. Materials and Methods:

2.1 Bacterial Growth Media:

For growth and isolation of bacteria both liquid and solid media were used. The media used were: MacConkey agar and Tryptic soy agar (TSA) (Difco, Detriot, MI), Luria-Bertani (LB) agar and LB broth (Mo-bio, Carlsburg, CA). MacConkey agar was prepared by adding 50g of powder per liter of deionized water and boiled to dissolve completely. TSA was prepared by dissolving 40 g of powder in a liter of deionized water. LB broth was prepared by adding 20g of powder per liter of deionized water. LB agar was prepared by mixing 35 g of powder in a liter of deionized water. All media were sterilized by autoclaving at 121°C and 15 psi for 15 minutes.

2.2 Preparation of Antibiotic Stock Solutions:

Antibiotics added to the growth media (either broth and agar plates) were made from the following powdered antibiotics: Ampicillin sodium salt, Tetracycline hydrochloride, Kanamycin, Nalidixic acid, Streptomycin sulfate salt, Chloramphenicol, Rifampin. All antibiotics were purchased from Sigma-Aldrich, St. Louis, MO. The antibiotic stock solutions were prepared as recommended by Sambrook *et al.,* 1989.

All antibiotic solutions were prepared with sterile deionized water in 1.5 ml sterile Eppendorf tubes. Ampicillin stock solution (100 mg / ml) was prepared by dissolving 100 mg of ampicillin sodium salt in 1 ml of deionized water in a 1.5 ml Eppendorf tube. Tetracycline stock solution (15 mg / ml) was prepared by adding 15 mg of Tetracycline hydrochloride to 1 ml of 50% Ethanol. Kanamycin solution (50 mg / ml) was prepared by dissolving 50 mg of Kanamycin in 1 ml of deionized water. Nalidixic acid solution (30 mg / ml) was prepared by dissolving 30 mg of Nalidixic acid in 1 ml of 1M NaOH. Streptomycin solution (100 mg / ml) was prepared by mixing

100 mg of Streptomycin sulfate salt in 1 ml of deionized water. Chloramphenicol stock solution (20 mg / ml) was prepared by adding 20 mg of Chloramphenicol to 1 ml of 95% Ethanol. Rifampin solution (100 mg / ml) was prepared by mixing 100 mg of Rifampin in 1 ml of Methanol and dissolved by adding 3 drops of 10N NaOH.

All antibiotics were dissolved by vortexing and the mixture was filter sterilized using a 0.2 μm syringe filter. The filtrate was collected in 1.5 ml sterile Eppendorf tubes and stored at -20°C until use. From the antibiotic stock solution, appropriate amount was mixed with LB broth and agar before use. The final concentration of antibiotics used were: ampicillin (100 μg / ml), tetracycline (15 μg / ml), streptomycin (100 μg / ml), kanamycin (50 μg / ml), rifampin (100 μg / ml), nalidixic acid (30 μg / ml) and chloramphenicol (20 μg / ml). These final concentrations were determined based on a recommendation by Sambrook *et al.* (1989).

2.3 Preparation of Buffer and Solutions:

Dulbecco's phosphate buffered saline (PBS) was prepared by mixing the following: 13.7 mM NaCl, 0.27 mM KCl, 1 mM Na_2HPO_4 and 0.2 mM KH_2PO_4 in 800 ml of deionized water. The pH was adjusted to 7.4 with HCl and the final volume was adjusted to 1 liter with additional water. Saline solution was prepared by dissolving 0.85 g of NaCl in 100 ml of deionized water. The PBS and Saline were sterilized by autoclaving at 121°C and 15 psi for 15 minutes before use. Both PBS buffer and saline solution (0.85 %) were used for making serial dilutions of biosolid samples. The gel electrophoresis buffer, Tris-Acetate EDTA (TAE) was prepared by mixing 0.04 M Tris-acetate and 0.001 M Ethylenediaminetetraacetic acid (EDTA), disodium salt in 800 ml of deionized water. The pH was adjusted to 8.0 with acetic acid and the final volume was adjusted to 1 liter with additional water. The TAE buffer was used for running an agarose gel electrophoresis.

2.4 Collection of Biosolids and Inoculation onto Selective Media:

Biosolid samples were collected from a wastewater treatment facility, Cheney, WA. Roughly 500 g of biosolids were collected in sterile glass bottles. They were placed on ice and transported to lab and stored in a 4°C cold room until use. From that, 10 g of biosolids were suspended in 90 ml of PBS and placed on a rotary shaker for 3 hours. The liquid was collected and serial dilutions (10^{-2} to 10^{-7}) were made. The diluted sample (100 μl) was spread on MacConkey agar and incubated at 37°C for 24-36 hours. Single isolated colonies were picked randomly with sterile toothpicks and streaked on antibiotic plates, and the plates were incubated at 37°C for 36-48 hours. Resistant bacterial strains were once again tested on the antibiotic plates using the same procedure to ensure that the isolates were truly resistant to the antibiotics used.

2.5 Bacterial Strains Used:

The bacterial strains and phages used in this study were: *Escherichia coli* MM294, *E. coli* DH5α, *E. coli* XK1502, *E. coli* MC4100 and phages Qβ and M13. The source and genotype for these organisms are shown in Table 4a and 4b.

Table 4a:

Control Bacteria	Strain Designation	Genotype	Source
Escherichia coli	MM294	F -, *glnV44*(AS), λ^-, *rfbC1*, *endA1*, *spoT1*, *thiE1*, *hsdR17*, *creC510*	Coli Genetic Stock Center (CGSC) #:6315
Escherichia coli	DH5α	*F -, φ80lacZΔM15 Δ(lacZYA-argF)U169, recA1 endA1, hsdR17(rk-, mk+), phoAsupE44 thi-1, gyrA96, relA1 λ-*	Biology department collection
Escherichia coli	MM294 transformed with pGLO	F -, *glnV44*(AS), λ^-, *rfbC1*, *endA1*, *spoT1*, *thiE1*, *hsdR17*, *creC510*	Biology department collection
Escherichia coli	MC4100	F -, $[araD139]_{B/r}$, *Δ(argF-lac)169*, λ^-, *e14-*, *flhD5301*, *Δ(fruK-yeiR)725(fruA25)*, *relA1*, *rpsL150*(strR), *rbsR22*, *Δ(fimB-fimE)632(::IS1)*, *deoC1*	Dr. Steve Moseley, Microbiology Department, UW
Escherichia coli	XK1502	*F', lacU169, traD8, nalA*	Dr. Steve Moseley, Microbiology Department, UW

Table 4b:

Control Phage	Strain Designation	Source
RNA Phage	Qβ	Dr. Steve Moseley, Microbiology Department, UW
Single-stranded (ss) DNA phage	M13	From our collection

2.6 Long Term Storage of Bacterial Isolates:

Resistant bacterial isolates were stored at -80°C in LB broth containing 15% (v/v) glycerol (Sigma Aldrich, St. Louis, MO). Bacterial isolates were streaked on LB plates containing antibiotics and incubated at 37°C for 24 hours to get individual colonies. A single isolated colony was picked and transferred to a 5ml LB broth containing antibiotics and placed on a 37°C shaker for 18 hours. Then, 0.7 ml of cell suspension was transferred to sterile 1.2 ml Cryovial (Midsci™, St. Louis, MO) and mixed with 0.3 ml of sterile 15% (v/v) glycerol. Samples were stored at -20°C for 48 hours and then moved to -80°C for long-term storage.

2.7 Plasmid DNA Extraction:

Plasmid DNA was extracted from resistant bacteria using the following kits: GeneJET™ Plasmid Miniprep Kit (Fermentas Life Sciences, Burlington, Ontario) and Zyppy™ Plasmid Miniprep Kit (Zymo Research, Irvine, CA). Plasmid DNA was extracted from resistant bacteria and their size and concentration were determined using 0.7% (w/v) agarose gel electrophoresis.

As per the manufacturer's protocol, an isolated colony was picked from an antibiotic plate and transferred to a 5 ml LB broth containing antibiotics and placed in a 37°C shaker for 16-18 hours. Then, 3 ml of cell suspension was transferred to two 1.5 ml sterile Eppendorf tubes and centrifuged at 10,000 X G for 4 minutes at room temperature. The supernatant was discarded and the cells were suspended with SDS/alkaline lysis buffer (supplied in kit) to release plasmid and genomic DNA. The resulting lysate was neutralized using neutralization buffer from the kit. Cell debris, SDS precipitate and the genomic DNA trapped in them were pelleted by

centrifugation and the supernatant containing the plasmid DNA was loaded onto the spin column membrane for further purification. The adsorbed DNA was washed to remove contaminants using wash buffer supplied in the kit. Finally, the plasmid DNA was eluted with elution buffer (from kit) and the DNA was stored at -20°C until use.

2.8 Agarose Gel Electrophoresis:

The agarose gel electrophoresis was used for the separation and visualization of plasmid DNA and also to determine the size and concentration of the DNA. The materials used for that purpose were: agarose (Invitrogen, Eugene, OR), TAE buffer, an electrophoresis chamber (Owl model B1A class II, Owl Separation Systems, Portsmouth, NH), 6X loading dye (New England Biolabs, Ipswich, MA) containing 5X SYBR gold (Invitrogen, Carlsbad, CA), Supercoiled DNA ladder (2-10 kb)(Cat#N04725, New England Biolabs, Ipswich, MA), 1 kb DNA ladder (0.5-10kb)(Cat#N3232S, New England Biolabs, Ipswich, MA), an Ultraviolet Transilluminator (Ultraviolet Products, Upland, CA) and Kodak EDAS 290 (Kodak, Rochester, NY) imaging system.

A 0.7% (w/v) gel was prepared by dissolving 0.35 g of agarose (Invitrogen, Eugene, OR) in 50 ml of TAE buffer and poured into an electrophoresis chamber (Owl model B1A class II, Owl Separation Systems, Portsmouth, NH). Samples (3 µl) were mixed with 1 µl of 6X loading dye (New England Biolabs, Ipswich, MA) containing 5X SYBR gold (Invitrogen, Carlsbad, CA) and the total mixture (4 µl) loaded into wells and allowed to run at 58-volt for 3 hours. A (2-10 kb) Supercoiled DNA ladder (Cat#N04725 New England Biolabs, Ipswich, MA) was used to determine the size and concentration of plasmid DNA. The DNA within the gel matrix was visualized with an Ultraviolet Transilluminator (Ultraviolet Products, Upland, CA) and photographed with a Kodak EDAS 290 (Kodak, Rochester, NY) imaging system. The size and concentration of the sample DNA were measured by comparing visually the fluoresecence of sample DNA band(s) with the

standard DNA ladder. Based on the size of sample DNA band(s) their corresponding concentrations were determined using the standard DNA concentration chart provided by the supplier.

2.9 Identification of Bacteria:

The bacterial isolates were identified using API-20E system and oxidase test. The materials used were: 0.85% saline solution, TSA plates, mineral oil, sterile tooth picks, sterile filter papers (Whatman No.1), API strips (BioMerieux, Durham, NC) and Oxidase reagent (Becton, Dickson and Company, Sparks, MD). Bacteria were grown on TSA plates at 37°C for 18-24 hours. A single well-isolated colony was picked and suspended in 0.85% saline solution and mixed thoroughly. Using a sterile pipette, bacterial suspension was added into the cupules of API strips and then mineral oil was added in recommended cupules and the strips were incubated at 37°C for 24-36 hours. Later, other reagents (BioMerieux supplies) were added and the color change was recorded as (+) and (–) signs. Based on the (+/-) score, an organism was identified using the Analytical Profile Index (BioMerieux, Durham, NC, 1999). For oxidase test, few drops of Oxidase test reagent (Becton, Dickson and Company, Sparks, MD) were added to a strip of Whatman filter paper. A single isolated colony was streaked on top of the reagent using a sterile toothpick and the color change from white to dark blue is considered as positive and no color change was considered as negative for the test.

2.10 Preparation of Competent Cells:

The competent cells were prepared as described in the MicroPulser lab manual (Bio-Rad, Hercules, CA). The *E. coli* MM294 was grown in a 5 ml LB broth on a 37°C rotary shaker for 18-24 hours. Then, 500 ml of fresh LB broth was inoculated with 5 ml of overnight *E. coli* culture and

grown on a 37°C shaker till the culture had reached the cell density of 0.3-0.5 at A_{550} using the SHIMADZU UV-1201 spectrophotometer (Columbia, MD). The culture was chilled on ice for 30 minutes and transferred to ice-cold centrifuge tubes and spun at 4000 X G for 15 minutes at 4°C using a Sorvall RC5B refrigerated centrifuge (Sorvall, Newtown, CT). The supernatant was discarded and the bacterial pellet was suspended with 500 ml of ice-cold MilliQ® water and spun at 4000 X G for 15 minutes at 4°C. This step was repeated twice with 250 ml ice-cold MilliQ® water and centrifuged in the same condition. The bacterial pellet was suspended in 20 ml ice-cold 10% (v/v) glycerol and spun at the same condition. Finally, the cell pellet was suspended in 2 ml of ice-cold 10% (v/v) glycerol and divided into small aliquots (50 μl) in 0.5 ml sterile Eppendorf tubes and stored at -80°C until needed.

2.11 Transformation:

Electroporation was carried out using a MicroPulser (Bio-Rad, Hercules, CA). The following items were used for that purpose: Super Optimal Broth (SOB) containing 10mM glucose, electroporation cuvette (0.2 cm gap), LB plates with appropriate antibiotics and a 37°C shaker.

The electro-competent cells were retrieved from a freezer and thawed on ice. Cell suspension (50 μl) was mixed with either 0.2 μg of pBR322 (positive control) or 0.3 μg of plasmid DNA extracted from the isolates. The mixture of cells and DNA were incubated on ice for 2 minutes and transferred to a cold electroporation cuvette. This mixture was subjected to one 2.5 kV pulse for 5 milli sec using a Bio-Rad MicroPulser (provided by Dr. Andrea Castillo, EWU Biology Dept). After a pulse, 1 ml of SOB medium was added to the cuvette and the entire content was transferred to a 5 ml test tube and incubated on a 37°C rotary shaker for 2 hours. Then, 100 μl of the cell suspension was spread on antibiotic plates and incubated at 37°C for 36-48 hours. The bacterial colonies or transformants formed on these antibiotic plates were tested again on

the same antibiotic to confirm their drug resistance. Later, plasmids were extracted from the transformants using the same procedure as mentioned in the section 'plasmid DNA extraction'. The presence of plasmids and their size and concentration were determined on an agarose gel following the same procedure. Plasmids extracted from the transformants were compared with the original plasmids used for transformation on an agarose gel to verify similarities between these two plasmids.

2.12 Restriction Digestion:

Restriction digestion was performed with the plasmids to confirm their size(s) after linearizing them. Plasmid DNA extracted from the transformants was digested with the restriction enzyme *Eco*RI (#FD0274, Fermentas, Glen Burnie, MD). The materials used were: sterile MilliQ® water, 10X FastDigest buffer (10mM potassium phosphate, 300mM Nacl, 1mM EDTA, 1mM DTT, 0.2mg/ml BSA, 0.15% Triton X-100 and 50% (v/v) glycerol), sterile 0.5 ml sterile Eppendorf tubes, 1 kb DNA ladder (N3232S, New England Biolabs, Ipswich, MA) and a 37°C heat block.

The reaction mixture (20 μl) was prepared by mixing 15 μl of sterile MilliQ® water, 2 μl of 10X FastDigest buffer, 1 μg of plasmid DNA and 10 units of *Eco*RI FastDigest enzyme in 0.5 ml sterile Eppendorf tubes. The mixture was spun at 3000 X G for a minute and incubated in a 37°C heat block for 3 hours. The digested DNA or 1kb DNA ladder were then mixed with 3 μl of 6X SYBR gold containing dye and loaded on a 0.7% (w/v) agarose gel and electrophorized.

2.13 Assay of Phage Sensitivity:

Phage sensitivity assay was performed to determine the presence of conjugative plasmid in the isolates. The materials used were Qβ and M13 phages, *Ecoli* MC4100 and XK1502, 15 ml

centrifuge tubes, LB broth, LB plates and chloroform (Sigma Aldrich, St. Louis, MO). The procedure for phage sensitivity was followed as suggested by Miller (1972).

The Qβ and M13 phage lysate was prepared as follows: *E. coli* XK1502 were grown in a 5 ml LB broth on a 37 °C rotary shaker for 18-24 hours. The overnight culture was diluted (1:100) with 5ml of fresh LB broth and allowed to grow for 3 hours on a 37°C rotary shaker. The Qβ and M13 phages were added to the bacterial cell suspension in two separate tubes in a ratio 1:10 and incubated in a 37°C water bath overnight. Three drops of chloroform were added to the cell suspension and centrifuged at 5000 X G for 10 minutes and the supernatant was carefully removed and stored at 4°C until use. The clear supernatant containing phages (phage lysate) was further used in phage sensitivity assay. The test isolates or *E.coli* XK1502 (positive control) or *E.coli* MC4100 (negative control) were grown to exponential phase and 100µl was plated on LB agar and let it dry for an hour. Then, 5µl of phage lysate was spotted on the LB plate containing test bacteria or positive control *E.coli* XK1502 or negative control *E.coli* MC4100 and the plates were incubated at 37 °C for 24 hours. The visible plaques formed on plates indicate the presence of conjugative plasmid in bacteria.

2.14 Tripartite Mating:

Tripartite mating process was used to identify non-conjugative but mobilizable plasmids. The materials used were: LB broth, LB plates containing antibiotics, control *E.coli* strains MC4100 and XK1502, a 37°C shaker. The procedure for tripartite mating was followed as per Miller (1972).

The tripartite mating process involve three different strains: a donor, a helper and a recipient. The donor is the strain isolated from biosolids, helper is the strain of *E.coli* XK1502 and the

recipient is the *E.coli* MC4100 strain. All the three strains were streaked on suitable antibiotic plates to get isolated colonies and incubated at 37°C for 24 hours. A single well-isolated colony from each plate was picked and transferred to a 5 ml LB broth and incubated at 37°C for 18-24 hours. The overnight culture was diluted with 5 ml of fresh LB broth in the ratio 1:40 for donor and helper, and 1:20 for recipient and allowed to grow for 3 hours on a 37°C rotary shaker. All the antibiotic plates were divided into four sections: 1. Donor (biosolid isolate), 2. Helper (*E.coli* XK1502), 3. Recipient (*E.coli* MC4100), and 4. Donor + Helper + Recipient. The recipient culture (5 µl) was first spotted on appropriate sections and let it dry for 30 minutes. The donor (5 µl) was then spotted directly on top of the recipient and let them dry for another 30 minutes. Finally, the helper (5 µl) was spotted on top of the recipient and donor spots and allowed to completely dry for 30 more minutes, and the plates were incubated at 37°C for 24 hours. The transconjugants or bacterial colonies formed on the antibiotic plates in a sector in which all three types were mixed together were then evaluated. Conjugation (bi-partite mating) was also performed simultanoeusly at the same time of tripartite mating to check whether the biosolid isolate carried conjugative plasmid. Conjugation is similar to tripartite mating except there are only two strains involved. The antibiotic plates were divided into three sections: 1. Donor (biosolid isolate), 2. Recipient (*E.coli* MC4100) and 3. Donor + Recipient. The diluted fresh cultures of donor and recipient as used in tripartite mating were used in this assay. The recipient culture (5 µl) was first spotted on appropriate sections and let it dry for 30 minutes. The donor (5 µl) was then spotted directly on top of the recipient and let it dry for another 30 minutes and the plates were incubated at 37°C for 24 hours. The transconjugants or bacterial colonies formed on the antibiotic plates were then evaluated.

3. Results and Discussion:

3.1 Collection of Biosolids and Isolation of Resistant Bacteria:

Wastewater serves as a reservoir of bacteria and viruses that may contribute to the selection and transfer of antibiotic resistance genes to other bacteria (Binh *et al.,* 2008; Lund *et al.,* 1996). Biosolids are the final product of the waste water treatment process, and they are used as fertilizer to promote plant growth all over the United States. They are used in public parks, agricultural lands and home gardens (U.S. EPA, 1994). People can be directly or indirectly affected by such application. For example, bioaerosols or microbes can be dispersed through wind, and people can inhale these organisms and get infected (Baerisch *et al.,* 2007). Pathogens can enter the human food chain by consuming food crops and vegetables grown on biosolid applied lands (Tamminga *et al.,* 1978; Wei *et al.,* 2010). Farm animals grazing on biosolid applied lands also contribute to the spread of pathogens to humans who consume meat products. It has been shown that addition of organic wastes to soil in the form of biosolids increases the growth and survival efficiency of *E.coli* and others (Unc *et al.,* 2006). Moreover, several studies reported the presence of drug resistant bacteria and viruses in biosolid samples (Binh *et al.,* 2008; Wong *et al.,* 2010). To my knowledge, Cheney biosolids are tested primarily

for the reduction of *Salmonella* and fecal coliforms such as *E.coli, Citrobacter, Enterobacter* and *Klebsiella.* It is not clear whether these biosolids are tested for other pathogens like drug resistant bacteria and viruses. In this study, we investigated the presence of drug resistant bacteria and their role in the transfer of drug resistance genes to other bacteria.

Biosolids were collected in June, 2012 from the wastewater facility, Cheney, WA. The samples were diluted and plated on MacConkey agar to select for gram-negative bacteria. From these plates, 100 colonies of varied colony morohology were randomly picked and tested against seven antibiotics representing a variety of antibiotic classes. Drugs from six different classes that are commonly used to prevent human infections were selected for this study. In aminoglycoside class, kanamycin and streptomycin were used. In the penicillin class, ampicillin was used. Nalidixic acid was selected from the quinolone class. In the tetracycline class of antibiotics, tetracycline was used. Other antibiotics such as rifampin and chloramphenicol were also tested. Of the 100 colonies tested, 68% of bacteria were resistant to two or more drugs (Table 5a and 5b). Of the 68 resistant isolates, 81% were resistant to ampicillin, 35% to kanamycin, 56% to streptomycin, 35% to chloramphenicol, 62% to nalidixic acid, 51% to tetracycline and 68% to rifampin (Figure 7). Overall, many of the bacteria screened were ampicillin and rifampin resistant which suggests the high usage of these antibiotics in the community (Figure 7). Ampicillin resistant isolates frequently exhibited tetracycline, streptomycin and rifampin resistance which is consistent with the study of others (Mirzaagha *et al.,* 2009; Van Donkersgoed *et al.,* 2003). We also found chloramphenicol resistant isolates

which is surprising because chloramphenicol is withdrawn in U.S based on their toxic effects. Macrolide antibiotics such as erythromycin inhibits protein synthesis by binding to 50S ribosomal subunit similar to chloramphenicol. Erythromycin resistant isolates have been isolated and identified from Cheney waste water facility (Marshall, 2009). This could be the reason for the presence of chloramphenicol resistance among bacteria. In addition, studies show resistance (R) plasmid that confer ampicillin and tetracycline resistance frequently confer chloramphenicol resistance (Haldar *et al.,* 1995; Mandal *et al.,* 2005). We found many ampicillin and tetracycline resistant isolates which also carried plasmid, so this might have conferred chloramphenicol resistance in bacteria. Of the 68 resistant isolates, 17.6% were resistant to two drugs, 20.5% to three drugs, 26.4% to four and five drugs and finally 8.8% were resistant to six drugs. None of the isolates were resistant to all the drugs tested (Figure 8). In this study, we found bacteria that were resistant to penicillin, tetracycline and quinolone class of antibiotics which is consistent with the previous reports (Marshall, 2009).

Table 5a: Resistance profile of the isolates X1-X36

Isolate #	Amp	Kan	Str	Chl	Nal	Tet	Rif
X1	+	-	+	-	+	+	-
X2	+	+	+	-	-	+	+
X3	+	-	-	+	-	-	+
X4	+	+	+	-	+	+	-
X5	+	+	+	+	+	+	-
X6	+	-	-	-	-	-	+
X7	+	-	-	+	-	+	-
X8	+	-	+	+	+	-	+
X9	+	-	+	-	-	-	+
X10	+	-	-	+	-	-	+
X11	+	-	+	-	+	+	-
X12	+	+	-	+	+	-	-
X13	+	-	-	+	-	-	+
X14	+	+	-	+	-	-	+
X15	+	-	-	-	+	-	+
X16	+	-	+	-	-	+	-
X17	+	-	-	-	-	+	+
X18	+	-	-	-	+	-	+
X19	+	-	-	-	+	-	-
X20	+	-	+	+	+	-	+
X21	-	-	+	-	-	-	+
X22	+	-	+	-	-	+	+
X23	+	-	+	+	-	+	+
X24	+	-	+	-	-	+	+
X25	+	+	+	-	-	+	+
X26	+	-	-	+	-	-	-
X27	+	-	+	-	-	+	+
X28	+	-	+	+	-	+	+
X29	+	-	-	-	-	-	+
X30	+	-	+	+	+	+	-
X31	+	+	+	-	-	+	+

X32	+	+	-	-	-	+	+
X33	+	+	+	+	-	+	+
X34	+	+	+	-	+	+	+
X35	+	-	+	-	+	+	-
X36	+	-	+	-	-	+	+

Table 5b: Resistance profile of the isolates X37-X68

Isolate #	Amp	Kan	Str	Chl	Nal	Tet	Rif
X37	+	+	-	-	+	+	+
X38	-	-	+	-	+	-	-
X39	+	-	-	-	+	+	+
X40	+	+	-	-	+	-	+
X41	+	-	+	-	+	+	+
X42	+	+	-	+	+	-	+
X43	+	+	-	-	+	-	+
X44	+	+	+	+	+	-	+
X45	+	+	+	+	+	-	+
X46	+	+	-	-	+	-	-
X47	+	+	+	-	+	-	-
X48	+	+	+	+		-	+
X49	+	+	+	-	+	-	+
X50	+	-	+	-	+	+	+
X51	-	-	-	-	+	-	+
X52	-	-	+	+	+	+	+
X53	+	-	+	-	-	-	+
X54	+	+	+	+	+	-	+
X55	-	-	-	+	+	-	-
X56	+	-	+	-	+	+	+
X57	-	+	+	+	+	-	-
X58	-	-	+	-	-	-	+
X59	-	-	-	-	+	+	-
X60	-	+	+	+	+	+	-
X61	-	-	-	-	+	+	+
X62	+	-	-	-	+	+	+
X63	-	-	-	-	+	+	-
X64	+	-	+	-	+	+	-
X65	-	-	-	+	+	+	-
X66	+	-	-	-	+	+	+
X67	+	-	-	-	+	-	+

X68	-	+	-	-	+	-	-

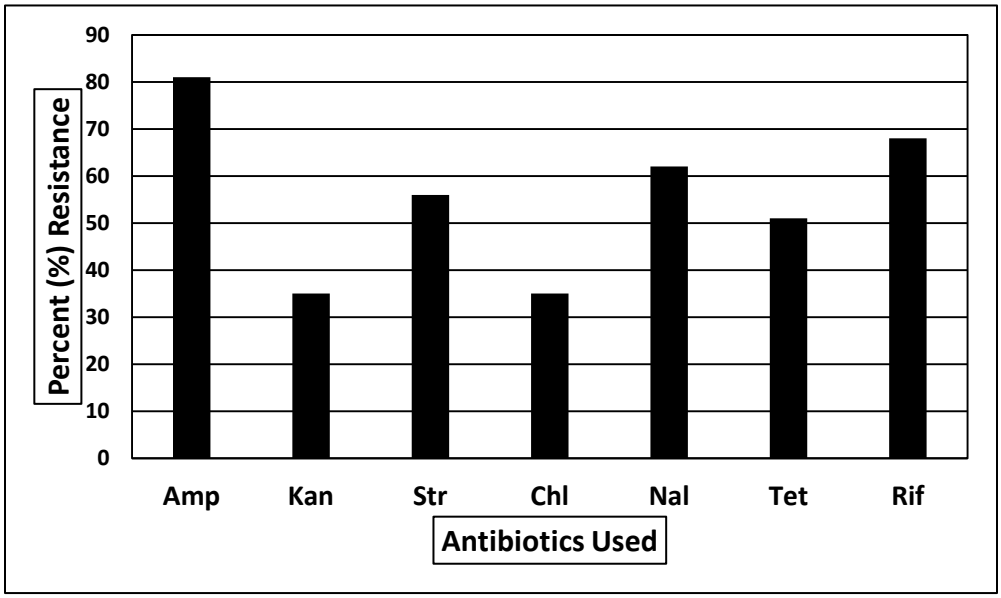


Figure 7: The graph represents the percent of isolates that were resistant to different antibiotics**.**

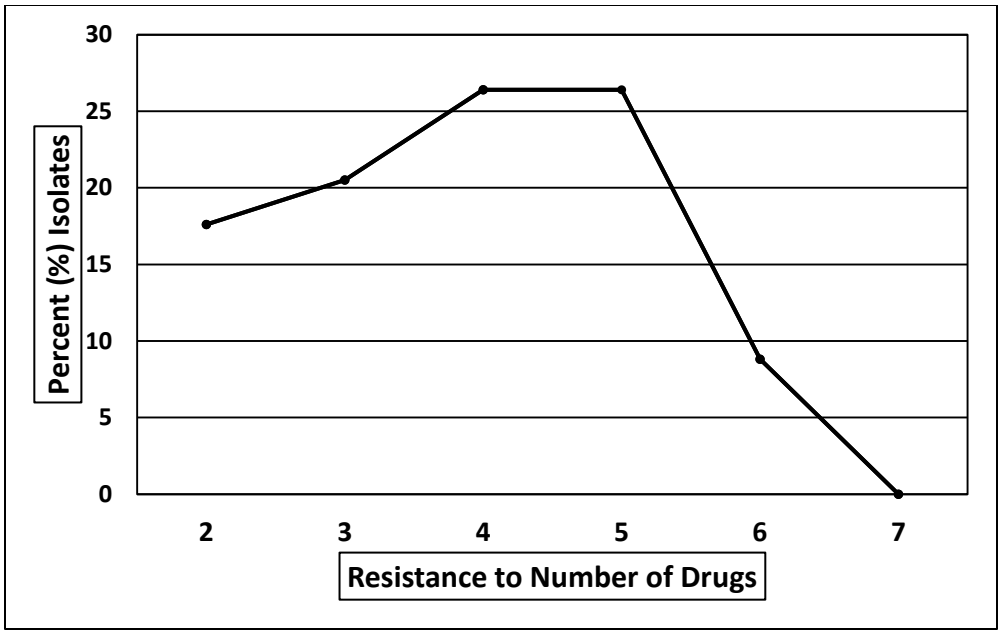


Figure 8: The graph represents the percent of isolates that were resistant to a given number of the antibiotics tested**.**

We did not do any correlation study regarding the usage of antibiotics by the local community and the presence of drug resistant bacteria in Cheney biosolids. Former student in our lab took survey from three pharmacies in Cheney local, and tried to do some correlation study (Marshall, 2009). However, she was not able to correlate the antibiotics sold by local pharmacies with the drug resistant bacteria present in Cheney biosolids (Marshall, 2009). Many people in Cheney commute to Spokane every day and vice versa, so they could obtain antibiotics from pharmacies outside the city limit. It is difficult to survey the amount of prescription antibiotics sold by all these pharmacies because of privacy issues. Amount of resistant bacteria and unadsorbed antibiotics

excreted by the people can also vary (Sarmah *et al.,* 2006). For these reasons, antibiotics that are commonly used by people are chosen for this study.

3.2 Isolation of Plasmids from Resistant Bacteria:

Multi-drug resistant bacteria are more likely to carry resistance plasmids (Nikaido, 2009; Maruyama *et al.,* 2006). To verify this possibility, plasmids were extracted from all the 68 resistant isolates X1 to X68 (data not shown here). Prior to plasmid extraction, resistant isolates were once again streaked on antibiotic plates to ensure their drug resistance (Figure 9). Using the manufacturer's protocol of plasmid isolation kit, plasmids were isolated, and their size and yield were determined on a 0.7% agarose gel. There were nine isolates X3, X9, X11, X16, X41, X52, X53, X58 and X66 that showed the presence of plasmids (Figure 10a, 10b and Table 6). Here, we showed that multi-drug

resistant bacteria carried resistance plasmid which is consistent with other studies (Bergstrom *et al.,* 2000; Kruse and Sorum, 1994; Nikaido 2009). There are two possible ways to explain the absence of plasmids in the remaining resistant isolates. One, the resistant bacteria carry resistance genes on their chromosome. It has been shown that drug resistance genes are often encoded on the bacterial chromosome (Carattoli, 2001; Drlica and Malik, 2003; Sharma and Mohan, 2006). Second, the resistant bacteria might have carried large molecular weight plasmids which were not processed in this assay. The plasmid preparation kit used was suitable for smaller size plasmids (<20 kb), so plasmids larger than that might not be isolated (Fermentas Life Sciences, Irvine, CA).

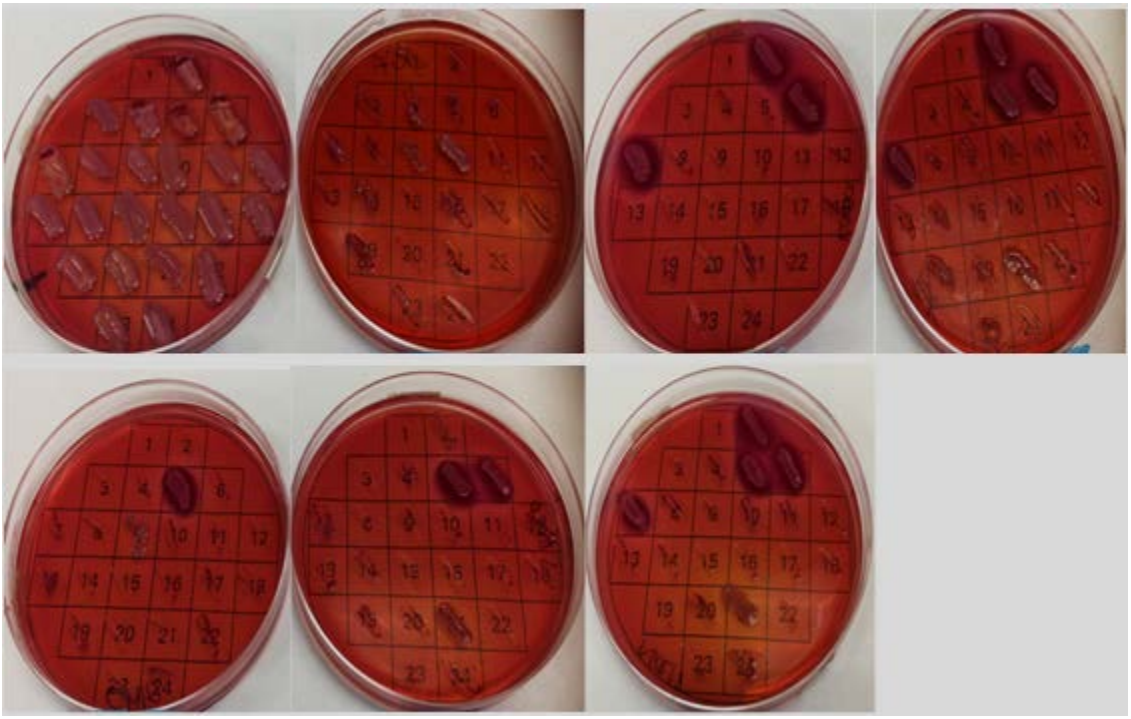


Figure 9: Antibiotic sensitivity testing of the isolates. At the top from left to right: the plates contain ampicillin, rifampin, kanamycin and tetracycline. At the bottom from left to right: the plates contain chloramphenicol, streptomycin and nalidixic acid. These antibiotic plates serve as a model to represent the sensitivity of bacteria to various drugs.

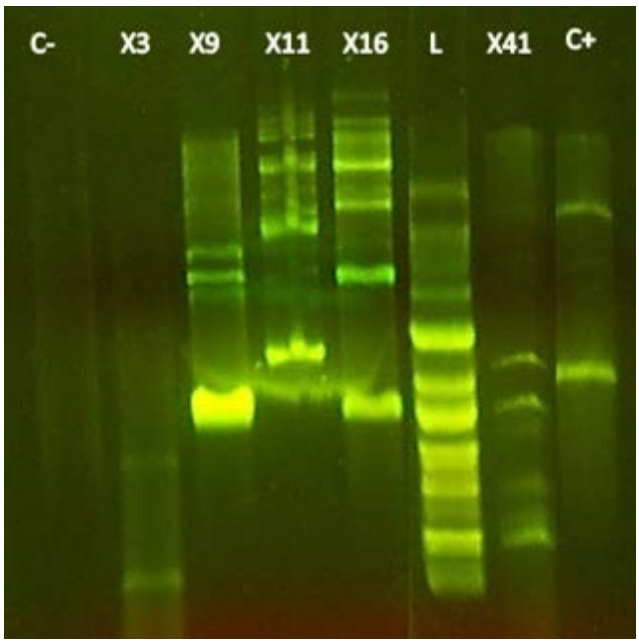


Figure 10a: Agarose gel showing the presence of plasmids.

Lane C-, *E.coli* DH5α lacking a plasmid. Lane L is a supercoiled DNA ladder. Lane C+, *E.coli* MM294 containing pGLO plasmid. Lanes X3, X9, X11, X16 and X41 are the isolates that showed the presence of plasmids.

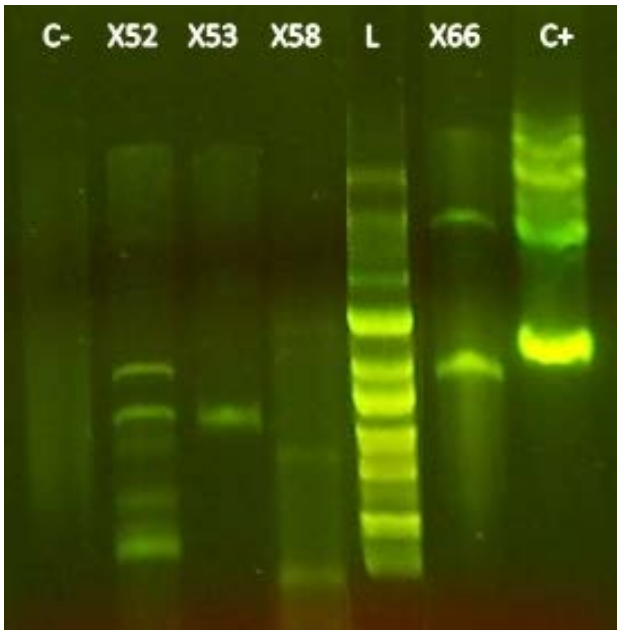


Figure 10b: Agarose gel showing the presence of plasmids.

Lane C-, *E.coli* DH5α lacking a plasmid. Lane L is a supercoiled DNA ladder. Lane C+, *E.coli* MM294 containing pGLO plasmid. Lanes X52, X53, X58 and X66 are the isolates that showed the presence of plasmids.

3.3 Identification of Resistant Bacteria:

The nine isolates (X3-I, X9-I, X11-I, X16-I, X41-I, X52-I, X53-I, X58-I and X66-I) that showed the presence of plasmids were identified using the API identification scheme (Figure 11) and oxidase test. The three isolates (X3-I, X52-I and X58-I) were identified as *Escherichia coli*, two isolates (X9-I and X66-I) as *Salmonella spp* and two isolates (X16-I and X53-I) as *Kluyvera spp.* There was one isolate (X11-I) identified as *Enterobacter aerogenes*. Finally, the remaining isolate (X41-I) could be either *Klebsiella pnuemoniae* or *Klebsiella planticola*. These data are summarized in Table 6. The isolates were identified based on some important biochemical tests. For instance, *E. coli* isolates were positive for indole production, beta-galactosidase and ornithinine decarboxylase reactions. The *Klebsiella pnuemoniae* isolates were positive for citrate utilization, urea hydrolysis and acetoin production. Similarly, *Kluyvera* isolates were positive for indole production, citrate utilization and glucose oxidation; the *Salmonella* isolates were positive for ornithinine decarboxylase and hydrogen sulfide production. Finally, the *Enterobacter aerogenes* was positive for citrate utilization, ornithinine decarboxylase and acetoin production. None of the isolates were positive for the oxidase test confirming that these isolates truly belong to *Enterobacteriaceae* family.

Figure 11: API-20E strips used for the identification of bacteria. At the top is the isolate X58-I identified as *E.coli*. At the middle is an isolate X16-I identified as *Kluyvera spp*. At the bottom: the isolate X11-I identified as *Enterobacter aerogenes*.

Table 6: Identity of resistant bacteria and their antibiotic resistance profile

Isolate #	Antibiotic Resistance	API Identification
X3-I	*Amp, Chl, Rif*	*E.coli*
X9-I	*Amp, Str, Rif*	*Salmonella spp.*
X11-I	*Amp, Str, Nal, Tet*	*Enterobacter aerogenes*
X16-I	*Amp, Str, Tet*	*Kluyvera spp.*
X41-I	*Amp, Str, Nal, Tet, Rif*	*K.pnueumoniae /K.planticola*
X52-I	*Str, Nal, Tet, Rif, Chl*	*E.coli*
X53-I	*Amp, Str, Rif*	*Kluyvera spp.*
X58-I	*Str, Rif*	*E.coli*
X66-I	*Amp, Tet, Nal, Rif*	*Salmonella spp.*

The API and oxidase test results indicated that all the bacteria identified (*E.coli, Salmonella, Enterobacter, Kluyvera* and *Klebsiella*) belong to *Enterobacteriaceae* family which is consistent with the results of others (Hoyle *et al.,* 2005; Van Donkersgoed *et al.,* 2003). Studies show that members of the *Enterobacteriaceae* family are frequently found in sewage samples because many of them are present in the human and animal gastro-intestinal tract (Hoyle *et al.,* 2005; Van Donkersgoed *et al.,* 2003). A correlation between antimicrobials used and the development of drug resistance in *E.coli* has been well documented (Van den Bogaard and Stobberingh, 2000). It has been shown that resistance determinants can be transferred from *E.coli* to enteric pathogens (Aslam and Service, 2006; Blake *et al.,* 2003). In this study, we identified multidrug resistant *E.coli* and other organisms. Many of these multidrug resistant organisms carried plasmid which is in agreement with the previous reports (Bergstrom *et al.,* 2000; Nikaido 2009). Furthermore, it has been reported that conjugative plasmids encoding multidrug resistance genes could be responsible for the transfer of resistance among *Enterobacteriaceae* (Paterson, 2006 and Poppe *et al.,* 2001).

3.4 Transformation:

Transformation was performed to identify the location of resistance genes. Plasmids isolated from nine isolates (X3-I, X9-I, X11-I, X16-I, X41-I, X52-I, X53-I, X58-I and X66-I) were introduced into *E.coli* MM294 (Figure 10a and 10b). These 9 plasmids are referred to as 'parental plasmids' in this work. The bacterial colonies formed on the antibiotic plates represent transformants that took the plasmid DNA. Of the 9 plasmid isolates, only 7 plasmids (77.7%) were successfully and experimentally introduced into *E.coli* MM294 (Table 7). The transformation efficiency of these seven isolates was in the range of 6.7 X 10^2 to 4.7 X 10^3 colony forming units per microgram of DNA (shown in Table 7). Based on transformation results, it is clear that tetracycline, ampicillin, streptomycin, nalidixic acid and rifampin resistance is carried on a plasmid (Table 7) which is consistent with other studies (Roberts, 2006). The resistance of these transformants was reconfirmed by streaking them on the appropriate antibiotic plates. Plasmids were then reisolated from 2-3 randomly selected transformants, and their presence was confirmed on an agarose gel (Figure 12a – 12f).

Table 7: Transformation efficiency of Parental Plasmids

Transformant Isolate #	Growth of Transformants on Antibiotic Plates	*No of Transformants / µg DNA*
X3-II	*No growth*	-
X9-II	*Amp*	*3.4 X* 10^3
X11-II	*Str, Tet*	*Str – 1.7 X* 10^3 *Tet – 3.4 X* 10^3
X16-II	*Tet*	*3.2 X* 10^3
X41-II	*Rif*	*7.4 X* 10^2
X52-II	*Nal, Str, Tet*	*Nal - 6.7 X* 10^2 *Str – 3.7 X* 10^3 *Tet – 4.7 X* 10^3
X53-II	*Rif*	*6.7 X* 10^2
X58-II	*No growth*	-
X66-II	*Tet*	*3.0 X* 10^3

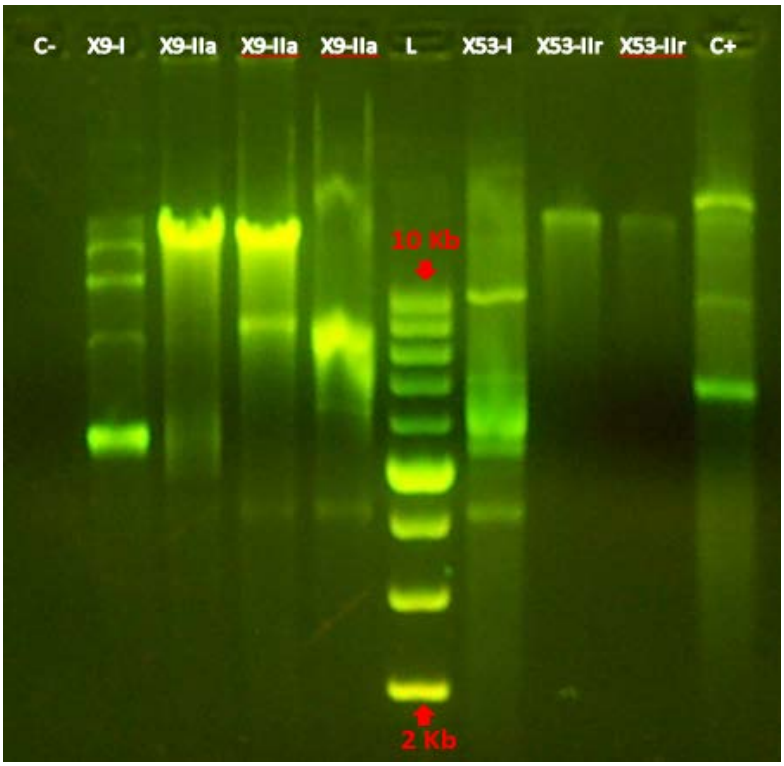


Figure 12a: A comparison between plasmids isolated from transformants with parental plasmids.

Lane C- is *E.coli* MM294 lacking a plasmid. Lane 2 is a parental X9-I plasmid. Lanes 3, 4 and 5 are *E.coli* transformants taken from an Ampicillin plate. Lane L is a supercoiled ladder. Lanes 7 is a parental X53-I plasmid. Lanes 8 and 9 are *E.coli* transformants taken from from a Rifampin plate. Lane C+ is *E.coli* MM294 transformed with pGLO plasmid.

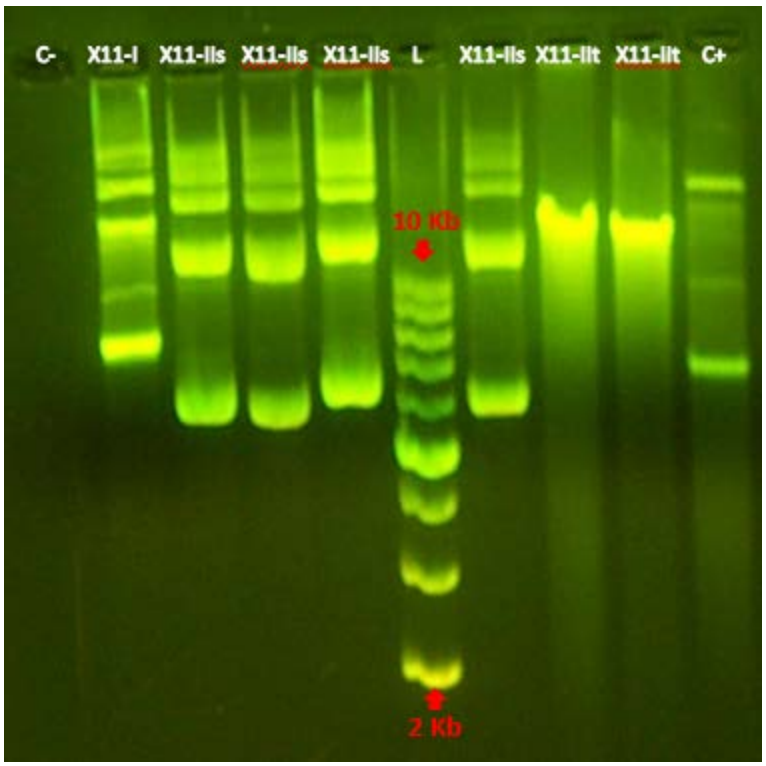


Figure 12b: A comparison between plasmids isolated from transformants with parental plasmids.

Lane C- is *E.coli* MM294 lacking a plasmid. Lane 2 is a parental X11-I plasmid. Lanes 3, 4, 5 and 7 are *E.coli* transformants taken from a Streptomycin plate. Lane L is a supercoiled ladder. Lanes 8 and 9 are *E.coli* transformants taken from a Tetracycline plate. Lane C+ is *E.coli* MM294 transformed with pGLO plasmid.

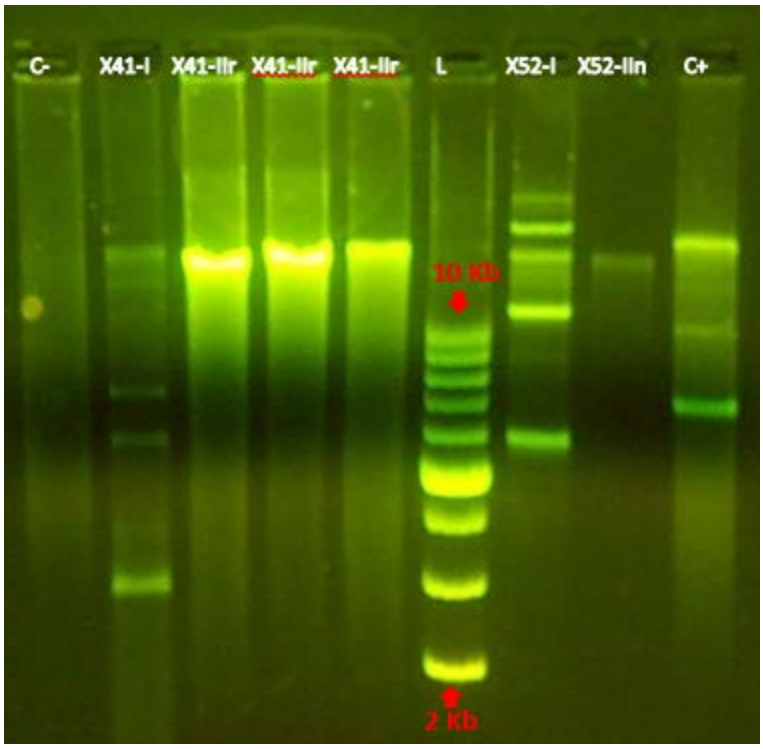


Figure 12c: A comparison between plasmids isolated from transformants with parental plasmids.

Lane C- is *E.coli* MM294 lacking a plasmid. Lane 2 is a parental X41-I plasmid. Lanes 3, 4 and 5 are *E.coli* transformants taken from a Rifampin plate. Lane L is a supercoiled ladder. Lanes 7 is a parental X52-I plasmid. Lane 8 is *E.coli* transformants taken from a Nalidixic acid plate. Lane C+ *E.coli* MM294 transformed with pGLO plasmid.

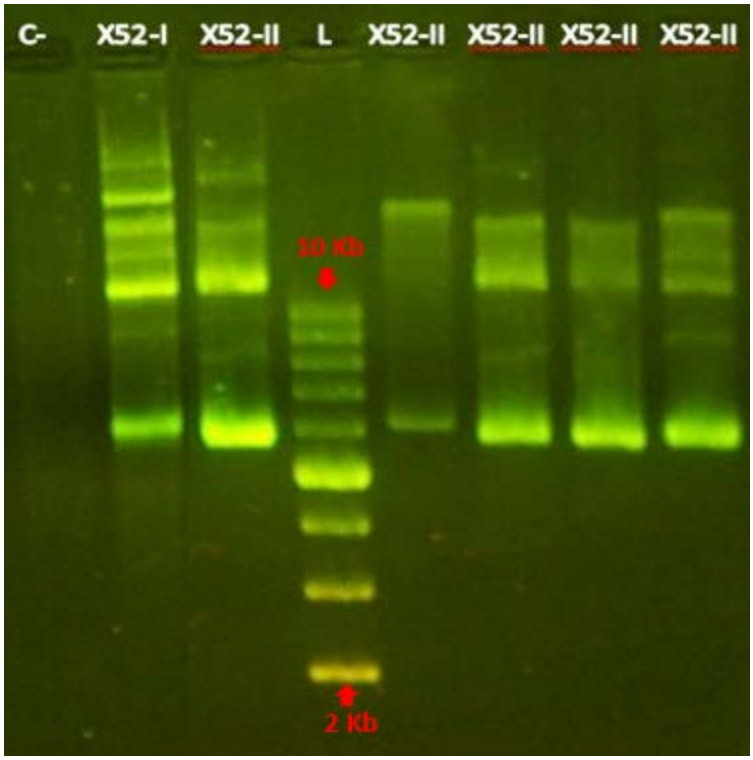
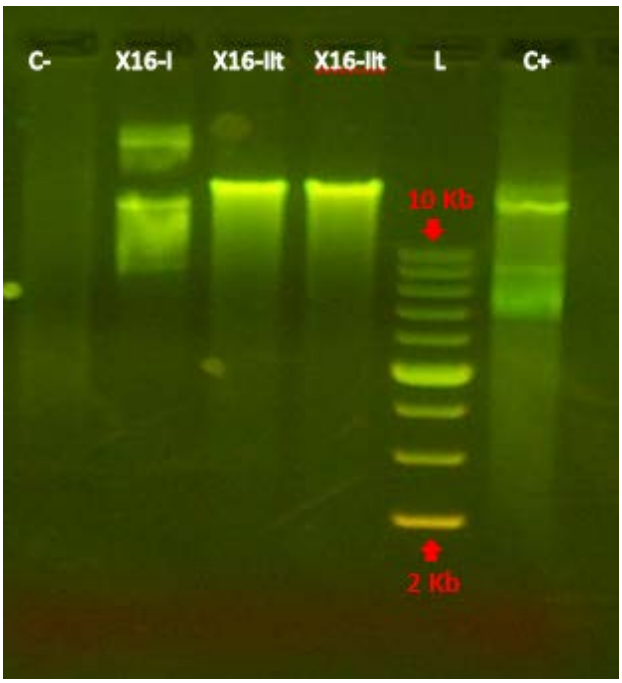


Figure 12d: A comparison between plasmids isolated from transformants with parental plasmids.

Lane C- is *E.coli* MM294 lacking a plasmid. Lane 2 is a parental X52-I plasmid. Lanes 3 and 5 are *E.coli* transformants taken from a Tetracycline plate. Lane L is a supercoiled ladder. Lanes 6, 7 and 8 are *E.coli*



transformants taken from a Streptomycin plate.

Figure 12e: A comparison between plasmids isolated from transformants with parental plasmids.

Lane C- is *E.coli* MM294 lacking a plasmid. Lane 2 is a parental X16-I plasmid. Lanes 3 and 4 are *E.coli* transformants taken from a Tetracycline plate. Lane L is a supercoiled ladder. Lane C+ is *E.coli* MM294 transformed with pGLO plasmid.

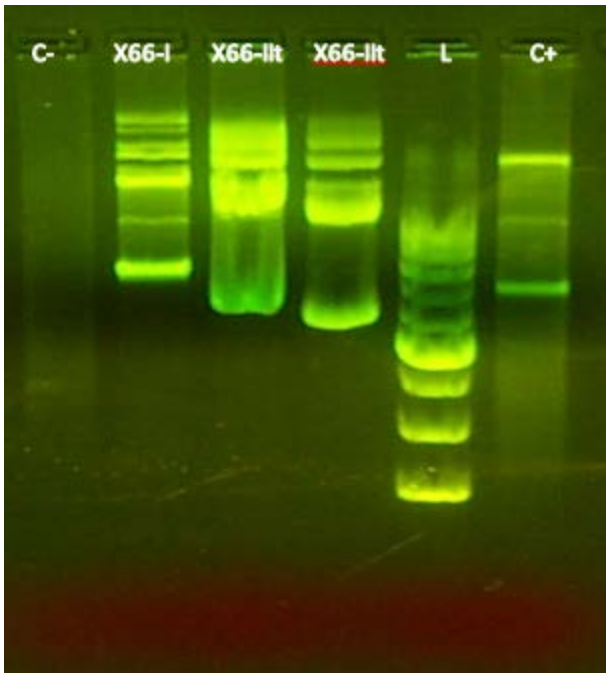


Figure 12f: A comparison between plasmids isolated from transformants with parental plasmids.

Lane C- is *E.coli* MM294 lacking a plasmid. Lane 2 is a parental X66-I plasmid. Lanes 3 and 4 are *E.coli* transformants taken from a

Tetracycline plate. Lane L is a supercoiled ladder. Lane C+ is *E.coli* MM294 transformed with pGLO plasmid.

The transformants (X9-II, X11-II, X16-II, X41-II, X52-II, X53-II and X66-II) showed resistance patterns similar to the parental strains (Table 6 and 7). For instance, the parent (X11-I) was resistant to four drugs: ampicillin, streptomycin, nalidixic acid, tetracycline whereas the transformant (X11-II) was resistant to streptomycin and tetracycline. That means, the streptomycin and tetracycline resistance were carried on a plasmid. These two resistance genes could be carried on a same plasmid or two different plasmids (Table 6 and 7). Similarly, X41-I was resistant to five drugs: ampicillin, streptomycin, nalidixic acid, tetracycline and rifampin. However, the transformant X41-II carried rifampin resistance on a plasmid (Table 6 and 7). The remaining or missing resistance might be carried on a chromosome. Many of the plasmids extracted from transformants (X41-IIr, X52-II and X16-IIt) migrated at the same distance as parental plasmids on a gel (Figure 12c, 12d and 12e). There were few exceptions; plasmids X9-IIa, X11-IIs and X66-IIt extracted from transformants migrated faster than the parental plasmids (Figure 12a, 12b and 12f). This is not surprising because *E. coli* will take only limited amount of DNA during transformation, and it is unlikely that *E. coli* take more than one plasmid. The different migration patterns of parent and transformants on the gel could be because *E. coli* took only one plasmid eventhough the parent contained multiple plasmids. Some of the transformants showed the presence of multiple plasmids with different sizes on a gel. The multiple plasmid bands may reflect the presence of plasmid in isomeric forms such as supercoiled, relaxed and nicked (Schmidt *et al.,* 1999).

To check whether these are truly different forms of a plasmid, we performed restriction digestion which is described in the next section (Figure 13a – 13g). The plasmids from two isolates (X3-I and X58-I) failed to transform in the *E.coli* strain. There could be a reason for that. The resistance genes might not be present on plasmids which prevented *E.coli* from growing on antibiotic plates. These two isolates might have carried resistance genes on the bacterial chromosome.

3.5 Restriction Digestion:

Restriction digestion was performed to verify whether the multiple DNA bands represent a single plasmid in different forms. It has been shown that plasmids exist in various forms and the supercoiled plasmid moves much faster than the linear and nicked plasmids on an agarose gel (Schmidt *et al.,* 1999). As mentioned in the previous section, seven out of nine plasmid preparations successfully produced transformants. These seven isolates were cross checked on antibiotic plates, and plasmids were extracted from them. These plasmids were further digested with the Type II restriction enzyme, *Eco*RI*.* Many of the isolates X9-II, X11-II, X16-II, X41-II, X52-II, X53-II, X66-II formed single band on an agarose gel after digestion with *Eco*RI, suggesting these plasmids contained a single *Eco*RI restriction site (Figure 13a, 13c, 13d, 13e, 13f and 13g). Only exception was X11-II, it formed 3 to 4 bands of smaller size than the original plasmid (Figure 13b). This is not surprising because an *Eco*RI site is predicted to occur once every ~4000 bp, and the size of parental plasmid was estimated to be >10 Kb, so it might have resulted in multiple small fragments.

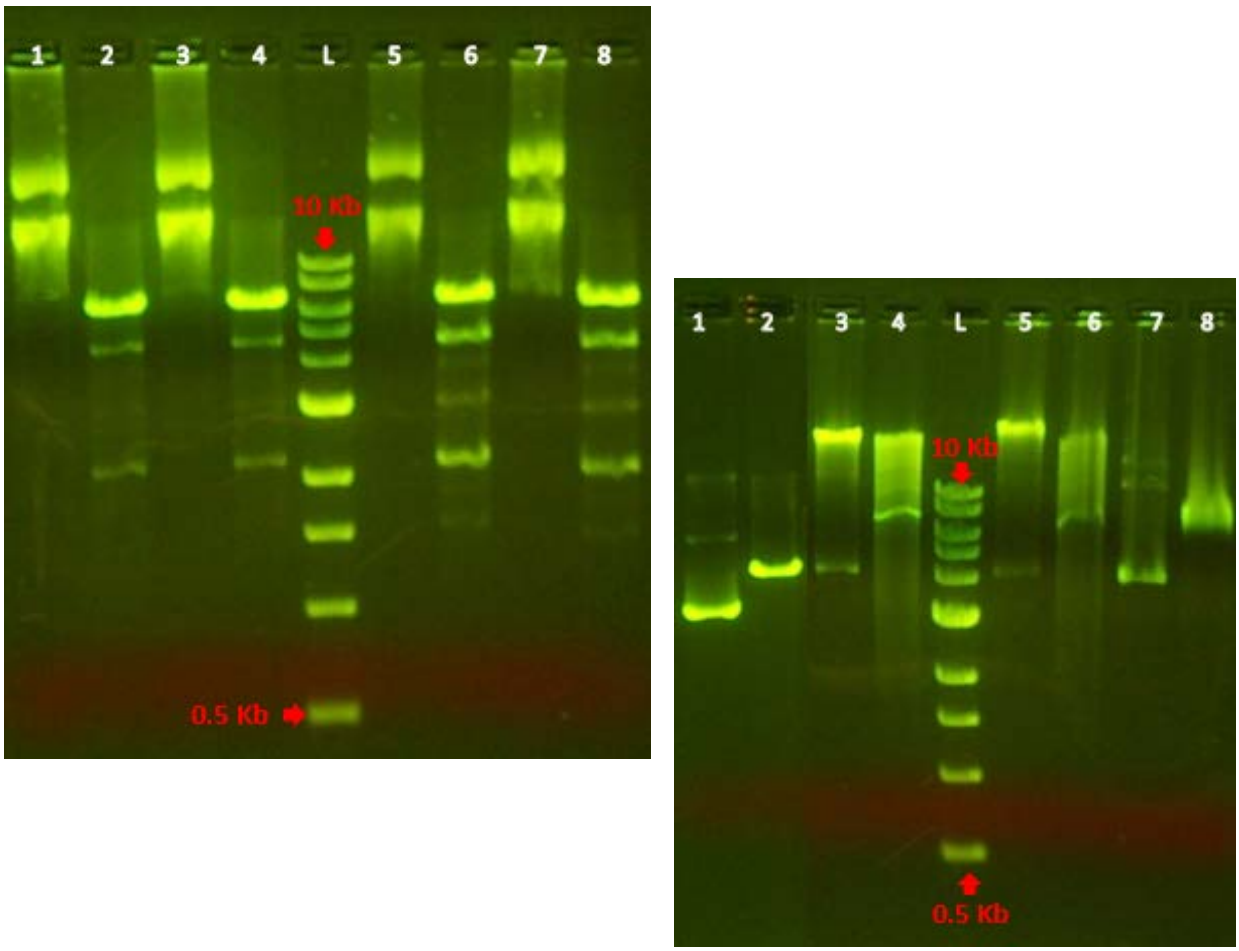


Figure 13a: Restriction digestion of the plasmids extracted from transformants.

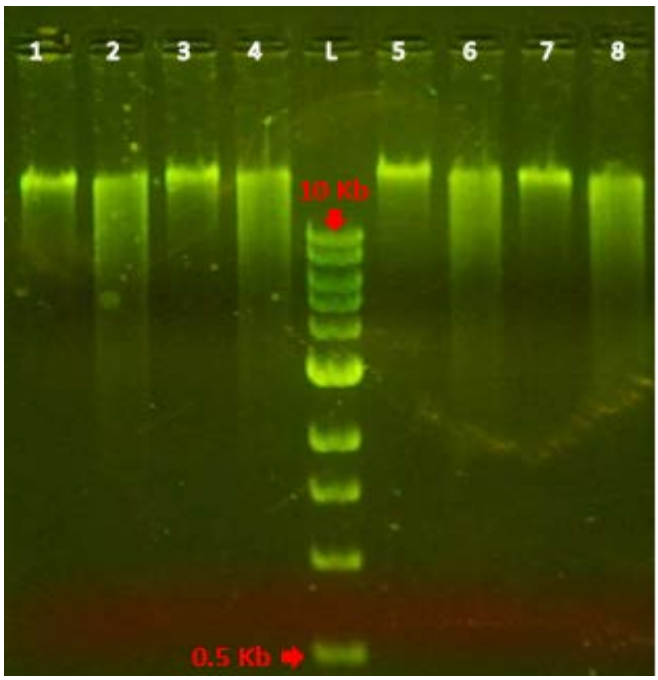
Lane 1 is an undigested plasmid, pBR322. Lane 2 is a plasmid pBR322 digested with *Eco*RI shows a single band as expected. Lanes 1 and 2 serve as a positive control. Lane L is a 1 Kb DNA ladder. Lanes 3, 5 and 7 are X9-II plasmid samples that are not subjected to any digestion. Lanes 4, 6 and 8 are the respective plasmids from X9-II that are digested with *Eco*RI.

Figure 13b: Restriction digestion of the plasmids extracted from transformants.

Lane L is a 1 Kb DNA ladder. Lanes 1, 3, 5 and 7 are X11-II plasmid samples that are not subjected to any digestion. Lanes 2, 4, 6 and 8 are the respective plasmids from X11-II that are digested with *Eco*RI.

Figure 13c: Restriction digestion of the plasmids extracted from transformants.

Lanes 1 and 3 are X11-II plasmid samples that are not digested. Lanes 2 and 4 are X11-II plasmid samples that are digested with *Eco*RI. Lane L is a 1 Kb DNA ladder. Lanes 5 and 7 are the respective plasmids from X16-II plasmid samples that are not subjected to any digestion. Lanes 6 and 8 are the respective plasmids from X16-II that are digested with *Eco*RI.



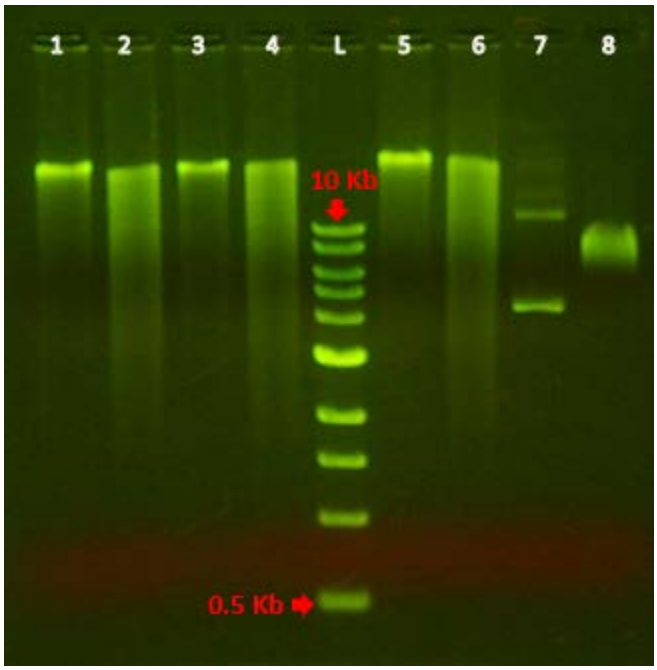


Figure 13d: Restriction digestion of the plasmids extracted from transformants.

Lanes 1, 3 and 5 are X41-II plasmid samples that are not digested. Lanes 2, 4 and 6 are X41-II plasmid samples that are digested with *Eco*RI. Lane L is a 1 Kb DNA ladder. Lane 7 is X52-II plasmid sample that is not subjected to any digestion. Lane 8 is the respective plasmid from X52-II digested with *Eco*RI.

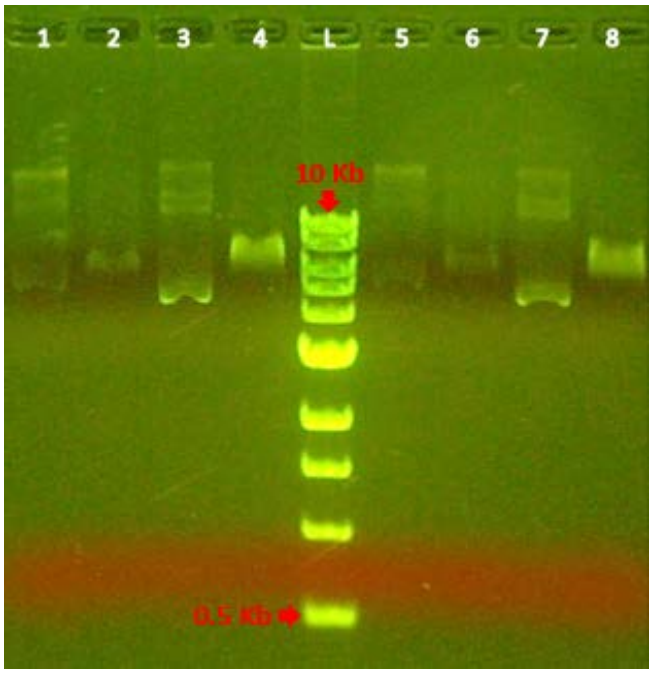


Figure 13e: Restriction digestion of the plasmids extracted from transformants.

Lanes 1, 3, 5 and 7 are X52-II plasmid samples that are not digested. Lanes 2, 4, 6 and 8 are the respective plasmids from X52-II that are digested with *Eco*RI. Lane L is a 1 Kb DNA ladder.

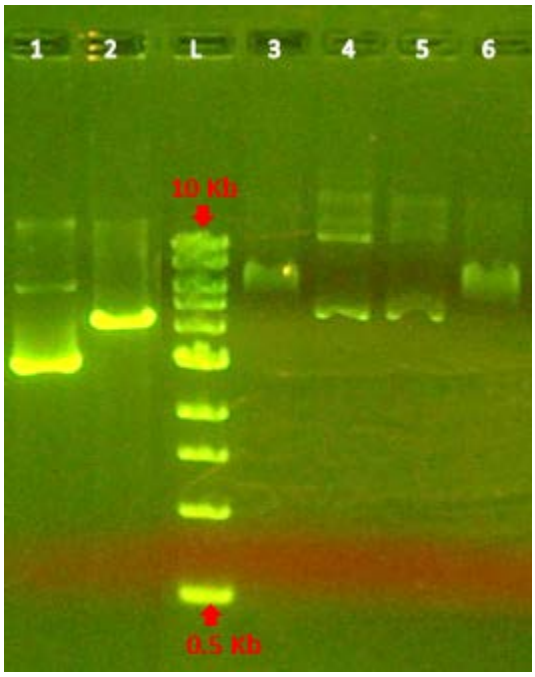


Figure 13f: Restriction digestion of the plasmids extracted from transformants.

Lane 1 is an undigested plasmid, pBR322. Lane 2 is a plasmid pBR322 digested with *Eco*RI. Lanes 1 and 2 serve as a positive control. Lane L is a 1 Kb DNA ladder. Lane 3 is an X66-II plasmid sample digested with *Eco*RI. Lane 4 is an X66-II plasmid sample that is not digested. Lane 5 is X66-II plasmid sample that is not digested. Lane 6 is the respective plasmid from X66-II digested with *Eco*RI.

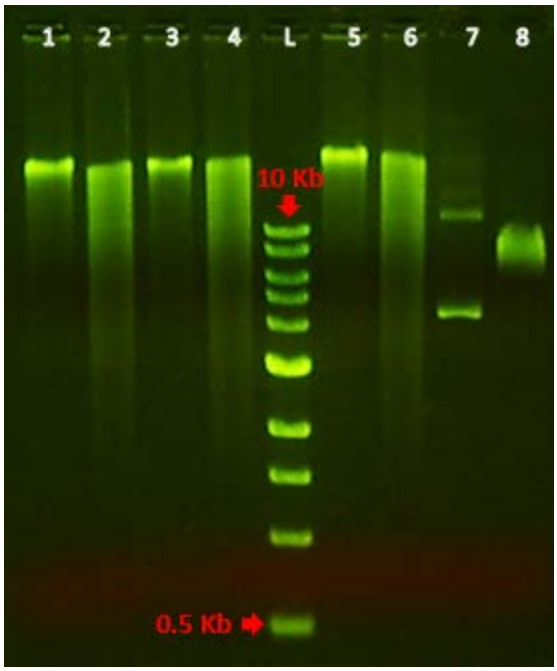


Figure 13g: Restriction digestion of the plasmids extracted from transformants.

Lane 1 is an X52-II plasmid sample that is not digested. Lane 2 is an X52-II plasmid sample digested with *Eco*RI. Lane L is a 1 Kb DNA ladder. Lanes 3 and 5 are X53-II plasmid samples that are not digested. Lanes 4 and 6 are X53-II plasmid samples that are digested with *Eco*RI. Lane 7 is an undigested plasmid, pBR322. Lane 8 is a plasmid pBR322 digested with *Eco*RI. Lanes 7 and 8 serve as a positive control.

3.6 Phage Sensitivity Testing:

Coliphages such as Qβ and M13 are known to infect *E.coli* strains carrying conjugative plasmids (Long *et al.,* 2005; Sobsey *et al.,* 1995). The nine isolates X3-I, X9-I, X11-I, X16-I, X41-I, X52-I, X53-I, X58-I and X66-I that showed the presence of plasmids were tested for the presence of conjugative plasmids using coliphages. The Qβ and M13 phages infect through conjugation pilus formed by the bacteria, so if the bacteria are infected, they have conjugative genes either on the plasmid or genomic DNA. Based on this concept, the nine isolates were tested for the sensitivity to Qβ and M13 phages. The natural host for Qβ and M13 phages are *E.coli*. Three *E.coli* isolates X3-I, X52-I and X58-I that showed the plasmid presence were tested for conjugative plasmid, but none of them were infected by the coliphages (Figure 14). We also tested other isolates (non *E. coli*) that showed plasmid presence even though they are not the natural host for these phages. As expected, none of them were infected (data not shown here).

There could be two reasons for the isolates not being infected. First, all these plasmids might not be conjugative; i.e. these isolates were not able to form conjugation pilus. Second, even if the plasmids were conjugative, the conjugative function was repressed for some reason. It has been shown that conjugative plasmids may carry fertility inhibition factor or *fin* genes which are able to suppress the formation of conjugation pilus in order to prevent infection caused by bacteriophages (Haft *et al.,* 2009). Conjugation adds stress to the host bacteria and these bacteria tend to grow relatively slowly compared to the one without conjugation factors. The fertility inhibition helps the host cells to multiply rapidly and dominate the population, so the *fin*

genes are turned on to reduce the cost to the host bacterium (Haft *et al.,* 2009; Mc Ginty and Rankin, 2012). Therefore, it could be possible that the *E.coli* isolates we tested might have carried *fin* genes which prevented them from being infected by Qβ and M13 phages.

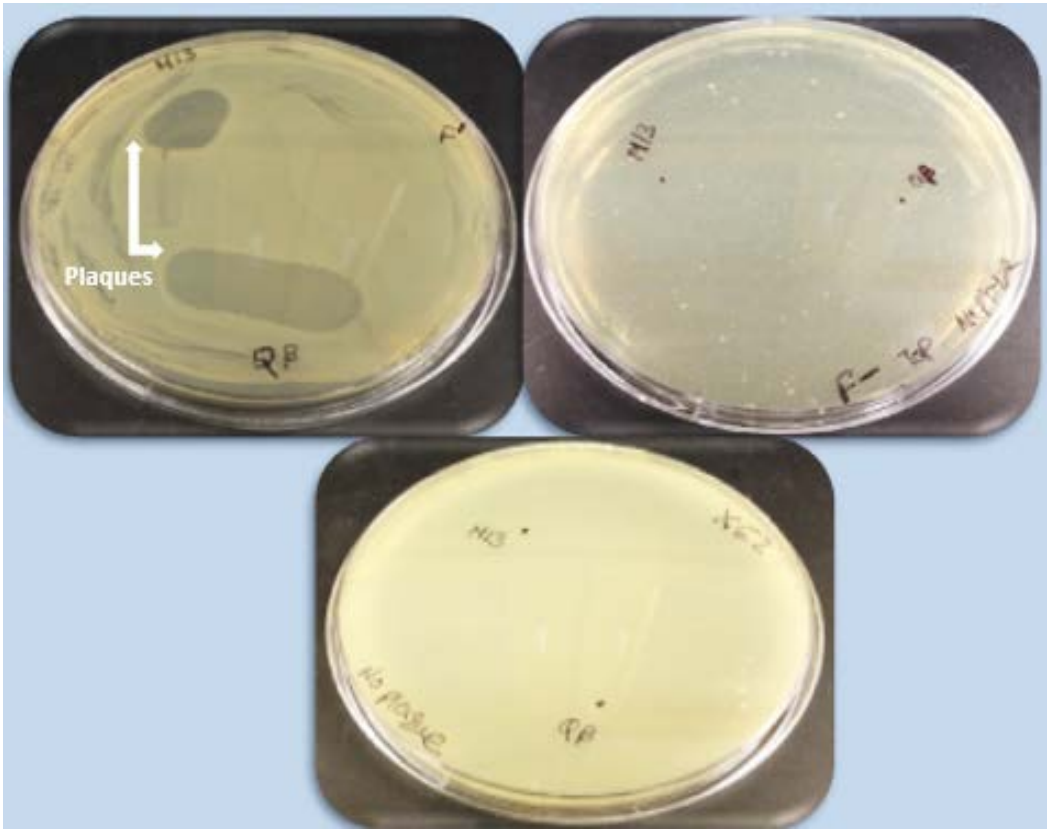


Figure 14: Phage sensitivity testing. On the left is a positive control, *E.coli* XK1502 infected with two phages: M13 and Qβ. The visible clearing or plaque formation on the lawn of bacteria represents the sensitivity of this bacteria to the phages. On the right is a negative control, *E.coli* MC4100 infected with the same phages but no plaques are produced. At the bottom is an isolate *E.coli* X52-I infected with the same phages. The absence of plaque formation indicates this isolate was not able to form conjugation pilus.

3.7 Tripartite Mating:

Tripartite mating was performed to identify mobilizable plasmids. This mating process requires three strains: a donor, a helper and a recipient. The donor may carry mobilizable plasmid but is incapable of forming conjugation pilus because of the absence of *tra* gene function. The helper will provide the missing *tra* function to the donor, so the donor will form conjugation pilus and transfer their gene to the recipient (Droge *et al.,* 2000; Schluter *et al.,* 2007). By this assay, mobilizable plasmids can be identified.

As mentioned in a previous section, the isolate *E.coli* X3-I was tested for the presence of conjugative plasmid using the phage sensitivity assay, but it was negative for conjugative plasmid. Before tripartite mating, the isolate X3-I was plated with the recipient to reconfirm the absence of conjugative plasmid (data not shown here). After reconfirmation, tripartite mating was performed with the isolate (X3-I) to identify the presence of mobilizable plasmid. The donor was resistant to 3 drugs (ampicillin, rifampin and chloramphenicol), the helper was nalidixic acid resistant, and the recipient was resistant to streptomycin (Figure 15a and 15b). The antibiotic plates were made in all different combinations of these 5 drugs, and each plate was divided into four sections: (1) donor alone, (2) recipient alone, (3) helper alone, and (4) recipient + donor + helper. Section 4 represents tripartite mating in which the donor isolate *E.coli* X3-I is plated together with the helper and recipient *E.coli* on all different antibiotic plates (Figure 15a and 15b). If the donor (X3-I) carried mobilizable plasmid, it will transfer the resistance plasmid to the recipient and form colonies on the antibiotic plates. Unfortunately, this

isolate did not form a transconjugant or bacterial colony on any of the antibiotic plates, so the donor lacks both the conjugative and mobilizable plasmids (Figure 15a and 15b). We tested only this isolate (X3-I) because it has a resistance marker different from the helper and recipient. We are not able to test other isolates because they are multi-drug resistant and carried the same resistance marker like the helper and recipient. If we test these isolates using the same procedure, then we would not be able to distinguish between the donor, helper and recipient. We had a trouble finding a strain that has a resistance marker different from the donor. Many of the strains available in CGSC and ATCC bacterial strain banks were not helpful because they carried the same resistance marker as the donor. We did not test the other isolates because of these complications.

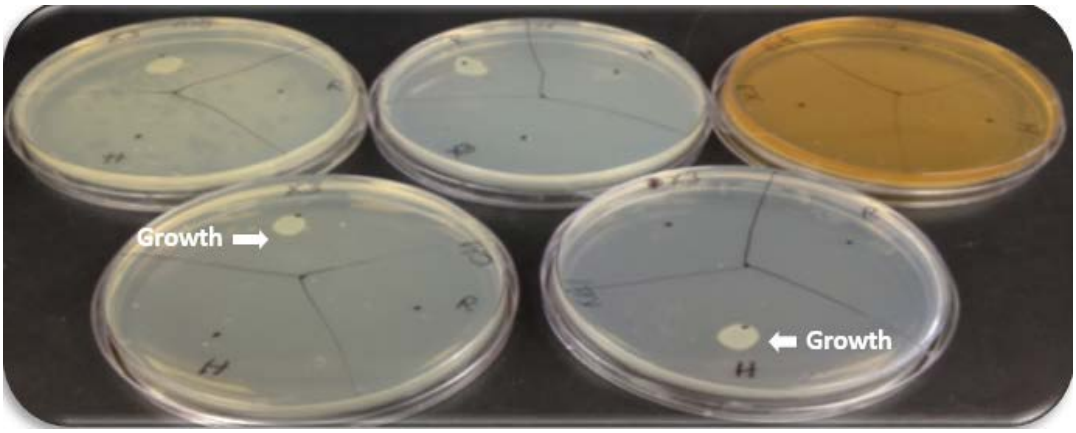


Figure 15a: Resistance profile of the donor, helper and recipient strains. The donor isolate X3-I is resistant to ampicillin, rifampin and chloramphenicol. The helper is resistant to nalidixic acid and the recipient is resistant to streptomycin. These antibiotics plates serve as a positive control for tripartite mating process and confirm the resistance pattern of the strains.

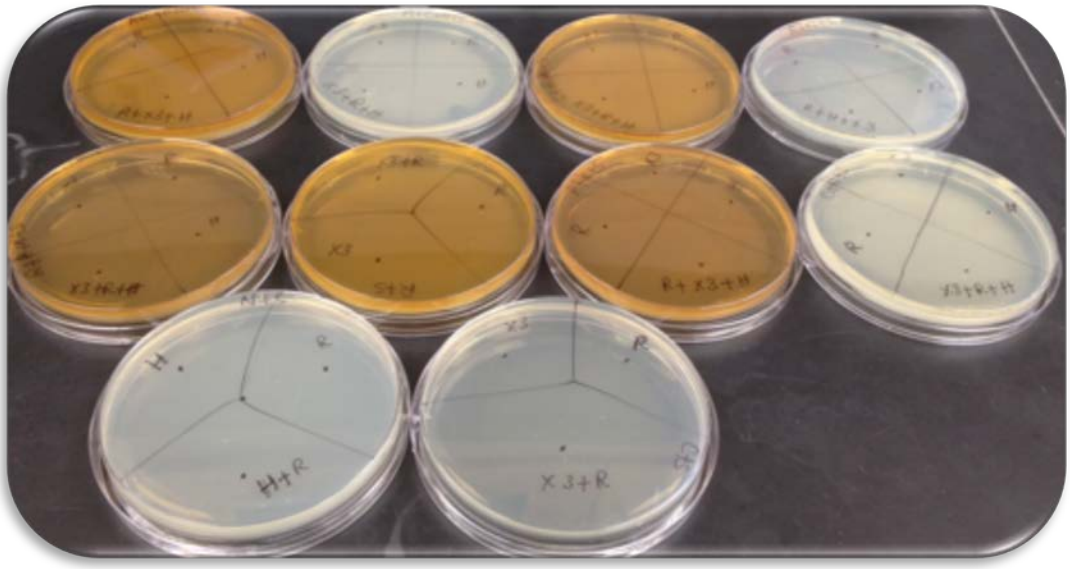


Figure 15b: Tripartite mating process. As mentioned in Fig 9a, the donor was resistant to 3 drugs, and both helper and recipient were resistant to one drug, so antibiotic plates were made in all different combinations of these 5 drugs. Each plate was divided into four sections: (1) donor alone, (2) recipient alone, (3) helper alone, and (4) recipient + donor + helper. Section 4 represents tripartite mating in which the donor isolate *E.coli* X3-I is plated together with the helper and recipient *E.coli* on all different antibiotic plates. None of them resulted in colony formation, so this isolate is neither conjugative nor mobilizable type.

4. Conclusions:

Biosolids are used as fertilizer in agricultural crops and home gardens all over the United States, but there is great concern regarding their application. It has been shown that antibiotics persist in the environment for a long time and reach ground water or aquatic sediments through application of liquid manure or sewage sludge as fertilizers (Kuhne *et al*., 2001; Kummerer, 2001). Antibiotics present in sewage select resistant bacteria, and there is increased risk of spreading drug resistance genes among members of bacterial community. Previous studies have shown that application of manure as fertilizer is unsafe because it can act as a reservoir for pathogens carrying drug resistance plasmids (Binh *et al.,* 2008). In addition, viruses present in sewage are maintained because they are more resistant to ammonia and heat treatments (Lund *et al.,* 1996). These studies show that biosolids contain resistant bacteria and viruses that can transfer their resistance genes to other bacteria. Currently, biosoilds are tested only for the indicator organisms such as fecal coliforms, but it is better to test the biosolids for the presence of drug resistant bacteria, human viruses and bacteriophages. Such studies should be carried out to avoid the risks of contaminating garden and agricultural soils with pathogens and to reduce infections in humans and animals. Alternately, the biosolids should be sterilized before being sold as fertilizer or soil conditioner.

The purpose of my study was to determine the presence of drug resistant bacteria and resistance (R) plasmids in Cheney biosolids. We identified bacteria (68%) were resistant to two or more drugs tested and many of these resistant bacteria (13.2%)

carried resistance plasmids. The R-plasmids were able to transfer successfully to a laboratory strain of *E.coli.* The resistant bacteria (13.2%) were tested for the presence of conjugative plasmid using Qβ and M13 phages, but none of the bacteria were conjugative type. In addition, resistant bacteria (13.2%) were tested for the presence of mobilizable plasmid by tripartite mating, but none of the bacteria were mobilizable type. As a result, the resistant bacteria tested were neither conjugative nor mobilizable type.

One of the obstacles for conjugation and tripartite mating is the availability of a suitable recipient bacteria with unique resistance marker. This problem can be solved by developing a unique recipient using recombinant DNA techniques. The presence of conjugative and mobilizable plasmids in biosolids can be further analysed by relaxase screening method (Alvarado *et al.,* 2012). The biosolids should be tested further for the presence of enteric viruses of health concern either by monitoring the presence of bacteriophages using PCR techniques (Fout *et al.,* 2003; Sobsey *et al.,* 1995). In conclusion, Cheney biosolids do contain drug resistant bacteria despite of all the waste water treatment processes, so it is better to enhance treatment regulations to avoid serious health effects.

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