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The potential for replication and transmission of antibiotic resistance plasmids in an *E. coli* population in agriculturally impacted stream sediment

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The Potential for Replication and Transmission of Antibiotic Resistance Plasmids in an
E. coli Population in Agriculturally Impacted Stream Sediment

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Abstract

The use of antibiotics in agriculture is thought to be a major cause of resistance in microorganisms found in the environment. Horizontal transfer of genetic information from transient to native and from native to transient bacterial populations may enhance the spread and recombination of resistance genes and might play a role in the formation of multi-resistant organisms in environmental reservoirs. Tetracycline resistance plasmids were compared using three isolation techniques -- traditional “endogenous” extraction from isolates, “exogenous” plasmid capture, and direct plasmid extraction from sediment --to determine the potential for plasmid born resistance in an *E. coli* population found in agriculturally-impacted stream sediment. Comparison of the number of tetracycline resistant *E. coli* in the sediment versus the water column identified the sediment as a more likely reservoir for resistance plasmids and therefore of genetic exchange. Exogenously captured, self-transmissible plasmids had a significantly greater incidence of resistance to multiple antibiotics than the endogenously isolated plasmids. Variation in plasmid extraction techniques eliminated some of the prejudice against the uncultivable environmental organisms and gave a more complete picture of the “mobilome” of tetracycline resistance plasmids that may circulate in populations of *E. coli* in stream sediments.

Introduction

The rising occurrence of antibiotic resistant bacteria has become a major public health concern. Pathogenic organisms have consistently become resistant to antibiotics soon after their introduction to clinical use (Shea, 2003). The development of resistance to antibiotics used to treat specific infections suggests a link between antibiotic use and the development of resistance (FDA, 2003). Several bacterial strains have even become untreatable due to resistance to multiple antibiotics (Andersson, 2003). There is evidence that the observed increase in resistance may be due not only to the overuse of antibiotics in humans, but also to antibiotic use in agriculture (Wegener, 2003).

It is estimated that as much as 80% of the antibiotics produced in the United States are used for agricultural purposes (Smith *et al.*, 2002). These purposes include both therapeutic and non-therapeutic applications (Wegener, 2003). "Therapeutic applications" of antibiotics refers to their use in the treatment and prevention of infection. Antibiotics are often administered to an entire flock or herd of animals rather than to an infected individual, particularly in poultry, which are not cost-effective to treat individually. (Mellon *et al.*, 2001). Non-therapeutic uses include low doses of antibiotics to promote growth and feeding efficiency without the intent to treat any specific disease (Hershberger *et al.*, 2005 Mellon *et al.*, 2001).

An estimated 24.6 million pounds of antibiotics are used each year for non-therapeutic purposes (Mellon *et al.*, 2001). To achieve higher growth rates, low levels of antibiotics (e.g., 35-100 mg of bacitracin per head per day) are incorporated into animal feed to increase feeding efficiency (Khachatourians, 1998). Increasing feeding efficiency

can promote the rate of weight gain 3% to 5% more than without antibiotic additives (Khachatourians, 1998). By increasing feeding rates, food-animal products become more cost efficient for both the producer and the consumer, which creates an increase in food availability (Wegener, 2003; FDA, 2003). Because of the success of antibiotics as growth enhancers, the recommended levels of antibiotics in feed has increased from 5-10 parts per million to a much greater amount (10 to 20 fold) since 1950 (Khachatourians, 1998). Antibiotic agents such as bacitracin, chlortetracycline, erythromycin, lincomycin, neomycin, oxytetracycline, penicillin, streptomycin, tylosin and virginiamycin are added to poultry feed in the amount of 1-400g per ton of feed to promote growth (Khachatourians, 1998).

Campylobacter is a prime example of how overuse of antibiotics in agriculture can have a strong effect on human health. The rise of fluoroquinolone-resistant *Campylobacter* (Lipstich *et al.*, 2002) has sparked suggestions for updating antibiotic distribution procedures due to the bacteria's role in gastroenteritis (food poisoning). Fluoroquinolone antibiotics are commonly prescribed to treat human gastroenteritis and are becoming ineffective due to resistance promoted by its overuse in agriculture (Lipstich *et al.*, 2002). The FDA is attempting to improve distribution procedures and regulations in hopes of reducing resistance complications caused by the overlap of significant medical and live-stock antibiotics (FDA, 2003). The most recent proposed amendments to the *Guidance for Industry*, issued in April of 2012, will work to phase out non-therapeutic uses of antibiotics that are of human significance and require veterinary consultation when medically significant antibiotics are needed for agricultural use (FDA, 2012).

Increased resistance rates of fecal bacteria species in antibiotic-treated livestock have been demonstrated in many studies, including research in our own lab (Brooks, 2005). Brooks found significantly higher levels of resistance to tetracycline in bacteria isolated from the litter of poultry that had been treated with tetracycline, as well as soil to which the litter had been applied, than in litter from poultry that had not been treated with tetracycline. In addition to increased levels of resistance, studies have identified high levels of multi-drug resistance in many different species of bacteria found in poultry litter (Kelley *et al.*, 1998; Brooks 2005). For example, resistance profiles created by Kelley *et al.* (1998) from poultry litter identified a strain of *Aeromonas hydrophila* that was resistant to ampicillin, bacitracin, penicillin, tetracycline, and streptomycin.

Antibiotic resistant bacteria have not only been found in antibiotic treated animals but also the resulting food products after slaughter, the farm workers that are in close contact with the livestock, and even the surrounding soil, water and other environments (Hershberger *et al.*, 2004; Wegener, 2003; Kelley *et al.*, 1998). Resistant organisms found on food products and in farm workers could be a result of poor sanitation and quality control methods. A study by Oppegard *et al.* (2001) found an identical multi-resistant plasmid in fecal coliforms collected from human farm inhabitants and from the cows on their farm. The ~65 kb plasmid found in both cases also shared the same resistance phenotype, with resistance to ampicillin, tetracycline, streptomycin, trimethoprim, and sulfonamides (Oppegard *et al.*, 2001).

The occurrence of antibiotic resistant bacteria, genes, and plasmids in soil and streams may be in part due to the widespread practice of using the fecal waste of treated animals on pastures and cropland as fertilizer (Wegener, 2003; Brooks, 2005). Once

released into the environment, resistance genes may spread to native environmental bacteria via horizontal gene transfer (HGT), increasing the ability of the resistant characteristics to transmit to bacteria populations in water and crops with the potential to reach consumers (Götz & Smalla, 1997; Binh, C. T. T., 2008).

Sediment as a reservoir for the microbial community. Sediments found in streams, estuaries, and lakes are nutrient rich reservoirs that provide niches for a huge variety of microbial populations. In contrast to the stream water itself, with its constant flow and shifts in nutrient concentrations, stream sediment has the potential to maintain a more persistent indigenous community (Nealson, 1997). The total mass of bacteria in sediment is normally greater than that of the water column (Nealson, 1997). In addition to stable nutrient concentrations, sediment offers greater surface area and microhabitat opportunities in which many varieties of organisms attach and flourish.

E. coli is commonly used as an indicator of freshwater fecal pollution, at least in part due to its inability to survive for more than a few days in freshwater (USEPA, 2005; Götz & Smalla, 1997). However, a number of studies have indicated that *E. coli* can persist in stream sediments. In 1979, after several federal law changes aimed at improving environmental quality, an analysis of *E. coli* populations in the sediment compared to the water was made in order to determine the sources of contamination (Stephenson & Rychert, 1982). The study indicated that the *E. coli* concentration was 2 to 760 times greater in the sediment when compared to that in the water (Stephenson & Rychert, 1982). The persistence of *E. coli* in freshwater (Davies *et al.*, 1995; An *et al.* 2002) and marine (Craig *et al.*, 2004) sediments suggests that they may act as a source of water contamination (Jamieson *et al.*, 2002). Stream sediments may therefore be an

important reservoir of persistent fecal bacteria such as *E. coli*, which may then contribute via HGT to the pool of genes, including antibiotic resistance genes, available to native and transient stream bacterial populations (Byappanahalli *et al.*, 2003).

Horizontal gene transfer and antibiotic resistance. Horizontal gene transfer has been described as the main vehicle for the rapid spread of antibiotic resistance worldwide (Davies, 1996). Bacteria use HGT, the transfer of DNA between mature cells, and vertical transfer, transfer of DNA to progeny cells, to adapt to changing pressures and exploit new environments (Davison, 1999; Viridi & Sachdeva, 2005). HGT can readily occur across species boundaries with the potential of genetic exchange between pathogenic and non-pathogenic organisms (Dahlberg *et al.*, 1998; Shoemaker *et al.*, 2001). Transfer of a resistance gene from a non-pathogenic species to a pathogenic species that can express the genetic trait is one of the most concerning results of HGT. Horizontal gene transfer from one organism to another occurs primarily in three different ways, via transduction, transformation, and conjugation (Kelly *et al.*, 2009).

Transduction is a method of horizontal gene transfer mediated by viruses that infect bacterial cells. These bacteria infecting viruses are called bacteriophage (phage). As the phage infects the cells they transfer and facilitate integration of genetic material, including genes encoding antibiotic resistance or virulence, between different bacterial cells (Davison, 1998). Transduction occurs frequently in spatially confined areas where the phage can spread and come into contact with multiple host cells that offer desirable characteristics for integration and reproduction. These “desirable” characteristics, most commonly surface proteins that facilitate attachment, may be limited to a very specific genetic configuration, potentially limiting a suitable host down to a specific strain.

Bacteriophage-mediated HGT has played a major role in the evolution of bacteria species regardless of the previously described limitations (Brabban *et al.*, 2005). The genes encoding Shiga toxin production in the pathogenic *E. coli* O157:H7, is one example of a change in a species due to virulence factors obtained through transduction (Wick *et al.*, 2005).

Transformation is dependent on specific genes located on the chromosome that direct the uptake and integration of free-floating DNA (Lorenz & Wackernagel, 1994). This DNA can be chromosomal or extra chromosomal (i.e. plasmid) DNA, and does not require a living donor; in fact most free DNA is a result of cell lysis (Lorenz *et al.*, 1991; Lorenz & Wackernagel, 1994). The free floating genetic information in the environment accepted into the cell through transformation can come from non-living cells and from cells that are not closely related or even closely located in space or time to the recipient (Lorenz & Wackernagel, 1994; Ochman, 2000).

Competence, the ability of a cell to take up DNA via transformation, can be present all the time, at certain times in a bacterium's life cycle, or induced, depending on the species of bacteria (Davison, 1999). Induced competence in *E. coli*, for example, can be achieved in several ways including treatment with CaCl₂, EDTA, and electro-shocks (also called electroporation) (Davison, 1999; Lorenz & Wackernagel, 1994). *E. coli* has also been shown to be naturally competent under certain environmentally-relevant conditions (Baur *et al.*, 1996)

Described as the most direct path for the horizontal transfer of genes, the third HGT mechanism, conjugation, requires physical contact between cells to initiate genetic

exchange between donor and recipient. (Götz & Smalla, 1997; Top *et al.*, 1994; Davison, 1999). Unlike transformation where a competent recipient is the determining factor of successful genetic uptake, conjugation is driven by the genetic information held by the donor, and in fact is encoded by the plasmid itself. The transferable genetic traits need to be part of a genetic element that possesses the information for transmission or mobilization between cells.

Many mobile genetic elements, such as transposons and plasmids, have been shown to have a broad host range with the ability to spread genetic information between species and even between Gram positive and negative organisms (Kelly *et al.*, 2009). Transposons are mobile DNA sequences that have the ability to remove or replicate and then insert themselves into chromosomal or plasmid DNA. A plasmid is a replicable, extra-chromosomal element that exists separate from a cell's chromosome. While some plasmids do not conjugate at all, most are thought to be either "self-transmissible" or "mobilizable". Self-transmissible plasmids contain the information necessary to promote their own transfer from cell to cell, while mobilizable plasmids require the transmission functions of a co-occurring plasmid. Conjugation of plasmids is the most common route for naturally occurring HGT in the environment (Kelly *et al.*, 2009).

Even in the absence of selection, HGT has been found to contribute to antibiotic resistance seen in populations of bacteria native to streams and soils (D'Costa *et al.*, 2006; Herrick *et al.*, 2003). With the exception of a few observations of natural behavior, HGT studies have for the most part been performed under laboratory conditions rather than *in situ* (Kruse & Sørum, 1994). Even fewer studies have examined resistance plasmids specifically in the environment. Cultivation and sampling techniques are a

limiting factor in the understanding of the environmental plasmid population and their role in the horizontal transfer of resistance genes (Stevenson *et al.*, 2004).

Many studies have used polymerase chain reaction (PCR) to assess the presence of antibiotic resistance genes in soils and sediment (e.g. Götz *et al.*, 1996; Pei *et al.*, 2006). Using PCR to amplify resistance genes is a good technique for studying gene availability in the environment without cultivation. While this technique is important for understanding the availability of resistance genes, it gives very little detail about the origin or potential mobility of the gene. By isolating entire, replicable, antibiotic resistance plasmids from the sediment the transmission potential of the resistance genes can be further studied.

Isolation and study of plasmids. Plasmids have traditionally been studied by isolation from cultured organisms. This method of studying plasmids – sometimes termed “endogenous” plasmid isolation – is advantageous because it allows for easy cell manipulation and a means by which to create more plasmid DNA for study. However, more than 99% of bacteria are un-cultivable in the laboratory due to growth requirements such as nutrient availability or temperature (Kaeberlein, T., 2002). The uncultivable nature of most environmental bacteria species is therefore a major disadvantage to the study of plasmids using the endogenous isolation technique alone.

PCR analysis of soil has demonstrated a vast diversity of genetic material not previously identified through strictly endogenous methods (Götz *et al.*, 1996). Community DNA extraction and “exogenous” plasmid capture techniques are allowing

for broader analysis of the plasmid population without depending on the cultivability of the plasmid host (Götz *et al.*, 1996; Smalla *et al.* 2000).

Exogenous plasmid capture, developed by Fry and Day (1990), samples the plasmid population by allowing plasmids from the environment to horizontally transfer, by conjugation, into a known recipient (Fry & Day,1990). By employing the natural capability of conjugation, cultivable cells can then be used to isolate a greater population of plasmids. Using selective media, this technique can be used to identify plasmids possessing a target characteristic such as antibiotic resistance. This technique has been used and manipulated to sample from various different locations and populations including manure, soil, sludge, and stream sediment (Smalla *et al.* 2000; Top *et al.*, 1993; Dahlberg *et al.*, 1997).

Exogenous plasmid capture only isolates self-transmissible or mobilizable plasmids because the method is dependent on the donor's ability to initiate conjugation. To attempt to overcome or at least complement this limitation we have also attempted in this study to develop a new technique that will also sample the "total" plasmid population independent of culturing or conjugation techniques. The term "total" plasmid is used in our work to define all plasmids available in the environment. This includes endogenous and exogenously captured plasmids, and all other plasmids that cannot be isolated using these methods. In this "total" method, DNA is directly extracted from sediment, the plasmid DNA separated from chromosomal using an alkaline lysis technique developed in our laboratory, and then the plasmid DNA is electroporated into a recipient and selected on appropriate media.

The more researchers understand the limitations of culturing, the more novel techniques are being employed to collect plasmids directly from the environment. One way this is being attempted is by using transposons to capture enteric plasmids without culturing (Jones, B.V. & Marchesi, J.R., 2007). Developing a technique to isolate all plasmids available in the sediment without the prejudice of culturing could reveal the full potential of the “mobileome” of environmental plasmids.

It is hypothesized that the three plasmid isolation techniques discussed – “endogenous”, “exogenous”, and “total” – samples from a somewhat different and potentially overlapping plasmid population. The endogenous method identifies only those plasmids found in an organism at the time of extraction. The exogenous capture method samples the self-transmissible and mobilizable plasmid population that can transfer to and express selectable genes in a specific recipient bacterium. Total plasmid extraction from the sediment, without the use of a recipient as in the exogenous method, could potentially draw from the entire plasmid population (culturable, unculturable, transmissible, and, non-transmissible) able to replicate and express a selectable marker. Currently, there are no published works utilizing a plasmid extraction method for collecting entire, replicable, plasmids from the sediment without cultivation. Considering that each method represents different, but overlapping snapshots of the so-called “mobileome”, a comparison in the plasmids isolated using each method would potentially develop a more complete picture of environmental plasmids.

In this study, a comparison of plasmids isolated with the three plasmid isolation techniques – exogenous, endogenous, and total – was done to assess the diversity of the tetracycline resistant plasmid population in the sediment of an agriculturally impacted

stream. The presence of a persistent tetracycline resistant *E. coli* population in the stream sediment was confirmed prior to plasmid analysis. Endogenous and exogenous isolation techniques sampled from seemingly different plasmid populations and new methods were employed to sample total plasmids from sediment.

Materials and Methods

Study site. Water and sediment samples were taken at the Shull Farm, located on Shull Run, a tributary of Mountain Run and Smith Creek in Rockingham County, Virginia. This study site was chosen due to the lack of farming upstream of the impacted site, and the high level of fecal contamination opportunities provided by cattle crossing points in the stream and adjacent poultry litter storage areas.

The cattle at the Shull farm had continuous access to the stream for water and passage from field to field with a primary passage at the furthest downstream point of the farm. In addition to the opportunity for fecal material from the cattle to enter the stream, the fields were treated with poultry litter, a mixture of manure and poultry house bedding materials. The litter was purchased from local poultry houses and stored in a pile in close proximity to Shull's Run prior to being used as fertilizer for the cattle field. During rain events, runoff from the treated fields and the litter storage flowed into the stream.

Sample collection. Sediment and water samples were collected at two points, designated "upstream" and "downstream" throughout this study. Upstream samples were collected just above a fence marking the boundary of the Shull Farm property. The downstream samples were taken at the primary cattle passage point described above, which was approximately 200 yards below the upstream sampling site. Samples collected in September, October, and November of 2006 and January of 2008.

Samples were collected in sterile 50 ml Falcon® tubes. For sediment samples, areas of fine sediment on the stream bed below areas of moving water were identified. The top layer of sediment was brushed away by hand prior to sampling. An open

Falcon® tube was inserted into the water column open top down so trapped air would prevent collection of excess water prior to contact with the sediment. Tubes were filled with sediment by a scooping motion and any water collected with the sediment was poured off prior to transport.

Water samples were taken in moving water using sterile 50 ml Falcon tubes. Tubes with lids were immersed in the column and lids removed approximately 5 cm under the surface, to avoid surface water and particulates, and replaced under water. Hands were kept downstream of sample bottles. Samples were transported on ice to the laboratory and refrigerated until time of use if not processed immediately upon return. Samples were stored no longer than 24 hrs before processing.

Bacterial strains. *E. coli* K-12 was used as a positive control for BOX fingerprinting. *Pseudomonas putida* L1-9, a strain KT2442-based transconjugant strain created by James Brooks (2005) containing a single ~58kb tetracycline resistant plasmid, was utilized to confirm successful plasmid extraction. *E. coli* strain LA61 was used as the recipient in all exogenous plasmid isolations. LA61 is a plasmid-free, tetracycline-sensitive, rifampin-resistant, *E. coli* isolated from Great Lakes beach sand (un-published work performed by Brendan Galagher, 2007). Electrocompetent *E. coli* strain EC100 (Epicenter Technologies, Madison, WI) cells were used for electroporation. EC100 was maintained and prepared for electroporation according to protocol 6.1.1 of the Bio-Rad® Gene pulser Xcell electroporator instruction manual.

Maintenance and storage of bacterial strains and plasmids. Tetracycline resistant strains were cultured overnight in trypticase soy broth (TSB) amended with 8

$\mu\text{g/ml}$ of tetracycline. Five-hundred microliters of the culture was transferred to a cryotube containing 500 μl sterile glycerol and frozen in duplicate at -80°C . Tetracycline resistant isolates, including control strain L1-9, were maintained on TSA amended with 25 $\mu\text{g ml}^{-1}$ of tetracycline to prevent spontaneous plasmid curing. Non-resistant strains and K-12 were maintained on TSA plates. Strain EC100 was stored in 10% glycerol at -80°C in 200 μl aliquots to limit freeze/thaw events.

***E.coli* isolation from sediment and water.** For the isolation of *E. coli* from stream sediment, 2 g of sediment was added to 200 ml of 1% sodium pyrophosphate and vortexed vigorously for 5 to 10 seconds to release cells (Holben et al., 1988). The solution was allowed to settle at room temperature for 5 min before ten 5 ml aliquots were removed from the supernatant. Five milliliters of sterile phosphate-buffered saline (PBS, 80mM Na_2HPO_4 , 70mM KH_2PO_4 , 145 mM NaCl, pH7) were added to each aliquot to increase the volume to 10 ml for more efficient filtration.

For isolation of *E. coli* from water, 10ml, 1ml, and 0.1ml sub-samples were taken directly from sample tubes. PBS was added to 1ml and 0.1 ml samples to reach a volume of 10 ml for filtration. Samples were filtered through sterile 0.45 μm cellulose acetate filters (Millipore corporation, Billerica, MA). Filters were transferred to Modified mTEC Agar plates. Modified mTEC Agar was prepared as described in EPA Method 1603 (2002). To prepare 1 L of modified mTEC the following ingredients were added to 1 L of ddH₂O: 5.0 g proteose peptone #3, 3.0 g yeast extract, 10.0 g lactose, 7.5 g sodium chloride, 3.3 g potassium phosphate (dibasic), 1.0 g potassium phosphate (monobasic), 0.2 g sodium lauryl sulfate, 0.1 g deoxycholic acid (sodium salt), 0.5 g 5-bromo-6-chloro-3-indoyl- β -D-glucuronide, and 15.0 g granulated agar. The solution was heated until all

ingredients dissolved and then was autoclaved at 121°C for 15 minutes to sterilize. Agar plates with applied filters were wrapped in Whirl-Pak bags and sealed in plastic water tight containers. The containers were incubated in a 45°C water bath for 24hrs.

Colonies exhibiting a blue coloration indicative of the production of β -glucuronidase, – the enzyme produced by most *E. coli* strains during the fermentation of lactose – were enumerated and randomly selected isolates were streaked and incubated onto Eosin Methylene Blue agar (EMB) (Becton, Dickinson and Company, Sparks, MD). Those isolates with a green sheen after 24 hours of incubation at 37°C were tentatively identified as *E.coli*. *E. coli* isolates were incubated for 24 hours at 37° C on trypticase soy agar (TSA) for temporary storage and use. Isolates were verified by fluorescence in Colilert® broth (indicating β -glucuronidase production, an enzyme characteristic of *E. coli*). Identifiers were given to the isolates based on location and sample date (for example Sh-B2-21: Shull Run, Downstream, 2nd sample date, and a numerical identifier). Each confirmed *E. coli* isolate was plated on tetracycline (25 $\mu\text{g ml}^{-1}$) - amended TSA to identify resistant isolates.

Repetitive sequence PCR for strain differentiation. Isolates were differentiated and biotyped using the BOX-PCR repetitive sequence fingerprinting method described by Rademaker *et al.* 1998. All *E.coli* (resistant and non-resistant isolates) used for BOX-PCR were grown on unamended TSA. For BOX-PCR fingerprinting, an inoculating needle was used to transfer a small number of cells from colonies grown overnight on TSA broth to 10 μl of ddH₂O in a sterile 0.2 ml PCR tube. Cells were lysed in a MiniCycler (MJ Research, Watertown, MA) at 95°C for 5 min. Three microliters of crude lysate were added to separate 0.2 ml PCR tubes containing 5 μl of Premix G

(Epicentre Technologies) and 2 µl of PCR master mix. The PCR master mix contained 1.3 µl of BOX primer (13 pmol), 0.1 µl Failsafe enzyme mix (Epicentre), and 0.6 µl of sterile ddH₂O. The sequence of the BOX primer was 5'-CTACGGCAAGGCGACGCTGACG-3' (Sigma Genosys, Woodland, TX) (Rademaker *et al.* 1998). PCR was carried out at 95°C for 2 min and then for 30 cycles of 94° C for 3 s, 92°C for 30 s, 50°C for 60 s, with a final extension step at 65°C for 8 min. Samples were refrigerated until electrophoresis.

Ten microliters of amplified DNA were loaded into a 1% agarose gel with 2 µl of 6X loading dye and run at 60 V for 2.5 hours. Gels were stained with 0.5% ethidium bromide for 15-20 min and destained with ddH₂O. Photographs of gels were taken with a Kodak DC 290 digital camera (Kodak, New Haven, CT) and analyzed using Kodak 1D Scientific Imaging System v.3.5.4.

BOX-PCR “fingerprints” were compared by visual inspection and defining band patterns identified to differentiate between each biotype (Fig. 1). Biotypes were given arbitrary numbers in order of their identification. Only *E. coli* strains isolated from sediment for the purpose of extraction of endogenous plasmids were biotyped.

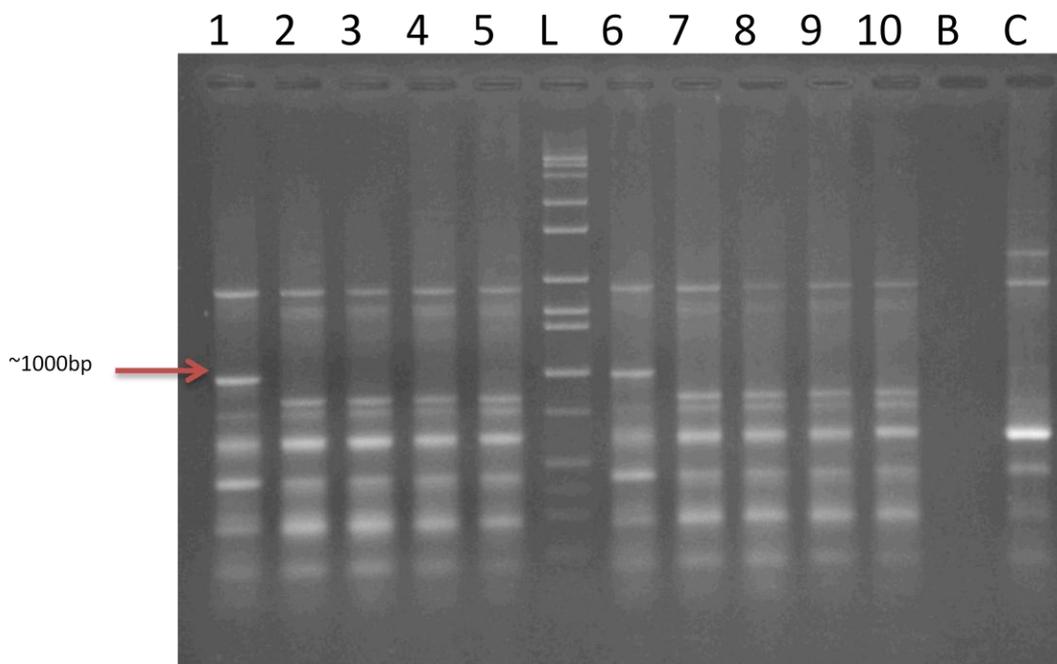


Figure 1. BOX-PCR fingerprint. “Endogenous” isolates run in duplicate, one set on either side of the Promega 1kb ladder (L). The endogenous isolates were run with the following lane assignments - Lane 1 and 6, pD-47; Lane 2 and 7, pD-72; lane 3 and 8, pD-90; Lane 4 and 9, pD-92, Lane 5 and 10, pD-95 -5. Arrow indicates a distinct ~1000bp band that differentiates biotypes 2 and 1. Lane B is blank and Lane C contains *E. coli* strain K-12 as a control.

Plasmid isolation. A plasmid preparation procedure developed in our laboratory for the isolation of large, native, single-copy plasmids was used to determine the presence of plasmids in tetracycline resistant (tet^R) *E. coli* isolates. Tetracycline resistant cells were cultured at 37 °C overnight in 10 ml of TSB. A 1.5 ml aliquot of turbid broth was centrifuged at 10,000 x g for 5 min and the supernatant was removed from the cell pellet by aspiration. The cell pellet was resuspended using 100 μ l of an alkaline resuspension buffer (10 mM EDTA; 50 mM dextrose; 10 mM Tris-Cl, pH 8.0). Two hundred microliters of 0.2 M NaOH/1% SDS was added to lyse the cells. The cells and SDS solution were mixed by inversion and allowed to set at room temperature for 5 minutes to

insure successful lysis. To denature the chromosomal DNA, 150 μ l of ammonium acetate was added to reduce pH. One hundred fifty microliters of chloroform was added to denature proteins. The mixture was centrifuged at 16,000 x g for 10 min and the supernatant removed and added to a 30% polyethylene glycol 8000/1.5 M NaCl solution to separate plasmid from chromosomal DNA. This was chilled on ice for 15 min and then centrifuged at 16,000 x g for 10 min. The supernatant containing chromosomal material was removed by aspiration and the remaining plasmid DNA pellet was reconstituted in 100 μ l of sterile ddH₂O. Plasmid DNA was stored at -20°C.

The presence of plasmid DNA was confirmed by agarose gel electrophoresis. Ten microliters of plasmid DNA mixed with 2 μ l of 6X loading dye was run on a 0.7% agarose gel at 90V for 2 hours. Gels were developed, photographed, and analyzed as described above.

For electroporation and restriction enzyme digestion, plasmid DNA was purified using a 1:1 phenol-chloroform extraction to remove residual proteins. The phenol-chloroform extraction was followed by an ethanol precipitation to concentrate plasmid DNA and remove residual salt that may inhibit electroporation. Plasmid DNA was precipitated by adding 2 volumes of cold (-20°C) ethanol and 5 μ l of 0.3 M sodium acetate. DNA was allowed to precipitate on ice for 20 minutes and then centrifuged at 16,000 x g for 10 min. The supernatant was removed by aspiration and the remaining DNA pellet was rinsed with 70% ethanol. The mixture was centrifuged for 2 minutes and the supernatant was removed by aspiration; care was taken to not disturb the (potentially) loose DNA pellet. The DNA pellet was allowed to air dry at room temperature until

residual ethanol evaporated; it was then resuspended gently in 100 μ l sterile ddH₂O.

Plasmid DNA was stored at -20°C.

Plasmids extracted from tetracycline resistant *E.coli* isolates collected from the sediment were considered “endogenous” to the sediment *E.coli* population. Once extracted from the endogenous isolate the plasmids were labeled by removing the “Sh” from the isolate name and replacing it with a “p” (example pB2-21)

Exogenous isolation of tet^R plasmids. Transmissible tet^R plasmids were captured using an “exogenous” method developed in our laboratory, modified from the method of Fry and Day (Fry & Day,1990). Cells were extracted from sediment samples to act as potential plasmid donors in a conjugation mating. Sediment was collected as described above. Ten grams of sediment were mixed with 90 ml of 0.1% sodium pyrophosphate and agitated for 40 sec. Sediment was allowed to settle at room temperature for 5 min. One milliliter of liquid was centrifuged for 10 min at 5,800 x g. The supernatant was removed and the remaining pellet was reconstituted with 1 ml of PBS and centrifuged for 10 min at 5,800 x g to remove remaining sediment. The supernatant was removed and the pellet was reconstituted on 500 μ l of PBS.

Recipient strain LA61 (tet^R, rif^R), was cultured overnight in TSB at 37°C with agitation (~170 RPM). One milliliter of the culture was centrifuged and washed with PBS in the same manner as the donor cells from the sediment. The resulting washed pellet was reconstituted in 500 μ l of PBS.

The 500 μ l volume of recipient cells was added to the centrifuge tube containing the 500 μ l volume of potential donor cells. Cells were gently mixed by inversion for 20

seconds. Two hundred microliters of donor/recipient cell mixture was transferred onto a sterile 0.45 μm cellulose acetate filter applied to a TSA plate. (Millipore Corporation, Billerica, MA). The cells were allowed to conjugate overnight, incubated at 37 °C. After incubation the filter was aseptically removed from the TSA plate and placed in a 50 ml Falcon® tube. Ten milliliters of PBS was added to the tube with the filter and vigorously vortexed for three minutes to release cells from the filter.

Two hundred microliters of the supernatant was pipetted onto TSA plates amended with rifampicin (50 $\mu\text{g ml}^{-1}$) and tetracycline (25 $\mu\text{g ml}^{-1}$) to select for transconjugants. Donors and recipients were individually plated onto plates containing rifampicin (50 $\mu\text{g ml}^{-1}$) and tetracycline (25 $\mu\text{g ml}^{-1}$) as negative controls (and to account for spontaneous rifampicin resistance), and onto TSA as a positive control. Putative transconjugants were verified as the recipient host strain by BOX rep-PCR fingerprinting as described above.

Plasmids from the transconjugants were extracted using the plasmid preparation technique described above and electroporated into a control recipient, EC100, to isolate single plasmids and to verify plasmid-borne tetracycline resistance.

Electroporation of tet^R plasmids. Plasmid DNA was electroporated using a Bio-Rad® Gene Pulser Xcell. Electroporation was carried out as described in the Bio-Rad® Gene Pulser Xcell instruction manual. Plasmid DNA, precipitated with ethanol to remove residual salt, was electroporated into the electrocompetent tetracycline susceptible (tet^S) *E. coli* strain EC100. Cells were plated on tetracycline (25 $\mu\text{g ml}^{-1}$) amended TSA plates. Plates were incubated for 48 hours at 36°C. Colony formation was considered indicative

of successful transformation. Electroporated transformants were used for antibiotic phenotyping. Plasmids from the tet^R transformant isolates were isolated using the plasmid preparation protocol listed above for classification and comparison by restriction digest.

Extraction of “total” tet^R plasmids from sediment. Five gram samples of sediment, collected from the study site, were added to four flasks, two containing 100 ml of 5% PTYG broth (0.25 g/L peptone, 0.25 g/L tryptone, 0.25 g/L yeast extract, 0.5 g/L glucose, 0.6 g/L MgSO₄, 0.07 g/L CaCl₂-2H₂O) and two containing 100 ml of a sterilized manure broth. Manure broth was made in the lab from cow manure collected from a deposit at the cattle crossing at the Shull farm. One hundred milliliters of cow manure / stream water slurry was thoroughly mixed with one liter of ddH₂O and filtered through doubled cheese cloth to remove large particles. The manure broth was autoclaved for 20 min to sterilize. Flasks were incubated with agitation at 24°C and one of each type (manure and PTYG medium) removed at 12 hours and at 24 hours and placed in refrigeration until samples could be processed. All samples were processed within 24 hours of removal from incubation.

For plasmid extraction, sediment was allowed to settle from the enrichment broth. In an attempt to increase the number of cells collected from each enrichment flask, cells suspended in the broth were combined with cells released from the sediment using the following technique. Forty five milliliters of broth from each of the four flasks was added to four 50 ml centrifuge tubes. Tubes were centrifuged at 7,000 x g for 5 min and supernatant was removed by aspiration. The remaining volume of enrichment broth from the flasks was added to the 50 ml centrifuge tubes containing the pellet from the first centrifugation. The second volume of enrichment broth was centrifuged and the

supernatant was removed using the same method as the first volume. After all of the broth was removed, 100 ml of 0.1% sodium pyrophosphate was added to sediment remaining in each flask. Flasks were agitated and allowed to settle, as described previously, to release cells from the sediment. Supernatant containing the released cells was removed and added to centrifuge tubes containing pelleted cells from respective broth cultures. The released cells were processed in two volumes using the same method as the broth. The resulting cell pellets, a combination of cells from the enrichment broth and cells released from the sediment, were resuspended in PBS and centrifuged at 7,000 x g for 5 min to aid in the removal of residual sediment. The supernatant was removed by aspiration.

The previously described plasmid preparation process was modified to accommodate the large pellet that resulted from the above cell extraction technique. Five hundred microliters of resuspension buffer was added to each pellet and vortexed until resuspended. One milliliter of SDS was added to the cells, mixed, and allowed to set at room temperature for 5 min. Each SDS/cell solution was divided into five 1.5 µl centrifuge tubes (approximately 300 µl of mixture per tube) for plasmid extraction as described in the previous paragraph. Resulting cell pellets were thoroughly reconstituted in 100 ml of ddH₂O and all five volumes were combined into one tube. Ethanol precipitation was used to concentrate plasmid DNA and remove any remaining sediment. DNA was electroporated using the method identified above and electrocompetent strain EC100. Cells were recovered on tet-amended (25 µg ml⁻¹) TSA plates. Resulting tet-resistant transformants were cultured for antibiotic phenotyping and plasmid prepped for restriction digestion.

Antibiotic susceptibility phenotyping. Resistance phenotypes for the antibiotics tetracycline (30µg), gentamicin (10 µg), ciprofloxacin (5 µg), trimethoprim / sulfamethoxazole (23.75 / 1.25 µg), imipenem (10 µg), tobramycin (10µg), kanamycin (30 µg), aztreonam (30 µg), ticarcillin (75 µg), piperacillin/ tazobactam (100 / 10 µg), piperacillin (100 µg), and cefepime (30 µg) were assessed using a Stokes disk diffusion assay (Acar & Goldstein, 1996) as modified by Brooks for assaying conjugated plasmid resistance (Brooks, 2005) . Recipient EC100 control and tet^R transformants resulting from electroporation were grown overnight in TSB. Sterile swabs were used to apply turbid broth cultures to Mueller-Hinton agar plates (Becton Dickinson). Mueller-Hinton plates were divided into thirds. Transformants were swabbed on the outer sections of the plate and the empty recipient control was swabbed in the center third (Fig. 2). Antibiotic diffusion disks were applied along the line where the control and transformant met (Fig. 2). Plates were incubated for 24 hours at 30 °C.

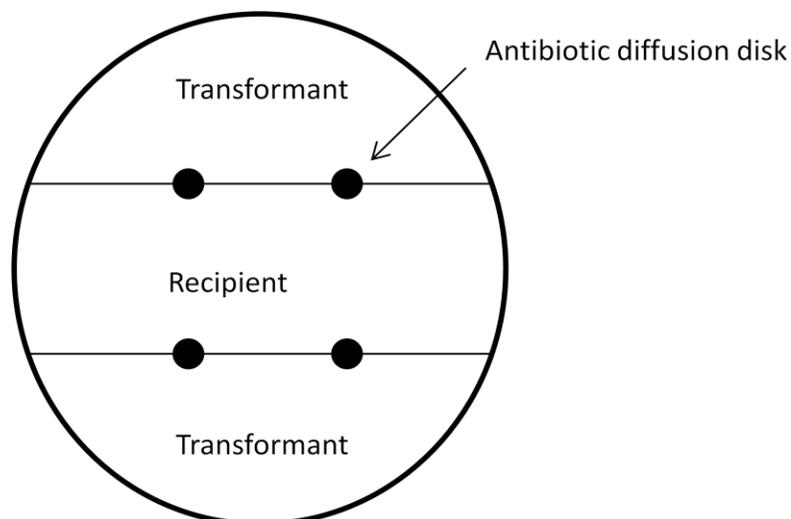


Figure 2: Modified Stokes Method for the assesment of antibiotic resistance in electroporated plasmids. Transformants and empty recipients (control) are applied to Mueller-Hinton media. Antibiotic diffusion disks are then placed at the point at which the transformants and recipients meet. Resulting zones of inhibition of the transformants and the recipient are compared to dtermine if the plasmid in the tranformant encodes resistance to the antibiotic in the diffusion disk. A reduction of ≥ 2 mm in the radius of the zone of inhibition was designated as “resistant” to the antibiotic.

The radii of clearing zones from the control side and the transformant side of the disks were manually measured and compared. A ≥ 2 mm reduction in the radium of the zone of inhibition was considered resistant to each antibiotic.

Differentiation of tet^R plasmids by restriction digest. tet^R plasmids extracted from electroporated transformants were sequentially digested using three restriction enzymes, EcoRI, BamHI, and HinDIII (Promega Corporation, Madison, WI). Three digestions were prepared per plasmid DNA sample, one for each of the three enzymes. Five microliters of sterile ddH₂O, 2 μ l of RE 10x Buffer (Promega), 2 μ l of acetylated BSA, and 10 μ l of plasmid DNA were added to separate 0.2 ml tubes. One microliter of enzyme, EcoRI, BamHI, or HinDIII, was added to the solution and mixed gently by

pipetting and a 10 sec centrifugation. Digests were incubated in a 37 ° C water bath for 2.5 hours.

Four microliters of 6x loading buffer were added to each 20 µl sample and the entire 24 µl sample was loaded into a 1% agarose electrophoresis gel. The gel was run at 90 V for 2 hours. Gels were developed and photographed as described above.

Data analysis. The diversity of water and sediment *E. coli* populations were compared using BOX fingerprint patterns. Visual interpretation was used to differentiate fingerprint patterns. Different patterns were given a type number for identification. The Shannon-Weaver index (Shannon, C.E. & Weaver, W., 1949) was used to evaluate the diversity of fingerprint types in both the water and sediment populations. A population's Shannon-Weaver diversity (H) value, based on the number of different individuals in a population, versus the maximum chance of diversity (H_{max}), calculated by assuming every individual is different, defines the level of diversity (D). The closer the sample population's H value is to the maximum possible H value, the greater the level of diversity.

The occurrence of resistance in downstream versus upstream *E. coli* isolates was compared using a Chi-square analysis. The expected value of resistance was calculated by assuming an equal number of resistant and susceptible isolates would be found in both locations. Values were compared by assuming 3 degrees of freedom based on the number of parameters utilized in the Chi-square. A probability value (*p*-value) was calculated based on the result of the Chi-square to show the level of significance between the expected and the actual findings. The number of cells collected from the sediment

upstream and downstream locations were also compared using a Chi-square. Equal distribution of cells upstream and downstream was assumed to calculate the expected value and 1 degree of freedom was utilized when determining the p -value.

Results

Enumeration of *E. coli* from sediment and water. To test the hypothesis that sediment contained more *E. coli* than water, on a weight per volume basis, samples were collected once a month for three months from locations upstream and downstream of the Shull farm. The sediment had a consistently higher number of *E. coli* colony forming units (CFU) than the water for each sample time and location. Assuming an even distribution of CFUs upstream and downstream of the farm in a Chi-square analysis, the upstream sediment samples contained significantly fewer CFUs than the downstream location ($p = 0.0001$) (Table 1).

Table 1. Enumeration of *E. coli* collected from Shull's Run over three consecutive months

Source	September 2006		October 2006		November 2006	
	Upstream	Downstream	Upstream	Downstream	Upstream	Downstream
Sediment (CFU/1g) ^a	204	330	10	134	38	380
Water (CFU/ 1ml) ^b	0.72	0.22	0.60	0.20	0.70	0.52

^a Upstream and downstream sediment samples were compared using Chi-square analysis. Expected values were calculated assuming even distribution between the two locations. The resulting p -value = 0.0001, indicating a significant difference in number of CFUs isolated upstream versus downstream.

^b Original data was measured by CFU/100ml because of the low level of *E. coli* found in the water. The values have been adjusted to CFU/1ml to make it comparable to the 1g sediment sample.

Biotype differentiation of sediment and water isolates. Sediment and water *E. coli* isolates were differentiated and their diversity assessed using rep-PCR amplification with a BOX primer, that amplifies intervening regions between repetitive BOX sequences (Sigma Genosys, Woodland, TX) (Rademaker *et al.* 1998). The resulting BOX rep-PCR banding patterns were used to assign a biotype to the isolates. Seventy-six water isolates

(unpublished data, Brandon Kocher, James Madison University 2008) and 139 sediment isolates were randomly selected for biotyping resulting in 46 different biotypes of *E. coli* in the water and 9 biotypes in the sediment.

The diversity of each *E. coli* population was compared using the Shannon-Weaver diversity index. The observed diversity of the water isolates was only 5.74% less diverse than the H_{\max} , (H_{\max} assumes all isolates are different). Singleton biotypes were frequently found in the water population. Conversely, multiple individuals of the same biotype were found more frequently in the sediment. The observed diversity of the isolates found in the sediment was 26.03% lower than the H_{\max} , suggesting that the sediment population is less diverse than the population in the water.

Although a much larger number of different biotypes were identified in the water, there were several incidences of overlapping types between the two substrates. The two most common biotypes found in the sediment, biotype-1 and biotype-8, were also identified in the water.

Biotype persistence in the sediment. Sediment biotypes from three consecutive months, September, October, and November of 2006, and an additional sample, taken in January of 2008, were compared (Table 2) to determine if biotypes persisted over time. Isolates from both the upstream and downstream locations were biotyped in September 2006 whereas only downstream isolates were biotyped from the other sampling dates.

Table 2. Number and distribution of BOX rep-PCR *E. coli* biotypes found in agriculturally impacted stream sediment collected from Shull's Run

Biotype	<i>September 2006</i> ^a		<i>October 2006</i> (n=12)	<i>November 2006</i> (n=28)	<i>January 2008</i> (n=23)
	<i>Upstream (n= 44)</i>	<i>Downstream (n=55)</i>			
1	36(82%)	25(45%)	-	-	-
2	-	10(18%)	-	-	-
3	4(9%)	6(11%)	-	-	21(91%)
4	1(2%)	9(16%)	-	-	-
5	1(2%)	-	-	-	-
6	1(2%)	-	-	-	-
7	1(2%)	-	-	-	-
8	-	5(9%)	12(100%)	3(11%)	-
9	-	-	-	25(89%)	-
10	-	-	-	-	2(9%)

^aDashes (-) indicate when a biotype was not identified on a sample date.

Isolation of tetracycline resistant *E. coli*. Sediment *E. coli* isolates sampled over three consecutive months (September, November, and October of 2006) were plated on TSA amended with 25 $\mu\text{g ml}^{-1}$ of tetracycline to enumerate those exhibiting resistance. At all three sampling times, there were significantly more tetracycline resistant isolates found downstream of the Shull Farm compared to the upstream “non-impacted” sample location (Table 3).

Table 3. Tetracycline resistant and susceptible *E. coli* isolates found upstream and downstream of Shull's Farm

	<i>September 2006</i> (n=139)		<i>October 2006</i> (n=68)		<i>November 2006</i> (n=99)	
	Upstream	Downstream	Upstream	Downstream	Upstream	Downstream
Susceptible	44	43	5	18	7	1
Resistant ^a	7	45	0	45	0	91

^a Resistance was defined as the ability to grow on TSA amended with 25 µg ml⁻¹ of tetracycline. The incidence of tetracycline resistant isolates was significantly higher in September downstream sediment samples than upstream samples (Chi-square test, $p = 0.0002$).

Plasmid borne tetracycline resistance. Tet^R isolates were biotyped and the plasmid DNA extracted using a large plasmid isolation method developed in our laboratory (“Endogenous” isolation; see methods). All isolates contained at least one plasmid and many had multiple plasmids (Figure 3A). Plasmids were electroporated into a tet^S control strain. Transformants were grown on tetracycline amended plates (25 µg ml⁻¹) to determine if a plasmid carrying the tetracycline resistant characteristic was transferred. It was assumed that growth on the tetracycline amended media was indicative of plasmid conferred tetracycline resistance rather than from genes integrated into the chromosome. All tet^R isolates had tetracycline resistance stemming from large, ~60kb, plasmids (Figure 3B). Once isolated, resistance to multiple antibiotics and restriction digest patterns were determined.

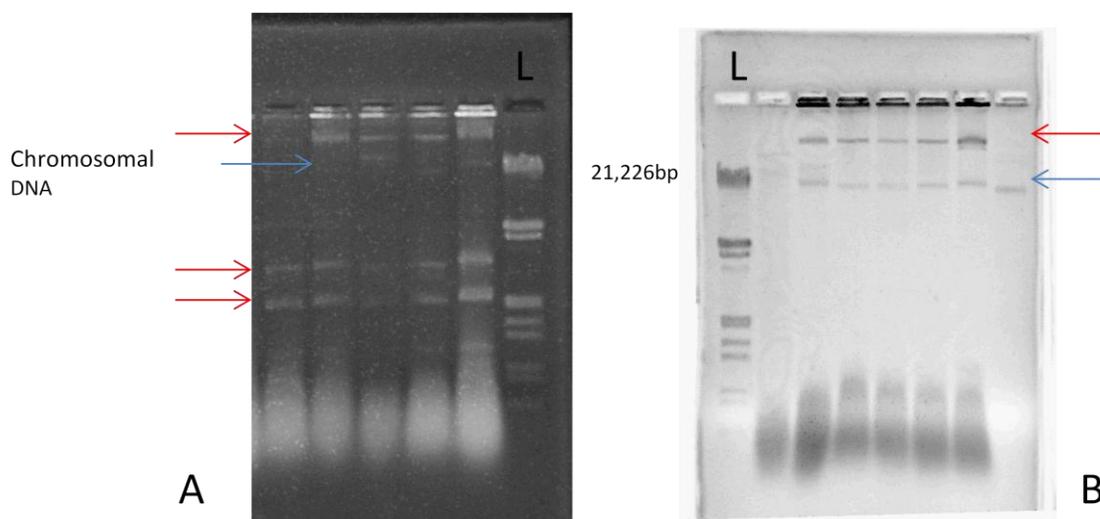


Figure 3. A. Example of plasmids extracted directly from stream sediment *E. coli*. Red arrows indicate plasmids and the blue arrow chromosomal DNA (~21kb). B. Example of plasmids extracted from electroporated transformants selected for on tetracycline amended plates. The red arrow indicates the tet^R plasmids and the blue arrow indicates residual chromosomal DNA.

Exogenous isolation of tetracycline resistant plasmids. Isolation of transmissible tet^R plasmids from the sediment was achieved using an exogenous plasmid capture method. This method captures transmissible plasmids into a control recipient without the requirement to culture the donor cells. Cells were extracted from two sediment samples, taken from the downstream location of the Shull Farm were combined with a rifampin resistant, tetracycline susceptible, control cell, LA61. The two samples were taken five months apart, the first sample taken in October 2006 and the second sample was taken in March of 2007. The two samples yielded a total of 44 transconjugants exhibiting resistance to tetracycline.

All cells capable of growing on plates amended with both rif and tet were verified as transconjugated LA61 using BOX rep-PCR. Positive and negative controls were employed to verify conjugation. LA-61 was verified as rifampin resistant and tetracycline

susceptible on a TSA plate amended with rifampin and a TSA plate amended with tetracycline, respectively. Cells extracted from the sediment were plated on rifampin-amended TSA to ensure there were no environmentally occurring rif^R isolates. Sediment isolates were plated on tetracycline-amended plates to screen for the presence of tet^R donors.

Thirty of the conjugated plasmids were electroporated into a tet-susceptible electrocompetent host cell, *E. coli* EC100. The presence of a single tet^R plasmid in each electroporated cell was verified by plasmid extraction and electrophoresis. Restriction digest and the modified Stokes test for assessing resistance to multiple antibiotics (Figure 2) were performed on each electroporated exogenous plasmid. Restriction digest patterns and resistance phenotypes were used to differentiate the tet^R plasmids (Table 5).

“Total” Plasmid extraction from sediment. To completely eliminate the need for any enrichment of cells containing plasmids, different techniques were employed to sample the total plasmid population in the sediment. Sediment samples from the “downstream” sample site were enriched at 24°C for 12 and 24 hours in PTYG and 12 and 24 hours in manure broth. Plasmid DNA from each enrichment was extracted and quantified (Table 4).

Table 4. Concentration and electroporation results of plasmid DNA extracted directly from enriched stream sediment.

Enrichment Method	DNA ng/ μ g	Tet ^R Electroporated Transformants (CFUs)
12 hour PTYG	2986	0
24 hour PTYG	2823	6
12 hour Manure Broth	0	arc ^a
24 hour Manure Broth	327	arc ^a

^a An electrical arc occurs when the electroporation substrate contains a contaminant that interrupts the conductivity of the electrical charge. Multiple unsuccessful attempts were made to complete electroporation of the samples that resulted in arcs.

Positive controls confirmed the presence of cultivable tet^R organisms in the sediment sample. An *E.coli* culture (K-12) was grown under optimal conditions in lab created manure media to ensure the media would promote a similar amount of growth as PTYG. This test was performed as part of the comparison of enrichment techniques on the isolation of total plasmids. Both the manure broth and PTYG yielded $\sim 6 \times 10^7$ CFUs/ml after a 24 hour incubation time at 37°C.

Two of the six successfully electroporated plasmids were characterized by antibiotic resistance profiling and restriction digest (Table 5).

Antibiotic susceptibility phenotyping. Plasmids exhibiting tetracycline resistance isolated using each of the three plasmid extraction techniques (“endogenous”, “exogenous”, and “total”) were assessed for resistance to multiple antibiotics using the modified Stokes disk diffusion assay (Fig. 2). Electroporated transformants were used in order to verify that resistance was due to plasmid-borne genes, as well as to rule out resistance due to a non-tet^R plasmid. Thirty-seven plasmids extracted from sediment *E.*

coli (endogenous plasmids), 30 exogenously captured plasmids, and two plasmids extracted directly from the sediment (“Total” plasmid isolation) were evaluated (Table 5).

Table 5. Classification and antibiotic resistance profiles of plasmids collected by endogenous, exogenous, and total plasmid isolation techniques.

Plasmid	<i>E. coli</i> host		Resistance Phenotype ^c
	Biotype ^a	Plasmid Type ^b	
<u>Endogenous^d</u>			
pU-30	6	9	TE
pU-44	1	10	TE, TIC
pU- 81	1	1	TE
pU-87	1	1	TE
pU-100	1	10	TE, NN, TZP
pD-3	1	1	TE
pD-33	2	1	TE
pD-46	1	2	TE
pD-47	8	2	TE
pD-72	2	1	TE
pD-78	8	2	TE
pD-84	8	2	TE
pD-90	2	1	TE
pD-92	2	1	TE
pD-95	2	1	TE
pD2-2	8	3	TE
pD2-12	8	3	TE
pD2-21	8	2	TE
pD2-29	8	2	TE
pD2-40	8	4	TE
pD2-65	8	2	TE
pD2-66	8	2	TE
pD3-1	9	10	TE
pD3-4	9	10	TE
pD3-11	8	2	TE
pD3-21	9	8	TE, K
pD3-26	9	8	TE
pD3-42	9	10	TE
pD3-48	9	10	TE
pD3-64	9	8	TE
pD3-84	9	8	TE
pD3-88	9	10	TE
pD4-2	3	6	TE
pD4-11	3	6	TE

Table 5 Continued.

Plasmid	<i>E. coli</i> host Biotype ^a	Plasmid Type ^b	Resistance Phenotype ^c
<u>Endogenous</u>			
<u>Continued</u> ^d			
pD4-14	3	6	TE
pD4-19	3	7	TE
pD4-20	3	6	TE
<u>Exogenous</u> ^d			
Ex1-1	-	9	TE, NN, K, TIC, TZP, PIP, FEP
Ex1-2	-	9	TE, NN, K, TIC, TZP, PIP, FEP
Ex1-6	-	9	TE, NN, K, TIC, TZP, PIP
Ex1-7	-	9	TE, K, TIC, PIP
Ex1-8	-	9	TE, K, TIC, PIP
Ex1-9	-	9	TE, K, TIC, TZP, PIP
Ex1-10	-	9	TE, K, TIC, PIP
Ex1-11	-	9	TE, K, TIC, PIP, FEP
Ex1-12	-	9	TE, K, TIC, PIP
Ex1-13	-	9	TE, K, TIC, PIP
Ex1-14	-	9	TE, CIP, K, TIC, PIP
Ex1-15	-	9	TE, K, TIC, PIP, FEP
Ex1-16	-	9	TE, K, TIC, PIP
Ex1-17	-	9	TE, K, TIC, PIP
Ex1-18	-	9	TE, K, TIC, PIP
Ex1-19	-	9	TE, K, TIC, PIP
Ex1-20	-	9	TE, CIP, K, TIC, PIP
Ex1-21	-	9	TE, CIP, K, TIC, PIP
Ex1-24	-	9	TE, CIP, K, TIC, PIP
Ex2-1	-	11	TE
Ex2-2	-	11	TE, AZT, FEP
Ex2-4	-	11	TE, FEP
Ex2-5	-	11	TE
Ex2-9	-	11	TE, TIC
Ex2-10	-	11	TE
Ex2-13	-	11	TE
Ex2-14	-	11	TE
Ex2-16	-	11	TE, AZT

Table 5 Continued.

Plasmid	<i>E. coli</i> host Biotype ^a	Plasmid Type ^b	Resistance Phenotype ^c
<u>Exogenous Continued^d</u>			
Ex2-17	-	11	TE
Ex2-19	-	13	TE
<u>Total^d</u>			
pTotal-1	-	12	TE
pTotal-6	-	12	TE, CIP, TIC

^a The host biotype is based on the differentiation of strains by a BOX repetitive sequence PCR fingerprint.

^b Plasmids identified grouped based on the restriction digest patterns of three enzymes, EcoRI, BamHI, HinDIII.

^c Tetracycline (TE), gentamicin (GM), ciprofloxacin (CIP), trimethoprim / sulfamethoxazole (SXT), imipenem (IPM), tobramycin (NN), kanamycin (K), aztreonam (AZT), ticarcillin (TIC), piperacillin/tazobactam (TZP), piperacillin (PIP), and cefepime (FEP).

^d Endogenous plasmids (p) are identified with a U (upstream) or D (downstream) to indicate sample location with a number to indicate sample time followed by a numerical indicator. Exogenous (Ex) plasmids are separated by sample time with a 1 or 2 followed by a numerical identifier. Total plasmids (p) are named by sample technique followed by a number.

Tetracycline resistance was confirmed in all plasmids. Decreased susceptibility to antibiotics other than tetracycline was conferred by only three of the 37 endogenous plasmids. However, twenty-three of the 30 exogenously captured plasmids exhibited decreased susceptibility to multiple antibiotics. Decreased susceptibility to seven antibiotics (tetracycline, tobramycin, kanamycin, ticarcillin, tazobactam, piperacillin, and cefepime) was identified in a single “exogenous” plasmid (Table 5). One of the two plasmids taken directly from the sediment (“total” plasmid extraction) had reduced susceptibility to multiple antibiotics (Table 5).

Restriction digest analysis of tetracycline resistance plasmids. Restriction digestion of plasmids using three different restriction endonucleases was used to group the plasmids according to similarity of plasmid “backbone”, as identified by the digest banding. Commonalities of the banding patterns were used to group the plasmids in the same “plasmid type”. Table 5 lists the plasmid identifications based on this plasmid “backbone”. Since most endogenously isolated plasmids only carried tetracycline resistance, the digest information was used to distinguish plasmid types.

Plasmids isolated endogenously had a greater diversity of plasmid types than those isolated exogenously: ten different plasmid types were observed in the endogenously isolated plasmids and only 3 types in the group isolated exogenously. Endogenous plasmid types were not specific to either the biotypes of the isolates they originated from or sample date. Conversely, exogenous plasmid types isolated were different depending on collection date. One plasmid type isolated endogenously was also seen in the exogenously capture plasmid group.

Discussion

The overuse of antibiotics in agriculture creates selective pressure that increases the incidence of resistance genes in streams. These genes can then be horizontally transferred between transient fecal and environmental organisms. This study used three different techniques to isolate and study stream sediment plasmids carrying resistance genes to illuminate the potential and diversity of plasmids in the environment. Differences between endogenously isolated and exogenously captured tet^R plasmid populations were identified and new techniques were applied in an attempt to assay a larger proportion of tet^R plasmids capable of replicating in *E. coli*.

***E. coli* in stream sediment.** Before investigating the plasmid population it was necessary to understand the ability of stream sediment to support an *E. coli* population and potentially provide a suitable setting for HGT. Stream sediment provides properties for supporting microbial life (surface area, nutrients, etc.) that a moving water environment cannot sustain. Moving water, on the other hand, has the potential to circulate organisms and genetic material between farm land, sediment, and other bodies of water.

Downstream of the Shull farm, the number of sediment *E. coli* and the incidence of resistant *E. coli* were greater than what was found upstream of the farm (Table 3). Spreading of poultry manure on adjacent banks in conjunction with rain events and direct fecal deposition by cattle in and along the stream may have increased the numbers of *E. coli* and tetracycline resistant genes available at the downstream location.

A greater number of cultivable *E. coli* was found in the stream sediment than in the water (Table 1). The biotypes of the isolates found in the sediment were less diverse than what was recovered from the water. This less diverse population in the sediment suggests that certain strains are more successful in that niche, which differs greatly from that of the water column. The higher overall numbers may mean that those biotypes that are more adapted for survival in sediment are stable enough to replicate. And, that would suggest that those *E. coli* strains adapted to the sediment may persist longer than do those in the moving water column. Indeed, our data shows similar biotypes in sediment samples taken months apart (Table 2).

Proximity to genetic material and time to access and incorporate that genetic material is necessary for successful horizontal gene transfer (HGT) (Lorenz & Wackernagel, 1994). The apparent stability of the *E. coli* population in the sediment over time (Table 2) suggests that the sediment is more conducive to supporting HGT than water. Because of this, stream sediment was the environmental medium used to assess the tet^R plasmid population in this study.

Plasmid diversity identified by three isolation techniques. Tetracycline resistance in all sediment *E. coli* isolates was encoded by large plasmids (larger than 23 kb). Similarly, the tet^R plasmids exogenously captured and the total tet^R plasmids isolated directly from the sediment were all about the same size as the endogenous plasmids. Although these plasmids were all similar in size, 10 different backbone types, as defined by restriction digestion, were identified among the endogenous plasmids, 3 different backbones were defined in the exogenous plasmids, and an additional backbone was found in the total plasmids.

Restriction digestion is probably a very conservative way to differentiate plasmids, as it samples only a small proportion of all the nucleotides on a plasmid. Therefore plasmids with the same restriction profile might differ considerably outside the specific restriction enzyme target sequences. It is likely that those plasmids with identical restriction digests, and antibiotic resistance phenotypes, coupled with the fact that they were isolated in close spatial and temporal proximity in this study, were probably closely related.

Several interesting patterns and correlations were identified between plasmid backbones from different origin biotypes, sample dates, sample locations, and isolation methods. The endogenously isolated plasmid backbone types did not correlate with the BOX-rep PCR biotypes of their host cell. Most biotypes supported more than one type of tet^R plasmid. Plasmids were also not specific to collection time of the isolates that they were extracted from. Plasmid backbone 2 was identified in *E. coli* from each sample from September, October, and November (Table 5) suggesting either that the plasmid persisted in the sediment *E. coli* population over time or was being repeatedly replenished from an external source such as fecal-contaminated stream water. Additionally, there were two different plasmid backbones that were found in both the upstream and downstream locations. This was somewhat unexpected as it was assumed that the plasmid DNA at the downstream location would be representative of the fecal influx of the farm and therefore different than *E. coli* plasmids from the comparatively pristine upstream location.

Two tet^R plasmids were isolated using an alkaline lysis protocol from total DNA extracted from a 24 hour PTYG enrichment. These were electroporated into *E. coli*. The

two tet^R plasmids that were successfully electroporated had the same plasmid backbone. This backbone pattern was, however, different from plasmids isolated with the other two techniques, which may indicate that this method accesses a different collection of environmental plasmids from the endogenous and exogenous plasmid isolation methods.

Comparison of resistance phenotypes of sediment plasmids. A modified Stokes disc-diffusion assay was used to develop a resistance profile for plasmids from each of the isolation techniques (Acar & Goldstein, 1996, modification by Brooks, 2005). All but three plasmids endogenously extracted possessed resistance to only tetracycline (Table 5). Two of the three multi-resistant endogenous plasmids were isolated from the upstream sample location. This was surprising considering there has been apparently little or no agricultural influence upstream of that sample location. One endogenous plasmid, plasmid backbone type 9, isolated from the upstream sample site, was also identified through exogenous plasmid capture at the downstream location. Interestingly, when found endogenously the isolate possessed resistance to only tetracycline and when the same plasmid type was found exogenously the plasmid backbone type had resistance to up to seven antibiotics. This could suggest that as this plasmid, containing singular resistance, traveled through the farm, additional resistance genes could have been picked up along the way.

The difference in this single common plasmid backbone was similar to what was found when the resistance profiles of all endogenous and exogenous plasmids were compared (Figure 4). Only 8% of the endogenous plasmids versus 77% of exogenous plasmids were resistant to more than one antibiotic. One interesting aspect of this finding is that, since the majority of plasmid studies to date have been based on endogenous

approaches (Kelly *et al.*, 2009), we may be seriously underestimating the extent of multi-resistance in stream sediments.

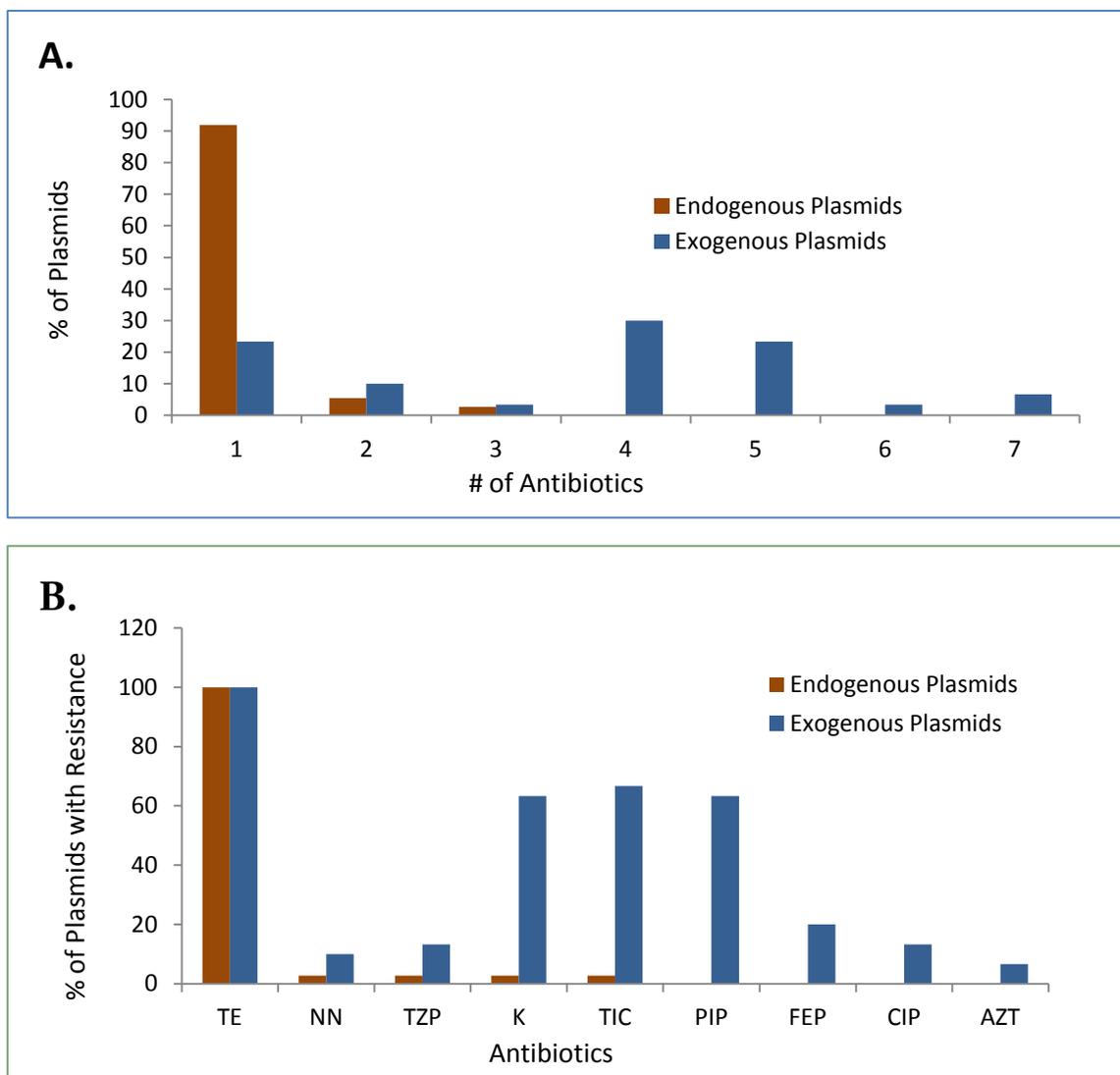


Figure 4. A. Resistance Phenotypes of Plasmids Collected from Stream Sediment. Comparison of occurrence of multiple resistances from endogenously isolated and exogenously captured plasmids from the sediment. **B. Antibiotic Resistance Found on Plasmids Collected From Stream Sediment.** Percent of plasmids carrying resistance to Tetracycline (TE), tobramycin (NN), piperacillin/ tazobactam (TZP), piperacillin (PIP), cefepime (FEP), ciprofloxacin (CIP), and trimethoprim / aztreonam (AZT).

The multi-resistant profiles found in the exogenous plasmids included resistance to a few medically significant and non-agricultural antibiotics, including piperacillin, cefepime, and ciprofloxacin (Figure 4 B). Piperacillin, and piperacillin / tazobactam, is part of a group of antibiotics that are used to treat food-borne illnesses and is one of the very limited treatments for *Pseudomonas aeruginosa* infections (FDA, 2003). Cefepime is a fourth generation cephalosporin that is used as a treatment for neutropenic fever (FDA, 2003). Ciprofloxacin is part of the fluoroquinolone family of antibiotics use to treat multidrug resistant gram negative rods common in enteric infections (FDA, 2003). Genes coding for resistance to antibiotics that are not used in agriculture found on a plasmid with resistance to common agricultural antibiotic suggests that these genes are "hitchhiking" along with the sequence encoding tetracycline resistance.

Exogenously captured plasmids contain the genes required for self replication and transmission. Additional resistance genes could be collected and recombined – perhaps via transposition and integration from other plasmids within host cells – as the plasmid is communicated from cell to cell. It is unknown if multi-resistance was present when the plasmid entered the stream or if it arose through recombination with native organisms. Transmissibility of the endogenous plasmids was not assessed in this study. The large size of the endogenous plasmids would, however, suggest that genes for self-transmissibility were present, so the prevalence of endogenous plasmids encoding resistance only to tet may be indicative of a new tet^R plasmid ready to accept genes encoding multi-resistance.

The resistance profiles of the two plasmids isolated directly from the sediment with limited enrichment had similar plasmid backbones. Although both plasmids had a

similar backbone, one plasmid encoded resistance to only tetracycline while the other was resistant to tetracycline, ciprofloxacin and ticarcillin. Ciprofloxacin and ticarcillin are classified as critically important for the treatment of gram negative rod and pseudomonas infections, respectively, by the FDA guidance for industry (FDA, 2003). As the methods for isolating plasmids directly from the sediment improve we hope to isolate more representatives of the total plasmid population that can be fully compared and contrasted to the other two methods.

Refining techniques for “total” plasmid extraction. One goal of this study was to isolate and identify all the tetracycline resistant plasmids in the sediment, which could be replicated and expressed in *E. coli* without culturing or selection. Extracting plasmids directly from the sediment without culturing will increase the visibility the “mobilome”. A low quantity of target plasmid DNA was the primary obstacle in extracting plasmids directly from the sediment. To overcome this obstacle different growth media were used for enrichment in an attempt to reduce selection. A lower incubation temperature (24°C) and limited nutrient media were chosen to create conditions closer to *in situ*. This was done so as not to select against native species that could be limited by typical lab culturing techniques. PTYG medium was utilized because it offers a limited nutrient range in small quantities that are similar to the nutrients available in a natural stream setting (Balkwell & Ghiorse, 1985). A sterile manure broth, made from manure collected from the study site, was also used for enrichment. This was thought to be a logical mimic of conditions in the study stream and others impacted by runoff since manure is periodically present in high concentrations in these streams. In addition, manure has been

shown to enhance plasmid mobilization and survival of *Pseudomonas* in soil (Gotz & Smalla, 1997).

Two, six, twelve, and twenty-four hour enrichment times were tested for the different growth media (PTYG and manure broth). Only the 12 and 24 hour incubation times for both media types yielded enough plasmid for visualization on an electrophoresis gel. The 24 hour enrichment in PTYG was the only combination that yielded plasmids that could be successfully electroporated into the control *E. coli*. The large amount of left over sediment from the isolation technique, unknown elements in the manure enrichment media, and low yield of DNA created difficulties with the electroporation process. Residual salts and impurities from the collection / enrichment technique disrupted electrical charge through the cells preventing successful electroporation. Because of the low DNA yield, typical DNA cleaning techniques, which typically reduce DNA quantity, were risky, in that a balance needs to be struck between purification and loss of product.

Conclusions. Sufficient numbers of cells as well as close physical proximity suitable for genetic exchange are important for successful horizontal gene transfer. Streams and other natural environments offer challenges to microbial communities such as limited nutrients and potentially inhospitable temperatures. Although laboratory conditions eliminate these challenges most microorganisms cannot be cultivated in the lab therefore potentially selecting against their use in laboratory-based experiments in HGT.

The three different techniques used for plasmid isolation targeted the plasmids present in *E. coli* at the time of strain isolation from sediment (endogenous), the

demonstrably self-transmissible (exogenous), and all other plasmids available (total) that could potentially replicate in *E. coli*. It was hypothesized that these three techniques would collect from different yet overlapping plasmid populations. The endogenous and exogenous techniques isolated two different plasmid populations with regard to resistance to specific antibiotics and, especially, the incidence of multiresistance. It is unknown if the endogenous plasmids isolated have self-transmissible characteristics. If having a mobile capability increases the chance of picking up resistance traits, as suggested by this study, a test of the endogenous plasmids for self-transmissibility could predict the potential for multi-resistant development.

Although there were very few plasmids taken directly from the sediment (i.e. via the ‘total’ isolation method), the different populations collected by the endogenous and exogenous isolation methods suggests that the method of plasmid isolation can have a large effect on the plasmid population isolated. The direct isolation of plasmids – without using a host cell – from DNA derived directly from an environmental sample shows particular promise for isolating and identifying previously unknown plasmids, as it completely bypasses the bias of culturing. This study identified multiresistant transmissible plasmids and plasmids conveying resistance to medically significant antibiotics. Other than tetracycline, the antibiotics employed in this study have either no use or strictly regulated use in agricultural practices. Environmentally occurring resistance to significant antibiotics such as these is starting to emerge worldwide despite their lack of use in agriculture (Po-Ren *et. al.* 2004). This would suggest that these presumably non-selected resistance genes could be “hitchhiking” with those resistance genes that are selected for by agricultural practices. The unexpected presence of these

genes begs the question; what other genes are available in the environmental
“mobilome”?

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