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Antibiotic Resistance Gene (ARG) Maintenance: Aerobic versus Anaerobic conditions and the correlation of plasmid loss to the intracellular redox environment

by

William R Mansfield

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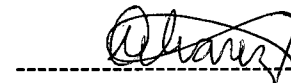

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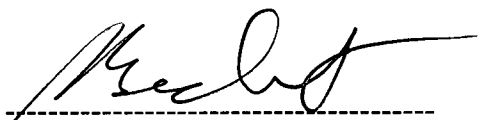

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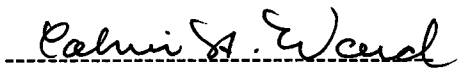

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ABSTRACT

Antibiotic Resistance Gene (ARG) Maintenance: Aerobic versus Anaerobic conditions and the correlation of plasmid loss to the intracellular redox environment

By

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Antibiotic resistance genes (ARGs) have become emerging contaminants through the overuse and misuse of antibiotics in animal agriculture. Propagation, development, and maintenance of such a contaminant through bacterial reservoirs are not well understood. Identifying environments to which ARGs are attenuated due to an inability to meet the metabolic burden of maintaining the plasmids that carry the ARGs, will give insight to possible solutions. An anaerobic environment was shown to cause the loss of tetracycline resistant (Tet^R) gene TetC which is carried on the pSC101 plasmid within the tested strain *Escherichia coli* c600. Fluctuation and attenuation of the ARG harboring plasmid was also correlated with the intracellular reduction potential of the cells, which was measured as the NADH/NAD+ ratio. This suggests a relationship between ARG plasmid maintenance and the energy state of the cells, possibly reflecting that the energy burden of ARG and associated plasmid maintenance is more difficult to meet under anaerobic conditions that are less favorable from an energy harvesting perspective. These results suggest that the use of anaerobic barriers (e.g., permeable anaerobic mulch

barriers or anaerobic lagoons) to intercept ARG-laden drainage from confined animal feeding operations may attenuate the propagation of ARGs into the environment.

ACKNOWLEDGEMENTS

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CONTENTS

INTRODUCTION

A growing concern surrounding the misuse of antibiotics in animal agriculture and in particular Confined Animal Feeding Operations (CAFOs) is the possible contamination of natural water supplies and soil environments with antimicrobial resistance determinants (Stuart B. Levy & Marshall, 2004; "New Books Received," 2003).

This research will attempt to explain a specific dynamic of Antibiotic Resistance Gene (ARG) maintenance known as plasmid curing (loss), under differing environmental conditions, aerobic versus anaerobic, by correlating plasmid loss to the redox environment (Schafer & Buettner, 2001) within bacteria cells. Specifically addressing the difference between anaerobic and aerobic conditions will add to the current knowledge of environmental factors that affect plasmid maintenance. Additionally, correlating the redox environment within antibiotic resistant bacteria will provide a more direct physiological link to understanding plasmid maintenance.

Antibiotic resistance to commercial antibiotics has been around since shortly after the mass production of penicillin in the early 1940s and over the past seven decades has grown into an epic battle between men and microbes. Today researchers continue to uncover as well as explain the mechanisms and dynamics of antibiotic resistance, in particular Tetracycline (TC) resistance.

Mechanisms of plasmid transfer

Plasmids are autonomous molecules of DNA which can propagate by way of conjugation, transformation, or cell division (Lipps, 2008) and are generally inherited with a high degree of stability (Zimmerman, 1986). Conjugation is by far the most common method and involves the use of pili, which are hair like structures found on bacteria that are used to make a direct connection and transfer genetic material (plasmids) between two bacteria of the same species (vertical gene transfer), but can also be used to connect two different species (horizontal gene transfer). This ability of bacterial species such as Escherichia coli (*E. coli*) to conjugate with other species such as Pseudomonas aeruginosa (*P. aeruginosa*) by way of horizontal gene transfer (HGT) and propagate ARG harboring plasmids, has lead to the increase of antibiotic resistance pathogens in the environment (Kruse & Sorum, 1994; Licht, Christensen, Krogfelt, & Molin, 1999; Pruden, Pei, Storteboom, & Carlson, 2006).

Intracellular redox environment

Microbial proliferation under varying environmental conditions requires a great deal of catabolic flexibility (Alexeeva, Hellingwerf, & Teixeira de Mattos, 2003). This flexibility allows organisms such as *E. coli* to adjust the expression levels of over one hundred different functional proteins in the cell responding to an environmental shift from aerobic to anaerobic (Sawers, 1988). These shifts in protein expression affect the intracellular oxidation reduction (redox) reactions, thus altering the redox environment inside the cell. Nicotinamide adenine dinucleotide (NAD) makes up the cofactor pair NADH/NAD+ and is responsible as a cofactor in over 300 redox reactions (Foster, Park,

Penfound, Fenger, & Spector, 1990). In a recent study, researchers revealed the profound influence that NADH can have on a bacterial culture (*E. coli*) by artificially increasing the amount of NADH causing a shift to fermentation in an aerobic environment (Berríos-Rivera, Bennett, & San, 2002). NAD+ is used to oxidize carbon sources during metabolism where it is reduced to NADH. For continued cell proliferation NADH must be oxidized back into NAD+. These dramatic effects and the central role that NADH/NAD+ cofactors have on the metabolic activity within bacteria cells, make it a useful tool to measure the intracellular redox environment (Alexeeva et al., 2003; Foster et al., 1990; Tseng, Martin, Nielsen, & Prather, 2009).

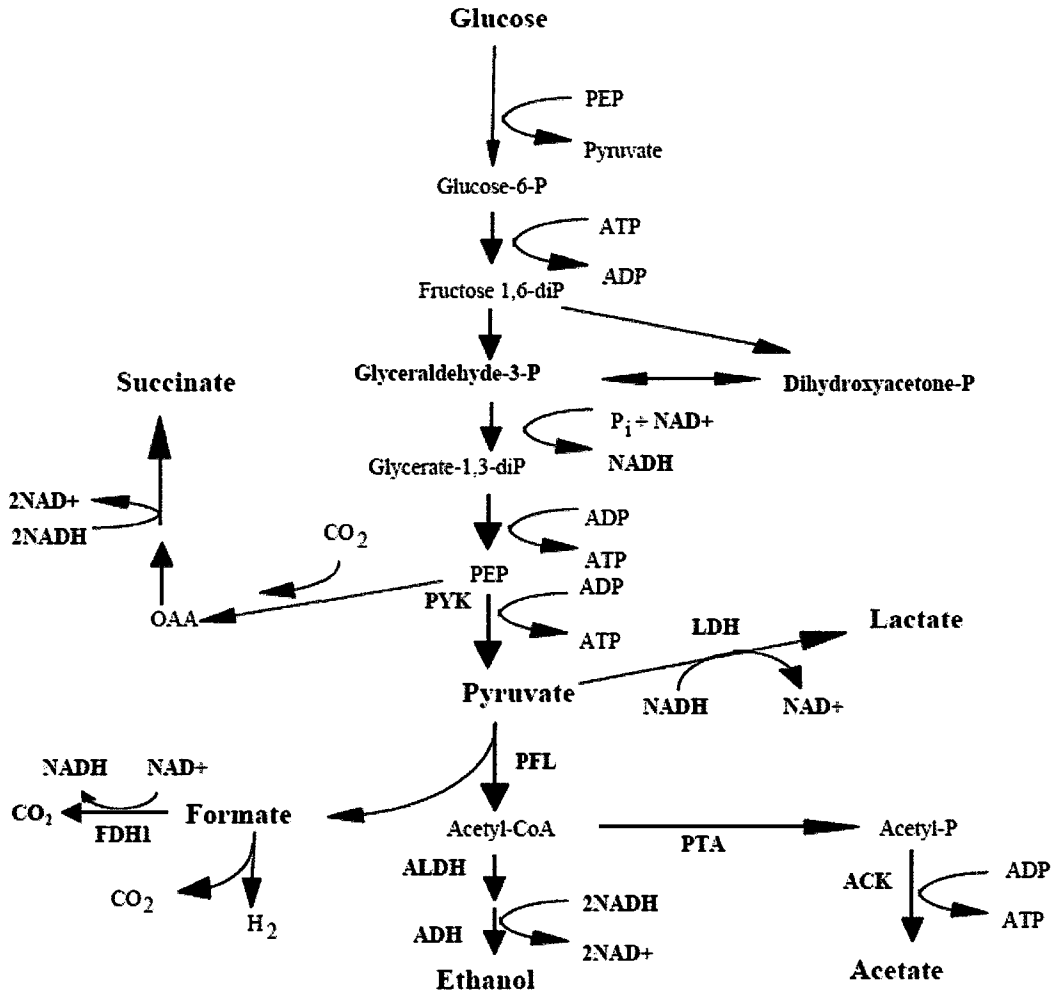
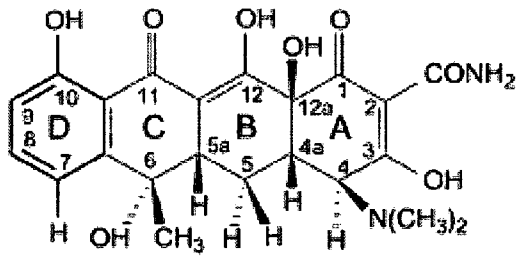


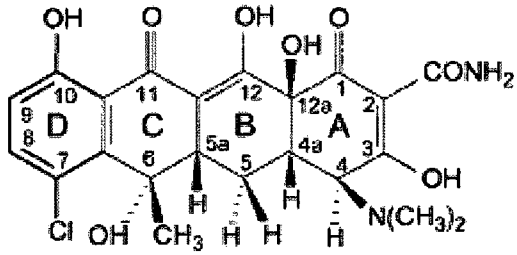
Figure L.1 Central anaerobic metabolic pathway of *Escherichia coli* showing generation of NADH and regeneration of NAD+ including the new NAD+-dependent formate dehydrogenase (FDH) from Candida boidinii.

Mechanisms of TC and TC resistance

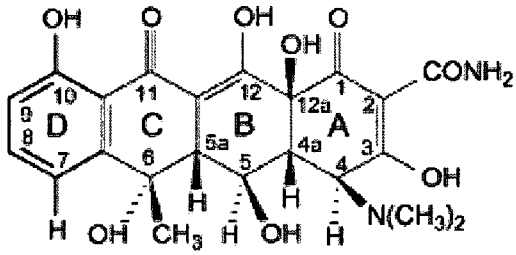
Tetracycline comes from a large family of naturally occurring antibiotics known as Tetracyclines and was first discovered in 1945. It is a bacteriostatic drug that inhibits protein synthesis by binding to the 30S ribosomal subunit of bacteria. This binding to the 30S subunit prevents the attachment of aminoacyl-tRNA which carries amino acids to the ribosome as the building blocks for protein synthesis. To counter this mechanism bacteria have adapted through natural selection the ability to inhibit the effects of TC. There are three known mechanisms of TC resistance which are produced by a list of TC resistance (TET^R) genes that are genetically and biochemically distinguishable (Roberts, 1996). The first is a rare mechanism that produces an enzyme capable of cleaving TC and thus inactivating it. The second is a set of genes that produce a ribosomal protection protein to inhibit the binding of TC to the 30S subunit, while the third includes a set containing the two genes used in this research (TetC and TetA) which code for efflux pumps. These efflux pumps comprised of membrane bound proteins are energy dependent and can expel antibiotics and other toxins out of the cell (Webber & Piddock, 2003). It's broad spectrum status and extensive use in every sector of medical and therapeutic treatment in humans and animals makes TC an effective model for antibiotic resistance research (Chopra & Roberts, 2001; Roberts, 1996).



Tetracycline (TC)



Chlortetracycline (CTC)



Oxytetracycline (OTC)

Figure L.1 Structures of most commonly used tetracyclines. Tetracycline MW 444.44 (g/mol), pKa 3.3, solubility in water (mg/L) 231, and logK_{ow} -1.3 (TC): R5=H, R7=H; Chlorotetracycline (CTC): R5=H, R7=Cl; Oxytetracycline (OTC): R5=OH, R7=H (Halling-Sorensen, Sengelov, & Tjornelund, 2002; Lindsey, Meyer, & Thurman, 2001)

Misuse of antibiotics

Providing food for a growing population increases the pressure placed on industrialized animal agriculture, forcing farmers to continually amplify the numbers and size of their animals. Increasing the number of animals and the use of Confined Animal Feeding Operations (CAFOs), concentrates livestock to a point where microbial transmission is inevitable,(Gilchrist, 2007) while increasing the size of animals through the non-therapeutic use of antibiotics produces antibiotic resistance (Stuart B. Levy & Marshall, 2004).

There are three uses for antibiotics in animal agriculture, therapeutic, sub-therapeutic, and growth promotion(Barza, Gorbach, & DeVincent, 2002; McEwen & Fedorka-Cray, 2002). Therapeutic treatment is used to treat animals that are actively infected with a microorganism, where as sub-therapeutic levels are used to prevent infection as well as promote growth. Microflora within the gastrointestinal (GI) systems of livestock provide nutrients for the animals through fermentative processes along with protection from the establishment of pathogenic strains of bacteria (Dibner & Richards, 2005). Antibiotic use for growth promotion in animal agriculture works by eliminating some of the microflora found in the animals GI system, which allows more of the food consumed by livestock to be converted into mass (Visek, 1978). Some studies involving swine production have shown increases in rate of weight gain, up to 16.4%, feeding efficiency (6.9%), and a 50% reduction in mortality (Zimmerman, 1986). Positive economic benefits provided by

therapeutic antibiotics, keeps farmers and pharmaceutical companies happy, creating a strong opposition to discontinue their use (Lathers, 2001; Zimmerman, 1986).

Studies have estimated the amount of antibiotics used at sub-therapeutic levels in animal agriculture to be eight times of that used in humans for medical treatment. That's 24.6 million pounds of antibiotics per year spread throughout the industry including poultry, swine, and cattle (Mellon, 2001).

The misuse of antibiotics in animal agriculture is not only in the amount used, but in the actual antibiotics used, such as penicillins, tetracyclines, and streptogramins which are all used in the medical treatment of humans (Stuart B. Levy & Marshall, 2004; "New Books Received," 2003; Singer et al., 2003). This leads to production of pathogens that are resistant to all of our common weapons against infection ("New Books Received," 2003).

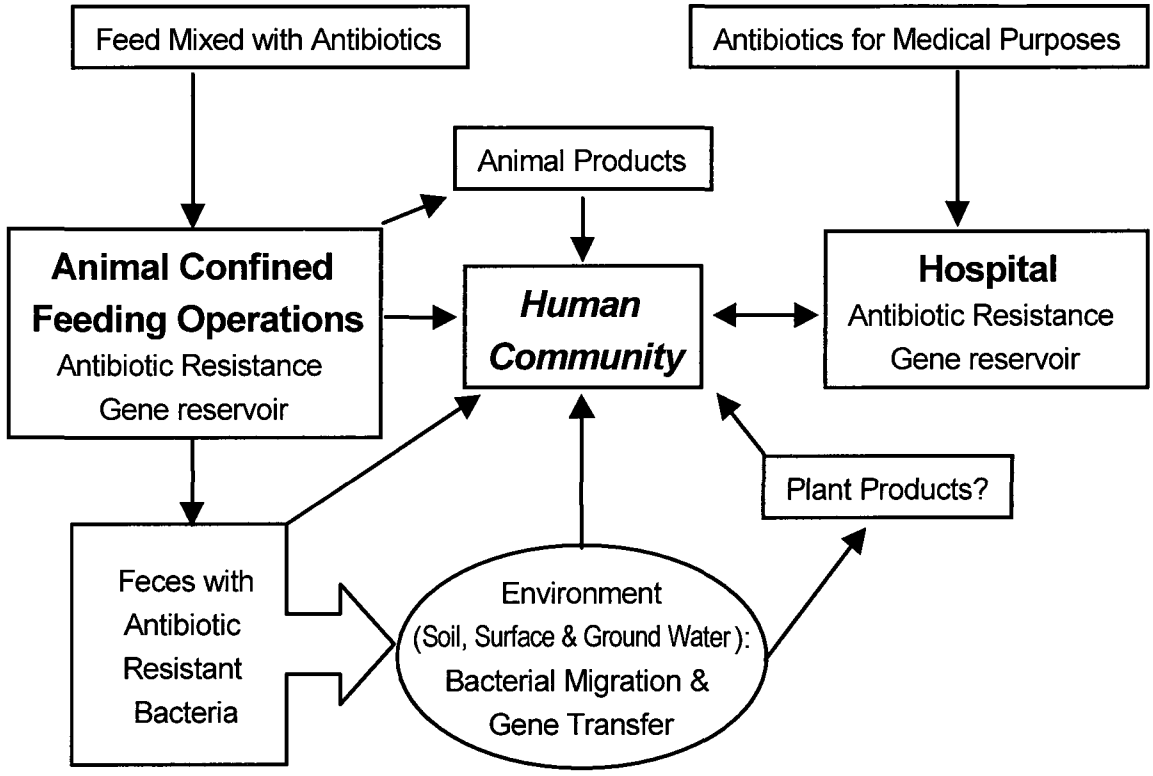


Figure L.2 Potential pathways for antibiotic resistance gene (ARG) propagation. Although hospitals can be a big contributor to ARG propagation, it is clear from the diagram that antibiotic use in animal agriculture has many more routes of transmission into the human population (Rysz, 2007).

In 1976, 31.3% of farmers running a poultry farm that began treating chickens with tetracycline, tested positive for tetracycline resistance in their intestinal flora along with 6.8% of the surrounding neighbors (S. B. Levy, FitzGerald, & Macone, 1976). In another study, researchers tested 200 samples of ground meat from supermarkets in Washington D.C. and found 34 of them to contain antibiotic resistant *Salmonella* (White et al., 2001). These cases and many others are becoming evidence to the transmission of antibiotic resistance through human-livestock interaction and the food supplied by animal agriculture (Angulo, 2000; Blaser, 2004; Hua et al., 2006; Witte, 1998).

A lesser known route of transmission that has been of growing concern is through the natural water supplies and soil environments surrounding Confined Animal Feeding Operations (CAFOs) (Chee-Sanford, 2001). Bacterial transport through soil and ground water has been studied extensively (Chee-Sanford, 2001; Furushita et al., 2003; Pei, Cha, Carlson, & Pruden, 2007) and although several groups have shown, through the use of real time quantitative PCR (RTqPCR), that ARGs are present in wastewater lagoons surrounding CAFOs (Peak et al., 2007; Smith et al., 2004) and up to 250m downstream (Chee-Sanford, 2001), little is known about the fate and transport of ARGs in the environment. This indicates the need for further research to better understand the environmental conditions that affect ARG plasmid maintenance and attenuation.

HYPOTHESIS

If the maintenance of ARG harboring plasmids confers a metabolic (energetic) burden, then measuring the NADH/NAD+ ratio as an indicator of intracellular redox state while monitoring the presence or absence of these plasmids will give an indication of the cells redox environment and thus their metabolic capacity under differing environmental conditions.

OBJECTIVES

Two main objectives will be addressed, the first being, to measure the curing (loss) and or maintenance of ARG harboring plasmids under anaerobic versus aerobic growing conditions. The second is to measure the NADH/NAD+ ratios within the bacteria cells and determine if there is a correlation between plasmid loss and redox potential within the cells.

METHODOLOGY

Bacterial strains and initial growth parameters

Two pure bacterial strains, both conferring tetracycline resistance were used for this research: an enteric *E. coli* c600 strain harboring the pSC101 plasmid (9.3 kb) containing the *tet*(C) resistance gene (ATCC#37032), and a common soil strain *Pseudomonas aeruginosa* PU21 carrying the RP1 plasmid (56 kb) containing the *tet*(A) resistance gene (NCTC050076). The *E. coli* strain was acquired from a glycerol stock in the lab from previous research and the *P. aeruginosa* strain was purchased from National Collection of Type Cultures in London (catalog # NC50183). Initial cultures were grown in LB medium (Difco™ catalog # 244620 containing 10g tryptone, 5g yeast extract, and 10g sodium chloride per liter) supplemented with 15 mg L^{-1} TC (T3383, Sigma Co) in the *E. coli* strain and 50 mg L^{-1} in the *P. aeruginosa* strain to enhance plasmid maintenance within the host bacteria. These cultures were then grown overnight and used to seed subsequent batches, both in the presence of TC (TC+, 50 $mg \cdot L^{-1}$) and without TC (TC-, 0 $mg \cdot L^{-1}$) as a control for the effect of antibiotic treatment on resistance plasmid maintenance. Overnight cultures were harvested and the optical density at wavelength 600 (OD_{600}) (Ultrospec 2100 pro spectrophotometer, Amersham Biosciences) was recorded.

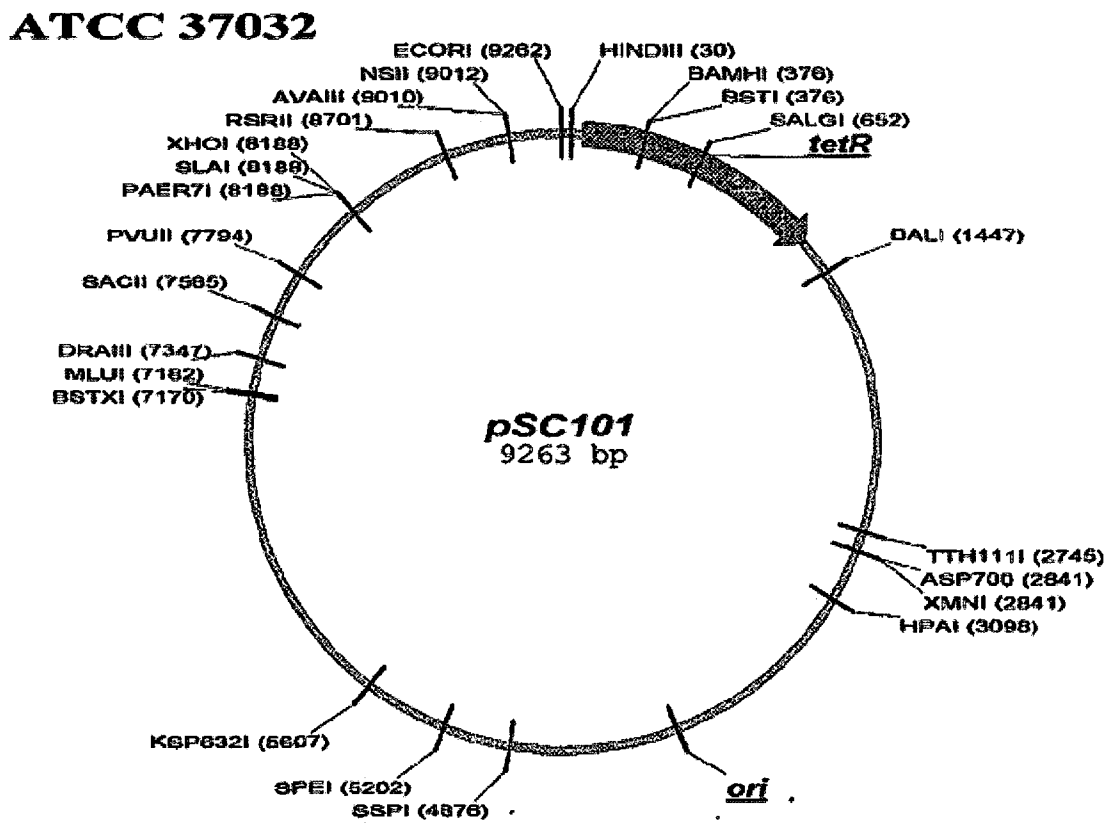


Figure 1 Restriction map of the pSC101 plasmid (9.2kb), carrying the TetC resistance gene. http://atcc.org/attachments/1464.jpg

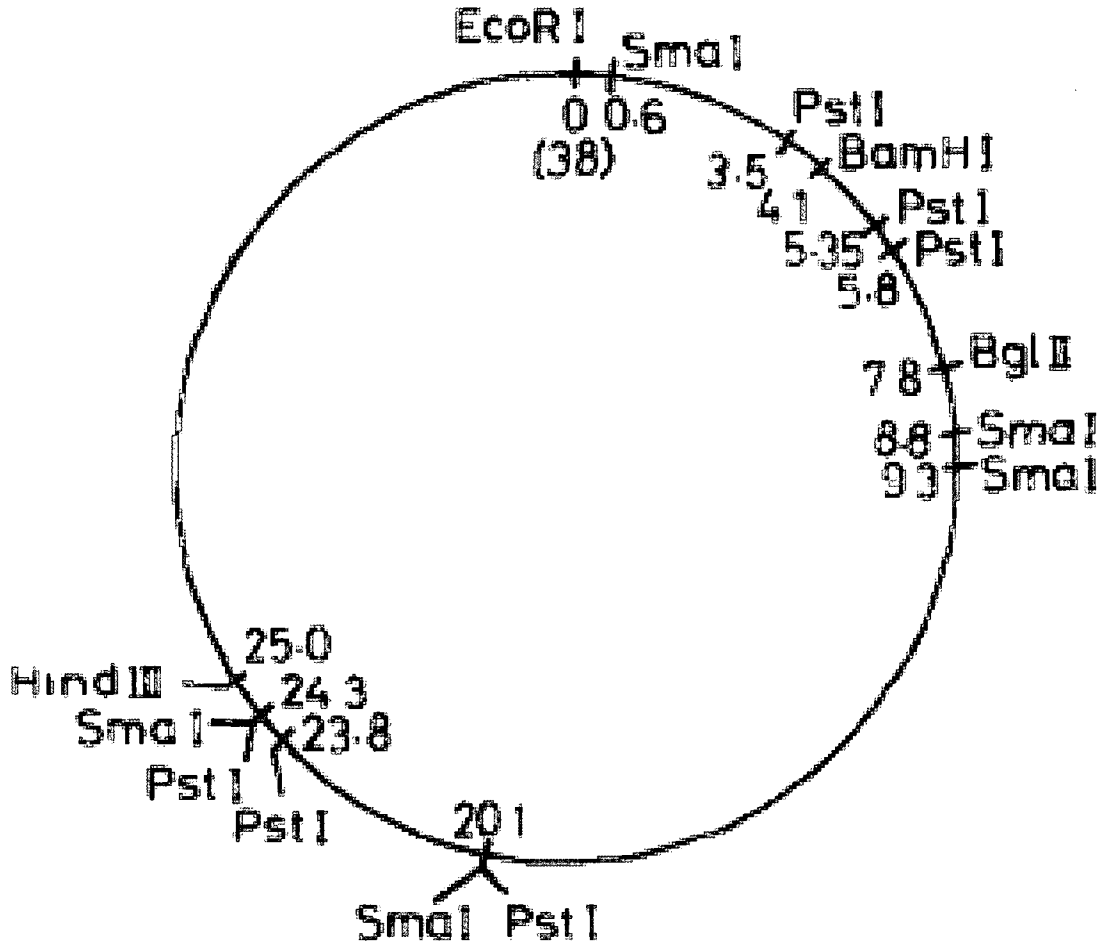


Figure 2 Restriction map of the RP1 plasmid (56kb), carrying the TetA resistance gene. (Grinsted, Bennett, & Richmond, 1977).

Standard curve for calculating concentration of bacterial cultures

Standard curves were created to measure the concentration of batch cultures and subsequently the number of generations between each sampling point. This was done by plotting the OD_{600} of the cultures against the actual concentration. Actual concentration was measured by Colony forming units (CFU) growing a set of initial cultures to stationary phase and then streaking an LB agar plate with 100µl of a 1:10 serial dilutions of the cultures. Plates were incubated overnight at 37°C and colonies were counted the following day. Number of colonies X the dilution factor gives the concentration or number of bacteria per micro liter.

Aliquots of the harvested cultures were used to seed subsequent reactors with and without TC for the duration of the experiment (approximately 230-300 generations for *E. coli*, and 80 generations for *P. aeruginosa*).

Standard curve for Real Time Quantitative PCR (RTQ-PCR)

Standard curves for (RTQ-PCR) were created by plotting the CT (cycle threshold) values against the concentrations of DNA from PCR products produced from bacterial template DNA and primers specific to the 16s gene for each strain and the corresponding Tetracycline resistance (TET^R) genes. DNA was extracted from batch cultures of each strain using UltraClean™ Microbial DNA Kit (MoBio Laboratories) according to manufacturer's protocol, and the concentrations of the total purified DNA were determined using an Ultrospec 2100 pro spectrophotometer prior to RTQ-PCR analysis. Extracted DNA from each strain was used as template DNA and mixed with PCR Master Mix (2X) from Fermentas along with primers for 16s and TET^R genes. The PCR reaction

mixtures consisted of 2.5µl of each primer normalized to 100nM, 12.5µl of PCR master mix (2X), 2.5µl of water and 5µl of template DNA normalized to 10ng/µl. Three sets of forward/reverse (For/Rev) primers were ordered from Integrated DNA Technologies (IDT). The first set targeted the 16S rDNA gene (For – 5'GTGSTGCAYGGYTGTCGTCA3', Rev 5'ACGTCRTCCMCACCTTCCTC3') (MAEDA ET AL., 2003), the second targeted the *tet*(C) gene (For – 5'GCCTATATCGCCGACATCAC3', Rev5'GTAGGTTGAGGCCGTTGAGC3') (Thaddeus B. Stanton & Samuel B. Humphrey, 2003), and the third targeted the *tet*(A) gene (For- 5'CCTGCGCGATCTGGTTCACT3', Rev-'5GCCAGCGAGACGAGCAAGA3') (T. B. Stanton & S. B. Humphrey, 2003). The PCR products were run on a 2% agarose gel and purified using the Wizard® SV Gel and PCR Clean-Up System from Promega (catalog # A9281). DNA was then measured and serial dilutions were made to provide an accurate concentration for each gene being detected to plot against the CT value.

Anaerobic versus Aerobic

E. coli c600 cultures were grown in 50 ml Falcon tubes both in the presence of TC (TC+, 15mg/L) and absence of TC (TC-, 0mg/L) in LB medium under aerobic and anaerobic (fermentative) conditions. Resazurin (Sigma-Aldrich® catalog # R7017) at 0.001% was used as an oxygen indicator (Masalha, Borovok, Schreiber, Aharonowitz, & Cohen, 2001) in both the aerobic and anaerobic cultures to ensure there was no oxygen present in the anaerobic cultures. Anaerobic cultures were prepared in a COY Laboratory Products Inc. anaerobic chamber using LB medium in a mixed gas atmosphere (10%CO_2, 10%

Hydrogen, Balanced with Nitrogen). Samples were tightly capped and parafilmed before removal from the anaerobic chamber and transferred to a 37°C incubator. Overnight cultures were removed from the incubator where aerobic samples were taken to the bench and anaerobic samples were transferred back into the anaerobic chamber. Aliquots from both aerobic and anaerobic samples were then used to seed subsequent reactors (10^7 and 10^4 dilution respectively). Cultures were then harvested for DNA extraction and NADH/NAD+ analysis.

Calculating Generations

OD_{600} was then measured and used to calculate the number of bacterial generations between each sampling period.

$$\text{Number of Generations} = \log\left(\left(\frac{X}{Y}\right)/\log(2)\right)$$

X=Total number of bacteria in the culture

Y=Number of bacteria used to inoculate culture

Real time quantitative PCR (RTQ-PCR)

Real time quantitative PCR (RTQ-PCR), with reported detection levels of 1.4×10^{-15} g of DNA per reaction (Skovhus, Ramsing, Holmstrom, Kjelleberg, & Dahllof, 2004), was used for antibiotic resistance gene monitoring. This allowed for sensitive detection of the targeted genes and the quantification of their relative abundance, while eliminating the bias associated with cell culturing techniques. The Tet^R strains were screened for 16S rDNA, *tet*(C) (for *E. coli*), and *tet*(A) (for *P. aerugionosa*) gene concentrations by RTQ-

PCR with the 7500 Real Time PCR System (Applied Biosystems) in 25 μL reaction mixtures on 96-well plates containing 1X SYBR® Green Master Mix (Applied Biosystems), 2μL aliquots of the DNA samples, forward and reverse primers (Same 3 sets mentioned earlier) at 500nM final reaction concentration. The cycling conditions were: 1 min at 50°C, 10 min at 95°C, 40 cycles of denaturation for 15 sec at 95°C, annealing for 1 min at 60°C, followed by the dissociation stage cycle (15 sec at 95°C, 1 min at 60°C, 15 sec at 95°C).

NADH/NAD+ measurement

NADH/NAD+ extraction was performed using EnzyChrom™ NADH/NAD+ Assay Kit (ECND-100) following manufactures protocol except for the addition of a 10 second sonication step (Sonic Ruptor 250 Omni International) with samples on ice (Tseng et al., 2009) at 50% power. Absorbance was measured (SpectraMax® plus, Molecular Devices) at 565nm for time zero and 15min. An 8 channel multichannel pipette was used to add the mixed reagents from the EnzyChrom™ kit into the 96 well plate (BD Biosciences catalog # 353070) containing the extracted NADH/NAD+. NAD+ oxidizes organic compounds to produce energy within the cell (Schafer & Buettner, 2001); therefore, measuring the ratio of NADH/NAD+ was used as an indicator of a cells energetic state. Resistance plasmid maintenance represents an energetic burden, thus, this assay was used to determine if there was a correlation between the energetic state of the cell and the maintenance of the ARG plasmids.

RESULTS AND DISCUSSION

Effect of environment (anaerobic versus aerobic) on ARG plasmid maintenance

Batch cultures were grown overnight in LB medium at 37°C. The initial plasmid loss in the anaerobic batches occurred between 0 and 29 generations dropping the [TetC]/[16s] gene ratio from 1.0 (± .026) to 0.35 (+ 0.002), whereas the aerobic batches maintain the resistance plasmid with a [TetC]/[16s] gene ratio of 0.67 (± .003) or higher until 302 generations, when the ratio drops to 0.37 (± .003). In the absence of TC, faster ARG loss (i.e., decreasing TetC/16s) occurred under anaerobic (fermentative, indicated by the use of resazurin) than aerobic conditions as a function of the number of cell generations (Fig 1), indicating that cells under less energetically favorable anaerobic conditions lose the resistance plasmid in fewer generations to alleviate the energetic burden associated with its maintenance. This suggests that intercepting drainage from animal farm operations using anaerobic barriers (e.g., anaerobic lagoons, permeable reactive barriers) might help attenuate ARG propagation from the source. The fluctuations in the graph are interesting and could indicate a natural homeostatic oscillation or a product of experimental procedure. For additional information it is important to consider the time in which plasmid loss in occurring. Each point on the two sets of data in the figure indicates a 24hr period, this shows that the anaerobic culture had the most significant plasmid loss in the first two days, in comparison the aerobic culture takes eight days to drop to its lowest point.

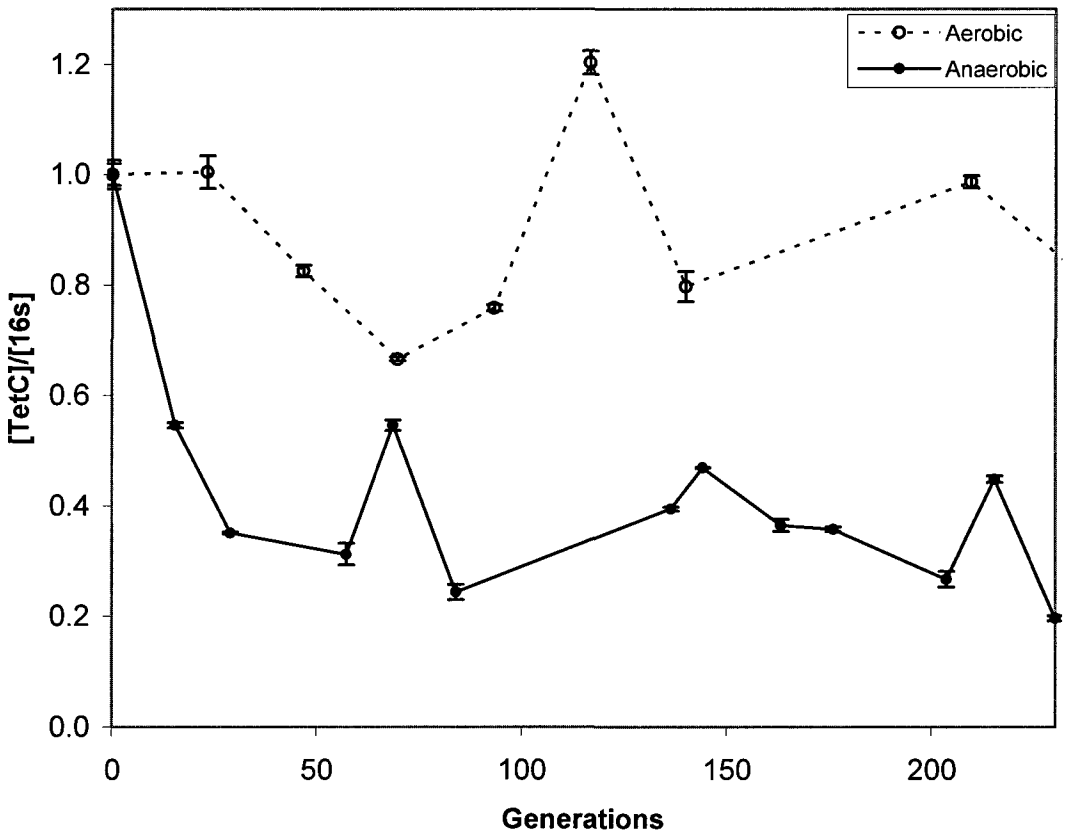


Figure R.1 [TetC]/[16s] plotted against the number of generations without TC, under aerobic and anaerobic conditions.

Effect on ARG plasmid maintenance in P. aeruginosa

Comparing the gene ratio ([16s]/[TetA]) to the number of generations under aerobic conditions with TC(50mg/L) and without TC(0mg/L) (Figure R.2). This data indicates the continued maintenance of the 56kb RP1 plasmid harboring the TetA gene under the pressure of continued TC treatment. Additionally it shows the attenuation of the RP1 plasmid when TC treatment is halted, dropping from the normalized initial value of 1 to 0.22 after 80 generations. These results confirm previous research done in the laboratory and show that in the absence of TC the ARG plasmid is indicating a drastic decrease in ARG plasmid maintenance. Anaerobic cultures of *P. aeruginosa* were also seeded, but failed to grow. Several attempts were made to grow the *P. aeruginosa* cultures in the anaerobic chamber including the seeding of cultures under aerobic conditions and subsequently transferring them into the chamber. An attempt to transfer a *P. aeruginosa* culture that was in log phase under aerobic conditions also failed These failed attempts could be an indication of this strains inability to proliferate under anaerobic conditions and possibly support the suggestion of anaerobic barriers as a treatment for the attenuation of ARG determinants.

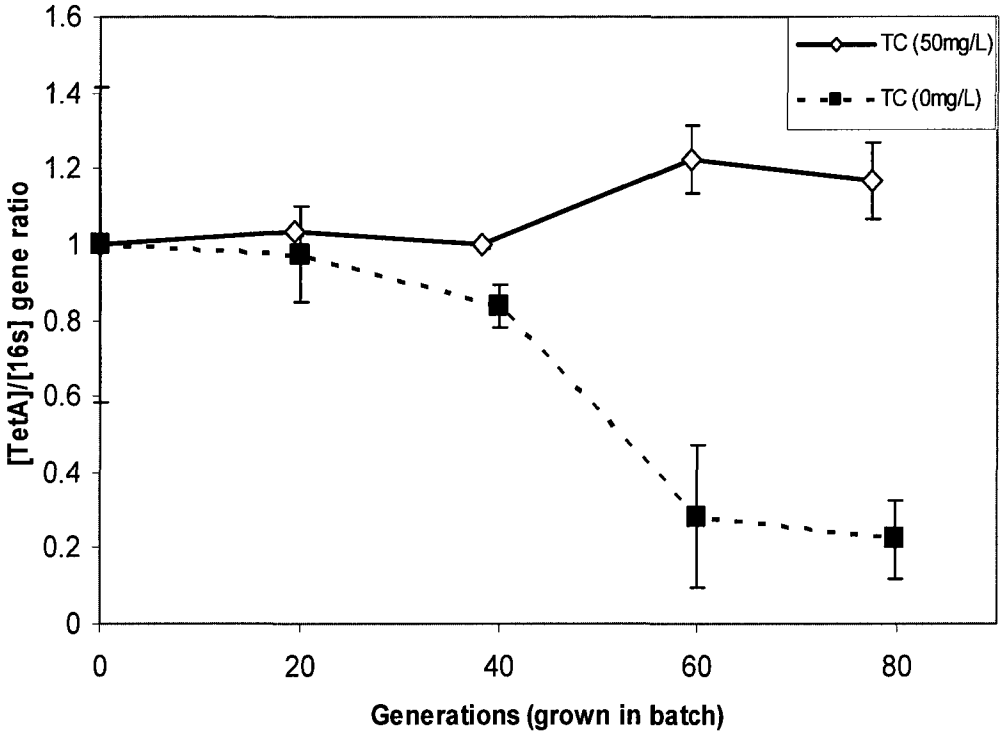


Figure R.2 [TetA]/[16s] gene ratio plotted against the number of generations under aerobic conditions with TC(50mg/L) and without TC(0mg/L)

Correlation between NADH/NAD+ and TetC/16S without TC

E. coli grown in batch culture under anaerobic conditions without TC (0mg/L), comparing ratios of TetC/16s and NADH/NAD+ (Figure R.3). Data shows a great deal of fluctuation in both ratios and strong correlation between the two data sets ($p < .05$, $R^2 = 0.827$) using student t-test and linear regression analysis respectively. This indicates that the intracellular redox environment within the cells is related to the maintenance of the antibiotic resistance plasmid.

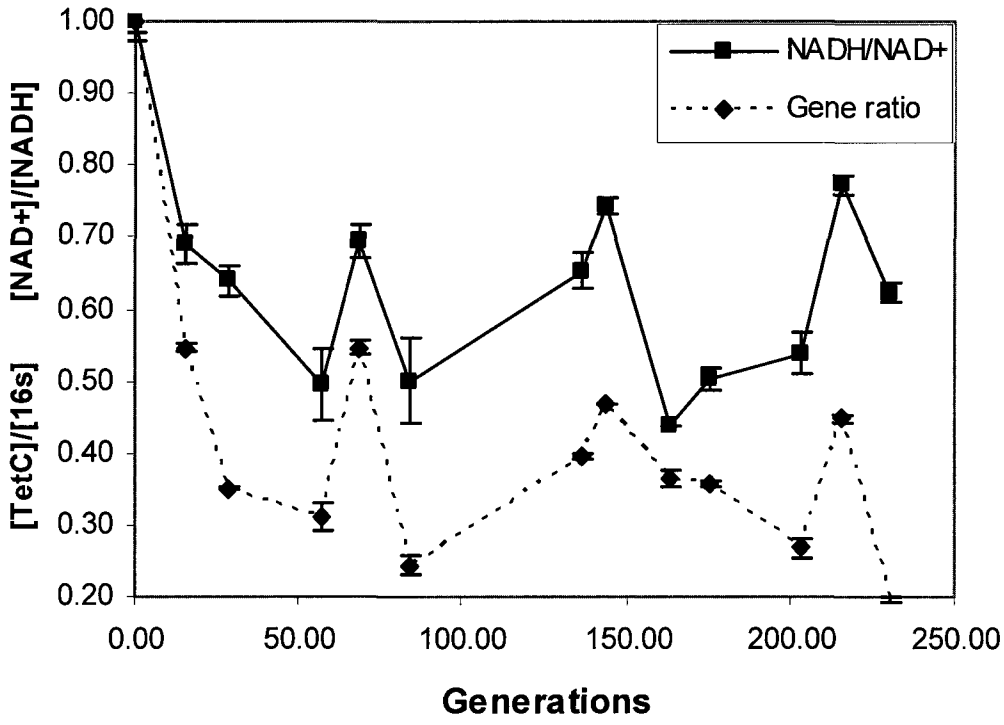


Figure R.3 Correlation between intracellular redox environment [NADH]/[NAD+] and the gene ratio [TetC]/[16s] against the number of generations using *E. coli* c600 without TC. Numerical results are normalized to 1.

Correlation between NADH/NAD+ and TetC/16S with TC

E. coli grown in batch culture under anaerobic conditions with TC (15mg/L), comparing ratios of TetC/16s and NADH/NAD+ (Figure R.4). Data shows a great deal of fluctuation in both ratios and strong correlation between the two data sets ($p < .05$, $R^2 = 0.842$) using the student t-test and linear regression analysis respectively. This indicates that the redox environment within the cells is related to the maintenance of the antibiotic resistance plasmid. Fluctuations in Figure R.3 and R.4 could be due to changes in the micro atmosphere of the anaerobic chamber. Each day, sample collection in the anaerobic chamber exposes the continuously maintained cultures to changes in the concentration of hydrogen gas.

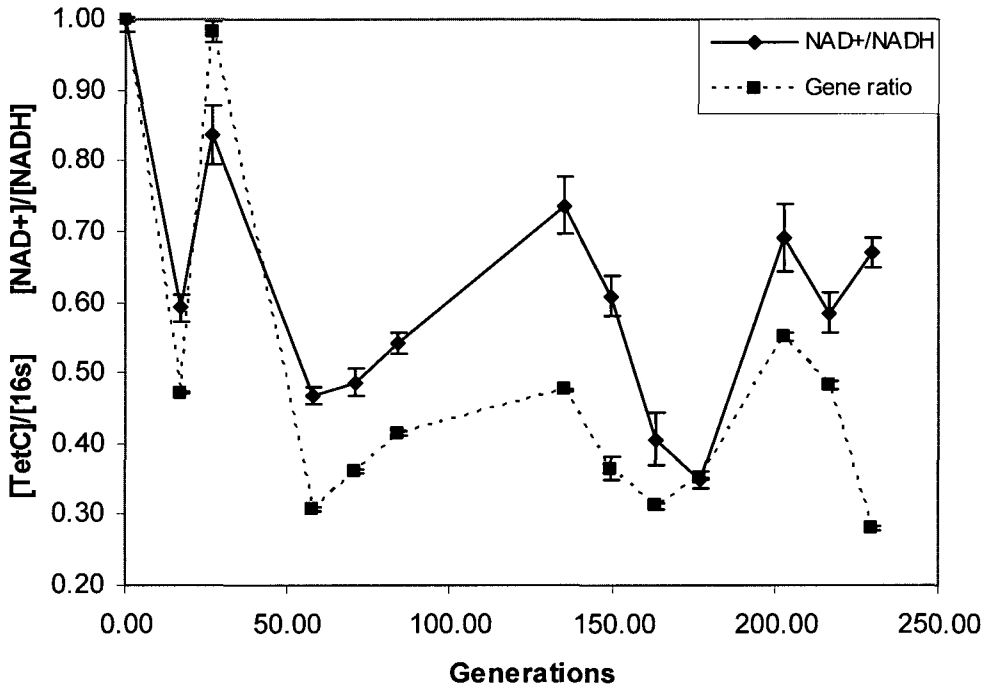


Figure R.4 Correlation between intracellular redox environment [NADH]/[NAD+] and the gene ratio [TetC]/[16s] against the number of generations using *E. coli* c600 with TC (15mg/L). Numerical results are normalized to 1.

These data support the notion that the loss of the resistance plasmid is associated with the energetic status of the cells, and suggests that environmental conditions conducive to low energy status (e.g., anaerobic environments, limited nutrient availability etc.) make it difficult for the cells to meet the metabolic burden of ARG plasmid maintenance and favor the loss of antibiotic resistance. As the ratio of NADH/NAD+ decreases the redox environment is shifting from a more reducing (anabolic) environment in which plasmid maintenance would occur (Schafer & Buettner, 2001), to a more oxidizing (catabolic) environment where cells are primed to breakdown available nutrients for energy production (Berríos-Rivera et al., 2002).

CONCLUSION

Knowledge gained

Continued use of antibiotics, in animal agriculture and especially CAFOs, that are also used in the medical treatment of humans will promote the spread of multi-drug resistant bacteria. This research has shown that under anaerobic conditions, ARG harboring plasmids are lost at a much faster rate, leading to the possible solution of anaerobic barriers downstream of the contamination. Correlation between the redox environment and ARG plasmid loss has strengthened the hypothesis that environments lower in energy (e.g., anaerobic) make it more difficult for bacteria to overcome the metabolic burden of plasmid maintenance and thus attenuating the ARG determinants.

Engineering significance

This research has increased the knowledge of ARG plasmid maintenance and will allow engineers to better understand the dynamics involved with microbial genetic determinants as they pertain to the overuse of antibiotics in animal agriculture. Recognizing antibiotic resistance determinants as emerging contaminants will affect the site characterization of contaminated CAFOs and identify the need for remediation to combat the spread of antibiotic resistance determinants harbored among common pathogenic bacteria in the soil and groundwater.

Future Research

Future research would include the measurement of additional redox pairs (e.g., NADPH/NADP+ and GSSG/2GSH) to elute further detail of the redox environment within the cells. In addition, ATP should be measured and compared to the redox environment so that definite correlation between redox state and energy can be achieved. Soil and groundwater samples from contaminated CAFO sites would also need to be attained so that indigenous microbial communities could be compared to that of the model bacteria strains previously used.

Practical implications

Anaerobic lagoons are a popular choice for treatment of animal manure (DeSutter, 2000) and could provide a possible treatment for antibiotic resistance propagation. However as mentioned previously, tetracycline resistant determinants have been detected downstream of waste water lagoons which could be a result of linear seepage or accidental overflow. Even if a lagoon was lined with plastic or concrete the risk of overflow would still exist, but if a lagoon is already in use it could be a valid first line of defense. Another option is mulch Permeable Reactive Barriers (PBRs) which provide a low cost low maintenance under ground anaerobic environment in which runoff from CAFOs can be intercepted (Fenton, Healy, & Schulte, 2008). However this technology is continuously exposed to the surrounding environment which will promote the transfer of genetic material between microbes. Anaerobic digesters have already been used in the animal agriculture environment as a possible means of using the waste produced by livestock to produce energy that can be converted into electricity to supply power to the animal facility

(Wilkie, 2000). The use of anaerobic digesters would allow the animal waste to be collected and transferred into a closed system, eliminating contact between the enteric microorganisms containing ARG harboring plasmids (emerging pollutant), from the surrounding environment. Additional drainage barriers would need to be in place where a possible plug flow style anaerobic digester could be applied (Mata-Alvarez, Macé, & Llabrés, 2000). These three technologies have the potential to attenuate the propagation of antibiotic resistance based on the results of this research in which ARG harboring plasmids were lost at much more rapid rate under anaerobic conditions. Additionally, using these technologies in combination would most likely increase their effectiveness.

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APPENDICES

Anaerobic TC						Anaerobic						Aerobic TC				Aerobic			
		NADH		[TetC]				NADH		[TetC]			[TetC]				[TetC]		
Sample	Gens	NAD+	stdev	[16s]	stdev	Sample	Gens	NAD+	stdev	[16s]	stdev	Sample	Gens	[16s]	stdev	Sample	Gens	[16s]	stdev
T3001	0	0.850	0.017		0.004	T3001	0	0.850	0.017	0.116	0.026	T2001	0	0.154	0.033	T2001	0	0.081	0.02
T3002	0	0.879		0.093		T3002	0	0.879		0.088		T2002	0	0.098		T2002	0	0.096	
T3003	0	0.849		0.087		T3003	0	0.849		0.065		T2003	0	0.096		T2003	0	0.056	
T3011	17.3	0.497	0.019	0.041	0.002	3011	15.4	0.609	0.027	0.043	0.005	T2011	23	0.061	0.019	2011	23.3	0.112	0.03
T3012	17.3	0.499		0.043		3012	15.4	0.561		0.052		T2012	23	0.067		2012	23.3	0.068	
T3013	17.3	0.531		0.044		3013	15.4	0.606		0.052		T2013	23	0.095		2013	23.3	0.055	
T3021	27.2	0.708	0.041	0.08	0.014	3021	29	0.544	0.02	0.033	0.002	T2021	46.8	0.062	0.014	2021	46.7	0.072	0.01
T3022	27.2	0.764		0.08		3022	29	0.531		0.031		T2022	46.8	0.071		2022	46.7	0.053	
T3023	27.2	0.683		0.105		3023	29	0.571		0.03		T2023	46.8	0.043		2023	46.7	0.068	
T3041	57.4	0.403	0.019	0.028	0.002	3041	57.3	0.414	0.023	0.051	0.02	T2031	69.7	0.111	0.077	2031	69.9	0.056	0.003
T3042	57.4	0.421		0.025		3042	57.3	0.410		0.018		T2032	69.7	0.064		2032	69.9	0.05	
T3043	57.4	0.383		0.03		3043	57.3	0.451		0.016		T2033	69.7	0.214		2033	69.9	0.049	
T3051	70.5	0.435	0.015	0.029	0.003	3051	68.7	0.550	0.06	0.051	0.009	T2041	93	0.055	0.002	2041	93.2	0.059	0.006
T3052	70.5	0.406		0.032		3052	68.7	0.573		0.039		T2042	93	0.053		2042	93.2	0.065	
T3053	70.5	0.412		0.036		3053	68.7	0.663		0.057		T2043	93	0.052		2043	93.2	0.053	
T3061	83.8	0.452	0.041	0.041	0.003	3061	84.1	0.405	0.027	0.006	0.014	T2051	117	0.114	0.012	2051	117	0.086	0.021
T3062	83.8	0.433		0.034		3062	84.1	0.458		0.028		T2052	117	0.089		2052	117	0.077	
T3063	83.8	0.511		0.037		3063	84.1	0.424		0.032		T2053	117	0.104		2053	117	0.118	
T3101	135	0.633	0.048	0.042	0.001	3101	136	0.588	0.029	0.033	0.003	T2061	140	0.067	0.015	2061	140	0.05	0.027
T3102	135	0.681		0.044		3102	136	0.531		0.034		T2062	140	0.047		2062	140	0.093	
T3103	135	0.584		0.042		3103	136	0.565		0.039		T2063	140	0.038		2063	140	0.043	
T3111	150	0.536	0.028	0.022	0.015	3111	144	0.640	0.015	0.042	8E-04	T2091	209	0.055	0.005	2091	210	0.086	0.011
T3112	150	0.540		0.05		3112	144	0.622		0.042		T2092	209	0.063		2092	210	0.08	
T3113	150	0.490		0.026		3113	144	0.651		0.043		T2093	209	0.062		2093	210	0.064	
T3121	163	0.374	0.021	0.024	0.005	3121	163	0.371	0.013	0.029	0.011	T2131	302	0.038	0.006	2131	302	0.03	0.004
T3122	163	0.341		0.034		3122	163	0.365		0.045		T2132	302	0.031		2132	302	0.024	
T3123	163	0.333		0.027		3123	163	0.390		0.024		T2133	302	0.026		2133	302	0.032	
T3131	177	0.285	0.013	0.033	0.002	3131	176	0.432	0.008	0.033	0.004	T2141	326	0.041	0.012	2141	326	0.035	0.007
T3132	177	0.307		0.032		3132	176	0.439		0.028		T2142	326	0.061		2142	326	0.045	
T3133	177	0.306		0.029		3133	176	0.423		0.036		T2143	326	0.061		2143	326	0.031	
T3151	203	0.630	0.037	0.054	0.006	3151	204	0.458	0.006	0.04	0.014	T2161	372	0.072	0.037	2161	372	0.055	0.007
T3152	203	0.556		0.043		3152	204			0.014		T2162	372	0.002		2162	372	0.065	
T3153	203	0.594		0.051		3153	204	0.466		0.018		T2163	372	0.058		2163	372	0.051	
T3161	216	0.526	0.02	0.041	0.007	3161	215	0.640	0.021	0.047	0.006	T2181	419	0.045	0.012	2181	419	0.054	0.015
T3162	216	0.491		0.038		3162	215	0.673		0.035		T2182	419	0.049		2182	419	0.03	
T3163	216	0.490		0.051		3163	215	0.678		0.039		T2183	419	0.068		2183	419	0.058	
T3171	230	0.555	0.02	0.027	0.003	3171	230	0.553	0.024	0.018	0.005	T2201	465	0.056	0.006	2201	465	0.045	0.017
T3172	230	0.618		0.021		3172	230	0.508		0.013		T2202	465	0.043		2202	465	0.07	
T3173	230	0.555		0.028		3173	230	0.542		0.022		T2203	465	0.052		2203	465	0.038	

Figure A.1 Excel data used to propagate the graphs to describe the *E. coli* experiments.

Gens=Generations

Aerobic TC				Aerobic			
		[TetA]				[TetA]	
Sample	Gens	[16s]	stdev	Sample	Gens	[16s]	stdev
T82-1	0	13.11	0.416377	T82-1	0	13.11	0.416377
T82-2	0	12.52		T82-2	0	12.52	
T83-1	19.50409	13.22	0.009269	83-1	20.04751	12.38	0.125969
T83-2	19.50409	13.2		83-2	20.04751	12.56	
T84-1	38.43111	12.79	0.011528	84-1	40.0061	10.77	0.054739
T84-2	38.43111	12.77		84-2	40.0061	10.69	
T85-1	59.29546	15.73	0.089313	85-1	59.9799	3.749	0.187945
T85-2	59.29546	15.6		85-2	59.9799	3.483	
T86-1	77.47053	14.9	0.103365	86-1	79.87217	2.916	0.102886
T86-2	77.47053	15.04		86-2	79.87217	2.771	

Figure A.2 Excel data used to propagate the graph for *P. aeruginosa*. Gens=Generations

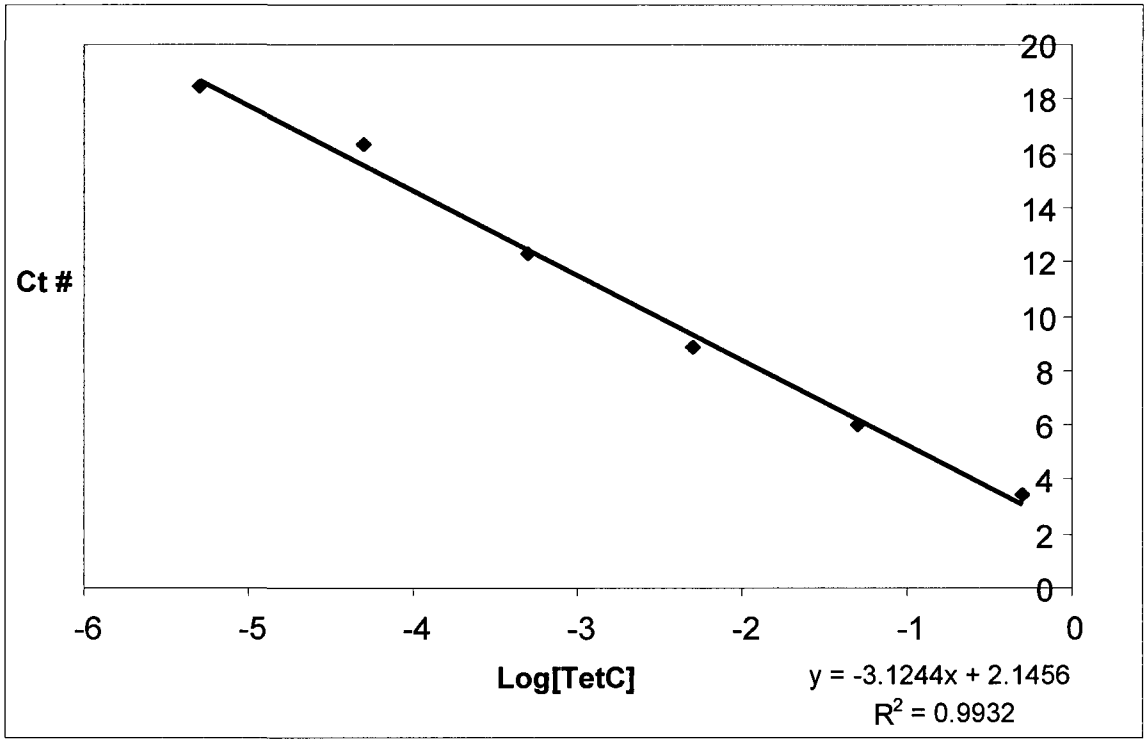

Figure A.3 Standard curve used to calculate the concentration (μg/ml) of TetC gene in *E. coli*. Ct # represents the RTQ-PCR cycle at which the increase in SYBR® Green dye fluorescence begins its exponential increase.

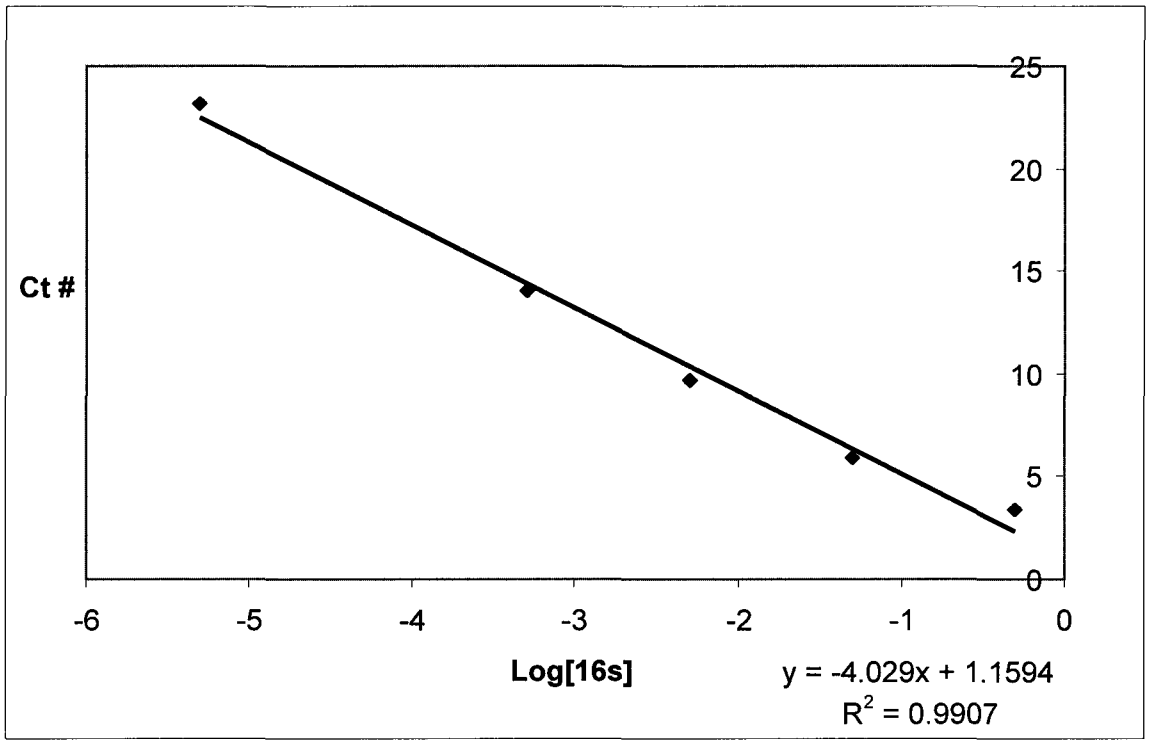

Figure A.4 Standard curve used to calculate the concentration (μg/ml) of 16s gene in *E. coli*. Ct # represents the RTQ-PCR cycle at which the increase in SYBR® Green dye fluorescence begins its exponential increase.

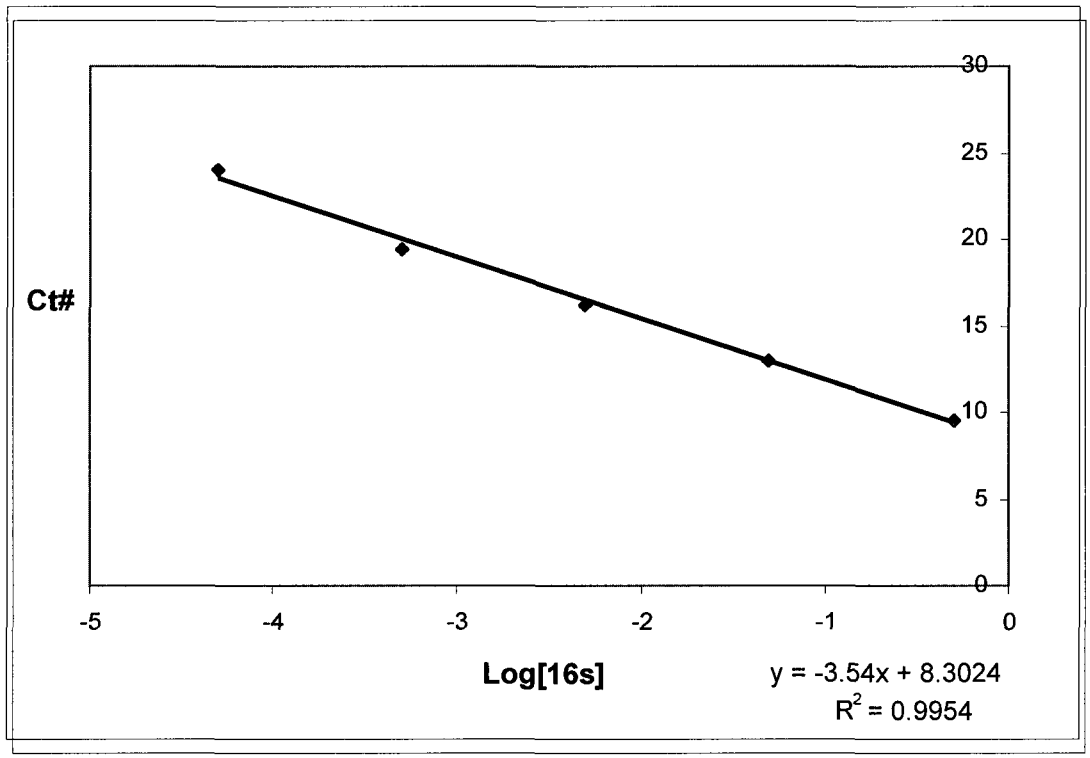

Figure A.5 Standard curve used to calculate the concentration (μg/ml) of 16s gene in *P. aeruginosa*. Ct # represents the RTQ-PCR cycle at which the increase in SYBR® Green dye fluorescence begins its exponential increase.

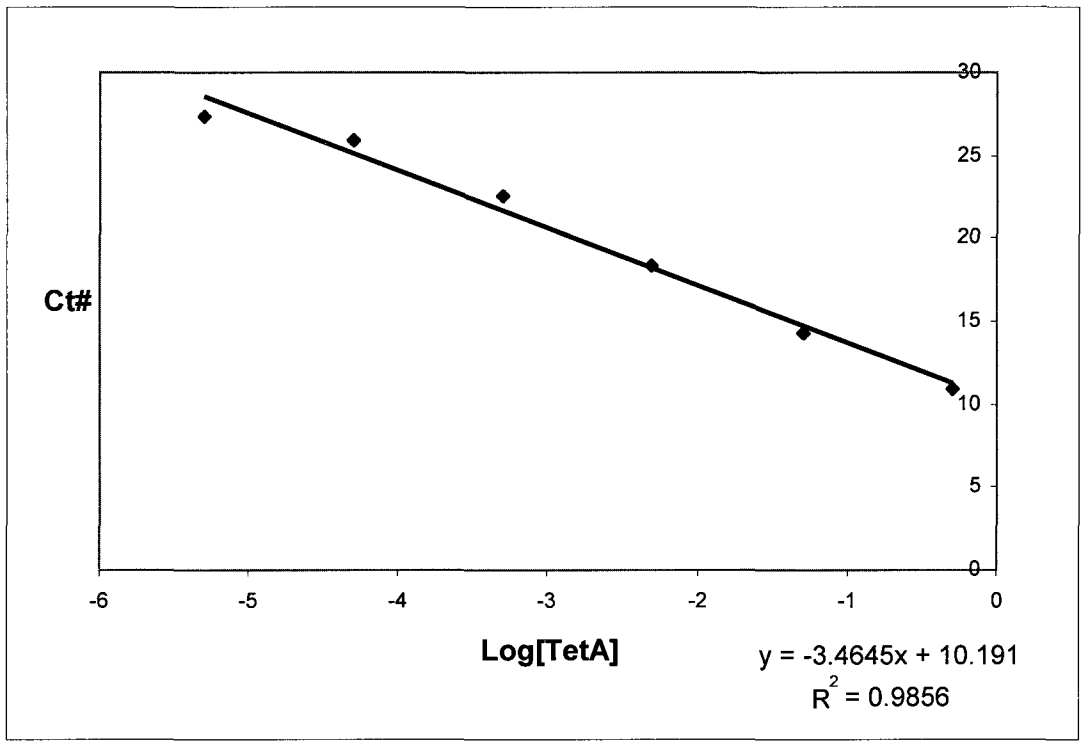

Figure A.6 Standard curve used to calculate the concentration (μg/ml) of TetA gene in *P. aeruginosa*. Ct # represents the RTQ-PCR cycle at which the increase in SYBR® Green dye fluorescence begins its exponential increase.