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CHARACTERIZATION OF TETRACYCLINE EFFLUX GENES IN SOIL BACTERIA AND AN ANALYSIS OF ENVIRONMENTAL FACTORS CONTROLLING THEIR EXPRESSION

presented by

BRIAN MARK CAMPBELL

has been accepted towards fulfillment
of the requirements for the

Master of Science	degree in	Microbiology & Molecular Genetics

Major Professor's Signature

August 7, 2008

Date

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By

Brian Mark Campbell

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Microbiology & Molecular Genetics

2008

ABSTRACT

CHARACTERIZATION OF TETRACYCLINE EFFLUX GENES IN SOIL BACTERIA AND AN ANALYSIS OF ENVIRONMENTAL FACTORS CONTROLLING THEIR EXPRESSION

By

Brian Mark Campbell

Un-altered tetracycline (TC) residues disseminate to soil environments through the practice of applying manure to cropland as fertilizer from animals receiving sub-therapeutic doses of tetracycline for growth promotion purposes. Such practice has created concern that these residues are enhancing the development and transfer of TC resistance in soil environments. However, multiple knowledge gaps exist that need to be examined to address such concerns. First, few investigators have studied tetracycline resistance in soils by molecular methods. Second, it is unknown if TC residues present in the soil environment are bioavailable to microbial cells to exert a selective pressure.

Two separate sets of experiments were conducted to bridge these gaps. TC resistance was examined in an agricultural soil, collected one week after manure application by both culture dependent and independent methods. Novel findings include the discovery of *tet* gene variants *tet*(30), and *tet*(31) in soil, and the first observations of *tet*(A) in *Microbacterium* spp., *tet*(Y) in *Pseudomonas* spp., *tet*(C) and *tet*(31) in *Stenotrophomonas* spp., and *tet*(A) and *tet*(C) in *Thermomonas* spp.

In the second set of experiments bioavailability of cation chelated TC in solution was examined with a tetracycline bioreporter containing a *gfp* gene fused to a tetracycline inducible promoter. Both TC-Mg^{2+} and TC-Ca^{2+} chelation significantly reduced TC efflux gene expression whereas monovalent cations (Na^{+} and K^{+}) had no effect.

This work is dedicated to my two grandfathers, George Campbell and Carl McLaughlin, who instilled in me the value of knowledge and hard work.

ACKNOWLEDGEMENTS

There are a number of people who deserve thanks for helping me throughout my time as a graduate student. In no particular order I would like to thank the following people affiliated with Michigan State University; Uri Levine, Zarraz Lee, Yujing Ding, Erick Cardenas, Jie Deng, Woo Jun Sul, Yu Yang, Ryan Penton, Jeff Urquart, and Drs. Jorge Rodriguez, Dion Antonopolous, Peter Bergholz, Stephan Gantner, Carlos Rodriguez-Minguela, Hui Li, Brian Teppen, Robert Britton, Stephanie Eichorst, Deborah Himes, Mary Beth Leigh, and Jennifer Auchtung. Without their expertise none of this would have been possible. Special thanks go to Dr. Louis King, Dr. Jeff Landgraf, and Shari Tjugum-Holland of the Research Technology Support Facility for their help with various projects. My family, friends, and specifically Rachel Kanine are especially deserving of thanks. Their continued support throughout my time as a graduate student was essential. Credit for this work also goes to current and previous members of my research guidance committee, Dr. Syed Hashsham, Dr. Jay Lennon, Dr. Thomas Schmidt, and Dr. Vince Young. I am grateful to have been able to work with you. I would like to thank the United States Department of Agriculture, and Michigan State University whose financial support made this work possible.

Lastly, and most importantly, I would like to thanks Dr. James Tiedje. His guidance and support as an advisor allowed me to grow as a student and a person and made this work possible.

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CHAPTER I

TETRACYCLINES AND TETRACYCLINE RESISTANCE IN THE ENVIRONMENT

DISCOVERY, STRUCTURE, AND MECHANISM OF ACTION

In 1948 the first tetracycline compound, aureomycin, was isolated from a soil-dwelling Actinomycete, *Streptomyces aureofaciens* (Duggar, 1948). Soon after in 1950 Finlay and coworkers isolated terramycin, the first tetracycline to have its structure solved, from *S. rimosus* (Durckheimer, 1975). The tetracyclines are four-ring molecules with partial conjugation and a carboxyamide functional group (Sarmah, Meyer, & Boxall, 2006). Tetracycline has three distinct functional groups: tricarbonyl methane (pKa 3.3); dimethyl ammonium cation (pKa 9.6); and phenolic diketone (pKa 7.7) (Sarmah et al., 2006). There are a number of structural features important for antibacterial activity. These include the (α) stereochemical configurations at the 4a, 12a (A-B ring junction), and 4 (dimethylamino group) positions, and conservation of the keto-enol system (11, 12, and 12a) in proximity to the phenolic D ring (Chopra & Roberts, 2001). For a review of tetracycline structures see (Oka, Ito, & Matsumoto, 2000). This compound and other derivatives inhibit bacterial translation by binding to the 30S ribosomal sub-unit. More specifically, tetracyclines exert their bacteriostatic effect by binding and inhibiting accommodation of amino-acyl tRNA into the A site (Mitscher, 1978). The tetracyclines have a broad antibacterial spectrum, broader than any other known antibiotic at their time of discovery, and as such they were quickly applied for a variety of purposes.

APPLICATIONS OF TETRACYCLINES

It has been estimated that total antibiotic consumption worldwide lies between 100,000 – 200,000 tons (Kummerer, 2003). Worldwide tetracycline (TC) usage

encompasses a wide variety of purposes including human therapy, aquaculture, animal husbandry, and plant agriculture. Accurate estimations of antibiotic usage are notoriously difficult to obtain, however it is known that agricultural purposes represent a large percentage of usage. For example, it has been estimated that 70% of the 16 million kg of antibiotics used in the United States (U.S.) is for non-therapeutic purposes (Sarmah et al., 2006).

Especially concerning is the fact that developing countries, which account for 25% of world meat production, have poor or absent policies regarding antibiotic use in agriculture (Witte, 1998). In African countries such as Tanzania and Uganda veterinary antimicrobials are easily accessible and under low levels of control from government authorities (Sarmah et al., 2006). Worldwide usage of tetracyclines has created an environment which is potentially enriching tetracycline resistant (TcR) bacteria. The foundation for understanding this problem begins with an understanding of the ways in which tetracyclines are commonly used.

Animal Husbandry. Tetracyclines are commonly used in animal husbandry for purposes of prophylaxis, chemotherapy, and growth promotion. The bulk of tetracycline usage in animal husbandry is for growth promotion, hence the focus of this discussion. Growth promotion refers to the practice of mixing antimicrobials, such as tetracyclines, into animal feed in order to rear a larger, healthier animal. The growth promoting benefits of tetracyclines were first discovered in 1949 when chickens fed by-products of tetracycline fermentation were found to grow faster than those not fed the antibiotic (Phillips et al., 2004). After recognition of the beneficial effects, their application to other animals quickly ensued. Today the greatest usage of tetracyclines is likely in swine

husbandry. In the United States there are 60 to 92 million swine and 40% are reported to be fed chlortetracycline (CTC) for ~ 2 months during the production cycle (Sarmah et al., 2006). The benefits of supplementing antibiotics into feed during swine production occur throughout the entire growth stage of the pig, however, the greatest benefit is in young pigs with an average growth rate improvement of 16.4% and an average increase in feed utilization efficiency of 6.9% (Cromwell, 2002). Overall, animals with antibiotics in their feed gain 4-5% more weight than those without (Witte, 1998).

Surprisingly the basis for why tetracyclines and other antibiotics achieve these increases is still not well understood. Four mechanisms have been proposed for how antibiotics improve animal growth: 1) inhibition of sub-clinical infections 2) reduction of microbial metabolites that reduce animal growth 3) reduced utilization of nutrients by microbes instead of the host animal and 4) nutrient uptake enhancement through thinner intestinal walls associated with antibiotic fed animals (Gaskins, Collier, & Anderson, 2002).

Human Therapy. The most obvious use of the tetracyclines, in the eyes of the public, is use in humans to treat infectious diseases. In the United States five tetracyclines are used for human therapeutic purposes – demeclocycline (DMC), doxycycline (DXC), minocycline (MCLN), oxytetracycline (OTC), and TC (Smilack, 1999). In 1997 wholesale cost of all TC prescriptions filled in U.S. retail pharmacies totaled $400 million (Smilack, 1999).

Use in human therapy has generally declined because of increasing pathogen resistance (Chopra & Roberts, 2001), however, there are still situations in which the tetracyclines are useful. Tetracyclines are still the drug of choice for infections with

Chlamydia trachomatis (nongonococcal urethritis, pelvic inflammatory disease, and lymphogranuloma venereum), *Rickettsiae* spp. (rocky mountain spotted fever, and endemic and epidemic typhus Q fever), *Borrelia recurrentis* (Brucellosis, Lyme borreliosis, Ehlrichosis, and Relapsing fever), *Vibrio* spp. (cholera), *Helicobacter pylori* (gastritis and peptic ulcer disease), and *Plasmodium falciparum* (malaria) (Chopra & Roberts, 2001; Smilack, 1999). Tetracycline is also still used for treatment of acne (Ross, Eady, Cove, & Cunliffe, 1998).

There has also been development in new applications of tetracyclines. The discovery the tetracycline can inhibit prion infectivity, infections associated with Alzheimer's disease and bovine spongiform encephalopathy (mad cow disease), has sparked interest in nontraditional uses (Tagliavini et al., 2000). The most recent development in tetracycline therapy is the approval of tigecycline, a third generation tetracycline derivative (more specifically a glycylcycline), for human therapy (Shlaes, 2006).

Aquaculture. In a span of 15 years, annual aquaculture production increased from 16.8 million tons in 1990 to 52.9 million tons in 2005, and is expected to reach 172 million tons by 2015 (A. Sapkota et al., 2008). OTC is commonly used to treat a wide range of bacterial infections in aquaculture. It is actually the most commonly used antibiotic in aquaculture (A. Sapkota et al., 2008), with 92% of the top 13 aquaculture producing countries employing its use. In the U.S., FDA approved uses for OTC include treatment of disease in Pacific salmon (250 mg/kg*day for 4 days), salmonids (2.5-3.75 g/100lb*day for 10 days), catfish (same as salmonids), and lobster (1g/lb medicated feed for 5 days) (Shao, 2001). In countries other than the U.S. commercial shrimp feeds are

commonly enriched with OTC (Graslund & Bengtsson, 2001). There are three major routes of administration; water treatment, incorporation into feed, and injection (Cabello, 2006).

Unfortunately, due to few antimicrobials approved for aquaculture in Norway and North America "off-label" usage can occur. "Off-label" refers to treatments at higher doses than approved, by a route not approved, treatment for a non-approved disease, or treatment of non-approved species (Burka et al., 1997). Furthermore, in Asian countries, which account for the bulk of world aquaculture production, there are few if any guidelines for OTC usage beyond residuals for exported products (A. Sapkota et al., 2008).

Plant Agriculture. A minor but significant use of tetracyclines is in the control of plant pathogens. The potential of antimicrobials to treat plant disease was recognized as early as the 1950s (McManus, 2000). OTC is registered in the U.S. for use on pear for control of *Erwinia amylovora* which causes fire blight, and on peach and nectarine for control of *Xanthomonas arboricola*, the causative agent of bacterial spot (McManus, Stockwell, Sundin, & Jones, 2002). In Mexico, OTC is used for *E. amylovora* on apples and in Latin America OTC is used on vegetable crops (McManus et al., 2002). OTC has also been used to control disease caused by phytoplasmas (McManus, 2000). Application rates of OTC are 3 gallons of 150 ppm solution per tree or 240 gallons per acre for peaches and at 200 ppm are 50 – 100 gallons of solution per acre for pears (Vidaver, 2002). The application rate for peaches can be increased for larger trees but is not to exceed 500 gallons per acre per application (Vidaver, 2002).

In the U.S., total antibiotic usage on plants ranges from 20,000 to 65,000 kg annually, which represents less than 0.5% of the total antibiotic production (McManus et al., 2002). In 1999, the USDA estimated that 40% of pear acreage received 12,000 lbs of OTC and apples received 3,000 lbs of OTC on 5% of the acreage (Vidaver, 2002). Throughout the 1990s OTC usage increased significantly, mainly due to increased streptomycin (the other antibiotic commonly used in plant agriculture) resistance in *E. amylovora* (Vidaver, 2002).

DEVELOPMENT OF RESISTANCE TO TETRACYCLINES

The development and spread of resistance to tetracycline includes the evolution of antibiotic resistance traits and the subsequent horizontal transfer to diverse microbiota. Below I provide an overview of the mechanisms of tetracycline resistance, followed by a discussion of the evolutionary origins that have resulted in the diversity of resistance genes today. This is followed by a discussion of horizontal transfer mechanisms that have allowed for the dissemination of resistance genes throughout the bacterial domain (Tables 1.1 to 1.5).

Mechanisms of Resistance. There are at least four types of resistances to tetracycline; tetracycline efflux, ribosomal protection, enzymatic inactivation, and ribosomal RNA mutation (Chopra & Roberts, 2001). For up to date information regarding the TC resistance genes see Dr. Marilyn C. Roberts' website:

http://faculty.washington.edu/marilynr/

The tetracycline efflux genes export tetracycline in an energy dependent manner from the cytoplasm thereby detoxifying the cell by reducing the intracellular concentration (Chopra & Roberts, 2001). Currently 23 tetracycline efflux genes have

been identified, the most recent of which was *tet*(41) found in an environmental strain of *Serratia marcescens* (Thompson, Maani, Lindell, King, & McArthur, 2007) (Table 1.1). Generally, the tetracycline efflux genes are found in Gram-negative bacteria, the exceptions being *tet*(A), *tet*(L), *tet*(K), *tet*(33), *tet*(Z), *tetA*(P), and *otr*(B).

The tetracycline efflux genes are regulated by a repressor protein, TetR, which controls expression of the efflux pump as well as its own expression (Chopra & Roberts, 2001). Upon binding a TC-Magnesium complex, TetR undergoes a conformational change allowing release from two operators that overlap the divergently orientated promoters for both the efflux pump, Tet(A), and the repressor protein, thereby allowing mRNA transcription to proceed (Chopra & Roberts, 2001). This regulatory system is the most sensitive, effector-inducible transcriptional regulation system described to date (Chopra & Roberts, 2001).

Ribosomal protection proteins (RPPs) represent the second most diverse mechanism of tetracycline resistance based on current data. RPPs mitigate tetracycline's effect on the bacterial cell by dislodging TC from the ribosome in a GTP dependent manner (Connell, Tracz, Nierhaus, & Taylor, 2003). Currently there are 11 recognized classes of RPPs. G+C% of sequence data suggests that the RPPs originated in Gram-positive bacteria (Chopra & Roberts, 2001). The RPPs are well distributed throughout both Gram-positive and Gram-negative organisms. Seven of the eleven RPPs (*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32), *tet*(Q), *tet*(36)) have been described in gram-negative bacteria (Tables 1.1 – 1.5).

Regulation of the expression of RPPs is not well understood; however, it is known to differ significantly from that for the efflux pumps. It is speculated that RPPs are

regulated by translational (or transcriptional) attenuation of mRNA (Chopra & Roberts, 2001).

Enzymatic inactivation has not been as extensively characterized as the previous two mechanisms of resistance. Currently only three enzymatic inactivation genes are described, *tet*(X), *tet*(34), and *tet*(37). Interestingly, each of these genes appears to be unique in their mechanism of resistance. The *tet*(34) and *tet*(37) genes are recently discovered and hence, are not as well described. *tet*(34), originally isolated in *Vibrio* spp., encodes a protein that has homology to bacterial xanthine-guanine phosphoribosyltransferases (Nonaka & Suzuki, 2002). The *tet*(37) gene was cloned from an oral metagenome, and is not homologous to *tet*(X), but appears to have a similar mechanism of resistance (Diaz-Torres et al., 2003).

The *tet*(X) gene was originally discovered in a *Bacteroides fragilis* strain (Guiney, Hasegawa, & Davis, 1984) but was inactive due to its requirement for oxygen to function. When transferred into aerobically grown *Escherichia coli*, *tet*(X) was able to detoxify tetracycline (Speer & Salyers, 1988). It was later shown that the Tet(X) protein is a flavin dependent monooxygenase requiring FAD, NADPH, Magnesium, and Oxygen to detoxify tetracyclines by regiospecifically hydroxylating carbon 11a which results in this product breaking down inside of the cell (Speer, Bedzyk, & Salyers, 1991; W. Yang et al., 2004).

The fourth major mechanism of tetracycline resistance is provided by bacteria acquiring mutations in their ribosomal RNA (*rrn*) genes. In the gram-positive bacterium *Propionibacterium acnes*, the suspected causative agent of acne, a G to C mutation at *E. coli* equivalent base 1058 occurring in helix 34 of the *rrn* gene was shown to confer

resistance to tetracycline (Ross et al., 1998). The gram-negative bacterium *Helicobacter pylori* has been shown to accumulate mutations conferring tetracycline resistance in the *rrn* gene at base pairs 965 – 967 in helix 31 (Dailidiene et al., 2002), and at base pairs 926 – 928 (Gerrits, de Zoete, Arents, Kuipers, & Kusters, 2002). It is likely that these mutations are similar in that they all, either directly or indirectly, affect the binding of tetracycline to the ribosome. This claim is supported by crystal structures of tetracycline bound to the 30S ribosomal subunit showing interaction with both the helix 31 and 34 loops of the 16S rRNA (Brodersen et al., 2000).

Evolutionary Origins of Tetracycline Resistance Genes. Multiple biosynthetic pathways for antibiotic synthesis have been estimated at being between 200 and 800 million years old (Wright, 2007). If antibiotics are ancient then it can be postulated that resistances to those antibiotics are ancient as well. Combine this with the recent knowledge that horizontal transfer is a major contributor in evolutionary processes (Koonin, 2003) and it is not surprising that resistance to tetracycline was discovered in *Shigella dysenteriae* only 5 years after the introduction of the antibiotic for human therapy purposes (Chopra & Roberts, 2001). This raises an interesting but simplistic question: Where did these genes come from?

It is generally thought that there are two main routes through which antibiotic resistance has evolved: (a) evolution of resistance in antibiotic producers as self protection and (b) in other microbes as protection against antibiotics produced by the organisms described in (a) (Wright, 2007).

Most clinically relevant antibiotics are produced by soil dwelling actinomycetes (D'Costa, McGrann, Hughes, & Wright, 2006), therefore, one may expect that these

Table 1.1. Bacteria Isolated with Tetracycline Resistance Genes from Water Environments

Division	Genus	Gram	Env.	Gene(s)
Actinobacteria	Microbacterium	P	GW	M
	Streptomyces	P	AC	Otr(B)
α-Proteobacteria	Afipia	N	GW	M
	Brevundimonas	N	AC	B
	Roseobacter (3)	N	SW	G, BC, EG
Bacteroidetes	Flavobacterium	N	AC	AEM
β-Proteobacteria	Alcaligenes	N	SD	E
	Alteromonas	N	AC	D
	Variovorax	N	GW	L
Firmicutes	Enterococcus (3)	P	AC	L, M, S
	Lactococcus	P	AC	M, S
γ-Proteobacteria	Acinetobacter (4)	N	AC, SC, SW	39, H, 39, G
	Aeromonas (17)	N	AC, HE, SD	31, A, B, D, E, AB, AE, AM, DM, EM, DEM, A, A, D, E, Y, AE
	Citrobacter (7)	N	AC, SW	A, B, D, AB, AC, A, B
	Edwardsiella (2)	N	AC	A, AM
	Enterobacter (3)	N	AC	A, B, D
	Escherichia (11)	N	AC, SD, ST, **TP**	A, B, D, AB, AC, E, A, B, **A, B, AB**
	Halomonas	N	SW	CD
	Klebsiella (5)	N	AC, SW	A, D, A, B, D
	Moraxella	N	AC	H
	Morganella	N	AC	L
	Photobacterium (4)	N	AC	B, M, Y, BY
	Plesiomonas (4)	N	AC	A, B, D, AB
	Proteus	N	SW	E
	Providencia	N	SD	E
	Pseudoalteromonas (3)	N	AC	B, M, DE
	Pseudomonas (7)	N	AC, SD, SW	A, B, B34, A, C, G, E
	Salmonella	N	AC	D
	Serratia (3)	N	AC, SD, ST	B34, E, 41

Table 1.1 Continued

Division	Genus	Gram	Env.	Gene(s)
γ-Proteobacteria	Shewanella (2)	N	SW	D, G
	Stenotrophomonas	N	AC	35
	Vibrio (17)	N	AC, SD, SW	34, A, B, D, M, AB, AD, BD, BM, EM, ABD, ABM, BDM, A. B, G, E

Key: **AC = Aquaculture**, SG = Sewage, **SD = Sediment**, ST = Stream, **TP = Tap Water**, SW = Seawater, **HE = Hospital Effluent**, **GW = Groundwater**

References (see pg 67)

Table 1.2. Bacterial Isolates from Soil with Tetracycline Resistance Genes

Division	Genus	Gram	Env.	Gene(s)
Actinobacteria	Arthrobacter	P	AS	33
	Corynebacterium (2)	P	AS, UN	Z, 33
	Streptomyces (5)	P	AS, UN	Otr(B), Otr(A) Otr(B), L, Otr(A), Otr(B)
Bacteroidetes	Chryseobacterium	N	AS	A
	Sphingobacterium	N	AS	X
Firmicutes	Aerococcus	P	AS	M
	Bacillus (5)	P	AS, NAS	L, M, AL, LM, L
	Enterococcus	P	AS	M
	Kurthia	P	AS	LM
	Lactococcus	P	AS	S
	Oceanobacillus	P	AS	L
	Paenibacillus (2)	P	AS, NAS	M, L
	Sporosarcina	P	AS	L
	Staphylococcus (6)	P	AS	A, K, L, M, AL, LM
	Virgibacillus (2)	P	AS, NAS	L, L
α-Proteobacteria	Brevundimonas	N	AS	G
	Ochrobactrum (3)	N	AS	G, AB, GL
β-Proteobacteria	Alcaligenes	N	AS	A
	Variovorax	N	AS	A
γ-Proteobacteria	Acinetobacter	N	AS	M
	Citrobacter (7)	N	AS, NAS	A, M, AM, BS, GM, MS, BMOW, OSW
	Enterobacter (5)	N	AS, NAS	A, B, M, AG, M
	Escherichia (9)	N	AS, B	L, M, W, AW, BW, AMW, A, C, E
	Hafnia (2)	N	NAS	M, W
	Klebsiella (4)	N	AS, NAS	A, S, M, MW
	Morganella	N	AS	D
	Pantoea	N	AS	B
	Proteus	N	AS	M
	Providencia	N	AS	M

Table 1.2 Continued

Division	Genus	Gram	Env.	Gene(s)
γ-Proteobacteria	Pseudomonas (6)	N	AS	A, C, G, AB, AL, AM
	Psychrobacter	N	AS	O
	Rahnella	N	AS	M
	Serratia (4)	N	AS, NAS	A, B, AM, C
	Shigella	N	AS	M
	Stenotrophomonas	N	AS	GL

Key: AS = Agricultural Soil, B = Beach, NAS = Non-Agricultural Soil, UN = Unclassifiable Soil (when soil type could not be determined from publication)
References (see pg 70).

Table 1.3. Tetracycline Resistant Bacteria Isolated from Concentrated Animal Feeding Operation (CAFO) Environments

Division	Genus	Gram	Env.	Gene(s)
Bacteroidetes	Bacteroides	N	MS	36
	Flavobacterium	N	MS	L
Firmicutes	Bacillus	P	F	L
	Enterococcus	P	IA, MS	L, M, LM, KLM, M
	Kurthia	P	MS	L
	Lactobacillus	P	MS	M
	Listeria	P	BD, F, MS	A, A, A
	Staphylococcus	P	MS	M
	Streptococcus	P	IA	L, M, LM, KLM
γ-Proteobacteria	Citrobacter	N	F	L
	Enterobacter	N	F	L
	Escherichia	N	MS	L
	Proteus	N	MS	L

Key: BD = Bedding, F = Feed, IA = Indoor Air of Swine CAFO, MS = Manure Storage
References (see pg 70).

Table 1.4. Tetracycline Resistant Bacteria Isolated from Food

Division	Genus	Gram	Env.	Gene(s)
Firmicutes	Enterococcus (6)	P	Food	L, M, O, S, LM, MO
	Lactobacillus	P	Food	M
	Listeria (3)	P	Food	K, M, KM
ε-Proteobacteria	Campylobacter	N	Food	O
γ-Proteobacteria	Aeromonas (2)	N	Food	A, E
	Salmonella	N	Food	A

References (see pg 71)

Table 1.5. Tetracycline Resistant Bacteria Isolated from Animal Sources

Division	Genus	Gram	Env.	Gene(s)
Actinobacteria	Arcanobacterium (2)	P	C, Sw	W, W
	Arthrobacter	P	Sw	33
	Bifidobacterium (2)	P	Ot	O, W
	Streptomyces	P	Ch	Otr(B)
Bacteroidetes	Bacteroides (3)	N	Sw	Q, W, 36
	Prevotella (3)	N	C, Ot, Sw	Q, Q, Q
Chlamydiae	Chlamydia	N	Sw	C
Firmicutes	Acidaminococcus	N	Sw	W
	Anaerovibrio (2)	N	Sw	O, OQW32
	Bacillus	P	Sw	L
	Butyrivibrio	P	C	W
	Clostridiaceae (2)	P	Sw	O, OQW32
	Clostridium (6)	P	Ot, Sw	TetA(P), TetA(P), TetB(P), W, OW, OQ32, OQW32
	Enterococcus (14)	P	C, Ch, Ot, Sw	O, L, M, O, M, O, LM, KLM, LMS, K, L, M, O, S
	Eubacterium (3)	P	Sw	O, O32, OQW32
	Lactobacillus (3)	P	Sw	36, OW, OW32
	Megasphaera (7)[a]	N	Sw	O, W, OW(mos), OWO-1(mos), OWO-2(mos)
	Mitsuokella (2)	N	Ot, Sw	W, W
	Ruminococcus (2)	P	Sw	O, OQW32
	Selenomonas (2)	N	C, Sw	W, W32
	Staphylococcus (12)	P	C, Ch, Ot, Sw	K, K, LM, K, M, K, L, M, KL, KM, LM, KLM
	Streptococcus (8)	P	C, Sw	L, M, O, KL, LO, MO, M, O
	Subdoligranulum	N	Sw	OQW
Fusobacteria	Fusobacterium	N	Sw	O
Spirochaetes	Treponema	N	Sw	OW
ε-Proteobacteria	Campylobacter (2)	N	Ch, Sw	O, O
γ-Proteobacteria	Actinobacillus	N	Sw	B

Table 1.5 Continued

Division	Genus	Gram	Env.	Gene(s)
γ-Proteobacteria	Enterobacter	N	Sw	A
	Escherichia (20)	N	C, Ch, Ot, Sw	A, B, C, AB, AC, A, B, D, AB, A, B, A, B, C, D, L, AB, AC, BC, BE
	Mannheimia (3)	N	C	G, H, L
	Pasteurella (8)	N	C, Ot, Sw	B, H, L, M, G, H, B, H
	Proteus	N	Sw	L
	Pseudomonas	N	Sw	L
	Rahnella	N	Sw	L
	Salmonella (6)	N	C, Ot, Sw	A, A, A, B, G, BE

Key: **C = Cattle, Ch = Chicken, Sw = Swine, Ot = Other** (Includes: sheep, poultry, bison, wild boar, dog, cat, turkey, laying hen, fowl, and when animals could not be differentiated). (mos) = These genes are not recognized nomenclature but are genes where recombination events have occurred between the O and W variants.

References (see pg 72)

microbes represent a reservoir of antimicrobial resistance genes. In fact, this hypothesis was first put forth by Walker & Walker in 1970 (Walker & Walker, 1970). D'Costa et al. (2006) recently tested this hypothesis. They screened a library of approximately 400 *Streptomyces* spp., which produce over half of all known antibiotics, isolated from various soil environments for their ability to evade 21 different antibiotics representing natural, semi-synthetic, and fully synthetic antibiotics that act on eight major bacterial targets. On average strains were resistant to between seven to eight drugs and two strains were resistant to 15 of the 21 tested (D'Costa et al., 2006). Sixty percent were resistant to tetracycline, 1% to minocycline, and 30% to tigecycline (D'Costa et al., 2006), indicating that soil microbes represents a reservoir for resistance even to drugs that have been recently introduced. Some of this resistance to tetracycline could indicate novel genes. More evidence that soil microbes are a reservoir of tetracycline resistance genes was provided by Dr. Handelsman's group when they isolated a novel tetracycline efflux gene, *tcr*, from a metagenomic library with DNA extracted and cloned from a pristine soil sample (Riesenfeld, Goodman, & Handelsman, 2004).

The above studies demonstrate that Walker & Walkers' postulation that antibiotic producing organisms harbor antibiotic resistance genes is in fact true, however, they do not provide an answer to the origins and mechanisms by which tetracycline resistance genes developed. Multiple groups have addressed the question of describing the origins of both the tetracycline efflux and the ribosomal protection genes.

The tetracycline efflux genes known today have 12 and 14 transmembrane sequences (TMS) (Paulsen, Brown, & Skurray, 1996). Today's efflux genes may be ancestral to a 6-TMS gene that once had the physiological important function of

transporting sodium and potassium out of the cell (Guillaume, Ledent, Moens, & Collard, 2004). This hypothesis is based on two observations. First, the gram-positive tetracycline resistance genes *tet*(K) and *tet*(L) confer low levels of resistance to tetracycline and also can catalyze transport of sodium and potassium ions, and second, the observation that a truncated Tet(K) protein can still transport potassium (Guillaume et al., 2004). If present day gene variants are ancestors of ancient ion pumps, then at some point, preference for tetracycline exclusion must have evolved. Monophyletic origin of the tetracycline efflux pumps has been demonstrated and it has been suggested that the specificity towards tetracycline appeared once, around the time they separated from the multi-drug efflux sub-cluster, and was maintained despite considerable sequence divergence resulting in present day gene variants (Aminov et al., 2002).

Considerably more work has been done regarding the evolution of the RPP genes. Sanchez-Pescador and colleagues first showed that the *tet*(M) gene has significant sequence identity, which implies homology, to the translation elongation factors EF-G and EF-Tu (Sanchez-Pescador, Brown, Roberts, & Urdea, 1988). Since then, multiple groups have worked to untangle the origins of these genes.

The observation has been made that, based on available sequences, there is no evidence for recent transfer of RPPs from antibiotic producing strains to other bacteria (Aminov, Garrigues-Jeanjean, & Mackie, 2001; Lau, Woo, To, Lau, & Yuen, 2004). Indeed it appears that ancestral RPPs were present before divergence of the three superkingdoms, and before the evolution of tetracycline production, and that EF-G and the RPPs share common ancestry (Aminov et al., 2001; Kobayashi, Nonaka, Maruyama, & Suzuki, 2007). Hence, the current theory is that the translational elongation factors

EF-G and EF-Tu shared a common ancestor with the present day RPPs. Over time specificity towards tetracycline developed in the RPPs and was maintained. Further evidence, besides considerable sequence identity in the N-terminal region of the two genes, is that both the translational elongation factors and the RPPs hydrolyze GTP in a ribosome dependent manner (Connell et al., 2003).

Recent recombination events between *tet*(O) and *tet*(W) genes in *Megasphaera elsdenii* and separately in the *tet*(M) gene variants has further driven the evolution of RPPs (Oggioni, Dowson, Smith, Provvedi, & Pozzi, 1996; Stanton & Humphrey, 2003; Stanton, McDowall, & Rasmussen, 2004). It is tempting to speculate that perhaps recombination events such as this could provide for higher levels of resistance and for resistance to newer tetracycline derivatives such as tigecycline which are active against bacteria harboring traditional tetracycline resistance genes.

In summary, soil-dwelling antibiotic producers as well as native soil flora represent reservoirs of tetracycline resistance genes. Both tetracycline efflux and RPP genes have long evolutionary histories, with ancestry common to physiologically important functions that likely predate the evolution of tetracycline biosynthesis. This is a striking illustration of the function of what Gerard D. Wright has termed the "antibiotic resistome". The resistome contains all resistance genes, including those in pathogens and non-pathogens, cryptic embedded resistance genes, such as *tet*(X) described above in *Bacteroides sp.* (Speer & Salyers, 1990), and precursor genes, as evidenced by genes ancestral to present day tetracycline efflux genes and RPPs, that given sufficient time and appropriate selective pressure could evolve resistance functions (Wright, 2007). This is not a static system, but an ever-evolving reservoir which with sufficient time, and

through horizontal transfers, allows diverse phyla to become resistant to nearly any antimicrobial compound.

Horizontal Transfer. Aminov et al. (2001) speculated that the rapid movement of RPPs to taxonomically divergent bacteria is probably attributable to horizontal transfer. They also suggested that specificity toward tetracycline in efflux pumps appeared once and was maintained (Aminov et al., 2002). It follows from this assumption that horizontal transfer is also responsible for the dissemination of tetracycline efflux genes. This begs the question: How do tetracycline resistance genes transfer to diverse bacteria?

There are three mechanisms that allow for horizontal transfer of DNA. These include conjugation, transduction, and transformation. Conjugation is a process in which two bacteria mate and DNA is transferred from one bacterium to another by the transfer mechanisms of a self-transmissible DNA element (generally a plasmid) (Snyder & Champness, 2003). Transduction refers to a process in which DNA is transferred between two bacteria by a phage. There are two types of transduction, generalized and specialized. In generalized transduction essentially any region of bacterial DNA can be transferred from one bacterium to another (Snyder & Champness, 2003). Transfer via specialized transduction is limited to genes located close to the attachment site of a lysogenic phage in the bacterial chromosome (Snyder & Champness, 2003). Lastly, transformation is the process in which bacterial cells take up DNA from the environment.

Horizontal dissemination of genes is further enhanced by mobile elements such as transposons and integrons that can both act in a bacterial chromosome or inside a plasmid framework. A transposon is a DNA sequence that can move from one location in a

bacterial chromosome to another, or to another DNA element present in the same cell such as a plasmid (Snyder & Champness, 2003). A special type of transposon, a conjugative transposon, contains genes that code for its own transfer (Snyder & Champness, 2003) and can also mobilize other elements in *cis* or *trans*. Integrons are the most recently described agent of genetic change. They are assembly platforms that can incorporate exogenous circular DNA (gene cassettes; containing a single gene and a 3' attachment site) through site-specific recombination and express them by their outward orientated Pc promoter (Mazel, 2006). Integrons can be present in a chromosome (super-integrons) or on mobile elements such as conjugative plasmids and transposons (Mazel, 2006).

The gram-negative tetracycline efflux genes are typically present on transposons inserted into diverse plasmids (Roberts, 2003). With respect to tetracycline resistant environmental isolates, multiple groups have found *tet*(A) located in the non-conjugative transposon Tn*1721* or Tn*1721*-like transposons in diverse plasmids from bacterial isolates from untreated hospital effluent (Rhodes et al., 2000), the normal flora of swine (Sunde & Sorum, 2001), and Michigan apple orchards (Schnabel & Jones, 1999). Less work has been done with regarding the Gram-positive TcR efflux genes in environmental isolates, however, they are typically found on small plasmids (Chopra & Roberts, 2001).

Generally speaking, the RPPs *tet*(S) and *tet*(O) have been found on conjugative plasmids or the chromosome whereas *tet*(M) and *tet*(Q) have been found on conjugative transposons (Chopra & Roberts, 2001). The *tet*(M) gene is often associated with Tn*916*/Tn*1545*-like conjugative transposons (Agerso, Pedersen, & Aarestrup, 2006; Billington & Jost, 2006; De Leener, Martel, Decostere, & Haesebrouck, 2004; Wilcks,

Andersen, & Licht, 2005). The Tn*916*/Tn*1545*-like transposons are the most promiscuous conjugative transposons described, and have a host range including both Gram-positive and Gram-negative genera (Roberts, 2005). Dr. Marilyn Roberts has hypothesized that the host-range of the mobile genetic element carrying a given *tet* gene will ultimately determine its distribution throughout the bacterial superkingdom (Roberts, 2003). This hypothesis appears to have support as *tet*(M) is one of the more broadly distributed resistance genes in bacterial isolates of environmental origin being found in 31 genera (Tables 1.1 – 1.5).

Unfortunately most work regarding horizontal transfer of these genes has been done with isolates of clinical origins. Publications in which the authors have characterized resistance genes as well as the mobile genetic elements on which they reside in environmental isolates are conspicuously absent from public databases. Especially absent is an understanding of the role of integrons in tetracycline resistance. Work in this area represents a knowledge gap that is necessary to fill to have a comprehensive view of the fate of tetracycline resistance genes in environmental compartments. Understanding this dynamic will allow a better judgment of the risk associated with the contamination of environments with tetracycline residues.

CROSS-RESISTANCE, CO-SELECTION, AND FITNESS

Multiple studies have shown that a bacterium carrying a plasmid with an antibiotic resistance gene is at a fitness disadvantage to an isogenic progenitor without the plasmid in the absence of antibiotic selective pressure (Nguyen, Phan, Duong, Bertrand, & Lenski, 1989). However, it is also commonly observed that in multiple environments, upon withdrawal of antibiotic usage resistance levels remain high (Ghosh

& LaPara, 2007; Langlois, Cromwell, Stahly, Dawson, & Hays, 1983). Clearly there are non-obvious factors that help maintain tetracycline resistance in environmental settings after it has developed.

Cross-resistance. Cross-resistance refers to the ability of one gene to encode resistance to multiple compounds. Cross-resistance is problematic because genes that can confer resistance to diverse compounds can be selected by the selective pressure of only one compound. With reference to tetracycline the best examples are multidrug efflux pumps. Multidrug efflux pumps (MDE) are membrane bound pumps which confer resistance to a wide array of compounds. One such example is the *acrAB* operon in *E. coli*. Expression of this operon increases with increasing concentrations of tetracyclines (Viveiros et al., 2007). Indeed the *acrAB* MDE pump has the ability to confer resistance to tetracycline as well as other compounds (Elkins & Nikaido, 2002). *Pseudomonas aeruginosa* has an MDE pump, the *mexXY-oprM* system, that is induced and exports tetracycline among other compounds (Poole, 2005). *Shewanella oneidensis* MR-1 also contains a similar pump that confers slight resistance to tetracycline (Groh, Luo, Ballard, & Krumholz, 2007).

Co-selection. Co-selection can occur when two separate genes conferring resistance to different compounds are resident on the same mobile genetic element. Selective pressure provided by one compound can select for one resistance gene and also maintain another co-resident, non-selected gene. Observance of this phenomenon has been fairly common with TcR elements and examples in the literature are numerous. For example, the conjugative plasmid R478 contains genes for both mercury and tetracycline resistance (Gilmour, Thomson, Sanders, Parkhill, & Taylor, 2004). An IncF plasmid,

pRSB107, isolated by a culture independent approach from a sewage-treatment plant encodes nine different antibiotic resistance genes (Szczepanowski et al., 2005). Schnabel & Jones (1999) observed that 100% of the TcR phylloplane bacteria analyzed from a Michigan apple orchard were linked with the streptomycin phosphotransferase genes *strB-strA*. These three studies exemplify the increasing awareness of the role of co-selection in tetracycline resistance gene dissemination.

Fitness Effects. As mentioned above, and as has been demonstrated with tetracycline resistance on a plasmid, in the absence of antibiotic, carriage of a plasmid results in reduced fitness (Nguyen et al., 1989). Given this situation, one might expect that with antibiotic withdrawal and sufficient time the plasmid would be cured and resistant bacteria would once again become susceptible. However, Dr. Richard Lenski's elegant work has shown that this may not always happen. Given sufficient generations, compensatory mutations can occur in cells which negate the ill effects of plasmid carriage. In fact, the tetracycline resistance gene (*tet*(B)) residing on the plasmid actually provided a competitive advantage to host cells that had acquired compensatory mutation(s) which allowed carriage of the plasmid without reduced fitness effects (Lenski, Simpson, & Nguyen, 1994). Another study by Dr. Lenski's group revealed that due to the tight regulation of the *tet*(B) tetracycline efflux gene on the Tn*10* transposon by the TetR protein, carriage of Tn*10* essentially poses no burden on the microbial cell (Nguyen et al., 1989). It is important to keep the scale in perspective as this study focused on the tetracycline gene and not the plasmid as a whole. The fitness disadvantage of a plasmid must be the sum of all fitness effects of each gene resident on that particular plasmid (Lenski et al., 1994).

Another mechanism by which bacterial fitness can be enhanced by carriage of genes that have the ability to confer resistance to tetracycline is if that gene can provide a physiological benefit to the host organism. As stated above in the evolution section, the Gram-positive tetracycline efflux genes *tet*(L) and *tet*(K) can transport sodium and potassium ions (Krulwich, Jin, Guffanti, & Bechhofer, 2001). Recently, Groh and colleagues (2007) were able to show that (a) the *mexF* gene in *S. oneidensis* MR-1 is associated with increased fitness in sediment environments and (b) this gene is required for resistance to tetracycline. Both of these examples demonstrate the recent realization that other factors are contributing to the maintenance of resistance in natural environments.

DISSEMINATION OF RESISTANCE IN THE ENVIRONMENT

The evolution of tetracycline resistance genes and mechanisms to horizontally transfer them between diverse phyla, along with the anthropogenic use of tetracyclines, and non-antibiotic selective pressures has likely contributed to the environmental dissemination of tetracycline resistant bacteria. How much impact humans have had on this process is hotly debated. The goal here is not to debate this issue, but to provide an overview of the major pathways through which this dissemination occurs.

The various uses of tetracycline antibiotics including human therapy, animal husbandry, aquaculture, and plant agriculture were described above. Inevitably tetracycline residues and resistant microbes make their way to the environment. Figure 1.1 shows the anthropogenic use of tetracyclines including the primary and secondary reservoirs of tetracyclines, resistance genes, resistant bacteria, and the major paths in which they can be transferred from one environment into another. Primary reservoirs

refer to environments that receive direct input of tetracyclines through human activity whereas secondary reservoirs refer to environments in which accumulation of tetracyclines occurs due to transfer from a primary reservoir. Figure 1.1 illustrates a cycle through which the development, maintenance, and transfer of tetracycline resistance genes could potentially be enhanced.

Evidence for tetracycline resistant microbes residing in different environmental compartments is provided by Tables 1.1 – 1.5 which shows results of a comprehensive literature review of genera that have been isolated from specific environments and which tetracycline resistance genes they harbor. Currently, empirical evidence may be lacking for some of the pathways presented in Figure 1.1. It is not intended to be a concrete presentation of every pathway, but rather, a guide in thinking about alternative pathways that may exist and have yet to be researched. Presented below is a discussion of relevant research in the major environmental reservoirs and pathways exhibited in Figure 1.1. The intention with this discussion was not to be comprehensive, but to illustrate the representative studies that have identified these pathways as contributors to spread of tetracycline resistance.

Air Transport. The major input of tetracycline residues and resistant microbes into the air is likely through transfer from feces of animals that have been treated with TCs, especially air indoor concentrated animal feeding operations (CAFOs). A recent empirical investigation of airborne particulate matter sampled for a 20 year period from a swine CAFO found that TCs were present in samples 12 out of 20 years (Hamscher, Pawelzick, Sczesny, Nau, & Hartung, 2003). Concentrations of TCs in particulate matter ranged from 0.2 to 5.2 mg/kg with a mean of 0.81 mg/kg. Maximum concentrations were

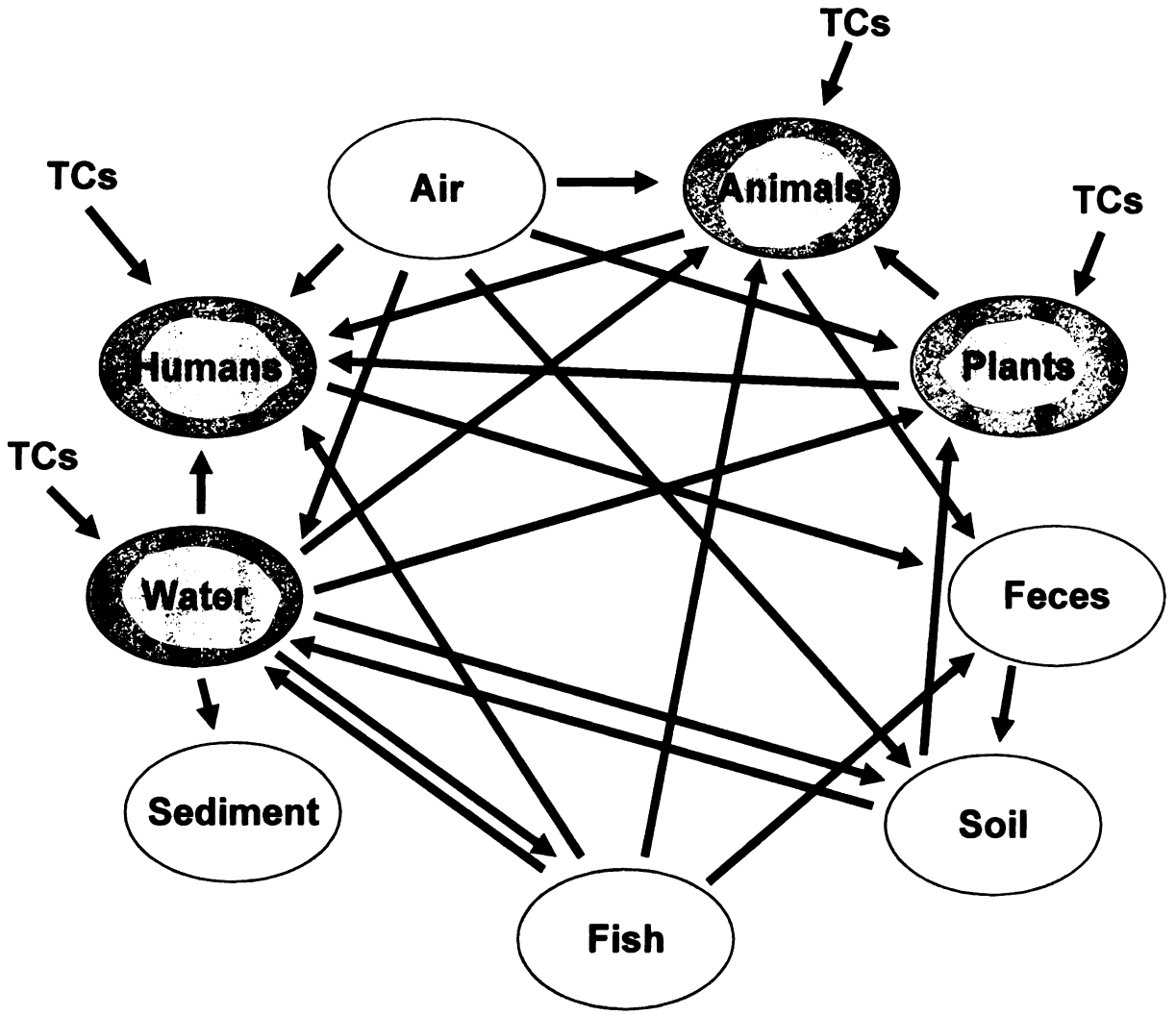

Figure 1.1. Pathways of tetracycline residues and resistant microbes to and between environmental reservoirs. TCs indicate direct human input of tetracyclines into primary reservoirs. Gray shading represents primary reservoirs whereas no shading indicates secondary reservoirs which receive tetracyclines and resistant bacteria from primary reservoirs.

found to be; 1.1 mg OTC/kg, 5.18 mg TC/kg, and 2.12 mg CTC/kg (Hamscher et al., 2003). The author's speculate that the drug residues originate from two sources, dried liquid manure particles from animal waste and dry powder animal feed supplemented with antibiotics.

Numerous studies have implied that TC resistant bacteria can become airborne through manure at CAFOs. To the best of the author's knowledge only one study has identified tetracycline resistance genes in such samples. The tetracycline resistance genes *tet*(K), *tet*(L), and *tet*(M) were found in various combinations in *Enterococcus* spp. and *Streptococcus* spp. isolates from air inside a swine CAFO (Table 1.3). Chapin et al. (2005) isolated *Enterococcus* spp., coagulase negative staphylococci, and viridians group streptococci from air inside a CAFO facility, 90% of which were resistant to tetracycline. Almost all of the tetracycline resistant bacteria also had evidence of cross-resistance to one or more antibiotics including erythromycin, clindamycin, and virginiamycin (Chapin, Rule, Gibson, Buckley, & Schwab, 2005). Another study by Gibbs et al. (2004) investigated external air both upwind and downwind of a swine CAFO that administered oxytetracycline via feed. They found that microbes isolated inside or downwind of the facility showed resistance to OTC whereas microbes isolated upwind never exhibited resistance. Furthermore, at one facility while swine were present in the barns 86% (50/56) were OTC resistant. When air was sampled downwind of this same barn after it was emptied, cleaned, and disinfected only 9% (5/57) of isolates were OTC resistant (Gibbs, Green, Tarwater, & Scarpino, 2004). In a follow up study Gibbs et al. (2006) showed once again that tetracycline resistant bacteria were significantly more dense in air up to 150 m downwind of a CAFO than upwind. *Staphylococcus aureus*, an important

human pathogen, represented 76% of the total bacteria isolated including samples from both inside and outside of the facility (Gibbs et al., 2006).

With sufficient wind carriage airborne TC resistant bacteria or particulate matter sorbed with TCs could easily be transferred to human and animal hosts, as well as water, plant, and soil environments (Figure 1.1). Deposition onto water, plants, and soil, or causation of animal infection by airborne tetracycline resistant bacteria from a CAFO would likely be difficult to determine. Not surprisingly there is a lack of published studies that have attempted such experiments. Separating natural background resistance from acquired resistance would likely prove to be especially problematic. However, Gibbs and co-workers finding that statistically similar amounts of TC and OTC resistant bacteria were found both inside and 150 m downwind of a swine CAFO strongly implies that aerial deposition onto the above mentioned environments is inevitable (Gibbs et al., 2006).

Many groups have attempted to establish a link between exposure to food production animals receiving antibiotics and antibiotic resistance in the human flora. It is difficult to establish a cause and effect relationship due to uncontrollable variables; however, such studies do provide compelling evidence that transfer of airborne resistant bacteria to farm workers is likely taking place. One study examined the resistance of bacteria isolated from 113 farm workers versus 113 non-farm workers and found that tetracycline resistant enterobacteria and *Escherichia coli* were isolated more frequently in pig farmers (Aubry-Damon et al., 2004). Another study found that slaughter plant workers are at higher risk of exhibiting multidrug resistant *Escherichia coli* than non-swine workers (Alali et al., 2008). Abigail Saylers' group found that the ribosomal

protection protein gene *tet*(Q) has transferred between animal commensal *Prevotella* spp., human colonic *Bacteroides* spp., and human oral *Prevotella* spp. strains (Nikolich, Hong, Shoemaker, & Salyers, 1994). Despite not being able to establish a direction of transfer, they were able to show a recent transfer of *tet*(Q) between a human oral strain of *Prevotella* spp. and a human colonic strain of *Bacteroides fragilis* (Nikolich et al., 1994). This suggests a link for how *tet*(Q) could transfer between animal ruminant *Prevotella* spp. and *Bacteroides* spp. resident in the human colon.

Water transport

Aquaculture. The most commonly recognized way in which tetracycline residues and resistant organisms can enter water environments is via aquaculture. As was mentioned above in the tetracycline usage section, OTC is used for therapeutic purposes in aquaculture and is the most commonly used antibiotic in this industry (A. Sapkota et al., 2008). Often the antibiotic is mixed directly into the water phase with feed at concentrations ranging from 50 to 2,000 ppm (Shao, 2001). Another, perhaps more intensive, way in which tetracyclines can reach aquaculture environments is through integrated fish farming, a practice in which manure from livestock production is used as feed in fish ponds (Petersen, Andersen, Kaewmak, Somsiri, & Dalsgaard, 2002). Asian countries, which account for 94% of global aquaculture production, have a history of administering wastewater, animal waste, and human waste to fish ponds (A. Sapkota et al., 2008). Any co-resident TCs with the waste are transferred to the aquaculture environment. These residues can bioaccumulate in cultured species, be deposited to sediments (which can be as high as 40% of medicated feed (Capone, Weston, Miller, & Shoemaker, 1996)), excreted via feces (as high as 90% can be excreted (Cravedi,

Chouber, & Delous, 1987)), and move from sediment back into the water column through dissolution (Graslund & Bengtsson, 2001).

An abundance of studies have traced OTC residues in cultured species and have found maximum muscle concentrations after OTC medication of between 4 and 20 ppm (Gomez-Jimenez, Espinosa-Plascencia, Valenzuela-Villa, & del Carmen Bermudez-Almada, 2008; Namdari, Abedini, & Law, 1996; Nogueira-Lima, Gesteira, & Mafezoli, 2006; Uno, Aoki, Kleechaya, Tanasomwang, & Ruangpan, 2006) depending on the species, water environment conditions, and amount of antimicrobial administered. One study revealed concentrations reaching as high as 10,050 ppm in hemolymph tissue of cultured shrimp (Gomez-Jimenez et al., 2008). In the United States, Japan, and European Union, regulatory agencies have set maximum residue limits of 0.2 ppm, 0.2 ppm, and 0.1 ppm, respectively, (Gomez-Jimenez et al., 2008) for OTC residues in edible food tissues brought to market. Generally farmed fish are given adequate withdrawal time to allow clearance of OTC from the animal. However, these three regions represent only 4% of world aquaculture production (A. Sapkota et al., 2008) and guidelines for antibiotic usage in developing countries, which account for the bulk of world aquaculture production, are poorly established. Due to this lack of information, combined with globalization, it is unclear to what extent contaminated aquaculture products are reaching the table in many countries. For example, in 1991 Japanese health authorities found unacceptable levels of OTC in farm-raised shrimp imported from Thailand (Graslund & Bengtsson, 2001). Similarly, in 1993 the Thai Medical Sciences Department found that 24% of shrimps exported contained OTC residues (Graslund & Bengtsson, 2001).

Ultimately some OTC residues accumulate in sediments below aquaculture cages. This process is dependent on four dominant factors: 1) the total amount of OTC added 2) the percentage of OTC that reaches the sediment 3) the area of sediment over which OTC is deposited and 4) the depth of the sediment through which OTC is distributed (Coyne et al., 1994). Concentrations found in marine sediments have ranged from 1 to 300 ppm and likely depend upon the characteristics of the local environment. Kerry et al. (1994) found peak OTC concentrations of 9.9 ppm with a half-life of 16 days in sediments below fish cages. At a depth of 8 cm OTC was still detectable by HPLC 19 days after cessation of medication, however, 33 days afterwards it was undetectable (Kerry et al., 1994). Samuelsen et al. (1992) found much higher OTC concentrations in sediments. After 10 days of OTC medication sediment concentrations ranged from 25 ppm to 300 ppm and stayed stable for 75 to 200 days at these concentrations. Residues were detectable even after as long as 550 days and persisted in sediment depths of 2 – 8 cm for at least 245 days at concentrations of approximately 30 ppm (Samuelsen, Torsvik, & Ervik, 1992). Furthermore, immediately after 10 days of feeding, 100% of culturable bacteria were OTC resistant. This percentage decreased during the first 75 days post medication and stabilized at a level of 10 to 50% for at least 550 days. Surprisingly few studies have recently been conducted on the persistence of OTC in aquaculture sediments. This is an area open to investigation. Similarly, no studies were found in which investigators measured soluble water concentrations of OTC in aquaculture cages. These could be appreciable considering that modeling studies estimated that 10 – 15% of OTC would be released as a pulse to receiving waters during medication and the first 5 days after (Rose & Pedersen, 2005).

Multiple studies have shown that OTC present in the upper layers of sediment can dissolve back into the water column (Capone et al., 1996; Hektoen, Berge, Hormazabal, & Yndestad, 1995). In one study boxes filled with sediment spiked with 200 ppm OTC were placed at a depth of 15 m in seawater (Hektoen et al., 1995). It was found that the OTC concentration decreased in the upper 2 cm from 200 ppm to approximately 30 ppm over the course of more than 200 days, however, OTC in the 6 to 7 cm range stayed at relatively high levels being measured at approximately150 ppm after more than 200 days (Hektoen et al., 1995). The half-life of OTC in 0 to 1 cm sediments was 151 days compared to more than 300 days in the 5 to 7 cm depth (Hektoen et al., 1995). Smith and Samuelsen (1996) used a modeling approach to predict that OTC concentrations in the bottom 1 cm of the water column would range between 0.016 μg/mL and 0.11 μg/mL depending on the sediment concentration. They further assumed that since previously approximately 10% of total OTC in seawater is bioavailable, the predicted bioavailable concentrations would range from 0.0016 μg/mL to 0.011 μg/mL (Smith & Samuelsen, 1996).

Tetracycline resistance in water environments is currently best characterized in aquaculture environments. Out of 115 unique isolate/gene combinations present in the literature from water environments 71 (62%) were isolated from aquaculture sources (Table 1.1). The two genera isolated harboring the most *tet* genes are *Aeromonas* spp. and *Vibrio* spp. likely because both are important fish pathogens. Other genera identified to contain more than three different *tet* genes include *Escherichia* spp. (11), *Citrobacter* spp. (7), *Pseudomonas* spp. (7), *Acinetobacter* spp. (4), *Photobacterium* spp. (4), and *Plesiomonas* spp (4). The bulk of resistance genes found in aquaculture environments to

date are efflux genes (Table 1.1). It is unknown whether this bias is due to the type of bacteria typically present in aquaculture environments, since efflux genes are historically more commonly associated with Gram-negative bacteria, or if the bias is due to lack of screening for RPPs. Nonetheless, aquaculture isolates continue to be a significant reservoir for tetracycline resistance genes exemplified by the discovery of new tetracycline gene variants. Three more recently discovered genes, *tet*(31), *tet*(34), and *tet*(39), discovered in 2000, 2002, and 2005, respectively, were isolated from *Aeromonas salmonidicia*, *Vibrio* spp., and *Acinetobacter* spp. from aquaculture sources (Agerso & Guardabassi, 2005; L'Abee-Lund & Sorum, 2000; Nonaka & Suzuki, 2002).

Few studies have specifically dealt with temporal and spatial dynamics of OTC resistance in aquaculture environments. As mentioned above, in some countries guidelines are established requiring a certain period of drug free rearing to keep OTC residues out of edible fish tissues. However, antibiotic resistant bacteria may persist in aquaculture environments after the cessation of medicated feeding (Samuelsen et al., 1992). This persistence creates an environment that could allow for the transfer of resistance between bacteria or the clonal expansion and dissemination of already resistant bacteria. In fact, resistance to CTC and OTC appears to be a worldwide phenomenon in aquaculture environments, as it has been reported in China, India, Japan, Philippines, Indonesia, South Korea, Bangladesh, Thailand, Chile, Norway, the United States, and Taiwan (A. Sapkota et al., 2008).

Kim et al. (2004) took a closer look at a regional examination of OTC resistance in aquaculture environments in Japan and Korea. Interestingly, they were able to see a contrasting distribution in *Vibrio* spp. isolated from healthy fish at the same location and

sampling time. In one set of healthy fish *Vibrio* spp. isolates were positive for the *tet*(M) gene and had a specific 16S rDNA RFLP pattern (Kim, Nonaka, & Suzuki, 2004). Another set of healthy fish isolated at the same time and location harbored *Vibrio* spp. isolates that were negative for *tet*(M) and had a different 16S rDNA RFLP profile (Kim, Nonaka, & Suzuki, 2004). This indicates that within the same farm differences can exist in the diversity of TC resistance genes on a microenvironment scale. Also, the observation was made that *Vibrio* spp. harboring *tet*(M) genes that shared the same 16S rDNA RFLP pattern while also lacking the *tet*(S) gene and a marker for Tn*1545*-Tn*916*-like transposons were isolated from healthy fish and seawater at two separate locations in Korea as well as in diseased fish in Japan (Kim et al., 2004). It is possible that there was a clonal expansion and dissemination of this *Vibrio* spp. strain throughout the region.

Resistance can also be prevalent in sediment environments below aquaculture sites. Petersen et al. (2002) investigated the temporal occurrence of OTC resistance in *Acinetobacter* spp. and *Enterococcus* spp. in an integrated fish farming environment. They found that there was a significant development of resistance during the first 2 months after fish production was initiated. Resistance to OTC in *Acinetobacter* spp. rose to 100% of cultured isolates, which is interesting because OTC was not fed to the broilers whose manure was used in the fish farm, indicating the probable co-selection of OTC resistance genes (Petersen et al., 2002). In contrast, *Enterococcus* spp. showed no significant change in resistance (Petersen et al., 2002).

The persistence of OTC resistant bacteria in the environment raises the concern that resistance could transfer to clinically relevant pathogens. Furushita et al. (2003) found that sequences of *tet*(C), *tet*(D), and *tet*(Y) from aquaculture isolates shared 100%

sequence identity to those of clinical strains. Furthermore strains of *Photobacterium* spp., *Vibrio* spp., *Alteromonas* spp., and *Pseudomonas* spp. could transfer their resistance determinants to *E. coli* via conjugation (Furushita et al., 2003) implying a potential pathway between environmental and human commensal bacteria. Similarly, it has been demonstrated that IncU R-plasmids previously only associated with aquaculture environments were also associated with human isolates and that this dissemination occurred in four separate countries (Norway, Scotland, England, and Germany) (Rhodes et al., 2000). In 2005 a new tetracycline efflux pump, *tet*(39), was discovered in isolates from fish farms as well as a clinical specimen from human urine collected in the Netherlands in 1986 (Agerso & Guardabassi, 2005). Although none of the above examples provide definitive proof that horizontal transfer is occurring they are consistent with this hypothesis.

Surface Water. Surface waters are prone to TC residue and resistant microbe contamination through wastewater treatment plant (WWTP) effluent discharges, direct discharges of sewage from households or hospitals, aquaculture sites, and runoff from soils with TC contaminated manure.

The presence and concentration of tetracyclines has been measured in surface waters by multiple investigators. Kolpin et al. (2002) tested 84 samples in a USGS national reconnaissance study. Even though their study was purposefully biased towards streams likely to be impacted by anthropogenic contamination they infrequently detected tetracyclines. CTC was detected in only 2 of 84 samples (median concentration of 0.42 ug/L) (Kolpin et al., 2002). Similarly, OTC and TC were detected at only one site (0.34 ug/L and 0.11 ug/mL, respectively) (Kolpin et al., 2002). In 2008 this same group

published another study in which they sampled surface waters that are sources for drinking water supplies. Of the 47 surface waters analyzed tetracyclines were never detected (Focazio et al., 2008). Similarly Batt and colleagues were unable to detect tetracyclines in river waters, although they were able to in WWTP outfalls (Batt, Bruce, & Aga, 2006).

However, TCs have been detected frequently in other surface waters. This is likely attributable to different site characteristics (e.g. organic C loads, flow rate etc.) and susceptibilities to TC contamination. It has been shown experimentally that TCs can be transported to surface waters through overland flow and field drainage after manure application (Davis, Truman, Kim, Ascough, & Carlson, 2006; Kay, Blackwell, & Boxall, 2004, 2005). Choi and colleagues detected OTC, minocycline (MCLN), doxycycline (DXC), meclocycline (MECN), democlocycline (DMC), and TC in river water at concentrations ranging from 0.01 to 0.2 μg/L (Choi, Kim, Kim, & Kim, 2007). Yang et al. (2003) also detected CTC, OTC, DMC, TC, and DXC in the Poudre River in Northern Colorado at concentrations ranging from 0.05 to 1.14 μg/L.

In fact, Dr. Kenneth Carlson's group at the Colorado State University has conducted the most extensive and comprehensive study of TC residues, resistant bacteria, and resistance genes in surface waters to date. They've studied the Poudre River in Northern Colorado temporally and spatially. Sampling at five separate sites revealed that TCs contamination in surface waters likely originated from anthropogenic sources including urban and agricultural impacts (Yang & Carlson, 2003). A pristine site located in the mountains upstream of any cities revealed no detectable tetracyclines (Yang & Carlson, 2003). As the sampling sites became more impacted by human activity, more

tetracyclines were detected. At a site directly downstream of a WWTP they detected a large spike in DMC (0.33 ug/L) (Yang & Carlson, 2003). Their data also show the impact of OTC and CTC, two commonly used antibiotics in animal husbandry, on the river at sites likely to be impacted by agricultural runoff. At the site directly downstream of the WWTP they only detected TC and DMC. OTC and CTC were also absent from the effluent coming from the WWTP. However, further downstream after the river flowed through areas where the land use is predominantly agricultural OTC and CTC were first detected (0.07 and 0.15 μg/L OTC and 0.19 μg/L CTC at site 5) (Yang & Carlson, 2003). In a follow up study they sampled the same five sites over a period of seven months and showed results consistent with the previous study indicating that this was not a one time event (Yang, Cha, & Carlson, 2004).

Further studies were conducted by Dr. Carlson's group that measured the concentrations of tetracyclines in sediments, the presence and quantity of specific tetracycline resistance genes, and of resistant bacteria. Sediment concentrations of TC residues were significantly higher than in surface waters (maximum total concentration of tetracyclines of 100.9 and 399.1 ppb for high and low flow events, respectively) (Pei, Kim, Carlson, & Pruden, 2006). This is not surprising considering the strong sorption of tetracyclines to soils (see the terrestrial section of this review). The *tet*(O) gene was the only gene detected at the pristine site, whereas *tet*B(P), *tet*(S), *tet*(W), and *tet*(O) were all detected at the impacted sites (Pei et al., 2006). The authors quantified *tet*(W) and *tet*(O) via RT-PCR and found that *tet*(O) concentrations were about an order of magnitude higher at impacted sites compared to pristine sites (Pei et al., 2006). They returned to this site for another study, this time more extensively analyzing *tet*(O) and *tet*(W) gene

concentrations by RT-PCR (Pruden, Pei, Storteboom, & Carlson, 2006) and also investigating surface runoff as sources of resistance genes. Gene concentrations followed the pattern (highest to lowest) of dairy lagoon water > irrigation ditch water > urban/agriculturally impacted sediment ($p < 0.0001$) (Pruden et al., 2006). Gene concentrations generally were between 10^{-7} to 10^{-5} gene copies/16S *rrn* copy. Although concentrations of TcR genes did not differ significantly from any of the sites along the river when normalized to 16S rRNA gene copy number, lowly impacted sites were significantly different from sites heavily impacted by urban and agricultural runoff without this normalization (Pruden et al., 2006). This may in fact be a better measure as it is known that a single bacterium can harbor one to fifteen copies of *rrn* genes. Ribosomal operon gene copy number varies with growth strategy as copiotrophs typically have higher copy numbers than oligotrophs (Klappenbach, Dunbar, & Schmidt, 2000). An earlier study indicated that the sites subject to human impact had higher organic C amounts indicative of significant surface runoff. It is possible that bacteria originating from a high nutrient environment would have higher *rrn* copies per bacterium which if transfered to surface water, could skew the data when TcR gene copy number is normalized per *rrn* copy.

Other investigators have examined the presence of tetracycline resistant bacteria and their genes in aquatic environments likely to be impacted by agricultural practices including the Jiazhou Bay in China (Dang, Ren, Song, Sun, & An, 2008) and the Mekong River in Southeast Asia (Kobayashi, Suehiro, Cach Tuyen, & Suzuki, 2007). Sapkota et al. (2007) examined surface waters impacted by a swine CAFO. They found that *Enterococcus* spp., *E. coli*, and fecal coliforms were significantly higher at surface water

sites downstream of the CAFO than upstream. Although only marginally significant (p = 0.06), bacteria downstream had a higher percentage resistance to TC supporting the possibility that the CAFO is the source of resistance (A. R. Sapkota, Curriero, Gibson, & Schwab, 2007).

Ground Water. Tetracyclines are rarely detected in groundwater samples. In a recent national reconnaissance study performed by the USGS, 47 groundwater sites were examined for a multitude of pharmaceuticals and personal health care products (Barnes et al., 2008). Tetracyclines were never detected at any of the 47 groundwater sites. A concurrent study sampled groundwater sources used for drinking water and again, no TCs were detected (Focazio et al., 2008). Hirsch and colleagues sampled groundwater wells in Germany and similarly did not detect any TCs (Hirsch, Ternes, Haberer, & Kratz, 1999). In a similar study, Campagnolo and colleagues only detected CTC at a concentration of 2 ug/L in a field well near a poultry farm that had used tetracyclines (Campagnolo et al., 2002). Mackie et al. (2006) sampled groundwater monitoring wells adjacent to swine manure lagoons at two CAFO sites in Illinois that had a history of TC usage. They were only able to detect OTC twice out of 45 samples at concentrations of 0.08 and 0.13 ug/L, TC once at a concentration of 0.4 ug/L, and the TC degradation products anhydrotetracycline, β-apooxytetracycline, and anhydrochlortetracycline three times at concentrations ranging from 0.1 μg/L to 0.3 μg/L (Mackie et al., 2006). Rare detection of TC residues in groundwater is not surprising considering that tetracyclines sorb strongly to soils and are rarely detected below depths of 30 cm (Table 1.6) (Pils & Laird, 2007; Sarmah et al., 2006).

Although TC residues are rarely detected in groundwater, TC resistant bacteria and genes have been commonly detected in groundwater impacted from CAFOs (Table 1.3) (Anderson & Sobsey, 2006; A. R. Sapkota et al., 2007). University of Illinois researchers have spent years characterizing the presence, persistence, and mobility of *tet* genes in aquifers located below swine manure lagoons at CAFOs with a history of TC use. In two separate studies, they were able to detect numerous RPPs and efflux genes in groundwater monitoring wells proximal to the swine lagoons (Aminov et al., 2002; Chee-Sanford, Aminov, Krapac, Garrigues-Jeanjean, & Mackie, 2001). Monitoring wells located behind the swine lagoons and upstream of the groundwater flow pattern did not contain *tet* genes. The *tet* genes may be mobile throughout the aquifer as they detected *tet*(B), *tet*(H), *tet*(Z), and *tet*(Q) 250 m downgradient from the swine lagoons (Aminov et al., 2002; Chee-Sanford et al., 2001). They also were able to culture isolates of *Enterococcus* spp., *Staphylococcus* spp., and *Lactobacillus* spp. containing *tet*(M) from groundwater samples (Chee-Sanford et al., 2001).

The authors subsequently conducted a long-term 3 year sampling of these sites to determine any patterns in resistant gene flow. The presence of RPPs *tet*(M), *tet*(O), *tet*(Q), and *tet*(W), and efflux genes *tet*(C), *tet*(H), and *tet*(Z) were monitored over six sampling dates (Koike et al., 2007). Two significant conclusions were made. First, a principle component analysis (PCA) determined how the monitoring wells grouped based on well *tet* gene profile compared to the *tet* gene profile of the swine lagoons. At one site five monitoring wells that were all located close and downgradient from the swine lagoon grouped with the lagoon in PCA (Koike et al., 2007). This result indicates that the swine lagoon is indeed the source of the *tet* gene contamination. This finding was corroborated

when they analyzed 100 sequences of *tet*(W) genes isolated from the various wells and lagoons. The *tet*(W) genes present in the groundwater monitoring wells shared over 99.8% identity with sequences from lagoon samples, whereas *tet*(W) genes isolated from control wells exhibited considerably less sequence identity (Koike et al., 2007). Although these studies confirmed that bacteria harboring *tet* resistance genes can contaminate groundwater samples via leeching from swine lagoons, they cannot determine if this is due to horizontal transfer of genes from lagoon bacteria to natural bacteria or if it is due to movement of lagoon bacteria into the groundwater phase.

Wastewater Treatment Plants. Wastewater treatment plants (WWTPs) are considered to be areas prone to antibiotic contamination and harboring resistance traits since they receive wastewater from households and hospitals that may be contaminated with these agents (Kummerer & Henninger, 2003; Rhodes et al., 2000; Thomas, Dye, Schlabach, & Langford, 2007).

Typically antibiotic concentrations in WWTP influent water are in the ppb range from less than 1 to 50 ppb whereas effluent concentrations typically are between 0.5 and 10 ppb if detectable. Karthikeyen et al. (2006) surveyed WWTPs in Wisconsin and found maximum influent concentrations of 48 ppb (TC), 0.31 ppb (CTC), 47 ppb (OTC), and 10 ppb (DXC) compared to maximum effluent concentrations of 3.6 ppb (TC), 0.42 ppb (CTC), 0.42 ppb (OTC), and 10.9 ppb (DXC). They measured soluble TC maximum influent concentrations of 1.2 ppb and maximum effluent concentrations of 0.85 ppb (Karthikeyan & Meyer, 2006). Yang and colleagues surveyed a WWTP in Fort Collins, CO and found maximum influent concentrations ranging from 0.1 to 0.3 ppb for TC, DMC, CTC, and DXC compared to maximum effluent concentrations of 0.06 ppb (CTC)

and 0.07 ppb (DXC) (Yang, Cha, & Carlson, 2005). TC and DMC were not detected in effluent. In China influent concentrations have been measured for TC at 1.3 ppb compared to 0.18 ppb in effluent (Gulkowska et al., 2008). Similarly, a WWTP in Canada was shown to have a maximum effluent concentration of 0.98 ppb during the study period (Miao, Bishay, Chen, & Metcalfe, 2004).

Given that TC concentrations in municipal WWTPs seem to be similar regardless of geographical location it is of interest to know if these environments are selecting or enriching TC resistant bacteria that could disseminate to the environment on a global scale. Ferreira da Silva et al. (2007) studied a WWTP in Portugal and they found that the percentage of tetracycline resistant *Escherichia* spp., *Shigella* spp., and *Klebsiella* spp. increased in effluent water compared to raw influent water. This could indicate either (a) the transfer of TcR determinants in the WWTP environment or (b) the enrichment of resistant bacteria. Furthermore, TC resistance had a positive correlation with ampicillin, ciprofloxacin, and mercury resistances indicating the potential for co-selection of TcR resistant determinants (Ferreira da Silva, Vaz-Moreira, Gonzalez-Pajuelo, Nunes, & Manaia, 2007). This is problematic because resistant bacteria could be discharged in the effluent water to surface waters. Support of this interpretation is provided by Goni-Urriza and colleagues study that sampled *Enterobacteriaceae* spp. and *Aeromonas* spp. upstream and downstream of a WWTP in Spain. Levels of resistance increased from 12.5% and 0% upstream to maximum values of 24.3% and 27.5% downstream for *Enterobacteriaceae* spp. and *Aeromonas* spp., respectively (Goni-Urriza et al., 2000).

As is commonly known to microbiologists, less than 1% of known bacteria are currently cultured (Torsvik, Goksoyr, & Daae, 1990; Torsvik & Ovreas, 2002). Hence,

studies using only cultured indicator organisms are used could drastically underestimate the extent of TcR in the environment. To avoid this bias a few research groups have tested by non-culture based methods for the presence and quantity of TcR genes in WWTP environments (Auerbach, Seyfried, & McMahon, 2007; Pruden et al., 2006). Pruden et al. (2006) tested for the presence of *tet*(O) and *tet*(W) by a PCR presence/absence assay throughout multiple stages in the WWTP process and detected both genes throughout all stages including treated drinking water (Pruden et al., 2006). Auerbach et al. (2007) tested for the presence of 10 *tet* genes encompassing both efflux and RPP determinants in WWTPs in Wisconsin. They consistently found a high diversity of *tet* genes and in multiple cases found all 10 genes to be present in WWTP environments (Auerbach et al., 2007) whereas in two control lake samples they only found *tet*(A). Concentrations of *tet*(G) and *tet*(Q), measured by RT-PCR, ranged from 1 x 10^7 – 1 x 10^9 copies per mL in influent, activated sludge, and biosolids (Auerbach et al., 2007). Effluent waters had concentrations ranging from 1 x 10^4 to 1 x 10^6 per mL, about three orders of magnitude lower (Auerbach et al., 2007). It is possible that this reduction in *tet* gene copy number is due to one or a combination of the following: cell death in the biosolid phase, absence of selective pressure due to decreased antibiotic bioavailability, or simply retention by the biosolid phase.

Terrestrial Transport

Soil. As mentioned above, tetracyclines are administered to animals for prophylactic, therapeutic, and growth promotion purposes. Multiple studies have demonstrated the development, selection, and maintainence of TC resistance traits in the animals (Table 1.5). Significant amounts of tetracyclines are excreted unaltered in urine

and feces (Sarmah et al., 2006). Concentrations of tetracyclines have been found up to 40 ppm in manure (Martinez-Carballo, Gonzalez-Barreiro, Scharf, & Gans, 2007). Not surprisingly, the presence of TC resistant microbes has been demonstrated in manure at all stages in the storage process (Stine et al., 2007). In the United States agricultural animals produce approximately 128 billion lbs of manure annually (Sarmah et al., 2006), most of which is recycled to fields as fertilizer. In recent years concern has developed that manure management practices may be enhancing the spread of TcR. TC residues and TcR bacteria may leach from manure storage areas into groundwater systems, runoff to surface waters after rainfall, or accumulate through land application of manure. In regards to land application, TcR enhancement could occur through TC residue transfer to soil and subsequent selection for resistant indigenous populations, soil deposition and enrichment of resistant microbes originating from animal sources, or horizontal transfer of TcR genes from animal commensal bacteria to indigenous soil bacteria.

Soils fertilized with TC contaminated manure typically contain concentrations up to 300 ppb (Table 1.6). Tetracyclines are known to have a strong capacity for sorption to soil that is dependent on humic material, pH, and cation exchange capacity (Gu, Karthikeyan, Sibley, & Pedersen, 2007; Pils & Laird, 2007; Sassman & Lee, 2005; Tolls, 2001). Correspondingly, tetracyclines detected in manured fields have been shown to accumulate in the top 30 cm and are infrequently detected below this depth (Hamscher, Pawelzick, Hoper, & Nau, 2005; Hamscher, Sczesny, Hoper, & Nau, 2002; Kay, Blackwell, & Boxall, 2004). Furthermore, these residues can persist in top-soil for years and accumulate with repeated manure applications (Hamscher et al., 2005).

Few studies have determined the *tet* gene variants in TC resistant cultured isolates originating from manured soils. In fact only six studies have been published to date (Fig. 1.2). Ghosh and LaPara (2007) have performed the most comprehensive study to date in which they cultured soil bacteria from swine farms using sub-therapeutic tetracyclines, dairy farms using TCs as therapeutics, and non-agricultural soils. They used both nutrient rich and nutrient poor media to culture a wider variety of soil bacteria than is typically isolated in soil studies. This study alone represents 50% of the unique isolate/TcR gene combinations from soil environments (Table 1.2). One of the swine farms sampled ceased operation during the study period and it was found that resistance levels remained high for at least 18 months (Ghosh & LaPara, 2007). Bacteria were sampled at various distances (5, 20, and 100 m) from a pen in which swine manure had been previously stored. Interestingly, there was considerable overlap in the types of resistant bacteria at 5 and 20 m from the pen, however, at 100 m *Streptomyces* spp. were most prevalent, representing approximately 50% of the isolates (Ghosh & LaPara, 2007). This indicates a shift from bacteria originating from the animal manure to soil indigenous bacteria as *Streptomyces* spp. are dominant members of soil communities and antibiotic producers likely to harbour resistance genes. Ultimately, a conclusion regarding the safety of the application of TC contaminated manure cannot be drawn from this study. However, the fact that this study alone represents 50% of unique gene/isolate combinations known in soil environments demonstrates that (a) soil is a significant reservoir of TcR and (b) the extent of horizontal dissemination of TcR genes is likely to be vastly underestimated.

Table 1.6. Tetracycline Antibiotic Concentrations found in Soils under Field Conditions

Antibiotic	Max. Conc. (ppb)	Depth (cm)	Sample Time (weeks post-app)	Method
TC	9,000[b]	0 – 0.2[b]		LC-MS/MS
TC	295	10 – 20	4	LC-MS/MS
TC	254	0 – 30	2	LC-MS/MS
TC	198.7	10 – 20	4	LC-MS/MS
TC	14.5	0 – 10		LC-MS/MS
TC	11.29	0 – 5	3	LC-MS
Total TCs[a]	2251	0 – 5	44	ELISA
OTC	254,000[b]	0 – 0.2[b]		LC-MS/MS
OTC	270.05	0 – 5	3	LC-MS
OTC	216	0 – 40		HPLC
CTC	4,000[b]	0 – 0.2[b]	4	LC-MS/MS
CTC	39	20 – 30	post-app[c]	LC-MS/MS
CTC	15.5	0 – 20	4	LC-MS/MS
CTC	7.3	20 – 30		LC-MS/MS
anhCTC	1.44	5 – 10	3	LC-MS
anhTC	1	10 – 15	3	LC-MS
β-apo-OTC	1.22	5 – 10	3	LC-MS

anhCTC = anhydrochlortetracycline, anhTC = anhydrotetracycline, β-apo-OTC = apotetracycline. [a]This was a non-specific ELISA test. [b]These amounts are extremely high because samples were taken only 0.2 cm deep since manure was not incorporated into the soil. [c]This sampling was completed immediately after fertilization with manure. Max. Conc. = maximum concentration measured. References (see pg 76).

No study to date has provided conclusive evidence that transfer of TC residues and resistant microbes into soil environments enhances the transfer of resistance to clinical pathogens. However, this is not to say that this process is not occurring. In fact, it is possible that a lack of evidence is due to a lack of appropriately-controlled, long-term studies. Field scale studies typically involve sites which have been subject to agricultural practices for many years. Adequate data regarding antimicrobial usage is rarely available to researchers and this makes untangling introduced resistance from background resistance a nearly impossible task. Furthermore, few studies have combined culture-independent molecular methods with culture methods. Both have inherent bias that could vastly underestimate the true extent of resistance. The fact that less than 1% of known bacteria have been cultured limits our understanding of the extent of antibiotic resistance. Culture-independent methods can be expensive and time-consuming. For example, examining the entire extent of TcR genes in any sample requires detection of 38 *tet* gene variants and this does not include genes yet to be discovered. Even when *tet* genes are identified by molecular methods, few studies adequately investigate the mechanisms of horizontal transfer that will allow judgment of the capability for further dissemination of resistance. Clearly TcR resistance in soil environments and the impact of animal agriculture on such warrants further investigation.

Plant. Plant agriculture represents the least researched terrestrial system with regards to tetracycline resistance. OTC is sprayed in orchards at concentrations of ~ 300 ppm to control various plant diseases (McManus et al., 2002). Ironically, this system represents the most direct application of OTC (OTC is sprayed directly onto plant

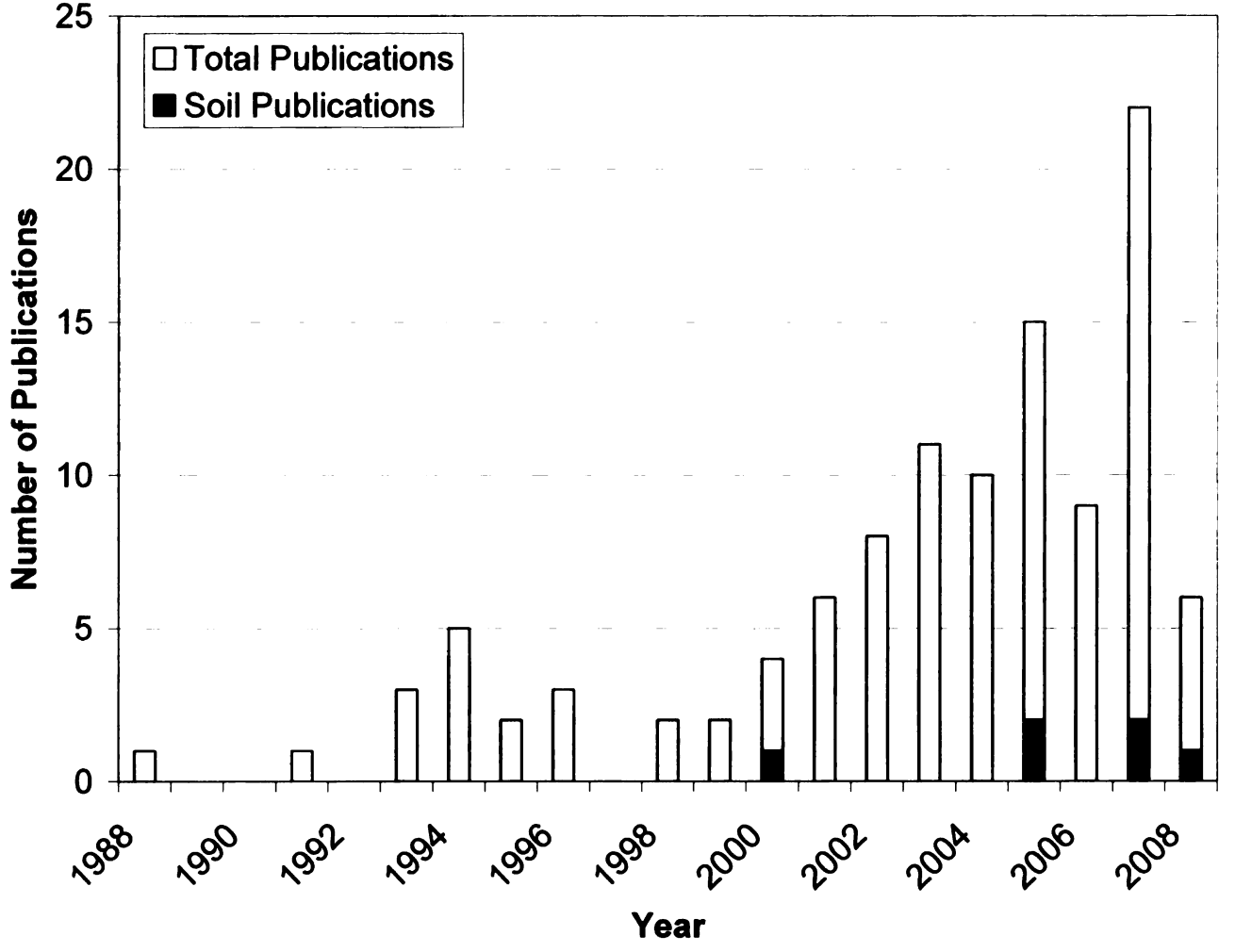

Figure 1.2. Publications that Identified Tetracycline Resistance Genes in Bacterial Isolates from Environmental, Food, and Animal Origin.

surfaces after dissolution into water), yet is the least well characterized.

Currently only one study has investigated the cultivatable TcR fraction from phylloplane bacteria associated with OTC treated plants. A survey of resistance in Michigan apple orchards revealed the presence of *tet*(A), *tet*(B), *tet*(C), and *tet*(G) in isolates mostly of *Pantoea agglomerans* or fluorescent and nonfluorescent *Pseudomonas* spp. (Schnabel & Jones, 1999). All TcR elements were located on plasmids. In particular *tet*(A) and *tet*(B) were exclusively associated with Tn*1721*-like and Tn*10* transposons, respectively (Schnabel & Jones, 1999). The author's go on to speculate that development of TcR in the major plant pathogen *Erwinia amylovora* (the causative agent of fire blight) will not be a short term consequence of OTC application in apple orchards (Schnabel & Jones, 1999). This conclusion was based on a number of astute observations; however, little is known regarding the transfer of TcR to other genera that may be of consequence to human health. Given the lack of studies on OTC usage and bacterial resistance that could ultimately end up in retail food it certainly represents an area that deserves future research consideration.

SIGNIFICANCE OF RESEARCH

It is my belief that in a world of limited time and resources, scientists should be able to justify the significance of their research to the community that a) provides funding and b) will reap the benefits from it. Considerable time has been spent describing the broader antibiotic resistance problem in the context of tetracycline resistance. Now the discussion narrows and shifts towards the relevancy of the topic of research, that is, tetracycline resistance in the soil agricultural environment.

Economic Impacts. As has been eluded to, there is heated debate as to whether or not antimicrobials should be eliminated for growth promotion purposes (Hileman, 2001). The best justification for researching antimicrobial resistance in the agricultural environment can be provided by an analysis of the potential economic impact of either withdrawal of antibiotics for growth promotion, or in continued use, which could potentially enhance the problem of treating antibiotic resistant pathogens.

The economic justification for the continued use of antimicrobials in farming practices (aquaculture, animal husbandry, and plant agriculture) is that usage will decrease costly bacterial infections that could decimate crops or animals. The benefits of therapeutic/prophylactic usage are obvious and their purpose is rarely debated. It is sub-therapeutic usage that takes the most scrutiny. Farmer's argue that sub-therapeutic antimicrobial usage allows them to use less feed that in turn helps keep food prices low. It has been estimated that there is an additional net return of $2.99 per pig if antimicrobials are used from weaning to market for each $0.7 invested in the antibiotic (Cromwell, 2002). The economic return if used in the breeding diet is $7.12 net return per litter and $2.63 if used in lactation feed per litter (Cromwell, 2002). On a broader scale it has been estimated that the complete withdrawal of antimicrobials for growth promotion in food production in the US would cost consumers $5 – 40 per capita per annum (Phillips et al., 2004). Another, often overlooked, factor is the added cropland that would need to be used because of the decrease in feed efficiency. This has been estimated to be an additional 2 million acres of cropland in the US alone (Phillips et al., 2004). Transition of more land to cropland for feed production would also have a significant environmental impact.

Concern has been raised that antimicrobial residues that ultimately end up being applied to land through contaminated manure can aid in selection of resistance determinants that may ultimately transfer to pathogens seen in the clinic. Potential for resistance transfer and enrichment has been demonstrated via consumption of TcR and TC contaminated food, water, and air (Chapin, Rule, Gibson, Buckley, & Schwab, 2005; Koksal, Oguzkurt, Samasti, & Altas, 2007; Zhang et al., 2007). If this occurrs a disastrous economic consequence is that infectious diseases will become more difficult and costly to treat. In 1992 it was estimated that approximately $120 billion (15%) of all healthcare expenditures were related to treating infectious disease and that antibiotics were the most commonly prescribed drugs (Cassell, 1997). Various sources have estimated that the annual cost of treating drug resistant infections in the US ranges from $1.5 to $ 4 billion (Cassell, 1997; Twomey, 2000).

What we have come to is a difficult tug of war between two different vested interests, with economic and ethical consequences on both sides. On the one hand the world's population is exponentially increasing and maintaining efficient and high levels of food production is essential to provide sufficient nourishment to the masses. Usage of antimicrobials for sub-therapeutic purposes may help this high level be maintained. On the other hand this usage could have consequences to infectious disease treatment, and this can become a quality of life issue. Therefore it becomes researcher's responsibility to find and fill the knowledge gaps to determine how much of a risk the practice of sub-therapeutic antimicrobial growth promotion presents.

Knowledge Gaps. Despite 60 years worth of research on tetracyclines, significant gaps in understanding regarding the environmental dissemination of

tetracycline resistance genes still exist. Two particularly relevant gaps are the molecular identification of tetracycline resistance genes in soil environment and the effect that tetracycline chemistry has on the bioavailability of the antibiotic in environmental matrices.

A comprehensive literature review was conducted to investigate the extent to which molecular investigation of TcR determinants has been researched. This survey revealed that 111 studies have characterized tetracycline resistance genes in cultured isolates originating from environmental, food, and animals by molecular methods (Fig. 1.2). Bacteria isolated from human clinical sources were omitted in this survey because this abundant data would obscure the environmental information. There has been a significant body of work completed since 2000. Eighty three percent (92/111) of the total studies have been published in the last eight years. Of all studies only 6/111 (6 %) are from soil environments. Despite this low number of studies genera/gene combinations unique to soil represent 17% of the total unique genera/gene combinations. Of these six studies 84% of the unique genera/gene combinations only come from two studies, one published in 2007 and the other in 2008 (Ghosh & LaPara, 2007; Srinivasan et al., 2008). This review demonstrates the strong likelihood that there is a significant amount of unexplored molecular diversity in present agricultural soil environments, especially those that have been impacted by manure contaminated with tetracycline residues.

A second major area that has been ignored is the effect of tetracyclines' chemistry on the bioavailability of the molecule and how this controls the expression of tetracycline resistance genes in environmental matrices. Bioavailability is defined as *the ability of*

tetracyclines to leave the environmental matrix, penetrate the microbial cell's membrane, and act on their targets.

There have been multiple investigators that have identified the gap in understanding of bioavailability in a natural context (Chopra & Roberts, 2001; D'Costa, Griffiths, & Wright, 2007; Kummerer, 2004). Despite public knowledge of this gap, few studies have attempted to address the topic of tetracycline bioavailability to microbial cells in natural environments (Chander, Kumar, Goyal, & Gupta, 2005; Verma, Robarts, & Headley, 2007). Furthermore, these two studies have only implicitly addressed the topic of tetracycline bioavailability in natural environments. To the best of the author's knowledge, no studies exist in which mechanistic examination of bioavailability and how it controls expression of the tetracycline efflux genes in the context of the relevant environmental tetracycline chemistry have been conducted.

Objectives –

Presented herein are the results of research on three specific objectives:

1) To determine the full-coding sequence of potentially novel RPP genes detected in an agricultural soil environment impacted with manure contaminated with tetracycline residues.
2) To examine the diversity of soil genera harboring tetracycline efflux pumps in the environment mentioned above.
3) To conduct mechanistic research into factors that would control bioavailability of tetracycline in environmental matrices, and hence, expression of tetracycline efflux genes through modulation of the TetR repressor protein.

REFERENCES

Agerso, Y., & Guardabassi, L. (2005). Identification of Tet 39, a novel class of tetracycline resistance determinant in Acinetobacter spp. of environmental and clinical origin. *J Antimicrob Chemother, 55*(4), 566-569.

Agerso, Y., Pedersen, A. G., & Aarestrup, F. M. (2006). Identification of Tn5397-like and Tn916-like transposons and diversity of the tetracycline resistance gene tet(M) in enterococci from humans, pigs and poultry. *J Antimicrob Chemother, 57*(5), 832-839.

Alali, W. Q., Scott, H. M., Harvey, R. B., Norby, B., Lawhorn, D. B., & Pillai, S. D. (2008). Longitudinal Study of Antimicrobial Resistance among Escherichia coli Isolated from Integrated Multi-site Cohorts of Humans and Swine. *Appl Environ Microbiol.*

Aminov, R. I., Chee-Sanford, J. C., Garrigues, N., Teferedegne, B., Krapac, I. J., White, B. A., et al. (2002). Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. *Appl Environ Microbiol, 68*(4), 1786-1793.

Aminov, R. I., Garrigues-Jeanjean, N., & Mackie, R. I. (2001). Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl Environ Microbiol, 67*(1), 22-32.

Aubry-Damon, H., Grenet, K., Sall-Ndiaye, P., Che, D., Cordeiro, E., Bougnoux, M. E., et al. (2004). Antimicrobial resistance in commensal flora of pig farmers. *Emerg Infect Dis, 10*(5), 873-879.

Auerbach, E. A., Seyfried, E. E., & McMahon, K. D. (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res, 41*(5), 1143-1151.

Barnes, K. K., Kolpin, D. W., Furlong, E. T., Zaugg, S. D., Meyer, M. T., & Barber, L. B. (2008). A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States - I) Groundwater. *Science of The Total Environment, in press.*

Batt, A. L., Bruce, I. B., & Aga, D. S. (2006). Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. *Environ Pollut, 142*(2), 295-302.

Billington, S. J., & Jost, B. H. (2006). Multiple genetic elements carry the tetracycline resistance gene tet(W) in the animal pathogen Arcanobacterium pyogenes. *Antimicrob Agents Chemother, 50*(11), 3580-3587.

Brodersen, D. E., Clemons, W. M., Jr., Carter, A. P., Morgan-Warren, R. J., Wimberly, B. T., & Ramakrishnan, V. (2000). The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30S ribosomal subunit. *Cell, 103*(7), 1143-1154.

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ Microbiol, 8*(7), 1137-1144.

Capone, D. G., Weston, D. P., Miller, V., & Shoemaker, C. (1996). Antibacterial residues in marine sediments and invertebrates following chemotherapy in aquaculture. *Aquaculture, 145*(1-4), 55-75.

Cassell, G. H. (1997). Emergent antibiotic resistance: health risks and economic impact. *FEMS Immunol Med Microbiol, 18*(4), 271-274.

Chander, Y., Kumar, K., Goyal, S. M., & Gupta, S. C. (2005). Antibacterial activity of soil-bound antibiotics. *J Environ Qual, 34*(6), 1952-1957.

Chapin, A., Rule, A., Gibson, K., Buckley, T., & Schwab, K. (2005). Airborne multidrug-resistant bacteria isolated from a concentrated swine feeding operation. *Environ Health Perspect, 113*(2), 137-142.

Choi, K. J., Kim, S. G., Kim, C. W., & Kim, S. H. (2007). Determination of antibiotic compounds in water by on-line SPE-LC/MSD. *Chemosphere, 66*(6), 977-984.

Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev, 65*(2), 232-260 ; second page, table of contents.

Connell, S. R., Tracz, D. M., Nierhaus, K. H., & Taylor, D. E. (2003). Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob Agents Chemother, 47*(12), 3675-3681.

Coyne, R., Hiney, M., O'connor, B., Kerry, J., Cazabon, D., & Smith, P. (1994). Concentration and persistence of oxytetracycline in sediments under a marine salmon farm. *Aquaculture, 123*(1-2), 31-42.

Cravedi, J. P., Chouber, G., & Delous, G. (1987). Digestibility of chloramphenicol, oxolinic acid, and oxytetracycline in rainbow trout and influence of these antibiotics on lipid digestibility. *Aquaculture, 60*, 133-141.

Cromwell, G. L. (2002). Why and how antibiotics are used in swine production. *Anim Biotechnol, 13*(1), 7-27.

D'Costa, V. M., Griffiths, E., & Wright, G. D. (2007). Expanding the soil antibiotic resistome: exploring environmental diversity. *Curr Opin Microbiol, 10*(5), 481-489.

D'Costa, V. M., McGrann, K. M., Hughes, D. W., & Wright, G. D. (2006). Sampling the antibiotic resistome. *Science, 311*(5759), 374-377.

Dailidiene, D., Bertoli, M. T., Miciuleviciene, J., Mukhopadhyay, A. K., Dailide, G., Pascasio, M. A., et al. (2002). Emergence of tetracycline resistance in Helicobacter pylori: multiple mutational changes in 16S ribosomal DNA and other genetic loci. *Antimicrob Agents Chemother, 46*(12), 3940-3946.

Dang, H., Ren, J., Song, L., Sun, S., & An, L. (2008). Diverse tetracycline resistant bacteria and resistance genes from coastal waters of Jiaozhou Bay. *Microb Ecol, 55*(2), 237-246.

De Leener, E., Martel, A., Decostere, A., & Haesebrouck, F. (2004). Distribution of the erm (B) gene, tetracycline resistance genes, and Tn1545-like transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. *Microb Drug Resist, 10*(4), 341-345.

Diaz-Torres, M. L., McNab, R., Spratt, D. A., Villedieu, A., Hunt, N., Wilson, M., et al. (2003). Novel tetracycline resistance determinant from the oral metagenome. *Antimicrob Agents Chemother, 47*(4), 1430-1432.

Duggar, B. M. (1948). Aureomycin; a product of the continuing search for new antibiotics. *Ann N Y Acad Sci, 51*(Art. 2), 177-181.

Durckheimer, W. (1975). Tetracyclines: chemistry, biochemistry, and structure-activity relations. *Angew Chem Int Ed Engl, 14*(11), 721-734.

Elkins, C. A., & Nikaido, H. (2002). Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of Escherichia coli is determined predominantly by two large periplasmic loops. *J Bacteriol, 184*(23), 6490-6498.

Ferreira da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O. C., & Manaia, C. M. (2007). Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiol Ecol, 60*(1), 166-176.

Focazio, M. J., Kolpin, D. W., Barnes, K. K., Furlong, E. T., Meyer, M. T., Zaugg, S. D., et al. (2008). A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States - II) Untreated drinking water sources. *Science of the Total Environment, in press.*

Furushita, M., Shiba, T., Maeda, T., Yahata, M., Kaneoka, A., Takahashi, Y., et al. (2003). Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl Environ Microbiol, 69*(9), 5336-5342.

Gaskins, H. R., Collier, C. T., & Anderson, D. B. (2002). Antibiotics as growth promotants: mode of action. *Anim Biotechnol, 13*(1), 29-42.

Gerrits, M. M., de Zoete, M. R., Arents, N. L., Kuipers, E. J., & Kusters, J. G. (2002). 16S rRNA mutation-mediated tetracycline resistance in Helicobacter pylori. *Antimicrob Agents Chemother, 46*(9), 2996-3000.

Ghosh, S., & LaPara, T. M. (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J, 1*(3), 191-203.

Gibbs, S. G., Green, C. F., Tarwater, P. M., Mota, L. C., Mena, K. D., & Scarpino, P. V. (2006). Isolation of antibiotic-resistant bacteria from the air plume downwind of a swine confined or concentrated animal feeding operation. *Environ Health Perspect, 114*(7), 1032-1037.

Gibbs, S. G., Green, C. F., Tarwater, P. M., & Scarpino, P. V. (2004). Airborne antibiotic resistant and nonresistant bacteria and fungi recovered from two swine herd confined animal feeding operations. *J Occup Environ Hyg, 1*(11), 699-706.

Gilmour, M. W., Thomson, N. R., Sanders, M., Parkhill, J., & Taylor, D. E. (2004). The complete nucleotide sequence of the resistance plasmid R478: defining the backbone components of incompatibility group H conjugative plasmids through comparative genomics. *Plasmid, 52*(3), 182-202.

Gomez-Jimenez, S., Espinosa-Plascencia, A., Valenzuela-Villa, F., & del Carmen Bermudez-Almada, M. (2008). Oxytetracycline (OTC) accumulation and elimination in hemolymph, muscle, and hepatopancreas of white shrimp *Litopenaeus vannamei* following an OTC-feed therapeutic treatment. *Aquaculture, 274*(1), 24-29.

Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P., & Quentin, C. (2000). Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and Aeromonas spp. *Appl Environ Microbiol, 66*(1), 125-132.

Graslund, S., & Bengtsson, B. E. (2001). Chemicals and biological products used in south-east Asian shrimp farming, and their potential impact on the environment--a review. *Sci Total Environ, 280*(1-3), 93-131.

Groh, J. L., Luo, Q., Ballard, J. D., & Krumholz, L. R. (2007). Genes that enhance the ecological fitness of Shewanella oneidensis MR-1 in sediments reveal the value of antibiotic resistance. *Appl Environ Microbiol, 73*(2), 492-498.

Gu, C., Karthikeyan, K. G., Sibley, S. D., & Pedersen, J. A. (2007). Complexation of the antibiotic tetracycline with humic acid. *Chemosphere, 66*(8), 1494-1501.

Guillaume, G., Ledent, V., Moens, W., & Collard, J. M. (2004). Phylogeny of efflux-mediated tetracycline resistance genes and related proteins revisited. *Microb Drug Resist, 10*(1), 11-26.

Guiney, D. G., Jr., Hasegawa, P., & Davis, C. E. (1984). Expression in Escherichia coli of cryptic tetracycline resistance genes from bacteroides R plasmids. *Plasmid, 11*(3), 248-252.

Gulkowska, A., Leung, H. W., So, M. K., Taniyasu, S., Yamashita, N., Yeung, L. W., et al. (2008). Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China. *Water Res, 42*(1-2), 395-403.

Hamscher, G., Pawelzick, H. T., Hoper, H., & Nau, H. (2005). Different behavior of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure. *Environ Toxicol Chem, 24*(4), 861-868.

Hamscher, G., Pawelzick, H. T., Sczesny, S., Nau, H., & Hartung, J. (2003). Antibiotics in dust originating from a pig-fattening farm: a new source of health hazard for farmers? *Environ Health Perspect, 111*(13), 1590-1594.

Hamscher, G., Sczesny, S., Hoper, H., & Nau, H. (2002). Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal Chem, 74*(7), 1509-1518.

Hektoen, H., Berge, J. A., Hormazabal, V., & Yndestad, M. (1995). Persistence of antibacterial agents in marine sediments. *Aquaculture, 133*(3-4), 175-184.

Hileman, B. (2001). Resistance is on the rise: FDA proposes criteria for restricting or banning certain antibiotics in livestock. *Chemical & Engineering News, 79*(8), 47-52.

Hirsch, R., Ternes, T., Haberer, K., & Kratz, K. L. (1999). Occurrence of antibiotics in the aquatic environment. *Sci Total Environ, 225*(1-2), 109-118.

Karthikeyan, K. G., & Meyer, M. T. (2006). Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Sci Total Environ, 361*(1-3), 196-207.

Kay, P., Blackwell, P. A., & Boxall, A. B. (2004). Fate of veterinary antibiotics in a macroporous tile drained clay soil. *Environ Toxicol Chem, 23*(5), 1136-1144.

Kerry, J., Hiney, M., Coyne, R., Cazabon, D., NicGabhainn, S., & Smith, P. (1994). Frequency and distribution of resistance to oxytetracycline in micro-organisms

isolated from marine fish farm sediments following therapeutic use of oxytetracycline. *Aquaculture, 123*(1-2), 43-54.

Kim, S. R., Nonaka, L., & Suzuki, S. (2004). Occurrence of tetracycline resistance genes tet(M) and tet(S) in bacteria from marine aquaculture sites. *FEMS Microbiol Lett, 237*(1), 147-156.

Klappenbach, J. A., Dunbar, J. M., & Schmidt, T. M. (2000). rRNA operon copy number reflects ecological strategies of bacteria. *Appl Environ Microbiol, 66*(4), 1328-1333.

Kobayashi, T., Nonaka, L., Maruyama, F., & Suzuki, S. (2007). Molecular evidence for the ancient origin of the ribosomal protection protein that mediates tetracycline resistance in bacteria. *J Mol Evol, 65*(3), 228-235.

Kobayashi, T., Suehiro, F., Cach Tuyen, B., & Suzuki, S. (2007). Distribution and diversity of tetracycline resistance genes encoding ribosomal protection proteins in Mekong river sediments in Vietnam. *FEMS Microbiol Ecol, 59*(3), 729-737.

Koike, S., Krapac, I. G., Oliver, H. D., Yannarell, A. C., Chee-Sanford, J. C., Aminov, R. I., et al. (2007). Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period. *Appl Environ Microbiol, 73*(15), 4813-4823.

Koksal, F., Oguzkurt, N., Samasti, M., & Altas, K. (2007). Prevalence and antimicrobial resistance patterns of Aeromonas strains isolated from drinking water samples in istanbul, Turkey. *Chemotherapy, 53*(1), 30-35.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., et al. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environ Sci Technol, 36*(6), 1202-1211.

Koonin, E. V. (2003). Horizontal gene transfer: the path to maturity. *Mol Microbiol, 50*(3), 725-727.

Krulwich, T. A., Jin, J., Guffanti, A. A., & Bechhofer, H. (2001). Functions of tetracycline efflux proteins that do not involve tetracycline. *J Mol Microbiol Biotechnol, 3*(2), 237-246.

Kummerer, K. (2003). Significance of antibiotics in the environment. *J Antimicrob Chemother, 52*(1), 5-7.

Kummerer, K. (2004). Resistance in the environment. *J Antimicrob Chemother, 54*(2), 311-320.

Kummerer, K., & Henninger, A. (2003). Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin Microbiol Infect, 9*(12), 1203-1214.

Langlois, B. E., Cromwell, G. L., Stahly, T. S., Dawson, K. A., & Hays, V. W. (1983). Antibiotic resistance of fecal coliforms after long-term withdrawal of therapeutic and subtherapeutic antibiotic use in a swine herd. *Appl Environ Microbiol, 46*(6), 1433-1434.

Lau, S. K., Woo, P. C., To, A. P., Lau, A. T., & Yuen, K. Y. (2004). Lack of evidence that DNA in antibiotic preparations is a source of antibiotic resistance genes in bacteria from animal or human sources. *Antimicrob Agents Chemother, 48*(8), 3141-3146.

Lenski, R. E., Simpson, S. C., & Nguyen, T. T. (1994). Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. *J Bacteriol, 176*(11), 3140-3147.

Martinez-Carballo, E., Gonzalez-Barreiro, C., Scharf, S., & Gans, O. (2007). Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. *Environ Pollut, 148*(2), 570-579.

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nat Rev Microbiol, 4*(8), 608-620.

McManus, P. S. (2000). Antibiotic Use and Microbial Resistance in Plant Agriculture. *ASM News, 66*(August), 448.

McManus, P. S., Stockwell, V. O., Sundin, G. W., & Jones, A. L. (2002). Antibiotic use in plant agriculture. *Annu Rev Phytopathol, 40*, 443-465.

Miao, X. S., Bishay, F., Chen, M., & Metcalfe, C. D. (2004). Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environ Sci Technol, 38*(13), 3533-3541.

Mitscher, L. A. (1978). *The chemistry of the tetracycline antibiotics*. New York: M. Dekker.

Namdari, R., Abedini, S., & Law, F. C. P. (1996). Tissue distribution and elimination of oxytetracycline in seawater chinook and coho salmon following medicated-feed treatment. *Aquaculture, 144*(1-3), 27-38.

Nguyen, T. N., Phan, Q. G., Duong, L. P., Bertrand, K. P., & Lenski, R. E. (1989). Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of Escherichia coli K12. *Mol Biol Evol, 6*(3), 213-225.

Nikolich, M. P., Hong, G., Shoemaker, N. B., & Salyers, A. A. (1994). Evidence for natural horizontal transfer of tetQ between bacteria that normally colonize humans and bacteria that normally colonize livestock. *Appl Environ Microbiol, 60*(9), 3255-3260.

Nogueira-Lima, A. C., Gesteira, T. C. V., & Mafezoli, J. (2006). Oxytetracycline residues in cultivated marine shrimp (*Litopenaeus vannamei* Boone, 1931) (Crustacea, Decapoda) submitted to antibiotic treatment. *Aquaculture, 254*(1-4), 748-757.

Nonaka, L., & Suzuki, S. (2002). New Mg2+-dependent oxytetracycline resistance determinant tet 34 in Vibrio isolates from marine fish intestinal contents. *Antimicrob Agents Chemother, 46*(5), 1550-1552.

Oggioni, M. R., Dowson, C. G., Smith, J. M., Provvedi, R., & Pozzi, G. (1996). The tetracycline resistance gene tet(M) exhibits mosaic structure. *Plasmid, 35*(3), 156-163.

Oka, H., Ito, Y., & Matsumoto, H. (2000). Chromatographic analysis of tetracycline antibiotics in foods. *J Chromatogr A, 882*(1-2), 109-133.

Paulsen, I. T., Brown, M. H., & Skurray, R. A. (1996). Proton-dependent multidrug efflux systems. *Microbiol Rev, 60*(4), 575-608.

Pei, R., Kim, S. C., Carlson, K. H., & Pruden, A. (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res, 40*(12), 2427-2435.

Petersen, A., Andersen, J. S., Kaewmak, T., Somsiri, T., & Dalsgaard, A. (2002). Impact of integrated fish farming on antimicrobial resistance in a pond environment. *Appl Environ Microbiol, 68*(12), 6036-6042.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., et al. (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother, 53*(1), 28-52.

Pils, J. R., & Laird, D. A. (2007). Sorption of tetracycline and chlortetracycline on K- and Ca-saturated soil clays, humic substances, and clay-humic complexes. *Environ Sci Technol, 41*(6), 1928-1933.

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *J Antimicrob Chemother, 56*(1), 20-51.

Pruden, A., Pei, R., Storteboom, H., & Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environ Sci Technol, 40*(23), 7445-7450.

Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Smith, P., et al. (2000). Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant tet A. *Appl Environ Microbiol, 66*(9), 3883-3890.

Riesenfeld, C. S., Goodman, R. M., & Handelsman, J. (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol, 6*(9), 981-989.

Roberts, M. C. (2003). Tetracycline therapy: update. *Clin Infect Dis, 36*(4), 462-467.

Roberts, M. C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiol Lett, 245*(2), 195-203.

Rose, P. E., & Pedersen, J. A. (2005). Fate of oxytetracycline in streams receiving aquaculture discharges: model simulations. *Environ Toxicol Chem, 24*(1), 40-50.

Ross, J. I., Eady, E. A., Cove, J. H., & Cunliffe, W. J. (1998). 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium. *Antimicrob Agents Chemother, 42*(7), 1702-1705.

Samuelsen, O. B., Torsvik, V., & Ervik, A. (1992). Long-range changes in oxytetracycline concentration and bacterial resistance toward oxytetracycline in a fish farm sediment after medication. *Sci Total Environ, 114*, 25-36.

Sanchez-Pescador, R., Brown, J. T., Roberts, M., & Urdea, M. S. (1988). Homology of the TetM with translational elongation factors: implications for potential modes of tetM-conferred tetracycline resistance. *Nucleic Acids Res, 16*(3), 1218.

Sapkota, A., Sapkota, A. R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., et al. (2008). Aquaculture practices and potential human health risks: Current knowledge and future priorities. *Environ Int.*

Sapkota, A. R., Curriero, F. C., Gibson, K. E., & Schwab, K. J. (2007). Antibiotic-resistant enterococci and fecal indicators in surface water and groundwater impacted by a concentrated Swine feeding operation. *Environ Health Perspect, 115*(7), 1040-1045.

Sarmah, A. K., Meyer, M. T., & Boxall, A. B. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere, 65*(5), 725-759.

Sassman, S. A., & Lee, L. S. (2005). Sorption of three tetracyclines by several soils: assessing the role of pH and cation exchange. *Environ Sci Technol, 39*(19), 7452-7459.

Schnabel, E. L., & Jones, A. L. (1999). Distribution of tetracycline resistance genes and transposons among phylloplane bacteria in Michigan apple orchards. *Appl Environ Microbiol, 65*(11), 4898-4907.

Shao, Z. J. (2001). Aquaculture pharmaceuticals and biologicals: current perspectives and future possibilities. *Adv Drug Deliv Rev, 50*(3), 229-243.

Shlaes, D. M. (2006). An update on tetracyclines. *Curr Opin Investig Drugs, 7*(2), 167-171.

Smilack, J. D. (1999). The tetracyclines. *Mayo Clin Proc, 74*(7), 727-729.

Smith, P., & Samuelsen, O. B. (1996). Estimates of the significance of out-washing of oxytetracycline from sediments under Atlantic salmon sea-cages. *Aquaculture, 144*(1-3), 17-26.

Snyder, L., & Champness, W. (2003). *Molecular genetics of bacteria* (2nd ed.). Washington, D.C.: ASM Press.

Speer, B. S., Bedzyk, L., & Salyers, A. A. (1991). Evidence that a novel tetracycline resistance gene found on two Bacteroides transposons encodes an NADP-requiring oxidoreductase. *J Bacteriol, 173*(1), 176-183.

Speer, B. S., & Salyers, A. A. (1988). Characterization of a novel tetracycline resistance that functions only in aerobically grown Escherichia coli. *J Bacteriol, 170*(4), 1423-1429.

Speer, B. S., & Salyers, A. A. (1990). A tetracycline efflux gene on Bacteroides transposon Tn4400 does not contribute to tetracycline resistance. *J Bacteriol, 172*(1), 292-298.

Srinivasan, V., Nam, H. M., Sawant, A. A., Headrick, S. I., Nguyen, L. T., & Oliver, S. P. (2008). Distribution of tetracycline and streptomycin resistance genes and class 1 integrons in Enterobacteriaceae isolated from dairy and nondairy farm soils. *Microb Ecol, 55*(2), 184-193.

Stanton, T. B., & Humphrey, S. B. (2003). Isolation of tetracycline-resistant Megasphaera elsdenii strains with novel mosaic gene combinations of tet(O) and tet(W) from swine. *Appl Environ Microbiol, 69*(7), 3874-3882.

Stanton, T. B., McDowall, J. S., & Rasmussen, M. A. (2004). Diverse tetracycline resistance genotypes of Megasphaera elsdenii strains selectively cultured from swine feces. *Appl Environ Microbiol, 70*(6), 3754-3757.

Stine, O. C., Johnson, J. A., Keefer-Norris, A., Perry, K. L., Tigno, J., Qaiyumi, S., et al. (2007). Widespread distribution of tetracycline resistance genes in a confined animal feeding facility. *Int J Antimicrob Agents, 29*(3), 348-352.

Sunde, M., & Sorum, H. (2001). Self-transmissible multidrug resistance plasmids in Escherichia coli of the normal intestinal flora of healthy swine. *Microb Drug Resist, 7*(2), 191-196.

Szczepanowski, R., Braun, S., Riedel, V., Schneiker, S., Krahn, I., Puhler, A., et al. (2005). The 120 592 bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antibiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated functions. *Microbiology, 151*(Pt 4), 1095-1111.

Tagliavini, F., Forloni, G., Colombo, L., Rossi, G., Girola, L., Canciani, B., et al. (2000). Tetracycline affects abnormal properties of synthetic PrP peptides and PrP(Sc) in vitro. *J Mol Biol, 300*(5), 1309-1322.

Thomas, K. V., Dye, C., Schlabach, M., & Langford, K. H. (2007). Source to sink tracking of selected human pharmaceuticals from two Oslo city hospitals and a wastewater treatment works. *J Environ Monit, 9*(12), 1410-1418.

Thompson, S. A., Maani, E. V., Lindell, A. H., King, C. J., & McArthur, J. V. (2007). Novel tetracycline resistance determinant isolated from an environmental strain of Serratia marcescens. *Appl Environ Microbiol, 73*(7), 2199-2206.

Tolls, J. (2001). Sorption of veterinary pharmaceuticals in soils: a review. *Environ Sci Technol, 35*(17), 3397-3406.

Torsvik, V., Goksoyr, J., & Daae, F. L. (1990). High diversity in DNA of soil bacteria. *Appl Environ Microbiol, 56*(3), 782-787.

Torsvik, V., & Ovreas, L. (2002). Microbial diversity and function in soil: from genes to ecosystems. *Curr Opin Microbiol, 5*(3), 240-245.

Twomey, C. (2000). Antibiotic resistance--an alarming health care issue. *AORN J, 72*(1), 64-66, 68-75 quiz 76-80.

Uno, K., Aoki, T., Kleechaya, W., Tanasomwang, V., & Ruangpan, L. (2006). Pharmacokinetics of oxytetracycline in black tiger shrimp, *Penaeus monodon*, and the effect of cooking on the residues. *Aquaculture, 254*(1-4), 24-31.

Verma, B., Robarts, R. D., & Headley, J. V. (2007). Impacts of tetracycline on planktonic bacterial production in prairie aquatic systems. *Microb Ecol, 54*(1), 52-55.

Vidaver, A. K. (2002). Uses of antimicrobials in plant agriculture. *Clin Infect Dis, 34 Suppl 3*, S107-110.

Viveiros, M., Dupont, M., Rodrigues, L., Couto, I., Davin-Regli, A., Martins, M., et al. (2007). Antibiotic stress, genetic response and altered permeability of E. coli. *PLoS ONE, 2*(4), e365.

Walker, M. S., & Walker, J. B. (1970). Streptomycin biosynthesis and metabolism. Enzymatic phosphorylation of dihydrostreptobiosamine moieties of dihydrostreptomycin-(streptidino) phosphate and dihydrostreptomycin by Streptomyces extracts. *J Biol Chem, 245*(24), 6683-6689.

Wilcks, A., Andersen, S. R., & Licht, T. R. (2005). Characterization of transferable tetracycline resistance genes in Enterococcus faecalis isolated from raw food. *FEMS Microbiol Lett, 243*(1), 15-19.

Witte, W. (1998). Medical consequences of antibiotic use in agriculture. *Science, 279*(5353), 996-997.

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol, 5*(3), 175-186.

Yang, S., & Carlson, K. (2003). Evolution of antibiotic occurrence in a river through pristine, urban and agricultural landscapes. *Water Res, 37*(19), 4645-4656.

Yang, S., Cha, J., & Carlson, K. (2004). Quantitative determination of trace concentrations of tetracycline and sulfonamide antibiotics in surface water using solid-phase extraction and liquid chromatography/ion trap tandem mass spectrometry. *Rapid Commun Mass Spectrom, 18*(18), 2131-2145.

Yang, S., Cha, J., & Carlson, K. (2005). Simultaneous extraction and analysis of 11 tetracycline and sulfonamide antibiotics in influent and effluent domestic wastewater by solid-phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr A, 1097*(1-2), 40-53.

Yang, W., Moore, I. F., Koteva, K. P., Bareich, D. C., Hughes, D. W., & Wright, G. D. (2004). TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. *J Biol Chem, 279*(50), 52346-52352.

Zhang, Y., Yeh, E., Hall, G., Cripe, J., Bhagwat, A. A., & Meng, J. (2007). Characterization of Listeria monocytogenes isolated from retail foods. *Int J Food Microbiol, 113*(1), 47-53.

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Agerso, Y., & Guardabassi, L. (2005). Identification of Tet 39, a novel class of tetracycline resistance determinant in Acinetobacter spp. of environmental and clinical origin. *J Antimicrob Chemother, 55*(4), 566-569.

Akinbowale, O. L., Peng, H., & Barton, M. D. (2007). Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *J Appl Microbiol, 103*(5), 2016-2025.

Andersen, S. R., & Sandaa, R. A. (1994). Distribution of tetracycline resistance determinants among gram-negative bacteria isolated from polluted and unpolluted marine sediments. *Appl Environ Microbiol, 60*(3), 908-912.

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jeanjean, N., & Mackie, R. I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol, 67*(4), 1494-1502.

Dang, H., Ren, J., Song, L., Sun, S., & An, L. (2008). Diverse tetracycline resistant bacteria and resistance genes from coastal waters of Jiaozhou Bay. *Microb Ecol, 55*(2), 237-246.

Dang, H., Zhang, X., Song, L., Chang, Y., & Yang, G. (2006). Molecular characterizations of oxytetracycline resistant bacteria and their resistance genes from mariculture waters of China. *Mar Pollut Bull, 52*(11), 1494-1503.

Dang, H., Zhang, X., Song, L., Chang, Y., & Yang, G. (2007). Molecular determination of oxytetracycline-resistant bacteria and their resistance genes from mariculture environments of China. *J Appl Microbiol, 103*(6), 2580-2592.

DePaola, A., Hill, W. E., & Harrell, F. M. (1993). Oligonucleotide probe determination of tetracycline-resistant bacteria isolated from catfish ponds. *Mol Cell Probes, 7*(5), 345-348.

DePaola, A., & Roberts, M. C. (1995). Class D and E tetracycline resistance determinants in gram-negative bacteria from catfish ponds. *Mol Cell Probes, 9*(5), 311-313.

Furushita, M., Shiba, T., Maeda, T., Yahata, M., Kaneoka, A., Takahashi, Y., et al. (2003). Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl Environ Microbiol, 69*(9), 5336-5342.

Gordon, L., Cloeckaert, A., Doublet, B., Schwarz, S., Bouju-Albert, A., Ganiere, J. P., et al. (2008). Complete sequence of the floR-carrying multiresistance plasmid pAB5S9 from freshwater Aeromonas bestiarum. *J Antimicrob Chemother, 62*(1), 65-71.

Jacobs, L., & Chenia, H. Y. (2007). Characterization of integrons and tetracycline resistance determinants in Aeromonas spp. isolated from South African aquaculture systems. *Int J Food Microbiol, 114*(3), 295-306.

Kim, S. R., Nonaka, L., & Suzuki, S. (2004). Occurrence of tetracycline resistance genes tet(M) and tet(S) in bacteria from marine aquaculture sites. *FEMS Microbiol Lett, 237*(1), 147-156.

L'Abee-Lund, T. M., & Sorum, H. (2000). Functional Tn5393-like transposon in the R plasmid pRAS2 from the fish pathogen Aeromonas salmonicida subspecies salmonicida isolated in Norway. *Appl Environ Microbiol, 66*(12), 5533-5535.

Miranda, C. D., Kehrenberg, C., Ulep, C., Schwarz, S., & Roberts, M. C. (2003). Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob Agents Chemother, 47*(3), 883-888.

Nikolakopoulou, T. L., Egan, S., van Overbeek, L. S., Guillaume, G., Heuer, H., Wellington, E. M., et al. (2005). PCR detection of oxytetracycline resistance genes otr(A) and otr(B) in tetracycline-resistant streptomycete isolates from diverse habitats. *Curr Microbiol, 51*(4), 211-216.

Nonaka, L., & Suzuki, S. (2002). New Mg2+-dependent oxytetracycline resistance determinant tet 34 in Vibrio isolates from marine fish intestinal contents. *Antimicrob Agents Chemother, 46*(5), 1550-1552.

Ozgumus, O. B., Celik-Sevim, E., Alpay-Karaoglu, S., Sandalli, C., & Sevim, A. (2007). Molecular characterization of antibiotic resistant Escherichia coli strains isolated from tap and spring waters in a coastal region in Turkey. *J Microbiol, 45*(5), 379-387.

Petersen, A., & Dalsgaard, A. (2003). Species composition and antimicrobial resistance genes of Enterococcus spp, isolated from integrated and traditional fish farms in Thailand. *Environ Microbiol, 5*(5), 395-402.

Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Smith, P., et al. (2000). Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant tet A. *Appl Environ Microbiol, 66*(9), 3883-3890.

Schmidt, A. S., Bruun, M. S., Dalsgaard, I., & Larsen, J. L. (2001). Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. *Appl Environ Microbiol, 67*(12), 5675-5682.

Stine, O. C., Johnson, J. A., Keefer-Norris, A., Perry, K. L., Tigno, J., Qaiyumi, S., et al. (2007). Widespread distribution of tetracycline resistance genes in a confined animal feeding facility. *Int J Antimicrob Agents, 29*(3), 348-352.

Thompson, S. A., Maani, E. V., Lindell, A. H., King, C. J., & McArthur, J. V. (2007). Novel tetracycline resistance determinant isolated from an environmental strain of Serratia marcescens. *Appl Environ Microbiol, 73*(7), 2199-2206.

References Table 1.2, pg 12

Agerso, Y., & Sandvang, D. (2005). Class 1 integrons and tetracycline resistance genes in alcaligenes, arthrobacter, and Pseudomonas spp. isolated from pigsties and manured soil. *Appl Environ Microbiol, 71*(12), 7941-7947.

Ghosh, S., & LaPara, T. M. (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J, 1*(3), 191-203.

Nikolakopoulou, T. L., Egan, S., van Overbeek, L. S., Guillaume, G., Heuer, H., Wellington, E. M., et al. (2005). PCR detection of oxytetracycline resistance genes otr(A) and otr(B) in tetracycline-resistant streptomycete isolates from diverse habitats. *Curr Microbiol, 51*(4), 211-216.

Srinivasan, V., Nam, H. M., Sawant, A. A., Headrick, S. I., Nguyen, L. T., & Oliver, S. P. (2008). Distribution of tetracycline and streptomycin resistance genes and class 1 integrons in Enterobacteriaceae isolated from dairy and nondairy farm soils. *Microb Ecol, 55*(2), 184-193.

Stine, O. C., Johnson, J. A., Keefer-Norris, A., Perry, K. L., Tigno, J., Qaiyumi, S., et al. (2007). Widespread distribution of tetracycline resistance genes in a confined animal feeding facility. *Int J Antimicrob Agents, 29*(3), 348-352.

Tauch, A., Puhler, A., Kalinowski, J., & Thierbach, G. (2000). TetZ, a new tetracycline resistance determinant discovered in gram-positive bacteria, shows high homology to gram-negative regulated efflux systems. *Plasmid, 44*(3), 285-291.

References Table 1.3, pg 14

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jeanjean, N., & Mackie, R. I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol, 67*(4), 1494-1502.

Sapkota, A. R., Ojo, K. K., Roberts, M. C., & Schwab, K. J. (2006). Antibiotic resistance genes in multidrug-resistant Enterococcus spp. and Streptococcus spp. recovered

from the indoor air of a large-scale swine-feeding operation. *Lett Appl Microbiol, 43*(5), 534-540.

Srinivasan, V., Nam, H. M., Nguyen, L. T., Tamilselvam, B., Murinda, S. E., & Oliver, S. P. (2005). Prevalence of antimicrobial resistance genes in Listeria monocytogenes isolated from dairy farms. *Foodborne Pathog Dis, 2*(3), 201-211.

Stine, O. C., Johnson, J. A., Keefer-Norris, A., Perry, K. L., Tigno, J., Qaiyumi, S., et al. (2007). Widespread distribution of tetracycline resistance genes in a confined animal feeding facility. *Int J Antimicrob Agents, 29*(3), 348-352.

Whittle, G., Whitehead, T. R., Hamburger, N., Shoemaker, N. B., Cotta, M. A., & Salyers, A. A. (2003). Identification of a new ribosomal protection type of tetracycline resistance gene, tet(36), from swine manure pits. *Appl Environ Microbiol, 69*(7), 4151-4158.

References Table 1.4, pg 15

Balassiano, I. T., Bastos Mdo, C., Madureira, D. J., Silva, I. G., Freitas-Almeida, A. C., & Oliveira, S. S. (2007). The involvement of tetA and tetE tetracycline resistance genes in plasmid and chromosomal resistance of Aeromonas in Brazilian strains. *Mem Inst Oswaldo Cruz, 102*(7), 861-866.

Facinelli, B., Roberts, M. C., Giovanetti, E., Casolari, C., Fabio, U., & Varaldo, P. E. (1993). Genetic basis of tetracycline resistance in food-borne isolates of Listeria innocua. *Appl Environ Microbiol, 59*(2), 614-616.

Gevers, D., Danielsen, M., Huys, G., & Swings, J. (2003). Molecular characterization of tet(M) genes in Lactobacillus isolates from different types of fermented dry sausage. *Appl Environ Microbiol, 69*(2), 1270-1275.

Lee, C. Y., Tai, C. L., Lin, S. C., & Chen, Y. T. (1994). Occurrence of plasmids and tetracycline resistance among Campylobacter jejuni and Campylobacter coli isolated from whole market chickens and clinical samples. *Int J Food Microbiol, 24*(1-2), 161-170.

Macovei, L., & Zurek, L. (2007). Influx of enterococci and associated antibiotic resistance and virulence genes from ready-to-eat food to the human digestive tract. *Appl Environ Microbiol, 73*(21), 6740-6747.

Nogrady, N., Toth, A., Kostyak, A., Paszti, J., & Nagy, B. (2007). Emergence of multidrug-resistant clones of Salmonella Infantis in broiler chickens and humans in Hungary. *J Antimicrob Chemother, 60*(3), 645-648.

Wilcks, A., Andersen, S. R., & Licht, T. R. (2005). Characterization of transferable tetracycline resistance genes in Enterococcus faecalis isolated from raw food. *FEMS Microbiol Lett, 243*(1), 15-19.

References for Table 1.5, pg 16

Aarestrup, F. M., Agers, L. Y., Ahrens, P., JC, J. L., Madsen, M., & Jensen, L. B. (2000). Antimicrobial susceptibility and presence of resistance genes in staphylococci from poultry. *Vet Microbiol, 74*(4), 353-364.

Aarestrup, F. M., Agerso, Y., Gerner-Smidt, P., Madsen, M., & Jensen, L. B. (2000). Comparison of antimicrobial resistance phenotypes and resistance genes in Enterococcus faecalis and Enterococcus faecium from humans in the community, broilers, and pigs in Denmark. *Diagn Microbiol Infect Dis, 37*(2), 127-137.

Aarestrup, F. M., & Jensen, L. B. (2002). Trends in antimicrobial susceptibility in relation to antimicrobial usage and presence of resistance genes in Staphylococcus hyicus isolated from exudative epidermitis in pigs. *Vet Microbiol, 89*(1), 83-94.

Agerso, Y., & Sandvang, D. (2005). Class 1 integrons and tetracycline resistance genes in alcaligenes, arthrobacter, and Pseudomonas spp. isolated from pigsties and manured soil. *Appl Environ Microbiol, 71*(12), 7941-7947.

Anderson, J. F., Parrish, T. D., Akhtar, M., Zurek, L., & Hirt, H. (2008). Antibiotic resistance of enterococci in American bison (Bison bison) from a nature preserve compared to that of Enterococci in pastured cattle. *Appl Environ Microbiol, 74*(6), 1726-1730.

Barbosa, T. M., Scott, K. P., & Flint, H. J. (1999). Evidence for recent intergeneric transfer of a new tetracycline resistance gene, tet(W), isolated from Butyrivibrio fibrisolvens, and the occurrence of tet(O) in ruminal bacteria. *Environ Microbiol, 1*(1), 53-64.

Billington, S. J., & Jost, B. H. (2006). Multiple genetic elements carry the tetracycline resistance gene tet(W) in the animal pathogen Arcanobacterium pyogenes. *Antimicrob Agents Chemother, 50*(11), 3580-3587.

Billington, S. J., Songer, J. G., & Jost, B. H. (2002). Widespread distribution of a tet W determinant among tetracycline-resistant isolates of the animal pathogen Arcanobacterium pyogenes. *Antimicrob Agents Chemother, 46*(5), 1281-1287.

Brown, M. B., & Roberts, M. C. (1991). Tetracycline resistance determinants in streptococcal species isolated from the bovine mammary gland. *Vet Microbiol, 29*(2), 173-180.

Chaslus-Dancla, E., Lesage-Descauses, M. C., Leroy-Setrin, S., Martel, J. L., & Lafont, J. P. (1995). Tetracycline resistance determinants, Tet B and Tet M, detected in Pasteurella haemolytica and Pasteurella multocida from bovine herds. *J Antimicrob Chemother, 36*(5), 815-819.

De Leener, E., Martel, A., Decostere, A., & Haesebrouck, F. (2004). Distribution of the erm (B) gene, tetracycline resistance genes, and Tn1545-like transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. *Microb Drug Resist, 10*(4), 341-345.

Duarte, R. S., Bellei, B. C., Miranda, O. P., Brito, M. A., & Teixeira, L. M. (2005). Distribution of antimicrobial resistance and virulence-related genes among Brazilian group B streptococci recovered from bovine and human sources. *Antimicrob Agents Chemother, 49*(1), 97-103.

Dugan, J., Rockey, D. D., Jones, L., & Andersen, A. A. (2004). Tetracycline resistance in Chlamydia suis mediated by genomic islands inserted into the chlamydial inv-like gene. *Antimicrob Agents Chemother, 48*(10), 3989-3995.

Gebreyes, W. A., & Altier, C. (2002). Molecular characterization of multidrug-resistant Salmonella enterica subsp. enterica serovar Typhimurium isolates from swine. *J Clin Microbiol, 40*(8), 2813-2822.

Guevremont, E., Nadeau, E., Sirois, M., & Quessy, S. (2006). Antimicrobial susceptibilities of thermophilic Campylobacter from humans, swine, and chicken broilers. *Can J Vet Res, 70*(2), 81-86.

Hansen, L. M., Blanchard, P. C., & Hirsh, D. C. (1996). Distribution of tet(H) among Pasteurella isolates from the United States and Canada. *Antimicrob Agents Chemother, 40*(6), 1558-1560.

Huys, G., D'Haene, K., Van Eldere, J., von Holy, A., & Swings, J. (2005). Molecular diversity and characterization of tetracycline-resistant Staphylococcus aureus isolates from a poultry processing plant. *Appl Environ Microbiol, 71*(1), 574-579.

Johansson, A., Greko, C., Engstrom, B. E., & Karlsson, M. (2004). Antimicrobial susceptibility of Swedish, Norwegian and Danish isolates of Clostridium perfringens from poultry, and distribution of tetracycline resistance genes. *Vet Microbiol, 99*(3-4), 251-257.

Kehrenberg, C., Catry, B., Haesebrouck, F., de Kruif, A., & Schwarz, S. (2005). tet(L)-mediated tetracycline resistance in bovine Mannheimia and Pasteurella isolates. *J Antimicrob Chemother, 56*(2), 403-406.

Kehrenberg, C., Salmon, S. A., Watts, J. L., & Schwarz, S. (2001). Tetracycline resistance genes in isolates of Pasteurella multocida, Mannheimia haemolytica,

Mannheimia glucosida and Mannheimia varigena from bovine and swine respiratory disease: intergeneric spread of the tet(H) plasmid pMHT1. *J Antimicrob Chemother, 48*(5), 631-640.

Kehrenberg, C., & Schwarz, S. (2001). Molecular analysis of tetracycline resistance in Pasteurella aerogenes. *Antimicrob Agents Chemother, 45*(10), 2885-2890.

Kim, T. E., Jeong, Y. W., Cho, S. H., Kim, S. J., & Kwon, H. J. (2007). Chronological study of antibiotic resistances and their relevant genes in Korean avian pathogenic Escherichia coli isolates. *J Clin Microbiol, 45*(10), 3309-3315.

Kumai, Y., Suzuki, Y., Tanaka, Y., Shima, K., Bhadra, R. K., Yamasaki, S., et al. (2005). Characterization of multidrug-resistance phenotypes and genotypes of Escherichia coli strains isolated from swine from an abattoir in Osaka, Japan. *Epidemiol Infect, 133*(1), 59-70.

Lanz, R., Kuhnert, P., & Boerlin, P. (2003). Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland. *Vet Microbiol, 91*(1), 73-84.

Lee, C., Langlois, B. E., & Dawson, K. A. (1993). Detection of tetracycline resistance determinants in pig isolates from three herds with different histories of antimicrobial agent exposure. *Appl Environ Microbiol, 59*(5), 1467-1472.

Mayrhofer, S., Domig, K. J., Amtmann, E., Van Hoek, A. H., Petersson, A., Mair, C., et al. (2007). Antibiotic susceptibility of Bifidobacterium thermophilum and Bifidobacterium pseudolongum isolates from animal sources. *J Food Prot, 70*(1), 119-124.

Michael, G. B., Cardoso, M., & Schwarz, S. (2005). Class 1 integron-associated gene cassettes in Salmonella enterica subsp. enterica serovar Agona isolated from pig carcasses in Brazil. *J Antimicrob Chemother, 55*(5), 776-779.

Miles, T. D., McLaughlin, W., & Brown, P. D. (2006). Antimicrobial resistance of Escherichia coli isolates from broiler chickens and humans. *BMC Vet Res, 2*, 7.

Nikolakopoulou, T. L., Egan, S., van Overbeek, L. S., Guillaume, G., Heuer, H., Wellington, E. M., et al. (2005). PCR detection of oxytetracycline resistance genes otr(A) and otr(B) in tetracycline-resistant streptomycete isolates from diverse habitats. *Curr Microbiol, 51*(4), 211-216.

Nikolich, M. P., Hong, G., Shoemaker, N. B., & Salyers, A. A. (1994). Evidence for natural horizontal transfer of tetQ between bacteria that normally colonize humans and bacteria that normally colonize livestock. *Appl Environ Microbiol, 60*(9), 3255-3260.

Olasz, F., Fekete, P. Z., Blum-Oehler, G., Boldogkoi, Z., & Nagy, B. (2005). Characterization of an F18+ enterotoxigenic Escherichia coli strain from post weaning diarrhoea of swine, and of its conjugative virulence plasmid pTC. *FEMS Microbiol Lett, 244*(2), 281-289.

Patterson, A. J., Colangeli, R., Spigaglia, P., & Scott, K. P. (2007). Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. *Environ Microbiol, 9*(3), 703-715.

Poeta, P., Costa, D., Igrejas, G., Rojo-Bezares, B., Saenz, Y., Zarazaga, M., et al. (2007). Characterization of vanA-containing Enterococcus faecium isolates carrying Tn5397-like and Tn916/Tn1545-like transposons in wild boars (Sus Scrofa). *Microb Drug Resist, 13*(3), 151-156.

Randall, L. P., Cooles, S. W., Osborn, M. K., Piddock, L. J., & Woodward, M. J. (2004). Antibiotic resistance genes, integrons and multiple antibiotic resistance in thirty-five serotypes of Salmonella enterica isolated from humans and animals in the UK. *J Antimicrob Chemother, 53*(2), 208-216.

Schwarz, S., Kadlec, K., & Strommenger, B. (2008). Methicillin-resistant Staphylococcus aureus and Staphylococcus pseudintermedius detected in the BfT-GermVet monitoring programme 2004-2006 in Germany. *J Antimicrob Chemother, 61*(2), 282-285.

Schwarz, S., & Noble, W. C. (1994). Tetracycline resistance genes in staphylococci from the skin of pigs. *J Appl Bacteriol, 76*(4), 320-326.

Sengelov, G., Halling-Sorensen, B., & Aarestrup, F. M. (2003). Susceptibility of Escherichia coli and Enterococcus faecium isolated from pigs and broiler chickens to tetracycline degradation products and distribution of tetracycline resistance determinants in E. coli from food animals. *Vet Microbiol, 95*(1-2), 91-101.

Srinivasan, V., Gillespie, B. E., Lewis, M. J., Nguyen, L. T., Headrick, S. I., Schukken, Y. H., et al. (2007). Phenotypic and genotypic antimicrobial resistance patterns of Escherichia coli isolated from dairy cows with mastitis. *Vet Microbiol, 124*(3-4), 319-328.

Srinivasan, V., Nguyen, L. T., Headrick, S. I., Murinda, S. E., & Oliver, S. P. (2007). Antimicrobial resistance patterns of Shiga toxin-producing Escherichia coli O157:H7 and O157:H7- from different origins. *Microb Drug Resist, 13*(1), 44-51.

Stanton, T. B., & Humphrey, S. B. (2003). Isolation of tetracycline-resistant Megasphaera elsdenii strains with novel mosaic gene combinations of tet(O) and tet(W) from swine. *Appl Environ Microbiol, 69*(7), 3874-3882.

Stanton, T. B., McDowall, J. S., & Rasmussen, M. A. (2004). Diverse tetracycline resistance genotypes of Megasphaera elsdenii strains selectively cultured from swine feces. *Appl Environ Microbiol, 70*(6), 3754-3757.

Stine, O. C., Johnson, J. A., Keefer-Norris, A., Perry, K. L., Tigno, J., Qaiyumi, S., et al. (2007). Widespread distribution of tetracycline resistance genes in a confined animal feeding facility. *Int J Antimicrob Agents, 29*(3), 348-352.

Sunde, M., Fossum, K., Solberg, A., & Sorum, H. (1998). Antibiotic resistance in Escherichia coli of the normal intestinal flora of swine. *Microb Drug Resist, 4*(4), 289-299.

Tian, Y., Aarestrup, F. M., & Lu, C. P. (2004). Characterization of Streptococcus suis serotype 7 isolates from diseased pigs in Denmark. *Vet Microbiol, 103*(1-2), 55-62.

Wasteson, Y., Roe, D. E., Falk, K., & Roberts, M. C. (1996). Characterization of tetracycline and erythromycin resistance in Actinobacillus pleuropneumoniae. *Vet Microbiol, 48*(1-2), 41-50.

Whittle, G., Whitehead, T. R., Hamburger, N., Shoemaker, N. B., Cotta, M. A., & Salyers, A. A. (2003). Identification of a new ribosomal protection type of tetracycline resistance gene, tet(36), from swine manure pits. *Appl Environ Microbiol, 69*(7), 4151-4158.

Wu, J. R., Shieh, H. K., Shien, J. H., Gong, S. R., & Chang, P. C. (2003). Molecular characterization of plasmids with antimicrobial resistant genes in avian isolates of Pasteurella multocida. *Avian Dis, 47*(4), 1384-1392.

Yazdankhah, S. P., Sorum, H., & Oppegaard, H. (2000). Comparison of genes involved in penicillin resistance in staphylococci of bovine origin. *Microb Drug Resist, 6*(1), 29-36.

References for Table 1.6, pg 48

Aga, D. S., O'Connor, S., Ensley, S., Payero, J. O., Snow, D., & Tarkalson, D. (2005). Determination of the persistence of tetracycline antibiotics and their degradates in manure-amended soil using enzyme-linked immunosorbent assay and liquid chromatography-mass spectrometry. *J Agric Food Chem, 53*(18), 7165-7171.

Brambilla, G., Patrizii, M., De Filippis, S. P., Bonazzi, G., Mantovi, P., Barchi, D., et al. (2007). Oxytetracycline as environmental contaminant in arable lands. *Anal Chim Acta, 586*(1-2), 326-329.

Hamscher, G., Pawelzick, H. T., Hoper, H., & Nau, H. (2005). Different behavior of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure. *Environ Toxicol Chem, 24*(4), 861-868.

Jacobsen, A. M., Halling-Sorensen, B., Ingerslev, F., & Hansen, S. H. (2004). Simultaneous extraction of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using pressurised liquid extraction, followed by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *J Chromatogr A, 1038*(1-2), 157-170.

Sczesny, S., Nau, H., & Hamscher, G. (2003). Residue analysis of tetracyclines and their metabolites in eggs and in the environment by HPLC coupled with a microbiological assay and tandem mass spectrometry. *J Agric Food Chem, 51*(3), 697-703.

Sengelov, G., Agerso, Y., Halling-Sorensen, B., Baloda, S. B., Andersen, J. S., & Jensen, L. B. (2003). Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environ Int, 28*(7), 587-595.

Zilles, J., Shimada, T., Jindal, A., Robert, M., & Raskin, L. (2005). Presence of macrolide-lincosamide-streptogramin B and tetracycline antimicrobials in swine waste treatment processes and amended soil. *Water Environ Res, 77*(1), 57-62.

CHAPTER II

CHARACTERIZATION OF TETRACYCLINE EFFLUX PUMPS IN A MANURED AGRICULTURAL SOIL BY BOTH CULTURE DEPENDENT AND INDEPENDENT METHODS

INTRODUCTION

In the United States tetracycline (TC), oxytetraycline (OTC), and chlortetracycline (CTC) are approved for and commonly given as growth promoting agents to swine (Sarmah, Meyer, & Boxall, 2006). Growth promotion refers to the sub-therapeutic use of an antibiotic, typically mixed in with feed, to improve pig weight gain and feed efficiency (Gaskins, Collier, & Anderson, 2002). It is estimated that about 24.6 million lbs of antimicrobials are given annually to animals for non-therapeutic purposes, whereas 3 million are given to humans (Gorbach, 2001). As much as 75% of a single dose can be excreted un-metabolized in urine or feces (Sarmah et al., 2006). Hence a significant percentage of the antibiotic makes its way into the environment. In the United States confined animal-feeding operations (CAFOs) generate 128 billion pounds of manure annually (Sarmah et al., 2006). This manure is stored for some time and ultimately spread onto fields. Multiple investigators have found manured soil concentrations of tetracyclines in the range of 200 ppb (Table 1.6). Correspondingly, tetracycline resistance has also been observed in soil environments (Table 1.2).

Few investigators have analyzed genes conferring tetracycline resistance in soil systems. Only six studies have cultured antibiotic resistant bacteria from soils and examined their tetracycline resistance elements by molecular methods (Fig. 1.2). Out of those six studies, four focused on agricultural soil systems (Agerso & Sandvang, 2005; Ghosh & LaPara, 2007; Srinivasan et al., 2008; Stine et al., 2007) and of these four

studies, 93% of the unique gene/isolate combinations come from two very recent studies (Ghosh & LaPara, 2007; Srinivasan et al., 2008). In other words, agricultural soils fertilized with TC contaminated manure are under-studied with regards to molecular tetracycline resistance. This is particularly surprising given that tetracycline has been studied for 60 years and soil has been hypothesized as a reservoir for antimicrobial resistance genes for at least 38 years (Walker & Walker, 1970).

Previously, Dr. Carlos Rodriguez-Minguela examined the extent of tetracycline resistance in an agricultural soil (Table 2.1) that was sampled 1 week after tetracycline contaminated manure was applied (Rodriguez-Minguela, 2005). His work focused on characterizing ribosomal protection proteins (RPPs), one of the four known mechanisms of tetracycline resistance, that confer resistance to tetracycline by dislodging it from the ribosome in a GTP-dependent manner (Connell, Tracz, Nierhaus, & Taylor, 2003). By utilizing degenerate RPP primers he found that *tet*(M) and *tet*(O/W) variants were most predominant 1 week post-application (Rodriguez-Minguela, 2005). Other genes detected included *tet*(O), *tet*(Q), *tet*(36) and two putative novel RPPs referred to herein as clone 397 and clone 492 (Rodriguez-Minguela, 2005). The partial sequence of clone 397 has 71% amino acid identity with its closest relative, Tet(32), and potentially represents a new RPP gene based on the definition of $\leq$ 80% amino acid identity to the closest characterized tetracycline resistance gene (Levy et al., 1999; Rodriguez-Minguela, 2005).

Table 2.1. Site # 21 Soil (Michigan Farm)

Source	Manure Application	Antibiotic Used	Soil Type
Wheat field	1 week before sampling	Cl-tetracycline	Capac loam

Cl-tetracycline was routinely used as swine feed supplement (growth promoting agent)

Clone 492 shared 85% amino acid identity to Tet(O/W), however, the last 135 amino acids were 98% identical to Tet(32) (Rodriguez-Minguela, 2005). Unfortunately the culture approaches used by Rodriguez-Minguela failed to yield a bacterial isolate harboring clone 397 or 492, and so definitive proof of functional tetracycline resistance remaines unknown.

The advent of culture independent methods, termed metagenomics, represents another approach to recover the full-coding sequences of clones 397 and 492. Metagenomics refers to any culture-independent sequence or expression based analysis of a microbial community (Schloss & Handelsman, 2005). Less than 1% of known bacterial species are currently cultivatable (Torsvik, Goksoyr, & Daae, 1990). Construction of metagenomic insert libraries (e.g. fosmid, cosmid, or bacterial artificial chromosome) of random community DNA can circumvent the need for cultured isolates to study the genomes of these bacteria. Three studies have isolated tetracycline resistance genes through construction of various types of metagenomic libraries (Diaz-Torres et al., 2003; Diaz-Torres et al., 2006; Riesenfeld, Goodman, & Handelsman, 2004). In two of these studies the use of metagenomics allowed the discovery of two novel tetracycline resistance genes, *tcr*, a tetracycline efflux gene, from a pristine soil (Riesenfeld et al., 2004) and *tet*(37), a tetracycline inactivating enzyme, from the human oral metagenome (Diaz-Torres et al., 2003). Given the success of previous investigators in isolating novel tetracycline resistance determinants and the problem of recovering full-coding sequences of clones 397 and 492, metagenomics was an appropriate method to pursue.

This metagenomic approach was supplemented with an analysis of the cultivatable fraction of bacterial isolates harboring tetracycline efflux genes in the same

soil sample previously used by Dr. Rodriguez-Minguela. He recovered *tet*(M) in *Paenibacillus* spp. and *Enterococcus* spp. and *tet*(O) in *Corynebacterium* spp. (Rodriguez-Minguela, 2005). Given the lack of studies examining molecular tetracycline resistance in agricultural soil environments it is also of interest to characterize the fraction resistant to tetracycline due to the other major mechanism, tetracycline efflux. The tetracycline efflux pumps are membrane bound proteins that exchange a tetracycline-magnesium complex in an energy dependent manner for a proton thereby reducing the intracellular concentration and detoxifying the cell (Chopra & Roberts, 2001).

The goals of my study were to:

1) To recover the full-coding sequences of clones 397 and 492 via metagenomic library construction and demonstrate functional tetracycline resistance
2) To analyze the extent of resistance to tetracycline via efflux pumps in the cultivable and non-cultivable (via metagenomics) bacterial fraction.

MATERIALS AND METHODS

Sample. Soil used for this study was a Capac loam sampled from a wheat field on a Michigan farm one week after application with manure from animals that received Cl-tetracycline routinely as a growth promoting agent (Table 2.1) (Rodriguez-Minguela, 2005).

Soil Microcosms. Samples of site 21 soil (5 g) were transferred into serum bottles. These samples were supplemented with 1 mL of a 40 μg/mL tetracycline (Sigma-Aldrich Co., St. Louis, MO) + 200 μg/mL cyclohexamide (Sigma-Aldrich Co., St. Louis, MO) 10 μg/mL tetracycline + 200 μg/mL cyclohexamide, or water + 200 μg/mL cyclohexamide solution. Serum bottles were sealed and incubated aerobically for

10 days. Every other day bottles were opened in a laminar flow hood for 10 minutes to allow atmosphere replacement.

DNA Extraction, Purification, Sizing, and Cloning. After 10 days incubation, DNA was extracted from 2.5 g samples from soil microcosms by a method modified from (Zhou, Bruns, & Tiedje, 1996). After this extraction step the soil DNA was amenable to PCR, however, attempts at library construction failed indicating that the DNA was not of sufficient quality for cloning purposes. Subsequently, a number of additional steps were taken to further purify the DNA. DNA was concentrated with YM-10 Microcon filters (Millipore Co., Billerica, MA) for about 30 min at a speed of 500 rcf in a table top centrifuge. After sufficient concentration the DNA was loaded onto a 1% pulse field certified (PFC) agarose (Bio-Rad, Hercules, CA) pulse field gel electrophoresis (PFGE) gel with the 2 cm of gel below the wells cut out and refilled with 1% PFC agarose + 2 % polyvinylpyrrolidone (PVP; Sigma-Aldrich Co., St. Louis, MO) in 1X tris-acetate-EDTA (TAE) buffer. The gel was run with the auto algorithm on a Bio-Rad CHEF Mapper^{TM} PFGE unit (Bio-Rad, Hercules, CA) to separate DNA in a size range from 10 – 100 Kb. After the PFGE run, the gel regions corresponding to the Low Range and Mid Range I PFG Markers (New England Biolabs Inc., Ipswich, MA) flanking the DNA was cut from the gel and stained in a bath with 100 mL 1X TAE and 15 µL SYBR® Safe (Invitrogen, Carlsbad, CA). Cut gel fragments containing PFG markers were visualized with an Eagle Eye II gel imaging system (Stratagene, La Jolla, CA) and cover slips were used to make small cuts in the gel corresponding to DNA sizes of 35 – 60 Kb. The gel slices were re-aligned to re-form the original gel and slices corresponding to soil DNA 35 – 60 Kb in size were cut. DNA was extracted from these

slices by electroelution in dialysis tubing (Size MC-18, 12,000 – 16,000 MWCO Daltons, 25 mm flat width; Sargent-Welch, Buffalo, NY) as described in (Sambrook & Russell, 2001). Bags containing DNA were dialyzed in 1 L sterilized water at 4°C for 20 minutes. After dialysis liquid containing the DNA was removed from the bags, concentrated with YM-100 Microcon filters, and finally subject to end-repaired and cloned as described in the CopyControl™ Fosmid Library Production Kit (EPICENTRE® Biotechnologies , Madison, WI).

Restriction Analysis and Library Storage. The FosmidMAX™ Kit (EPICENTRE® Biotechnologies , Madison, WI) was used according to the manufacturer's protocol to extract fosmid DNA. Two micrograms of fosmid DNA from fourteen randomly chosen clones were digested with *Eco*RI (New England Biolabs Inc., Ipswich, MA) according to the manufacturer's protocol and run on a PFGE set to separate DNA in the 1 to 20 kb size range.

The library was amplified and stored as liquid gel pools as described in (Hrvatin & Piel, 2007).

Construction of Control Strain EPI300™-397. Before PCR based screening for the presence of clone 397 a control strain (referred to as EPI300™-397) was constructed to test the sensitivity of the PCR screen. The 1.3 kb fragment of clone 397 was PCR amplified from pCR®4-TOPO® vector (Invitrogen, Carlsbad, CA) containing the clone 397 insert (Rodriguez-Minguela, 2005). PCR was conducted with primer set 1 (Table 2.2) as described in Rodriguez Minguela (2005). After amplification the PCR mixture containing the amplified fragment was cleaned using ExoSAP-IT® (USB Co., Cleveland, OH) according to the manufacturer's protocol. This purified 1.3 kb fragment

was then end-repaired and cloned into pCC1FOS™ (EPICENTRE® Biotechnologies, Madison, WI) according to the manufacturer's protocol. Vector containing insert DNA was electroporated into electrocompetent EPI300™ cells prepared as described in Sambrook & Russell (2001). Transformants were plated on LB + 12.5 μg/mL chloramphenicol (Sigma-Aldrich Co., St. Louis, MO). Ten randomly chosen clones were further analyzed by PCR for the presence of clone 397 with primer set 15 (Table 2.2). The PCR master mix contained 5 μL 5X GoTaq® Buffer (Promega, Madison, WI), 1.5 μL MgCl2 (25 mM stock; Promega, Madison, WI), 0.5 μL dNTPs (100 μM Mix; Promega, Madison, WI), 0.375 μL 397(FWD) (10 μM stock), 0.375 μL 397(REV) (10 μM stock), 1 μL 100X bovine serum albumin (BSA; New England Biolabs, Ipswich, MA), 0.25 μL GoTaq® DNA Polymerase (Promega, Madison, WI), and 15 μL sterilized water per reaction giving a total volume of 24 μL. To this 1 μL of target DNA (100 ng) was added. Reactions were cycled on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA) thermocycler. Cycling conditions were as follows: initial denaturation at 98°C for 2 min, followed by 25 cycles of denaturation at 95°C for 40 s, annealing at 60°C for 40 s, and extension at 60°C for 25 s. This was followed by a final extension of 1 min at 60°C. Presence of amplicons were verified on 35 mL 3% MetaPhor Agarose (Cambrex Co., East Rutherford, NJ) gels in 1 X TAE supplemented with 1 μL SYBR® Safe (Invitrogen, Carlsbad, CA) run at 100 volts for 45 mins.

PCR Screen for Clone 397. Before conducting the PCR based screen for clone 397, the method was tested for its sensitivity. This was done by diluting the EPI300™-397 control strain in various amounts and adding it to a clone pool of 99 randomly picked soil DNA fosmid clones constructed with the same vector (courtesy of Dr. Thomas

Schmidt, Michigan State University). Ratios tested included one positive in 100 clones, one positive in 1,000 clones, and one positive in 10,000 clones (Fig. 2.1). Dilutions of pools were also tested for inhibitory effects of an overabundance of cell material. PCR was done on cell lysate after lysis in a thermocycler at 95°C for 10 mins. The PCR protocol used was as described above for primer set 15 with the exception that 50 cycles were used to increase the sensitivity of the assay. This test verified that the method is sensitive enough to detect one positive in 10,000 clones.

To screen the library liquid gel pools were used as inoculum for forty-one 1 mL overnight cultures of LB + 12.5 μg/mL chloroamphenicol. After growth, aliquots of cells were lysed at 95°C for 10 min in a thermocycler and subject to PCR with primer set 15 as described above.

Functional Screen of Soil Fosmid Library and Reduction of Redundancy. The soil fosmid library was screened by activity for the presence of expressed tetracycline resistance genes. Liquid gel pools (Hrvatin & Piel, 2007) were used as inoculum for forty-one 1 mL overnight cultures (37°C, 150 rpm) in LB + 12.5 μg/mL chloramphenicol. After growth, 41 individual LB agar plates containing 12.5 μg/mL chloramphenicol + 5 μg/mL tetracycline were plated with 15 μL from each overnight culture and grown at 37°C overnight. Colonies (262) were picked into 96 well plates with LB + 12.5 μg/mL chloramphenicol + 5 μg/mL tetracycline and grown at 37°C overnight with shaking at 150 rpm. It was expected that using liquid gel pools as inoculum for overnight cultures would introduce redundancy due to clonal growth of fosmid clones. As such, it was necessary to reduce this redundancy before screening the 262 isolated colonies for the presence of tetracycline resistance genes. This was done by

Table 2.2. Primers Used

Set	Name	Application	Sequence (5' to 3')	Amplicon Size (bp)	Reference
1	tet1 (fwd) tet2 (rev)	Amplification of RPP genes	GCT CAY GTT GAY GCA GGA A AGG ATT TGG CGG SAC TTC KA	~ 1300	(Barbosa, Scott, & Flint, 1999)
2	8F 1492R	Amplification of 16S genes	AGA GTT TGA TCC TGG CTC AG GGT TAC CTT GTT ACG ACT T	~1400	(Giovannoni, 1991)
3	tetA-FW tetA-RV	Amplification of tet(A)	CGC CGA TCT GGT TCA CTC G AGT CGA CAG YRG CGC CGG C	164	(Aminov et al., 2002)
4	tetB-FW tetB-RV	Amplification of tet(B)	TAC GTG AAT TTA TTG CTT CGG ATA CAG CAT CCA AAG CGC AC	206	(Aminov et al., 2002)
5	tetC-FW tetC-RV	Amplification of tet(C)	GCG GGA TAT CGT CCA TTC CG GCG TAG AGG ATC CAC AGG ACG	207	(Aminov et al., 2002)
6	tetD-FW tetD-RV	Amplification of tet(D)	GGA ATA TCT CCC GGA AGC GG CAC ATT GGA CAG TGC CAG CAG	187	(Aminov et al., 2002)
7	tetE-FW tetE-RV	Amplification of tet(E)	GTT ATT ACG GGA GTT TGT TGG AAT ACA ACA CCC ACA CTA CGC	199	(Aminov et al., 2002)
8	tetG-FW tetG-RV	Amplification of tet(G)	GCA GAG CAG GTC GCT GG CCY GCA AGA GAA GCC AGA AG	134	(Aminov et al., 2002)
9	tetH-FW tetH-RV	Amplification of tet(H)	CAG TGA AAA TTC ACT GGC AAC ATC CAA AGT GTG GTT GAG AAT	185	(Aminov et al., 2002)
10	tetJ-FW tetJ-RV	Amplification of tet(J)	CGA AAA CAG ACT CGC CAA TC TCC ATA ATG AGG TGG GGC	184	(Aminov et al., 2002)
11	tetY-FW tetY-RV	Amplification of tet(Y)	ATT TGT ACC GGC AGA GCA AAC GGC GCT GCC GCC ATT ATG C	181	(Aminov et al., 2002)

Table 2.2. Primers Used Continued

Set	Name	Application	Sequence (5' to 3')	Amplicon Size (bp)	Reference
12	tetZ-FW tetZ-RV	Amplification of tet(Z)	CCT TCT CGA CCA GGT CGG ACC CAC AGC GTG TCC GTC	204	(Aminov et al., 2002)
13	tet30-FW tet30-RV	Amplification of tet(30)	CAT CTT GGT CGA GGT GAC TGG ACG AGC ACC CAG CCG AGC	210	(Aminov et al., 2002)
14	tet31-FW tet31-RV	Amplification of tet(31)	GCT CTA TCT AGG GAG AAT GA GCT AAC CAT GAT ACC TTG TA	652	(Agerso & Sandvang, 2005)
15	397(FWD) 397(REV)	Amplification of clone 397	CAA TCG GAA CTG TGC GGG TAT GTA CGG TGT CTG TTG GGA CGA TTT CTC	187	(Rodriguez-Minguela, 2005)
16	492(FWD) 492(REV)	Amplification of clone 492	CAC AGA GAT GCG TAT TCC ATC CA TCT ACC GTT GTC CGA AGT AAT GG	180	(Rodriguez-Minguela, 2005)
17	T7	Sequencing of fosmid ends	TAA TAC GAC TCA CTA TAG GG	n/a	See Epicentre's CopyControl™ Fosmid Library Manual
18	pEpiFOS-RSP	Sequencing of fosmid ends	CTC GTA TGT TGT GTG GAA TTG TGA GC	n/a	See Epicentre's CopyControl™ Fosmid Library Manual
19	tetX(FWD) tetX(REV)	Amplification of tet(X)		468	(Ng, Martin, Alfa, & Mulvey, 2001)

pooling 100 μL of culture from rows of 96 well plates (for example A1 to A12) into a total volume of 1.2 mL, extracting fosmid DNA (as described above), and subjecting this pool to DNA restriction analysis with *Eco*RI (New England Biolabs Inc., Ipswich, MA) according to manufacturer's protocols. This pooled restriction digest was then run on a 125 mL 1% Ultrapure™ agarose (Invitrogen, Carlsbad, CA) gel with 9 uL SYBR® Safe (Invitrogen, Carlsbad, CA) at 4°C for 4 h at 75 volts. The pooled digest patterns were compared to the digest pattern of the fosmid clones on the end of the rows (for example A1 and A12, each digested individually). If overlapping patterns were detected in the pooled digest each clone in an entire row was analyzed individually to identify unique clones. This method allowed the identification of seven (referred to as TC1 – TC7) fosmid clones with unique digest patterns selected for further study (Fig. 2.5).

Sub-Cloning of Fosmids TC1, TC3, TC5, and TC6. Fosmids TC1, TC3, TC5, and TC6 were selected for sub-cloning to identify the tetracycline resistance gene resident on insert DNA. Fosmid DNA to be sub-cloned was extracted with Qiagen's Plasmid Midi Kit (Valencia, CA) according to the manufacturer's protocol. DNA was randomly sheared by sonication (done in a bath with the following conditions: continuous, 50% duty cycle, output level = 7; W-385 Heat Systems-Ultrasonics Inc., Plainsview, NY) for 15 s to a size range of 2 to 4 kb. This DNA was then used for cloning into the pCC1FOS™ vector as described in the manufacturer's protocol with the exception that electroporation was used instead of transfection to transfer the ligated vectors into EPI300™ host cells. Preparation of electrocompetent cells and electroporation was performed as described in Sambrook & Russell (2001). Transformed cells were plated on LB containing 12.5 μg/mL chloramphenicol and 5 μg/mL

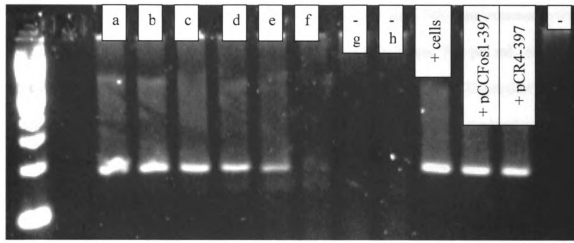


Figure 2.1. Test of PCR Screening Sensitivity for Clone 397. EPI300™ host cells containing the pCC1FOS™ vector with a fragment of clone 397 were diluted with 99 randomly picked soil fosmid library clones to give positive to background ratios as described. (a) 1 positive cell to 100 total cells. (b) 1/10th dilution of (a). (c) 1 positive to 1000 total cells. (d) 1/10th dilution of (c). (e) 1 positive to 10,000 total cells. (f) 1/10th dilution of (e). (g) negative control including the 99 cells used as background. (h) 1/10th dilution of (g). + cells = EPI300™-397 cell lysate. + pCCFOS1™-397 = vector DNA. + pCR4®-397 = original source of clone 397. – = negative PCR control. The ladder is Invitrogen's 100 bp ladder.

tetracycline to select clones with tetracycline resistance genes inserted. For each fosmid ten clones were randomly selected and digested with *Eco*RI to find the smallest insert conferring tetracycline resistance. The ends of this clone were then sequenced as described below.

Cultivation of Tetracycline Resistant Soil Isolates. Soil samples (5 g) prepared as described above were added to 20 mL of phosphate saline buffer (pH 7.5) supplemented with sodium pyrophosphate (Sigma-Aldrich Co., St. Louis, MO) and dithiothreitol (DTT; Sigma-Aldrich Co., St. Louis, MO) and diluted for plating as previously described (Stevenson, Eichorst, Wertz, Schmidt, & Breznak, 2004). Media used included LB agar (Becton Dickinson and Company, Franklin Lakes, NJ), 1/10th

strength R2A (Becton Dickinson and Company, Franklin Lakes, NJ) supplemented with 15 g Bacto Agar (Becton Dickinson and Company, Franklin Lakes, NJ) per mL, and Soil Media (SM = 25 mL of soil extract, 8 g of phytagel, and 0.6 mmol CaCl2 per liter of media). Plates were incubated at 25°C for 9 days before colonies were randomly selected and transferred to liquid culture of the same medium. DNA from liquid cultures was extracted by the Joint Genome Institute (JGI) standard operation procedure available at the following web address:

http://my.jgi.doe.gov/general/protocols/DNA_Isolation_Bacterial_CTAB_Protocol.doc

PCR of Tetracycline Resistance and 16S rRNA Genes. The general master mix for PCR of tetracycline efflux genes *tet*(A) – *tet*(30) (primer sets 3 – 13) included 5 µL 5X GoTaq® Buffer (Promega, Madison, WI), 2.5 µL 1 mM dNTPs (Promega, Madison, WI), 0.8 µL 25 mM Magnesium Chloride (Promega, Madison, WI), 1 µL GoTaq® DNA Polymerase (Promega, Madison, WI), and 9.7 µL sterilized water. To this 2.5 µL of each primer was added giving a final volume of 24 µL. Thermocycling conditions were performed as previously described (Aminov et al., 2002). Clones 397 and 492 were amplified as described for clone 397 above. The *tet*(31) and *tet*(X) genes were amplified as previously described (Agerso & Sandvang, 2005; Ghosh & LaPara, 2007; Ng et al., 2001). Amplification of 16S rRNA genes was performed as previously described (Eichorst, 2007).

Sequencing of Fosmid Ends and 16S rRNA Genes. All sequencing was conducted by staff at the Michigan State University Research Technology Support Facility – Genomics Core (East Lansing, MI). Sequencing was performed on an ABI PRISM® 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Primers used

for sequencing included T7, 8F, and pEpiFOS-RSP (Table 2.2). Sequences were automatically quality trimmed based on a Q20 value. Basic Local Alignment Search Tool (BLAST) searching was done with both BLASTN and BLASTP algorithms against the non-redundant database (Altschul et al., 1997). Nucleotide sequences were translated to protein at the following website: http://www.expasy.ch/tools/dna.html

RESULTS AND DISCUSSION

Fosmid Library Construction. A fosmid library was constructed with DNA extracted from site # 21 soil (Table 2.1) as described in materials and methods. Prior to cloning, PCR analyses of the final DNA preparation verified the presence of clone 397 but not clone 492 in purified DNA (Fig. 2.3). The absence of clone 492 may be due to it being located on a plasmid (or other DNA) less than 30 Kb in size. *Eco*RI restriction analysis of 14 randomly selected fosmid clones indicated an average insert size of approximately 30 kb (Fig. 2.4) and based on an estimated 27,000 to 30,000 clones, this library represents approximately 810 – 900 Mb of soil DNA; 135 to 150 genome equivalents assuming an average genome size of 6 Mb.

Sequence and Functional Based Screening for Cloned Tetracycline Resistance Genes. Unfortunately, no positive gel pools were identified for clone 397 by PCR based screens (Materials and Methods). Absence of a positive hit for clone 397 was not due to detection limit problems as tests for the sensitivity of the method revealed that positive detection was still obtainable with one positive clone in 10,000 (Fig. 2.1). It appears that the inability to obtain a fosmid containing clone 397 was due to insufficient enrichment of the target gene. Based on previous results, the highest copy number per gram of soil achieved for clone 397 was 10,000 (Rodriguez-Minguela, 2005). It is

commonly assumed that 1 g of soil contains 1 billion bacteria. This would correspond to one in 100,000 bacteria harboring clone 397 if a single gene was present on the chromosome. Given that the library represents 135 to 150 genome equivalents it appears that recovery of the full coding sequence of clone 397 will remain elusive until either better enrichment can be achieved, or an alternative, more appropriate method is used.

To complement the sequence based screening, a functional based screen of the fosmid library was also conducted. This involved growing overnight cultures of the clone pools and plating on LB plates with 5 μg/mL TC and 12.5 μg/mL CAM. It was expected that the overnight "pre-growth" step prior to plating would introduce a considerable amount of redundant TC resistant clones. This redundancy was eliminated as described in the materials and methods. Restriction analysis with *Eco*RI (Fig. 2.5) revealed seven TC resistant fosmid clones (TC1 – TC7) with unique restriction patterns and varying relative minimum inhibitory concentrations (MICs). It is important to note that I cannot rule out the possibility that two clones could contain fragments of the same DNA molecule cloned in different locations giving rise to different restriction digest patterns.

Tetracycline Resistance Genes Present on Fosmids. The seven TC resistant fosmid clones were then subject to either sub-cloning or PCR to determine the tetracycline resistance gene(s) present. Figure 2.6 summarizes the results. Three clones contain *tet*(Y) (TC4, TC6, and TC7), two clones contain *tet*(A) (TC1 and TC5), one clone contains *tet*(31) (TC3) and the last clone contains *tet*(C) (TC2). In addition to containing *tet*(A), clone TC1 also contains *tet*(30). Interestingly, all of these genes are tetracycline efflux pumps. This could be due to the fact that ribosomal protection proteins are

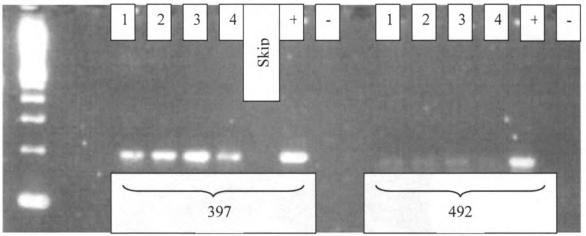


Figure 2.2. PCR Verification of Presence of Clones 397 and 492 in Soil DNA. 3% Metaphor Agarose gel in 1X TAE stained with SYBR Safe. Ladder is Invitrogen's 100 bp ladder. 1, 2, 3, & 4 refer to 4 separate soil DNA samples, + and – are positive and negative controls respectively.

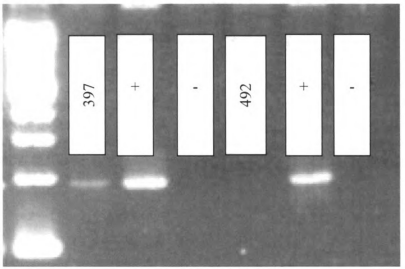


Figure 2.3. Verification of Presence of Clones 397 and 492 in Purified Soil DNA to be Cloned. 3% Metaphor Agarose gel in 1X TAE stained with SYBR Safe. Ladder is Invitrogen's 100 bp ladder. 397 and 492 refer to sample DNA. + and – are positive and negative controls respectively.

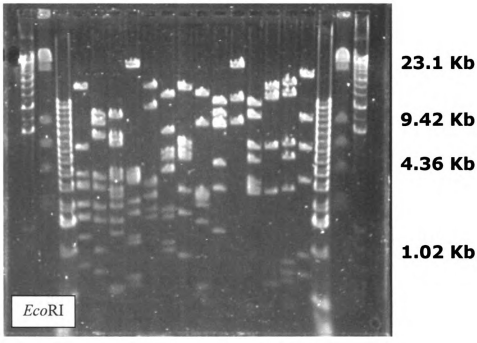
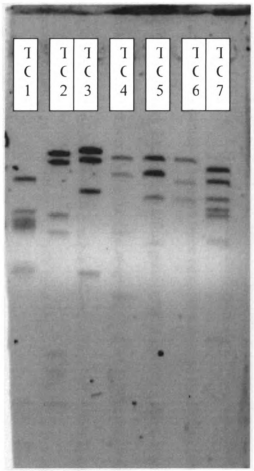


Figure 2.4. Average Insert Size of Soil Fosmid Library. Gel showing the EcoRI restriction patterns of fourteen randomly chosen fosmid clones. The outermost ladder is Invitrogen's λ phage digest ladder, the middle ladder is NEB's Low Range PFG ladder and the innermost ladder is Invitrogen's 1 Kb DNA ladder. Average insert size equals 29.94 Kb. Library size was between 27,000 and 30,000 clones.



Insert Size	TC MIC (relative)
TC1 = 21.2 kb	TC1 = 80 ug/mL
TC2 = 31 kb	TC2 = 20 ug/mL
TC3 = 26 kb	TC3 = 80 ug/mL
TC4 = 24.5 kb	TC4 = 80 ug/mL
TC5 = 27.5 kb	TC5 = 10 ug/mL
TC6 = 22 kb	TC6 = 80 ug/mL
TC7 = 32 kb	TC7 = 40 ug/mL

Figure 2.5. Size and Tetracycline Minimum Inhibitory Concentration of Isolated Fosmid Clones. The EcoRI restriction patterns, average insert sizes, and relative minimum inhibitory concentrations for 7 tetracycline resistant fosmid clones isolated by a functional screen. Clone T7 is also highly resistant to kanamycin.

generally found in Gram-positive organisms (Chopra & Roberts, 2001) and may not be efficiently expressed in a Gram-negative host such as *E. coli*. Heterologous gene expression is one of the known limitations of functional metagenomics (Handelsman, 2004) and usage of a Gram-positive host cell strain may permit the isolation of more RPPs. To date, only one study has isolated RPPs via a metagenomic method (Diaz-Torres et al., 2006).

The finding of *tet*(A) and *tet*(30) co-resident on the insert present in fosmid TC1 is of particular interest. Previously *tet*(30) has only been found in *Agrobacterium tumefaciens* C58 (Luo & Farrand, 1999) and detected by DNA:DNA hybridization in various garden and farm soils (Patterson, Colangeli, Spigaglia, & Scott, 2007). Resistance to tetracycline in *Agrobacterium tumefaciens* C58 was only identified after insertion of IS*426* into the *tetR* gene. Luo & Farrand (1999) went on to show that this *tetR* variant could bind the P*tet* promoter but was unable to be de-repressed by tetracycline, explaining why resistance was only detected after mutation of the *tetR* gene. This non-inducible TetR protein could also interact with promoters from other *tet* gene variants and was partially dominant when two *tet* variants were present in the same cell resulting in low level TC resistance (Luo & Farrand, 1999). Interestingly, relative MIC tests with TC1 (Fig. 2.5) indicated that carriage of this fosmid conferred high levels of tetracycline resistance. There are three possible scenarios explaining how TC1 can carry *tet*(30) and another tetracycline efflux gene (*tet*(A)) and still exhibit high levels of resistance. First, this could be the first observation of a fully functional *tetR* variant for *tet*(30). Second, perhaps *tet*(30) is not associated with a *tetR* in this fosmid capable of operator binding, or alternatively no *tetR* gene at all. And third, it is also possible that

only partial sequence of the *tet*(30) and/or *tetR* gene(s) were cloned, and hence are non-functioning. However, the third scenario is not likely since sequencing revealed a *tnpA* gene and an area with no significant homology flanking the ends of the pCC1FOS™ vector (Fig. 2.6). The observation of co-carriage of *tet*(A) and *tet*(30) warrants further investigation as it may be the first discovery of a functional TetR(30) variant.

The *tet*(Y) variant was the most frequently isolated efflux gene (Fig. 2.6, Table 2.3). This efflux gene was first discovered from the exogenous isolation of plasmids via biparental matings with bacteria from piggery manure as donors and *Escherichia coli* CV601 and *Pseudomonas putida* UWC1 as recipients (Smalla et al., 2000). *Aeromonas* spp. and *Photobacterium* spp., both from water environments, have been found to harbor *tet*(Y) (Table 1.1) (Furushita et al., 2003; Gordon et al., 2008b). Sub-cloning of TC6 revealed a sequence with 100% amino acid identity to the TetR(Y) repressor protein variant from plasmid pAB5S9 isolated from *Aeromonas bestarium* from a river in Brittany, France (Gordon et al., 2008a). Interestingly, Gordon et al. (2008) recently reported that this was, to their knowledge, the first discovery of a functional repressor protein for the *tet*(Y) gene. Therefore, the discovery of *tetR*(Y) in fosmid TC6 may represent the second report of a functional *tet*(Y) repressor protein. Similar to pAB5S9 the *tet*(Y) variant in fosmid TC6 was linked with the streptomycin resistance gene *strB*. However, it is unlikely that the insert in fosmid TC6 is from plasmid pAB5S9 since end sequencing of TC6 revealed *merA* and *merP* genes involved in mercury resistance (discussed below) that were not present on pAB5S9 (Gordon et al., 2008a). It is unknown if the *tet*(Y) genes present in fosmids TC4 and TC7 also contain functional *tetR* genes because the presence of *tet*(Y) was confirmed by PCR instead of sub-cloning as

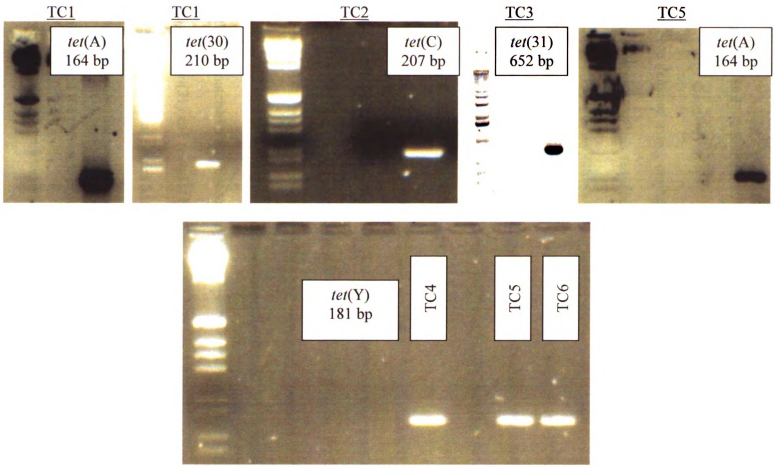


Figure 2.6. PCR Verification of the Presence of *tet* Genes on Fosmids. Each gene amplified and the proper amplicon size is listed on each gel corresponding to various fosmids. The well to the right of each positive amplicon is a negative control (except for *tet*(31) which is to the left). The ladder in each gel is Invitrogen's 1 Kb DNA ladder.

was done for TC6. The finding of *tet*(Y) in fosmids TC4, TC6, and TC7 represents only the second report of this gene being isolated from a soil environment (Schmitt, Stoob, Hamscher, Smit, & Seinen, 2006).

The discovery of *tet*(31) on fosmid TC3 represents only the second time this gene has been detected in any environment and the first identification in soil. This is likely not due to the rarity of this sequence in the environment, but the lack of studies that exhaustively search for tetracycline resistance gene variants. Out of the five studies that analyzed tetracycline resistance by culture dependent methods and PCR or DNA hybridization only one (Agerso & Sandvang, 2005) screened for the *tet*(31) gene, and this was only after isolates first lacked *tet*(A), *tet*(B), or *tet*(C) meaning that isolates carrying the A, B, or C variants and 31 would have been missed.

Currently little is known regarding the *tet*(31) variant. It was originally discovered on plasmid pRAS2 isolated from the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida* in Norway (L'Abee-Lund & Sorum, 2000) and hence, the finding of *tet*(31) on fosmid TC3 is quite interesting. When sub-clone end sequences of TC3 were matched against NCBI's non-redundant nucleotide database with BLAST (Altschul et al., 1997), both ends matched a sequenced fragment of pRAS2. Surprisingly, homology was not only over *tet*(31) and *tetR*(31) but also over ~ 600 bp of flanking sequence. The sequence corresponding to base-pairs 350 – 950 in pRAS2 (accession number AJ250203) was not annotated and so the sequence was translated and subject to protein BLAST analysis. This revealed the presence of what looks to be gene fragments possibly formerly involved in phenazine biosynthesis (Table 2.3). Phenazines are organic molecules that exhibit broad spectrum activity against bacteria, fungi, and parasites that are produced by

Table 2.3. Fosmid Sequences with Homology to Genes Listed.

Fosmid	T7 end	sub-clone (T7)[a]	TcR gene	sub-clone (RSP)[a]	RSP end
TC1	*tnpA*	*tetR*(A)	*tet*(A), *tet*(30)[b]	isochorismatase hydrolase	No sig. hits
TC2*	*tet*(C)		(see T7 end)		*iclR*
TC3	*repC*	*phzC/phzF*, *tetR*(31)	(see sub-clone RSP)	*tet*(31)	*hisC*
TC4*	*intI*		*tet*(Y)		*topA*
TC5	*cysE*	*tetR*(A)	(see sub-clone RSP)	*tet*(A), *strB*	*glnQ*, argininosuccinate lyase
TC6	Cons. Hyp.	*strB*, *intI*	*tet*(Y)	*tetR*(Y)	*merA*, *merP*
TC7*	*tnpA*, *lysR*		*tet*(Y)		*glnA*

[a] The orientation of these genes cannot be established relative to the T7 and RSP ends and is presented this way for convenience
[b] The orientation of these genes cannot be established. It could be reversed or one gene could be within this segment and another outside. * These fosmids were not sub-cloned.

beneficial root colonizing *Pseudomonas* spp (Blankenfeldt et al., 2004). Further upstream of bp 350 in the pRAS2 sequence is the presence of a transposase gene (*tnpA*). This implies that *tet*(31) may be resident on a transposon. Given that little is known regarding the dissemination of *tet*(31) it is of further interest to investigate if the sequence isolated on fosmid TC3 is resident on a transposon.

Other Genes of Interest Isolated on Fosmid Clones TC1-TC7. A number of other genes of interest were sequenced from the ends of the original or sub-cloned fosmids. In particular, transposase sequences indicative of the presence of a transposon were identified near the end of fosmids TC1 and TC7 (Table 2.3). Furthermore, two putative class 1 integrases were detected on TC4 and TC6. Integrases belong to the tyrosine-recombinase family and facilitate the insertion of gene cassettes (containing a gene and an *attC* site) into integrons at the *attI* site allowing expression driven by the *P*c promoter (Mazel, 2006). Although no tetracycline resistance genes have been found residing inside integrons the finding of these sequences are consistent with previous studies noting the linkage of integrons and transposons with tetracycline resistance (Agerso & Sandvang, 2005; Bahl, Hansen, Goesmann, & Sorensen, 2007; Chopra & Roberts, 2001).

Two *strB* genes encoding streptomycin phosphotransferases that confer resistance to streptomycin were partially sequenced. Often *strB* is linked with *strA* and this gene is required for high level resistance (Chiou & Jones, 1995; Sundin, 2002). Multiple investigators have found the *strA-strB* genes linked to tetracycline resistance. The finding of *tet*(31) in *Aeromonas salmonicida* plasmid pRAS2 was originally discovered due to an investigation into *strA-strB* streptomycin resistance (L'Abee-Lund & Sorum, 2000).

Also, the study that discovered the first *tetR*(Y) repressor variant found *strA-strB* linked in plasmid pAB5S9 (Gordon et al., 2008b). Resistance linkage has also been observed in isolates of *E. coli* with *tet*(B) on transposon Tn*10* (Khachatryan, Besser, & Call, 2008), with *tet*(M) in *Citrobacter braakii*, *E. coli*, and *Providencia rettgeri*, with *tet*(A) and *tet*(W) in *E. coli*, and simultaneously with *tet*(O), *tet*(S), and *tet*(W) in *Citrobacter freundii* (Srinivasan et al., 2008).

Fosmid TC6 has sequences with 100% amino acid identity to MerA and MerP near one end (Table 2.3). These genes act in an operon, with a general structure of *merRTPAD*, that confers mercury resistance by uptaking Hg(II) and reducing it to gaseous Hg(0) allowing it to diffuse out of the bacterial cell (Barkay, Miller, & Summers, 2003). Specifically, *merA* encodes a cytosolic flavin disulfide oxidoreductase that uses NAD(P)H as a reductant to reduce a Hg(II) dithiol derivative to Hg(0) (Barkay et al., 2003). MerP is a small periplasmic mercury binding protein that is thought to act in Hg(II) transfer by exchanging Hg(II) to two cysteine residues in a transmembrane helice of MerT, an inner (cytosolic) membrane protein (Barkay et al., 2003).

Finding sequences of all of the above genes is consistent with the current understanding of tetracycline resistance being: (a) commonly transferred by transposons (Chopra & Roberts, 2001), (b) often linked with integrons and/or streptomycin resistance genes (Srinivasan et al., 2008), and (c) the more recent understanding of co-selecting for tetracycline resistance through metal resistance (Stepanauskas et al., 2006).

TC Resistant Bacterial Isolates. Bacterial isolates were cultured to complement the metagenomic analysis of tetracycline resistance. As stated above, few investigators have cultured resistant bacteria and identified the tetracycline resistance genes they carry.

Of the four studies that have attempted this, only one cultured isolates on agar media with low nutrient concentrations (Ghosh & LaPara, 2007). This study alone represents 56% of the unique gene/isolate combinations identified to date. The other three studies used solely nutrient rich media (including LB, MacConkey agar, and TSA), short incubation times (24-48 h), and high temperatures (35 – 37°C) (Agerso & Sandvang, 2005; Srinivasan et al., 2008; Stine et al., 2007). Based on the observation that low nutrient media and extended incubation time can increase viable cell counts (Davis, Joseph, & Janssen, 2005) and recovered bacterial diversity, a strategy using such medias was employed to try and recover greater breadth of tetracycline resistant bacteria from the site # 21 soil.

With this in mind I attempted to recover isolates carrying *tet*(A), *tet*(C), *tet*(Y), *tet*(30), and *tet*(31). As can be seen from Figure 2.7, LB yielded in the highest total accumulation of colonies, followed by $1/10^{th}$ R2A, and lastly, the SM media. Individual analysis of each different media type revealed that pre-incubation of soil with the highest level of tetracycline (1 mL of 40 μg/mL solution) consistently resulted in the highest accumulation of resistant colonies demonstrating an enrichment of tetracycline resistant microbes. For both LB and $1/10^{th}$ R2A media, 50% of the colonies appeared on day 3, the first day of visible cell growth. For media SM colonies did not appear until day 6 which is consistent with low nutrient concentrations. The observation that no visible colonies appeared for the first 48 h on LB or $1/10^{th}$ R2A indicates that 5 μg/mL tetracycline was too high a concentration for many bacteria. Subsequent studies with lower tetracycline concentrations may reveal a greater diversity of isolates.

Table 2.4 displays the 32 isolates selected for further analysis and their phylogenetic assignment based on the Ribosomal Database Project (RDP) Classifier (Cole et al., 2007). The diversity of isolates cultured was rather limited with only 11 genera represented. These included *Microbacterium*, *Brevundimonas*, *Devosia*, *Streptomyces*, *Pedobacter*, *Sphingobacterium*, *Luteimonas*, *Pseudomonas*, *Stenotrophomonas*, *Thermomonas*, and one unknown. Isolates were subsequently screened by PCR for the presence of *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(X), *tet*(Y), *tet*(Z), *tet*(30), *tet*(31), and clone 397. Five genes were found in the isolates including *tet*(A), *tet*(C), *tet*(Y), *tet*(X), and *tet*(31).

Given the limited scope of this investigation a surprising number of novel observations were made. This study represents the first isolation of *tet*(A) in *Microbacterium* spp. (or any member of the *Actinobacteria*), *tet*(Y) in *Pseudomonas* spp., *tet*(C) and *tet*(31) in *Stenotrophomonas* spp., and *tet*(A) and *tet*(C) in *Thermomonas* spp., the first report of TC resistance in this microbe. This is also the first report of *tet*(Y) and *tet*(31) being isolated from any cultured soil bacterium (Table 1.2).

The two *Sphingobacterium* spp. isolates were screened for *tet*(X) based on Ghosh & LaPara's (2007) finding that a soil-borne *Sphingobacterium* spp. harbored *tet*(X). My discovery of *tet*(X) in *Sphingobacterium* spp. marks the second such finding. The *tet*(X) gene was originally isolated from a *Bacteroides fragilis* R plasmid but was inactive in this strict anaerobe due to the protein's requirement for oxygen (Guiney, Hasegawa, & Davis, 1984). Transfer of *tet*(X) into aerobically grown *E. coli* allows the protein to detoxify tetracycline (Speer & Salyers, 1988). This 44-kDa cytoplasmic soluble protein is a flavin dependent monooxygenase requiring FAD, NADPH,

Magnesium, and Oxygen to detoxify tetracyclines by regiospecifically hydroxylating carbon 11a which causes this product to break down intracellularily (Yang et al., 2004).

Interestingly, carriage in *E. coli* has been shown to confer increases in tigecycline resistance. This resistance is conferred by hydroxylation of carbon 11a, resulting in reduced affinity for magnesium and hence, reduced affinity for the ribosome (Moore, Hughes, & Wright, 2005). This is concerning since tigecycline, a tetracycline (more specifically a glycylcycline), was recently approved for use by the FDA in (Shlaes, 2006). Although resistance increases were slight, it has been hypothesized that mutations leading to increased enzymatic activity and transfer to clinically relevant organisms could be detrimental to tigecycline's effectiveness (Moore et al., 2005).

Originally *tet*(X) was hypothesized to have little clinical relevance since it is inactive in *Bacteroides* spp. (Chopra & Roberts, 2001), however its discovery in *Sphingobacterium* spp. isolates from geographically distinct locations, though both from farm soil impacted with tetracycline contaminated manure, should spark renewed interest in this gene. The G+C% content of *B. fragilis*, the organism within which *tet*(X) was originally isolated is approximately 42%, considerably higher than the 37% G+C content of it's *tet*(X) variant. Because of this Speers and colleagues hypothesized that *B. fragilis* was not the original host (Speer, Bedzyk, & Salyers, 1991). The %G+C of the genomes of studied members of the genus *Sphingobacterium* ranges from 37.3 to 44.2% (Yoo et al., 2007) and this may be the first clue in determining if indeed *Sphingobacterium* spp. are the native host for *tet*(X). Currently it is unknown if this gene is resident on a mobile genetic element in *Sphingobacterium* spp. and hence warrants future investigation.

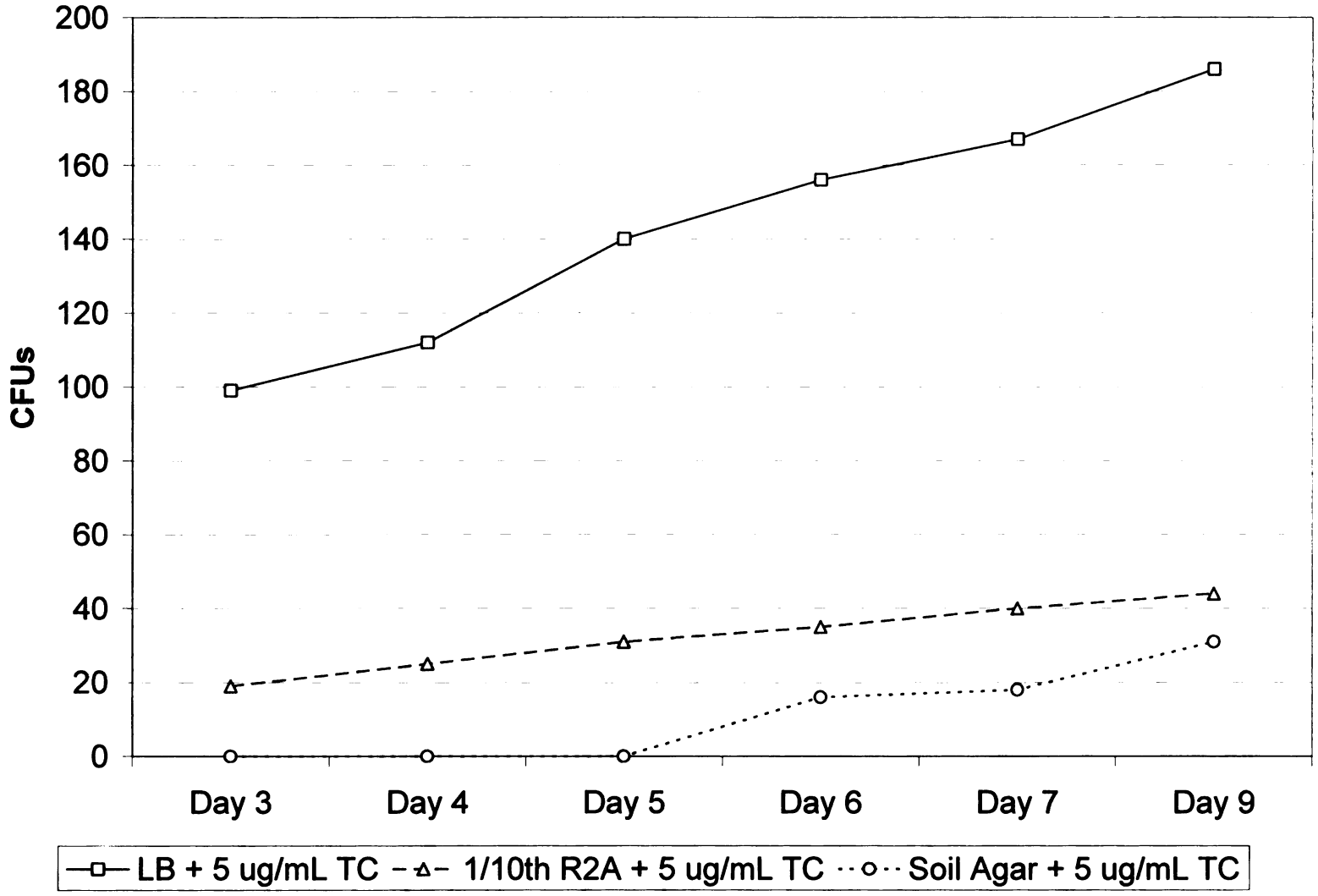


Figure 2.7. Accumulation of Tetracycline Resistant Colonies on Various Media Types.

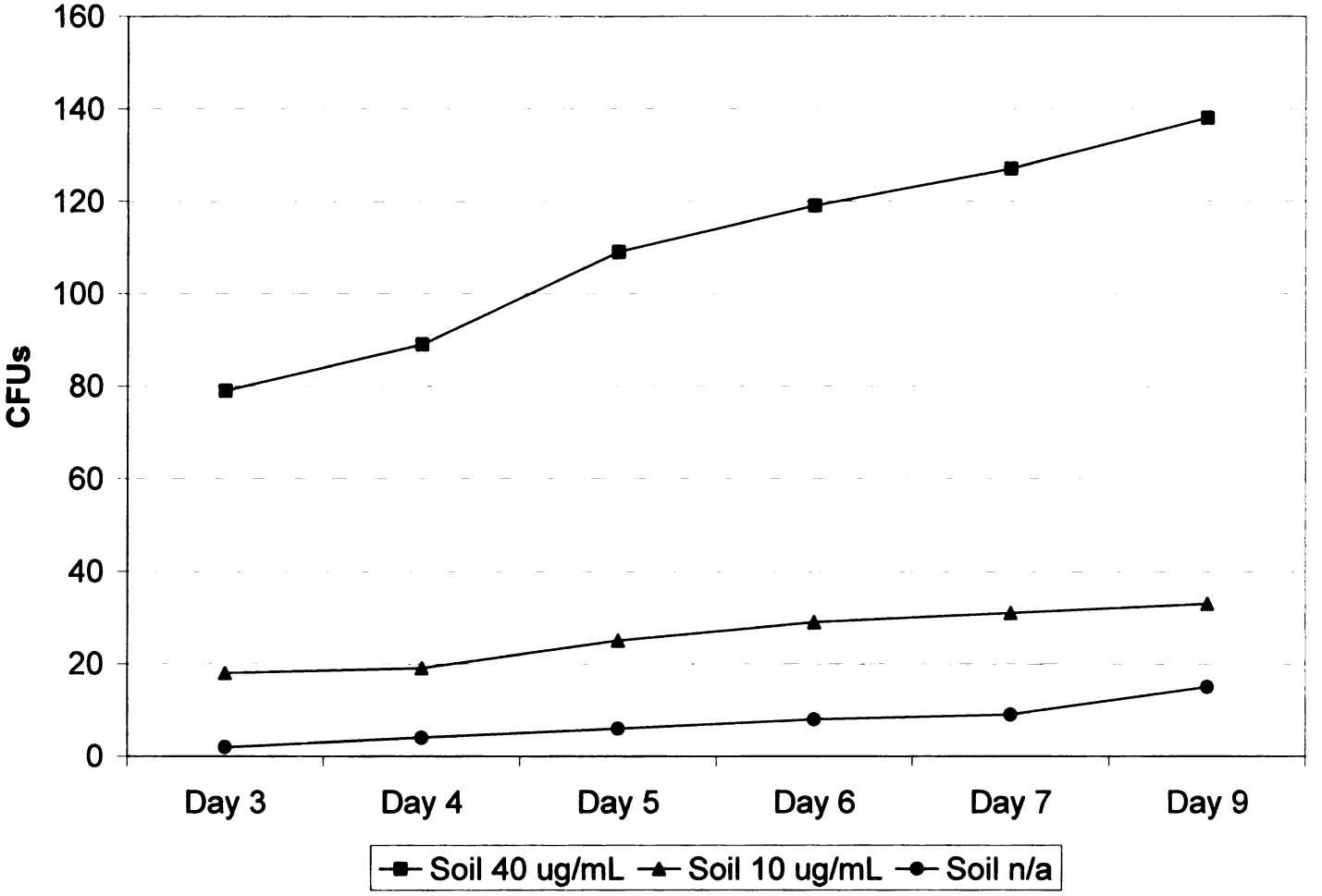


Figure 2.8. Accumulation of Colonies on LB Plates with 5 µg/mL Tetracycline. Symbols represent different soil microcosm conditions. n/a refers to no tetracycline added.

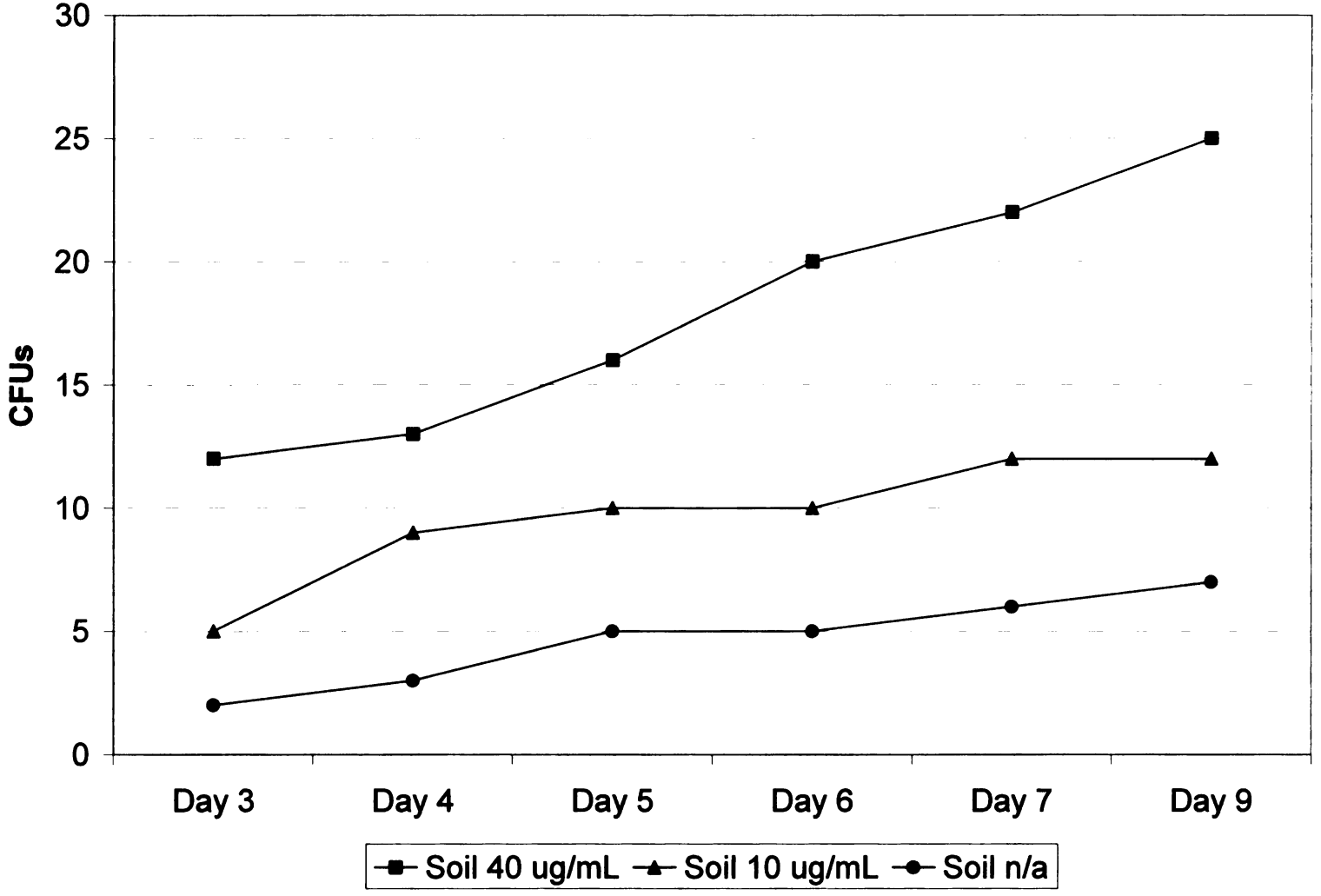


Figure 2.9. Accumulation of Colonies on 1/10th R2A Plates with 5 µg/mL Tetracycline. Symbols represent different soil microcosm conditions. n/a refers to no tetracycline added.

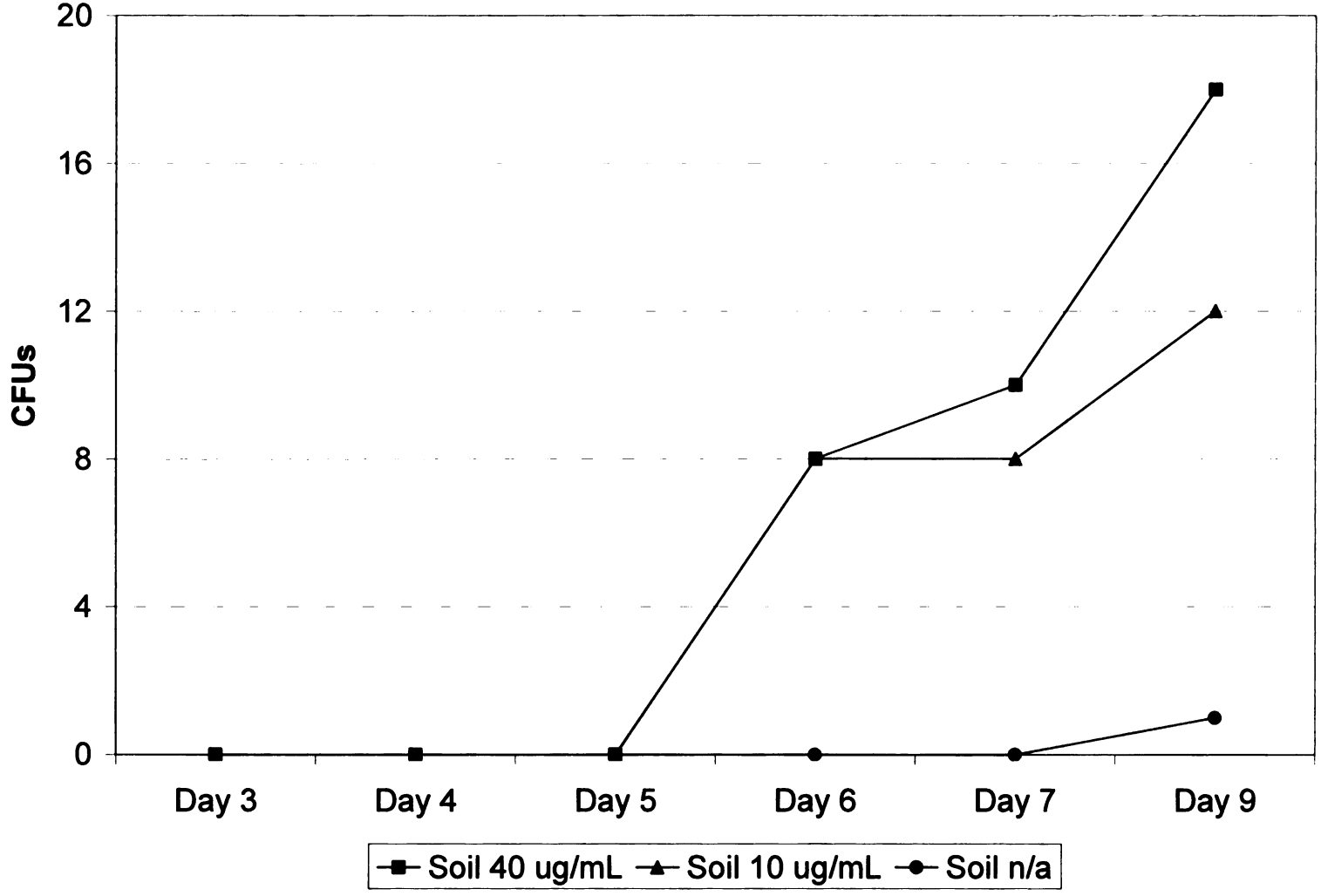


Figure 2.10. Accumulation of Colonies on Soil Media Plates with 5 µg/mL Tetracycline. Symbols represent different soil microcosm conditions. n/a refers to no tetracycline added.

L'Abee-Lund & Sorum (2000) first reported *tet*(31) in an R plasmid (pRAS2) from the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida*. The finding of *tet*(31) in *Stenotrophomonas* spp. is the second discovery of this gene in a bacterial isolate and the first from soil. This demonstrates how little is known regarding the distribution of many of the *tet* resistance elements. The *tet*(C) gene was also found in *Stenotrophomonas* spp. which brings the total number of *tet* genes found in this genus to five (*tet*(C), *tet*(G), *tet*(L), *tet*(31), and *tet*(35)), all efflux genes (Table 1.1 and 1.2). Tetracycline resistance in *Stenotrophomonas* spp. is particularly concerning given that one member of the genus, *Stenotrophomonas maltophilia*, is an opportunistic nosocomial pathogen of increasing importance. Infection with this organism is associated with significant morbidity and mortality, particularly in immunocompromised or immunosuppressed patients (Safdar & Rolston, 2007). Isolation rates of *S. maltophilia* associated with infectious diseases have been increasing since the early 1970s (Senol, 2004). The two most common clinical manifestations are bacteraemia and pneumonia, however, infection is also associated with a number of other diseases (Senol, 2004).

CONCLUSIONS AND FUTURE DIRECTIONS

Although this study failed in its goal of recovering full coding sequences of clones 397 and 492, it did achieve the goal of expanding the current understanding of the dissemination of tetracycline resistance elements in soil environments. A number of novel observations were made. The discovery of *tet*(30) on fosmid TC1 represents the first time this gene has been linked with another TC resistance gene (*tet*(A)) and possibly the first time a functional repressor protein has been identified for this gene. Similarly, the finding of *tet*(31) on fosmid TC3 is only the second time this gene has been isolated

Table 2.4. C% is the confidence for genus assignment with a 95% confidence threshold set for RDP's seqmatch tool. Best type match are the closest type culture relatives. S. S. is the similarity score. It corresponds to the number of unique oligomers shared between the query and a given RDP sequence divided by the lowest number of oligos in either of the two sequences. L is the length of the partial 16S sequence.

Table 2.4. Tetracycline Resistant Colonies Cultured. See previous page for description of table.

Plate	Day	Division	Genus	C%	Gene(s)	Best Type Match (Accession No.)	S.S.	L
LB	3	Actinobacteria	Microbacterium sp.	100		Microbacterium aerolatum (AJ309929)	0.979	733
LB	5	Actinobacteria	Microbacterium sp.	100	A	Microbacterium esteraromaticum (Y17231)	0.982	796
LB	5	Actinobacteria	Microbacterium sp.	100		Microbacterium esteraromaticum (Y17231)	0.986	729
LB	2	Actinobacteria	Microbacterium sp.	100		Microbacterium esteraromaticum (Y17231)	0.993	732
R2A	7	Actinobacteria	Streptomyces sp.	46		Streptomyces tauricus (AB045879)	0.977	603
LB	1	α-Proteobacteria	Brevundimonas sp.	100		Brevundimonas diminuta (M59064)	0.974	656
LB	3	α-Proteobacteria	Brevundimonas sp.	100		Brevundimonas diminuta (M59064)	0.977	756
LB	5	α-Proteobacteria	Brevundimonas sp.	100		Brevundimonas diminuta (M59064)	0.979	847
LB	7	α-Proteobacteria	Brevundimonas sp.	100		Brevundimonas diminuta (M59064)	0.981	786
R2A	2	α-Proteobacteria	Brevundimonas sp.	100		Brevundimonas intermedia (AB023784)	0.967	727
LB	1	α-Proteobacteria	Brevundimonas sp.	100		Brevundimonas intermedia (AB023784)	0.976	768
LB	3	α-Proteobacteria	Devosia sp.	100		Devosia neptuniae (AF469072)	0.971	765
R2A	5	Bacteroidetes	Pedobacter sp.	57		Pedobacter cryoconitis (AJ438170)	0.915	787
R2A	1	Bacteroidetes	Sphingobacterium sp.	100	X	Flavobacterium mizutaii (M58796)	0.997	807
LB	1	Bacteroidetes	Sphingobacterium sp.	100	X	Flavobacterium mizutaii (M58796)	0.989	750
R2A	2	γ-Proteobacteria	Luteimonas sp.	100		Luteimonas mephitis (AJ012228)	1.000	769
R2A	2	γ-Proteobacteria	Luteimonas sp.	100		Luteimonas mephitis (AJ012228)	0.982	540
R2A	7	γ-Proteobacteria	Luteimonas sp.	100		Luteimonas mephitis (AJ012228)	0.999	763
R2A	1	γ-Proteobacteria	Pseudomonas sp.	100		Pseudomonas pseudoalcaligenes (Z76675)	0.977	806
R2A	1	γ-Proteobacteria	Pseudomonas sp.	100		Pseudomonas stutzeri (U26262)	0.991	810
R2A	1	γ-Proteobacteria	Pseudomonas sp.	100	AC	Pseudomonas stutzeri (U26262)	0.994	809
R2A	1	γ-Proteobacteria	Pseudomonas sp.	100	CY	Pseudomonas stutzeri (U26262)	0.997	709
LB	1	γ-Proteobacteria	Pseudomonas sp.	100		Pseudomonas stutzeri (U26262)	0.996	786
LB	1	γ-Proteobacteria	Pseudomonas sp.	100	A	Pseudomonas stutzeri (U26262)	0.997	665
LB	7	γ-Proteobacteria	Stenotrophomonas sp.	100	31	Stenotrophomonas nitrireducens (AJ012229)	0.975	749
LB	7	γ-Proteobacteria	Stenotrophomonas sp.	100		Stenotrophomonas nitrireducens (AJ012229)	0.974	818
R2A	4	γ-Proteobacteria	Stenotrophomonas sp.	100	C	Pseudomonas pictorum (AB021392)	0.953	670
LB	1	γ-Proteobacteria	Stenotrophomonas sp.	100	C	Pseudomonas pictorum (AB021392)	0.980	732
LB	1	γ-Proteobacteria	Stenotrophomonas sp.	100	31	Stenotrophomonas nitrireducens (AJ012229)	0.980	773
LB	2	γ-Proteobacteria	Stenotrophomonas sp.	100	31	Pseudomonas pictorum (AB021392)	0.961	577
R2A	1	γ-Proteobacteria	Thermomonas sp.	100	AC	Thermomonas koreensis (DQ154906)	0.996	785
R2A	1	γ-Proteobacteria	Xylella sp.	14	AC	Pseudomonas pictorum (AB021392)	0.867	523

and the first time from soil.

A number of other new gene/isolate associations were made. This study represents the first time *tet*(Y) has been discovered in a culturable soil isolate (Table 1.2) and the first identification of this gene in *Pseudomonas* spp. The finding of *tet*(A) and *tet*(C) in *Thermomonas* spp. is the first identification of any tetracycline resistance elements in any member of this genus. Discovery of *tet*(C) and *tet*(31) marks the first finding of these genes in *Stenotrophomonas* spp. This is also the first time *tet*(31) has been discovered in a genus other than *Aeromonas* spp. Lastly, the identification of *tet*(A) in *Microbacterium* spp. is novel.

The reason that such a small survey was able to identify so many novel findings is most likely due to the lack of studies that investigate soil environments and use molecular methods to identify tetracycline resistance elements. Furthermore, when such studies do identify resistance elements the search is rarely exhaustive. Few studies come close to attempting to identify the presence of the 38 known tetracycline resistance elements. In fact, in the current literature there is only one study that actively screened tetracycline resistant soil isolates for the presence of *tet*(31) (Agerso & Sandvang, 2005) and no studies that screened for *tet*(30) or *tet*(Y). Exhaustive search is time consuming and expensive. I believe that the culture independent metagenomic approach used in this study was the key to identifying these genes in soil environments. By first attempting recovery of functional genes by this method the bias and problem of work reduction to identifying genes by PCR was eliminated. It must be noted that although metagenomic library construction eliminates primer bias, its downside is the potential inefficiency of heterologous expression in the host cell. The presence of any RPPs are conspicuously

absent from the metagenomic library. These genes are thought to have originated from Gram-positive microbes (Connell et al., 2003) and the lack of presence in this library may be due to inefficient gene expression in *E. coli*.

It was postulated that cultivating bacteria on lower nutrient media would allow recovery of novel gene/isolate combinations. Again, this has only been attempted once in a survey of the cultivatable tetracycline resistant fraction (Ghosh & LaPara, 2007). In my study few isolates were recovered on $1/10^{th}$ R2A media that were not recovered on LB media (Table 2.4). Selection of appropriate media is a difficult decision and each type has its own inherent biases. Unfortunately no isolates grown on the SM medium grew when transferred to liquid culture. Future studies should use a more diverse selection of low nutrient media to attempt to recover a breadth of novel isolates.

This investigation has opened the door for a number of interesting follow-up studies. Of particular interest are:

1) Given the length of sequence coverage between the *tet*(31) region on fosmid TC3 and plasmid pRAS2, determining whether this gene resides on a mobile element.

2) Investigate if the *tet*(30) gene resident on fosmid TC1 has a functional repressor protein.

3) Determine if the genes identified in isolates are present on mobile elements such as transposons and plasmids. The knowledge of whether *tet*(X) and *tet*(31) reside on mobile elements in *Sphingobacterium* spp. and *Stenotrophomonas* spp. would be useful. The linkage of *tet*(A) and *tet*(C) in *Pseudomonas* spp. and *Thermomonas* spp. implies that these

genes are on a mobile element. Further investigation of this is warranted.

4) Given that *tet*(X) has been shown to confer slight resistance to tigecycline in *E. coli* (Moore et al., 2005), it would be interesting to conduct evolution experiments to determine if mutations can confer higher levels of resistance, and if so, by what mechanism.

In conclusion, this study further exemplifies the role of soil environments as a reservoir of tetracycline resistance genes. The finding of a number of novel observations with a study of such limited scope demonstrates the lack of information regarding the environmental distribution of tetracycline resistance, particularly in soil systems. It appears that microbiologists are still at the tip of the ice-berg regarding the role of resistance in the environment. Further characterization is necessary before any comment can be made on the role of tetracycline residues promoting tetracycline resistance in the environment.

REFERENCES

Agerso, Y., & Sandvang, D. (2005). Class 1 integrons and tetracycline resistance genes in alcaligenes, arthrobacter, and Pseudomonas spp. isolated from pigsties and manured soil. *Appl Environ Microbiol, 71*(12), 7941-7947.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res, 25*(17), 3389-3402.

Aminov, R. I., Chee-Sanford, J. C., Garrigues, N., Teferedegne, B., Krapac, I. J., White, B. A., et al. (2002). Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. *Appl Environ Microbiol, 68*(4), 1786-1793.

Bahl, M. I., Hansen, L. H., Goesmann, A., & Sorensen, S. J. (2007). The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established alpha, beta and delta sub-groups. *Plasmid, 58*(1), 31-43.

Barbosa, T. M., Scott, K. P., & Flint, H. J. (1999). Evidence for recent intergeneric transfer of a new tetracycline resistance gene, tet(W), isolated from Butyrivibrio fibrisolvens, and the occurrence of tet(O) in ruminal bacteria. *Environ Microbiol, 1*(1), 53-64.

Barkay, T., Miller, S. M., & Summers, A. O. (2003). Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiol Rev, 27*(2-3), 355-384.

Blankenfeldt, W., Kuzin, A. P., Skarina, T., Korniyenko, Y., Tong, L., Bayer, P., et al. (2004). Structure and function of the phenazine biosynthetic protein PhzF from Pseudomonas fluorescens. *Proc Natl Acad Sci U S A, 101*(47), 16431-16436.

Chiou, C. S., & Jones, A. L. (1995). Expression and identification of the strA-strB gene pair from streptomycin-resistant Erwinia amylovora. *Gene, 152*(1), 47-51.

Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev, 65*(2), 232-260 ; second page, table of contents.

Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., et al. (2007). The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res, 35*(Database issue), D169-172.

Connell, S. R., Tracz, D. M., Nierhaus, K. H., & Taylor, D. E. (2003). Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob Agents Chemother, 47*(12), 3675-3681.

Davis, K. E., Joseph, S. J., & Janssen, P. H. (2005). Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. *Appl Environ Microbiol, 71*(2), 826-834.

Diaz-Torres, M. L., McNab, R., Spratt, D. A., Villedieu, A., Hunt, N., Wilson, M., et al. (2003). Novel tetracycline resistance determinant from the oral metagenome. *Antimicrob Agents Chemother, 47*(4), 1430-1432.

Diaz-Torres, M. L., Villedieu, A., Hunt, N., McNab, R., Spratt, D. A., Allan, E., et al. (2006). Determining the antibiotic resistance potential of the indigenous oral microbiota of humans using a metagenomic approach. *FEMS Microbiol Lett, 258*(2), 257-262.

Eichorst, S. A. (2007). Isolation and Characterization of Members of the Phylum *Acidobacteria* from Soils. [Dissertation]. *Unpublished Ph.D. Dissertation - Michigan State University.*

Furushita, M., Shiba, T., Maeda, T., Yahata, M., Kaneoka, A., Takahashi, Y., et al. (2003). Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl Environ Microbiol, 69*(9), 5336-5342.

Gaskins, H. R., Collier, C. T., & Anderson, D. B. (2002). Antibiotics as growth promotants: mode of action. *Anim Biotechnol, 13*(1), 29-42.

Ghosh, S., & LaPara, T. M. (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J, 1*(3), 191-203.

Giovannoni, S. J. (1991). The Polymerase Chain Reaction. *In* Nucleic Acids Techniques in Bacterial Systematics. E. Stackenbrandt. N.Y. John Wiley & Sons, 177-201.

Gorbach, S. L. (2001). Antimicrobial use in animal feed--time to stop. *N Engl J Med, 345*(16), 1202-1203.

Gordon, L., Cloeckaert, A., Doublet, B., Schwarz, S., Bouju-Albert, A., Ganiere, J. P., et al. (2008a). Complete sequence of the floR-carrying multiresistance plasmid pAB5S9 from freshwater Aeromonas bestiarum. *J Antimicrob Chemother, 62*(1), 65-71.

Gordon, L., Cloeckaert, A., Doublet, B., Schwarz, S., Bouju-Albert, A., Ganiere, J. P., et al. (2008b). Complete sequence of the floR-carrying multiresistance plasmid pAB5S9 from freshwater Aeromonas bestiarum. *J Antimicrob Chemother.*

Guiney, D. G., Jr., Hasegawa, P., & Davis, C. E. (1984). Expression in Escherichia coli of cryptic tetracycline resistance genes from bacteroides R plasmids. *Plasmid, 11*(3), 248-252.

Handelsman, J. (2004). Metagenomics: application of genomics to uncultured microorganisms. *Microbiol Mol Biol Rev, 68*(4), 669-685.

Hrvatin, S., & Piel, J. (2007). Rapid isolation of rare clones from highly complex DNA libraries by PCR analysis of liquid gel pools. *J Microbiol Methods, 68*(2), 434-436.

Khachatryan, A. R., Besser, T. E., & Call, D. R. (2008). The streptomycin-sulfadiazine-tetracycline antimicrobial resistance element of calf-adapted Escherichia coli is widely distributed among isolates from Washington state cattle. *Appl Environ Microbiol, 74*(2), 391-395.

L'Abee-Lund, T. M., & Sorum, H. (2000). Functional Tn5393-like transposon in the R plasmid pRAS2 from the fish pathogen Aeromonas salmonicida subspecies salmonicida isolated in Norway. *Appl Environ Microbiol, 66*(12), 5533-5535.

Levy, S. B., McMurry, L. M., Barbosa, T. M., Burdett, V., Courvalin, P., Hillen, W., et al. (1999). Nomenclature for new tetracycline resistance determinants. *Antimicrob Agents Chemother, 43*(6), 1523-1524.

Luo, Z. Q., & Farrand, S. K. (1999). Cloning and characterization of a tetracycline resistance determinant present in Agrobacterium tumefaciens C58. *J Bacteriol, 181*(2), 618-626.

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nat Rev Microbiol, 4*(8), 608-620.

Moore, I. F., Hughes, D. W., & Wright, G. D. (2005). Tigecycline is modified by the flavin-dependent monooxygenase TetX. *Biochemistry, 44*(35), 11829-11835.

Ng, L. K., Martin, I., Alfa, M., & Mulvey, M. (2001). Multiplex PCR for the detection of tetracycline resistant genes. *Mol Cell Probes, 15*(4), 209-215.

Patterson, A. J., Colangeli, R., Spigaglia, P., & Scott, K. P. (2007). Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. *Environ Microbiol, 9*(3), 703-715.

Riesenfeld, C. S., Goodman, R. M., & Handelsman, J. (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol, 6*(9), 981-989.

Rodriguez-Minguela, C. M. (2005). A molecular approach for the detection and characterization of novel tetracycline resistance genes, integrons, and integron-endcoded antibiotic resistance determinants in the environment. [Dissertation]. *Unpublished Ph.D. Dissertation - Michigan State University.*

Safdar, A., & Rolston, K. V. (2007). Stenotrophomonas maltophilia: changing spectrum of a serious bacterial pathogen in patients with cancer. *Clin Infect Dis, 45*(12), 1602-1609.

Sambrook, J., & Russell, D. W. (2001). Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory Press. .

Sarmah, A. K., Meyer, M. T., & Boxall, A. B. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere, 65*(5), 725-759.

Schloss, P. D., & Handelsman, J. (2005). Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. *Genome Biol, 6*(8), 229.

Schmitt, H., Stoob, K., Hamscher, G., Smit, E., & Seinen, W. (2006). Tetracyclines and tetracycline resistance in agricultural soils: microcosm and field studies. *Microb Ecol, 51*(3), 267-276.

Senol, E. (2004). Stenotrophomonas maltophilia: the significance and role as a nosocomial pathogen. *J Hosp Infect, 57*(1), 1-7.

Shlaes, D. M. (2006). An update on tetracyclines. *Curr Opin Investig Drugs, 7*(2), 167-171.

Smalla, K., Heuer, H., Gotz, A., Niemeyer, D., Krogerrecklenfort, E., & Tietze, E. (2000). Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. *Appl Environ Microbiol, 66*(11), 4854-4862.

Speer, B. S., Bedzyk, L., & Salyers, A. A. (1991). Evidence that a novel tetracycline resistance gene found on two Bacteroides transposons encodes an NADP-requiring oxidoreductase. *J Bacteriol, 173*(1), 176-183.

Speer, B. S., & Salyers, A. A. (1988). Characterization of a novel tetracycline resistance that functions only in aerobically grown Escherichia coli. *J Bacteriol, 170*(4), 1423-1429.

Srinivasan, V., Nam, H. M., Sawant, A. A., Headrick, S. I., Nguyen, L. T., & Oliver, S. P. (2008). Distribution of tetracycline and streptomycin resistance genes and class 1 integrons in Enterobacteriaceae isolated from dairy and nondairy farm soils. *Microb Ecol, 55*(2), 184-193.

Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., King, C. J., et al. (2006). Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ Microbiol, 8*(9), 1510-1514.

Stevenson, B. S., Eichorst, S. A., Wertz, J. T., Schmidt, T. M., & Breznak, J. A. (2004). New strategies for cultivation and detection of previously uncultured microbes. *Appl Environ Microbiol, 70*(8), 4748-4755.

Stine, O. C., Johnson, J. A., Keefer-Norris, A., Perry, K. L., Tigno, J., Qaiyumi, S., et al. (2007). Widespread distribution of tetracycline resistance genes in a confined animal feeding facility. *Int J Antimicrob Agents, 29*(3), 348-352.

Sundin, G. W. (2002). Distinct recent lineages of the strA- strB streptomycin-resistance genes in clinical and environmental bacteria. *Curr Microbiol, 45*(1), 63-69.

Torsvik, V., Goksoyr, J., & Daae, F. L. (1990). High diversity in DNA of soil bacteria. *Appl Environ Microbiol, 56*(3), 782-787.

Walker, M. S., & Walker, J. B. (1970). Streptomycin biosynthesis and metabolism. Enzymatic phosphorylation of dihydrostreptobiosamine moieties of dihydro-streptomycin-(streptidino) phosphate and dihydrostreptomycin by Streptomyces extracts. *J Biol Chem, 245*(24), 6683-6689.

Yang, W., Moore, I. F., Koteva, K. P., Bareich, D. C., Hughes, D. W., & Wright, G. D. (2004). TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. *J Biol Chem, 279*(50), 52346-52352.

Yoo, S. H., Weon, H. Y., Jang, H. B., Kim, B. Y., Kwon, S. W., Go, S. J., et al. (2007). Sphingobacterium composti sp. nov., isolated from cotton-waste composts. *Int J Syst Evol Microbiol, 57*(Pt 7), 1590-1593.

Zhou, J., Bruns, M. A., & Tiedje, J. M. (1996). DNA recovery from soils of diverse composition. *Appl Environ Microbiol, 62*(2), 316-322.

CHAPTER III

AN ANALYSIS OF TETRACYCLINE – CATION CHELATION AND ITS RELATION TO BIOAVAILABILITY AND TETRACYCLINE EFFLUX GENE EXPRESSION

INTRODUCTION

Tetracycline antibiotics are used for a variety of purposes including human therapy (Smilack, 1999), animal therapy and growth promotion (Gaskins, Collier, & Anderson, 2002), aquaculture (Cabello, 2006), and plant agriculture (McManus, Stockwell, Sundin, & Jones, 2002). Ultimately un-altered tetracyclines make their way to the environment through a variety of pathways including surface runoff (Pruden, Pei, Storteboom, & Carlson, 2006), WWTP discharges (Karthikeyan & Meyer, 2006), and manure application as fertilizer (Hamscher, Sczesny, Hoper, & Nau, 2002). Not surprisingly, multiple investigators have measured tetracycline (TC) accumulation and the presence of resistant bacteria and their genes in terrestrial and aquatic environments including; soil (Ghosh & LaPara, 2007), surface water (Goni-Urriza et al., 2000), groundwater (Chee-Sanford, Aminov, Krapac, Garrigues-Jeanjean, & Mackie, 2001), sediment (Neela, Nonaka, & Suzuki, 2007), and even dust particles inside concentrated animal feeding operations (CAFOs) (Chapin, Rule, Gibson, Buckley, & Schwab, 2005; Hamscher, Pawelzick, Sczesny, Nau, & Hartung, 2003) (Tables 1.1 – 1.5). These observations have created concern that the environmental accumulation of TC residues may be enhancing the development, maintenance, and transfer of TC resistance to human and animal pathogens (Kummerer, 2004). This scenario would be especially problematic because of (a) reduced effectiveness of TC to eliminate bacterial infections and (b) the economic consequences of pathogen resistance.

Tetracyclines cross the outer membrane of gram-negative enteric bacteria through OmpF and OmpC porin channels as positively charged metal-tetracycline coordination complexes (Chopra & Roberts, 2001). In the periplasm the metal-tetracycline complexes dissociate and the slightly lipophilic tetracycline molecules diffuse through the cytoplasmic membrane (Chopra & Roberts, 2001). Transfer across Gram-positive cytoplasmic membranes is assumed to occur in the same manner with the electroneutral, lipophilic form being the species transferred (Chopra & Roberts, 2001). Uptake across the cytoplasmic membrane is energy dependent and driven by the ΔpH component of the proton motive force (Chopra & Roberts, 2001). Once in the cytoplasm, tetracyclines reversibly bind to the 16S ribosomal RNA (rRNA) of bacterial ribosomes near the acceptor A site, preventing aminoacyl-tRNA binding (White, Alekshun, McDermott, & Levy, 2005). Peptide elongation is halted, and growth is subsequently inhibited.

To evade this growth inhibition, bacteria have developed, maintained, and transferred tetracycline resistance genes (TcR) over the course of evolution. Currently four genetic mechanisms are known; the two most widely disseminated being ribosomal protection (RPPs), and tetracycline efflux (Chopra & Roberts, 2001). Tetracycline efflux genes encode membrane bound pumps, regulated by tetracycline inducible repressor-operator systems (TetR-*tetO*), that confer resistance to tetracycline by an energy-dependent efflux of a magnesium chelated tetracycline molecule in exchange for a proton (Chopra & Roberts, 2001). The RPPs quench TC's effect on the bacterial cell by dislodging TC from the ribosome in a GTP dependent manner (Connell, Tracz, Nierhaus, & Taylor, 2003). The presence of these genes on mobile genetic elements such as conjugative plasmids and transposons (Chopra & Roberts, 2001) has allowed the

horizontal transfer of resistance to diverse genera (Tables 1.1 – 1.5). Selective pressure, provided by TC, can enhance horizontal transfer of TcR genes (Whittle, Shoemaker, & Salyers, 2002). Furthermore, it has been demonstrated that once established tetracycline resistance can persist even after the antibiotic is withdrawn (Langlois, Cromwell, Stahly, Dawson, & Hays, 1983). Phenomenon such as compensatory mutations (Nguyen, Phan, Duong, Bertrand, & Lenski, 1989), co-selection (Tuckfield & McArthur, 2007), cross-resistance (Levy, 2002), and beneficial physiological functions of genes (Krulwich, Jin, Guffanti, & Bechhofer, 2001) have all aided in the maintenance of resistance in the absence of selective pressure.

In order for TC residues to exert their selective pressure they must be bioavailable to the indigenous bacteria. Here, bioavailability is defined as *the ability of tetracycline to leave the environmental matrix, penetrate the microbial cell's membrane, and act on its target*. Currently it is unknown what factors control the bioavailability of TC residues found in environmental systems.

Tetracyclines are amphoteric, adaptable molecules that can form a variety of different tautomers in solution (Sassman & Lee, 2005). They consist of a linear fused tetracyclic nucleus (A, B, C, D) to which a variety of functional groups are attached (Mitscher, 1978). 6-deoxy-6-demethyltetracycline is the minimum pharmacore that retains antibacterial activity (Chopra & Roberts, 2001). The presence of three ionizable functional groups allow tetracyclines to exist as four different species in solution (cationic, zwitterionic, and two anionic species) with the relative distribution dependent upon pH. For a review of pH dependent TC speciation see Sassman & Lee, 2005. A significant amount of research has been conducted on tetracyclines' ability to chelate

cations in solution. Current theory suggests that chelation occurs in the lower peripheral region between O12 and O1 oxygen groups on the BA rings and the O10 and O11 oxygen of the DC rings for calcium; whereas the O11 carbonyl group and O12 enol group is thought to bind magnesium (Nelson, 1998). The presence of divalent and trivalent cations in solution with tetracycline tends to raise the minimum inhibitory concentration (MIC) of diverse bacteria (Avery, Goddard, Sumner, & Avery, 2004; Chopra & Howe, 1978; Nanavaty, Mortensen, & Shryock, 1998). Furthermore, it has been demonstrated that magnesium inhibits bacterial uptake of TC and that this effect is pH dependent (Yamaguchi, Ohmori, Kaneko-Ohdera, Nomura, & Sawai, 1991). Therefore, it seems likely that TC's ability to chelate cation's would prevent the antibiotic from entering the cell and activating expression of the TC-efflux genes by de-repression the TetR protein. All of the above evidence is qualitative and no studies have attempted to quantify the TC – cation chelation effect and how it relates to gene expression in a live microbial cell. Given that calcium, magnesium, sodium, and potassium are the predominant cations present in soil systems it seems that the geochemical characteristics of a given environment could modulate TC bioavailability and hence the effect on the native microbial flora. Characterizing this dynamic is essential to understanding the risk of the environmental accumulation of tetracyclines.

At present date only two studies have implicitly addressed the concept of TC bioavailability in natural systems (Chander, Kumar, Goyal, & Gupta, 2005; Verma, Robarts, & Headley, 2007). Neither of these studies provides any mechanistic understanding of the factors that control tetracycline bioavailability. The development of bioreporter technology over the past 15 years allows the question of what factors control

bioavailability to be addressed. A bioreporter (a.k.a. biosensor), in simple terms, is a cell that has a reporter gene (typically *gfp*, *lacZ*, or bacterial luciferase) that responds to an environmental stimulus through a fused promoter (Fig. 3.2). Bioreporters have been employed to measure metals, antibiotics, temperature, water potential, and quorem sensing compounds (Leveau & Lindow, 2002).

This study was designed to provide insight into how solution phase TC-cation chemistry affects microbial cell TcR gene expression. This was accomplished by the use of a whole-cell tetracycline biosensor developed by Dr. Soren Sorensen's group at the University of Copenhagen (Bahl, Hansen, & Sorensen, 2005), and a quantitative model for promoter activity developed by Dr. Steven Lindow's group at UC-Berkeley (Leveau & Lindow, 2001). Briefly, the biosensor used in this work is a live *Escherichia coli* cell containing a plasmid, pTGM, with a transcriptional fusion between a tetracycline inducible promoter (P*tet(A)*) and a *gfp* gene (Bahl et al., 2005). Two important aspects of this construct include:

- The architecture of the P*tet(A)* promoter is completely maintained as it was originally isolated from Tn*10*. Therefore, *gfp* expression is proportional to the P*tet(A)* activity that drives antibiotic resistance gene expression in a natural setting.
- The pTGM construct contains a tetracycline resistance gene, *tet*(M), to inhibit tetracyclines from killing the cell (Bahl et al., 2005). This gene is a ribosomal protection protein that dissociates TC from the ribosome upon GTP hydrolysis (Connell et al., 2003). Therefore the intracellular concentration of TC remains unchanged.

Figure 3.1. Epifluorescent Microscopy Images of the Tetracycline Bioreporter. a) uninduced b) induced with 0.1 µg/mL TC and c) induced with 1 µg/mL TC.

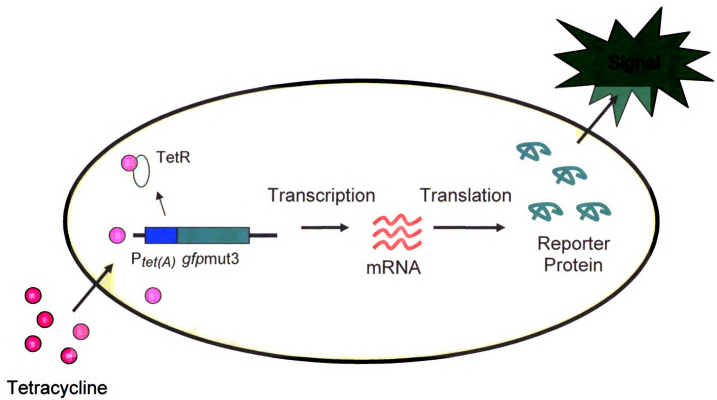


Figure 3.2. Schematic of Bioreporter Cells. Tetracycline enters the cell, de-represses the TetR repressor protein from the P*tet(A)* promoter, and activates *gfp*mut3 transcription. When the GFP protein is translated and matures the cell gives off a fluorescent signal proportional to the amount of tetracycline that entered the cell.

Bioavailable tetracyclines, once in the cytoplasm, will bind the transcriptional regulator, TetR, inducing a conformational change releasing it from the two operator regions of the P*tet(A)* promoter (Ramos et al., 2005). As a result the *gfp* gene is expressed (Figs. 3.1 and 3.2). In the present study this biosensor strain has been used to quantitatively analyze the activity of the P*tet(A)* promoter (which mimicks tetracycline efflux gene expression) in the presence of tetracycline complexed with environmentally relevant cations (magnesium, calcium, sodium, and potassium) at environmentally relevant pH's (5, 7 and 9) in the solution phase.

MATERIALS AND METHODS

Bacterial Strain & Culture Conditions. *Escherichia coli* strain MC4100/pTGM (Bahl et al., 2005) was used as a whole-cell bacterial biosensor for this study. Plasmid pTGM contains the P*tet(A)* promoter originating from Tn*10* fused to *gfp*mut3 encoding a flow cytometry optimized *gfp* gene.

Culture media was as follows: 10 g tryptone (Accumedia, Lansing, MI), 5 g yeast extract (Accumedia, Lansing, MI), and 0.5 g NaCl (J.T. Baker, Phillipsburg, NJ) per liter. To this pH 5.0 included 100 mM 2-(N-morpholino)ethanesulfonic acid (MES, free acid; Calbiochem, Darmstadt, Germany) buffer, pH 7.0 included 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS; Sigma-Aldrich Inc., St. Louis, MO) buffer, and pH 9.0 included 100 mM 3-[(1,1-dimethyl-2-hydroxyethly)amino]-2-hydroxypropanesulfonic acid (AMPSO, free acid; Research Organics, Cleveland, OH) buffer. Good's buffers were chosen because of their low interaction with metal ions (Good et al., 1966).

For promoter activity experiments cells were inoculated and incubated at 30°C overnight in the same type of media to be used for the assay the following day with 100 μg/mL ampicillin (Sigma-Aldrich Inc., St. Louis, MO) to maintain the pTGM plasmid. The following morning an appropriate amount of media was transferred to a sterilized 1000 mL Erlenmeyer flask. To this 100 μg/mL ampicllin was added. The flask was stirred well and three 25 mL aliquots were transferred to sterile 125 mL Erlenmeyer flasks to measure leaky expression of the P*tet(A)* promoter in the absence of tetracycline . To the remaining media an appropriate amount of tetracycline (Sigma-Aldrich Inc., St. Louis, MO) was diluted from a 5 mg/mL stock solution and added to achieve a final concentration of 0.1 μg/mL (100 ppb). The flask was again stirred and 25 mL aliquots were transferred to three sterile 125 mL flasks representing 100% P*tet(A)* expression at 0.1 μg/mL tetracycline. Media was then subdivided to five 80 mL fractions in 250 mL Erlenmeyer flasks. To these appropriate amounts of 1M stock solutions of cations ($MgCl_2$, $CaCl_2$, NaCl, KCl; J.T. Baker, Phillipsburg, NJ) were added to achieve final cation concentrations of 0.1 mM, 0.125 mM, 0.25 mM, 0.375 mM, 0.5 mM, 1 mM, 5 mM, and 10 mM. Media from these 80 mL flasks was then subdivided into three 125 mL Erlenmeyer flasks each receiving 25 mL of media. This extensive procedure was done to reduce variation introduced into the assay due to pipeting error. After all flasks were aliquoted for the days' experiment they were incubated in a horizontal shaker (150 rpm) at 30°C to equilibrate for 30 minutes.

After equilibration a 1/100 dilution of the overnight culture of the *Escherichia coli* strain MC4100/pTGM bioreporter was made into each 125 mL Erlenmeyer flask with the appropriate assay media. Cultures were then grown in triplicate exponentially in

125 mL Erlenmeyer flasks in at 30°C while shaking at 150 rpm. 1 mL aliquots were periodically taken for analysis by spectroflourimetry (*gfp*mut3 excitation = 488; emission = 511) and spectrometry ($O.D._{600}$) performed with a SpectraMax M2 spectroflourimeter (Molecular Devices, Sunnyvale, CA).

Experiments in which $MgCl_2$ and EDTA ((ethylenedinitrilo)tetraacetic acid, disodium salt, dehydrate; J.T. Baker, Phillipsburg, NJ) were added mid-culture were set up as described above. At appropriate times (as indicated in Figure 3.14) magnesium chloride was added from a 1M stock to achieve a final concentration of 10 mM. Similarly, EDTA was added from a 250 mM stock to achieve a final concentration of 10 mM.

Promoter Activity Calculations. Promoter activity is arrived at by using equation 13 (P = fss x μ x (1 + (μ/m))) from Leveau & Lindow (2001). Fluorescent steady state (fss) is calculated by the slope of an F'OD plot (OD600 plotted against relative fluorescence units (RFU)). Growth rate (μ) is determined by plotting the log of OD_{600} by time (hours) and taking the slope of that line. A value of 1.54 h^{-1} was used for the GFP maturation constant (m) (Leveau & Lindow, 2001). A value for promoter activity is arrived at with units RNU/OD/h (relative non-fluorescent units of GFP per OD unit X unit time). Scatter plots were made and linear regression was performed with Microsoft Excel. All R-squared values ranged from 0.95 – 0.999 (data not shown). All graphs for the present study were created with Microsoft Excel.

Analysis of intracellular TC. *Reagents and Chemicals.* All chemicals were at least of analytical reagent grade, and deionized water was used throughout, unless stated otherwise. Tetracycline hydrochloride was purchased from Sigma-Aldrich Inc., St. Louis,

MO. HPLC grade Methanol was purchased from J.T.Baker, Phillipsburg, NJ. McIlvain buffer (pH 3.8) consisted of 10.9 g of disodium hydrogen phosphate, 37.2 g ethylenediaminetetraacetic acid (EDTA) sodium salt, and 12.9 g citric acid monohydrate per 1L of water. All tetracycline hydrochloride solutions were protected from light and prepared fresh before each experiment.

Tetracycline was extracted from cell pellets with an Oasis HLB solid phase extraction (SPE) column (Waters Inc., Milford MA) and analyzed with an LC-20 solvent delivery unit (Shimadzu, Kyoto, Japan) coupled with an API 3200^{TM} LC/MS/MS (Applied Biosystems, Foster City, CA).

Sample Preparation. After cell cultures' promoter activity was analyzed (OD600 of approximately 0.8) cell cultures were centrifuged at 10,000 rpm to pellet cells and were subsequently washed twice with PBS buffer. Supernatant was removed and cell pellets were freeze dried overnight. Freeze dried cells were mixed with 10mL of McIlvain buffer, vortexed for 1 min, and then sonicated for 10 min. This solution was centrifuged at 10,500 rpm for 20 min. Supernatants were filtered through membranes (0.45 μm pore size) to eliminate cell material. Centrifugation and filter steps were repeated and filtrate was used for analysis.

Solid Phase Extraction. Before tetracycline extractions, SPE cartridges were preconditioned with 3 mL of methanol, followed by 3 mL of 0.1N of hydrochloride acid and 6 mL of pure water. Sample filtrate (10 mL) was passed through cartridges at the flow rate of 2 mL per min. After sample loading, the cartridges were washed with 3 mL of pure water and dried with nitrogen gas for 30 min. Tetracycline was eluted with 10 mL of a methanol – water mixture (1:1) containing 150 ppm EDTA.

Liquid Chromatograpy-Mass Spectrometry. The tetracycline elutions (10 μL) were separated by an LC-20 solvent delivery unit (Shimadzu, Kyoto, Japan) using a Gemini C18 column (5 μm, 50 * 2.00 mm; Phenomenex Inc., Torrance, CA). LC mobile phase A consisted of 95% water, 5% methanol, 20mM heptafluorobutyric acid (HFBA), and 2 mM ammonium acetate. LC mobile phase B consisted of 95% methanol, 20 mM heptafluorobutyric acid (HFBA), and 2 mM ammonium acetate. The flow rate was 0.5 mL per min. The gradient is shown in Table 3.1. LC was directly interfaced to the electrospray ionization source (ESI) coupled with Applied Biosystems API 3200^{TM} LC/MS/MS system. Two ion pairs were used to confirm the analyte (tetracycline), and one ion pair (445.000/410.000) was used for the purpose of quantitation.

Epiflourescent Microscopy. Overnight cultures of *E. coli* strain MC4100/pTGM were grown at 30°C and 150 rpm in standard LB (Accumedia, Lansing, MI) supplemented with an appropriate amount of tetracycline (Sigma-Aldrich Inc., St. Louis, MO) and magnesium chloride (J.T. Baker, Phillipsburg, NJ) (Figs. 3.1 and 3.3). Cultures were then diluted appropriately and epifluorescent images of 10 μL wet mounts were taken with a Zeiss Axioskop 2 Microscope (Carl Zeiss Inc., Thornwood, NY) fitted with a Spot 2 cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI), a Zeiss Universal Arc-lamp Power Supply with mercury burner (Carl Zeiss Inc., Thornwood, NY), and using Zeiss Filter set 09 (Carl Zeiss Inc., Thornwood, NY) for GFP detection.

Table 3.1. Liquid Chromatography Separation Gradient for Analysis of Intracellular Tetracycline.

Time	%B
0	5
1	5
3	95
5.5	100
6	5
8	7

RESULTS AND DISCUSSION

Verification of Functionality of Bioreporter. Upon receiving the *E. coli* MC4100/pTGM biosensor (Courtesy of Dr. Lars Hansen, University of Copenhagen) it was necessary to demonstrate that the GFP response was dependent on the concentration of tetracycline present, and simply not an on-off switch. To do this the biosensor was grown overnight in the presence of increasing concentrations of tetracycline. The following morning cultures were diluted and imaged by epifluorescent microscopy. Figure 3.1 shows a typical result of this experiment. As expected the brightness of GFP increases with increasing tetracycline concentration.

Multiple observations have been made that suggest that TC – cation complexes increase the minimum inhibitory concentrations (MICs) because tetracycline is unable to enter the cell under such conditions (Avery et al., 2004; Nanavaty et al., 1998). If this is true, then *gfp*mut3 gene expression from the pTGM plasmid should be reduced when the bioreporter is grown in media with tetracycline and cations. Furthermore, cation concentration dependent titration of this response should be possible. To confirm this assumption, overnight cultures of the *E. coli* strain MC4100/pTGM whole cell biosensor were grown in the presence of 0.1 μg/mL (100 ppb) tetracycline and various concentrations of magnesium chloride (0, 0.1, 0.5, 1, 5, and 10 mM). Again, the following morning cultures were diluted and imaged via epifluorescent microscopy (Fig. 3.3). As expected, the brightness of GFP decreases with increasing magnesium chloride concentration.

Determining the Range of Linear Response in Promoter Activity at pH 5.0, 7.0 and 9.0. Given that the substrate being measured (TC) has the ability at sufficient

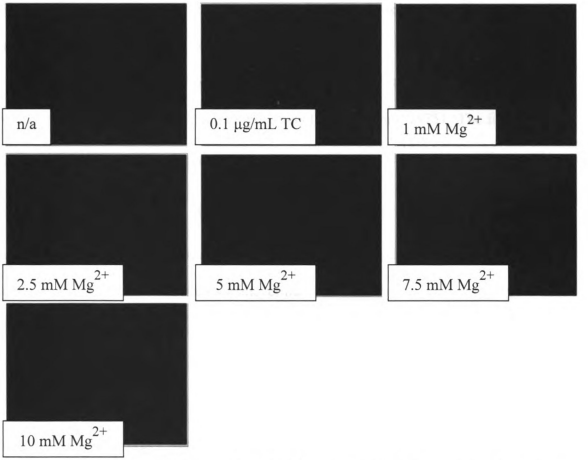


Figure 3.3. Fluorescent Images of pTGM Bioreporter in the Presence of Increasing Magnesium Chloride Concentrations.

concentration to inhibit the response (GFP protein), it was first necessary to determine the range of TC concentrations in which the response remained linear. Based on previous investigations into the induction of TetR from the *tet* operator (*tetO*) it is expected that the plot of TC concentration versus promoter activity (*P*) (calculated as described in Materials and Methods) should have a sigmoid shape (Lederer, Takahashi, & Hillen, 1995). If experiments were conducted at tetracycline concentrations outside of the linear range it would under or overestimate the percentage change in promoter activity due to decreasing or increasing cation concentrations, respectively. As expected the plots at pH 5.0, 7.0 and 9.0 all are sigmoid (Figs. 3.4 to 3.6) and demonstrate a clear linear range.

Based on these plots 0.1 μg/mL (100 ppb) tetracycline was selected as the concentration at which further experiments were conducted.

Cation Chelation Effect on Promoter Activity. Tetracycline in solution can complex with cations, anions, and biopolymers (Durckheimer, 1975). The following cations complex in order of decreasing affinity: $Fe^{3+} > Al^{3+} = Cu^{2+} > Co^{2+} = Fe^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$ (Nelson, 1998). Previous observations of multivalent cation's ability to raise minimum inhibitor concentrations (MICs) of tetracyclines suggest that cation complexation prevents tetracycline from entering the cell and acting on the bacterial ribosome. From these observations it can be postulated that at a constant TC concentration increasing the cation concentration should decrease promoter activity. Solution chemistry studies have shown that at pH 5.0 the predominant tetracycline species is H_2TC. As pH increases towards 7.0 a greater percentage of the TC is in the form of HTC^-, and further pH increases towards 9.0 results in a mix of HTC^- and TC^{2-} species. Furthermore, studies by Werner et al. (2006) demonstrate that a raise in pH corresponds with a raise in cation chelation by TC. Based on these observations it can be predicted that at pH 5.0 cations would have minimal effect on promoter activity because they would be unable to complex with tetracycline, and hence, decrease uptake across the membrane. However, as pH shifts higher reduction of promoter activity due to cation chelation would become stronger and measurable. Given the observation of cation affinity for TCs (Nelson, 1998) and that sodium was unable to raise TC MICs (Nanavaty, Mortensen, & Shryock, 1998), the following experiment was conducted with the expectation that the effect in decrease in promoter activity would be as follows for pH

7.0 and 9.0 (greatest effect to least effect): $Mg^{2+} > Ca^{2+} > Na^{+} = K^{+}$. It was also expected that at pH 5.0 Mg^{2+} and Ca^{2+} would be unable to repress promoter activity.

To determine the effect that these cations have on tetracycline bioavailability in solution, 12 separate experiments were conducted (Fig. 3.7 – 3.10). Promoter activity is plotted as a percentage of full induction on the y-axis (full induction, or 100%, meaning promoter activity at 0.1 μg/mL TC with no cation) and cation concentration is plotted on the x-axis. Cation concentrations tested included 0, 0.1, 0.5, 1, 5, and 10 mM.

As expected, at pH 7.0 and 9.0 the presence of divalent cations calcium and magnesium strongly decreases promoter activity. Comparison of the magnesium and calcium plots at pH 7.0 reveals that the effect of magnesium is stronger than that of calcium (discussed below). At a glance, it appears that at both pH 7.0 and 9.0 a 50% reduction in promoter activity is reached earlier when magnesium is present in the medium than when calcium is present. Given that at pH 9.0 the TC solution species become predominantly more negatively charged, and hence should have a greater affinity for cations, one might expect that at pH 9.0 the effect of magnesium should be greater than the effect of magnesium at pH 7.0, and similarly for calcium. It appears that there may be some slight evidence of this happening with magnesium; however, large measurement variability at pH 9.0 prevents this comparison from being made. This increase in relative variability at pH 9.0 and 5.0 for normalized values is due to the decrease in raw promoter activity values. It is possible that cellular stress responses due to growth at pH extremes is causing the significant decrease in promoter activity at pH 5.0 and 9.0 as compared to pH 7.0 at the same TC concentration (Maurer, Yohannes, Bondurant, Radmacher, & Slonczewski, 2005) (Fig. 3.11). Despite this, it is still clear that at pH 5.0 the effect of magnesium and

calcium is strongly minimized (Fig. 3.7 – 3.10). This is expected given that affinity for cations towards TC reduces with reducing pH (Jin et al., 2007). The predominant species of TC at pH 5 would be H_2TC, a neutral species in which pKa1 is negatively charged, pKa2 is neutral, and pKa3 is protonated. As pKa2 and pKa3's functional groups become deprotonated at higher pH there is a corresponding increase in cation affinity. It is also observed that sodium and potassium fail to reduce promoter activity at all pH's, as would be expected if they were unable to complex with TC.

Effect of Magnesium Chloride on Tetracycline Uptake. To conclusively determine that the reason added magnesium chloride reduced promoter activity was due to decreased cellular uptake of TC, an experiment was conducted in which LC-MS/MS was used to measure the intracellular TC concentration after promoter activity measurement in the presence of magnesium chloride. Cells were grown in the presence of TC or TC + 10 mM magnesium chloride in LB at pH 7.0 and promoter activity was measured. As can be seen from Figure 3.12 and 3.13, the presence of 10 mM magnesium chloride in the media greatly decreased the promoter activity and the intracellular TC concentration, as would be expected if reduced promoter activity was due to reduced TC accumulation inside cells. The intracellular concentrations for cells grown in the presence of 10 mM magnesium chloride were 6.72 μg TC/g dry cells (standard deviation of 0.23) compared to 105.37 μg TC/g dry cells (standard deviation of 8.69). Based on the percentage decrease in promoter activity, an intracellular concentration of 0.70 ug TC/g dry cells (in other words 0.66% of fully induced) would have been expected for the cells grown in the presence of 10 mM magnesium chloride. It is known that biopolymers bind TC and it is possible that the presence of magnesium increases this effect making

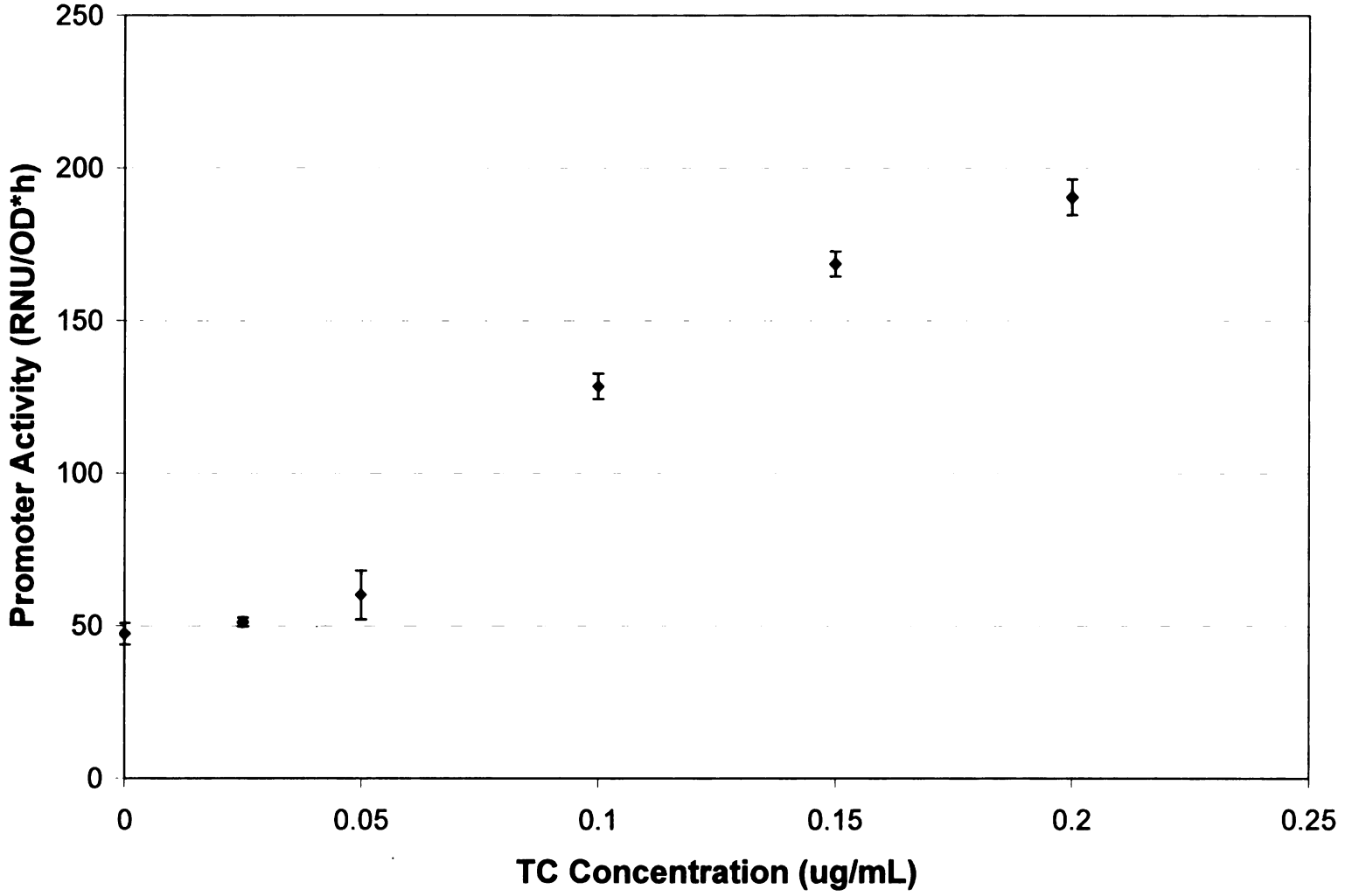


Figure 3.4. Range of Linearity of Promoter Activity at pH 5.0. Triplicate samples, error bars = 1 standard deviation.

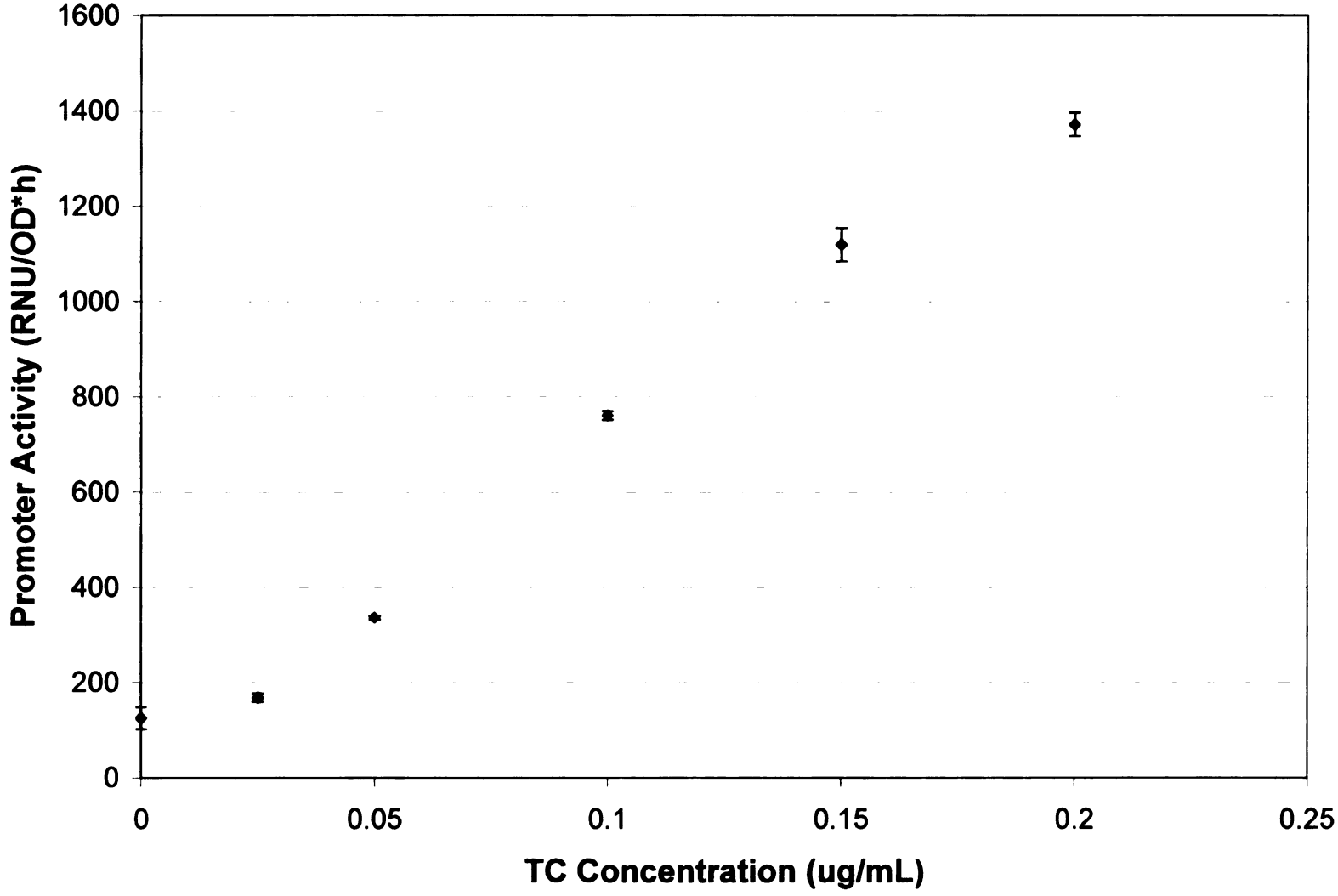


Figure 3.5. Range of Linearity of Promoter Activity at pH 7.0. Triplicate samples, error bars = 1 standard deviation.

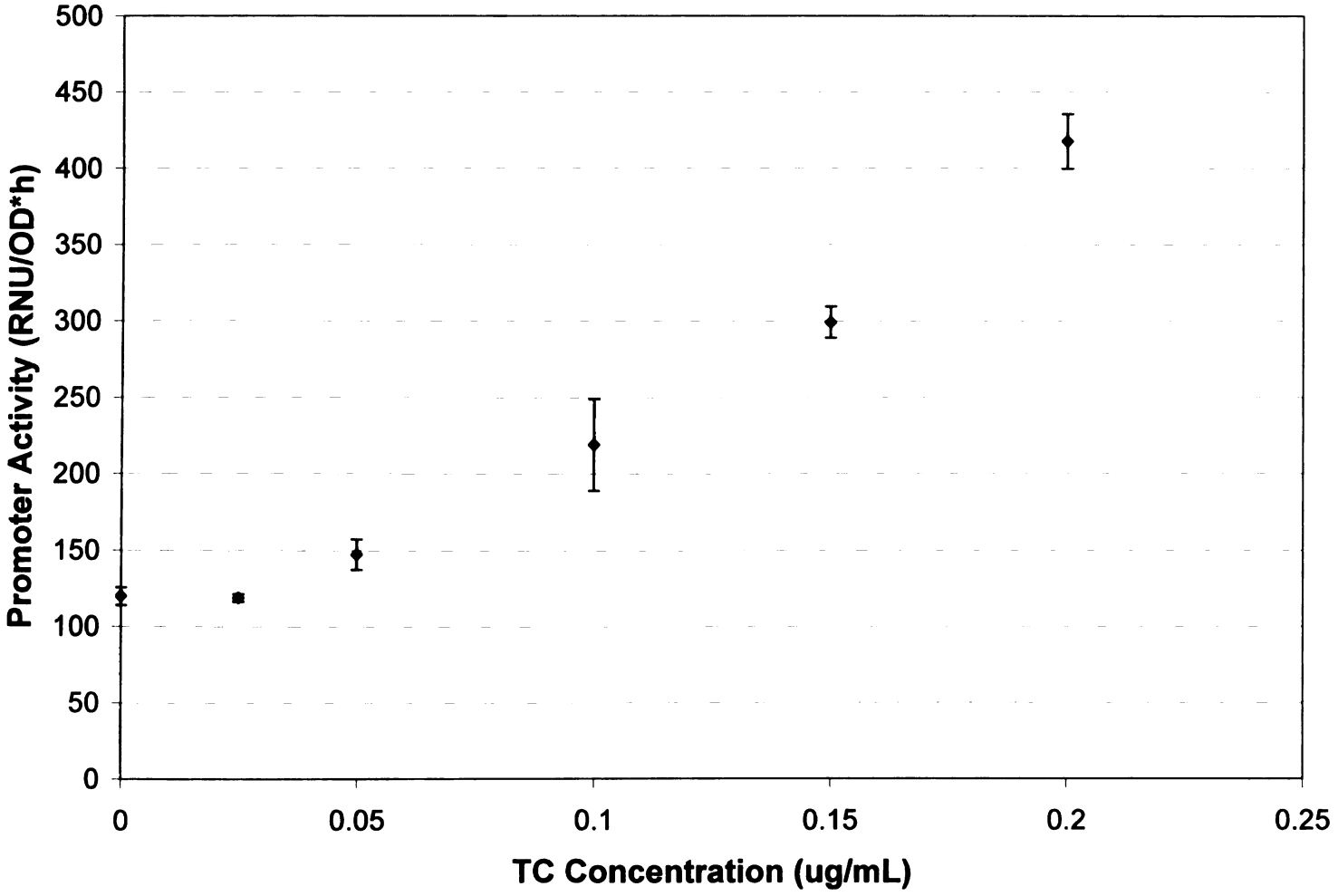


Figure 3.6. Range of Linearity of Promoter Activity at pH 9.0. Triplicate samples, error bars = 1 standard deviation.

washing steps unable to remove extracellular bound TC leading to a greater measured intracellular TC concentration (Durckheimer, 1975).

Modulation of Promoter Activity by addition of Magnesium Chloride or EDTA Mid-Growth. Another experiment was designed to corroborate the results of experiments displayed in Figures 3.12 and 3.13 with the expectation that if magnesium can chelate tetracycline and reduce cellular uptake, one should be able to modulate GFP expression by adding magnesium chloride during mid-culture growth. Magnesium chloride (final concentration of 10 mM) was spiked into medium containing 0.1 μg/mL TC with the expectation that upon entry, the magnesium ions would complex with TC and *gfp* expression would decrease to a level similar to a culture that was grown continuously in the presence of 0.1 μg/mL TC and 10 mM magnesium chloride. Similarly, if EDTA (known for its ability to chelate divalent cations) was spiked into a culture grown in the presence of 0.1 μg/mL TC plus 10 mM magnesium chloride, it would be expected that after EDTA addition *gfp* expression would increase. This would be due to EDTA chelating magnesium stronger than TC thereby freeing TC to enter the cell and activate gene expression.

Promoter activity was analyzed in 4 different conditions: no TC (negative control; black diamonds, solid line), 0.1 μg/mL TC (black squares, solid line), 0.1 μg/mL TC plus 10 mM magnesium chloride (black triangles, solid line), and 0.1 μg/mL TC plus 10 mM magnesium chloride plus 10 mM EDTA (black circles, solid line). Two separate cultures were started for the 0.1 μg/mL TC and 0.1 μg/mL TC plus 10 mM magnesium chloride conditions. At an appropriate time (indicated by the arrows, Fig. 3.14) 10 mM magnesium chloride was added to one of the 0.1 μg/mL TC cultures (open squares,

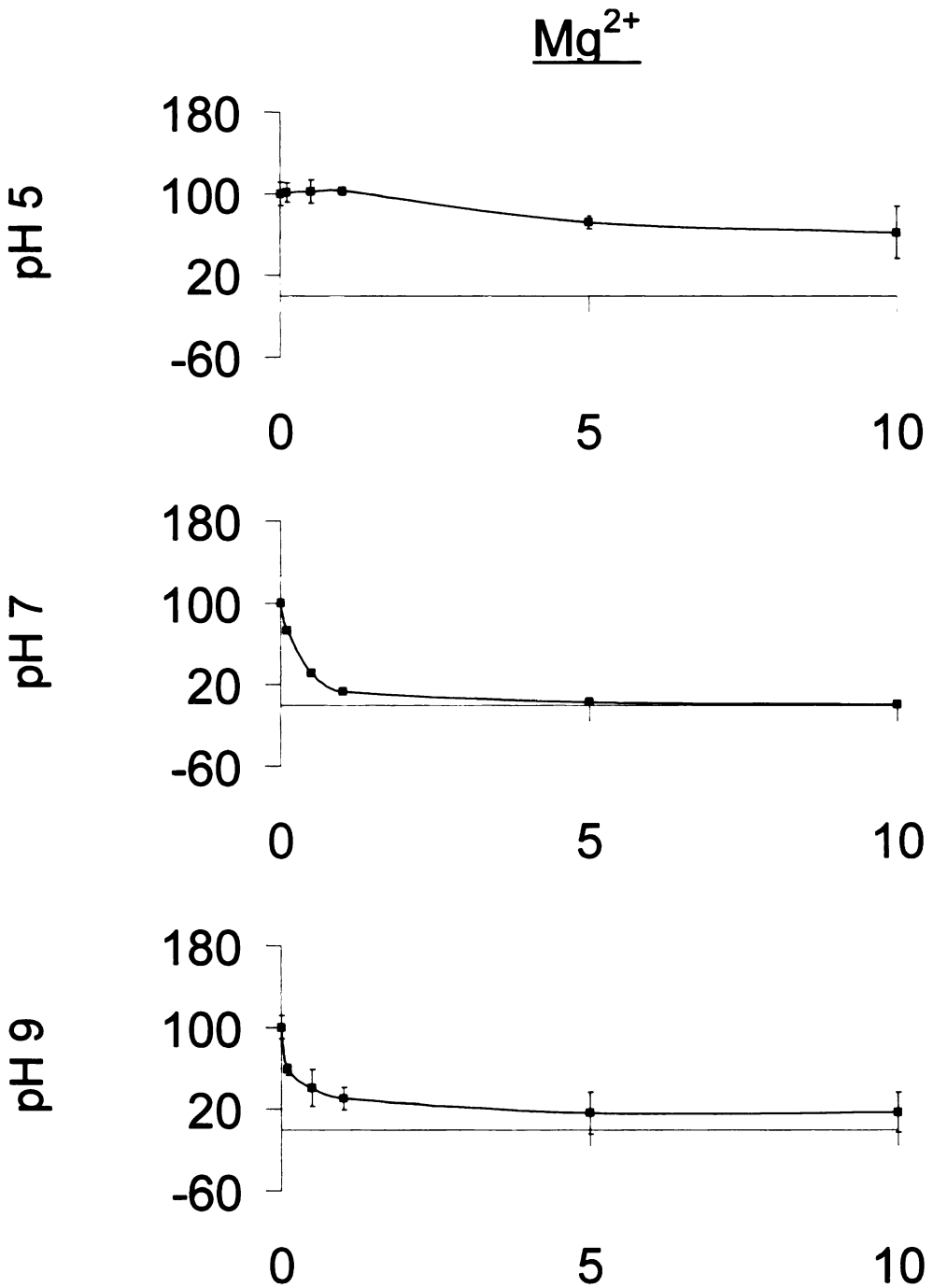


Figure 3.7. Promoter Activity Data for Magnesium at pH 5, 7, and 9. % promoter activity on the Y axis, mM concentration of corresponding X axis. Performed in triplicate, error bars represent 1 standard deviation.

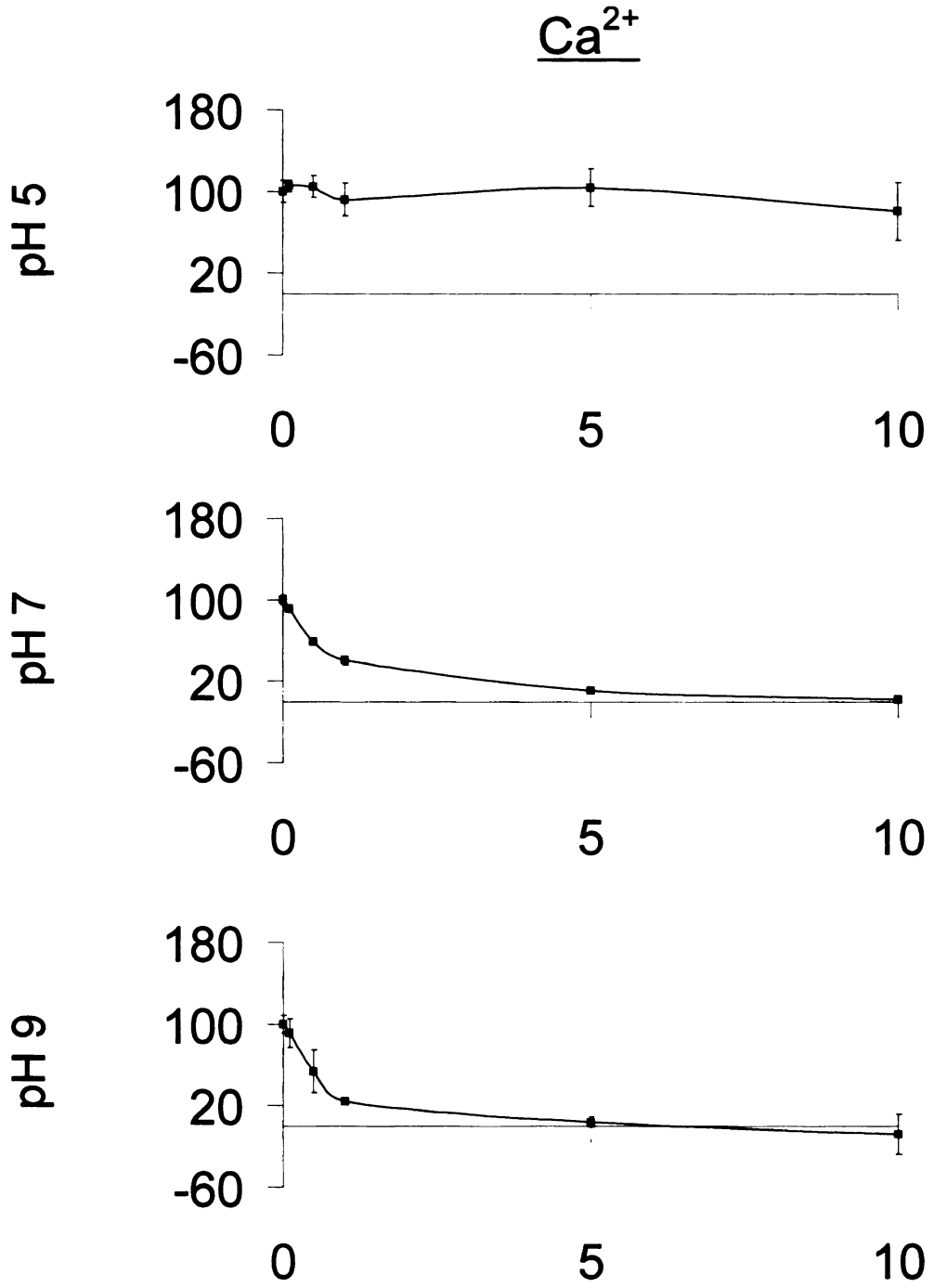


Figure 3.8. Promoter Activity Data for Calcium at pH 5, 7, and 9. % promoter activity on the Y axis, mM concentration of corresponding X axis. Performed in triplicate, error bars represent 1 standard deviation.

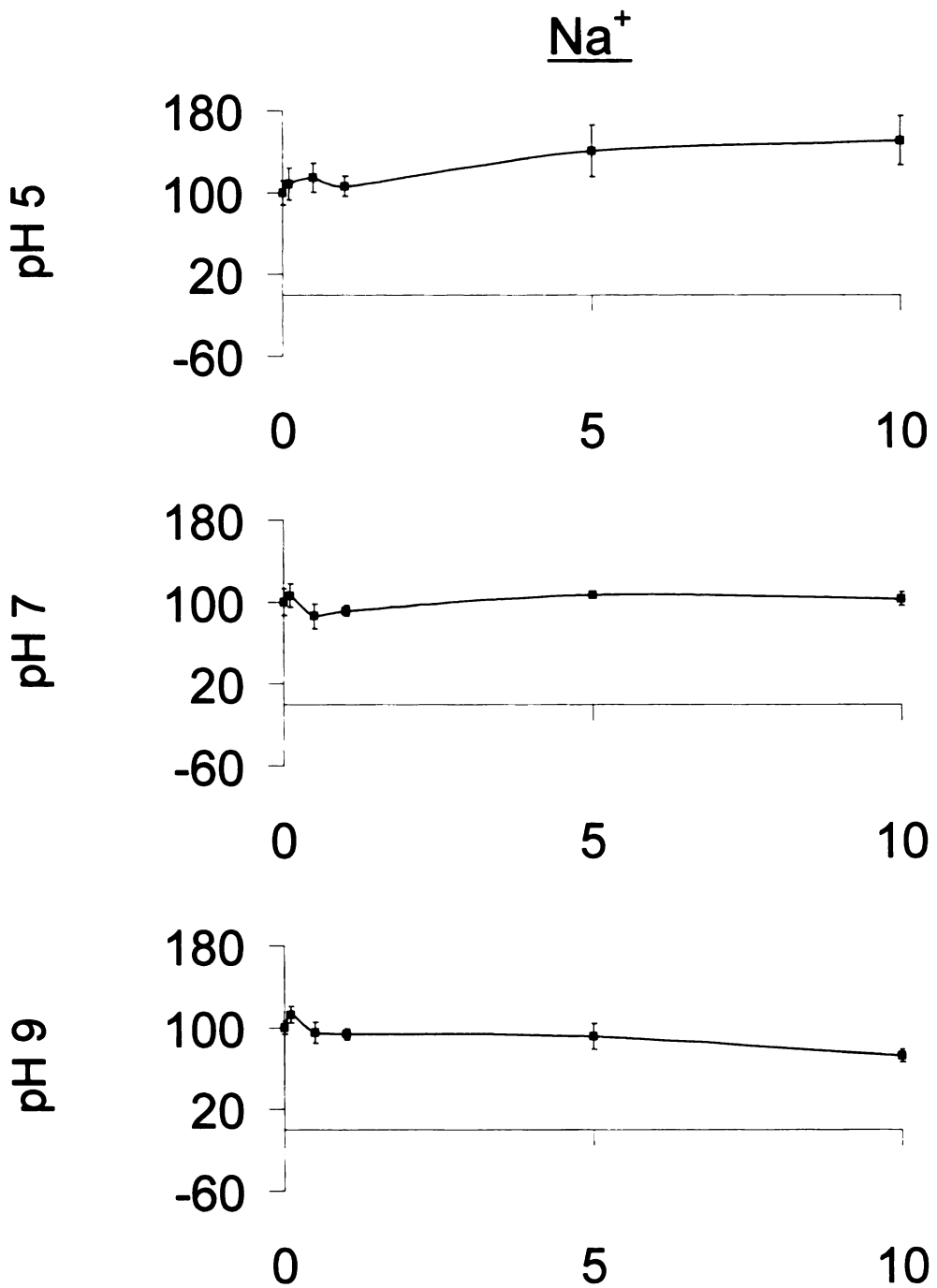


Figure 3.9. Promoter Activity Data for Sodium at pH 5, 7, and 9. % promoter activity on the Y axis, mM concentration of corresponding X axis. Performed in triplicate, error bars represent 1 standard deviation.

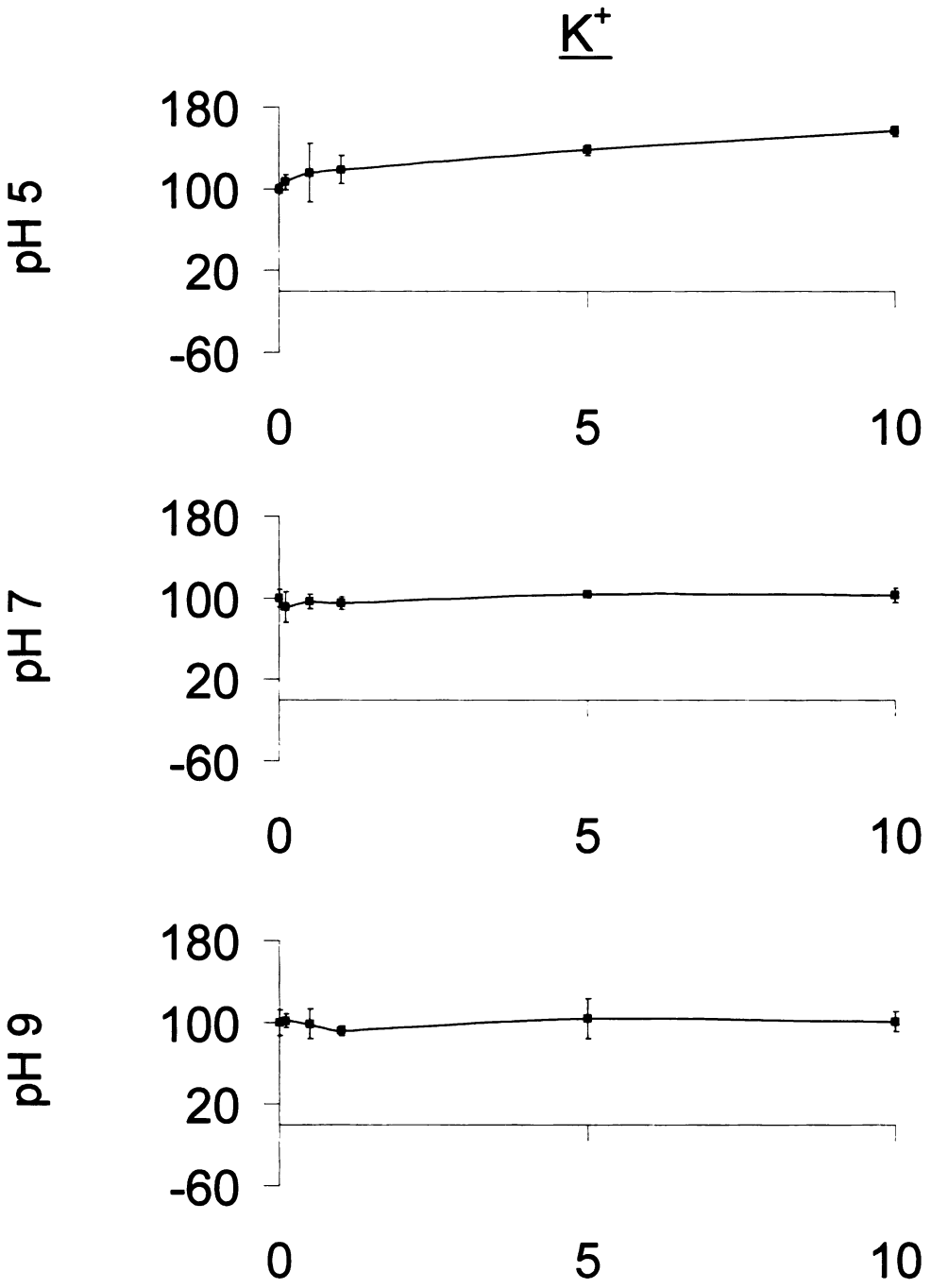


Figure 3.10. Promoter Activity Data for Potassium at pH 5, 7, and 9. % promoter activity on the Y axis, mM concentration of corresponding X axis. Performed in triplicate, error bars represent 1 standard deviation.

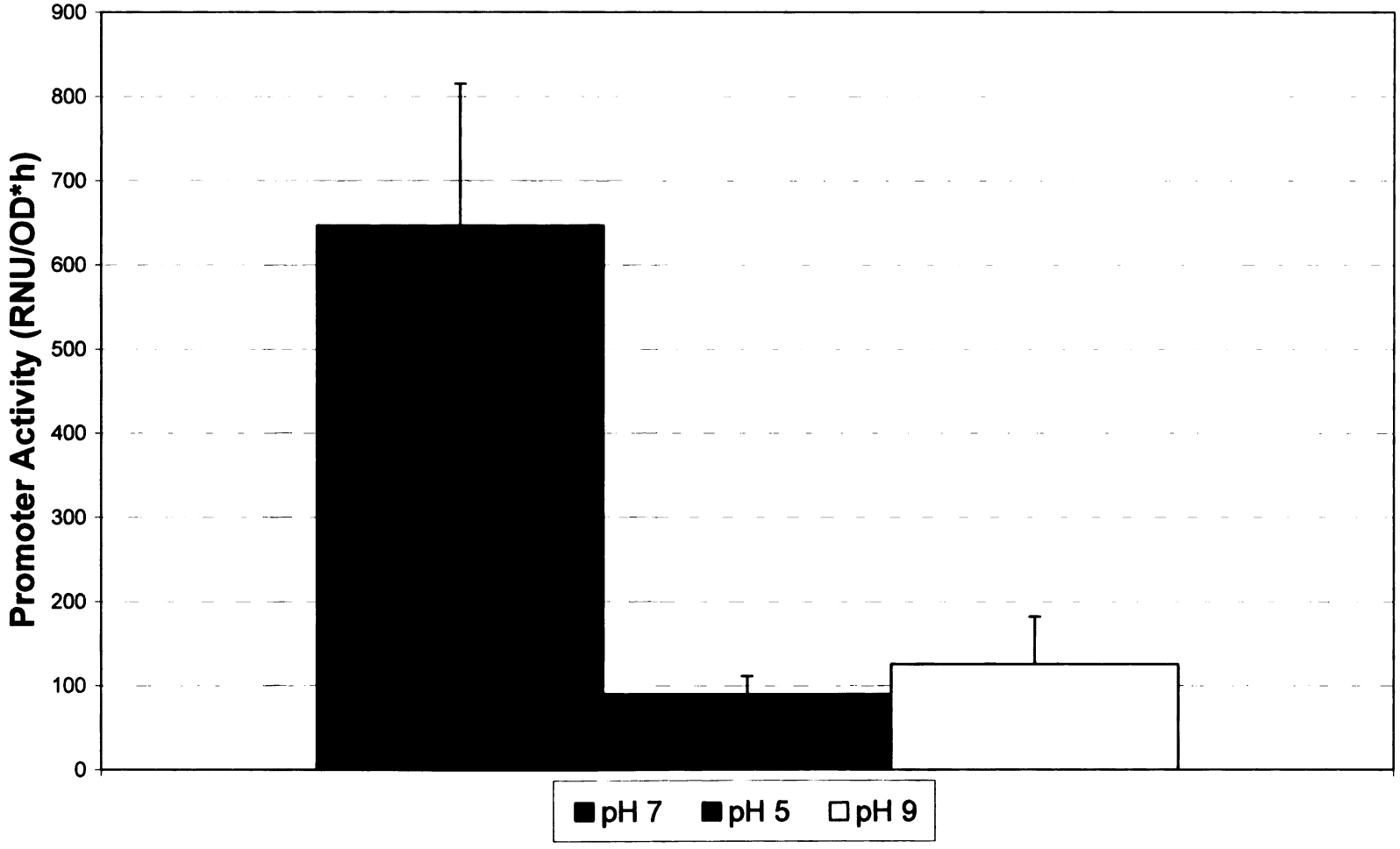


Figure 3.11. Promoter Activity of Fully Induced pTGM (0.1 µg/mL TC) versus pH.

dashed line) and 10 mM EDTA was added to one of the 0.1 μg/mL plus 10 mM magnesium chloride cultures (open circles, dashed line).

As expected, after addition of either magnesium chloride or EDTA the slope of the F'OD plot (representing fluorescent steady state) immediately changes to a level similar to cultures grown continuously under the same conditions (Fig. 3.14). The presence of 10 mM EDTA greatly increases the slope of the F'OD plot (black circles and open circles) compared to induction at 0.1 μg/mL TC (black squares). This is likely due to EDTA's ability to chelate cations that are not added, but are present in the assay media that complex with some amount of the TC reducing gene expression (black squares).

A Closer Comparison of the effect of Magnesium and Caclium at pH 7.0. It is known that TC – cation complexation is reversible (Durckheimer, 1975). Based on this observation it is hypothesized that at a constant TC concentration each incremental increase in the concentration of divalent cation should have incrementally less effect on the percentage reduction in promoter activity (i.e. exponential decay). This would occur because each increase in cation concentration corresponds with a decrease in the amount of "free" TC molecules surrounding each individual cation. Therefore, each incremental cation has less opportunity to interact with free, un-complexed TC than the previous cation.

Recently, Jin et al. (2007) used isothermal titration calorimetry to study the binding of calcium and magnesium to TC. They found that each cation binds with distinct stoichiometry; one calcium per TC and one magnesium per two TC molecules. Furthemore, they conclude that this stoichiometry is invariant with pH (Jin et al., 2007). This means that at a given TC concentration the cation concentration required to give any

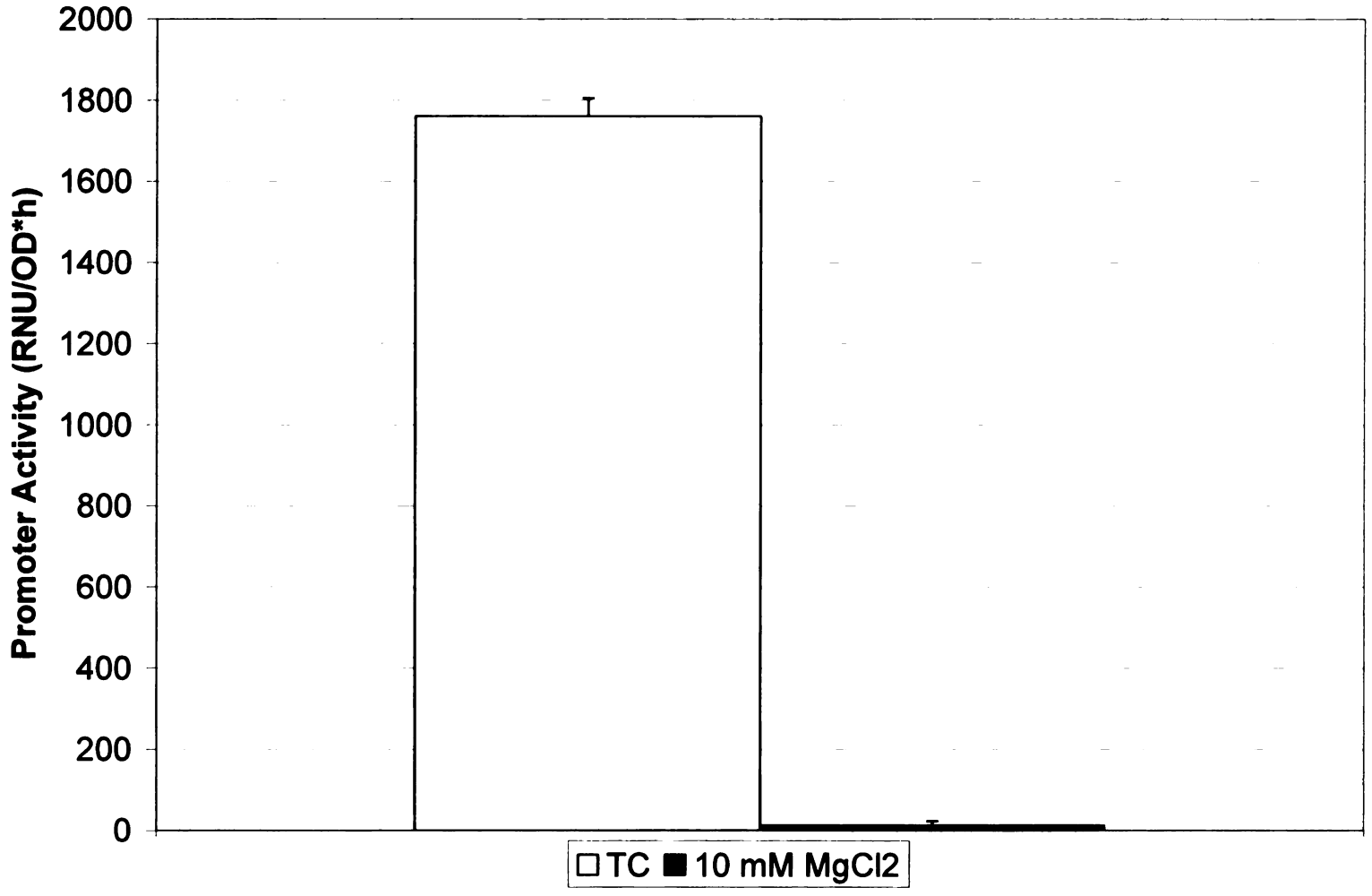


Figure 3.12. Promoter Activity of Cells Grown in 0.1 µg/mL TC (White Bar) and 0.1 µg/mL TC + 10 mM Magnesium Chloride (Black Bar). Error bars represent one standard deviation.

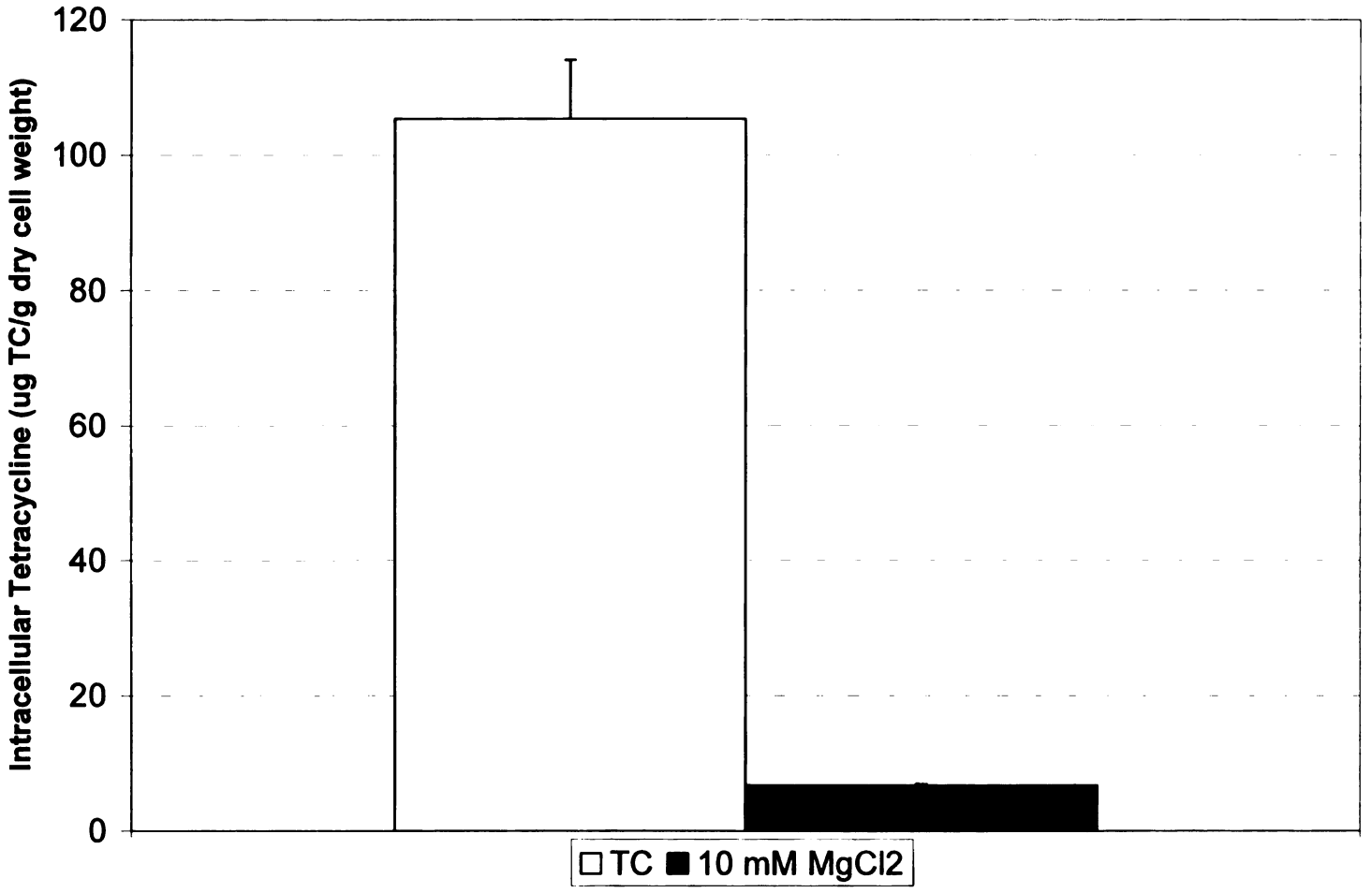


Figure 3.13. Intracellular TC Concentration (Measured by LC-MS/MS) of Cells Grown in the Presence of 0.1 µg/mL TC (White Bar) or 0.1 µg/mL TC + 10 mM Magnesium Chloride (Black Bar). Error bars represent one standard deviation.

percentage decrease in promoter activity should be twice as much for calcium as compared to magnesium. If this holds true under the present experimental conditions, and measurements are made in the linear response range for promoter activity, then if one fits exponential decay functions to scatter plots of cation concentration (x-axis) vs. percentage promoter activity(y-axis) for both magnesium and calcium it can be predicted that the ratio of calcium to magnesium concentration should equal 2 at any given y-value.

To examine this relationship an experiment was conducted at pH 7.0 with cation concentrations of 0.125, 0.25, 0.375, and 0.5 mM. Higher concentrations (i.e. 5 and 10 mM) were not used because at high cation concentrations the response (promoter activity) is depressed enough that it is outside of the linear range (Fig. 3.5). As expected plots of the data yielded curves that fit an exponential decay model well (Fig. 3.15). The equation for magnesium: $y = 100e^{-2.144x}$. The equation for calcium: $y = 100e^{-1.1693x}$. The ratio of -2.144x to -1.1693x equals 1.83 with a standard deviation of 0.27 and a 95% confidence interval of [1.65, 2.01] ($\alpha = 0.05$). This is in good agreement with Jin et al. (2007) and as can be seen from the exponential decay plots for magnesium and calcium at any y-value the x-value for calcium is always 1.83 times magnesium (Fig. 3.15).

There are multiple possible explanations for our inaccuracy and deviation from a value of 2.0. First, Jin et al. (2007) observed that the presence of sodium can have an effect on calcium and magnesium affinity for TC depending on the pH. Although the assay media is standard at any given pH, if sodium affects calcium and magnesium's affinity for TC differentially (as is demonstrated by Jin et al. (2007)) this would skew the shape of the exponential decay curves. Also, even though the culture media is buffered the *E. coli* MC4100/pTGM it has been observed that throughout exponential growth the

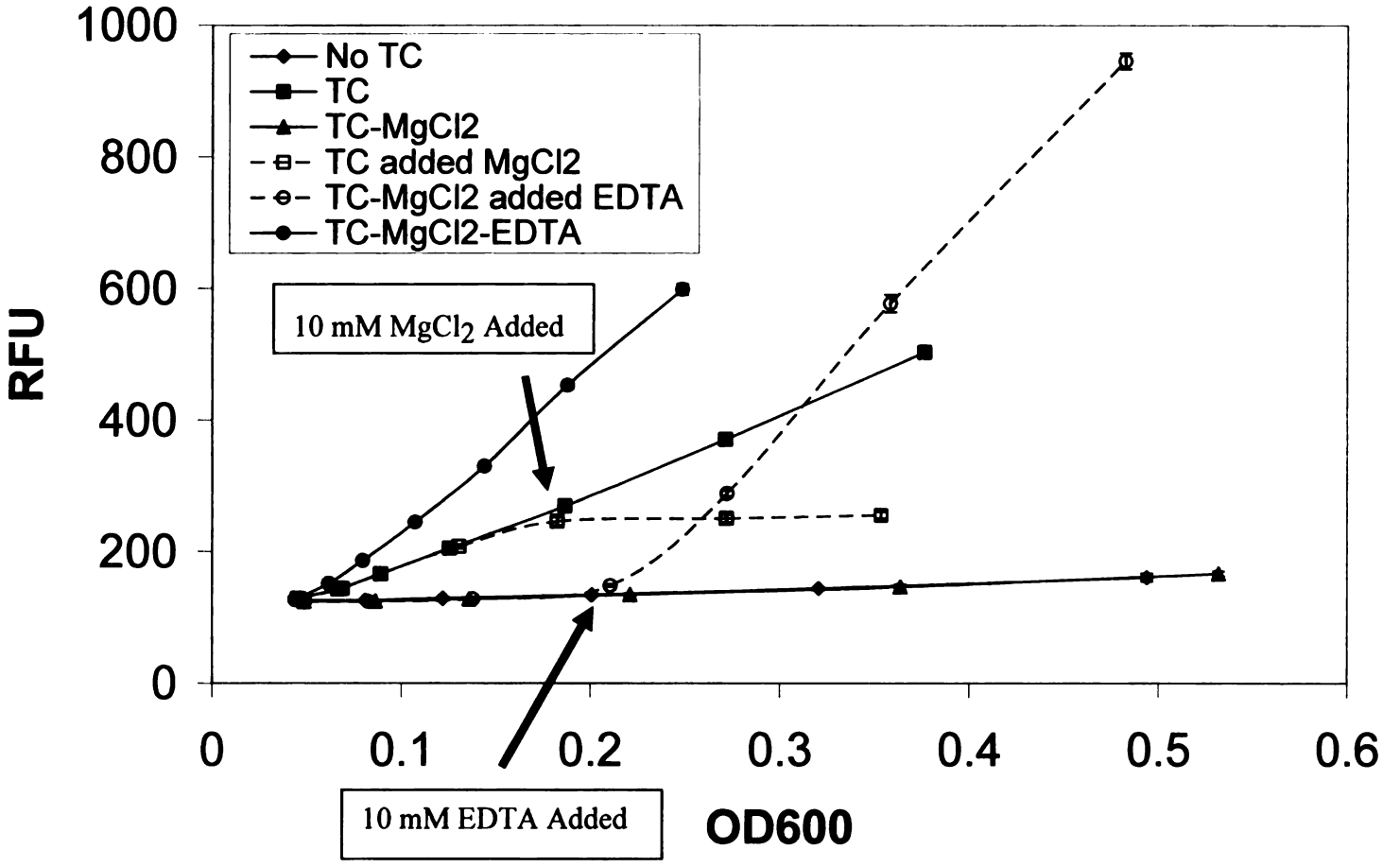


Figure 3.14. Addition of 10 mM Magnesium Chloride and 10 mM EDTA Mid-Growth. Experiment done in triplicate, error bars represent one standard deviation.

cells raise the medium pH to final values of approximately 7.1 (data not shown). Differences between pH changes in separate batch cultures would introduce variance. It is also possible that stoichastic fluctuations in gene expression in cell populations between cultures could introduce variance. There is growing evidence that a growing population of cells' gene expression is not uniform and random fluctuations likely help populations adapt to various environmental stresses (Losick & Desplan, 2008). Observation of induced cells via flow cytometry at multiple time-points taken throughout exponential growth confirms the presence of distinct populations; cells that express *gfp* and cells that do not (data not shown). Between culture variation in the proportion of cells expressing *gfp* will introduce variance. Furthermore, TC is known to bind biopolymers (Durckheimer, 1975). The assay was conducted in a rich media (see Materials and Methods) and it is possible that media components binding free TC led to an uneven distribution of TC upon subdividing the media to separate culture flasks.

If indeed the binding stoichiometry for calcium and magnesium is invariant with pH then this relationship should also hold at pH 5.0 and 9.0. However, at pH 5.0 TC's affinity for cations would be extremely low given that the predominant species in solution is the neutral form H_2TC. It is likely impossible to detect this relationship at pH 5.0 with the *E. coli* MC4100/pTGM bioreporter. In fact, Jin et al. (2007) were unable to detect any binding of calcium and magnesium at pH 5.0 with a 20-fold excess amount of cation using isothermal titration calorimetry. At pH 9.0 TC would be more negatively charged than at pH 7.0 with HTC^- and TC^{2-} being the solution species present. Given the observation that calcium and magnesium affinity is higher for TC at higher pH due to more negative charge it would be expected that the exponential decay curves would be

steeper than at pH 7.0. A 50% reduction in promoter activity should occur at a lower cation concentration at pH 9.0, when compared to the same cation at pH 7.0. Unfortunately, the cellular stress imposed on the bioreporter cells by growth at pH 9.0 precludes any accurate measurement of this effect. Visual comparison of the pH 7.0 and 9.0 graphs for magnesium in Figures 3.7 – 3.10 indicates that this may be occurring, however, this does not appear to hold for calcium. This may be simply due to the inability to obtain sufficiently accurate measurements at pH 9.0. If accurate measurement were possible under present assay conditions it is expected that at pH 9.0 the ratio of calcium to magnesium concentration would be less than 2.0. Jin et al. (2007) were able to show that the presence of sodium alters the stoichiometry of magnesium binding slightly at pH 8.5 and exhibits a larger increase at pH 9.5.

CONCLUSIONS AND FUTURE DIRECTIONS

It has been demonstrated that the *E. coli* MC4100/pTGM whole-cell biosensor is an effective tool for investigating the bioavailability of tetracycline in the solution phase under varying conditions (pH, cation, cation concentration). Most importantly, the activity of the biosensor responds in a predictable manner based on tetracycline solution chemistry. In summary, lowering pH increases the bioavailability of tetracycline in the presence of calcium and magnesium. As pH increases, magnesium and calcium bind to tetracycline and decrease its bioavailability. Futhermore, at pH 7.0 and 9.0 magnesium exerts a stronger effect than calcium. In contrast, monovalent cations such as sodium and potassium fail to bind TC at any pH and reduce bioavailability.

Often, bioreporters are looked at as a fast and cost-effective solution to determining the presence and quantity of the substrate of interest in various matrices.

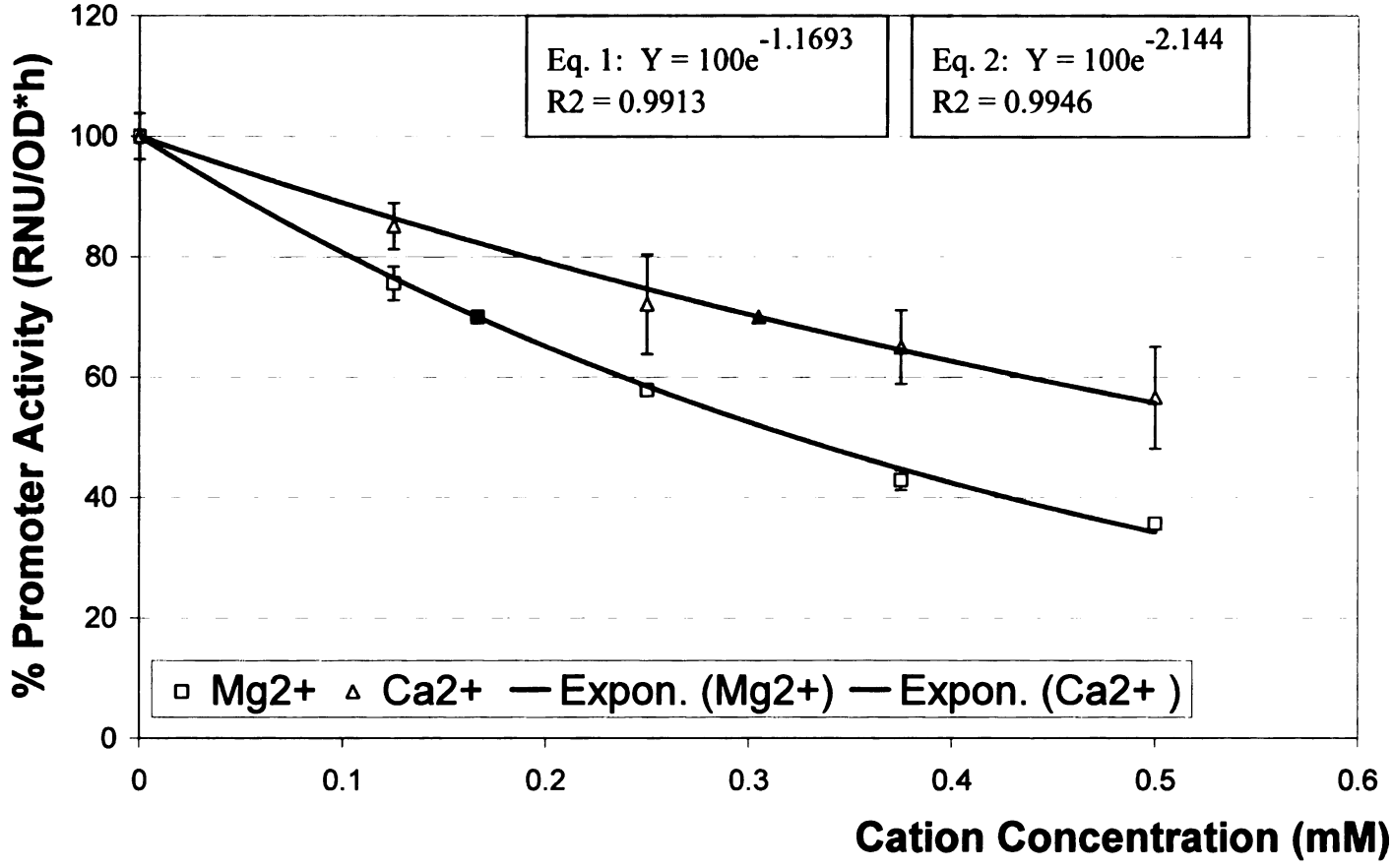


Figure 3.15. Comparison of Effects of Calcium and Magnesium on Promoter Activity. The black square and triangle represent the theoretical cation concentration to achieve a 70% reduction in promoter activity based on equation 1 (Calcium) and 2 (Magnesium). Error bars represent one standard deviation.

Multiple groups have genetically modified bacterial cells to achieve lower detection limits (Nivens et al., 2004). However, this overlooks the advantage inherent in utilizing a live bacterial cell to assay bioavailability. That is, the conditions affecting gene expression in a cell residing in a natural context can be examined quantitatively.

Tetracyclines have been measured in manure, soil, sediment, surface water, and groundwater. Typically concentrations range from less than 1 ppb in groundwater and surface waters to approximately 300 ppb in soil and up to 40 ppm in manure (Hamscher et al., 2002; Kay, Blackwell, & Boxall, 2004; Kolpin et al., 2002). Minimum inhibitory concentrations of tetracyclines for microbial cells are commonly in the ppm range. In other words, with the exception of manure, three orders of magnitude more concentrated than is found in environmental systems. Because of this difference, and the observation that tetracyclines bind strongly to various clay minerals (Sassman & Lee, 2005), it is often assumed that concentrations in environmental systems are too low to exert a significant effect on microbial cells. However, this assumption may not always be valid. Verma et al. (2007) spiked river and wetland water with tetracycline and analyzed inhibition of protein synthesis of the native microbial flora by 3H leucine incorporation. They were able to demonstrate a highly significant reduction in protein production ($p < 0.001$) at a concentration as low as 10 ppb. Interestingly, it took a concentration of 4000 ppb to achieve a significant ($p < 0.02$) reduction in protein synthesis in wetland water (Verma, Robarts, & Headley, 2007). Furthermore, they estimated that the actual "free" concentration of tetracycline was approximately 50% of the spiked concentration. This study demonstrates two points. First, differences in complex environmental matrices can have vast consequences to tetracycline bioavailability. Second, the assumption that low

tetracycline concentrations fail to exert any effect on the native microbial flora may be false depending on the characteristics of the natural system.

Not only is it possible that environmentally relevant concentrations of tetracyclines can inhibit protein synthesis, but it has also been demonstrated that low levels of tetracycline can stimulate the horizontal transfer of some tetracycline resistance elements (Doucet-Populaire, Trieu-Cuot, Dosbaa, Andremont, & Courvalin, 1991; Rice, Marshall, & Carias, 1992; Salyers, Shoemaker, & Li, 1995). Dr. Abigail Salyer's group has studied conjugative transposons in *Bacteroides* spp. and observed that low levels of tetracycline can increase transfer of these transposons harboring the *tet*(Q) gene 100 to 1000-fold (Salyers, Shoemaker, & Li, 1995). The mechanism is not yet well understood, and little research regarding similar transfer in environmental isolates has been conducted, however, it seems plausible that this may occur in natural settings outside of human and animal gastrointestinal tracts. This should raise concern that low level TC contamination may facilitate the dissemination of resistance genes throughout the bacterial domain.

It is also unknown if low TC levels in environmental systems can induce tetracycline resistance gene expression. As described before, expression of the tetracycline efflux pumps is controlled by the tetracycline inducible repressor protein TetR. This type of control is conserved in all tetracycline-resistance encoding efflux pumps in gram-negative bacteria (Roberts, 1996). Over-expression of these pumps will destroy membrane potential and kill the cell (Eckert & Beck, 1989). Therefore, it is imperative that expression is tightly regulated and only induced upon binding to tetracyclines. This has selected for the evolution of a sensitive expression system in

which tetracycline binds to TetR with a 1000-fold higher affinity than to the bacterial ribosome (Hinrichs et al., 1994), thereby allowing the cell to express the efflux pump and reduce the intracellular tetracycline concentration before it can exert its negative effect on the cell. Combined with the observation that 10 ppb tetracycline can significantly inhibit protein synthesis of native microbial flora in a specific aquatic system (Verma et al., 2007) it can be presumed that resistance gene expression would likely occur at a bioavailable concentration much lower than 10 ppb.

It is also of interest to test the effect that cations have on various tetracycline degradation products. Particularly relevant is anhydrotetracycline which has been demonstrated to be present in soil (Aga et al., 2005). Anhydrotetracycline is an atypical tetracycline that exhibits modest antibacterial activity but does not bind the ribosome. Current research suggests that it disrupts the bacterial cytoplasmic membrane to exert is bacteriocidal effect (Chopra & Roberts, 2001). The structural differences of anhydrotetracycline reduce its binding capacity to ribosomes, however, it retains affinity for TetR and induces gene expression roughly 1000X stronger than TC (Lanig, Othersen, Beierlein, Seidel, & Clark, 2006). Although seemingly counterintuitive, *E. coli* strains harboring multicopy plasmids with *tet*(B) are less resistant to 5a,6-anhydrotetracycline than TC sensitive strains (Moyed, Nguyen, & Bertrand, 1983). This decreased resistance is likely due to over-expression of efflux pumps that then destroy the membrane potential. By using anhydrotetracycline (which does not affect protein synthesis) to induce *tet*(B) expression Lenski et al. (1989) demonstrated that there was a fitness reduction associated even with low-level expression of the *tet*(B) gene. In the absence of induction the *tet*(B) gene had no detectable effect on fitness (Nguyen et al., 1989). Oddly

enough, it appears that some TC degradation products in soil, if bioavailable, could actually exert a negative selection pressure on carriage of the tetracycline efflux genes.

The following are proposed as further objectives to add light to the phenomenon presented above:

1) Testing the effect of clay-mineral systems on cation complexed tetracycline bioavailability.

Multiple investigators have documented the presence of TC residues in manured agricultural soils (Brambilla et al., 2007; Hamscher, Pawelzick, Hoper, & Nau, 2005). TC residues remain in soil for long periods and repeated manure application can cause TC residues to accumulate (Hamscher et al., 2002). The observation that TCs can exist in cationic complexes with divalent cations in solution at environmentally relevant pH, and that soil sorption of TC is dependent on cation exchange capacity as well as pH (Sassman & Lee, 2005) implies that geochemical characteristics of a given soil can have vast consequences on the bioavailability of TC to the indigenous microbial flora.

2) Repeating the experiments presented above with other tetracyclines and degradation products to determine their bioavailability (e.g. OTC, CTC, and anhydrotetracycline).

It has been demonstrated that OTC, CTC, and anhydrotetracycline all bind cations different than TC (Lambs, Decock-Le Reverend, Kozlowski, & Berthon, 1988; Wessels, Ford, Szymczak, & Schneider, 1998). Therefore it is likely that these differences have significant effects on bioavailability. Specifically, it is relevant to study the characteristics of OTC and CTC since these are the two antibiotics most commonly used

in agriculture and aquaculture (McManus et al., 2002; Sarmah, Meyer, & Boxall, 2006) and anhydrotetracycline for reasons stated above.

3) Transferring the P*tet(A)-gfp*mut3 construct into a broad-host range plasmid and mobilizing it into diverse Gram-negative genera to test if, how, and what genetic differences affect bioavailability.

4) Developing a Gram-positive tetracycline biosensor.

One obvious bias of the *E. coli* MC4100/pTGM reporter strain is that the *gfp* construct is expressed in a Gram-negative bacterium. This is reasonable considering that the majority of tetracycline efflux genes (which the reporter mimics the expression of) are found in Gram-negatives, however, it is possible that membrane differences between separate Gram-negative genera could affect tetracycline bioavailability. Most of the work regarding tetracycline uptake by bacterial cells has been done with *E. coli* and this bacterium will not be dominant in the majority of environmental settings. It is also likely that differences exist between tetracycline uptake of Gram-negative and Gram-positive genera.

There are two obvious ways to extend the observations presented in the present study to other bacteria. First, transferring the pTGM bioreporter construct into a broad-host range plasmid such as RSF1010 (Labes, Puhler, & Simon, 1990) and expressing it in diverse Gram-negative bacteria. The second way is to construct a new reporter to test bioavailability of TCs to Gram-positive cells. The recent discovery that the Gram-positive efflux genes *tet*(Z) and *tet*(33) are regulated by repressor proteins with homology to TetR suggests that one should be able to construct a biosensor similar to *E. coli*

MC4100/pTGM for study of Gram-positives (Guillaume, Ledent, Moens, & Collard, 2004).

5) Testing how tetracycline resistance gene expression works in stressed, dormant cells.

Lastly, it is important to realize that microbial cells present in natural settings are not growing similar to exponential growth in the lab. As such, experiments should be designed to delineate what stress factors control the expression or repression of tetracycline resistance genes in the natural environment.

In conclusion, the utilization of whole-cell tetracycline biosensors can add to the scientific community's understanding of the phenomenon described above. Although traditionally biosensors have been employed as an efficient way to detect contaminants they also are effective tools for examining the intersection of geochemistry and biology that is likely to determine gene expression in natural systems. Ultimately, further studies such as those outlined above will allow a better determination of the risk associated with the environmental contamination of tetracycline residues.

REFERENCES

Aga, D. S., O'Connor, S., Ensley, S., Payero, J. O., Snow, D., & Tarkalson, D. (2005). Determination of the persistence of tetracycline antibiotics and their degradates in manure-amended soil using enzyme-linked immunosorbent assay and liquid chromatography-mass spectrometry. *J Agric Food Chem, 53*(18), 7165-7171.

Bahl, M. I., Hansen, L. H., & Sorensen, S. J. (2005). Construction of an extended range whole-cell tetracycline biosensor by use of the tet(M) resistance gene. *FEMS Microbiol Lett, 253*(2), 201-205.

Brambilla, G., Patrizii, M., De Filippis, S. P., Bonazzi, G., Mantovi, P., Barchi, D., et al. (2007). Oxytetracycline as environmental contaminant in arable lands. *Anal Chim Acta, 586*(1-2), 326-329.

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ Microbiol, 8*(7), 1137-1144.

Chapin, A., Rule, A., Gibson, K., Buckley, T., & Schwab, K. (2005). Airborne multidrug-resistant bacteria isolated from a concentrated swine feeding operation. *Environ Health Perspect, 113*(2), 137-142.

Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev, 65*(2), 232-260 ; second page, table of contents.

Connell, S. R., Tracz, D. M., Nierhaus, K. H., & Taylor, D. E. (2003). Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob Agents Chemother, 47*(12), 3675-3681.

Durckheimer, W. (1975). Tetracyclines: chemistry, biochemistry, and structure-activity relations. *Angew Chem Int Ed Engl, 14*(11), 721-734.

Eckert, B., & Beck, C. F. (1989). Overproduction of transposon Tn10-encoded tetracycline resistance protein results in cell death and loss of membrane potential. *J Bacteriol, 171*(6), 3557-3559.

Gaskins, H. R., Collier, C. T., & Anderson, D. B. (2002). Antibiotics as growth promotants: mode of action. *Anim Biotechnol, 13*(1), 29-42.

Ghosh, S., & LaPara, T. M. (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J, 1*(3), 191-203.

Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P., & Quentin, C. (2000). Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and Aeromonas spp. *Appl Environ Microbiol, 66*(1), 125-132.

Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., & Singh, R. M. (1966). Hydrogen ion buffers for biological research. *Biochemistry, 5*(2), 467-477.

Guillaume, G., Ledent, V., Moens, W., & Collard, J. M. (2004). Phylogeny of efflux-mediated tetracycline resistance genes and related proteins revisited. *Microb Drug Resist, 10*(1), 11-26.

Hamscher, G., Pawelzick, H. T., Hoper, H., & Nau, H. (2005). Different behavior of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure. *Environ Toxicol Chem, 24*(4), 861-868.

Hamscher, G., Pawelzick, H. T., Sczesny, S., Nau, H., & Hartung, J. (2003). Antibiotics in dust originating from a pig-fattening farm: a new source of health hazard for farmers? *Environ Health Perspect, 111*(13), 1590-1594.

Hamscher, G., Sczesny, S., Hoper, H., & Nau, H. (2002). Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal Chem, 74*(7), 1509-1518.

Hinrichs, W., Kisker, C., Duvel, M., Muller, A., Tovar, K., Hillen, W., et al. (1994). Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. *Science, 264*(5157), 418-420.

Jin, L., Amaya-Mazo, X., Apel, M. E., Sankisa, S. S., Johnson, E., Zbyszynska, M. A., et al. (2007). Ca2+ and Mg2+ bind tetracycline with distinct stoichiometries and linked deprotonation. *Biophys Chem, 128*(2-3), 185-196.

Karthikeyan, K. G., & Meyer, M. T. (2006). Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Sci Total Environ, 361*(1-3), 196-207.

Krulwich, T. A., Jin, J., Guffanti, A. A., & Bechhofer, H. (2001). Functions of tetracycline efflux proteins that do not involve tetracycline. *J Mol Microbiol Biotechnol, 3*(2), 237-246.

Kummerer, K. (2004). Resistance in the environment. *J Antimicrob Chemother, 54*(2), 311-320.

Labes, M., Puhler, A., & Simon, R. (1990). A new family of RSF1010-derived expression and lac-fusion broad-host-range vectors for gram-negative bacteria. *Gene, 89*(1), 37-46.

Lambs, L., Decock-Le Reverend, B., Kozlowski, H., & Berthon, G. (1988). Metal Ion-Tetracycline Interactions in Biological Fluids. 9. Circular Dichroism Spectra of Calcium and Magnesium Complexes with Tetracycline, Oxytetracycline, Doxycycline, and Chlortetracycline and Discussion of Their Binding Modes. *Inorganic Chemistry, 27*(17), 3001-30012.

Langlois, B. E., Cromwell, G. L., Stahly, T. S., Dawson, K. A., & Hays, V. W. (1983). Antibiotic resistance of fecal coliforms after long-term withdrawal of therapeutic and subtherapeutic antibiotic use in a swine herd. *Appl Environ Microbiol, 46*(6), 1433-1434.

Lanig, H., Othersen, O. G., Beierlein, F. R., Seidel, U., & Clark, T. (2006). Molecular dynamics simulations of the tetracycline-repressor protein: the mechanism of induction. *J Mol Biol, 359*(4), 1125-1136.

Lederer, T., Takahashi, M., & Hillen, W. (1995). Thermodynamic analysis of tetracycline-mediated induction of Tet repressor by a quantitative methylation protection assay. *Anal Biochem, 232*(2), 190-196.

Leveau, J. H., & Lindow, S. E. (2001). Predictive and interpretive simulation of green fluorescent protein expression in reporter bacteria. *J Bacteriol, 183*(23), 6752-6762.

Leveau, J. H., & Lindow, S. E. (2002). Bioreporters in microbial ecology. *Curr Opin Microbiol, 5*(3), 259-265.

Levy, S. B. (2002). Active efflux, a common mechanism for biocide and antibiotic resistance. *Symp Ser Soc Appl Microbiol*(31), 65S-71S.

Losick, R., & Desplan, C. (2008). Stochasticity and cell fate. *Science, 320*(5872), 65-68.

Maurer, L. M., Yohannes, E., Bondurant, S. S., Radmacher, M., & Slonczewski, J. L. (2005). pH regulates genes for flagellar motility, catabolism, and oxidative stress in Escherichia coli K-12. *J Bacteriol, 187*(1), 304-319.

McManus, P. S., Stockwell, V. O., Sundin, G. W., & Jones, A. L. (2002). Antibiotic use in plant agriculture. *Annu Rev Phytopathol, 40*, 443-465.

Mitscher, L. A. (1978). *The chemistry of the tetracycline antibiotics*. New York: M. Dekker.

Moyed, H. S., Nguyen, T. T., & Bertrand, K. P. (1983). Multicopy Tn10 tet plasmids confer sensitivity to induction of tet gene expression. *J Bacteriol, 155*(2), 549-556.

Nanavaty, J., Mortensen, J. E., & Shryock, T. R. (1998). The effects of environmental conditions on the in vitro activity of selected antimicrobial agents against Escherichia coli. *Curr Microbiol, 36*(4), 212-215.

Neela, F. A., Nonaka, L., & Suzuki, S. (2007). The diversity of multi-drug resistance profiles in tetracycline-resistant Vibrio species isolated from coastal sediments and seawater. *J Microbiol, 45*(1), 64-68.

Nelson, M. L. (1998). Chemical and biological dynamics of tetracyclines. *Adv Dent Res, 12*(2), 5-11.

Nguyen, T. N., Phan, Q. G., Duong, L. P., Bertrand, K. P., & Lenski, R. E. (1989). Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of Escherichia coli K12. *Mol Biol Evol, 6*(3), 213-225.

Nivens, D. E., McKnight, T. E., Moser, S. A., Osbourn, S. J., Simpson, M. L., & Sayler, G. S. (2004). Bioluminescent bioreporter integrated circuits: potentially small, rugged and inexpensive whole-cell biosensors for remote environmental monitoring. *J Appl Microbiol, 96*(1), 33-46.

Pruden, A., Pei, R., Storteboom, H., & Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environ Sci Technol, 40*(23), 7445-7450.

Roberts, M. C. (1996). Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol Rev, 19*(1), 1-24.

Salyers, A. A., Shoemaker, N. B., & Li, L. Y. (1995). In the driver's seat: the Bacteroides conjugative transposons and the elements they mobilize. *J Bacteriol, 177*(20), 5727-5731.

Sarmah, A. K., Meyer, M. T., & Boxall, A. B. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere, 65*(5), 725-759.

Sassman, S. A., & Lee, L. S. (2005). Sorption of three tetracyclines by several soils: assessing the role of pH and cation exchange. *Environ Sci Technol, 39*(19), 7452-7459.

Smilack, J. D. (1999). The tetracyclines. *Mayo Clin Proc, 74*(7), 727-729.

Tuckfield, R. C., & McArthur, J. V. (2007). Spatial Analysis of Antibiotic Resistance Along Metal Contaminated Streams. *Microb Ecol.*

Verma, B., Robarts, R. D., & Headley, J. V. (2007). Impacts of tetracycline on planktonic bacterial production in prairie aquatic systems. *Microb Ecol, 54*(1), 52-55.

Wessels, J. M., Ford, W. E., Szymczak, W., & Schneider, S. (1998). The Complexation of Tetracycline and Anhydrotetracycline with Mg2+ and Ca2+: A Spectroscopic Study. *Journal of Physical Chemistry B, 102*(46), 9323-9331.

White, D. G., Alekshun, M. N., McDermott, P. F., & Levy, S. B. (2005). *Frontiers in antimicrobial resistance : a tribute to Stuart B. Levy*. Washington, DC: American Society for Microbiology.

Whittle, G., Shoemaker, N. B., & Salyers, A. A. (2002). The role of Bacteroides conjugative transposons in the dissemination of antibiotic resistance genes. *Cell Mol Life Sci, 59*(12), 2044-2054.

Yamaguchi, A., Ohmori, H., Kaneko-Ohdera, M., Nomura, T., & Sawai, T. (1991). Delta pH-dependent accumulation of tetracycline in Escherichia coli. *Antimicrob Agents Chemother, 35*(1), 53-56.