DIVERSITY OF TETX-LIKE PROTEINS

By

Laura Thompson, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2007) McMaster University

(Biochemistry) Hamilton, ON

TITLE: DIVERSITY OF TETX-LIKE PROTEINS

AUTHOR: Laura R. Thompson, B.Sc.(Lake Superior State University)

SUPERVISOR: Dr. G.D. Wright

NUMBER OF PAGES: 95

Abstract

The most uncommon form of tetracycline resistance is enzymatic inactivation of the drug. The first protein characterized that was shown to have this ability was TetX, a 44-kDa cytoplasmic protein responsible for inactivating tetracycline in *E.coli*. The associated gene, *tetX,* was found on a transposon in the bacterium *Bacteroides fragilis*, and encodes an NADP-requiring, FAD-dependent monooxygenase. TetX modifies the structure of tetracycline by the addition of a hydroxyl to the C-11a position, altering the β-diketone system of the tetracycline that is responsible for antibiotic activity. This project was designed to search for novel tetracycline inactivators, and to determine the origin of the *tetX*. Initially, the search for TetX-like proteins in *S. coelicolor* and *C. hutchinsonii* was performed using homologous protein sequences found using BLAST searches. Each of the genes encoding the homologous protein sequences was cloned, over-expressed and purified, then analyzed using HPLC and LC/MS methods to determine their tetracycline inactivating ability. Next, the published tetracycline inactivators Tet34 and Tet37 were tested for their ability to inactivate the drug using HPLC and LC/MS methods, after being cloned, over-expressed and purified. Finally, a search of the Actinomycete library belonging to the Wright Laboratory was conducted looking for novel tetracycline inactivators. Bioassays were the first step in a series of experiments done, with HPLC and LC/MS assays eventually being used to

determine if an inactivation event was occurring. The homologous sequences from *S. coelicolor* and *C. hutchinsonii* did not inactivate tetracycline as determined by HPLC and LC/MS data. The potential inactivators, Tet34 and Tet37, were also found to be void of tetracycline inactivating activity. Finally, one isolate in the actinomycete library was thought to be inactivating the drug, however, upon further inspection via HPLC and LC/MS methods this inactivation event was dismissed. Future research should focus on the search for novel enzymes capable of modifying the structure of tetracycline, as well as the origin of the only known tetracycline inactivator, *tetX*.

Acknowledgements

First of all, I would like to thank Dr. Gerry Wright without whom this research would not have been possible. I am very grateful for being given the chance to work for such a brilliant man and I have really enjoyed my time in his lab. His constant words of encouragement and support not only helped me get through some very difficult patches when scientifically nothing seemed to work, but also allowed me to learn an immense number of lab skills and grow as a scientist. Thank you for making what we do fun and for pushing me to become better.

I would also like to thank Dr. Marie Elliot and Dr. Justin Nodwell for being members of my committee and for their support and advice along the way. Thank you to Dr. Brian Coombs also for agreeing to be my external examiner and providing helpful insight into how to become a better writer.

The Wright lab crew has been the backbone for this work and I could not have finished this without each of them. Specifically, Dr. Ishac Nazi for his help with purifying proteins, Dr. Kalinka Koteva for being the smartest chemist ever, and Laura Rossi for always just being there when I needed to think something through or vent. The rest of the lab (and they know who they are) have kept me sane for the past three years, helping me laugh when all I could think about was crying, and sharing a huge amount of knowledge with me. Thank you all.

Thank you to my best friends; Keyvan, and Sara for always being there to listen to me whine and cheer me up. Without their support and company I would never have gotten through the tough days. You guys mean the world to me, thank you for always being there, I love you.

Lastly, I would like to thank my parents for always believing in me even when I didn't. Their constant reassurance and praise has gotten me through the worst days of my life and there aren't words to describe how much they mean to me. My mom has been the one constant in my life that I know will always be there, and I would be nothing without her. Her support of everything I have done has been a huge source of happiness in my life. I love you mom, thank you for everything. My dad has been my mentor, my teacher, and above all my friend. His belief in me is unwavering and he always pushes me to be better person. Although he continually tells me that I did this all on my own, I know that I could not have done it without him. Thank you Papa, I love you so very much.

Table of Contents

List of Figures

List of Tables

1. Introduction

1.1 - Introduction to the Antibiotic Resistome

Since the first discovery of antibiotics, resistance has emerged in bacteria to counteract their use. Antibiotic resistance in pathogenic bacteria commands the attention of researchers; however a plethora of resistance genes in non-pathogenic and environmental bacteria suggest that effort should be spent examining these as well. A relatively small number of known resistance genes are of clinical significance, whereas the information that can be obtained from other resistance genes could prove extremely useful in predicting antibiotic resistance and the path it will follow. The collection of all antibiotic resistance genes in all microbes has been termed the antibiotic resistome [1] and we believe that this resistome potentially holds the key to understanding resistance distribution throughout bacterial communities.

1.2 - History of Tetracyclines

The family of antibiotics known as the tetracyclines was first discovered in the late 1940s, as a product of *Streptomycetes*, a genus of soil-dwelling bacteria [2-7]. This was the first group of antibiotics classified as broad spectrum, meaning they exert their effect on both Gram-positive and

Gram-negative bacteria. Most tetracyclines are bacteriostatic agents and have shown activity against numerous free-living bacteria, as well as against some intracellular pathogens, such as *Chlamydiae* and certain protozoan parasites; the latter activity has been attributed to the ability of tetracyclines to easily cross biological membranes and enter the cell [2-7].

Clinically, typical tetracycline therapy has minimal side effects in patients, and the antibiotics can be administered orally making them an attractive option for treatment of infections in human hosts [3, 4]. Resistance to tetracyclines developed soon after the first use of these drugs as antibacterial agents [2, 8-10]. Since tetracyclines are produced by bacteria, it is appropriate that such bacteria possess a method of self-protection against these antimicrobials. Given the presence of intrinsic resistance mechanisms in nature, it is perhaps not surprising that resistance was seen soon after the first application of the drug in the clinics [5, 11-13].

1.2.1 - General Characteristics

Tetracyclines are comprised of a tetracyclic core modified with various functional groups (hydroxyl, amine, ketone, halogen) (Figure 1.1A) [3, 4, 6, 7, 10, 14-18]. The core is composed of the β-diketone system in rings B

and C (Figure 1.1B), which is essential for antibiotic activity of the compound [4, 6, 7]. This β-diketone system serves as a metal chelating centre that assists in transmembrane drug transport and is essential for interaction of the antibiotic with the target rRNA [7, 19]. The functional groups are responsible for the different forms of tetracycline.

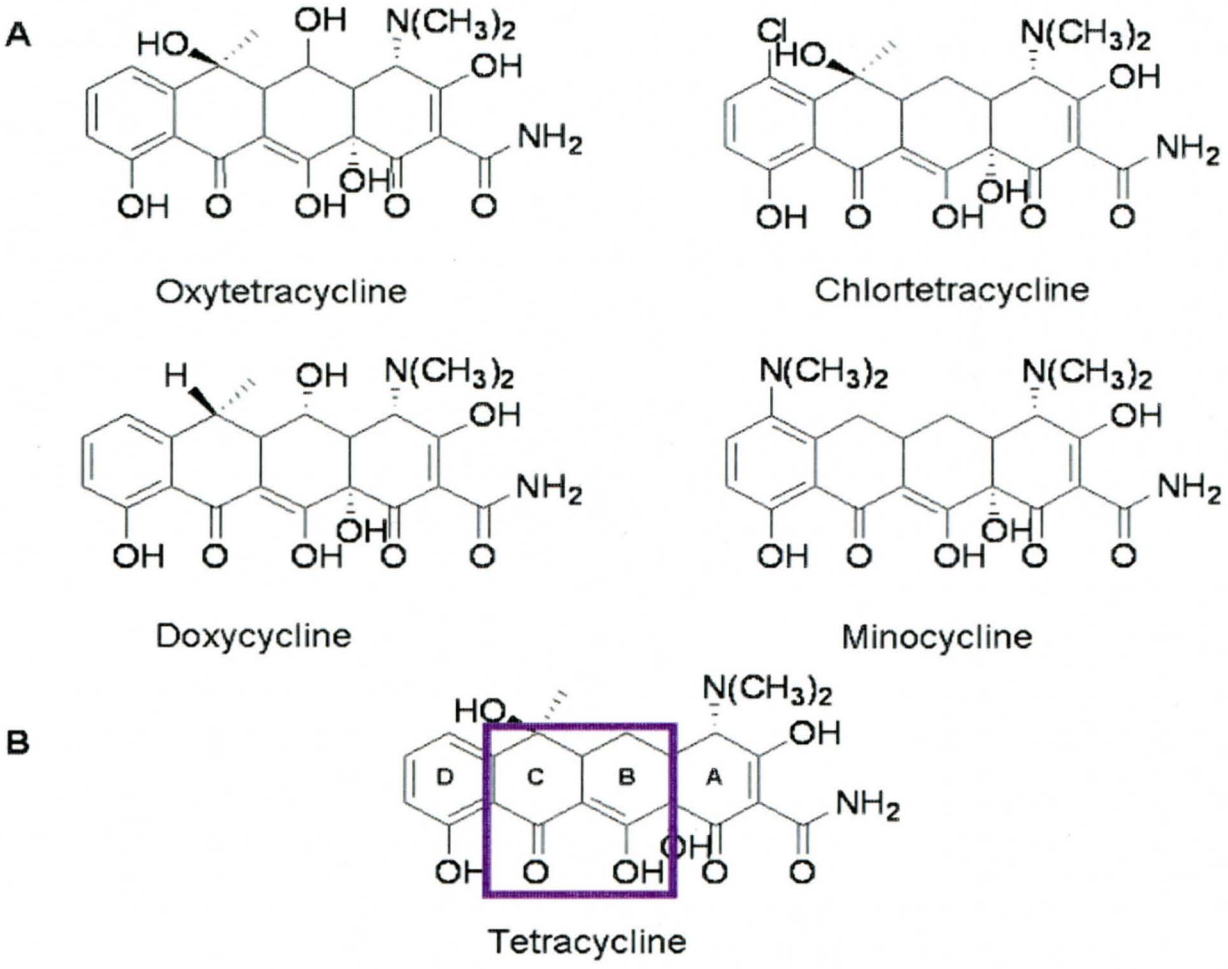


Figure 1.1: Chemical Structures of Some Members of the Tetracycline Family. (A) Commonly used tetracyclines, (B) Structure of Tetracycline. The β-diketone system (boxed) in rings B and C is essential for antibiotic activity [3, 4, 6, 7, 10, 14-18].

1.2.2 – Mode of Action

Tetracyclines exert their bacteriostatic effect on both Gram-positive and Gram-negative bacteria by inhibiting protein synthesis. Normally, protein synthesis involves three steps, initiation, elongation, and termination. This event is coordinated by a complex series of cytoplasmic factors that associate with ribosomes. Ribosomes are composed of two subunits (the 30S and the 50S) that associate at the onset of initiation. Once the two subunits unite, the ribosome can be considered in terms of three sites within the entity. The A site is responsible for the binding and decoding of amino-acyl tRNA, the P site for binding peptidyl-tRNA, and the E site accommodates the exit of the uncharged tRNA out of the ribosome. Elongation occurs when an elongation factor EF-Tu bound with a GTP delivers an aa-tRNA to the A site. A series of decoding events then takes place to ensure the correct amino acid incorporation. Once the correct aa-tRNA has been delivered to the A site, the EF-Tu undergoes conformational changes that cause the ribosome to induce GTP hydrolysis and subsequent EF-Tu release. The release of the EF-Tu allows for the peptidyl transferase reaction between the aa-tRNA and the peptidyl-tRNA to take place. Translocation then moves the tRNA from the A site in a ratchet-like movement down the strand of mRNA to the P site. This process is repeated under normal conditions within a cell until the mRNA reaches a stop codon at which time cytoplasmic factors aid in the

disassociation of the ribosome and other components involved in this process. Tetracyclines act by blocking the first step in this process through an interaction among three residues in the 30S ribosomal subunit and the β-diketone system, by way of a magnesium molecule (Figure 1.2) [4]. When tetracycline binds to the 30S subunit of the ribosome, the bound tetracycline effectively blocks entry of new aa-tRNA into the A site, preventing further protein synthesis from occurring (Figure 1.3).

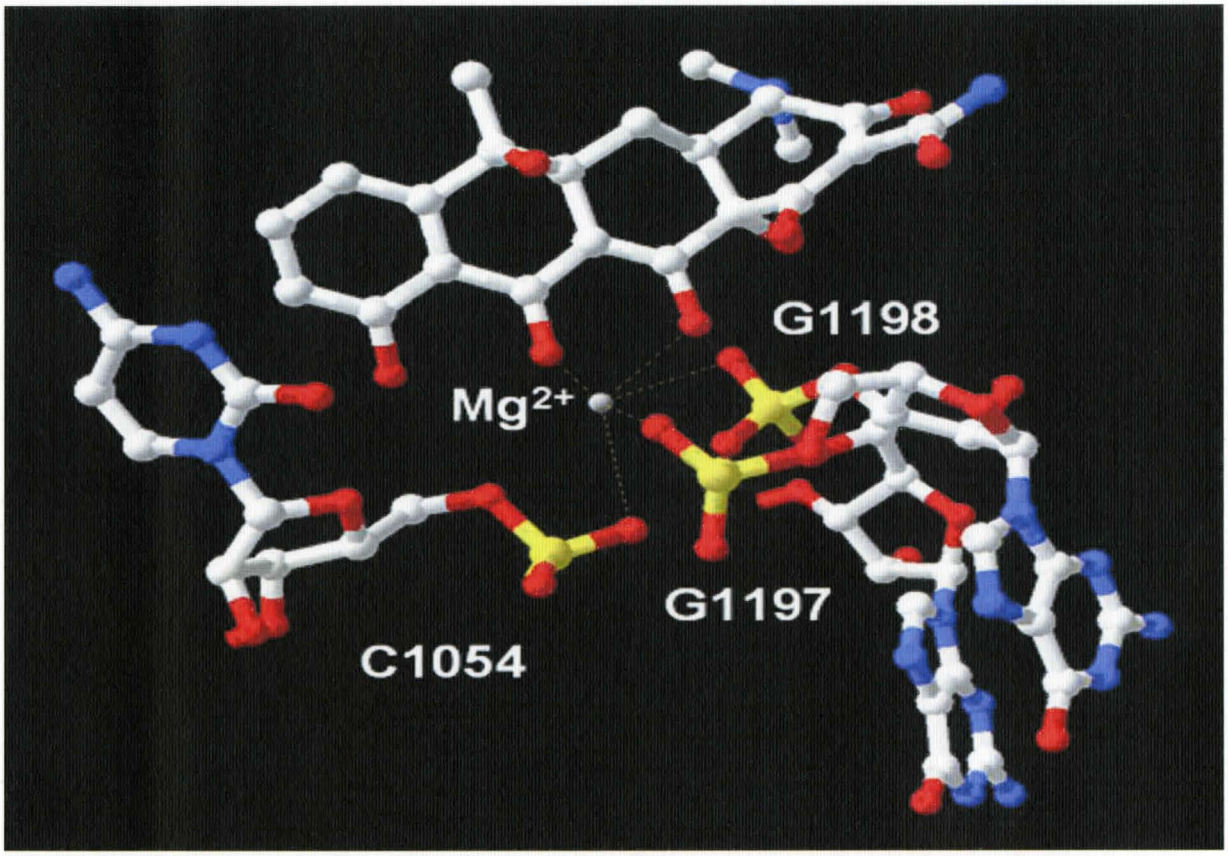


Figure 1.2: Tetracycline Bound to the Ribosome. Crystal structure demonstrating how tetracycline interacts with the 30s ribosomal subunit. A magnesium molecule binds the tetracycline through a cysteine, and two gylcine residues on the ribosome. (Dr. Ian Moore)

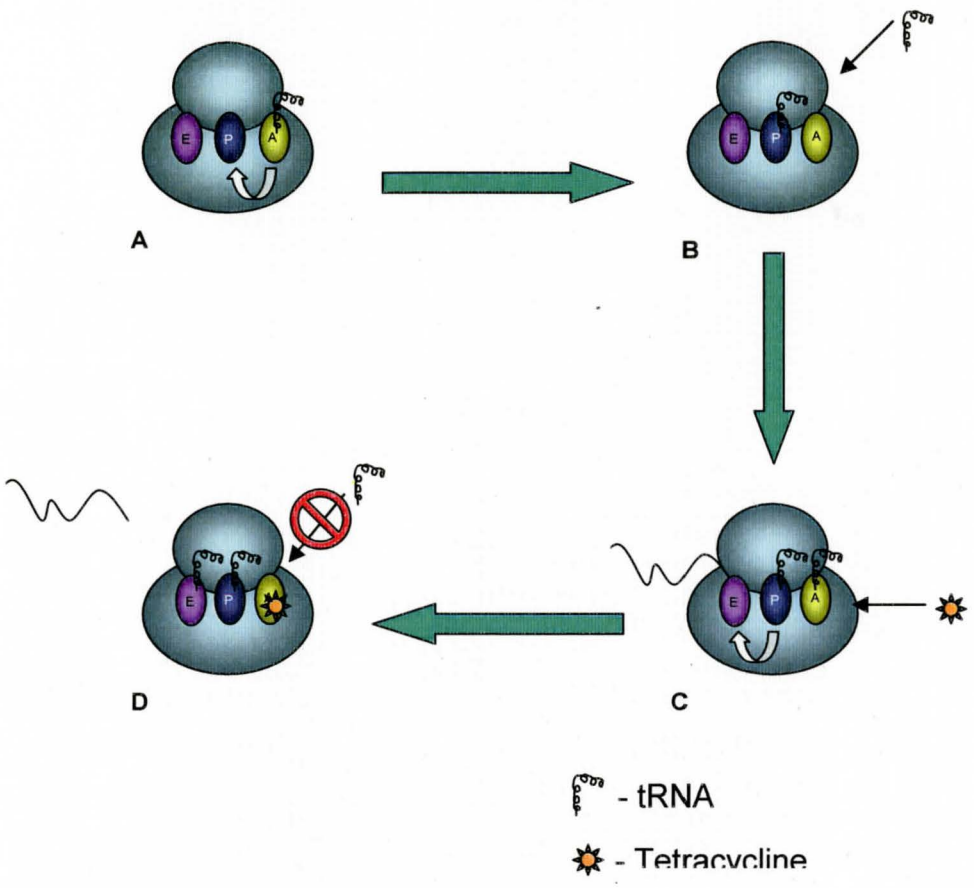


Figure 1.3: Tetracycline Disrupts Protein Synthesis in Bacterial Cells. Elongation (shown in steps A-D) is disrupted by the addition of tetracycline. Tetracycline binds to the 30S subunit of the ribosome, functionally blocking aa-tRNA from entering the A site.

1.3 - Mechanisms of Tetracycline Resistance

Resistance occurs when the tetracycline is not able to bind to the 30S ribosomal subunit, and this can occur via three main mechanisms [7, 11, 13, 20]. In drug efflux, once tetracycline has entered the cell, proteins are produced that will pump the tetracycline out of the cell, reducing the

cellular concentration of tetracycline and allowing protein synthesis to occur (Figure 1.4B) [4, 7, 8, 11, 13, 20-24]. Ribosomal protection proteins are also produced in the presence of tetracycline, and are thought to function by binding to the tetracycline-ribosome complex and releasing the tetracycline from the ribosome (Figure 1.4 C) [25-27]. The last case involves the modification of the tetracycline structure by an enzyme produced within the bacteria that causes the drug to lose its antibiotic properties (Figure 1.4D) [9, 28-32]. Each of these mechanisms will be discussed in greater detail below. In many cases, the genes involved in these mechanisms are found on mobile elements such as transposons and plasmids, which can explain the high amount of resistance genes in the population [11, 33]. Furthermore, many bacteria will have genes for both efflux and ribosomal protection resistance [11, 20]. Thus far, the only forms of tetracycline resistance that are clinically relevant involve drug efflux and ribosomal protection.

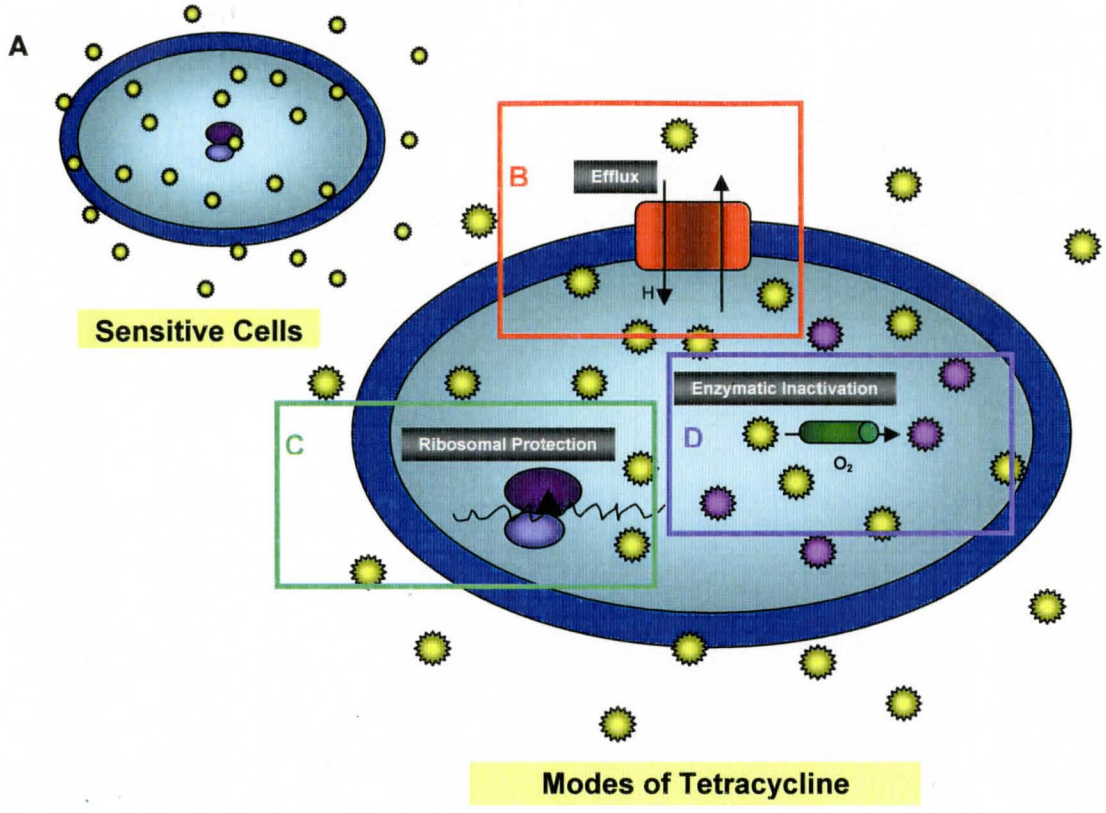


Figure 1.4: Three Avenues of Tetracycline Resistance. A) normal, tetracycline sensitive cells, B) resistance via efflux, C) resistance via ribosomal protection proteins, D) resistance via enzymatic inactivation. Figure adapted from [34].

1.3.1 - Efflux-mediated Resistance

Active drug efflux is the most prevalent method of tetracycline resistance. There are specific classes of efflux proteins with those associated with tetracycline resistance mainly being from the major facilitator superfamily. Proteins from this family typically have 12 alpha helical transmembrane domains. These domains can further be characterized into three groups: four are completely embedded in the hydrophobic interior, four face a putative water filled channel along their length, and four face the channel

for half their length with the other half being embedded in the hydrophobic interior. In the case of Gram-negative cells, tetracycline efflux pumps belonging to the MFS have 12 transmembrane fragments (TMS), but have 14 TMS in their Gram-positive counterparts. The pumps function as an electroneutral antiport system which catalyzes the exchange of a tetracycline-divalent-metal-cation complex for a proton (Figure 1.5) [23, 35-37]. The efflux proteins that accommodate the gylcylcyclines belong to another family called the resistance-nodulation-division superfamily [23]. These efflux proteins function similarly to MFS proteins as they catalyze efflux by an antiport H^+ mechanism. Structurally, the RND superfamily also has 12 transmembrane fragments, however two large extracytoplasmic domains are found between TM1 and TM2 as well as TM7 and TM8 [38].

To date, 23 genes encoding tetracycline efflux proteins have been documented. Efflux proteins are found in both Gram-negative and Gram-positive bacteria [20, 23]. In the case of Gram-negative bacteria, the efflux genes are often under the control of a TetR protein, such that in the presence of tetracycline, the proteins involved in tetracycline export are expressed. The genes involved in efflux-mediated resistance are usually found adjacent to *tetR* genes that directly control the expression of both. The region between the two genes contains the operators for each, and

the TetR protein binds to these operators and prevents transcription from the promoters. When tetracycline is present in the cell, it binds TetR with Mg^{+2}, causing a conformational change in the protein rendering it unable to bind DNA [23, 39-41]. The genes encoding the efflux proteins are then transcribed, allowing for insertion of these pumps in the cell membrane. In the case of Gram-positive bacteria however, the *tet* genes responsible for efflux are controlled by a translational attenuation mechanism: the mRNA of the efflux protein is continuously transcribed within the cell, but will only be translated into a functional protein in the presence of tetracycline [23].

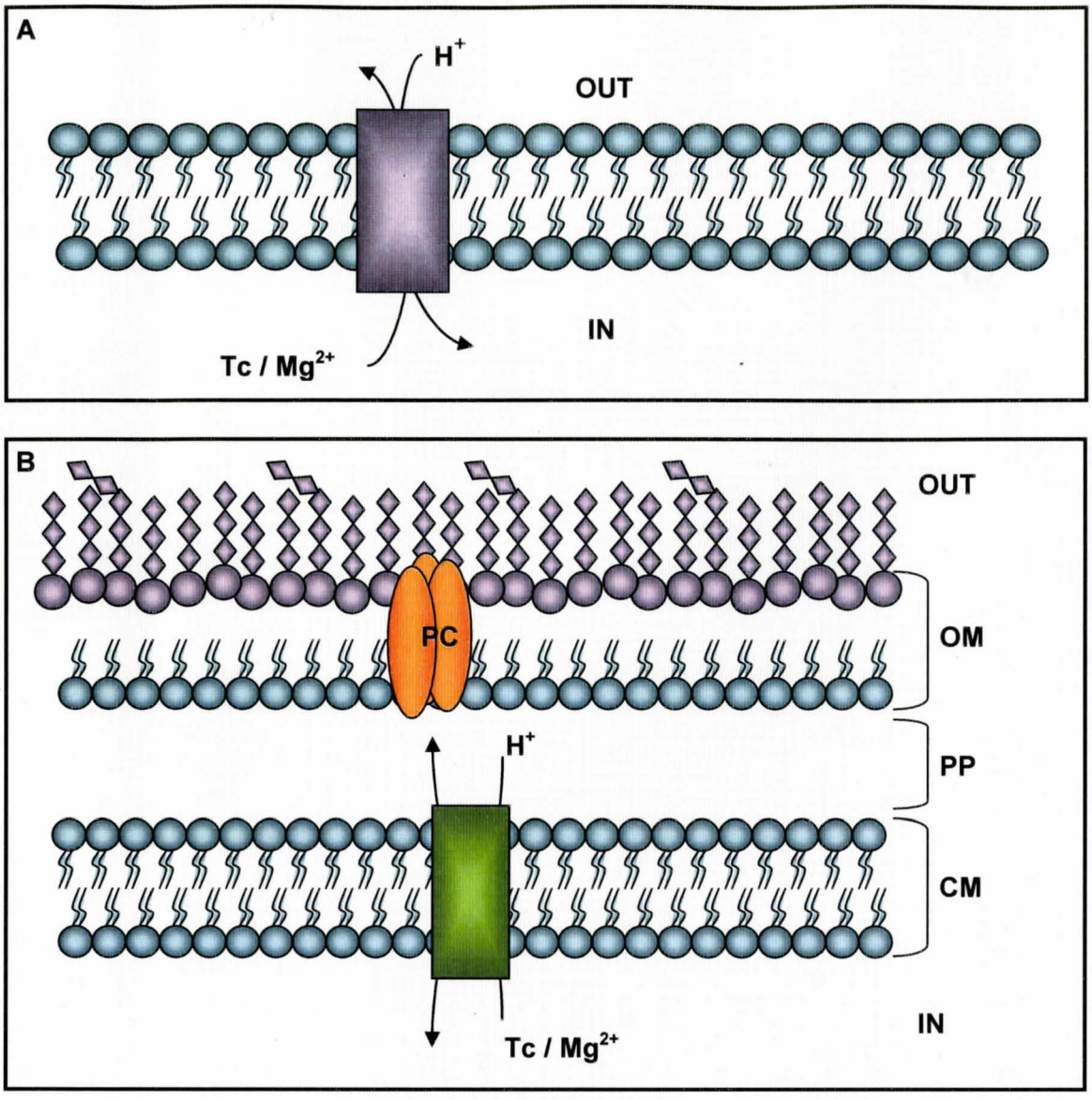


Figure 1.5: Diagram of efflux systems in both Gram-positive and Gram-negative bacteria. Efflux proteins function by exchanging a tetracycline/cation complex within the cell for a proton outside the cell. (A) Efflux system in Gram-positive bacteria. (B) Efflux system in Gram-negative bacteria. Porins are found in the outer membrane of Gram-negative bacteria and aid in transmembrane transport. Tetracycline (Tc), Outer membrane (OM), Periplasmic space (PP), cell membrane (CM), Porin Channel (PC).

It has been suggested that these pumps are very specific, as some pumps function specifically with tetracycline but not other members of this family of antibiotics [20, 23]. It has also recently been discovered that some *tet* genes encoding efflux proteins are specific to Gram-positive or Gram-negative species. Tet proteins A-E, G-J, Z, and Tet30 are exclusive to Gram-negative bacteria, where TetK and TetL are found predominantly in Gram-positive bacteria.

Although new genes encoding efflux proteins continue to be discovered as time progresses, tetracyclines remain a routine prescription in many clinical settings. Research targeting efflux as a means of combating tetracycline resistance is currently underway. Recently, inhibitors of Tet-mediated efflux resistance have been investigated, with plant extracts showing the ability to increase tetracycline activity in some species tested [23]. Work needs to continue in this area to determine if other classes of efflux proteins can also be inhibited. Inhibition of these efflux pumps could potentially allow for the continued clinical use of tetracyclines.

1.3.2 - Ribosomal Protection Proteins

Ribosomal protection proteins (RPPs) confer resistance through a less familiar mode of action. RPPs are cytoplasmic proteins that interact with

the ribosome and prevent the ribosome-tetracycline interaction, as previously described. To date, 11 genes encoding RPPs have been identified, with *tet(M)* and *tet(O)* being the most studied and the best understood. Although *tet(M)* and *tet(O)* classes share limited homology to other RPPs such as *tet(S)*, *tet(T)*, *tet(Q)*, *tetB(P)*, *tet(W)*, and *otr(A)*, all resultant protein products confer resistance to tetracycline derivatives [20, 26, 27]. It is interesting to note that all protection proteins share primary sequence similarity with elongation factor G, in the region of the protein that contains the GTP-binding site. Therefore, it has been speculated that these RPPs may have evolved from genes that encode bacterial elongation factors [26].

Typically, elongation factors are responsible for the translocation of amino acids from the A to P sites during protein synthesis. These elongation factors bind between the 30S and 50S ribosomal subunits, and function by providing the GTP required for hydrolysis that aids the in movement of amino acids. RPPs are responsible for binding to the 30S portion of the ribosome, causing a lower affinity for tetracycline binding (Figure 1.6). However, RPPs do not block the GTPase centre where the elongation factors bind and so protein synthesis is not affected. There is considerable debate over how these RPPs function in bacteria. One theory, emerging

from protein structural studies using cryoelectron microscopy and chemical probing, suggests that RPPs bind to the 30S ribosomal subunit only once a tetracycline has blocked the entry of t-RNA to the A-site. Once the RPP has bound to the tetracycline/ribosome complex, the tetracycline is released, leaving the ribosome ready to proceed with protein synthesis [26]. The mechanism by which RPPs find tetracycline-bound ribosomes and release tetracycline has not been established leaving the field open for investigation. As more becomes known about how these proteins function within cells, further work can be done to search for potential inhibitors. Ultimately, such inhibitors would limit RPP function and allow tetracycline to remain bound, exerting its bacteriostatic effect within the cell.

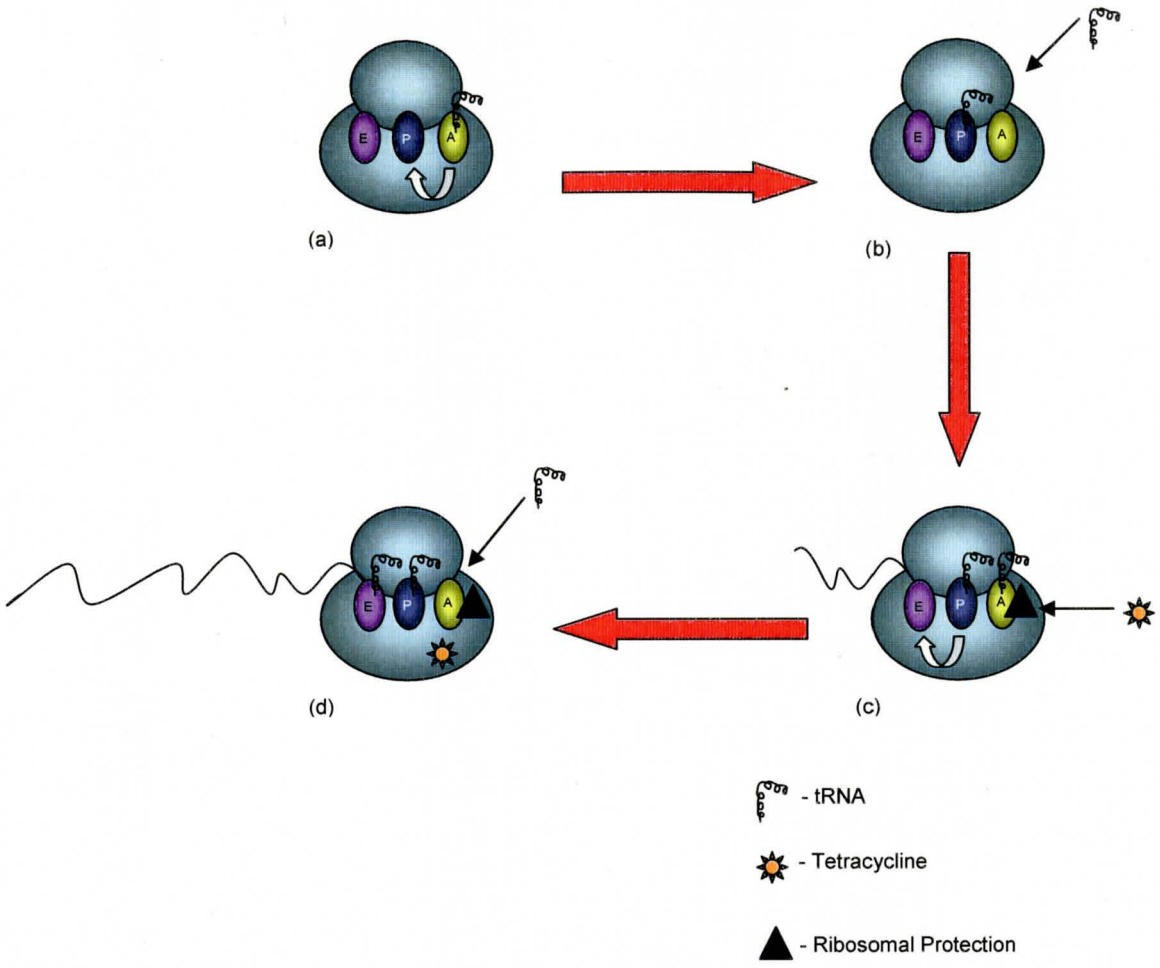


Figure 1.6: Diagram of the Mechanism of Ribosomal Protection Proteins. A ribosomal protection protein binds to the 30s subunit of the ribosome (c) functionally blocking tetracycline from binding thus allowing protein synthesis to occur as usual. RPPs specifically decrease the affinity for tetracycline, therefore stopping the antibiotic from binding to the ribosome, but allowing entry of amino acyl tRNAs.

1.3.3 - Enzymatic Inactivation

The most uncommon form of tetracycline resistance is enzymatic inactivation of the drug. The first protein characterized that was shown to have the ability to perform this task was TetX, a 44-kDa cytoplasmic

protein responsible for inactivating tetracycline in *E. coli* (Figure 1.7A). The associated gene, *tetX,* was found on a transposon in the bacterium *Bacteroides fragilis*, and encodes an NADPH-requiring, FAD-dependent monooxygenase [28, 42, 43]. TetX modifies the structure of tetracycline by the addition of a hydroxyl to the C-11a position (Figure 1.7C) [28]. This addition alters the β-diketone system of the tetracycline which is responsible for antibiotic activity. This modification can be monitored by the decrease in tetracycline peak and appearance of a secondary product peak on HPLC (Figure 1.7B) [28]. TetX has the ability to inactivate many members of the tetracycline family, including new semi-synthetic antibiotics such as tigecycline, via the same mechanism of hydroxyl addition at the C-11a position [28].

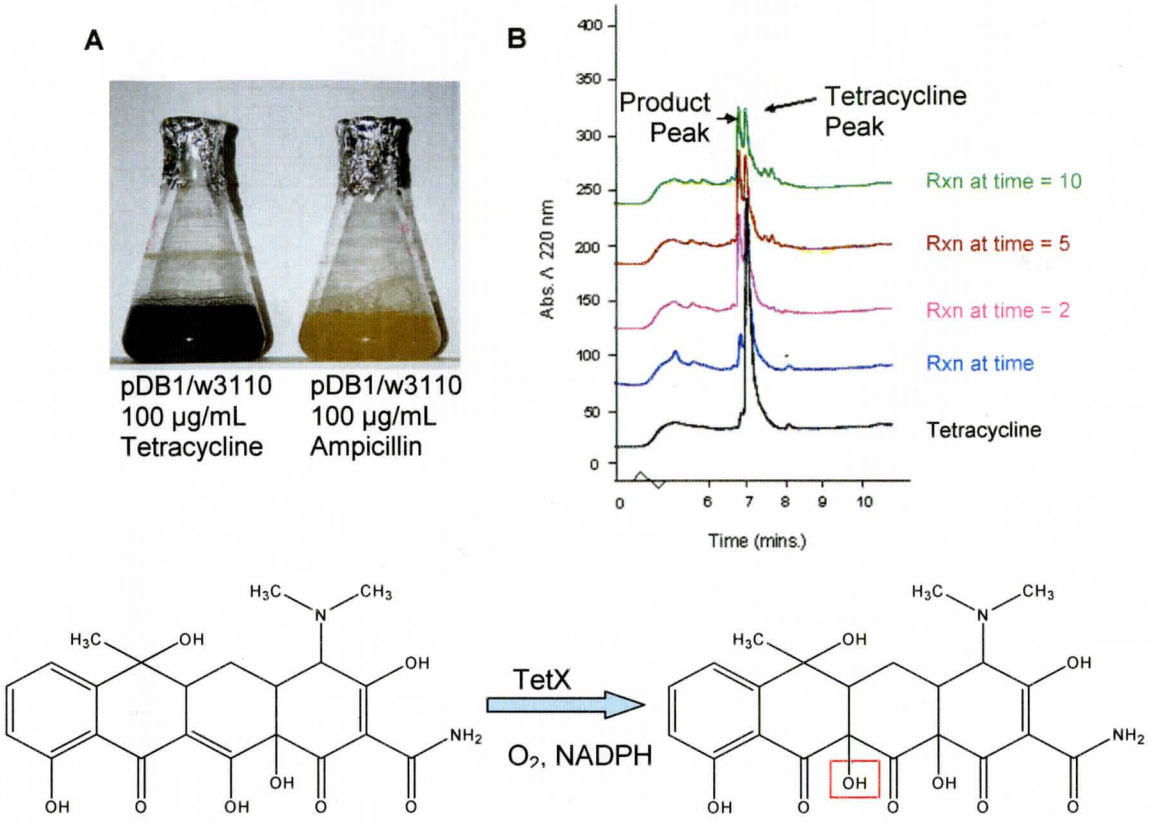


Figure 1.7: Enzymatic Inactivation of Tetracycline. A) Tetracycline inactivation associated with black colour in medium when *tetX* gene in *E. coli* W3110 is grown in the presence of tetracycline. (Taken from Yang et al. 2004). B) HPLC chromatogram demonstrating inactivation of tetracycline by TetX. Reaction was stopped at time points 0, 2, 5, and 10 minutes with the addition of 0.6M HCl. C) Structural representation of TetX catalyzed reaction.

Though the mechanisms remain unknown, *tet37* and *tet34* genes are also associated with the inactivation of tetracycline. The *tet37* gene codes for a predicted NADPH-requiring oxidoreductase, and was cloned from the oral metagenome in 2003, but the predicted protein has no sequence similarity to TetX [44]. *tet34* is a gene predicted to encode a protein similar to xanthine-guanine phosphoribosyl transferases, and interestingly is not an

NADPH-requiring oxidoreductase, that none-the-less has been shown to inactivate tetracycline [45]. This form of resistance has not yet been seen clinically, and therefore knowledge about this and other enzymes that function to inactivate tetracyclines could prove important for avoiding or treating this kind of resistance in the future.

1.4 - Project Objectives

This project was designed to search for novel tetracycline inactivators, and to provide insight into the origin of the *tetX* gene. Initially, a search for TetX-like proteins was performed using BLAST. This identified possible homologues in *S. coelicolor* and *C. hutchinsonii*.. Each of the genes encoding the similar protein sequences was cloned, over-expressed and purified, then their tetracycline inactivating activity was analyzed using HPLC and LC/MS. Similarly, the published tetracycline inactivators Tet34 and Tet37 were tested for their ability to inactivate the drug using HPLC and LC/MS methods. Finally, a screen of the Actinomycete library belonging to the Wright Laboratory was conducted looking for novel tetracycline inactivators. Bioassays were the first step in a series of experiments done, with HPLC and LC/MS assays eventually being used to determine if an inactivation event was occurring.

2. Materials and Methods

2.1 - General Materials and Methods

Liquid media used to grow various bacterial cultures included: Dubos Media (0.5 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4*7H_20$, 0.5 g KCl, 0.01 g $FeSO_4*7H_2O$, 1.0 L dH_2O), LB (Luria Broth – 10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl per 1.0 L), SOC (10.0 g tryptone, 3.5 g yeast extract, 0.3 g NaCl, 0.1 g KCl, 1.0 g $MgCl_2$-$6H_2O$, 0.6 g $MgSO_4$, 1.8 g glucose per 500 mL), TSB (Tryptone Soya Broth, Oxoid TSB 30.0 g/L – 17.0 g pancreatic digest of casein, 3.0 g papatic digest of soybean meal, 5.0 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose), MHB (Mueller-Hinton Broth, Difco MHB 21 g/L – 300g infusion from beef, 17.5 g casamino acids, 1.5 g soluble starch), Streptomyces Isolation Media (0.4 g casein, 1.0 g starch, 0.5 g KNO_3, 0.2 g K_2HPO_4, 0.1 g $MgPO_4$, 0.1 g $CaCO_3$), SVM (Soygrit Vegetative Media – 1.5 g glucose, 2.0 g potato starch, 1.5 g ground soygrits (Archer Daniels Midland Company), 1.0 g yeast extract, 0.2 g $CaCO_3$, and 1 mL corn steep liquor per 100 mL).

Solid media used included Dubos Agar (Dubos media supplemented with 15 g of agar per 1 L), LB Agar (LB supplemented with 7.5 g of agar per

500 mL), TSA (Oxoid TSB supplemented with 15 g of agar per 1 L), Streptomyces Isolation Media Plates (Streptomyces Isolation Media supplemented with 15 g of agar per 1 L), Bennet's Agar (5.0 g potato starch, 1.0 g casamino acids, 0.9 g yeast extract, 1.0 mL Czapek mineral mix (10 g KCl, 10 g $MgSO_4$-$7H_20$, 12 g $NaNO_3$, 0.2 g $FeSO_4$-$7H_2O$, 200 µL concentrated HCl per 100 mL) supplemented with 7.5 g of agar per 500 mL).

All media were sterilized by autoclaving at 121°C and 15 psi for 20 minutes. Media were supplemented with the appropriate antibiotics to final concentrations of 100 µg/mL ampicillin, 40 µg/mL chloretetracycline, 40 µg/mL demeclocycline, 40 µg/mL doxycycline, 50 µg/mL kanamycin, 40 µg/mL methacycline, 40 µg/mL minocycline, 40 µg/mL oxytetracycline, 40 µg/mL tetracycline, and 40 µg/mL tigecycline.

Streptomyces species were grown at 30°C and 250 rpm (for liquid cultures) in single-baffled flasks. Cytophaga cultures were also grown at 30°C, whereas all other bacterial cultures were grown at 37°C and 250 rpm (for liquid cultures). Minimum inhibitory concentrations were performed using the standard National Committee for Clinical Laboratory Standards (NCCLS) protocol.

Plasmid DNA was isolated from 5 mL overnight *Escherichia coli* cultures grown at 37°C in LB using the Qiagen QIAprep Spin Miniprep kit (Mississauga, Ontario). Restriction digests were performed in the appropriate MBI Fermentas buffer at recommended temperatures. DNA was analyzed using agarose gel electrophoresis and SYBRsafe staining. DNA bands were removed from agarose gels using the Qiagen QIAEX II Gel Extraction kit. Electrocompetent cells were transformed using a BioRad Micropulser with 0.2 mm cuvettes. Following electropulse, 0.5 mL of cold SOC was added to the sample and incubated at 37°C for 1 hr. The sample was then plated on LB supplemented with the appropriate antibiotic and allowed to grow overnight at 37°C.

DNA primers were designed using the Primer Select software (DNAstar). Sequence alignments were carried out using the Seqman software (DNAstar). Oligonucleotide synthesis and DNA sequencing were performed by the Mobix Central Facility at McMaster University (Hamilton, Ontario).

2.2 - Characterization of TetX-like Proteins

2.2.1 - Cloning, Overexpression, and Purification of *S. coelicolor*, and *C. hutchinsonii* homologs.

Previously, the genes SCO0484, SCO7223, SCO7625, and SCO7705 of *Streptomyces coelicolor* were cloned by Linda Ejim and Tejal Patel of the Wright laboratory. The fifth gene encoding a TetX-like protein, SCO0252, was originally cloned with an N-terminal His tag, but did not purify well, and was therefore amplified from *S. coelicolor* genomic DNA (previously prepared by Tejal Patel of the Wright Laboratory) by the polymerase chain reaction (PCR) and recloned with a C-terminal His tag. Similarly, the gene encoding the TetX-like protein from *Cytophaga hutchinsonii* was also amplified by PCR. Each 50 µL PCR reaction contained 500 ng genomic DNA, 1 µM of each of the forward and reverse primers designed for use with the Gateway Cloning System (Invitrogen Life Technologies) listed in Table 2.1, 1X Biotools DNA polymerase reaction buffer ($MgCl_2$ free), 0.4 µM of each deoxynucleotide 5'-triphosphate (dNTP), 5% dimethoxysulfide, and 2 mM $MgCl_2$. Reactions were incubated for 10 mins. at 94°C before the addition of 1 µL of Biotools DNA polymerase (Interscience Inc.). Each reaction underwent 30 cycles of 94°C for 1 min., the appropriate annealing temperature (Table 2.1) for 1 min., and 72°C for 1.5 mins., followed by 10

mins. at 72°C. The size of the amplified product was detected by agarose-gel electrophoresis.

Table 2.1: Primer sequences and PCR conditions used to amplify the TetX-like proteins from *S. coelicolor*, *C. hutchinsonii*, as well as from genes *tet34* and *tet37*.

Gene	Primers (5' → 3')	Annealing Temp. (°C)
SCO0252	Forward:GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGGCGACCCTCATGGCCAACACCTC Reverse:GGGGACCACTTTGTACAAGAAAGCTGGGTCATGGGCATGGCGAGCTTCAGGAG	65
SCO0484	Forward:CGTCTACACATATGAACTCTCCGACACCCGCC Reverse:CGGAATTCAAGCTTTCACTCGCCGGGTCCGCCGT	48
SCO7223	Forward:GCTCTACACATATGACCACGCACGTCACGA Reverse:CGGAATTCAAGCTTCTACTCCTGGCCGCTGAACAT	50
SCO7625	Forward:GCTCTAGACATATGAGACAACGTATCGCCGTGGT Reverse:CGGAATTCAAGCTTTCACCGGGACGGCTGGAC	59
SCO7705	Forward:GCTCTAGACATATGTTCGTTACGCTGGAGGTGT Reverse:CGGAATTCAAGCTTTGCCTGGTCACGCCTCTG	55
Tet34	Forward:GGGGACAAGTTTGTACAAAAAAAGCAGGCTTCGACGACGAAGATTGCGATA Reverse:GGGGACCACTTTGTACAAGAAAGCTGGGTCTAATACGGGGATGCAAACTTCAAA	40
Tet37	Forward:GGGGACAAGTTTGTACAAAAAAAGCAGGCTTCGTTCGCTATTACTCTAACATTGTAGG Reverse:GGGGACCACTTTGTACAAGAAAGCTGGGTCCATCTACAAATTATCGCCCATCGGCC	70
Cytophaga	Forward:GGGGACAAGTTTGTACAAAAAAAGCAGGCTTCGAACAATCAGCAATCAATAC Reverse:GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTACAGTTGTGGGTTGATTAGC	53

Cloning of genes was performed using the Gateway Cloning System. Once a gene was successfully cloned into a vector suitable for expression, the plasmid was used to transform *E.coli* BL21 cells for purification. Proteins were over-expressed and purified from a 1 L culture of LB supplemented with 100 µg/mL ampicillin inoculated with 10 mL of an overnight culture of the cloned gene. Cells were grown at 37°C to mid-log

phase (OD_{600} ~ 0.6) and were then induced with 1 mM isopropyl beta-D-thiogalactopyranoside (IPTG). Cells were grown for 20 hours at 16°C before being harvested. Cell pellets were resuspended in 20 mL lysis buffer containing 50 mM N-2-hydroxyethylpeperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 0.1 mM dithiothreitol (DTT). Lysis was performed by passing the resuspended cells through a large French press cell at a pressure of 10,000 psi a minimum of three times. Centrifugation at 15,000 X g for 15 minutes removed cellular debris from the lysate. The clarified lysate was then applied to a 5 mL Ni-NTA (nickel-nitrilotriacetic acid) column using an automated Fast Protein Liquid Chromatography (FPLC) system. Proteins were eluted with a linear gradient of elution buffer containing 50 mM Hepes, 250 mM NaCl, 250 mM Imidazole, pH 7.5). Fractions containing significant absorbance at 280 nm were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with coomassie brilliant blue, to assess protein purity (Figure 3.1). Fractions containing pure protein were then pooled and dialyzed (50 mM Hepes, pH 7.5) to remove excess salt from the solution. Protein concentration was determined by the standard Bradford assay [46]. Flavin content was determined for each of the purified proteins by denaturing a 1 mL sample of the protein through boiling for 10 mins., followed by a short centrifugation step to remove any precipitate. Absorbance spectra was

then monitored on a Cary 300 Bio UV-Visible spectrophotometer from 200 – 500 nm. Calculations using Beers law ($A = \varepsilon / bC$), the extinction coefficient for FAD, and the absorbance at 375 nm (the max. absorbance of flavin) were used to determine the amount of flavin cofactor in each sample.

2.2.2 - Assays of Tetracycline Inactivation

Reactions (1 mL) containing 10 µL 250 mM N-tris-(hydroxylmethyl)methyl-3-amino-propanesulfonic acid (TAPS) buffer pH 8.5, 1 mM magnesium chloride ($MgCl_2$), 1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.3 mM tetracycline, 8.7 µg protein, and water up to a final volume of 100 µL were incubated at room temperature for 1 hour. TetX from *Bacteroides fragilis* [28] was used as a positive control. Reactions were monitored by reverse phase high performance liquid chromatography (HPLC) using a Dionex Acclaim 120 C18 column (250 * 22 mm, 10 µm particle size). The column was equilibrated with H_2O plus 0.05% trifluoroacetic acid. Products were eluted using a linear gradient to 95% CH_3CN plus 0.05% trifluoroacetic acid over 14 mins. at a flow rate of 1 mL/min. Aliquots of the reaction samples were also subjected to liquid chromatography-mass spectrometry (LC/MS) performed by Kalinka Koteva of the Wright Laboratory. A Dionex Acclaim 120 C18 column was

equilibrated with 95% water and 5% acetonitrile both containing 0.05% formic acid. A linear gradient was developed over 20 mins. to 3% water and 97% acetonitrile.

2.3 – Characterization of Potential Tetracycline Inactivators

2.3.1 - Cloning, Overexpression, and Purification of *tet34* and *tet37*.

Tet34 was cloned using the same method outlined for each of the TetX-like proteins. Tet37 was not successfully amplified and will not be referred to again here. The original sample of DNA obtained by Gloria Yang of the Wright Laboratory was not successfully amplified after trying various conditions and the work was abandoned. A new sample of DNA was obtained in the hopes of continuing this work, however despite numerous attempts at PCR optimization reactions varying temperature, amounts of DNA, DMSO, dNTPs, primers, and $MgCl_2$, none of the reactions amplified the gene. For this reason, it is reasonable to assume that the sample of DNA obtained was not correct, or that this gene was not in the bacterial gDNA as previously thought. Each 50 µL PCR reaction contained 500 ng genomic DNA, 1 µM of each of the forward and reverse primers designed for use with the gateway cloning system listed in Table 2.1, 1X Biotools DNA polymerase reaction buffer ($MgCl_2$ free), 0.4 µM of each deoxynucleotide 5'-triphosphate (dNTP), 5% dimethoxysulfide, and 2 mM

$MgCl_2$. Reactions were incubated for 10 mins. at 94°C before the addition of 1 µL of Biotools DNA polymerase (Interscience Inc.). Each reaction underwent 30 cycles of 94°C for 1 min., the appropriate annealing temperature (Table 2.1) for 1 min., and 72°C for 1.5 mins., followed by 10 mins. at 72°C. The size of the amplified product was detected by agarose-gel electrophoresis. Cloning was performed using the Gateway Cloning System. The gene (*tet34*) was cloned and the plasmid was used to transform *E.coli* BL21 cells for purification. The gene product was over-expressed and purified from a 1 L culture of LB supplemented with 100 µg/mL ampicillin inoculated with 10 mL of an overnight culture of the cloned gene. Cells were grown at 37°C to mid-log phase (OD_{600} ~ 0.6) and were then induced with 1 mM isopropyl beta-D-thiogalactopyranoside (IPTG). Cells were grown for 20 hours at 16°C before being harvested. Cell pellets were resuspended in 20 mL lysis buffer containing 50 mM N-2-hydroxyethylpeperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 0.1 mM dithiothreitol (DTT). Lysis was performed by passing the resuspended cells through a large French press cell at a pressure of 10,000 psi a minimum of three times. Centrifugation at 15,000 X g for 15 minutes removed cellular debris from the lysate. The clarified lysate was then applied to a 5 mL Ni-NTA (nickel-nitrilotriacetic acid) column using an automated Fast Protein Liquid Chromatography (FPLC) system. Proteins were eluted with a linear

gradient of elution buffer containing 50 mM Hepes, 250 mM NaCl, 250 mM Imidazole, pH 7.5). Fractions containing pure protein were then pooled and dialyzed (50 mM Hepes, pH 7.5) to remove excess salt from the solution. Protein concentration was determined by standard Bradford assays [ ]. Flavin content was determined for each of the purified proteins by denaturing a 1 mL sample of the protein through boiling for 10 minutes, followed by a short centrifugation to remove any precipitate. Absorbance spectra were then monitored on a Cary 300 Bio UV-Visible spectrophotometer from 200 – 500 nm. Calculations using Beers law ($A = \varepsilon / bC$), the extinction coefficient for FAD, and the absorbance at 375 nm (the max. absorbance of flavin) were used to determine the amount of flavin cofactor in each sample (Table 4.1).

2.3.2 - Assays of Tetracycline Inactivation

Each reaction was performed in the same manner as those conducted for the *S. coelicolor* and *C. hutchinsonii* proteins (section 2.2.2). Reactions were incubated at room temperature for 1 hour, with TetX as a positive control (Figure 3.4). Reactions were monitored by reverse phase high performance liquid chromatography (HPLC) using a Dionex Acclaim 120 C18 column (250 * 22 mm, 10 µm particle size), equilibrated as previously stated (section 2.2.2). Aliquots of the reaction samples were also subjected to liquid chromatography-mass spectrometry (LC/MS) performed

by Kalinka Koteva of the Wright Laboratory, using similar gradients and columns as previously mentioned.

2.4 – Examination of Activity of TetX-like proteins Against Various Tetracycline Analogs

The reaction mixtures to be monitored via NADPH absorbance were carried out as reported in the previous section (2.2.2). In this case, however, 100 µL was allowed to react in a 96-well microtitre plate upon the addition of NADPH. Decreases in the absorbance at 340 nm (NADPH oxidation) were monitored and recorded using a Molecular Devices SpectraMax Plus microtitre plate reader for each protein being reacted against various tetracycline analogs. The tetracycline analogs (Figure 2.1) were used at a final concentration of 0.3 mM in each case and were subjected to reaction with each of the purified TetX-like proteins.

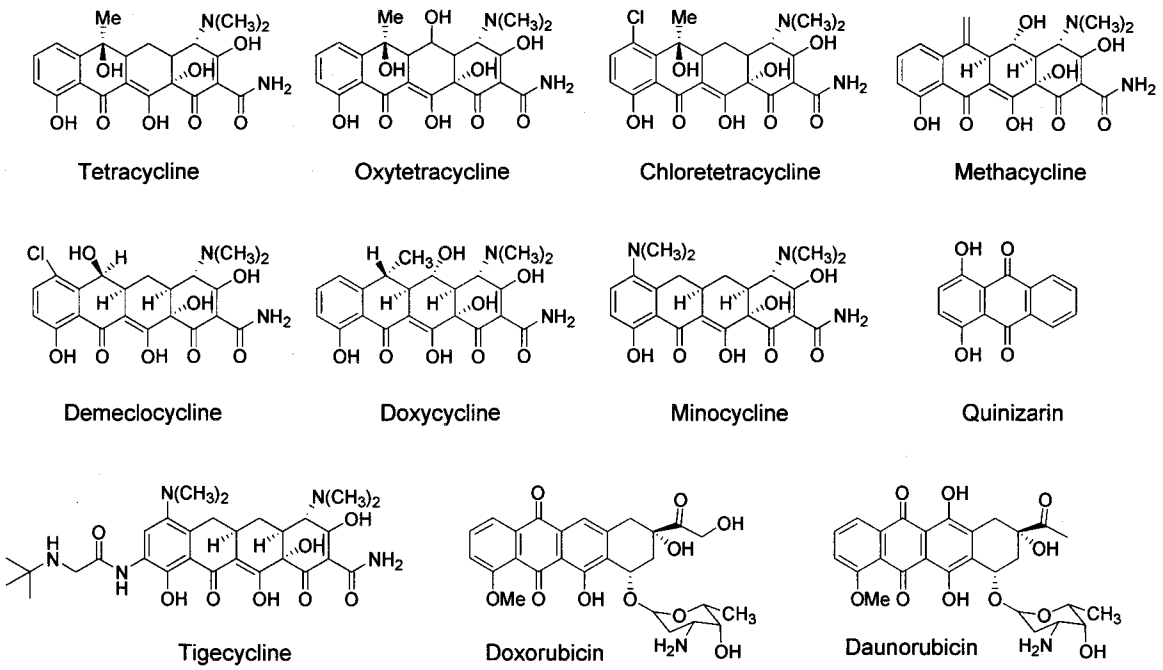


Figure 2.1: Structures of Various Tetracycline Analogs. Analogs were reacted against TetX-like proteins in order to determine if any chemical modifications were occurring.

2.5 – Screen of Actinomycete Library for Tetracycline Inactivation

2.5.1 – Phenotypic Characterization and Identification of Actinomycete Library Isolate

The tetracycline inactivating isolate, Kar21 was streaked on isolation media and grown at 30°C for 3-5 days to allow for sporulation. The plate was then submitted to the Integrated Microscopy Services (McMaster University, Hamilton, Canada) for scanning electron microscopy analysis.

Images, at 5000X and 10,000X magnification were taken using a JEOL840 Scanning Electron Microscope.

In an effort to determine the identity of this isolate, the gene encoding the 16S rRNA was amplified by PCR and sequenced (Mobix Central Facility, McMaster University, Hamilton, Canada). The 50 µL PCR reaction contained ~ 500 ng genomic DNA template, 1 µM of each of the forward and reverse primers (Table 2.2), 1X Biotools DNA polymerase reaction buffer with 2 mM $MgCl_2$, 0.4 mM of each dNTP, 10% DMSO, and 10 mM $MgCl_2$. In total, 30 cycles were done for this PCR at 94, 45, and 72°C for 1, 1, and 1.5 minutes respectively. The amplified gene was then confirmed by agarose gel electrophoresis. The PCR product was excised from the gel and purified before sequencing. The sequences were submitted as a query in a standard nucleotide-nucleotide BLAST (National Centre for Biotechnology Information, NCBI) search to obtain a number of genes encoding 16S rRNA from various organisms. These sequences were compiled and aligned using the Seqman software (DNAStar).

Table 2.2: Primers used for the Amplification of the Gene in Question for 16S rRNA Sequencing (BAC).

Gene	Primers (5' → 3')	Annealing Temp. (°C)
Kar21	Forward: AGAGTTTGATCMTGGCTCAG Reverse: TACGGYTACCTTGTTACGACTT	50

2.5.2 – Bioassays of Bacterial Isolates

Using 96-well microtitre plates, 200 µL cultures of each of the previously determined tetracycline resistant isolates from the Wright Laboratory Actinomycete Library [47] were grown at 30°C at 250 rpm for 2 days in liquid streptomyces isolation media supplemented with tetracycline to a final concentration of 40 µg/mL. Centrifugation of a 90 µL sample removed cellular debris so that the remaining media could be separated into two 45 µL aliquots in a new 96-well microtiter plate. Additional tetracycline was added to one of the samples at a final concentration of 40 µg/mL and the reaction was allowed to proceed at 30°C and 250 rpm for 20 hours. *Micrococcus luteus* was streaked onto TSA and grown for 48 hours at 30°C to obtain single colonies. Using sterile 0.85% saline, a suspension was prepared using the single *M. luteus* colonies to an OD_{625} of 0.08 – 0.1. The resulting suspension was used to swab a plate of TSA to produce a lawn of growth. Sterile paper disks were placed on the plates, and 15 µL of each reaction was pipetted onto the disks. The plates were then

incubated for 48 hours at 30°C so that zones of clearance could be observed around each disk and compared to a control (Figure 2.2).

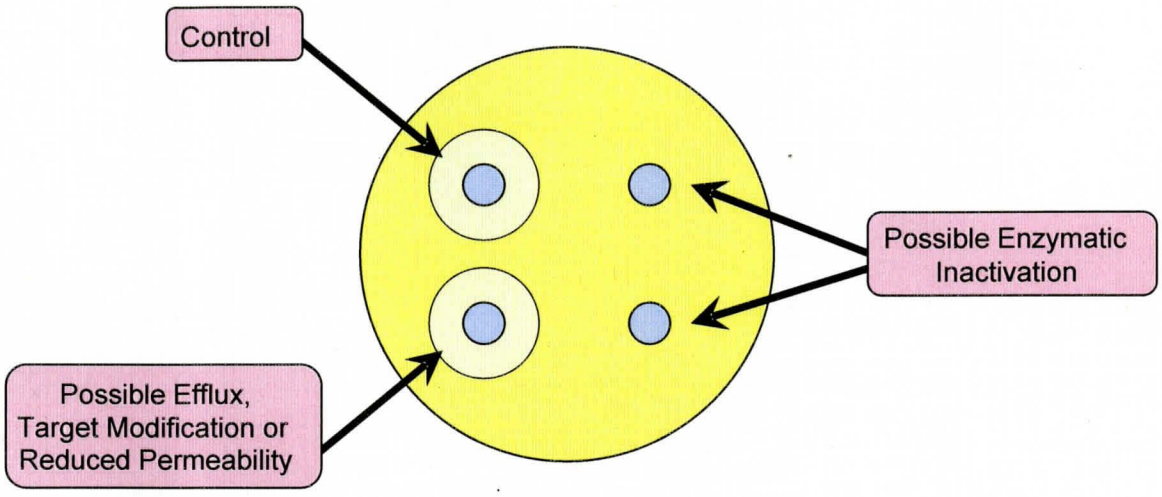


Figure 2.2 Diagram of Possible Enzymatic Inactivation.
Disappearance of a zone of clearing around a disk indicates a possible inactivation of the drug. Figure taken from [34].

2.5.3 – HPLC Assay of a Potential Tetracycline Inactivator

After identification of the possible inactivation of tetracycline by the soil isolate Kar21 via bioassay, an HPLC assay was performed to confirm this initial result. Cultures (5 mL) of the isolate in question were grown at 30°C and 250 rpm for 3 days, at which point 1 mL of the sample was centrifuged to remove cellular debris. Automated injection of 100 µL of sample was monitored via HPLC for changes in the tetracycline absorbance at 340 nm. Similar solvents and columns were used as in previous HPLC assays.

2.5.4 – HPLC Assay Monitorring [^{3}H] – Tetracycline

This assay was performed in the same manner as the HPLC assay mentioned above, however [^{3}H] -tetracycline (DuPont NEN Research Products) was used as the substrate. The Waters HPLC system from the Brown laboratory (McMaster University) was used for this experiment as it is equipped with a 717 autosampler, 486 absorbance detector, 600 pump and controller with an inline radiomatic flow scintillation analyzer (Packard) to measure the radioactivity of the samples.

2.5.5 – LC/MS Examination of Kar21

The cultures used for HPLC testing were used in the LC/MS experiments as well. Samples from each of the cultures were filtered using a 0.2 µm Acrodisc Supor syringe filter (Pall Lifes Sciences) to remove bacteria. Samples (100 µL) of media, media supplemented with tetracycline, media inoculated with Kar21, and media supplemented with tetracycline and inoculated with Kar21 were subjected to LC/MS using a Dionex Acclaim 120 C18 column. The column was equilibrated with 95% water and 5% acetonitrile both containing 0.05% formic acid. A linear gradient was developed over 20 min to 3% water and 97% acetonitrile.

3. Results

3.1 – Characterization of TetX-like Proteins

3.1.1 – Cloning, Overexpression and Purification

The proteins from *S. coelicolor*, and TetX (as a positive control) were over-expressed in *E. coli* BL21 (DE3) cells to facilitate purification on a Ni-NTA column using a linear imidazole gradient. Over-expression in *E. coli* BL21 (DE3) cells was obtained easily for most of the proteins, but expression of the *C. hutchinsonii* clone showed a greater yield in *E. coli* BL21 Star (DE3) cells. This strain carries a mutated *rne* gene that encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA [48].

Expression levels of these proteins varied, but sufficient amounts of protein were obtained from 1 L cultures of each to perform enzymatic assays (Table 3.1). Purification of these proteins using both gravity and FPLC systems gave relatively pure samples, but higher yields were obtained with the FPLC system and it was therefore used for the remaining purifications. The majority of the proteins were judged sufficiently pure based on Coomassie blue stained SDS-polyacrylamide gels (Figure 3.1) after a single pass through a NTA column. However a few of the proteins,

SCO7625 for example, repurified through a second NTA column. The SCO0252 protein was not pure after a secondary purification and was re-cloned with a C-terminal His tag. Purification of C-terminally tagged SCO0252 was performed in the same manner as for the other TetX-like proteins. Purification appeared to yield a clean sample with the C-terminal His tag and so these samples were used in the reactions with tetracycline and its analogs. The fractions containing SCO0484, SCO0252 and the Cytophaga protein were light yellow in solution suggesting the co-purification with flavin. The other proteins were all found to contain bound flavin when calculations using Beer's law were performed, however due to the low concentrations of proteins in these samples, they did not appear yellow.

Table 3.1: Comparison of TetX-like proteins.

Gene	% Similarity	Nucleotide Sequence Size	Predicted Protein Size	Amount of Bound Flavin	Ratio of Bound Flavin
tetX	100	1164 bp	44 kDa	$1.208 * 10^{-8}$ mol	1:1
SCO0252	39	1248 bp	46 kDa	$1.72 * 10^{-8}$ mol	2:1
SCO0484	40	1167 bp	41 kDa	$0.74 * 10^{-8}$ mol	1:3
SCO7223	36	1122 bp	39 kDa	$2.752 * 10^{-8}$ mol	1:2
SCO7625	40	1122 bp	40 kDa	$8.56 * 10^{-9}$ mol	1:5
SCO7705	39	1191 bp	42 kDa	$4.304 * 10^{-9}$ mol	1:3
Cytophaga	42	1164 bp	42 kDa	$1.288 * 10^{-8}$ mol	1:2

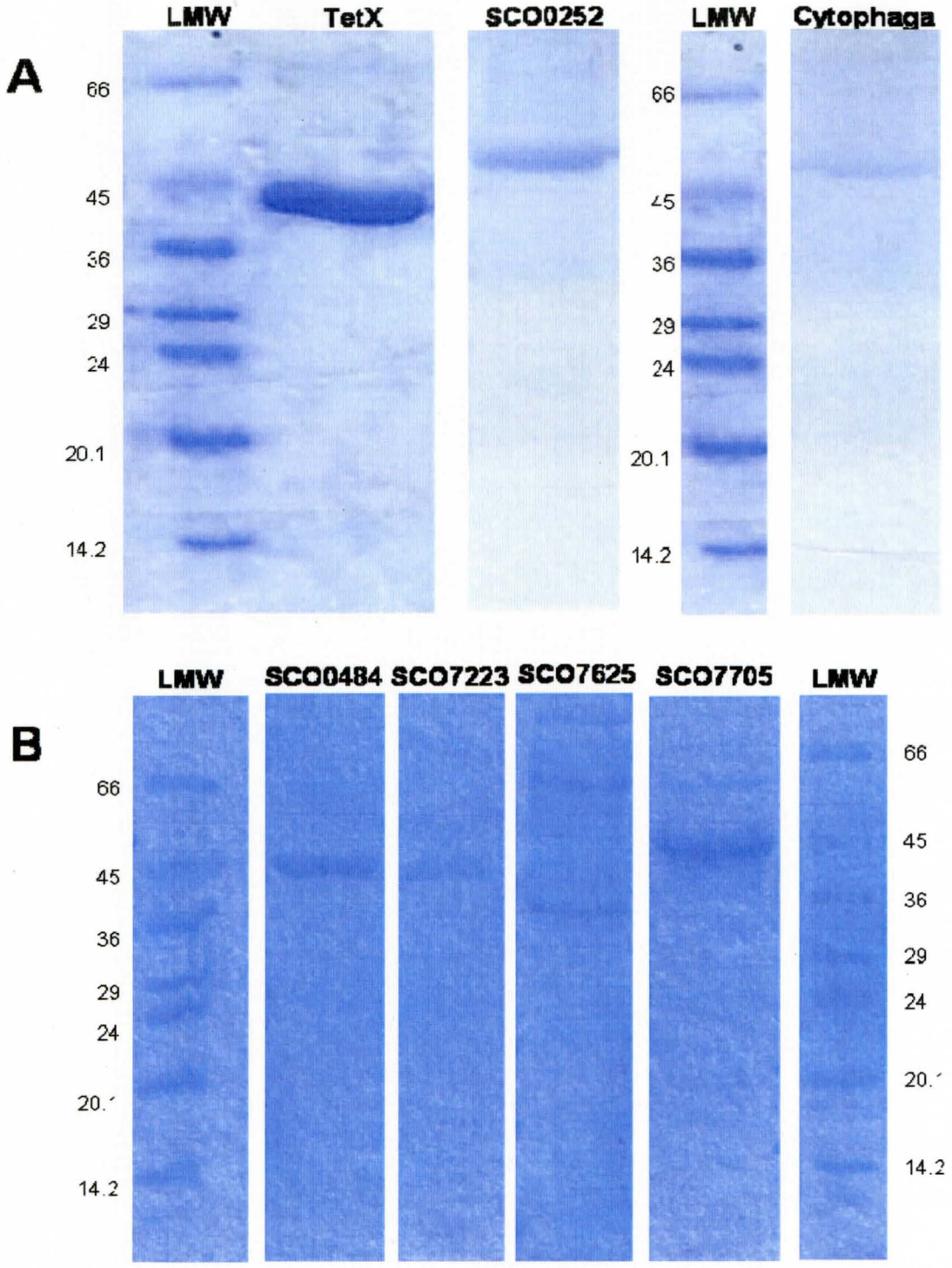


Figure 3.1: SDS-Polyacrylamide Gel Stained with Coomassie Blue Shows each of the Purified TetX-like Proteins. A) TetX, SCO0252 and Cytophaga proteins. B) Proteins from *S. coelicolor*. Each homolog is seen to be the appropriate predicted size, and sufficiently pure in solution to be used for further analysis.

No significant tetracycline resistance was observed in any case based on MIC values for each of the clones (Table 3.2).

Table 3.2: MICs of Tetracycline Against TetX-like Proteins. TetX used as a positive control.

Strain	Gene	Plasmid Backbone	MIC (μg/mL)
E. coli (DE3)	*SCO0252*	pDest17	4
E. coli (DE3)	*SCO0484*	pET28	4
E. coli (DE3)	*SCO7223*	pET28	4
E. coli (DE3)	*SCO7625*	pET28	4
E. coli (DE3)	*SCO7705*	pET28	4
E. coli Star (DE3)	*Cytophaga*	pDest17	< 1
E. coli (DE3)	*tet34*	pDest17	< 1
E. coli (DE3)	*tetX*	pET28	256
E. coli (DE3)	*pUC18*	pET28	< 2

Sequence alignments were constructed for the TetX-like proteins using ClustalW (Figure 3.2), and were used to construct a phylogenetic tree (Figure 3.3). Alignments were done using the ClustalW software, and an unrooted tree was constructed from these alignments using the neighbour joining method.

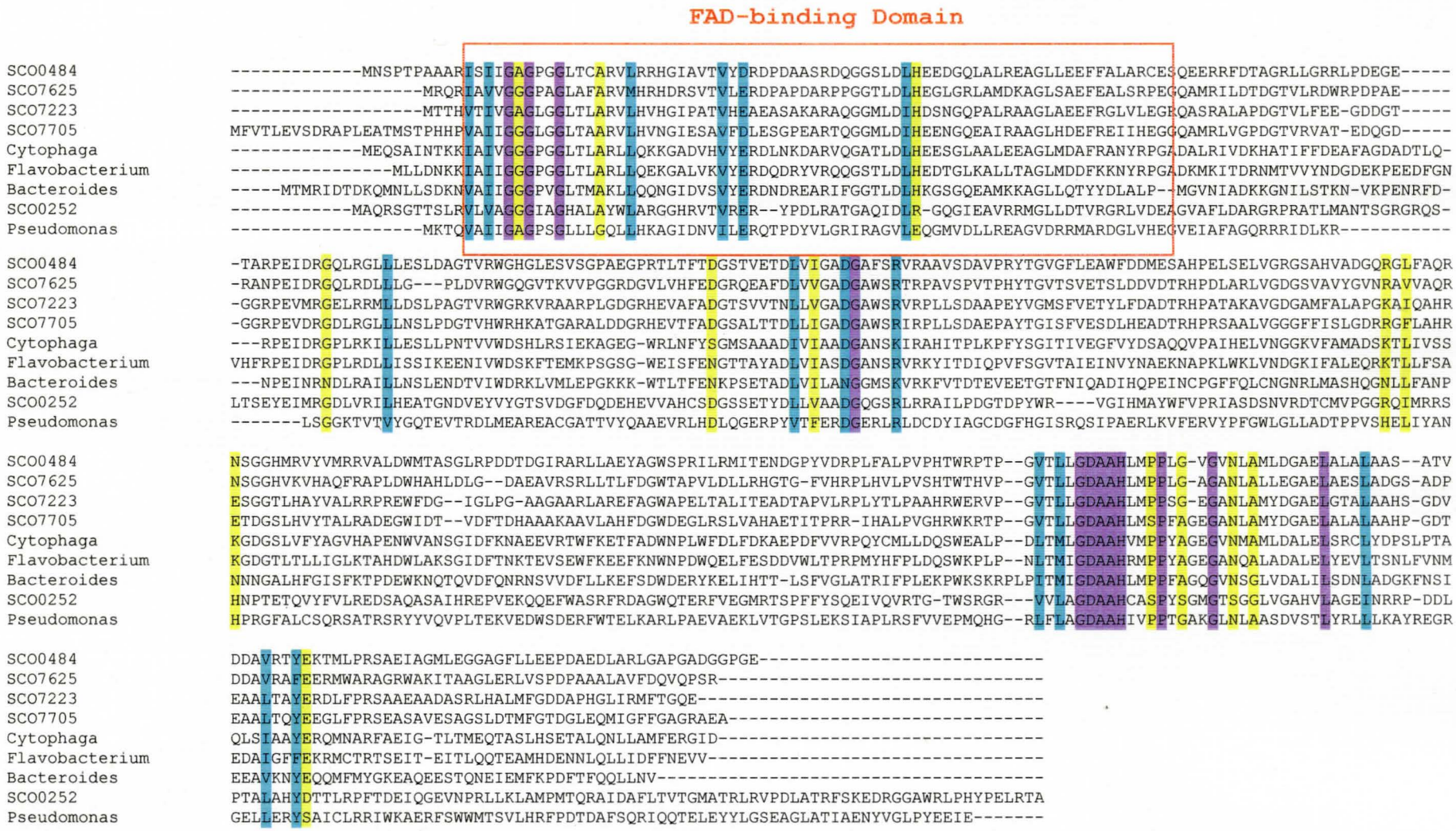


Figure 3.2: ClustalW Sequence Alignments of TetX-like Proteins. Multiple sequence alignment of putative monooxygenases sharing amino acid homology with TetX. All sequences contain a FAD-binding domain (boxed). Residues that are conserved in all sequences are shown in pink, similarly charged residues are shown in teal, and similar residues are shown in yellow.

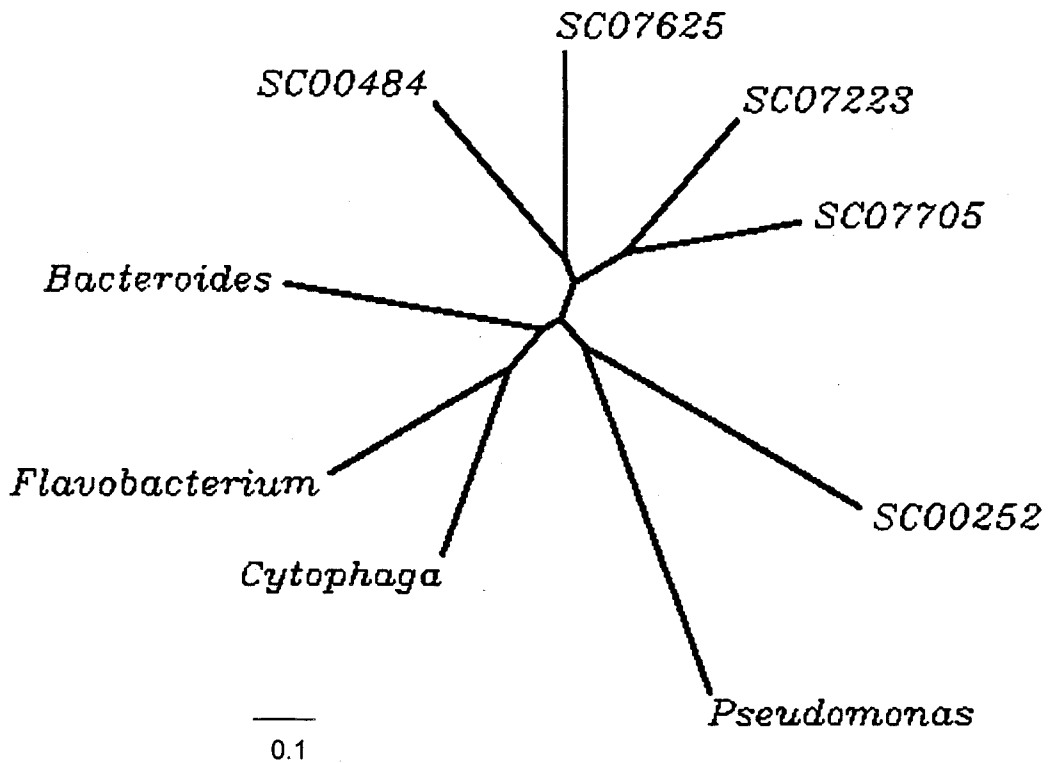


Figure 3.3: Unrooted Phylogenetic Tree of TetX-like Proteins. ClustalW alignment files were used to construct this tree via the neighbor joining method. Unrooted tree details the relatedness of the homologous sequences, however no conclusions can be drawn about ancestry. Scale bar refers to 0.1 amino acid substitutions per site

3.1.2 – Tetracycline Degradation Activity

The ability of the purified TetX-like proteins from *S. coelicolor,* and *C. hutchinsonii* to inactivate tetracycline was assessed by monitoring the reactions on HPLC and LC/MS. TetX was used as a positive control in all cases (Figure 3.4, Figure 3.5). No chemical modification of tetracycline

was detected with any of the purified TetX-like proteins in either the HPLC assays (Figure 3.6, Figure 3.7) or the LC/MS data (Figure 3.8).

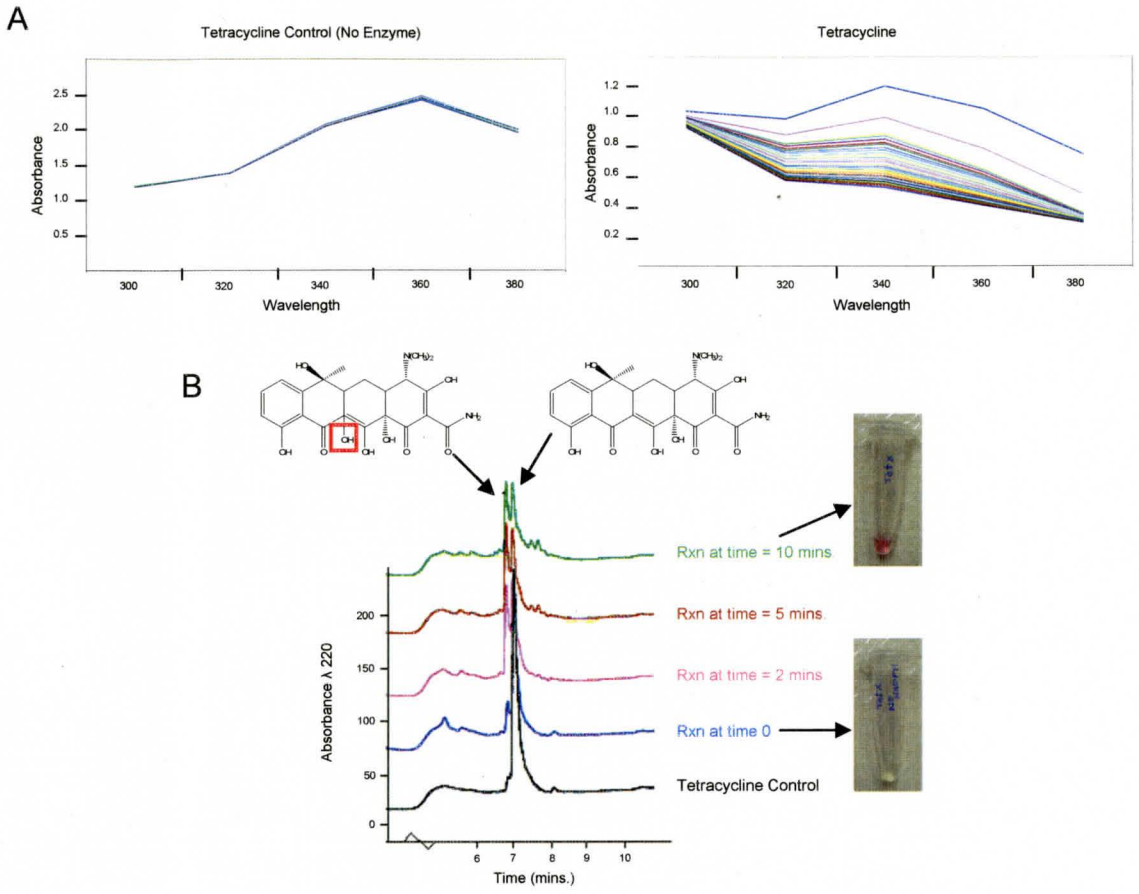


Figure 3.4: Reactions Performed using TetX as a Positive Control. When grown in LB media in an *E. coli* cell line in the presence of oxygen the breakdown of tetracycline results in a black product. (not shown) A) Decrease in NADPH absorbance associated with TetX reaction. B) HPLC chromatogram of TetX reaction showing a decrease in the tetracycline peak and the appearance of a second product peak. Colour change seen in eppendorf tube can also be used to follow the degradation of tetracycline.

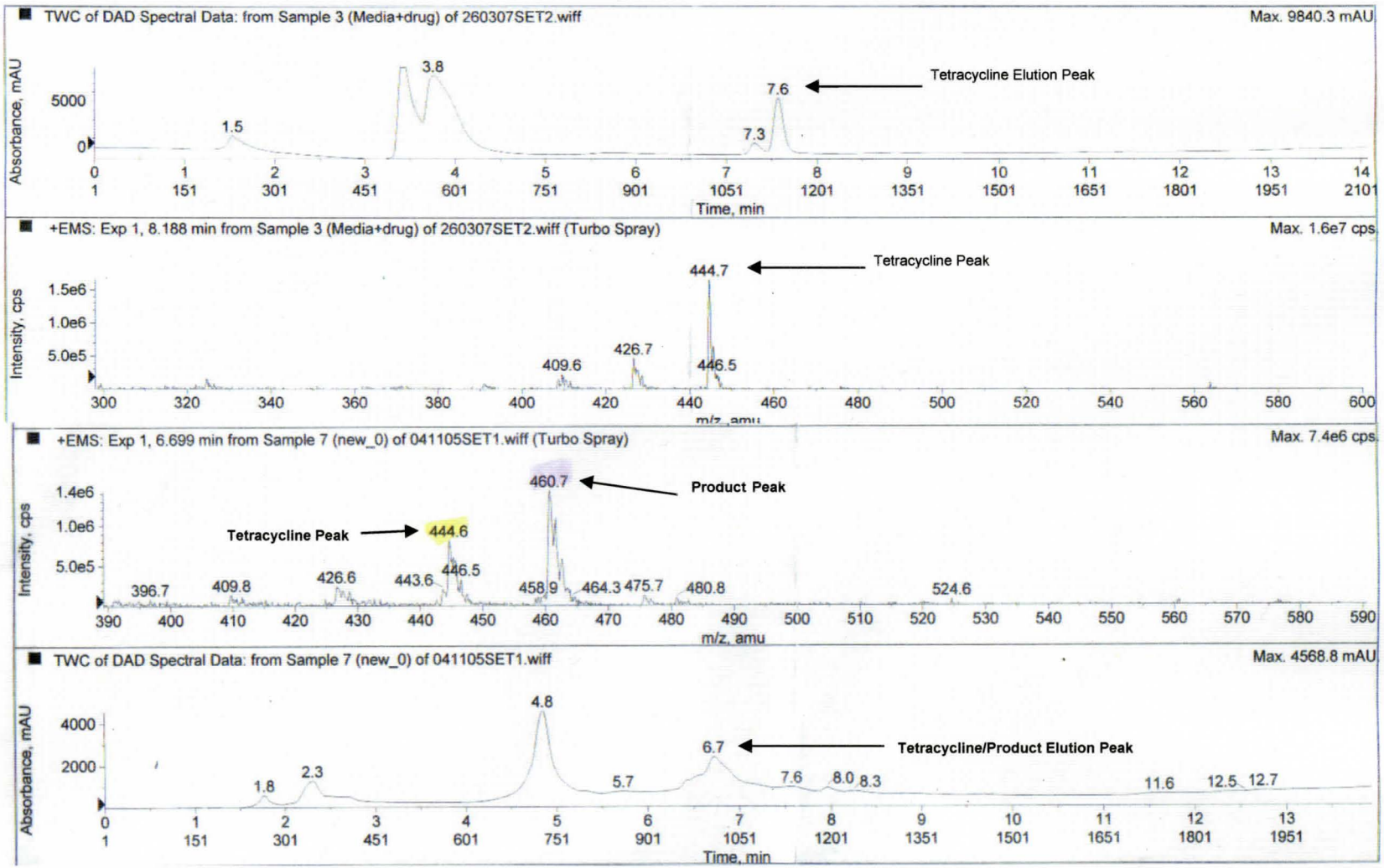


Figure 3.5: LC/MS Chromatogram of TetX reaction with Tetracycline. LC/MS chromatogram indicates through a shift in elution time, and a change in the mass of tetracycline, indicating that a chemical modification is occurring. A) LC chromatogram of tetracycline control. B) MS trace of tetracycline control. C) LC chromatogram of TetX reaction. D) MS trace of TetX reaction.

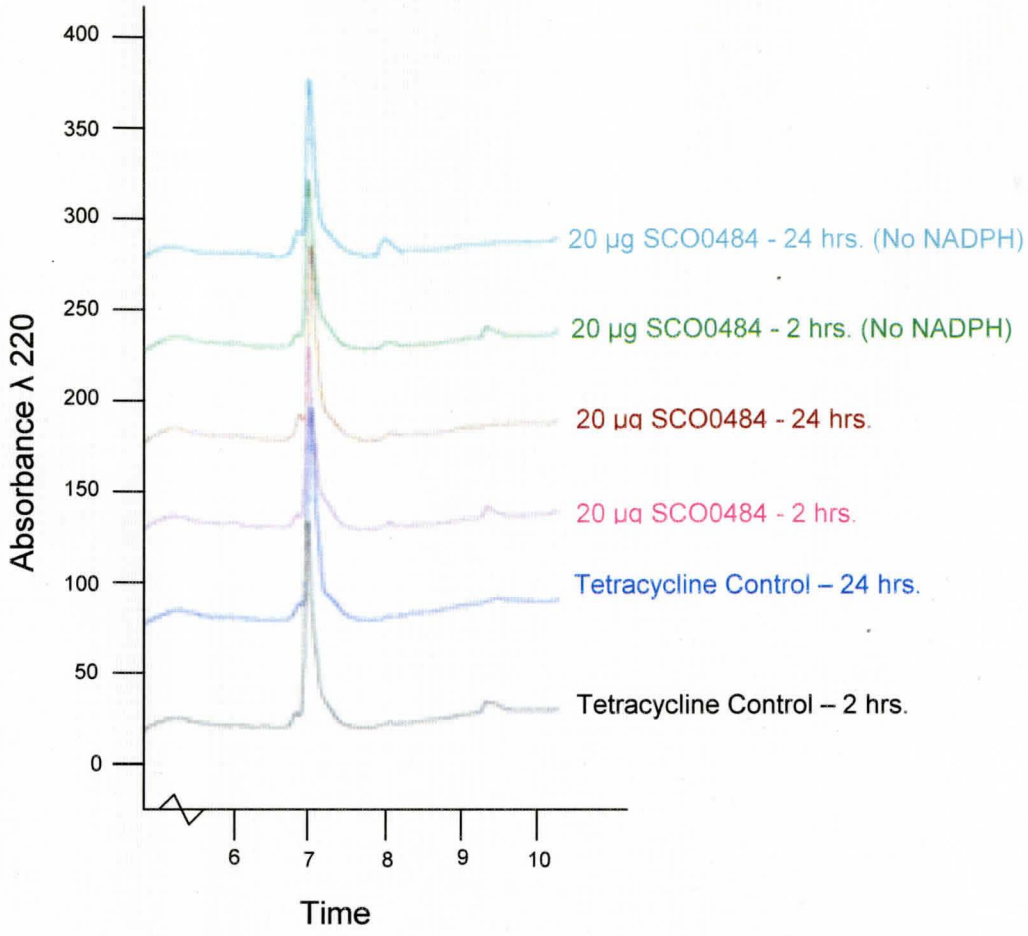


Figure 3.6: Representative HPLC Chromatogram of the TetX-Like Proteins. SCO0484 showed no change in the tetracycline peak after 24 hours under various conditions. Each of the proteins were tested in a similar fashion, however none of the over-expressed homologous proteins possessed tetracycline inactivating activity as seen by the lack of change in the tetracycline peak.

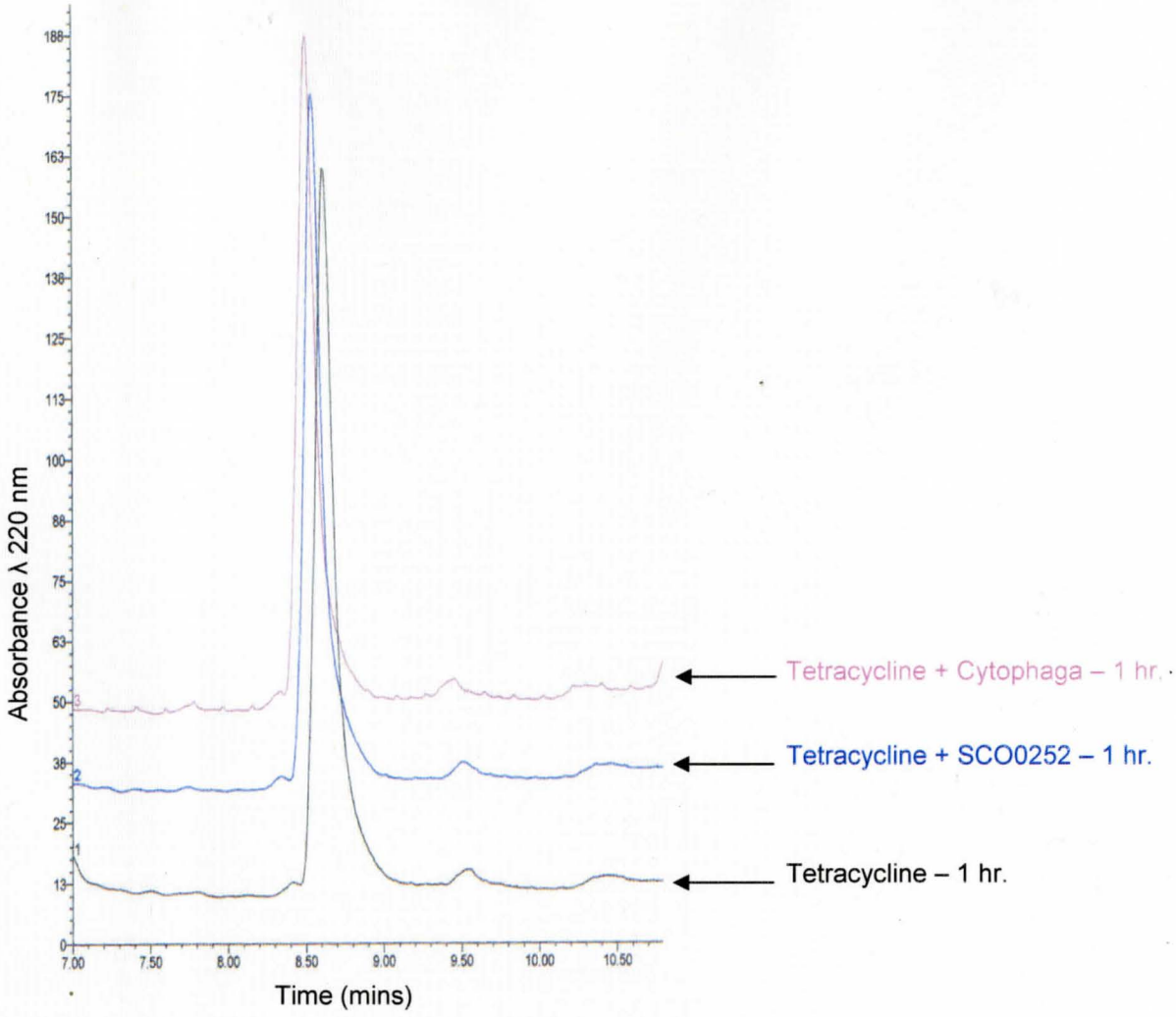


Figure 3.7: HPLC Chromatogram of SCO0252 and Cytophaga proteins. No inactivation seen with either of the proteins as indicated by the lack of change in the tetracycline peak.

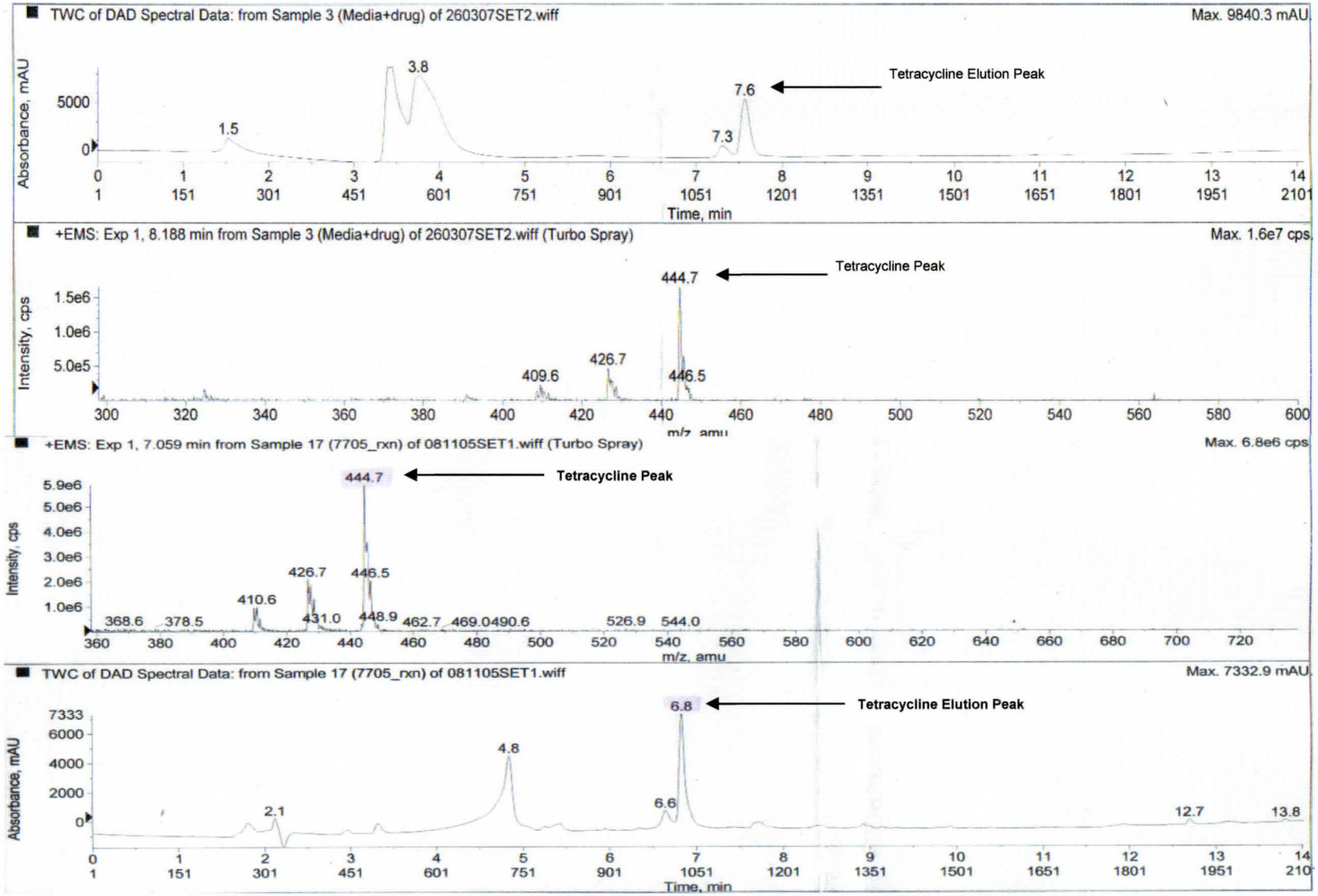


Figure 3.8: LC/MS Chromatogram of the *S. coelicolor* TetX-like Proteins. The over-expressed homologous proteins did not possess tetracycline inactivating activity as seen by the lack of change in the tetracycline mass. A) LC chromatogram of tetracycline control. B) MS trace of tetracycline control. C) LC chromatogram of SCO7705 reaction with Tetracycline. D) MS trace of SCO7705 reaction with Tetracycline.

3.2 – Characterization of Potential Tetracycline Inactivators

3.2.1 – Cloning, Overexpression and Purification

The *tet37* gene was not successfully amplified from *pUC119*, and was therefore not cloned and tested in the same manner as the other potential tetracycline inactivators.

The gene product from *tet34* was over-expressed in *E. coli* BL21 (DE3) cells to facilitate purification on a Ni-NTA column using a linear imidazole gradient. Over-expression in *E.coli* BL21 (DE3) cells was obtained easily for the clone, and sufficient amounts of protein were obtained from 1 L culture to perform enzymatic assays. Purification of this protein was done using a 5 mL Ni-NTA column on an FPLC system. The protein appeared sufficiently pure based on a Coomassie blue stained SDS-polyacrylamide gel after purification, but did not show any activity against tetracycline. Various amounts of protein were used in the reactions and each of the concentrations of protein were reacted against various tetracycline analogs to determine if this protein might be acting specifically. Oxytetracycline was the first tetracycline tested based on the conclusions drawn by Nonaka et al. who stated Tet34 is an oxytetracycline resistance determinant [45]. Several tetracyclines including anyhydrotetracycline,

chloretetracycline, methacycline, demeclocycline, doxycycline, minocycline and tigecycline were tested following this negative result, but the protein showed no inactivating activity against any of the analogs.

3.2.2 – Tetracycline Degradation Activity

The ability of Tet34 to inactivate tetracycline was also assessed by monitoring the reactions on HPLC and LC/MS. TetX was used as a positive control in all cases (Figure 3.4, Figure 3.5). No inactivation of tetracycline was seen with Tet34 in the HPLC assay or LC/MS data (Figure 3.9). Cultures for *tet34* in *E. coli* on *pDest17* and *tet37* in *E. coli* on *pUC119* were grown in 10 mL flasks of LB containing 40 µg/mL tetracycline. No growth was observed suggesting that these genes are not responsible for the inactivation of tetracycline.

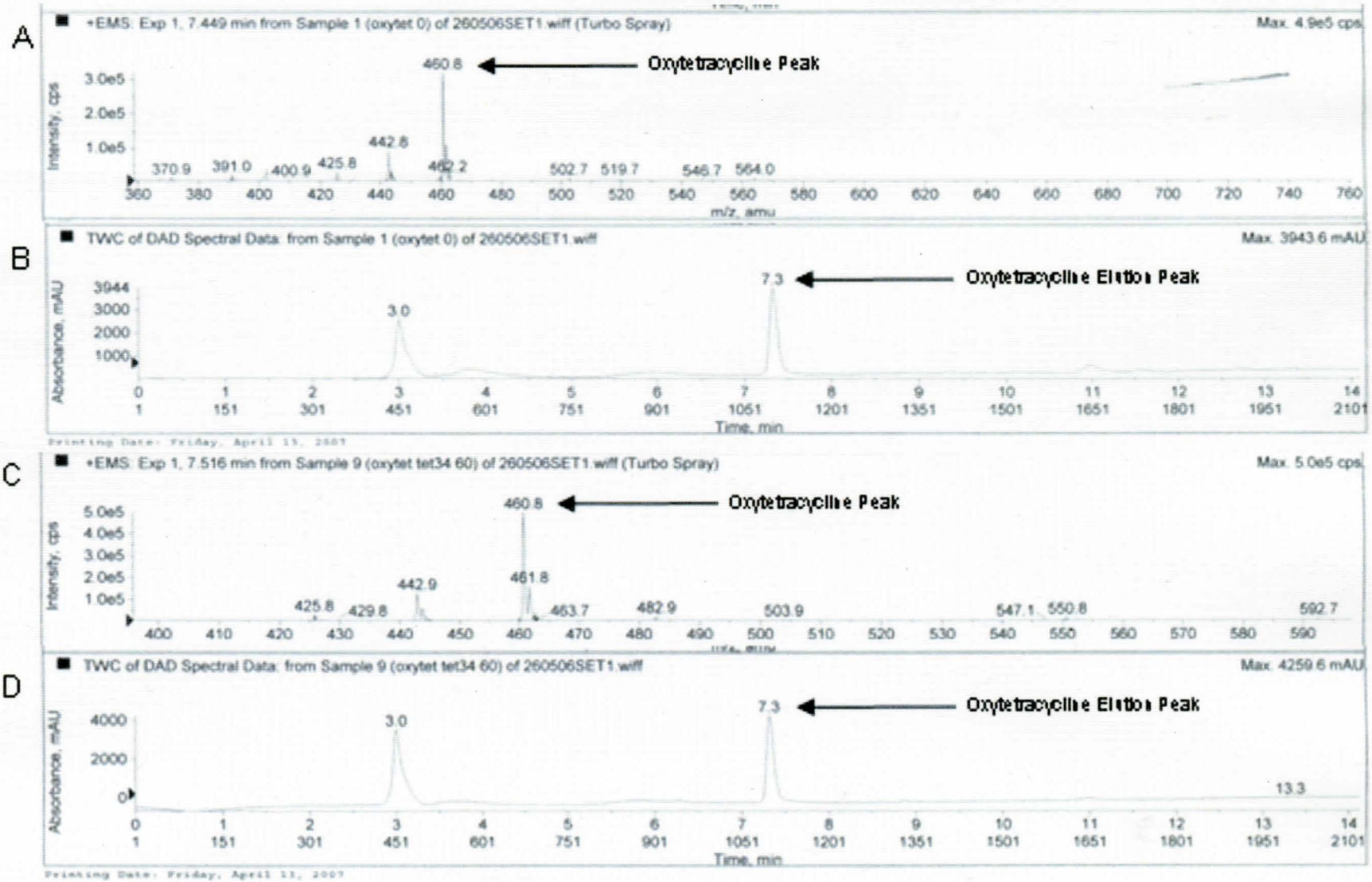


Figure 3.9: LC/MS Chromatogram of the Potential Tetracycline Inactivator Tet34. The over-expressed protein did not possess tetracycline inactivating activity as seen by the lack of change in the tetracycline mass. A) MS trace of tetracycline control. B) LC chromatogram of tetracycline control. C) MS trace of Tet34 reaction with Tetracycline.D) LC chromatogram of Tet34 reaction with Tetracycline.

3.3 – Activity of Similar Proteins Against Tetracycline Analogs

3.3.1 – NADPH Monitorring using Plate Reader

The TetX-like proteins SCO0252, SCO0484, SCO7223, SCO7625, SCO7705, Cytophaga, as well as the potential tetracycline inactivating enzyme Tet34 were reacted against various tetracycline analogs (Figure 2.1) and the absorbance of NADPH was monitored using a SpectraMax plate reader (Figure 3.10). The control experiment showing the reaction of tetracycline and TetX showed a decrease in the absorbance of NADPH over time, indicative of NADPH oxidation (Figure 3.4).

Each of the purified proteins was reacted against a series of tetracycline analogs in a 96 well format but none showed the characteristic decrease in the NADPH peak at 340nm (Figure 3.10 B-D).

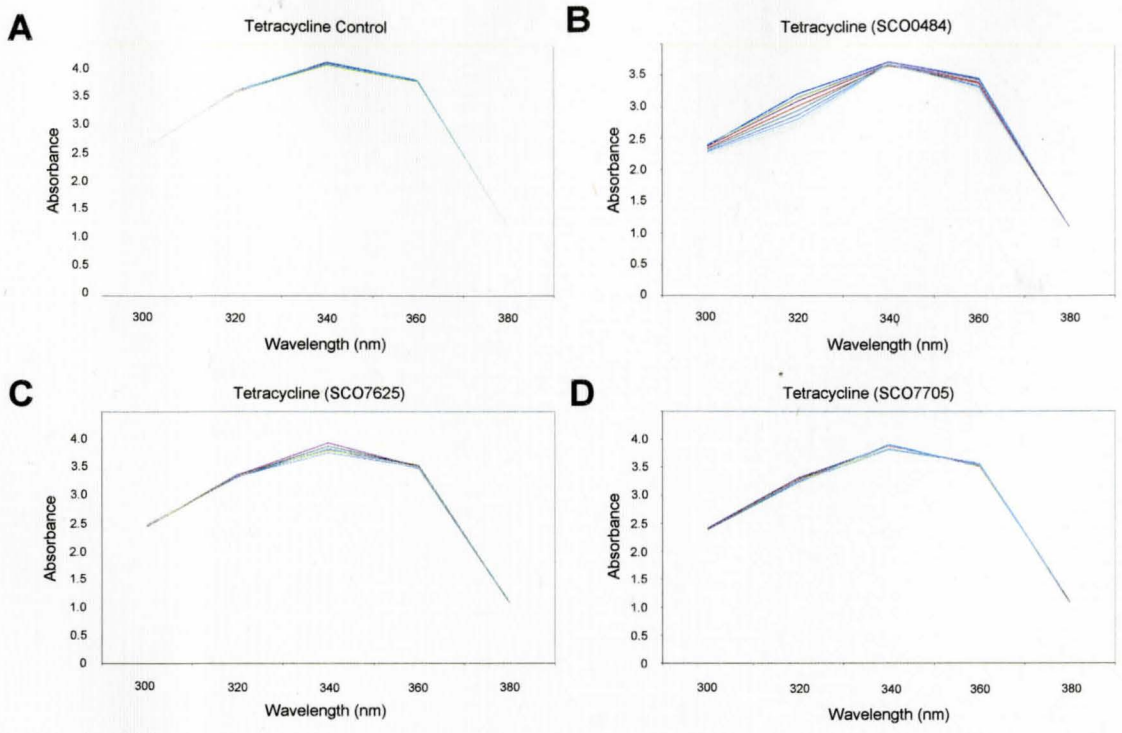


Figure 3.10: Representative Diagram of Reactions of TetX-like Proteins Against Various Tetracycline Analogs. Absorbance of NADPH was monitored at 340 nm on a spectramax plate reader. A) Tetracycline control. No change in absorbance was seen for TetX-like proteinous proteins SCO0484 (B), SCO7625 (C), or SCO7705 (D) suggesting that no chemical reaction had occurred.

3.4 – Actinomycete Library Screen

3.4.1 – Identification of Inactivating Isolates

Bioassays performed on each of the tetracycline resistant isolates from the actinomycete library revealed one potential inactivator. The bioassays were repeated a minimum of three times to ensure reproducibility. The isolate named Kar21 showed no zone of inhibition after 2 days of

incubation with tetracycline, indicating a possible inactivation event (Figure 3.11).

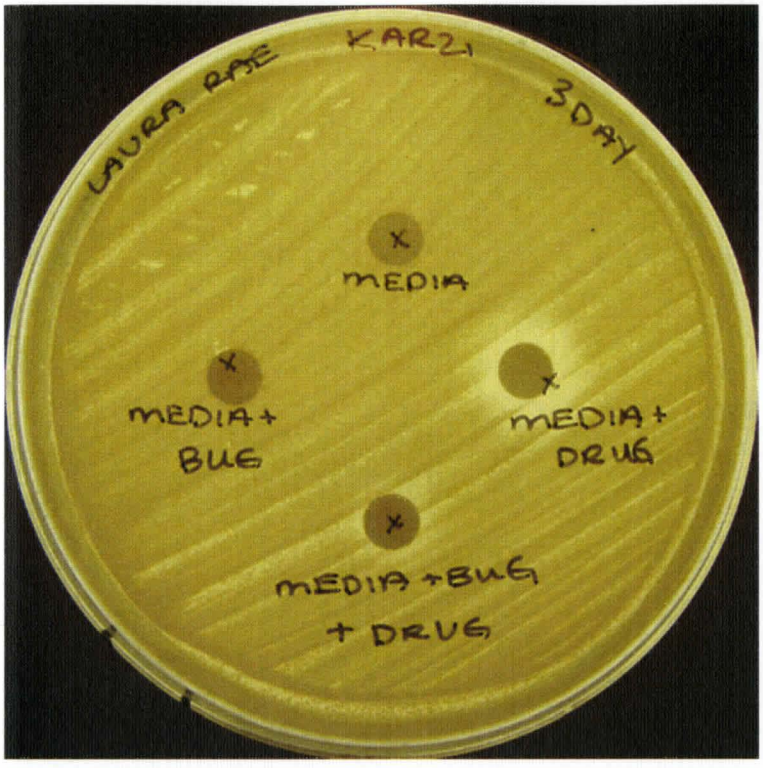


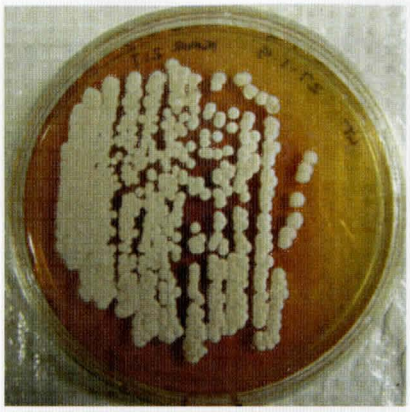
Figure 3.11: Potential Inactivation of Tetracycline by the Isolate Kar21. The decreased zone of inhibition indicates a possible modification of the drug.

3.4.2 – Characterization of Inactivating Isolates

Studies were performed to examine the physical characteristics of isolate Kar21, to better understand its identity. The isolate grew similarly to other streptomyces species when grown on minimal streptomyces isolation media (Figure 3.12A) [49]. Scanning electron microscopy was also used to visualize the isolate at a higher magnification (Figure 3.12B). From the

SEM pictures, this bacteria appeared to sporulate similarly to other members of the genus Streptomyces, but the spores had a distinct cylindrical shape, making it distinguishable from other species. 16s rRNA sequencing was performed on this isolate using the degenerate primers BACF and BACR designed for use with multiple Streptomycete species. Once sequencing results were obtained, an alignment was made that determined, due to the highly conserved sequences aligned, that this isolate is a member of the genus Streptomycetes, distinguishing it from other members of the Actinomycete family, with the closest species being *S. hirsutus* [49-51] (Figure 3.13).

A



B

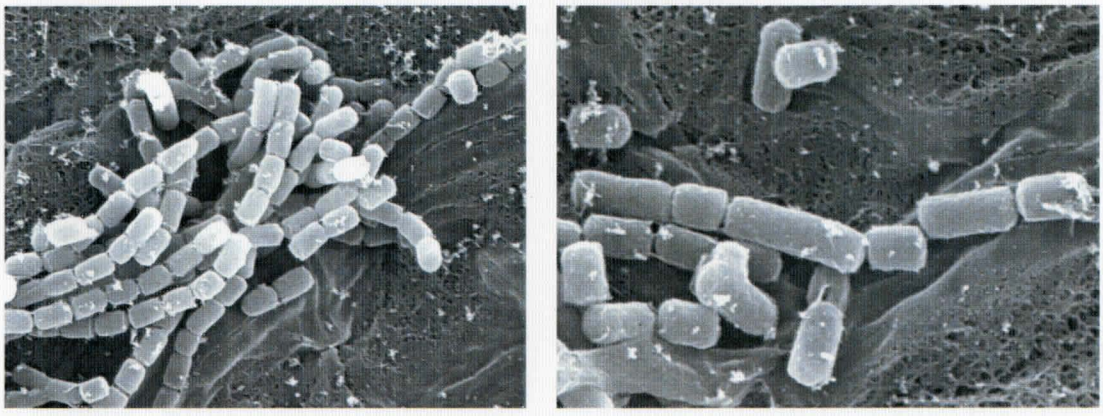


Figure 3.12: Phenotypic Examination of a Potential Tetracycline Inactivator from the Actinomycete Library. A) Growth similar to that of other Streptomycete species exhibited on isolation media. B) Scanning electron micrographs exhibit the isolate in the sporulating stage of its growth cycle. SEM images were obtained at 5000X and 10000X magnification respectively.

```
Kar21                -------------------------NNNNNNNNNGNNGNNNNNNNNNNTGCNAGTCGAACGATGAAGCCCTTCGG
S.hirsutus           ------------------AGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGG
S.violaceorectus     --------------------ACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGG
S.phaeochromogenes   ---------------CTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGG
S.termitum           -------------------GACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGG
S.bikiniensis        AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGG

Kar21                GGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGG
S.hirsutus           GGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGG
S.violaceorectus     GGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGG
S.phaeochromogenes   GGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGG
S.termitum           GGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGG
S.bikiniensis        GGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGG

Kar21                GTCTAATACCGGATACGACCTGGGAAGGCATCTTCCCGGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGC
S.hirsutus           GTCTAATACCGGATACGACCTGGGAAGGCATCTTCCCGGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGC
S.violaceorectus     GTCTAATACCGGATACGACCTGGGAAGGCATCTTCNCGGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGC
S.phaeochromogenes   GTCTAATACCGGATACGACCTGGGAAGGCATCTTCCCGGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGC
S.termitum           GTCTAATACCGGATACGACCTGGGAAGGCATCTTCCCGGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGC
S.bikiniensis        GTCTAATACCGGATACGACCTGGGAAGGCATCTTCTCGGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGC

Kar21                CTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACA
S.hirsutus           CTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACA
S.violaceorectus     CTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACA
S.phaeochromogenes   CTATCAGCTTGTTGGTGGGGTAACGGCCCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACA
S.termitum           CTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACA
S.bikiniensis        CTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACA

Kar21                CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG-CGCAAGCCTGA
S.hirsutus           CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACCAATGGGCGCAAGCCTGA
S.violaceorectus     CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG-CGCAAGCCTGA
S.phaeochromogenes   CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG-CGCAAGCCTGA
S.termitum           CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG-CGCAAGCCTGA
S.bikiniensis        CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG-CGCAAGCCTGA
```

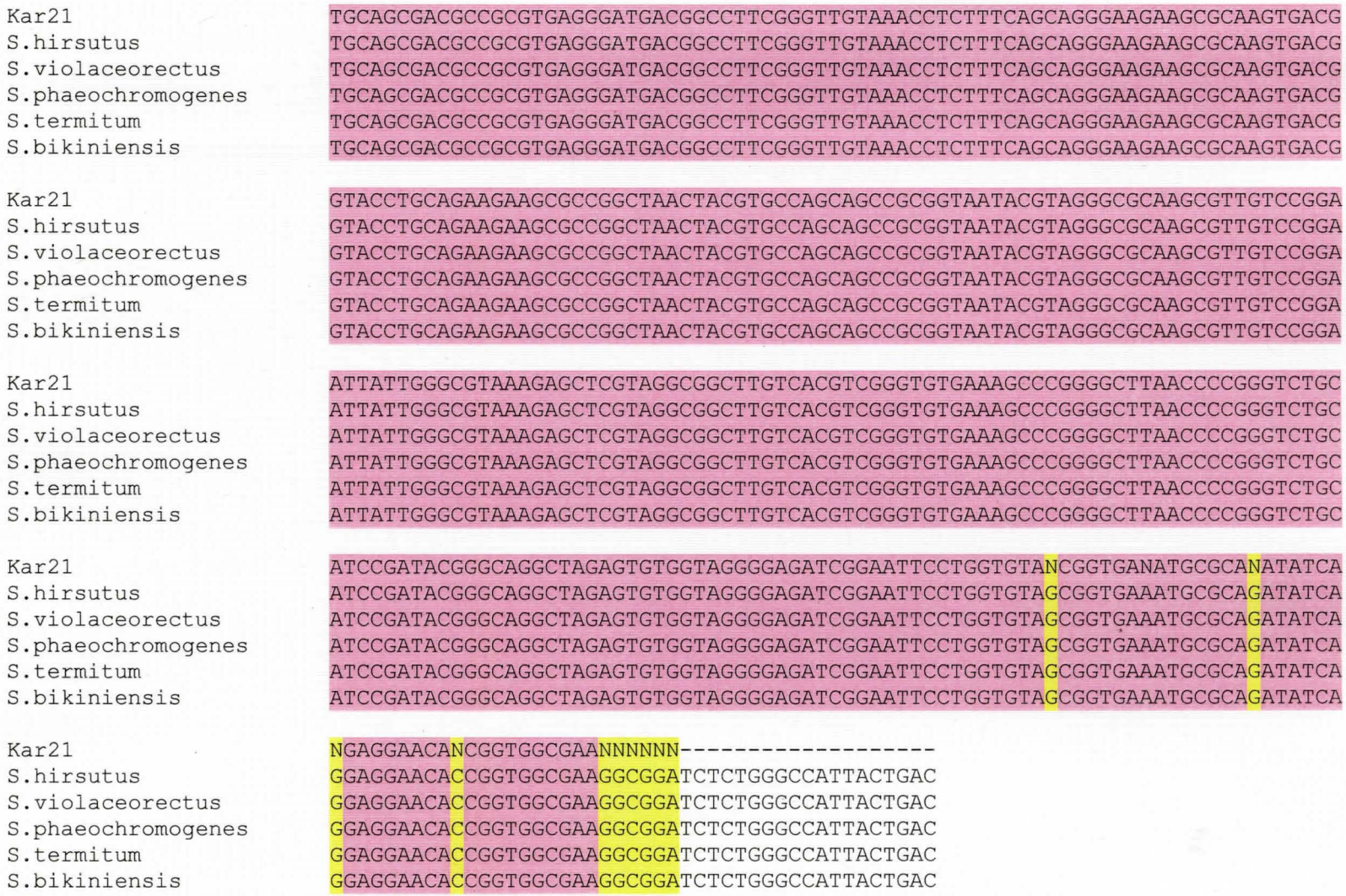


Figure 3.13: 16S RNA Alignments done for Comparision with Kar21. Highly conserved sequences from previously sequenced Streptomyces and the isolate Kar21 indicate that this bacterium is a member of the genus Streptomyces.

3.4.3 – Examination of Activity of Potential Tetracycline Inactivator

Further analysis of Kar21 using HPLC to detect changes in inoculated cultures grown in the presence of tetracycline, indicated no change in the antibiotic peak (Figure 3.14). Use of the Waters HPLC system in the Brown Laboratory (McMaster University) was required when the series of reactions was repeated using [^{3}H] -tetracycline as it is equipped with an inline radiomatic flow scintillation analyzer (Packard). Analysis of the data obtained from this work also indicated that no inactivation of tetracycline was achieved (Figure 3.15). The samples used for HPLC analysis were also filtered and analysed on the LC/MS. These data were consistent with those found on the HPLC, indicating no inactivation of the drug had occurred (Figure 3.16).

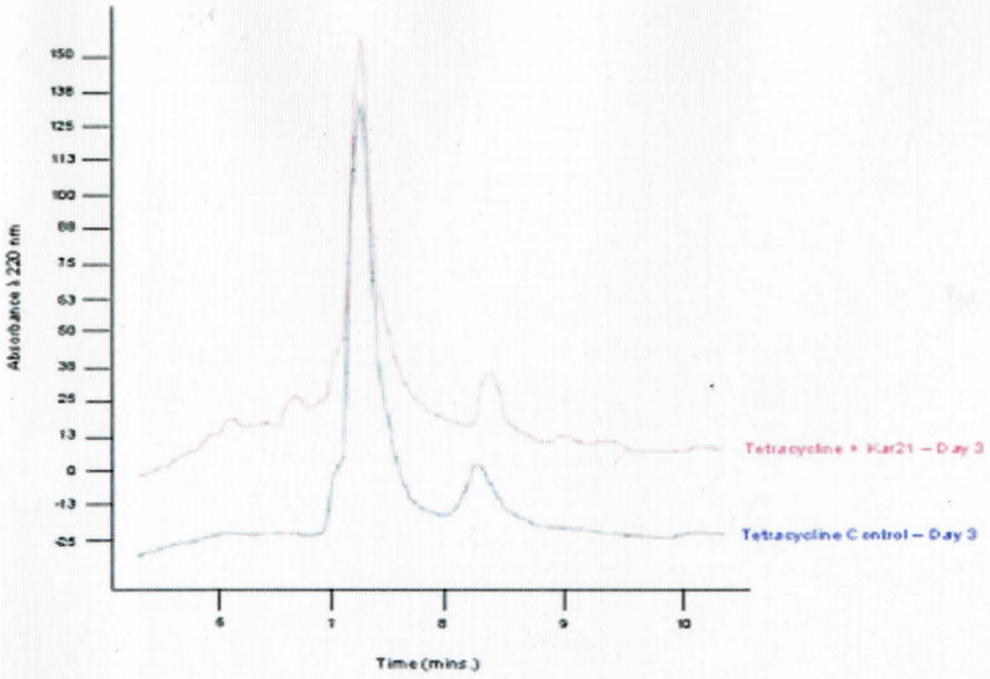


Figure 3.14: Inactivation Assay of Tetracycline by the Kar21 Isolate from the Actinomycete Library. HPLC chromatogram does not indicate inactivation of tetracycline as the tetracycline peak does not undergo significant changes.

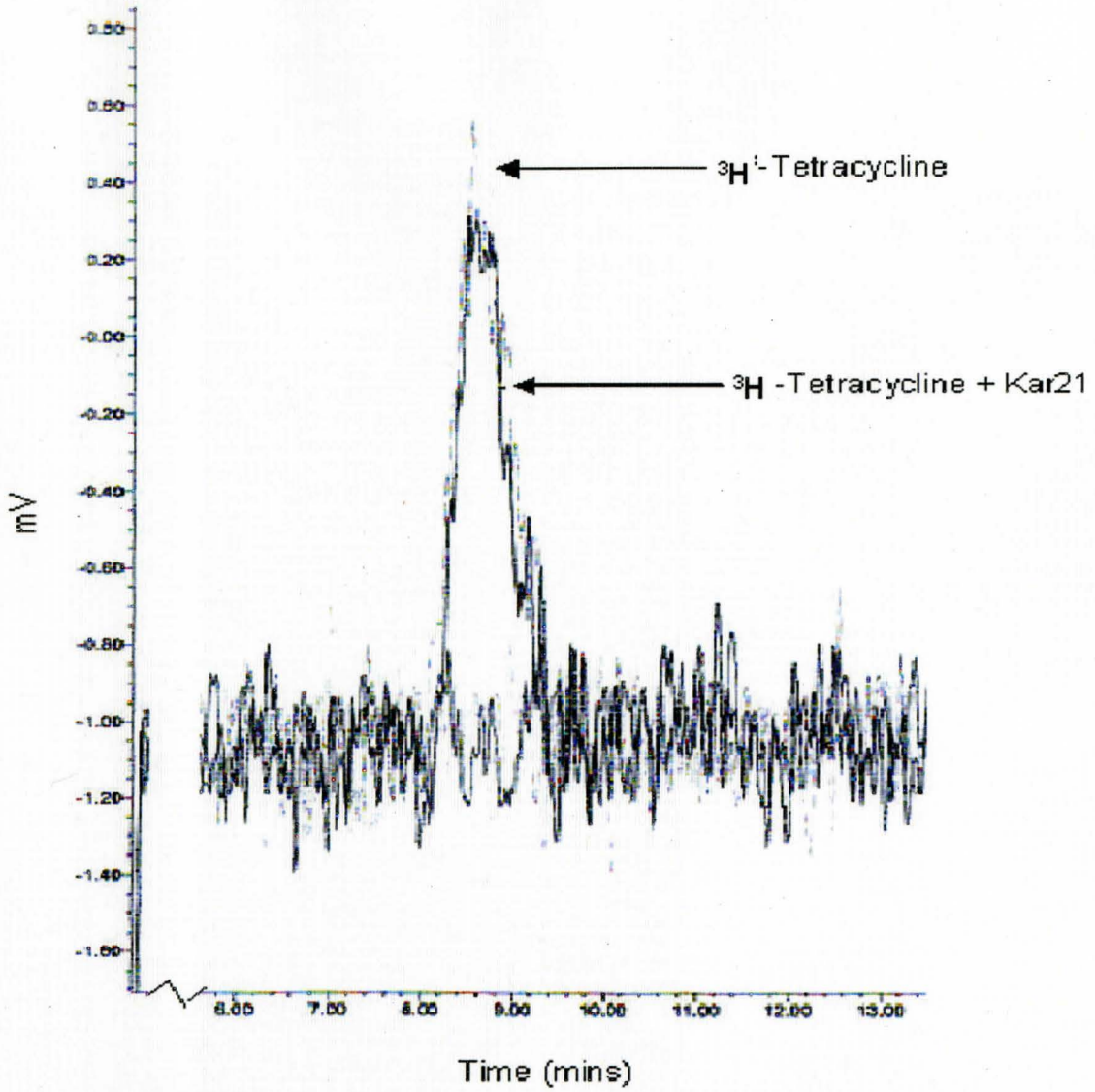


Figure 3.15: Following [^{3}H]-Tetracycline to Determine the Inactivation potential of Tetracycline by the isolate Kar21. Radioactive trace does not indicate inactivation of tetracycline as radioactivity is not detected anywhere other than in the tetracycline peak.

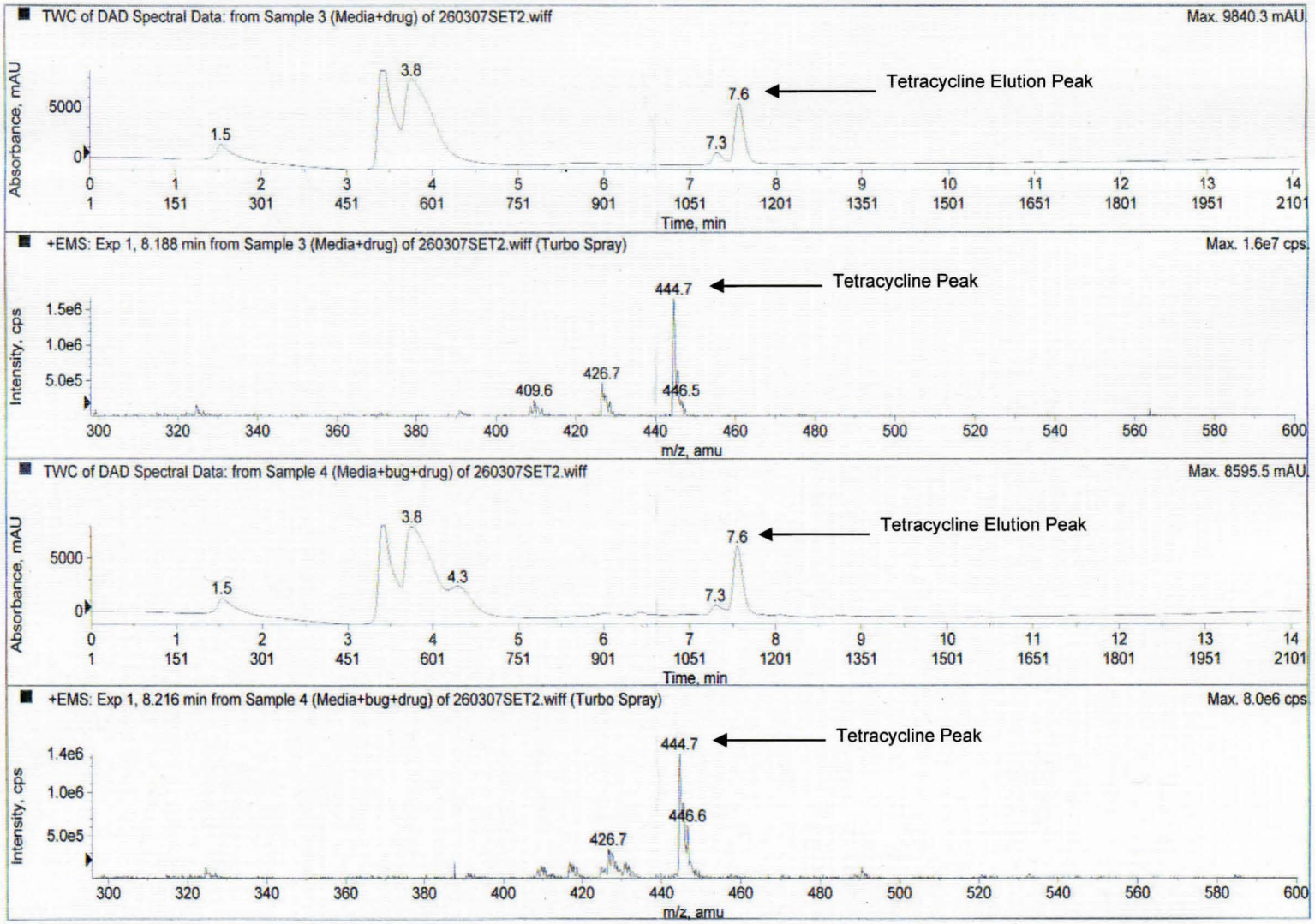


Figure 3.16: LC/MS Chromatograms Examining Tetracycline Inactivation by the isolate Kar21 from the Actinomycete Library. A) Elution peak of tetracycline control. B) Mass of tetracycline control. C) No shift in tetracycline elution peak in the presence of the Kar21 isolate. D) Mass of Tetracycline in the presence of Kar21 does not change indicating no chemical modification.

4. Discussion

4.1 – Characterization of TetX-Like Proteins in *Cytophaga hutchinsonii*, and *Streptomyces coelicolor*

4.1.1 – *S. coelicolor*

Tetracycline resistance is a common phenomenon, with efflux and ribosomal protection resistance being extremely widespread in pathogenic and environmental bacteria [1, 4, 7, 11, 12, 20, 26, 47, 52]. The latter is presumably due to the fact that this class of antibiotics is primarily produced by various actinomycete species. Enzymatic inactivation of the drug, however, has only been shown in an anaerobic *Bacteroides* species. This bacterium produces a 44 kDa cytoplasmic protein that has the ability to chemically modify the structure of various tetracyclines at the β-diketone ring system, rendering the antibiotic inactive (Figure 4.1) [28]. Work done in the Wright laboratory by Gloria Yang showed that this protein requires oxygen, NADPH, and flavin to modify the structure of tetracycline. It was also determined by G. Yang that when expressed in *E. coli* growing in the presence of tetracycline, that this protein has the ability to turn the media a black colour (Figure 1.6A). In an attempt to identify homologous proteins, BLAST searches were performed.

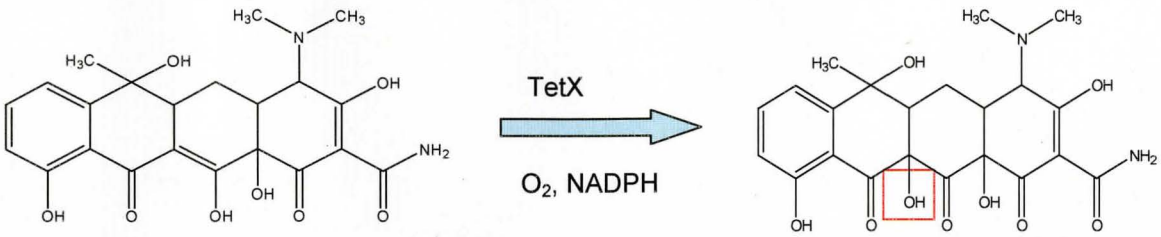


Figure. 4.1: Reaction of TetX with tetracycline. Addition of a hydroxyl group at the C-11a position renders the antibiotic void of antibiotic activity.

The original BLAST search was done using the TetX protein sequence and revealed five similar proteins encoded in the *S. coelicolor* genome. Homologous sequence similarity ranged from 36 to 42% with E-values of 4e-38 through 2e-33. Of particular interest, was that each of the five genes were found to have a putative TetR homolog either directly upstream or downstream of the gene in question (Figure 4.2). TetR proteins function as a set of well-defined regulators of tetracycline resistance and other gene expression mechanisms. In the case of tetracycline resistance, when tetracycline is present, TetR is bound by the antibiotic relieving transcriptional repression of the resistance gene [41]. Because of the proximity of the putative TetR proteins to the genes of interest in this study, it was thought that perhaps these genes could be

regulated in a similar fashion.

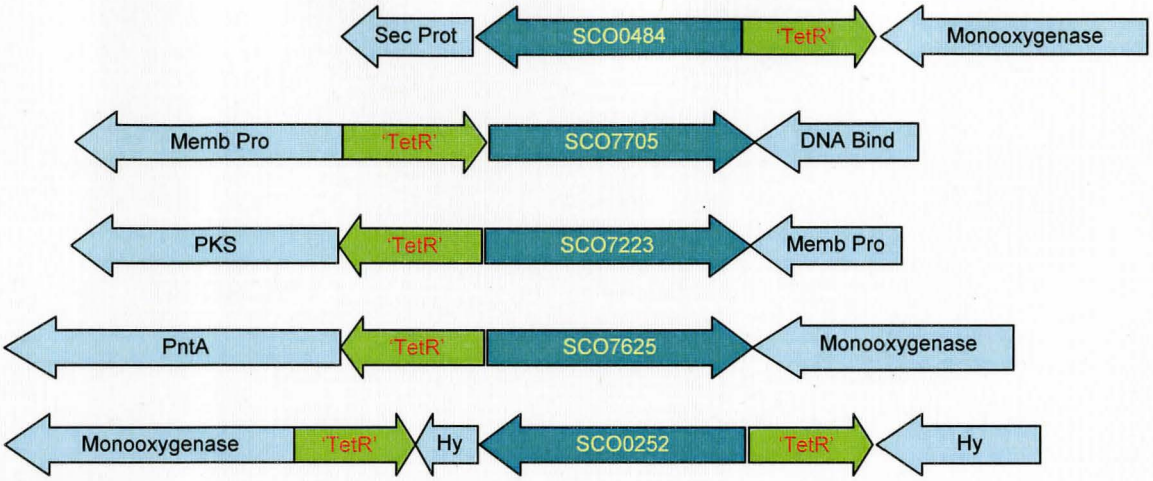


Figure 4.2: TetX-like Proteins in S. coelicolor. Gene map shows a TetR directly up or downstream of each of the five genes in question.

Initial cloning of these genes had been done by past members of the Wright lab, L. Ejim and T. Patel, therefore over-expression and purification was easily performed in most cases. In the case of the gene SCO0252 purification did not yield a sufficiently pure protein as determined by SDS-polyacrylamide gels. A new clone was therefore made for this gene with a C-terminal Histag. Possibly due to the high GC content of Streptomyces, cloning was more difficult than expected. After purification, the sample was pure and was therefore used in the remaining reactions.

Each of the homologs was over-expressed, purified and shown to purify with bound flavin. This was a positive result as TetX is a flavin-dependent

monooxygenase, which suggested that these proteins might have a similar function.

4.1.1.1 – Activity Against Tetracycline and its Analogs

Once initial calculations had been performed to determine amounts of protein and the ratio of bound flavin, the homologous proteins were reacted with tetracycline to determine if any modification would occur. Amounts of protein were determined using the standard Bradford technique [46]. Amount of flavin was determined by measuring the absorbance of the sample at 375 nm, followed by calculations using Beers Law (Table 4.1). In each of the five cases, reactions were left for a minimum of 24 hours, but no change in the tetracycline structure could be observed through our methods. Multiple methods were used to verify this result in each case including bioassay, HPLC, and LC/MS. The homologous TetX proteins were reacted with 11 various tetracycline analogs to determine if chemical modifications would occur. No changes were observed with any of the proteins. It should be noted however, that various concentrations of each of the analogs were used and that each of the subsequent reactions were allowed to proceed for variable lengths of time in order to determine without a doubt that no inactivation of the drug was occurring.

In retrospect, the fact that these proteins do not function similarly to TetX was not unexpected considering the % amino acid identities are not extremely high, and *S. coelicolor* is tetracycline sensitive. Furthermore, expression of the genes in an *E. coli* system showed no tetracycline resistance. Expression was optimized for each of the clones and the proteins were shown to be expressed via detection using Western Blot (results not shown). Each of these proteins was annotated as a putative monooxygenase, and sequence analysis suggested the ability to bind flavin. As a result of the amino acid sequence homology and the predicted functions these proteins were chosen for further analysis. It is apparent that these proteins are not involved in tetracycline inactivation in *S. coelicolor* and therefore must have another function. Recently it was shown that SCO7223 is linked to the synthesis of a novel siderophore, coelichelin [53]. Due to the *tetR* genes in close proximity in the gene clusters, it is probable that these genes have some role in the cell involving tetracycline-like molecules, but more work needs to be done in this area to elucidate that function.

4.1.2 – *C. hutchinsonii*

At the beginning of this study, the highest amino acid similarity TetX-like protein identified by BLAST search was from *Cytophaga hutchinsonii* (42%). Because *Cytophaga* and *Bacteroides* are within the same bacterial

group, we speculated that this would be the most likely gene to have similar function. The gene location showed differences from the *S. coelicolor* similar sequences with no TetR present, but a DNA binding protein is adjacent (Figure 4.3). Growing this bacterium was more difficult than expected, as *Cytophaga* has a growth requirement of cellulose [54]. Initially, growth was achieved by growing a small culture in liquid Dubos media with a piece of filter paper in the tube (Figure 4.4A). This culture was then used to prepare plates. Plates of Dubos media were prepared, and a piece of filter paper was cut to fit on top of the agar. The liquid culture was then pipetted onto the filter paper and allowed to grow for up to one week to obtain a useful amount of growth (Figure 4.4B). Once growth conditions had been optimized and gDNA had been prepared, cloning of this gene was done using the Gateway Cloning System with an N-terminal His tag.

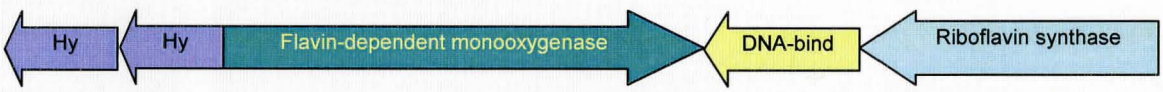


Figure 4.3: TetX-like protein in *C. hutchinsonii*. Gene map of the homologous sequence found via BLAST search. The flavin binding domain is well conserved in the sequence. Hypothetical proteins shown in purple (Hy).

Over-expression and purification of the protein yielded the same results as with the *S. coelicolor* homologs.

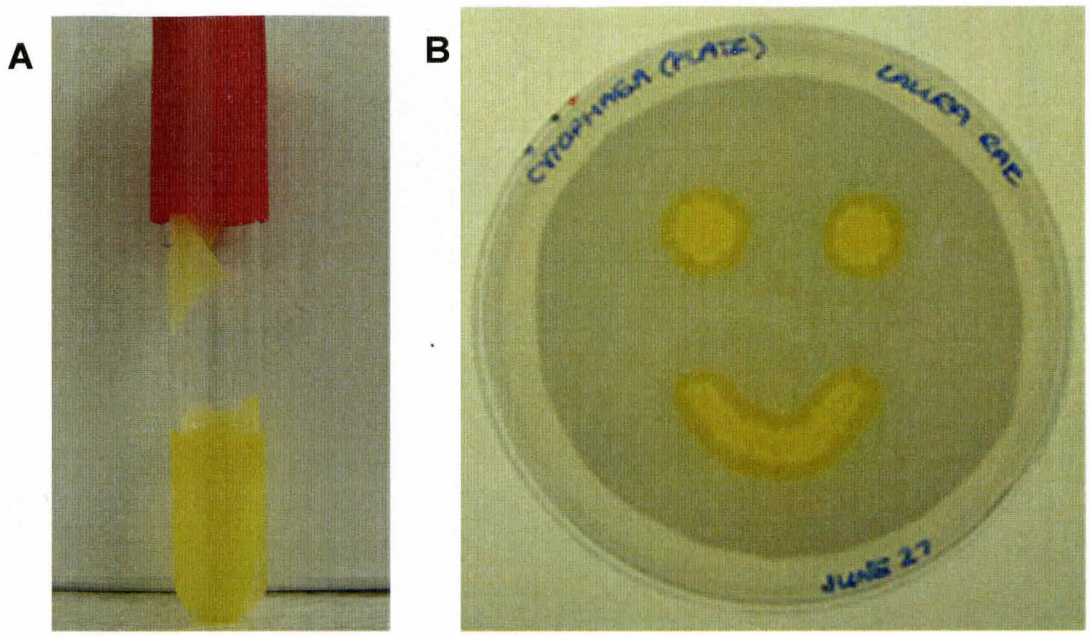


Figure 4.4: Growth of Cytophaga hutchinsonii requires cellulose. A) Initial liquid cultures were grown using Dubos media with a piece of filter paper in the test tube. B) Solid cultures were then made by pipetting the liquid culture onto Dubos plates covered with a piece of filter paper.

4.1.2.1 – Activity Against Tetracycline and its Analogs

Reaction of this protein with tetracycline had no effect on the structure of the antibiotic. Bioassays, HPLC chromatograms and LC/MS traces all verified that no reaction was occurring. Similarly, reactions were done with tetracycline analogs to determine if this protein could modify their structures, but no positive results were obtained.

4.1.3 – Characterization of *tet34*

Cloning of *tet34* was carried out with the Gateway cloning system. Expression in a *E. coli* BL21 DE3 cell line resulted in a pure sample that was then used in reactions with tetracycline and its analogs. Reactions were monitored using the HPLC and LC/MS systems, but no change in the structure of tetracycline was detected in either case. Tet34 does not share sequence homology to TetX, but rather the protein sequence is similar to xanthine-guanine phosphoribosyl transferases. Interestingly, this protein is not an NADPH-requiring oxidoreductase, but was characterized in 2002 as having the ability to inactivate tetracycline by an unknown mechanism [45]. The latter result was not duplicated in this study and for this reason it appears that this gene does not encode for a protein responsible for the structural modification of tetracycline.

The gene *tet37* was characterized in 2003 as encoding a potential tetracycline inactivator after the oral metagenome was sequenced [44]. A sample of the DNA in question (*pUC119*) was obtained from the UCL Eastman Dental Institute, University College London, UK, but amplification of the gene via PCR was never successful and testing of this protein could not be completed. Sequence homology suggested that this gene was from a fusobacterium species, but it has no homology to that of TetX. Further

studies need to be conducted to justify the claims that this gene product modifies the structure of tetracycline.

4.2 – Characterization of Inactivator from Actinomycete Library

After an intensive screen of the 480 isolates from the actinomycete library belonging to the Wright laboratory, it was found that of the 286 isolates (59.6%) were resistant to tetracycline [47]. Bioassays testing for tetracycline inactivation showed only one potential hit in this latter regard. This screen was repeated several times to rule out false positives, and once this had been determined, further tests were performed to better understand what was occurring to the tetracycline in the presence of this bacterium. Bioassays indicated the reduction of a zone of clearance when compared to a drug control when the isolate was incubated in the presence of tetracycline. Follow up to these assays included HPLC tests that demonstrated no change in the tetracycline peak indicating that no reaction was occurring. Radio-labelled tetracycline was also used and monitored with an HPLC system, and gave similar results. The discrepancy between the bioassay and HPLC information with this isolate makes it difficult to establish what was occurring. The lack of change to the tetracycline structure, as determined by HPLC and LC/MS, indicated that there should be no reduction in zone of clearing on the bioassays, yet this result occurred. Characterization of Kar21 via Gram-stain and

scanning electron micrograph was done to attempt to better understand this isolate. Gram-stain indicated a Gram-positive, filamentous bacterium. The fuzzy appearance and the characteristic soil-like smell when grown on solid media, suggested that this isolate belongs to the genus *Streptomyces* [49]. Scanning electron micrograph indicated a sporulating bacterium, with cylindrical shaped spores, also suggesting that this isolate belongs to the genus *Streptomyces*. Following 16s rRNA sequencing it was determined that the isolate Kar21 shared enough identity with other known Streptomyces to be able to classify it as a member of the Streptomyces genus.

5.0 - Conclusion

The problem of resistance to antibiotics is as old as antibiotics themselves, owing to the rapid evolution of various mechanisms to counteract or defend against these drugs. Bacteria have developed many mechanisms to defend themselves from antibiotics, and have the ability to exchange genetic material easily, making resistance an even larger problem [1, 4, 5, 7, 11, 13, 23-26, 31, 44-47, 52, 55, 56]. It is important to understand resistance mechanisms in order to develop new drugs or inhibitors so that bacterial infections can be combated. In the case of tetracycline, bacteria have developed three kinds of resistance mechanisms. Efflux and ribosomal protection proteins disrupt the normal function of the antibiotic

within the cell [23, 26, 52, 57], whereas enzymatic inactivation chemically modifies the structure of the drug rendering it void of antibiotic activity (TetX) [28].

This research was done to test various TetX-like protein sequences found by BLAST search in *Streptomyces coelicolor* and *Cytophaga hutchinsonii* to determine if these proteins may have similar function to that of TetX. Testing was done by expressing the different proteins and subjecting each of them to reactions with various tetracyclines. We hypothesized that these proteins were orthologues of TetX and could inactivate tetracycline through a chemical modification of its structure. This work was formulated to probe the distribution of TetX-like elements in bacteria and investigate the possible origin for this gene.

We would expect to find proteins of similar function in bacteria that are closely related and could easily share genetic material. Of the genes in question, *tetX* was originally found in a species of *Bacteroides,* which has been phylogenetically grouped with the bacteria *Cytophaga*. For this reason, it would seem a logical place to search for a common enzyme. It is known that many of the tetracyclines are produced by *Streptomyces* species and so searching for resistance mechanisms in this population would also seem logical. Because various forms of tetracyclines are

produced by Streptomycetes, it would be reasonable to assume that these bacteria possess enzymes capable of modifying the structure of tetracycline as is necessary when decorating the various analogs. For these reasons, we expected to find enzymes in both of these bacteria responsible for the inactivation of tetracycline.

In an attempt to understand a very specific type of resistance, specifically tetracycline resistance due to enzymatic inactivation of the drug, BLAST searches were performed to find homologous amino acid sequences to that of the only known enzyme responsible for the chemical modification of tetracycline. TetX was first described in 1989 by Speer et al. but was not assessed fully until Yang et al. examined the protein in 2003. They discovered that TetX was a flavin-dependent monooxygenase responsible for the addition of a hydroxyl group to the C-11a position of various members of the tetracycline family. Over the past 2 years, research was conducted with homologous protein sequences in an attempt to find novel tetracycline inactivating enzymes that could help to better understand the origin of this type of resistance.

Genes from *S. coelicolor* and *C. hutchinsonii* were cloned into expression vectors, over-expressed and purified, and tested for tetracycline inactivating activity. Although none of these proteins showed any ability to

modify the structure of tetracycline, their function in the cell still remains unclear. In the case of the genes from *S. coelicolor*, each of the genes is found either directly upstream or downstream of a TetR. This is interesting as the family of TetR proteins are involved with compound-dependent regulation of gene expression [41]. Therefore these genes may be involved with small molecule metabolism or response in some way, and further work should be carried out to determine the functional role of these genes.

This work demonstrates that enzymatic inactivation of tetracycline is not a property of the genes examined from *S. coelicolor* and *C. hutchinsonii*. This is consistent with the fact that this resistance mechanism has not been reported in the literature outside of TetX and the fact that both these organisms are tetracycline sensitive. Perhaps then this is a relatively recent property evolved from a homolog of TetX. This work shows that many bacteria harbour proteins with Flavin binding domains similar to this enzyme and therefore these may have served as progenitor to TetX. Efforts to mutate tetracycline inactivating homologs by error prone PCR, followed by selection on tetracycline could shed light on the evolutionary pathway that leads to resistance.

Nonaka et al.(2002) and Diaz-Torres et al.(2003) suggested the existence of other tetracycline inactivating enzymes. Nonaka et al. described a novel oxytetracycline resistance determinant in a species of Vibrio in the intestinal contents of yellowtail tuna (*Seriola quinqueradiata)* which they named Tet34. This determinant was found to have a requirement for Mg^{2+}, but shared no sequence homology to that of TetX [45]. After cloning the gene and testing the resulting protein for tetracycline inactivation, it was found that this protein had no effect on the structure of tetracycline, with or without the presence of Mg^{2+}. The second of the published tetracycline inactivators, Tet37, described in 2003 when the oral metagenome was sequenced by Diaz-Torres et al., proved extremely difficult to work with. A sample of DNA was sent by Dr. Diaz-Torres but it was not successfully amplified by PCR and so work on this protein was abandoned. Initial tests, however, indicate the gene is not responsible for tetracycline resistance as indicated by MICs of less than 1 μg/mL.

The final route taken to search for novel tetracycline inactivators was through the Actinomycete library made by V. D'Costa of the Wright Laboratory. A library of 480 soil isolates was screened against tetracycline and it was found that 286 of these isolates (59.6%) showed tetracycline resistance of some type, allowing the bacteria to grow in the presence of the antibiotic. Each of the isolates that showed tetracycline resistance was

tested for inactivation. Bioassays were performed for each isolate, however only one showed potential for inactivation due to the disappearance of a zone of clearing. Further studies on this isolate using HPLC and LC/MS methods indicated no change was occurring in the structure of tetracycline and therefore that something else must be occurring. Further studies to determine why the bioassays indicate an inactivation event should be pursued. It is possible that this bacterium is sequestering the drug which would account for the loss of zone of inhibition in the bioassays, or perhaps a subtle modification by a redox mechanism is responsible for this occurence.

We concluded that our hypothesis that TetX orthologs would be found in *S. coelicolor* and *C. hutchinsonii* were incorrect because we found no capacity of any of the homologous proteins to inactivate tetracycline. No change in the tetracycline structure was seen when reacted with any of the *S. coelicolor*, *C. hutchinsonii*, or Tet34 proteins when monitored using various methods. This leads us to suspect that the proteins we tested must have other functions within the cell. Although these proteins did not show inactivating potential, this does not mean that other homologous protein sequences could not. The idea behind this research remains sound, and could provide valuable knowledge about where this gene originated and how it is passed between bacterial species. It would be

beneficial to continue to search for other tetracycline inactivating enzymes through screening other homologous sequences found through BLAST searches. This research, however, has not given any indication as to the source of this kind of resistance or allowed any insight about where to search next. We concluded that the TetX-like protein sequences from *S. coelicolor* and *C. hutchinsonii*, as well as the putative tetracycline inactivating enzymes Tet34 and Tet37, are not responsible for the inactivation of tetracycline and therefore do not shed any insight as to the origin of the tetX gene or how it may have come to reside in a species of Bacteroides. Further research should be conducted to search for other tetracycline inactivating enzymes as well as where this gene came from. This information could be a beneficial predictive tool with regards to clinical resistance.

6.0 – Literature Cited

1. Wright, G., *The Antibiotic Resistome: The nexus of chemical and genetic diversity.* Nat Rev Microbiol., 2007. **5**(3): p. 175-186.
2. Chopra, I., Hower, T.G.B., Linton, A.H., Linton, K.B., Richmond, M.H., Speller, D.C.E., *The tetracyclines: prospects at the beginning of the 1980s.* J. Antimicrobial Chemotherapy, 1981. **8**: p. 5-21.
3. Chopra, I., Hawkey, P.M., Hinton, M., *Tetracyclines, molecular and clinical aspects.* J. Antimicrobial Chemotherapy, 1992. **29**: p. 245-277.
4. Chopra, I., Roberts, M., *Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance.* Microbiology and Molecular Biology Reviews, 2001: p. 232-260.
5. Kelch, W.J., Lee, J.S., *Antibiotic Resistant Patterns of Gram-Negative Bacteria Isolated from Environmental Sources.* App. Enviro. Micro., 1978. **36**: p. 450-456.
6. Nelson, M.L., *The Chemistry and Biology of the Tetracyclines.* Annual Reports Med. Chem., 2002. **37**: p. 105-114.
7. Schnappinger, D., Hillen, W., *Tetracyclines: antibiotic action, uptake, and resistance mechanisms.* Archives of Microbiology, 1996. **165**: p. 359-369.
8. Franklin, T.J., *Mode of action of tetracyclines.* Biochemical studies of antimicrobial drugs. Sixteenth Symposium of the Society for General

Microbiology., ed. B.A. Newton, Reynolds, P.E. 1966, Cambridge, United Kingdom: Cambridge University Press. 20.

9. Meyers, E., Smith, D.A., *Microbiological Degradation of Tetracyclines.* J. Bacteriology, 1962. **84**: p. 797-802.

10. Mitscher, L.A., *The Chemistry of tetracycline antibiotics.* 1978, New York, NY: Marcel Dekker Inc.

11. Roberts, M.C., *Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution.* FEMS Microbiological Review, 1996. **19**: p. 1-24.

12. Schmitt, H., Stoob, K., Hamscher, G., Smit, E., Seinen, W., *Tetracyclines and Tetracycline Resistance in Agricultural Soils: Microcosm and Field Studies.* Micro. Eco., 2006. **Online publication**.

13. Speer, B.S., Shoemaker, N.B., Salyers, A.A., *Bacterial Resistance to Tetracycline: Mechanisms, Transfer, and Clinical Significance.* Clin. Micro. Reviews, 1992. **5**: p. 387-399.

14. Chopra, I., *Tetracycline Analogs Whose Primary Target Is Not the Bacterial Ribosome.* Antimicrobial Agents and Chemotherapy, 1994. **38**(4): p. 637-640.

15. Finch, R.G., *Tetracyclines.* 7 ed. Antibiotic and Chemotherapy, 7th ed., ed. F. O'Grady, Lambert, H.P., Finch, R.G., Greenwood, D. 1997, New York, NY: Churchill Livingstone Ltd. 15.

16. Hlavka, J.J., Ellestad, G.A., Chopra, I., *Tetracyclines.* 4 ed. Kirk-Othmer encyclopedia of chemical technology, 4th ed., ed. J.I. Kroschwitz, Howe-Grant, M. Vol. 3. 1992, New York, NY: John Wiley & Sons. 15.

17. Klein, N.C., Cunha, B.A., *Tetracyclines.* Med. Clin. North Am., 1995. **79**: p. 789-801.

18. Williams, D.N., *Tetracyclines*, in *Infectious Diseases*, S.L. Gorbach, Bartlett, J.G., Blacklow, N.R., Editor. 1992, The W.B. Saunders Co.: Philadelphia, PA. p. 211-214.

19. Brodersen, D.E., Clemons Jr., W.M., Carter, A.P., Morgan-Warren, R.J., Wimberly, B.T., Ramakrishnan, V., *The Structural Basis for the Action of the Antibiotics Tetracycline, Pactamycin, and Mygromycin B on the 30S Ribosomal Subunit.* Cell, 2000. **103**: p. 1143-1154.

20. Roberts, M.C., *MiniReview: Update on acquired tetracycline resistance genes.* FEMS Microbiology Letters, 2005. **245**: p. 195-203.

21. Chopra, I., *Penetration of Antibiotics to their target sites.* Antimicrobial Agents and Chemotherapy, 1990. **26**: p. 607-611.

22. Park, B.H., Levy, S.B., *The Cryptic Tetracycline Resistance Determinant on Tn4400 Mediates Tetracycline Degradation as well as Tetracycline Efflux.* Antimicrobial Agents and Chemotherapy, 1988. **32**(12): p. 1797-1800.

23. Poole, K., *Efflux-mediated antimicrobial resistance.* J. Antimicrobial Chemotherapy, 2005. **56**: p. 20-51.

24. Tenover, F.C., *Mechanisms of antimicrobial resistance in bacteria.* Am. J. Med. - Discussion., 2006. **S3**.

25. Aminov, R.I., Garrigues-Jeanjean, N., Mackie, R.I., *Molecular Ecology of Tetracycline Resistance: Development and Validation of Primers for Detection of Tetracycline Resistance Genes Encoding Ribosomal Protection Proteins.* App. Enviro. Micro., 2001. **67**: p. 22-32.

26. Connell, S.R., Tracz, D.M., Nierhaus, K.H., Taylor, D.E., *Ribosomal Protection Proteins and Their Mechanism of Tetracycline Resistance.* J. Antimicrobial Chemotherapy, 2003. **47**: p. 3675-3681.

27. Whittle, G., Whitehead, T.R., Hamburger, N., Shoemaker, N.B., Cotta, M.A., Salyers, A.A., *Identification of a New Ribosomal Protection Type of Tetracycline Resistance Gene, tet(36), from Swine Manure Pits.* App. Enviro. Micro., 2003. **69**(7): p. 4151-4158.

28. Yang, W., Moore, I.F., Koteva, K.P., Bareich, D., Hughes, D., Wright, G., *TetX Is a Flavin-dependent Monooxygenase Conferring Resistance to Tetracycline Antibiotics.* J. Biol. Chem., 2004. **279**(50): p. 52346-52352.

29. Wright, G., *Bacterial Resistance to Antibiotics: Enzymatic Degradtion and Modification.* Advanced Drug Delivery Reviews, 2005. **57**: p. 1451-1470.

30. Speer, B.S., Bedzyk, L., Salyers, A.A., *Evidence that a Novel Tetracycline Resistance Gene Found on Two Bacteroides Transposons Encodes an NADP-Requiring Oxidoreductase.* J. Bacteriology, 1991. **173**(1): p. 176-183.

31. Speer, B.S., Salyers, A.A., *Novel Aerobic Tetracycline Resistance Gene That Chemically Modifies Tetracycline.* J. Bacteriology, 1989. **171**(1): p. 148-153.

32. Speer, B.S., Salyers, A.A., *Characterization of a Novel Tetracycline Resistance that Functions Only in Aerobically Grown Escherichia coli.* J. Bacteriology, 1988. **171**: p. 148-153.

33. Shoemaker, N.B., Vlamakis, V., Hayes, K., Salyers, A.A., *Evidence for Extensive Resistance Gene Transfer among Bacteroides spp. and among Bacteroides and Other Genera in the Human Colon.* App. Enviro. Micro., 2001. **67**(2): p. 561-568.

34. D'Costa, V.M., McGrann, K.M., Hughes, D.W., Ghadaki, B., Wright, G.D. *Antibiotic Resistance in the Soil: Establishing an Early Warning System for Clinical Resistance.* in *ICAAC*. 2005.

35. Weinglass, A.B., Kaback, H.R., *The central cytoplasmic loop of the major facilitator superfamily of transport proteins governs efficient membrane insertion.* PNAS, 2000. **97**(16): p. 8938-8943.

36. Tamura, N., Konishi, S., Iwaki, S., Kimura-Someya, T., Nada, S., Yamaguchi, A., *Complete Cysteine-scanning Mutagenesis and Site-directed Metal-Tetracycline/H+ Antiporter.* Journal of Biological Chemistry, 2001. **276**(23): p. 20320-20329.

37. Hirai, T., Heymann, J.A.W., Maloney, P.C., Subramaniam, S., *Structural Model for 12-Helix Transporters Belonging to the Major Facilitator Superfamily.* Journal of Bacteriology, 2003. **185**(5): p. 1712-1718.

38. Tseng, T.T., Gratwick, K.S., Kollman, J., Park, D., Nies, D.H., Goffeau, A., Saier, M.H. Jr., *The RND Permease Superfamily: An Ancient, Ubiquitous and Diverse Family that includes Human Disease and Development Proteins.* Journal of Molecular Microbial Biotechnology, 1999. **1**(1): p. 107-125.

39. Rodriguez-Garcia, A., Combes, P., Perez-Redondo, R., Smith, M.C.A., Smith, M.C.M., *Natural and synthetic tetracycline-inducible promoters for use in the antibiotic-producing bacteria Streptomyces.* Nucleic Acids Research, 2005. **33**(9).

40. Kisker, C., Hinrichs, W., Tovar, K., Hillen, W., Saenger, W., *The complex formed between Tet repressor and tetracycline-Mg^{2+} reveals mechanism of antibiotic resistance.* J. Mol. Biol., 1995. **247**: p. 260-280.

41. Ramos, J.L., Martinez-Bueno, M., Molina-Henares, A.J., Teran, W., Watanabe, K., Zhang, X., Trinidad Gallegos, M., Brennan, R., Tobes, R., *The TetR Family of Transcriptional Repressors.* Micro. Mol. Bio. Reviews, 2005. **69**(2): p. 326-356.

42. Speer, B.S., Salyers, A.A., *Novel Aerobic Tetracycline Resistance Gene That Chemically Modifies Tetracycline.* Journal of Bacteriology, 1988. **171**: p. 148-153.

43. Speer, B.S., Bedzyk, L., Salyers, A.A., *Evidence that a Novel Tetracycline Resistance Gene Found on Two Bacteroides Transposons Encodes an NADP-Requiring Oxidoreductase.* Journal of Bacteriology, 1990. **173**: p. 176-183.

44. Diaz-Torres, M.L., McNab, R., Spratt, D.A., Villedieu, A., Hunt, N., Wilson, M., Mullany, P., *Novel Tetracycline Resistance Determinant from the Oral Metagenome.* Antimicrobial Agents and Chemotherapy, 2003. **47**: p. 1430-1432.

45. Nonaka, L., Suzuki, S., *New Mg2+-Dependent Oxytetracycline Resistance Determinant Tet34 in Vibrio Isolates from Marine Fish Intentinal Contents.* Antimicrobial Agents and Chemotherapy, 2002. **46**: p. 1550-1552.

46. Bradford, M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal. Biochem., 1976. **72**: p. 248-254.

47. D'Costa, V.M., McGrann, K.M., Hughes, D.W., Wright, G.D., *Sampling the Antibiotic Resistome.* Science, 2006. **311**: p. 374-377.

48. Technologies, I.L., *BL21 Star™ Chemically Competent Cells.* Online: https://catalog.invitrogen.com/index.cfm?fuseaction=viewCatalog.viewProductDetails&productDescription=617&catname=North%20America%20Main, 2007.

49. Hopwood, D.A., *Practical Streptomyces Genetics.* 2000, Norwich: Crowes.

50. Lanoot, B., Vancanneyt, M., Hoste, B., Vandemeulebroecke, K., Cnockaert, M.C., Dawyndt, P., Liu, Z., Huang, Y., Swings, J., *Grouping of streptomycetes using 16S-ITS RFLP fingerprinting.* Res Microbiol., 2005. **156**(5-6): p. 755-762.

51. Mehling, A., Wehmeier, U.F., Piepersberg, W., *Nucleotide Sequences of streptomycete 16S ribosomal DNA: towrds a specific idenification system for streptomycetes using PCR.* Microbiology, 1995. **141**: p. 2139-2147.

52. Taylor, D.E., Chau, A., *Tetracycline Resistance Mediated by Ribosomal Protection.* Antimicrobial Agents and Chemotherapy, 1996. **40**: p. 1-5.

53. Lautru, S., Oves-Costales, D., Pernodet, J.L., Challis, G.L., *MbtH-like protein-mediated cross-talk between non-ribosomal peptide antibiotic and siderophore biosynthetic pathways in Streptomyces coelicolor M145.* Microbiology, 2007. **153**(5): p. 1405-1412.

54. McBride, M.J., Baker, S.A., *Development of techniques to genetically manipulate members of the genera Cytophaga, Flavobacterium, Flexibacter, and Sporocytophaga.* Appl Environ Microbiol., 1996. **62**(8): p. 3017-3022.

55. Pang, Y., Brown, B.A., Steingrube, V.A., Wallace, Jr., R.J., Roberts, M.C., *Tetracycline resistance determinants in Mycobacterium and Streptomyces species.* Antimicrobial Agents and Chemotherapy, 1994. **38**: p. 1408-1412.

56. Villedieu, A., Diaz-Torres, M.L., Hunt, N., McNab, R., Spratt, D.A., Wilson, M., Mullany, P., *Prevalence of Tetracycline Resistance Genes in Oral Bacteria.* Antimicrobial Agents and Chemotherapy, 2003. **47**(3): p. 878-882.

57. Trieber, C.A., Burkhardt, N., Nierhaus, K.H., Taylor, T.E., *Ribosomal Protection from tetracycline mediated by Tet(O): Tet (O) interaction with ribosomes is GTP-dependent.* Biol. Chem., 1998. **379**: p. 847-855.