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Deletion or Substitution of Conserved Amino Acid Residues at the Tip of the Domain IV of Tet(O) Impairs Tetracycline Resistance

by

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Abstract

Resistance to tetracycline (Tc), an inhibitor of protein synthesis, decreases its effectiveness for the treatment of bacterial infections. Tc resistance (Tc^R) can be mediated by the ribosomal protection protein, Tet(O), which was first reported in *Campylobacter jejuni*, a cause of bacterial diarrhea worldwide. Tet(O) confers Tc^R by mediating Tc release from 70S ribosomes, thus restoring protein synthesis. Tet(O) is widely distributed in a variety of bacterial genera, restraining the clinical use of Tc. This thesis is the first investigation into the role of the conserved set of amino acid residues, YSPVST, occupying positions 507-512 at the tip of domain IV of Tet(O). Impaired Tc release from 70S ribosomes observed with $Tet(O)^{mutants}$ lacking one or more of these conserved residues suggests residues at positions 509-512 play a role in Tet(O)-mediated Tc^R. This study provides insight into the molecular mechanism of Tc^R, which is essential for the development of novel therapeutics.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

A	Alanine
Å	Angstrom
AA	Double alanine
aa-tRNA	Aminoacyl t-RNA
APS	Ammonium Persulphate
ARDS	Acute Respiratory Distress Syndrome
A-site	Aminoacyl site or the decoding site on the 70S ribosome
ATP	Adenosine triphosphate
Bmax	Maximal Binding
BSA	Bovine Serum Albumin
cfu	Colony forming unit
CLSI guidelines	Clinical Laboratory Standards Institute guidelines
Cme	*Campylobacter* multidrug efflux
croy-EM	Cryo Electron Microscopy
D	Aspartate
DMG	N,N-dimethyl glycylamido
DMG-MINO	N,N-dimethyl glycylamido derivative of minocycline
DMS	Dimethyl Sulphate
DNA	Deoxyribonucleic Acid
dpm	decay per minute
DTT	Dithiothreitol
E	Glutamate

EF	Elongation Factor
EF-G	Elongation factor G
EF-Tu	Elongation factor Tu
EK	Enterokinase
ESI MS	Electro Spray Ionization Mass Spectrometry
E-site	Exit site on the 70S ribosome
FKBP	FK506 Binding Protein
G+C	Guanosine + Cytosine content of DNA
GDP	Guanosine diphosphate
GFP	Green Fluorescent Protein
GMP	Guanosine Monophosphate
GMPPCP	Guanosine 5'-[(β,γ)-methyleno]-triphosphate, nonhydrolysable GTP analogue
GTP	Guanosine 5'-Triphosphate
h	helices of 16S rRNA, e.g. h34
H	Helices of 23S and 5S rRNA, e.g H69
H/His	Histidine
H_2O_2	Hydrogen Peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His6/6xHis	A stretch of 6 histidine residues
HOCl	Hydroxy Chloride
HPLC	High Performance Liquid Chromatography
ICU	Intensive Care Unit
IL-1β	Interleukin-1β

IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropylthio-β-D-galactosidase
IR	Inverted Repeat
Kan	Kanamycin
k_{cat}	Turn over number
Kd	Dissociation constant, a measure of binding affinity
kDa	Kilo Dalton = 10^3 Dalton
Km	Michaelis Menten constant
L	Large 50S subunit associated proteins
LB	Luria Bertani
MALDI-MS	Matrix Assisted Laser Desorption Ionization Mass Spectrometry
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MDa	Mega Dalton =10^6 Dalton
MDR	Multi Drug Resistance
MFS	Major Facilitator Superfamily
Mg^{2+}	Divalent cation of magnesium
MIC	Minimal Inhibitory Concentration
MICs	Minimal Inhibitory Concentrations
\MMPs	Matrix Metalloproteases
mRNA	messenger RNA
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry

MW	Molecular Weight
NESP	National Enteric Surveillance Program
NO	Nitric Oxide
$ONOO^-$	peroxynitrite
ORF	Open Reading Frame
otr	oxytetracycline resistance genes
P	Proline (as in YSPVST)
PCR	Polymerase Chain Reaction
Ram	Ribosome ambiguity state
RF-1	Release Factor-1
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell-division
ROS	Reactive Oxygen Species
RPP	Ribosomal Protection Protein
RPPs	Ribosomal Protection Proteins
rRNA	Ribosomal Ribonucleic acid
RT	Room Temperature
s	Small 30S subunit associated proteins
S	Svedberg unit = 10^{-13} seconds, e.g .70S, 50S, 30S, 23S, 16S, 5S
S	Serine (as in YSPVST or H(E/D)VDSS)
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrlamide Gel Electrophoresis
SEM	Standard Error Mean

SMR	Small Multidrug Resistance
SOC	Super Optimal Broth with Catabolite Repression
T	Threonine (as in YSPVST)
Tc	Tetracycline
Tc^R	Teracycline resistance/ Teracycline resistant
Tcs	Tetracyclines
Tc^S	Tetracycline susceptible
TEMED	N,N,N',N'-tetramethyl ethylene diamine
tet	Tetracycline resistance genes
TLC	Thin Layer Chromatography
Tn	Transposon (example Tn916-1545)
tRNA	Transfer RNA
V	Valine
VRE	Vancomycin resistant *Enterococci*
WT	Wild Type
Y	Tyrosine

Chapter 1 Introduction

1.1 *Campylobacter jejuni*

Campylobacter jejuni is a member of the epsilon class of proteobacteria and shares the Campylobacteriales order along with *Helicobacter* and *Wolinella*. All three genera of bacteria (*Campylobacter*, *Helicobacter* and *Wolinella*) are capable of building a long-term association with their host, some of which are pathogenic [1]. *C. jejuni* is a major food-and water-borne pathogen and a leading cause of acute bacterial entercolitis in humans [2, 3]. *Campylobacter* is a Gram negative, thermotolerant, microaerophilic, helical (spiral or curved), motile, entero-pathogen [4]. As early as 1886, Theodor Escherich reported spiral shaped organisms that resembled *Campylobacter* morphologically and were found to be associated with enteric infections in neonates and infants [3, 5]. In 1913, John McFadyean and Stewart Stockman first isolated what we now know as *Campylobacter* species from the uterine fetus of a pregnant sheep from a flock of 150 Devon longwoolled ewes that experienced a 33% abortion rate [6, 7]. In 1919, Theobald Smith and Marian Taylor isolated identical organisms from the aborting cattle and named them *Vibrio fetus* [7, 8]. In 1957, Elizabeth King isolated 'related *Vibrio'* from the blood samples of children with diarrhea [7, 9]. The 'related *Vibrio*' described by King was apparently identical to the bacteria isolated from the feces of calf by Jones *et al.* in 1931 [7, 10]. The microaerophilic *Vibrio fetus* was classified into a distinct bacterial group and renamed as *Campylobacter fetus* by French workers in 1973 [7, 11]. *Campylobacter* was first successfully isolated from the blood specimen of a nurse with acute diarrhea in 1973 by Butzler *et al.* in Belgium [7, 12]. Two subspecies are recognized within *C. jejuni* namely, *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*. The pathogenic role of the latter is unknown [1].

1.1.1 Symptoms and complications

Campylobacteriosis is an inflammatory enteritis. In the initial stages of the infection *Campylobacter* affects the small bowel and in the later stages of infection it affects the colon and the rectum of humans [13, 14]. The incubation time for *Campylobacter* is longer than many other enteric pathogens and ranges from 1-7 days [13]. The major symptoms of the campylobacteriosis include diarrhea (watery/sticky, which might contain blood), dehydration, accompanied by fever, nausea (only 15% vomit), muscle pain and headache [13, 15]. Although the diarrhea usually subsides 2-3 days following the onset of infection, *Campylobacter* remains present in the feces of the infected individual for several weeks [16]. A study by Kapperaud *et al.* [16] reported 16% of the individuals carried *Campylobacter* during convalescence with a mean carriage time of 31 days. A prodrome of influenza-like symptoms (fever, headache, dizziness and myalgia) can precede diarrhea in 30% of cases of campylobacteriosis. For some unknown reasons, patients with these early symptoms tend to have more serious infections [13, 15].

C. jejuni is also associated with post-gastrointestinal infection manifestations, such as irritable bowel syndrome, Guillain–Barre syndrome [17, 18], reported in 0.1% of the cases [19] and reactive arthritis, reported in 1.0% of the cases [19]. The latter two are autoimmune diseases attacking the peripheral nervous system or the joints respectively.

The watery/bloody diarrhea produced during campylobacteriosis arises by two proposed mechanisms [13] (i) watery diarrhea is caused by the production of toxins following adherence of *Campylobacter* to the intestine, and (ii) bloody diarrhea stems from an inflammatory response following the invasion and replication of the *Campylobacter* within the intestinal mucosa.

Humoral response to *Campylobacter* antigens is common among most people infected with *Campylobacter.* Antibody specificity studies have revealed antibodies against the potential virulence factors in *Campylobacter* that are responsible for infection in humans [13]. These immunogenic factors of *Campylobacter* include flagellin protein of the flagella [20, 21], some major outer

membrane proteins (OMA, OMF, OMPIP, LPS-EP, MomP) the periplasmic/membrane-associated proteins (PEB1, PEB3), capsular polysaccharide antigens [22], and the cytolethal distending toxin that initiates the production of antibodies that can neutralize toxins [23].

1.1.2 Incidence and transmission of Campylobacteriosis

1.1.2.1 Incidence in industrialized countries

Statistics show that globally, 5-14% of reported cases of diarrhea are caused by infection with *Campylobacter* [24]. *C. jejuni* causes more than 90% of gastrointestinal infections in developed world [19]. In Canada 95% of the cases of Campylobacteriosis are caused by *C. jejuni,* 4% by *C. coli* and 1% by other *Campylobacter* species [25]. A national integrated enteric pathogen surveillance proGram implemented in 2007 in two sentinel sites (Region of Waterloo, Ontario and Fraser Health Authority, British Columbia) in Canada reported a total of 177 (35.6/100,000) cases of *Campylobacter* infection. Of these, 26% (46/177) were travel-related, and 74% (131) were endemic (26.4/100,000). The endemic (present in a community at all times but at a low frequency versus pandemic which is an epidemic that becomes widespread and affects a whole region continent or world) incidence rates were highest in males <5 years of age. Of the reported endemic cases 52 were female (20.9/100,000) and 79 were male (31.9/100,000) 2007 [26].

Significant improvement in slaughterhouse hygiene and disinfection of water used in the poultry chill tanks has resulted in a steady decline in the incidence of *Campylobacter* infections in the US from 25.2 in 1997 to 17.3 per 100,000 in 1999 and to 12.7 per 100,000 in 2006 [27]. Campylobacteriosis incidence rates have increased sharply in other industrialized countries like New Zealand, (from 14 to 120 per 100,000 between 1981 and 1990 to 363 per 100,000 by 1998 and 396 per 100, 000 in 2005 reported by FoodNet 2005) and Denmark where the infection rates increased nearly threefold from 1990-1998 [28].

A total of 9345 (30.2 per 100,000) cases of campylobacteriosis were reported in Canada in 2004 as compared to 4953 (16.0 per 100,000) cases of

salmonellosis [25]. In Alberta, *Campylobacter* causes over 1000 cases of human enteric infections every year which is 4 times higher than enteric infections caused by *E. coli* and 50% higher than Salmonellosis [29].

In contrast to developed countries, enteric infections in developing countries are usually polymicrobial and co-infection with enteric pathogens such as, *Shigella, Salmonella, Escherichia coli, Giardia lamblia,* and *Rotavirus is* commonly reported along with *Campylobacter* enteritis [30]. The lack of national surveillance programs for campylobacteriosis in developing countries is a major bottleneck for the correct monitoring of the sporadic cases or the outbreak of human campylobacteriosis [30].

1.1.2.2 Transmission of *Campylobacter*

Campylobacter is a common intestinal flora of a wide variety of healthy domestic and wild animals (e.g. chickens, cattle, sheep, geese, ducks, pigs, goats, wild birds, dogs, cats, rodents, and marine mammals) and are shed in the animal's feces [31]. *Campylobacter* colonizes the intestine of poultry, cattle and pigs as a harmless commensal [4]. *Campylobacter fetus* subsp. *fetus* is one type that may cause abortions in livestock [32]. Campylobacteriosis is a zoonotic infection [2] which is transmitted to humans in a fecal oral mode through the ingestion of improperly handled or contaminated food like improperly cooked meat, raw milk and untreated drinking water.

The basis for the different outcomes of *C. jejuni* infections in chickens versus humans is not well understood due to the absence of a good animal model which reproduces the disease. *C. jejuni* colonizes the deep crypts of the caecum in chicken in the mucus layer close to the epithelial cells, in extremely high numbers (up to 10^{10} colony forming units per gram of infected intestine) [1]. An inhibition of the human epithelial cell invasion by *C. jejuni* in the presence of chicken intestinal mucus led to the suggestion that mucus contributes to the asymptomatic nature of *C. jejuni* infections in chicken [33]. The increased body temperature of chickens (41-45°C) in contrast to 37°C in humans results in an alteration of the transcription profile of *C. jejuni* and upregulation of genes involved in transport

and binding proteins which modifies the *C. jejuni* membrane structure and cell wall and envelope components. This trait could contribute to the different outcome of *C. jejuni* infection in chicken and humans [1].

1.1.2.2.1 Raw and undercooked meat

Raw and undercooked meat is a critical source of *Campylobacter* [34-36]. Contamination of food can occur during animal slaughter and processing when the edible portions come into contact with animal feces. Ingestion of as few as 500 organisms of *Campylobacter* can cause infection [37, 38]. Cross-contamination of food with raw meat at local restaurants may lead to *C. jejuni* infection outbreaks. For instance the group of *Campylobacter* infections reported in Oklahama, US, August 1996 resulted from lettuce cross-contaminated with raw chicken and affected 16-20 persons [39]. This report suggested that it is important to keep certain food and utensils separate during food handling [39]. A recent review to evaluate the change in the prevalence of *Campylobacter* on dead chicken carcasses during processing demonstrated a decline in the prevalence of *Campylobacter* in chickens sampled before and after scalding (20 to 40%) and a 26.6% increase in the prevalence of *Campylobacter* in chickens after chilling. Defeathering and evisceration increased the prevalence of *Campylobacter* upto 72% and 15% respectively [40]. During the summers of 2005 and 2006, 41%–59% of flocks in British Columbia, were infected with *Campylobacter* [25]. From 2002-2005 about 37%–51% of retail chickens in Canada were contaminated with Campylobacter as opposed to 10%-17% infection with *Salmonella* [25].

1.1.2.2.2 Raw milk

Campylobacter is readily destroyed by pasteurization of dairy products. An investigation launched by the Kansas Department of Health and Environment determined the source and extent of *Campylobacter jejuni* infection associated with unpasterurized milk and cheese in Kansas in 2007 [41]. It was found that 66% (67/101) people who consumed fresh cheese at a community fair obtained at

a local dairy fell ill. The pulsed-field gel electrophoresis patterns of *C. jejuni* isolates from two ill persons were identical, and the isolate from a third ill person was similar to the other two. Although all samples of cheese tested negative for *Campylobacter*, results of the epidemiologic investigation found an association between illness and consumption of fresh cheese made from unpasteurized milk [41]. A survey of the bulk tank milk samples from 248 herds in 16 counties in Pennsylvania detected *Campylobacter* in 2.2% (5/248) samples [42]. A survey for food-borne pathogens in bulk tank milk from 131 dairy herds in eastern South Dakota and western Minnesota detected *Campylobacter* in 9.2% (12/131) samples [43].

1.1.2.2.3 Untreated drinking water

Untreated drinking water is another source of *Campylobacter* infections [44-46]. The Walkerton, Ontario outbreak of gastroenteritis was associated with *E. coli* O157:H7 and *Campylobacter* contamination of the municipal water supply. Stool samples from 174 people showed laboratory evidence of *E. coli* O157. 167/174 samples were *E. coli* O157:H7. Stools from 116 patients were confirmed with *Campylobacter* spp. infection. 65 patients were admitted to the hospital. 27/65 developed haemolytic uremia and 6 infected with *E. coli* O157:H7 died [47]. Poor hygiene and sanitation contribute to acquisition of the enteric pathogen, *Campylobacter,* in developing countries [30].

1.1.2.2.4 Age of individuals

In developing countries infants and children below 2 years are most susceptible to campylobacteriosis [48]. About 40,000-60,000 out of 100,000 children < 5 years of age are estimated to be affected by *C. jejuni* infections in developing countries [30]. In the industrialized countries children more than 5 years of age and adults (in particular men aged between 20-29 years) are more prone to the infection [48]. The estimated number of cases for infection among children below 5 years of age in developed countries is only 300 cases out of 100, 000 individuals [30].

1.1.2.2.5 Travel

Campylobacter is also the causative agent for Travelers' diarrhea - a clinical syndrome - which commonly affects travelers, traveling from developed countries with proper hygiene infrastructure to countries where the infrastructure is less developed. The acquired cases of campylobacteriosis contribute to 5-10% of the cases in the US, 50-65% of the cases in Sweden and Norway and 10-15% of the cases in Great Britain and Denmark [28]. In Canada between 2000 and 2004, 67 travel-acquired cases of *Campylobacter* were reported to the National Enteric Surveillance Program (NESP) [29]. A history of travel was provided for approximately 1% of all *Campylobacter* infections reported to the NESP. Travel to Asia, Mexico and the Caribbean accounted for the majority of these infections, (Table 1.1) [29].

1.1.2.2.6 Occupational exposure

Contact with infected animals in farms and slaughterhouses are yet another mode of transmission of *Campylobacter* [49]. Use of unsafe laboratory techniques makes laboratory workers prone to *Campylobacter* infections.

1.1.2.2.7 Living in close quarters

At home improper washing of hands following contact with feces of infected human or contact with feces of animals (especially pet dogs and cats) can spread *Campylobacter* infections among other members living in close quarters. Neonates of infected mother are also prone to infection [49]. Outbreaks may also occur in homosexual men [25].

1.1.3 Morbidity and mortality

In Canada, about 5%-10% of the affected individuals need to be hospitalized and 5 in 10,000 die [25]. In contrast in the US, approximately 2,400,000 *Campylobacter* infections are estimated to occur every year, resulting in an estimated 13,000 hospitalizations and 124 deaths each year [28].

1.1.4 Treatment and prophylaxis of *Campylobacter* infections

Campylobacter infections are primarily clinically mild and of relatively short duration in which case they do not require any treatment [50-52]. Treatment with antibiotics becomes essential for infections in immuno-compromised patients, or systemic infections, and in severe and long lasting infections [51, 53].

Macrolides (erythromycin and azithromycin) are the antibiotic of choice and tetracyclines (doxycycline) and fluoroquinolones (ciprofloxacin) are used alternatively for prophylaxis of traveler's diarrhea [15, 54-55]. Recently a new conjugative vaccine composed of the capsule polysaccharide from two different strains of *C. jeuni* (81-76 and CG8486) was shown to effectively protect mice and the New World monkey (*Aotus nancymaee*) against *C. jejuni* infection [56]. Bioniche Life Sciences Incorporation, a Canadian pharmaceutical company first released a vaccine, Econiche^{TM}, following studies by Dr. Brett Finlay. The vaccine could reduce the shedding of *E. coli* O157 by cattle. *E. coli* O157 resides harmlessly in domestic cattles (cows) but is released in their fecal matter from where they spread to human food and water supplies. Ingestion of *E. coli* O157 contaminated vegetables, meat and water by humans can cause severe illness in humans and also prove to be fatal [57]. The research by Dr. Finlay suggests that development of a similar vaccine in chickens, which are the major carriers of *C. jejuni* may help to reduce the shedding of *C. jejuni* in their feces which in turn may result in a reduction of *C. jejuni* infections in humans.

Antibiotics are 'wonder drugs' that inhibit (static) or prevent (cidal) the growth of bacteria [58]. They are 'magic bullets' that can target the microbe without affecting the host [58] either by affecting bacterial (i) cell wall biosynthesis (β-lactamases, vancomycin, cephalosporins); (ii) cell membrane synthesis (polymixin); (iii) protein synthesis (macrolides, aminoglycosides, oxazolidinones and tetracyclines); (iv) nucleic acid synthesis (rifampicin, quinolones); or by (v) competitive inhibition (trimethoprim, sulphonamides) [58, 59].

Over the past few years there has been a remarkable increase in the number of antibiotic resistant *C. jejuni* strains; particularly the increasing number

of tetracycline resistant (Tc^R) strains has raised concerns [60-62]. In Alberta, the number of *C. jejuni* strains that are resistant to tetracycline (Tc) have increased from 6.8%-49.8% from 1980-2002 [63], which strictly restrained the clinical use of the drug to treat *Campylobacter* infections.

This introduction will review the use of tetracyclines for treating *Campylobacter* and other infections, their mode of action, their interaction with the bacterial ribosome in detail, and how bacteria have gained resistance to tetracycline, which has been used widely in human and veterinary medicines.

1.2. Tetracyclines

1.2.1 Chemical structure of tetracycline

Tetracyclines (Tcs), discovered in the 1940s are the first group of antibiotics to which the term broad spectrum was used. In 1948, Benjamin M Duggar of Lederle laboratories discovered 7-chlorotetracyline (Aureomycin) from the culture broth of the soil microbe *Streptomyces aurofaciens* [64]. In 1950, a research team from Charles Pfizer and Company isolated 5-hydroxytetracyline (Terramycin) following the fermentation of the actinomycete *Streptomyces rimosus* [65]. These microorganisms that produce antibiotics are resistant to the action of their own antibiotic and the reason as to how or why bacteria are resistant to their own antibiotics is however unknown. Julian Davis suggests that antibiotics (such as Tc) could either act as "weapons in intracellular warfare" or they act as "signaling molecules" that stabilize interaction between bacterial communities and plants in different environments by probably binding to ribosomes or transcription complexes in receptor bacteria [66]. In 1953, tetracycline (Achromycin) was produced by the catalytic hydrogenolysis of the carbon-chlorine bond in chlorotetracycline by Lloyd Conover of Pfizer [67] (Figures 1.1a-c). The broad spectrum nature of tetracycline (Tc) makes it effective against both Gram positive and Gram negative bacteria and a whole series of atypical microorganisms such as chlamydiae, rickettsiae, mycoplasmas and protozoal parasites [68]. Tcs may occur naturally, such as, oxytetracycline and

chlortetracycline or produced semi-synthetically (e.g. doxycycline, mithocycline and minocycline) [68]. The simplest Tc with an antibacterial property is 6-deoxy-6-demethyltetracycline (Figure 1.2a). They are composed of linear fused tetracyclic rings/nucleus designated as A, B, C and D, to which a number of polar functional groups remain bound along one side. These groups form charged/hydrophilic interactions with the ribosomal ribonucleic acid (rRNA) residues of the bacterial 70S ribosome while hydrophobic interactions occur along the other face of the Tc (Figure 1.2b) [69]. The minimum pharmacophore critical for antibacterial activity includes the hexamembered, carbocyclic, fused, linear tetracycle, the α-stereochemical configuration at A-B ring junction (positions 4a and 12a), conserved keto enol system in proximity to the D ring (positions 11, 12 and 12a) and the conservation of dimethylamino group at position 4 [68].

1.2.2 Classification of tetracyclines

Tetracyclines, chlorotetraccylines, minocycline and doxycyclines (Figures 1.3a-b) are classified as "typical tetracyclines" and are bacteriostatic in nature [68]. *In vitro* studies indicate that typical Tcs inhibit bacterial growth by binding to the bacterial 70S ribosomes and inhibiting protein synthesis which inhibits the growth of the bacteria [70-71].

The "atypical tetracyclines" such as, chelocardin, anhydrotetracycline, 6-thiatetracycline and anhydrochlorotetracycline are bactericidal. They interfere with bacterial membrane permeability, damage cells which results in cell lysis and death [72-73]. 6-thiatetracycline and chelocardin both have toxic side effects due to their ability to interact with mammalian membranes [74].

A more potent microbiologically active analogue of Tc discovered in the 1990s is glycylcycline which is N,N-dimethylglycylamido (DMG) derivatives of minocycline (DMG-MINO) or 6-dimethyl-6-deoxytetracycline [75, 238]. It carries a DMG substituent at position 9 of C-atom of D-ring of Tc nucleus. Tigecycline (formerly called GAR-936, Trademark: Tygacil; Wyeth Pharmaceuticals, Inc., Philadelphia, PA, USA) is a 9-(t-butylglycylamido)-minocycline and was discovered in 1993 [75, 238]. These third generation Tcs

(Figures 1.4a-c) are less toxic than the atypical tetracyclines. The N-alkyl glycyclamido side chain at C-9 arms tigecycline with its improved antimicrobial activity (i) the bulky glycylamido group enhances the lipid solubility of tigecycline, (ii) creates steric hindrance that prevents its efflux out of the cell by membrane-bound efflux proteins and (iii) also allows it to bind more tightly to the 70S ribosomes compared to first generation Tcs [75].

1.2.3 Interaction of tetracycline with the bacterial ribosomes

Current understanding of the possible Tc binding sites on the bacterial 70S ribosome is based on the data presented by two independent groups of researchers [69, 76]. The bacterial 70S ribosome is assembled from a large 50S subunit and a small 30S subunit. Antibiotics like Tc interact only with the 30S subunit of the 70S ribosome [77]. The two research groups studied the X-ray crystal structure of the 30S subunit of *Thermus thermophilus* complexed with Tc. Crystal structure of the 30S subunit of this thermophilic bacteria has revealed a wealth of information on the details of the molecular interaction of antibiotics like Tc with the components of the 30S subunit.

1.2.3.1 Bacterial 70S ribosome

Ribosomes are tiny cellular organelles that catalyze protein synthesis [78]. Ribosomes catalyze the synthesis of functional polypeptides of proteins, using transcribed messenger ribonucleicacids (mRNAs) as a template and consuming aminoacyl transfer RNAs (aa-tRNAs) as substrate. Chemically 70S ribosomes are macromolecular machines of ribonucleoprotein complexes, composed of 65% ribosomal RNAs (rRNAs) and 35% ribosomal proteins [79]. The 2.5 MDa 70S complexes are composed of a large 50S subunit and a small 30S subunit. The 50S subunit of *E. coli* has a molecular mass of 1.6 MDa and is assembled from a 2904 nucleotide long 23S rRNA, a 120 nucleotide long 5S rRNA and 33 proteins designated as L1-L33 (L=large) (Figure 1.5). The small 30S subunit of *E. coli* has a molecular mass of 0.9 MDa and is assembled from a 1542 nucleotide long 16S rRNA and 21 different proteins [80] designated as S1-S21(S=small) (Figure 1.5).

The ribosomes and their subunits are named according to their sedimentation during ultracentrifugation. The sedimentation properties depend on the molecular mass, the geometric shape and the physical properties of the solution through which the molecule sediments. The sedimentation factor is expressed in Svedberg (S) units. It is named after the discoverer Theodore Svedberg, the Swedish chemist who won a Nobel Prize in 1926 for developing the centrifugation technique for studying macromolecules and colloids (1 Svedberg unit=10^{-13} seconds). The two bacterial ribosomal subunits have sedimentation coefficients at $30x10^{-13}$ and $50x10^{-13}$ and are hence referred to as 30S and 50S ribosomal subunits [81].

1.2.3.1.1 Fine structure of the bacterial ribosome

The 30S subunit has been crystallised and studied at a resolution of 3.0Å in *Thermus thermophilus* [82]. The 16S rRNA gene sequences contain conserved sequences that alternate with and flank the hypervariable regions. The hypervariable regions provide species-specific signature sequences useful for bacterial identification [83]. The X-ray crystal structure studies illustrated the secondary structure of the 16S rRNA is composed of extensive stem loops and can be divided into 3 distinct domains (Figure 1.6a). Domain I is formed by the 5' end of the rRNA, domain II by the central part of 16S rRNA, and domain III (composed of two sub-domains: major and minor), the 3' end of the 16S rRNA. The secondary structure folds upon itself to form the tertiary structure (Fig. 1.6b). The tertiary structure is composed of several helices which have been designated using a small h (e.g. h32, h44 etc). The three domains can be easily detected in the structure of the 30S subunit. Domain I comprise the body, domain II makes up the platform and the 3' major domain of domain III forms the head [84]. The 3' minor domain of the domain III subunit stretches all the way from the base of the head (the neck region) to the bottom of the body along the 30S interface [82].

The 50S subunit has been crystallised and studied at 2.4 Å resolutions in *Holoarcula marismortui* (Figure 1.7) [85]. The helices of the 23S and 5S rRNA are designated using H (e.g. H69). The 23S rRNA exist as a single homogenous structure within the 50S subunit, there being no clear demarcation between its

domains (i.e. the domains do not correspond well to the structural tertiary domains) [86].

The contact between the 50S and the 30S subunit is small and discrete and free from proteins [87] and provides space for binding of mRNA and tRNAs. No proteins are present within 18Å of the active *petidyl transferase* site [85] which is responsible for catalyzing the growth of the polypeptide chains at the time of protein synthesis. This indicates the rRNA molecules contribute to the catalytic activity of ribosomes and hence they are called *ribozymes*. The 30S subunit is also composed of a 'spur', a 'channel' that runs through its neck, and a 'tunnel' through which the polypeptide chain passes [88], [Figure 1.8c]. The inter-subunit cavity carries the aminoacyl (A), peptidyl (P) and exit (E) sites for binding the aminoacyl, peptidyl and deacylated tRNAs respectively and the mRNA passes through the channel in the 30S subunit (Figures 1.8a, c and d). The floor of the cavity is composed of seven bridges (designated as B1-B7) which is a complex interaction of the rRNA residues which holds the 50S and the 30S subunits together [87], (Figures 1.8 b, 1.9a-c).

When protein synthesis initiates, mRNA and tRNA are positioned in the 30S subunit with the help of initiation factors, before association with the 50S subunit to form a complete 70S initiation complex [89]. A Shine-Dalgarno (SD) sequence (AGGAGGU) which lies upstream of the initiation codon on the mRNA has to base pair with an anti SD sequence in the 3' end of the 16S rRNA to allow protein synthesis to begin from the correct reading frame [90, 231]. A recent study by Korostelev *et al*. [90], helped to gain insight into the interactions and dynamics of the SD helix in the 70S ribosome. They determined the structure of the 70S initiation like ribosomal complex at 3.8Å resolution that contained the initiator $tRNA^{fMet}$ bound to the P-site, endogenous elongator tRNAs bound to the E-site and and a 27-nucleotide long mRNA containing the Shine Dalgarno sequence. They found the SD helix contacts the helices 23a, 26 and and the neck of the 30S subunit composed of helix 28 of 16S rRNA. Contact with the neck region of the 30S affects the positioning of the 30S head and allows it to interact optimally with the initiator tRNA. The bulged U2723 in helix 23a which is well conserved in

archaea and bacteria interacts with the minor groove of the SD helix at the C1539•G-10 base pair.

X-ray crystal structure of the 70S ribosomes of *Thermus thermophilus* complexed with tRNA was studied at a resolution of 3.7Å [91]. This study revealed a wealth of information on the interaction between the ribosome, mRNA and tRNA at the P and the E sites, also how the tRNA is deformed while it interacts with the ribosome. Conformation of the 70S ribosome changed following tRNA binding and also resulted in changes in the petidyl-transferase site that catalyzes the transfer of the amino acids from the P-site into the A-site. An ever better understanding of the complex 70S ribsomes was possible recently by the use of multistart simulated annealing crystallographic refinement to a 70S-RF1 (release factor 1) translation termination complex which was crystallized and studied at 3.2Å. This technique improved the interpretation of the electron density map of the 70S ribosomes [92].

There is no convention for the use of h and H to designate the helices of rRNA components of the small 30S subunit and the large 50S subunit of a bacterial 70S ribosome. This reference pattern was used in this thesis for the sake of clarity for the reader.

1.2.3.2 Comparison of Brodersen's and Pioletti's models of tetracycline binding sites on the 70S ribosome

While Brodersen's model [69] classifies Tc binding sites as primary and secondary sites, Pioletti's model suggests six distinct Tc binding sites (designated as Tet-1 to Tet-6) on the 30S subunit of *Thermus thermophilus* that was co-crystallized with Tc [76]. A closer look at the two models reveals a distinct overlap between the primary Tc binding site of Brodersen and Tet-1 site of Pioletti, and Brodersen's secondary Tc binding site is analogous to Pioletti's Tet-5 site (Figures 1.10a-b) (Table 1.2). Unlike previous biochemical studies by Buck *et al.* [93] and Oehler *et al.* [94], Brodersen's [69] model does not show Tc interacting with any of the protein components of the constituent subunits of the bacterial ribosome [95].

1.2.3.2.1 Primary/Tet-1 site

The primary Tc binding site/Tet-1 site (Figures 1.11a-b) is an inhibitory Tc binding site to which Tc binds with a high affinity (Kd ~1-20μM) [96]. The dissociation constant (Kd) is defined as the concentration of Tc (in μM) at which one half of the maximal amount of Tc remains bound to the 70S ribosomes. The primary Tc binding site lies between the head and the shoulder of the 30S subunit and in close proximity to the ribosomal A-site [69, 76] in the h34 of 16S rRNA. Thus it mediates the inhibitory effect of Tc on A-site occupation [95]. The most important interactions that occur in the primary Tc binding site are hydrophilic interactions. Interaction with the phosphate oxygen atoms of residues G1197 and G1198 in h34 and the hydrophilic residues of Tc is mediated through a Mg^{2+} ion which is known to be important for Tc binding [69, 76] (Figure 1.11a).The hydrophilic side chains of Tc interact with the sugar phosphate backbone of the 16S rRNA residues 1053-1056:1196-1200, which lies within the irregular minor groove of helix34 (h34) (Figure 1.11a) [69, 76]. Tc also forms charged interactions with residues 964-967 of helix31 (h31) [69, 76] (Fig. 1.11b).

In order to identify the interaction of Tc with the 16S rRNA residues, dimethyl sulphate (DMS) has been used as a chemical probe. It is an alkylating agent that adds methyl group to the N1 and N3 positions of adenosine and cytosine respectively and modifies them [95]. An increased reactivity of C1054 to DMS [97] in the presence of Tc suggests a shift in the position of this residue to accommodate Tc [95].

The h34 of 16S rRNA is a well conserved region which is involved in translation accuracy and chain termination during protein synthesis in bacteria. A mutation in helix 34 may weaken the binding of tetracycline to the ribosome or allow access of tRNAs in the presence of tetracycline [77]. For instance mutation of a critical guanosine residue (G1058) can disrupt the shape of h34 by disrupting G1058 and U1199 base pairing and significantly inhibit Tc binding making bacterial cells resistant to the antibiotic [69, 77]. The conservation of h34 in all 16S rRNA is most likely responsible for making all bacterial ribosomes susceptible to Tc (unless the bacteria carry a Tc^R determinant).

1.2.3.2.2 Secondary/Tet-5 site

In the secondary/Tet-5 Tc binding site Tc contacts the residues constituting the h11 and switch helix (h27) of 16S rRNA (Figure 1.11c-d). The h27 is a functional hot spot and switches the conformation of the 30S subunit from an error prone *ram* state to a hyper-accurate *restrictive* state [98, 99]. *Ram* refers to 'ribosome ambiguity' state which allows translation of mRNA with a level of misreading significantly higher than the restrictive state when there are minimal mistakes made in translating the mRNA. These two states correspond to two different conformations of the 30S subunit. The 30S subunit adopts a closed conformation in the *ram* state and is open in the *restrictive* state. The crystallography studies by Brodersen [69] supported the idea that binding of Tc locked h27 in the *ram* state which indirectly disrupted the function of the 30S subunit and inhibiting protein synthesis. Hence Tc is an error inducing antibiotic [100]. The primary interactions that hold Tc in place at the secondary site are hydrogen bonding interactions that occur between the 2'-OH and 3'-OH groups of the ribose moiety of the 16S rRNA residues and the oxygen and nitrogen atoms attached to the side chain of the ring A of Tc. Some sequence specific hydrogen bonding contacts include C893:O2 and A892:N1 (Figure 1.11c) [69]. Since the base A892 interacts with Tc it is protected from being methylated by DMS in the presence of the drug [97].

Tc interacts with the small subunit proteins S4, S9, S17 and S7/ S9 at Tet-2, Tet-3, Tet-4, and Tet-6 sites respectively [101]. These are primary rRNA binding proteins that assist the assembly of the 30S subunit [102], suggesting that the binding of Tc at Tet-2, Tet-3, Tet-4, and Tet-6 sites do not affect decoding directly [95]. Tc occupation of these binding sites inhibits the initial assembly of the 30S subunit and thus contributes to the *bacteriostatic* effect of Tc [76].

Eukaryotes carry 80S ribosomes which are assembled from a small 40S subunit and a large 60S subunit. A study by Budkevich *et al.* [103] compared the binding of Tc to the bacterial 70S versus the eukaryotic 80S ribosomes. They found Tc bound the 70S ribosomes with an affinity (Kd) of 1-2 μM while the binding affinity of Tc for the 80S ribosomes was 15 fold lower (Kd= 30 μM).

They speculated the reduced binding affinity of Tc to the eukaryotic ribosomes resulted from structural differences in the primary Tc binding site between bacterial (*E. coli*) and eukaryotic (*Sacharomyces cerevisiae*) rRNAs at h34 around residue 1200. An extra nucleotide sequence is present in h34 of all eukaryotic sequences before the universally conserved A1204 residue. This altered the tertiary organization of the phosphate backbone around h34 and is possibly responsible for reduced affinity of Tc to the h34.

1.2.3.3 Proposed mechanism of tetracycline action

Tcs bind to the 30S subunit [77] of the bacterial 70S ribosomes and inhibit the accommodation of aminoacyl-tRNA into the decoding A-site of the ribosome which in turn inhibits the elongation step of protein synthesis) [104] (Figures 1.12a-c). Tcs, however, do not interfere with the initial binding of the ternary complex (EF-Tu•GTP•aa-tRNA) to the A-site (Figure 1.12b) of the ribosome, as is evident from the ability of EF-Tu to hydrolyse GTP in the presence of Tc [105]. Quite a few hypotheses have been proposed as to how the binding of Tc inhibits the accommodation of aa-tRNA to the A-site (Figure 1.12c) and locks the ribosome in a postranslocational state [95]. Biochemical data by Noah *et al.* [106], suggests that binding of Tc induces a conformational change in the h44 of the 30S subunit. They found UV can induce crosslinking of the bases C1402 and C1501, located at the top of h44 in the presence of Tc, which implies that Tc possibly encourages a subtle reorganization in the 30S functional centre that favours C1402-C1501 crosslink. This conformational change is, however, not evident in the studies of Brodersen [69] and Pioletti [76]. The crystal structure [69, 76] of Tc bound to 30S suggests aa-tRNA is prevented from being accommodated simply through a steric clash between the accommodating aa-tRNA and the bound Tc making the structural rearrangement of 30S redundant.

A model described by Spahn *et al.* [107] proposes how the 70S ribosome proceeds through the different phases of the protein elongation cycle (Figure 1.13, steps a to e), in the absence of Tc. Studies by Brodersen *et al.* [69] has revealed that decoding by the ternary complex (EF-Tu•GTP •aa-tRNA) begins subsequent

to the binding of Tc to the primary site (Step f). This indicates that the interaction between the codon and the anticodon of the EF-Tu bound aminoacyl tRNA (aa tRNA) is unaffected by the presence of Tc (Figure 1.13, step a). Accommodation (Figure 1.13, step b) of the aa-tRNA into the A-site in the consequent step is inhibited by steric clash of the anticodon loop of the aa-tRNA with the drug [69]. This locks the ribosome in a non constructive cycle of ternary complex binding and release (Figure 1.13, steps i-j) which brings elongation to a halt and stalls the ribosome [107].

Tc has been co-crystallized (1:1) with *E. coli* trypsin modified EF-Tu-Mg-GDP complex suggesting Tc inhibits protein synthesis most likely by binding to EF-Tu and inhibiting it from delivering aa-tRNA to the A-site [108]. Molecular dynamic simulations of 30S subunit has revealed that Tet-1 site is the predominant Tc binding site and steric interference of the aa-tRNA with Tc is the major mechanism of Tc action [109]. In a similar study binding of both Tc and tigecycline to EF-Tu did not have any significant role in inhibiting protein synthesis [110].

1.2.4 Applications of tetracycline

Tcs were one of the most widely used antibiotics throughout the world for a number of different reasons. The broad spectrum nature of Tc made it effective against bacteria and atypical microbes [68]. Tcs were cost effective, safe and had relatively few side effects. They could be administered orally, and, were used for outpatient therapy. Tcs ranked second among the most frequently used antibiotic every year, the first being penicillin. Tcs were used to treat sexually transmitted diseases caused by *Neisseria gonorrhoea* until the late 1980s, when the first Tc^R strain of *N. gonorrhoea* first appeared [111-113]. Tcs have been discontinued as the primary antibiotic for treating bacterial infections of the lower respiratory tract, since the emergence of Tc^R Gram positive cocci (*Staphylococci*, *Streptococci*) and mycoplasmas [113-115]. Tcs were also used to treat maternal infection in pregnant women following the establishment of placental transfer of Tc in 1950 [116]. Use of Tc in pregnant women is associated with several adverse

effects in the fetus (such as yellowing of teeth [117], inhibition of fibula growth [118] and maternal liver toxicity [119]) has greatly minimized the use of Tc in pregnant women in recent years.

The ability of chlorotetracycline to promote animal growth (by inhibiting the growth of microbes that cause infectious disease which may lead to death of poultry or livestock) was first observed in chickens in 1949 [120] and gradually extended to cattle and swine [121]. Since then, chlorotetracycline and oxytetracycline have been widely used non-clinically as a livestock feed additive to stimulate weight gain in cattle and poultry. In the US, the food and drug administration approved the use of chlorotetracycline (1951) and oxytetracyline (1953) as feed additives [239]. A report by Swann in 1969 [122] expressed concern, for the first time, over the increase in the number of Tc^R human isolates due to the extensive use of Tc as a feed-additive. Consequently, the use of Tc was banned in the European Union in 1970 [68].

Doxycycline is used in combination with other drugs to treat anthrax (*Bacillus anthracis)*, tularemia (*Francisella tularensis)* and the plague (*Yersinia pestis)*. Oxytetracycline and Tc are still used to treat chlamydial infections and non-gonococcal urethritis. Rickettsial infections such as lyme disease, typhus, brucellosis, are also still treated by Tcs. Cholera, syphilis, periodontal infections (gingivitis) and community-acquired pneumonia are treated by doxycycline [123]. Certain lipophilic derivatives of Tc, such as thiatetracycline, may readily cross the cell membrane, and have antiparasitic activity that inhibits the growth of protozoa such as, *Giardia lamblia, Entamoeba histolytica, Plasmodium falciparum, Trichomonas vaginalis* and *Leishmania major* [68, 113].

Tcs as an emerging class of pleiotropic compounds which, apart from having antimicrobial property, also possesses anti-protease, anti-apoptotic, anti-inflammatory, and anti-cancer properties has been extensively reviewed by Griffin *et al.* [123]. The best characterized anti-protease property of Tcs is their ability to inhibit the activity of matrix metalloproteases (MMPs), which are zinc-dependent proteases that are involved in many physiological (embryogenesis and tissue remodeling) and pathophysiological (tumor invasion, inflammation)

processes [124]. Tcs chelate Zn^{2+} metal ions required for the structure and catalytic activity of the MMPs and therefore inhibit the pathological activity of MMPs. The phenol ring of Tcs have the ability to scavenge the highly reactive oxygen species (ROS) [125] (such as superoxides: O_2^- and hydroxyl radicals: $OH^{\bullet}$, hydrogen peroxides: H_2O_2, hypochloride: HOCl and peroxynitrite: $ONOO^-$) [126]. ROS are produced in excess under pathological conditions and can result in oxidative destruction and malfunction of cell constituents.

The anti-apoptotic property of Tcs has been suggested to have a neuroprotective role in the prevention of stroke and multiple sclerosis. The biochemical pathways that link mitochondria, oxidative stress, poly (ADP-ribose) polymerase-1 and apoptosis are proposed to be the prime targets of Tc that are linked to neurodegeneration [127]. Survival of hippocampal neurons following global brain ischemia was increased by minocycline and doxycycline by inhibition of the activity of caspase-1 in the apoptotic pathway [128]. Minocycline was also found to protect against Huntington's disease [129], traumatic brain injury [130] and Parkinson's disease [131] by inhibiting the expression of caspase-1 and/or caspase-3 in the apopototic cascade. Doxycycline and minocycline have the ability to enhance the lifespan of scrapie-infected animals, by binding with and reversing the protease resistance of the pathological prion [Prp(Sc)] proteins, hence, lowering the prion titre [132], suggesting doxycycline and minocycline have therapeutic potential for encephalopathy-or variant Creutzfeldt-Jacob disease [123].

Doxycyclines are used in the treatment of acne and rosaceae due to their anti-inflammatory properties. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) levels are increased in the patients with acne [133]. These cytokines induce the expression of MMPs. ROS and NO play a role in the pathophysiology of rosacea [134]. Doxycyclines are capable of inhibiting the levels of pro-inflammatory cytokines, MMPs and ROS. Other anti-inflammatory properties of doxycycline and to a lesser extent minocycline include their ability to inhibit pancreatic and non-pancreatic phospholipase A2, which plays a role in joint inflammation [135]. Doxycyclines mediate their anti-

inflammatory effect by inhibiting the proliferation of lymphocytes [136], neutrophil migration [137] and their adherence [138].

A study by Steinberg *et al.* [139] evidenced the prophylactic use of the chemically modified Tc: COL-3, in an animal model of sepsis-induced acute respiratory disease syndrome (ARDS), where COL-3 prevented ARDS and septic shock. COL-3 also has anti-cancer properties. MMP is overexpressed in Kaposis's sarcoma cells and are involved in tumor metastasis and angiogenesis. COL-3 administered at 50 mg/day reduced MMP-2 and MMP-9 plasma levels from baseline to minimum and displayed anti-tumor activity in a phase II study [139].

1.3 Tetracycline resistance

1.3.1 Development of tetracycline resistance

The first Tc^R bacterium, *Shigella dysenteriae* was isolated in 1953. The cause of Tc^R in *Shigella dysenteriae* was not known at that time. Within two years time, 0.02% of *Shigella* strains tested were resistant to multiple drugs (Tc, streptomycin and chloramphenicol). By 1960, 10% of the *Shigella* strains in Japan were found to be multidrug resistant [68], and by 1993, the incidence of Tc^R increased to 60% [140]. It is now known that multidrug resistance in *Shigella* spp. is caused by the efflux pump genes [141]. Since then, multidrug resistance, which includes Tc^R, has been identified in a variety of Gram-negative and Gram-positive pathogens and opportunistic bacteria, even though Tc was never used to treat the bacteria. Since Tc^R determinants are present along with other antibiotic resistance genes on plasmids or transposons that result in the transfer of Tc^R determinants along with other drug resistance genes [68]. A study conducted in 1994 reported 90% of methicillin resistant *Staphylococcus aureus,* 70% of multidrug resistant *Enterococcus faecalis* and 60% of multidrug resistant *Streptococcus pneumoniae* were Tc^R [68, 142].

The excessive use and industrial scale production of antibiotics such as Tc has resulted in a dramatic increase of Tc in the environment which consequently

resulted in an increase in the appearance of Tc^R bacteria [143]. Unlike other antibiotics which are degraded partially in sewage treated plants [144-145], Tcs are not degraded by this process [143]. Rate of degradation of Tc varies between 10-180 days depending on the environmental conditions [146]. A study by Aga *et al.* [147] reported the presence of low levels of Tcs in manured soil for up to 28 days. Soil concentrations of Tc can reach approximately 200 μg/kg of soil fertilized with liquid manure upto first 30 cm of soil depth and not beyond [148]. Tc released into the environment can result in any of the following [143] (i) the spread of Tc^R in both pathogenic and non-pathogenic bacteria, which mostly occurs by horizontal gene transfer (by plasmids, integrons and transposons), (ii) removal of defined pools of bacteria (both beneficial and pathogenic) and affect the natural ecological balance in the environment, (iii) contaminated soil and water in its primary (Tcs produced artificially) and secondary metabolite (Tcs produced naturally by the bacteria) form and (iv) unfavourable for plant growth [149]. A recent study by Popowska *et al.* [143] examined the effect of Tc on the ability of microbes to inhabit three different types of soil: forest soil, compost and agricultural soil. The Tc minimal inhibotory concentrations (MICs) of bacteria were 20-180 μg/mL for forest soil, 20-120 μg/mL for compost and 40-120 μg/mL for agricultural soil. Tc concentations of 7 mg/kg of soil reduced the number of bacteria capable of growing in media in the laboratory conditions by 85%. Highest Tc^R were exhibited by *Rhizobium radiobacter*, *Pasteurella multocida*, *Burkholderia cepacia* and *Brevundimonas vesicularis.* Agricultural soils that are constantly subject to Tc pressure from biosolids and animal manures carried the most Tc^R bacteria, some of which were opportunistic pathogens of humans and/or animals that causes a variety of infections ranging from inflammation to sepsis. The constant pressure of persistent levels of Tc in the environment might be a reason for inherent resistance to Tc by the Tc producing strains (e.g. actinomycetes) [143].

1.3.2 Tetracycline resistance determinants

The major determinants of Tc^R in both Gram positive and Gram negative bacteria involve (i) efflux of Tc across the cell membrane with the help of ATP to prevent its cytoplasmic accumulation [150] and/or (ii) ribosomal protection mediated by soluble, cytosolic ribosomal protection proteins (RPPs) that interact directly with bacterial ribosomes to promote the release of the ribosome bound drug [151-152]. Other less prevalent mechanisms of Tc^R include (iii) enzymatic inactivation of Tc, as seen in *Bacteroides* [153] (iv) chemical modification of Tc to inactivate it or mimic the structure of elongation factors which would impede the release of the ribosome bound drug; and (v) mutation of 16S rRNA altering the binding site of Tc, as seen in *H. pylori* [154-155] (Figure 1.14). The Tc and the oxytetracycline resistance genes are commonly referred to as *tet* genes. There are currently 43 different *tet* and *otr* genes, 27 of which encode for energy dependent efflux proteins, 12 genes encode for RPPs, 3 encode for an inactivating enzyme, one of which is *tet(X)* and the mechanism of resistance by the remaining 1 gene is not known [156] (Table 1.3).The details of the distribution of the *tet* genes in bacteria are updated twice a year in the website of MC Roberts [156].

1.3.2.1 Efflux pumps

The efflux pumps of bacteria that transport molecules in and out of the cell have been reviewed by Chopra *et al.* [68]. They have been classified into 3 groups which include the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance-nodulation-cell division (RND) and the ATP-binding cassette transport family. Some and not all of these efflux systems confer resistance to Tc. Proton motive force is used by RND, SMR and MFS families to drive molecules out of the cell. The energy derived from ATP hydrolysis is used by the ATP-binding cassette transporters for the efflux of molecules. Members of the RND efflux pumps such as multidrug efflux (Mex) systems of *Pseudomonas aeruginosa* [157], Acr system in *E. coli* [158], multidrug resistance transport systems (Mtr) system in *N. gonorrhoeae* [159] are capable of transporting Tc. The *E. coli* emrE gene encodes for SMR that are capable of conferring Tc^R [160].

The *tet* efflux genes that are responsible for pumping out Tc are members of the MFS [68]. The MFS are a 46 kDa membrane spanning protein, which exchanges a proton against a Tc-cation complex against a concentration gradient [161]. This reduces the intracellular concentration of Tc and protects the bacterial ribosome *in vivo*. Currently, 27 *tet* genes encoding for efflux proteins have been identified which are distributed in both Gram positive and Gram negative bacteria [156]. Genetic organization of the efflux determinants in Gram negative bacteria (Tet(A), Tet(B), Tet(C), Tet(D), Tet(E), Tet(G), Tet(H), Tet(35), Tet(39)) differ from that found in Gram positive bacteria. Gram negative *tet* efflux genes are regulated by a structural gene and a repressor that are expressed in the opposite directions from operator regions that overlap. The regulation of Gram positive *tet(k)* and *tet(l)* genes occurs by mRNA attenuation (formation of a hair pin loop in the mRNA that inhibits protein synthesis) as observed for *erm* and *cat* genes of Gram positive bacteria encoding for rRNA methylase and chloramphenicol acetyltransferase, respectively [162]. The *tet* efflux genes only confer resistance to Tc but not Tc derivatives (except *tet(B)* which can confer resistance to minocycline along with Tc) [68].

Multidrug resistance (MDR) through efflux was first described in *C. jejuni* in 1995 [163]. The complete sequence annotation of *C. jejuni* NCTC 11168 [164] has revealed 13 putative MDR transporter genes which functions in extruding a wide variety of structurally unrelated antimicrobial compounds including tetracycline that the bacteria stumbles upon in the intestinal tract [165]. The two functionally well-characterized, *Campylobacter* multidrug efflux pump (Cme) members are, CmeABC [166-167] and CmeDEF [172]. Recent evidence has shown that interaction between CmeABC and CmeDEF is responsible for conferring resistance to Tc in some strains of *C. jejuni* [172]. These pumps belong to the resistance nodulation division family (RND) of drug transporters that are encoded by the chromosomally located operon composed of three-genes [166, 168-169], and share significant sequence and structural homology to recognized tripartite multidrug efflux transporters of other Gram negative bacteria (e.g. MexAB-OprM system in *P. aeruginosa*) [170-171]. CmeDEF plays a secondary

role in imparting resistance to antimicrobials in a cell intrinsically, while CmeABC is the predominant efflux pump in *C. jejuni* [172]. CmeABC is a tripartite drug transporter [166] composed of inner membrane energy-dependent efflux pump, CmeB, a periplasmic membrane adaptor protein CmeA, and an outer membrane channel forming protein CmeC [166,168]. CmeABC is also responsible for protecting the bacteria from the action of the bile salts thus allowing their successful colonization as demonstrated by the failure of *cmeb* null mutants to colonize chicken [173]. Inactivation of CmeABC pumps by point mutations (A2074C and A2075G) significantly increased susceptibility of *C. jejuni* to several structurally diverse antimicrobials including Tc [174], indicating that CmeABC plays a critical role of in the intrinsic resistance of *C. jejuni* to antimicrobials [166].

CmeR, a two domain protein which shares structural similarity to TetR regulator family of proteins [175-177] functions as a transcriptional repressor of CmeABC, by binding to a 16 bp inverted repeat (IR) region located between *cmeR* and *cmeABC* [177]. Insertional mutagenesis of CmeR or single mutation in the IR region results in over expression of *cmeABC* which indicates CmeR functions as a repressor of *cmeABC* [165].

Use of CmeABC efflux pump inhibitors in poultry may reverse the resistance of *C. jejuni* to antibiotics like tetracycline and minimise both the emergence and transfer of tetracycline resistant *C. jejuni* strains to humans through the food chain [240].

1.3.2.2 Ribosomal protection proteins

Ribosomal protection proteins (RPPs) can provide ribosome protection by releasing the 70S ribosome bound Tc from the A-site and allowing protein synthesis to continue [95, 107, 178]. RPPs offer resistance to Tc, oxytetracycline and minocycline [68]. There are 12 different *tet* genes encoding for RPPs [156]. Tet(M) was the first ribosomal protection protein (RPP) identified to protect the bacterial ribosomes from the action of Tc and was isolated from *Streptococcus faecalis* in 1986 [230]. Tet(O) was first reported in *C. jejuni* [179-181]. The best

characterized RPPs to date are Tet(O) and Tet(M) [182-187]. It is assumed that other RPPs function in the same way as Tet(O) and Tet(M).

The average G+C content in bacteria varies between 25%-75%. Gram positive bacteria have a low G+C content (25-42)% (such as *Bacillus subtilis* (42%), *Lactobacillus viridescens* (40%), *Staphylococcus aureus* (33%) *Clostridium perfringens* (38%)) or a high G+C content (69-75)% (such as *Micrococcus luteus* (75%), *Streptomyces griseus* (73%), *Mycobacterium tuberculosis* (67%)). Gram negative bacteria have a moderate G+C content (50-60)% (such as *E. coli* (51%), *Salmonella typhimurian* (51%), *Pseudomonas fluorescens* (60%)) [188]. The G+C content is well conserved among the same bacterial species (for instance *E. coli* has a G+C-content of 51%, *Bacillus subtilis* has a G+C content of 43%). The G+C content is also similar among phylogenetically related bacterial species (both *E. coli* and closely related *Shigella flexineri* have a G+C-content of 51%) [189-190]. Variation in the mutation rates at the 4 nucloetides ATGC, are responsible for a variation in the GC content [189].

All the RPPs except Tet(W) have a <50% G+C content which suggests they most likely originated from Gram positive bacteria [191]. Genes encoding RPPs are widely distributed in both Gram positive and Gram negative bacteria. The *tet(32)* and *tet(36)* genes which are recently identified members of this group, were reported in anaerobes *Clostridium* spp. and *Bacteroides* spp. respectively, unlike other members which were reported to occur in aerobes only [191]. RPPs are often present with efflux genes which synergistically confer Tc^R.

1.3.2.3 Enzymatic inactivation

Tc can be inactivated by the NADPH-dependent oxidoreductase gene *tet (X)* gene. Interestingly this gene is only present in the anaerobe *Bacteriodes* which grows in the absence of oxygen, hence this Tc^R determinant cannot function [191]. The G+C content of *tet(X)* gene is 37.4% suggesting its origin from the Gram positive aerobe *E. coli* [191-192]. However, due to the lack of activity of the *tet(X)* gene in *E. coli*, not much work has been done to explore its function

further. The newly reported *tet(37)* gene having a G+C content of 37.9% was found to encode for another NADPH-requiring oxidoreductase. This gene was isolated from an oral metagenome and no bacteria were found to specifically carry it [191-192]. Another recently identified member of this group includes the *tet(34)* gene which, unlike *tet(X)* and *tet(37)*, encodes for a xanthine-guanine phosphoribosyl transferase from *Vibrio cholerae* [191, 193].

1.3.2.4 *tet(U)* gene

The *tet(U)* gene is composed of 105 amino acids and shares 21% sequence similarity with Tet(M). The absence of the consensus GTP-binding sequences in *tet(U),* which is proposed to be critical for Tc^R [191], suggests *tet(U)* might not be able to function as strongly as Tet(M) in conferring Tc^R. However, the recent identification of the *tet(U)* gene in the vancomycin and tetracycline resistant *Staphylococcus aureus* in the absence of the common Tc^R determinants, such as the efflux (*tet(K), tet(L)*) and RPP genes (*tet(M)*,*tet(O)*), suggest *tet(U)* is an emerging Tc^R determinant [191, 194].

1.3.3 Association of *tet* genes with mobile elements

The *tet* genes very commonly remain associated with mobile genetic elements in bacteria, such as plasmids, transposons and conjugative transposons, which may carry other antibiotic resistance genes or heavy metal resistance genes. Therefore selection of Tc^R may also select for multidrug resistance. Tc^R genes also remain associated with class I integrons in self-transmissible plasmids in both Gram positive (*Corynebacterium*) and Gram negative bacteria (*Salmonella*, *Aeromonas*) [195]. Integrons are genetic elements composed of a gene encoding an integrase, gene cassettes and an integration site for a gene cassette (*att*) [196] and are classified into two categories as reviewed by Fluit *et al.* [196]. Resistance integrons, There are three classes of resistance integrons (Classes 1, 2 and 3). These integrons carry less than 10 gene cassettes that encode for resistance to disinfectants and antibiotics, located on plasmids, transposons and chromosomes. Superintegrons, first isolated in *Vibrio cholerae,* are classified as a class 4

integron. They carry more than 100 gene cassettes which encode for a variety of different functions and are located on the chromosomes [196]. A study by Agerso *et al.* [195] investigated the presence of commonly found Tc^R genes and Class 1 resistance integrons in bacteria isolated from pig sty environment and manured soil. Class 1 integrons were found in 7% of soil isolates and 25% of pig sty isolates. All isolates that contained *tet* genes were also found to carry Class 1 integron, except a Gram negative pig sty isolate with *tet(B)*.The *tet(33)* was present in all Gram positive isolates with Class 1 integron.

The *tet(O)* gene encoding for RPPs are often associated linked with transmissible conjugative plasmids [63, 182, 197-198]. Other RPP genes may also be associated with transposons (*tet(M)*) or in promiscuous conjugative Tn916-Tn1545 transposons (*tet(M), tet(S)* and *tet(W)*). The Tn916-Tn1545 family of transposons currently has more than 10 members and a remarkably broad host range. Due to their promiscuous nature they naturally occur in or have been transferred into 52 different species of bacteria from 24 different genera [199]. The presence of RPP genes on mobile bacterial elements has facilitated their distribution to a variety of other bacterial genera by horizontal gene transfer. For instance, the *tet(M)* gene has the widest host range and is found in 72 genera. The tet(W) and tet(O) genes follow next, and are found in 22 genera [156]. Plasmids in bacteria such as *Enterococcus faecalis* and *Streptococcus spp*., have similar size and restriction profiles to those isolated from *C. jejuni*, and also carry the *tet(O)* determinant [152, 200]. The presence of *tet(O)* on conjugative plasmids explains the widespread propagation of this resistance determinant by horizontal gene transfer [68]. The other *tet* genes are not so commonly distributed in bacteria. This review will focus on the ribosomal protection protein Tet(O) which is a Tc^R determinant first reported in *Campylobacter* .

1.3.4 Importance of studying tetracycline resistance

The desirable properties of Tc such as their low cost, minor side effects and broad spectrum activity make them prime drug candidates. The selection pressure exerted by the extensive use of Tc in prophylaxis and therapy for humans

and animals has resulted in the emergence of Tc^R organisms. Acquired Tc^R has increased dramatically since the 1950s which has reduced the efficacy and the use of Tc as a therapeutic. A better understanding of the molecular mechanism of Tc^R has paved the way for the development of new generation of Tcs - glycyclcyclines [238]- such as the tigecycline (9-t-butylglycylamido derivative of the second generation Tc, minocycline) [68]. The US Food and Drug Administration allowed its fast track approval, and intravenous use began in adults in June 2005 [201]. Tigecyclines were observed to bind to the 70S ribosomes with a 5-fold higher binding affinity than Tcs and were resistant to the two major determinants of bacterial Tc^R: efflux pumps and RPPs [202]. Burgeron *et al*. [202] also reported that the binding of tigecycline to the 70S occurs in a unique orientation relative to the binding observed with Tc. A study by Bauer *et al*. [203] compared the binding of Tc and tigecycline to the 70S ribosomes by DMS footprinting and drug-directed Fe^{2+} cleavage of 16S rRNA. This study revealed that both Tc and tigecyclines bind to overlapping sites on the 70S and most likely have the same mode of action. The large substituent (t-butylglycylamido) at position 9 of tigecycline (Figure 1.4c) is most likely responsible for its ability to overcome the Tc^R by efflux pumps and RPPs [203]. Tigecyclines were developed in response to the growing prevalence of multidrug resistance in superbugs common in hospitals (methicillin resistant *Staphylococcus aureus*, *Acinetobacter baumanii* and vancomycin resistant *Enterococci*) and are commonly used to treat infections in intensive care unit (ICU) patients [201]. The New Delhi metallo-β-lactamase multidrug resistant Enterobacteriaceae has also shown to be susceptible to tigecycline [204]. Unfortunately, tigecycline resistance has already been reported in *Acinetobacter baumanii* in a hospital study in Israel [205] and in *Enterococcus faecalis* isolated from a 65 year old ICU patient in Germany [206]. Tigecycline resistance in *E. faecalis* was not caused by the *tet(X)* gene or efflux pumps or 16S rRNA mutations. The multidrug resistance efflux pump: resistance-nodulation-division (RND) found in *P. aeruginosa*, *Proteus* spp., *Morganella* spp., and *Providencia* spp. confers tigecycline resistance [75]. This suggests a new

generation of Tcs should be developed soon to develop alternative strategies of therapy for these bacteria.

Understanding the mechanism of Tc^R at the molecular level can allow the development of novel strategies for the treatment of infections. Tc molecules may be used in combination with other molecules, or Tc molecules may be modified in a way to interfere with RPPs such as Tet(O) (present within the bacterial cell) to allow Tc to maintain its bacteriostatic activity. Development of such effective therapeutics may be possible by collaboration with pharmaceutical companies whose key proprietary platform technology is the development of novel Tc analogues. Paratek Pharmaceuticals Incorporation (started by Dr. Stuart B. Levy, professor of Molecular Biology and Microbiology and of Medicines at Tufts University School of Medicine, and Dr. Walter Gilbert, the Nobel Prize winning professor at Harvard University) developed a database of more than 1,000 novel, proprietary Tcs designed to address widespread resistance to the drug, as well as potentially offering new therapies for a wide range of conditions from arthritis to cancer [unpublished data].

1.4 Tet(O)

1.4.1 Ribosomal protection by Tet(O)

The ribosomal protection protein Tet(O) is one of the major determinants of Tc^R [151-152]. RPPs vary in length between 639-663 amino acids. They have been classified into 3 groups based on their amino acid sequence similarity [68, 191, 207]. The best characterized RPPs, Tet(M) and Tet(O) share 75% sequence similarity and have a molecular mass of 72.5 kDa [184]. Both these proteins along with Tet(S), Tet(32) and Tet(W) share 67-77% amino acid sequence similarity [68, 191, 207] and belong to group I (Table 1.4).

The *tet(O)* gene was first cloned from the pUA466 plasmid of *C. jejuni* UA466 strain [179]. pUA466, a 45-kilobase transmissible Tc^R plasmid was mapped with *Ava*I, *Ava*II, *Bcl*I, *Hinc*II, *Pst*I, *Xho*I and *Xba*I. The Tc^R determinant was cloned and expressed in *E. coli* and its homology with *tet(M)* Tc^R determinant

from *Streptococcus* spp. [230] was compared. The *tet(O)* gene from *C. jejuni* UA466 strain was sequenced by Manavathu *et al.* [208] and so far is the only reported *tet(O)* gene sequence from *C. jejuni*. However, in several Gram positive bacteria [209] and in some strains of *Campylobacter* (*C. coli*) residing within the intestine of poultry, Tet(O) may also be encoded by chromosomal genes [200]. A study by Kos *et al*. [197] reported that 72/104 *C. jejuni* isolates obtained from peptone washes of retail broilers from Alberta, Canada were Tc^R (Tc MIC 64-256 μg/mL). All the Tc^R isolates carried the *tet(O)* gene which in 93% of the *C. jejuni* isolates (67/72) was present on the plasmids and 7% (5/72) of the isolates was found on the chromosome. Likewise, studies by Lee *et al.* [198] reported 96% of Tc^R *C. jejuni* isolates from chicken, and 88% of clinical Tc^R *C. jejuni* isolates harbored the *tet(O)* gene in the plasmids. These results are also consistent with the studies by Gibreel *et al*. [63] who reported the presence of plasmid encoded *tet(O)* gene in 67% (67.67/101) clinical Tc^R *C. jejuni* isolates in Alberta, Canada. However a study by Pratt *et al.* [210] reported the presence of *tet(O)* gene in the chromosome of 76% of Tc^R Australian *Campylobacter* strains while only 32.3% carried a *tet(O)*-harbouring plasmid. The occurrence of a plasmid-free Tc^R *tet(O)* determinant may be explained by recombination events between a Tc^R plasmid and chromosome or the integration of such plasmids into the chromosome. The Tc^R determinant *tet(O)* requires a 300 bp upstream DNA sequence for its full expression and function [211]. Tet(O), like Tet(M), protects the ribosome from the inhibitory action of Tc by dislodging Tc from its primary binding site (near the A-site) on the ribosome, thus conferring Tc^R [178].

1.4.2 Structural similarity of Tet(O) and elongation factor proteins

All RPPs including Tet(O) shows a high degree of homology with the bacterial elongation factor (EF) superfamily GTPases [212], EF-G and EF-Tu [213] and exhibit a ribosome-dependent GTPase activity [183, 185, 187]. The maximum homology is observed in the N terminal GTP binding domain, which is composed of 5 distinct motifs, G1 to G5 [184, 214] Guanine moiety of GTP binds to the G4 motif which comprises of two highly conserved residues asparagine

and aspartate. Substitution of Asparagine-128 by other amino acids abolishes Tc release activity of Tet(O), suggesting GTP binding is essential for Tet(O)-mediated Tc^R [184, 214]. The amino acid sequences of all RPPs are remarkably similar to the five different domains [184, 213] of EF-G, and suggest an evolutionary link among them. RPPs might have arisen from an ancestral EF-G gene, which lost its original function and now functions as antibiotic resistant determinant. As a consequence of this homology, RPPs were considered to function like tetracycline resistant elongation factors by Sanchez-Pescador *et al.* [213]. Studies by Burdett [187] and Deming *et al.* [35] illustrated the RPP Tet(M) failed to substitute the function of bacterial elongation factor proteins *in vivo* and *in vitro,* ruling out the possibility that RPPs have arisen from ancestral EF-G gene.

1.4.3 Localization of Tet(O) binding sites on the ribosome

The binding sites for RPPs on the 70S ribosomes have been studied by three different techniques namely: biochemical [215], cryo-electron microscopy (cryo-EM) [68] and chemical probing by dimethyl sulphate (DMS) [178, 216].While chemical probing and cryo EM studies were done to show the interaction between Tet(O) and the 70S, the biochemical studies focused on the contacts of Tet(M) with 70S**.**

The biochemical assay by Dantley *et al.* [215] revealed Tet(M) and EF-G compete for binding to similar sites on the 70S ribosome. The antibiotic thiostrepton binding L11 region of the 50S subunit was a constituent of this site. Binding of thiostrepton alters the conformation of the 70S that inhibits binding of both EF-G and Tet(M) to the ribosome.

Cryo-EM by Spahn *et al.* [68] has led to the visualization of Tet(O) complexed to *E. coli* 70S ribosome at a resolution of 16Å (Figure 1.15), in an unfixed and unstained condition, and in its native environment, in the presence of an non-hydrolyzable GTP analogue. Comparison of the three dimensional Tet(O)-ribosome complex with EF-G-ribosome complex revealed that both Tet(O) and EF-G had very similar shape and bound to similar sites within the intersubunit cavity of 70S ribosome, on the A site, at the base of L7/L12 stalk [95]. This

agrees well with the biochemical evidence [215] that showed both EF-G and Tet(M) bind to the L11 stalk of the 70S ribosome. The similarity of the Tet(O) and EF-G cryo EM reconstructions with 70S ribosomes is substantiated by the 51% sequence homology shared between the two proteins [184]. This similarity in sequence has resulted in the identification of tentative domains of Tet(O) based on its homology with domains of EF-G [217] (Figure 1.16).

Cryo-EM studies [68] also revealed that domains I (G), II, III and V of both EF-G and Tet(O) interact similarly with the different components of the bacterial ribosome. Tet(O) mostly interacts with the rRNA components of the 70S, with the exception of domain III which interacts with protein S12 (Table 1.5). The domain I of Tet(O) that binds to GTP interacts with the α-sarcin stem loop of the 50S subunit of 70S ribosome. Domain II interacts with the helix 5 of 16S rRNA that connects shoulder to the lower part of the body of the 30S subunit [82].

DMS studies by Connell *et al*. [178] revealed that Tet(O) preferentially interacts with the post-translocational ribosome (Figure 1.13). Conformational changes most likely around the ribosomal GTPase-associated centre is induced following Tet(O) binding. This in turn is reflected in the altered DMS modification pattern around the L11 region of the 50S subunit (Figure 1.17).

1.4.4 Differences in the interaction of the domains of Tet(O) and EF-G with 70S ribosomes

The difference in the functions of EF-G and Tet(O) is dictated by the differences in the way the Domain IV of the two proteins interact with the ribosome. In EF-G domain IV contacts bridge B2a of H69 of 23S rRNA (Figure 1.18b) [218-219] that connects the 50S with h44 of 16S rRNA of the 30S subunit and extends into the A site in a way that the tip of domain IV overlaps the anticodon arm of the tRNA [218, 220] (Figure 1.19b). This close contact of EF-G with the anticodon arm of the A-site tRNA is essential for the translocation of tRNAs.

The tip of the domain IV of Tet(O) does not contact the bridge B2a (Figure 1.18a) or overlap the anticodon arm of tRNA (Fig.1.19a) in the A-site [107]. It contacts the joint between the head and the shoulder of the 30S subunit composed of the minor groove of h34 (1051-53:1208-10) and 530 pseudoknot of h18 (517-19:531-33) [107] which lies close to primary Tc binding site [69] (Figure 1.20). The interaction of domain IV of Tet(O) with h34 of the 30S subunit, which constitutes the primary Tc binding site, is compatible with its role in Tc release.

In EF-G, domains I and II rotate relative to domains III, IV and V following the binding of EF-G to the 70S [218, 220]. This rotation introduces subtle differences in the interaction of domains III and V of GTP-bound EF-G with 70S. For instance, the domain V of EF-G comes close to the base A2477 in the loop of H89 of 23S rRNA. This rotation movement is not observed in Tet(O) interactions with 70S. The domain V of Tet(O) lies close to the N-terminal domain of the L11 protein, a component of the thiostrepton binding domain of 23S rRNA of the 50S subunit.

The absence of the relative rotation of the domains in Tet(O) compared to EF-G, allows domain III of Tet(O) to interact more intimately with the S12 protein of the 30S subunit [82, 107, 221]. Point mutations in the S12 protein reduced Tc^R mediated by Tet(O) or Tet(M) [222]. These studies showed that one particular mutation in the S12 protein (Lys42Gln) almost abolished Tc^R mediated by Tet(O). This suggests that interaction of Tet(O) with S12 may be critical for its ability to release Tc.

Connell *et al*. [178] reported that the L11 and α-sarcin loop are critical for Tet(O) function, as is illustrated by alterations in DMS reactivity of bases in this region [Figure 1.17]. EF-G protein was also found to contact the L11 and α-sarcin loop in the 50S subunit, which were critical for the GTP-ase activity of EF-G [226-228]. This indicates both Tet(O) and EF-G contact similar ribosomal elements for GTP-ase activity. However, the DMS studies [178] demonstrated that Tet(O) and EF-G interacted with different bases in the H42/43/44 regions [Figure 1.17a-c, 178]. This likely arises because EF-G interacts with

pretranslocation ribosomes while Tet(O) interacts with posttranslocation ribosomes. During translocation the L11 region undergoes conformational changes [225], following which Tet(O) binds to the 70S and hence recognizes a conformation of the 50S different from that recognized by EF-G.

1.4.5 Changes in ribosome conformations introduced by Tet(O) and EF-G

The conformational changes induced in the 70S ribosome by EF-G in the presence of GTP or GDP have been studied [218, 223]. Binding of EF-G•GMPPCP (a noncleavable GTP analogue) to the 70S principally caused a bifurcation of the L7/L12 stalk of the 50S subunit, introduced a "ratchet movement" of the 50S subunit relative to the 30S subunit coupled with relative rearrangements within the 30S subunit (change in the shape of 30S subunit head [218, 223] and its movement [107, 218, 223] relative to the platform and the body), and affected the L1 protuberance of 50S. When EF-G•GTP complex binds to the 70S, the C-terminal domain of L9 is detached from the 50S sub unit and the N-terminal domain of L9 stays attached below the L1 protein (Figure 1.18b**).**

Spahn *et al.* [107] showed Tet(O)•GMPPCP complex binds to the 70S without introducing any conformational changes in the 70S that are observed with EF-G•GMPPCP. The only alteration observed is the extension, instead of bifurcation, of the L7/L12 stalk. An"arc-like" connection exists between the G' domain of the fusidic acid-stalled EF-G•GMPPCP•70S complexes or kirromycin-stalled EF-Tu•GMPPCP•70S complex and the base of L7/L12 stalk of 50S [220, 224]. This "arc-like" connection is not found in Tet(O)•GMPPCP•70S complexes.

Absence of such conformational changes in 70S induced by Tet(O) explains why RPPs such as Tet(O), cannot substitute for the translocation function of EF-G [185, 187, 218, 223].

Cryo-EM studies by Spahn *et al.* [107] suggested the tip of domain IV of Tet(O) closely approaches the primary Tc binding sites in the 70S (Figure 1.20). Tc in its primary site contacts h34 and h31 of 16S rRNA. The tip of domain IV of Tet(O) contacts h34, h18 and S12 protein. DMS studies by Connell *et al.* [216] localized the sites of interaction of Tet(O) with the 30S subunit (Figure 1.21). On

the 30S subunit, Tet(O) contacts h34 (C1214) and h44 (A1408) which occupies the A-site, close to the primary Tc binding site. The DMS sensitivity of a base can be either enhanced or reduced following the binding of Tet(O). Increased susceptibility of a base to DMS can arise only through a conformational change induced by Tet(O) binding. Protection of a base from DMS arises from a conformational change or direct shielding of the base by Tet(O). The C1214 base was protected from DMS following the binding of Tet(O). Cryo-EM data illustrated Tet(O) domain IV closely approaches C1214, indicating Tet(O) either interacts with C1214 directly or indirectly via other bases in the h34. The cryo-EM data of Spahn *et al.* [107] exemplified that domain IV of Tet(O) did not approach A1408 in h44 which was therefore more susceptible to DMS. This demonstrates Tet(O) binding to the 70S induces "long-range rearrangements" in the 70S. These changes may be facilitated by the S12 protein which lies on the top of h44 and also appears to interact with Tet(O) [107].

1.4.6 Mechanism of Tet(O)-mediated tetracycline release

In order to circumvent the problem of Tc^R, it is important to understand how the activity of Tc is inhibited by Tet(O). The Tc-stalled ribosome is recognized by Tet(O), either by the virtue of its altered conformation or the presence of a vacant A-site, which allows Tet(O) to bind to the ribosome [95]. DMS-probing experiments [216] and cryo-EM studies [107] had revealed Tet(O) triggers release of Tc by binding to a site close to the primary Tc binding site, while previous work by Trieber *et al.* [229] has shown that Tet(O) in its GTP bound state can release Tc. Moreover the tip of Domain IV of Tet(O) was found to be located within 6Å of the primary Tc binding site, which is too large for a direct interaction between Tet(O) and Tc on the ribosome [107]. A model for Tet(O)-mediated Tc release was proposed by Spahn *et al.*[107] (see section 1.2.3.2.1, Figure 1.13 f to j). Binding of Tet(O) at the base of the h34 minor groove and pseudoknot of h18, causes a disturbance in h34 (around residues 1053-1056 which lies in the sugar phosphate backbone of h34 bulge). This disturbance is propagated to the primary Tc binding site which releases Tc by an

allosteric mechanism [107]. Tet(O), however, is unable to release Tc from the secondary binding sites, which supports the idea that the primary Tc binding site is the protein elongation inhibitory site [216].

The most recent model for Tet(O)-mediated Tc release was proposed by Connell *et al*. [178] (Figure 1.22). Their data suggested that both Tc and Tet(O) induce conformational changes in the ribosome. The increased GTP hydrolysis by EF-Tu in the presence of $Tet(O)^{D131N}$ mutant (can hydrolyze XTP but not GTP) relative to its absence suggested that the conformational change induced by Tet(O) continues even after it has been released from the ribosome. This prevents Tc from reassociating with the ribosome. DMS studies by Connell *et al.* [178] suggested the conformational change induced by Tet(O) occurs around the L11 and α-sarcin loop of the 50S subunit of the ribosome, critical for the GTP-ase activity of Tet(O) [178] (see section 1.4.4). Thus the ternary complex (EF-Tu•aa-tRNA•GTP) can efficiently compete with Tc and occupy the A- site and thereby restore protein synthesis. However when the A-site is empty following translocation, Tc may once again bind to the 70S ribosomes.

Antibiotics other than Tc also interfere with protein synthesis in bacteria. For instance, aminoglycosides (such as gentamicin, streptomycin, kanamycin, tobramycin, neomycin, apramycin, paromycin, etc.) have the ability to inhibit protein synthesis in bacteria, by one of the following three ways: (i) blocking the formation of the 70S initiation complex formation prior to the initiation of protein synthesis, (ii) binding to the A-site of the 30S subunit and increasing the affinity of the A-site for ternary complex whose anticodons do not match the codon in the mRNA. This causes miscoding of the amino acids in the emerging nascent polypeptide chain, or (iii) by blocking the translocation of the A-site tRNA into the P-site [232]. Tet(O) protein however is likely unable to confer resistance to aminoglycosides because Tet(O) is capable of binding only to a post-translocation ribosome, which has a vacant A-site.

1.5. Rationale of the study

The *C. jejuni* Tet(O) shares 51% sequence similarity with *Thermus thermophilus* EF-G [184] and a similar tertiary structure as substantiated by cryo-EM studies [107]. Tet(O) and EF-G are both ribosome-dependant GTPases [183, 185, 187] that contact the ribosome at similar sites [107], yet they perform very different functions. Domains I (GTPase) and II of both Tet(O) and EF-G interact similarly with the ribosome, whereas, domains III, IV and V occupy discrete positions in the ribosome cavity (see section 1.4.4). Of particular importance is that the tip of domain IV of EF-G and Tet(O) interact very differently with the ribosome. While domain IV of EF-G contacts the 50S subunit only, Tet(O) domain IV exclusively contacts the 30S subunit of the bacterial 70S ribosome [107]. The amino acid residues H(E/D)VDSS constitute the domain IV distal tip of EF-G were found to be conserved at positions 583-588 in all EF-Gs studied [217] and were critical for the translocation activity of EF-G [233-234]. Similar conserved residues are however not present in the eukaryotic elongation factor-2 (e-EF2) that is responsible for translocation in eukaryotes or in the mitochondrial EF-G of rats (rEF-Gmt) which shares 62% sequence homology with *Thermus thermophilus* EF-G [235]. Interestingly, aligning the RPPs (including Tet(O)) showed that the amino acids YSPVST in the equivalent domain IV loop sequence are well conserved at positions 507-512 [217]. These observations suggest that YSPVST residues may be functionally critical for RPPs such as Tet(O), for releasing Tc bound to 70S ribosomes. Accordingly, these amino acids may be responsible for the functional difference between Tet(O) and EF-G. To date no studies have reported whether the residues YSPVST are critical for Tet(O)-mediated Tc resistance.

In order to study the *C. jejuni* UA466 Tet(O) protein, it is necessary to overexpress and purify it using a suitable model organism. *C. jejuni* is a level 2 pathogen and requires special safety precautions for working in the lab. It grows optimally under microaerophilic (5-10% O_2 and 2-10% CO_2) and thermophilic (42°C) conditions. Although the genome of *C. jejuni* NCTC 11168 has been fully sequenced, the fastidious growth conditions of *C. jejuni* and their increased

sensitivity to environmental stresses like aerobic conditions, acidic conditions, heat (above 48°C), dessication and disinfectants does not make it a suitable model organism to work with. For these reasons *E. coli* BL21(DE3) was used as a model organism for our study. *E. coli,* unlike *C. jejuni,* has a rapid growth rate and grows optimally with simple nutritional supplements. *E. coli* can be easily grown at 37°C under aerobic conditions. Genetic manipulations can be easily performed in *E. coli* whose genome has been sequenced andterized system. Accordingly, *E. coli* will allow effective overexpression and purification of Tet(O).

The purified Tet(O) protein may be studied *in vitro* with purified 70S ribosomes of *E.coli*. Under *in vivo* conditions, 70S monomers do not exist unless the bacterial cells are actively translating mRNA [236]. *In vivo* conditions may be artificially simulated by forcing the 30S and 50S subunits to stay together in the presence of a high concentration of Mg^{2+} [237]. With the successful overexpression and purification of Tet(O), it has become feasible to carry out *in vitro* studies of Tc release from 70S ribosomes.

1.5.1 Hypothesis

This thesis tested the hypothesis that amino acid residues YSPVST, occupying positions 507-512 at the tip of domain IV of Tet(O), are critical for Tet(O)-mediated Tc release from its primary binding site on the 70S ribosome.

1.5.2 Objectives

1.5.2.1 To determine how changes in the structure of the domain IV tip of Tet(O)^{WT}, introduced by the substitution or deletion of residues YSPVST, can alter the ability of Tet(O)^{mutants} to release Tc from its primary binding site on 70S ribosomes

A series of Tet(O) single amino acid substitution and deletion mutants were previously generated by site-directed mutagenesis (Nehal Thakor and Breanna Baranec) of Tet(O)^{WT} to assess the impact of these changes on the interactions between Tet(O) and the 70S ribosome as reflected by changes in Tc susceptibility and Tc release. The *tet(O)^{WT}* gene isolated from the *C. jejuni* UA466 strain carries an intact YSPVST sequence at the loop tip of the domain IV and is capable of releasing 70S-bound Tc and has a Tc^{R} phenotype.

i) Alanine scanning mutagenesis

Alanine scanning introduces subtle changes in the conformation of a protein which may or may not affect its function. A single alanine replacement of the YSPVST residues at the tip of domain IV did not affect the Tc susceptibility (Tc^{S}) of the Tet(O) single alanine mutants. Double alanine scanning mutagenesis was proposed to be a more sensitive indicator of the impact of changes in the interactions of Tet(O) with 70S.

ii) Deletion mutants

Sequential deletion of the YSPVST residues was proposed to produce a dramatic change in the conformation of the loop structure of domain IV of Tet(O)^{WT}. Deletion of the individual amino acid residues in the loop of Tet(O) may collapse the loop and affect the interactions of Tet(O) with 70S.

iii) *Sequentially substitute Tet(O) domain IV amino acids with those of EF*-G

The Tet(O)^{WT} domain IV residues YSPVST, were replaced sequentially by the equivalent EF-G residues, H(E/D)VDSS, to generate Tet(O) polar/non polar mutants. Substitution with these residues was proposed to alter the loop conformation by creating a closer inter-helical packing by virtue of their polar side chains which may in turn affect the interactions of Tet(O) with 70S.

1.5.2.1.1 To determine the degree of structural change in Tet(O) needed to detect a change in the Tc^{S} phenotype

Screening for the Tc^{S} phenotype allows for the interpretation of the impact for the structural change in the generated Tet(O)^{mutants} and thereby identify candidate mutations that alter the ability of Tet(O)^{WT} to release Tc.

1.5.2.1.2 To correlate the ability of Tc^{S} phenotype of the Tet(O)^{mutants} with their ability/inability to release Tc from its primary binding site by *in vitro* Tc-70S-Tet(O) interaction studies

In vitro Tc binding assays conducted in the presence of purified Tet(O)$^{WT/mutant}$ proteins and isolated 70S ribosome complexes provides information on the binding affinity of Tc to the 70S. Tc binding to the 70S in the absence of Tet(O)$^{WT/mutants}$ reflects maximal Tc binding. The calculation of kinetic parameters assesses the binding affinity of Tc to the primary binding site on the 70S ribosome. Comparison of the Tc binding curves of Tet(O)$^{WT/mutants}$ versus 70S alone provides a measure of Tc release by the Tet(O)$^{WT/mutant}$ protein. The correlation of Tc release with the Tc^{S} phenotype will offer a molecular basis for the observed Tc^{S} phenotype.

Table 1.1: Number of reported travel acquired cases of *Campylobacter* infections in Canada from 2000-2004 [29]

Area	**2000**	**2001**	**2002**	**2003**	**2004**	**# of Travel Acquired Campylobacteriosis Cases**
Mexico & Caribbean	2	5	10	7	-	**24**
Asia	5	4	4	3	5	**21**
Europe	2	-	2	1	2	**7**
Central & S.America	1	1	1	1	-	**4**
Africa	1	-	-	1	1	**3**
United States	-	1	-	1	1	**3**
Multiple regions	2	1	-	-	-	**3**
Australia & Pacific	1	1	-	-	-	**2**
Total Cases	**14**	**13**	**17**	**14**	**9**	**67**

Table 1.2 Comparison of Brodersen's and Pioletti's Models of Tc Binding Sites on the 70S Ribosomes

Model	Technique used	Resolution	Tc Binding sites
Brodersen *et al*. [69]	*X crystallography* of *T. thermophilus* 30S bound to Tc	3.3 Å	Primary=high affinity Secondary= low affinity Tc interacts only with 16S rRNA
Pioletti *et al.* [76]	*X crystallography* of *T. thermophilus* 30S bound to Tc	4.2 Å	Tet1-Tet6 Tc binding sites Tet1= high affinity, Tet5 = low affinity, Tet2, 3,4 and 6 interacts with, S4,S9,S17 and S7/S9

Table 1.3 The different types of tetracycline resistance determinants [156]

Efflux (27)	**Ribosomal Protection (12)**	**Enzymatic[c] (3)**	**Unknown[a]**
Tet(A), Tet(B), Tet(C), Tet(D), Tet(E)	Tet(M), Tet(O),Tet(S), Tet(W), Tet(32)	Tet(X)	Tet(U)
Tet(G), Tet(H), Tet(J), Tet(V), Tet(Y)	Tet(Q), Tet(T), Tet(36),	Tet(37)	
Tet(Z), Tet(30), Tet(31), Tet(33)	Otr(A), Tetb(P)[b],Tet	Tet(34)	
Tet(35)[d]	Tet(44)		
Tet(39), Tet(41)			
Tet(K), Tet(L),Tet(38)			
Teta(P), Tet(40)			
Otr(B), Otr(C))			
Tcr			
Tet(42)			
Tet(43)			

[a] *tet* (U) does not appear to be related to either efflux or ribosomal protection proteins
[b] *tetB*(P) is not found alone, *tetA*(P) and *tetB*(P) are counted as one operon
[c] *tet*(X) and *tet*(37) are unrelated but both are NADP-requiring oxidoreductases, *tet*(34) similar to the xanthine-guanine phosphoribosyl transferase genes of *V. cholerae*;
[d] Not related to other *tet* efflux genes
Data obtained from the website of Roberts MC [156] which is updated twice a year.

Table 1.4 Classification of Ribosomal Protection Proteins [107,191]

Group	RPPs	aa Sequence Identity
I	Tet(M), **Tet(O)**, Tet(S), Tet(W), Tet(32)	67-77%
II	Otr(A), TetB(P)	36%
III	Tet(Q), Tet(T), Tet(36)	49-60%
Unknown	Tet, Tet(44)	-

Table 1.5 EF-G and Tet(O) interactions with the ribosome [95, 107]

Domain	Interaction with 70S	
	EF-G	**Tet(O)**
I	H95	H95
II	h5	h5
III	S12	S12
IV	**H69**	**h34**
V	H43/44	H43/44

Helix is abbreviated with a lowercase h when referring to a helix within the 16S rRNA, whereas an uppercase H refers to a helix within the 23S rRNA.
Numbers in bold highlight the different contact sites of Tet(O) and EF-G domain IV with the 70S ribosome.

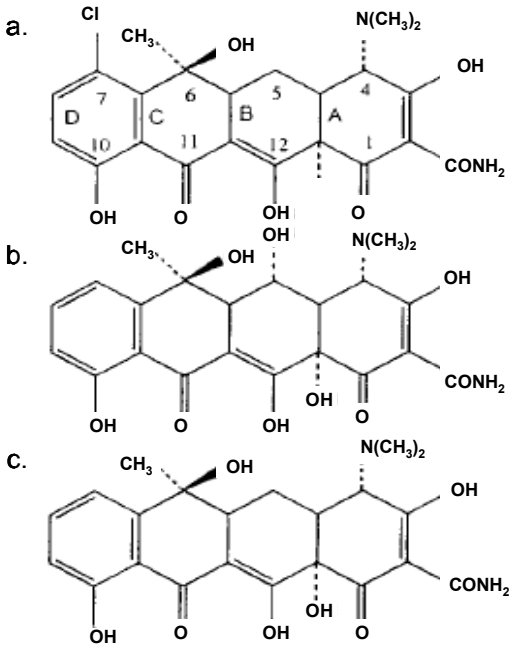

Figure 1.1 The first generation typical tetracyclines [68]

(a) 7-chlorotetracycline (Aureomycin) first discovered in 1948;
(b) 5-Hydroxytetracycline (Terramycin), discovered in 1950 and;
(c) Tetracycline (Achromycin) discovered in 1953.

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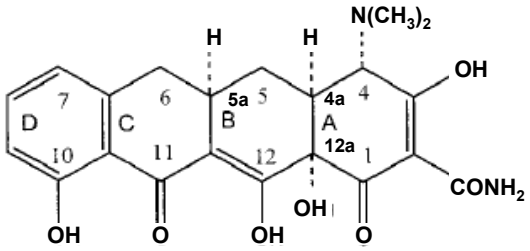

Figure 1.2a Structure of 6-deoxy-6-demthyltetracycline [68]

The antibacterial property is imparted by the hexamembered, carbocyclic, fused, linear tetracyclic nucleus (A, B, C and D), the α-stereochemical configuration at A-B ring junction (positions 4a and 12a), conserved keto-enol system at positions 11, 12 and 12a and the conservation of dimethylamino [$N(CH3)_2$] group at position 4.

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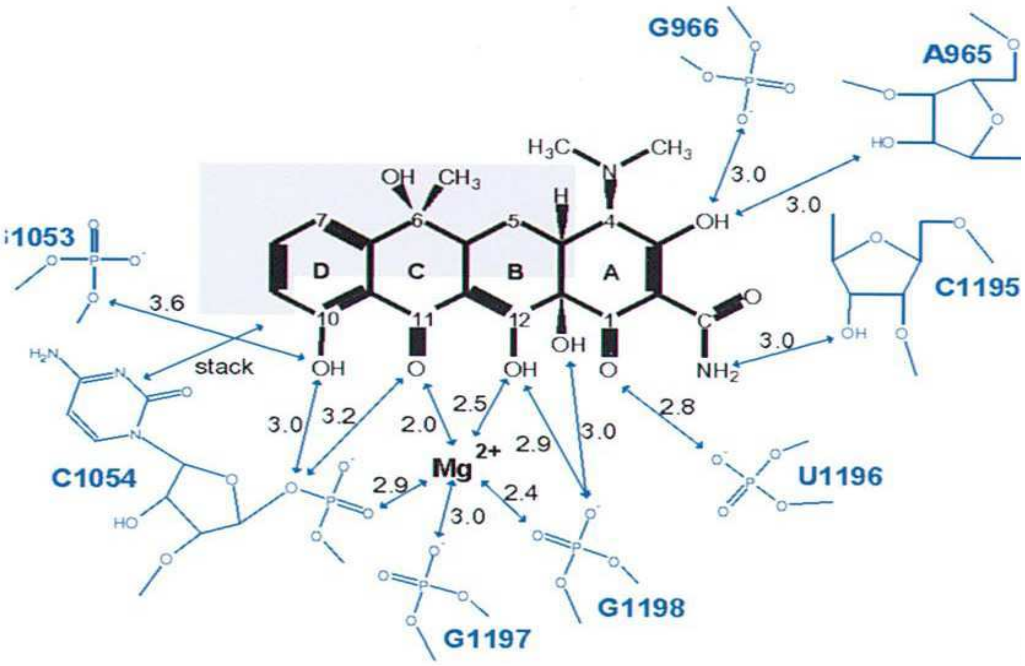

Figure 1.2b Interaction of the polar side chains of Tc with the 16S rRNA residues [69]

The residues C1054 and U1196 most likely shift to accommodate Tc, making C1054 more susceptible to dimethyl sulphate [97]. Some of the contacts between Tc and the 16S rRNA are sequence specific. Most of the interactions that hold Tc in position are the hydrogen bondings that occur between the 2'-OH and 3'-OH groups of the ribose moiety of the 16S rRNA residues and the oxygen and nitrogen atoms attached to the side chain of the ring A of Tc [69].

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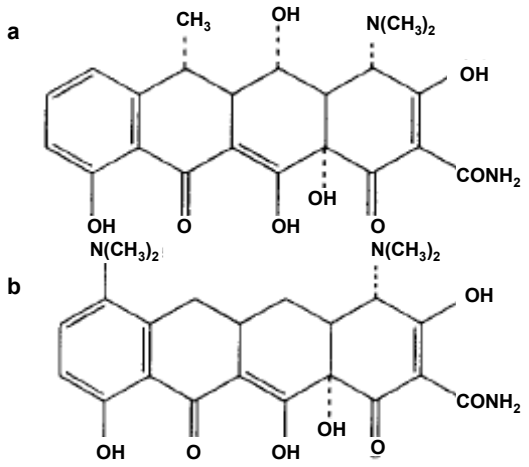

Figure 1.3 The structure of second generation typical tetracyclines [68]

(a) doxycycline and; (b) minocycline.

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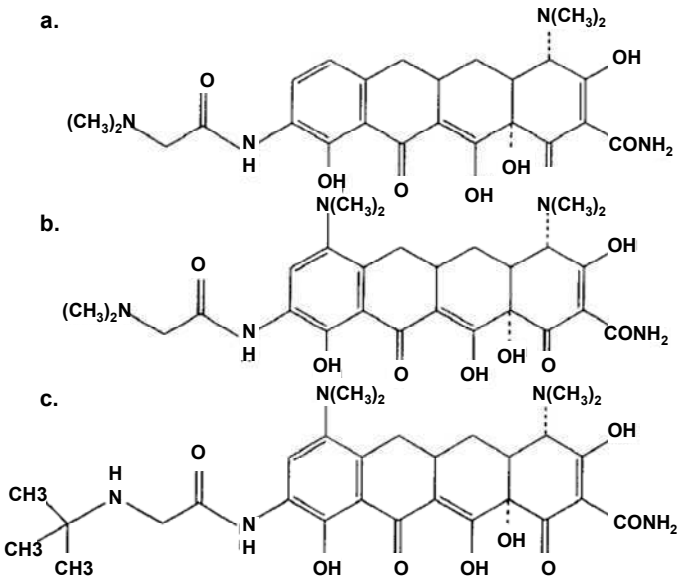

Figure 1.4 The structure of the third generation tetracyclines, Glycylcyclines [68]

(a) 9-(N,N-Dimethylglycylamido)-6-demethyl-6-deoxytetracycline;
(b) 9-(N,N-Dimethylglycylamido)-minocycline and;
(c) 9-(t-Butylglycylamido)-minocycline (tigecycline).

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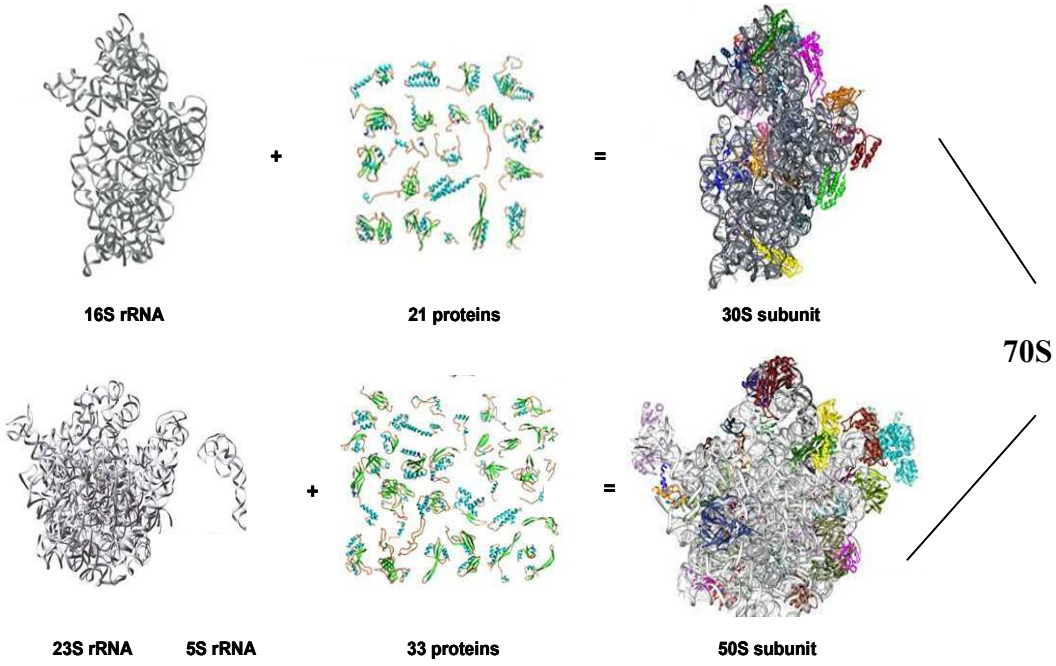

Figure 1.5 The constituents of the 70S ribosome [Modified from http://www.riboworld.com/structure/struktur-eng.html]

The 30S subunit is composed of 16S rRNA and 21 different proteins. The 50S subunit is composed of 23S rRNA and 33 different proteins. The 50S and 30S together constitute the 70S ribosomes at the time of protein synthesis.

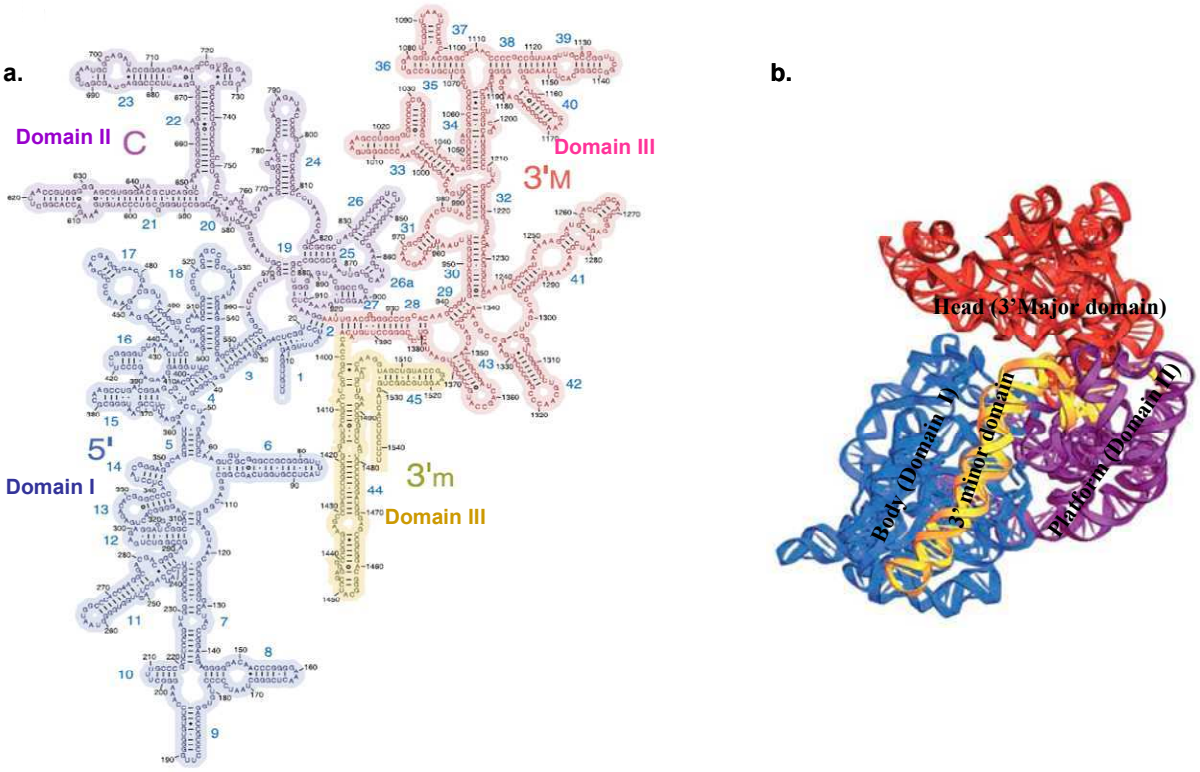

Figure 1.6 Structure of the 30S subunit in *Thermus thermophilus* [241]

(a) Secondary structure of *T.thermophilus* 16*S* rRNA, with its 5'domain (blue) central domain (magenta), 3' major domain (red), and 3'minor domain(yellow) [241]; (b) 3-dimensional folding of 16*S* rRNA in 30*S* ribosomes, with its domains colored as in (a).

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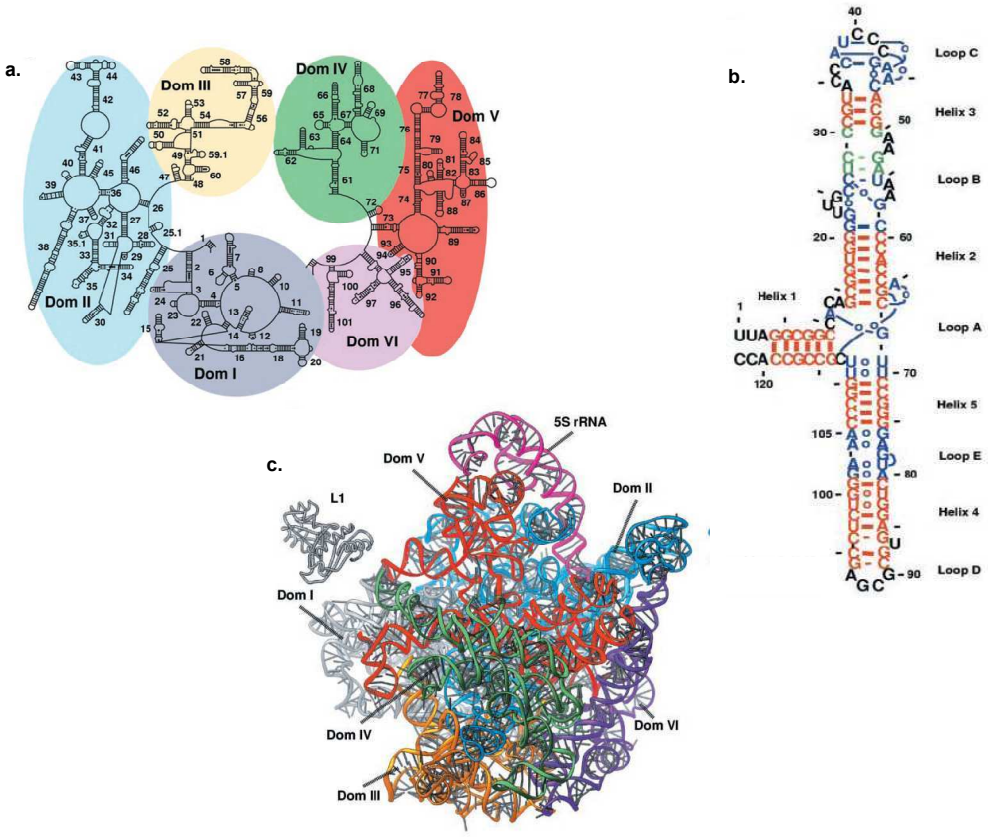

Figure 1.7 Secondary and tertiary structure of the 23S and 5S rRNA in *Holoarcula marismortui* [85]

(a) Schematic secondary structure diaGram of 23*S* rRNA from *H. marismortui;* (b) The secondary structure of 5*S* rRNA from *H. marismortui;* (c) The 50S subunit illustrating the tertiary folding of the 23S and 5S rRNA. Domains are color-coded as shown in the schematic (a) and (b).

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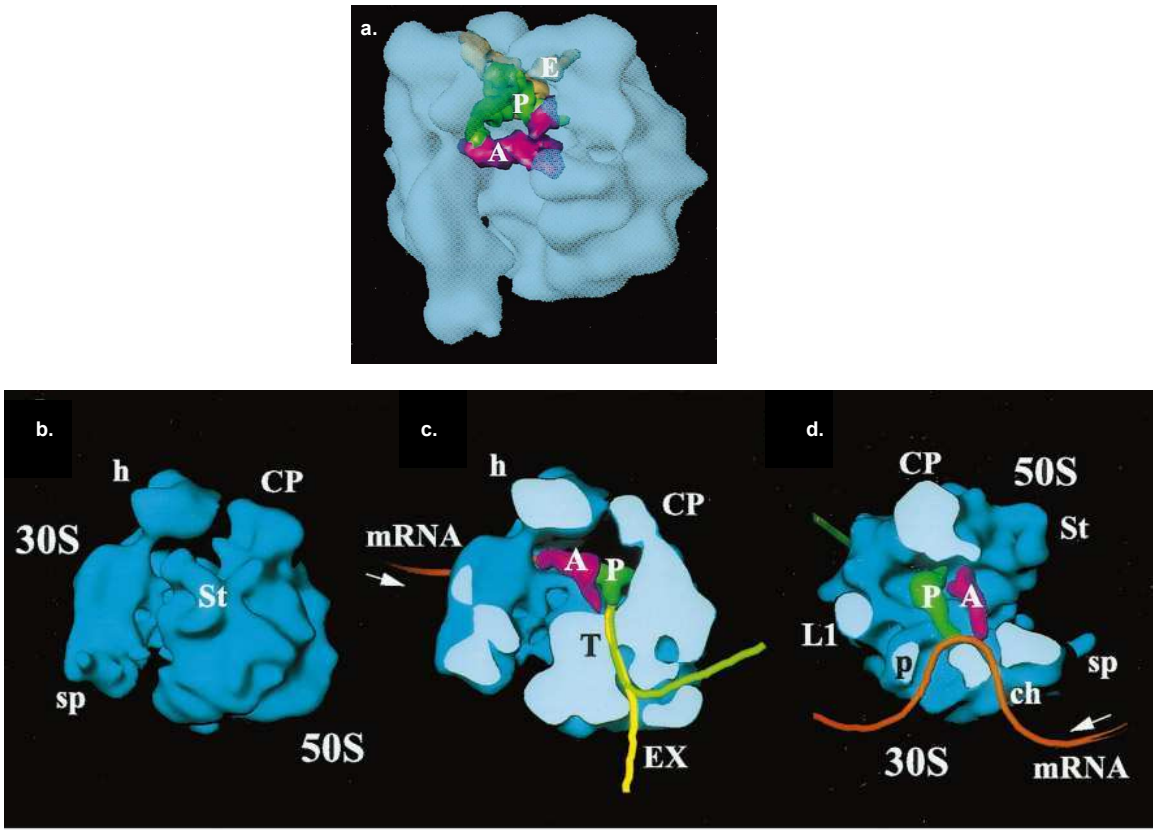

Figure 1.8 Cryo-EM model of the the 70S at 25-Å resolution illustrating the positions of tRNAs and mRNAs in the intersubunit space of the 30S and 50S subunits [242]

(a) The 3 tRNA binding sites at the interface of the 50S and 30S subunits in the 70S ribosome; (b-d) 25 Å cryo-EM map of *E. coli* 70S ribosome demonstrating protein synthesis; (b) Side view of the 70S, showing the 30S and 50S subunits connected by a number of bridges; (c) the placement of tRNAs in A- and P-site positions and the possible pathway of the polypeptide chain through a tunnel in the 50S subunit; (d) the intersubunit space, tRNAs and possible path of mRNA through a channel in the neck region of the small subunit.
Landmarks on 30S: h, head; ch, channel; sp, spur; p, platform. 50S: CP, central protuberance; T, tunnel; EX, exit of tunnel; L1, L1 protein; St, L7/L12 stalk.

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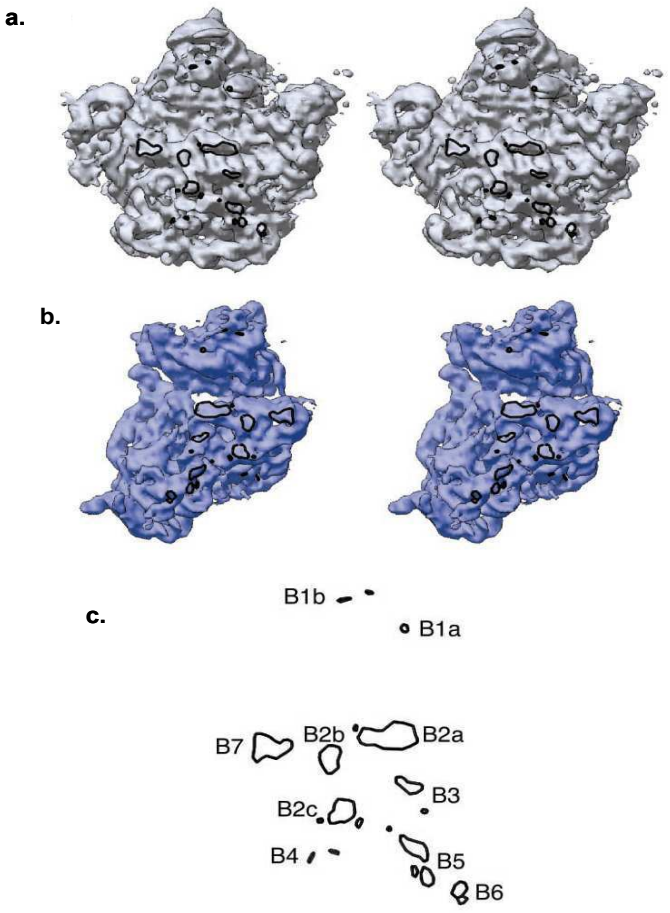

Figure 1.9 The interface of the 30S and 50S subunits formed of 7 bridges [87]

The contact point between the 50S and the 30S subunits are composed of 7 bridges which is a complex interaction between the rRNA molecules which criss-cross each other and holds the 50S and 30S subunits in place in the 70S ribosome. (a) Stereo view of 50S ribosomal subunit interface. Regions of contact between the 50S and 30S ribosomal subunits are surrounded by bridges (indicated as thick black cirlcles); (b) Stereo view of 30S ribosomal subunit interface, marked as in (a). Bridges are the mirror image of those in (a); (c) Cryo-EM reconstructions of the 7 bridges.

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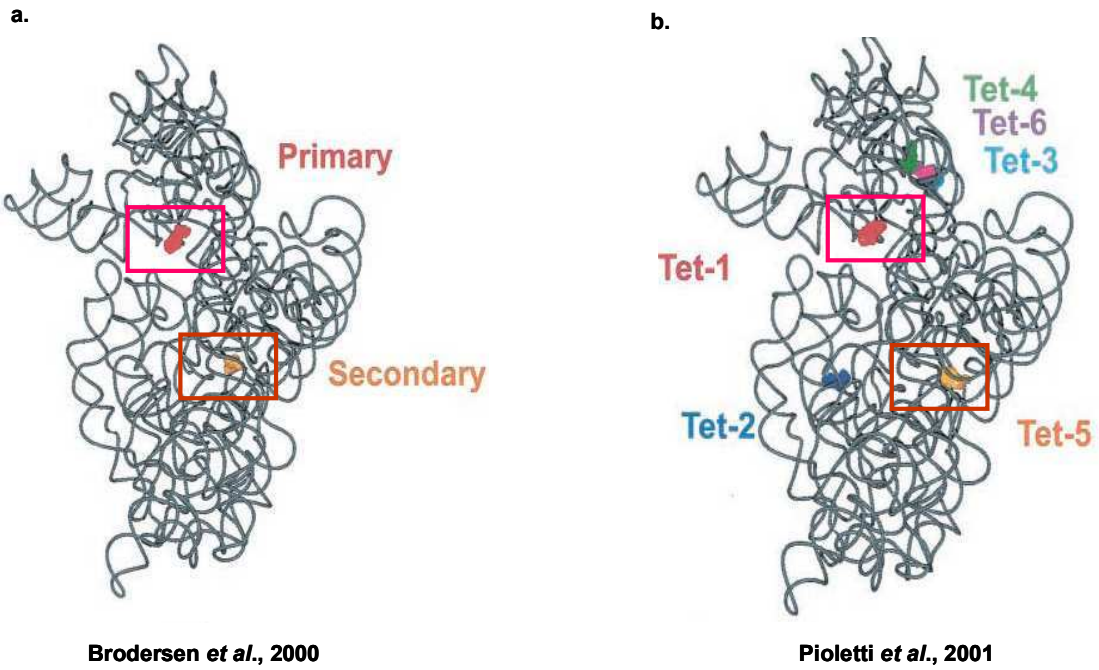

Figure 1.10 Tc binding sites on the 70S as illustrated by the X-ray crystallography studies [95]

(a) The positions of the Tc binding sites determined by Brodersen *et al*. [69]. Primary Tc binding site is shown in red and secondary Tc binding site is shown in orange; (b) The positions of the Tc binding sites determined by Pioletti *et al*.[76]. Tc binding sites are designated Tet-1-Tet-6. Tet-1 site is shown in red, Tet-2 site in dark blue, Tet-3 in cyan, Tet-4 in green, Tet-5 in orange, and Tet-6 site is shown in purple. The boxes represent the common Tc binding to the 70S demonstrated by both studies. Tet-1 site of Piloetti *et al.* [76] is analogous to the primary Tc binding site of Brodersen *et al.* [69].The Tet-5 site in Pioletti's model [76] is similar to the secondary Tc binding site of Brodersen's model [69].

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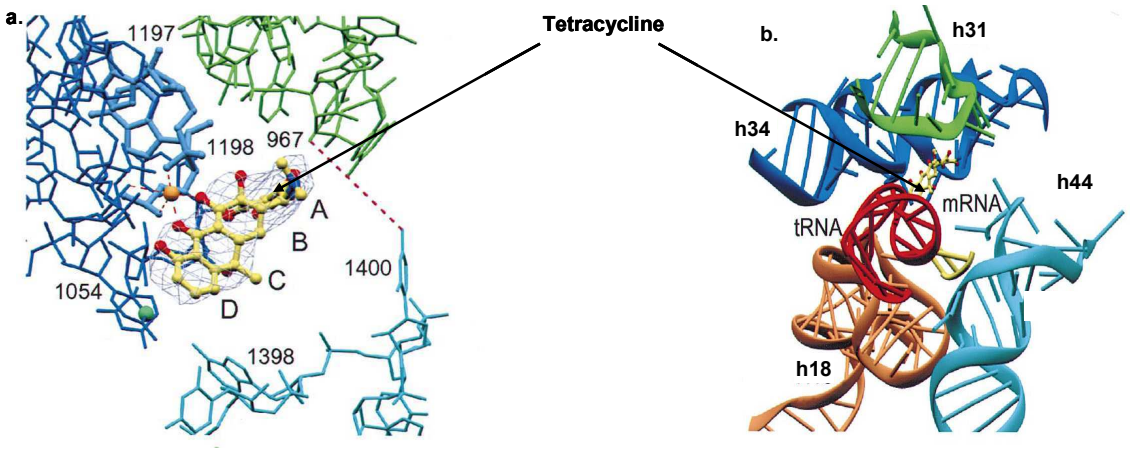

Figure 1.11 Molecular details of primary Tc binding on the 30S subunit and its interaction with the 16S rRNA residues [69]

(a) Tc contacts h34 (blue) and h31 (green) in its primary site. The hydrophilic side chains of Tc interact with phosphate backbone of 1053-1056:1196-1200 16S rRNA residues within h34. Tc forms charged interaction with 964-967 residues of 16S rRNA in h31. C1054 (green sphere) is more reactive to dimethyl sulphate following Tc binding. The bound magnesium ion (gold sphere) mediates contact with G1197 and G1198 16S rRNA residues in h34; (b) Summary of the Tc primary binding site illustrating the interaction with A site tRNA, h34 (blue), h31 (green), h18 (orange), and h44 (cyan). A site tRNA is indicated in red and mRNA is indicated in yellow.

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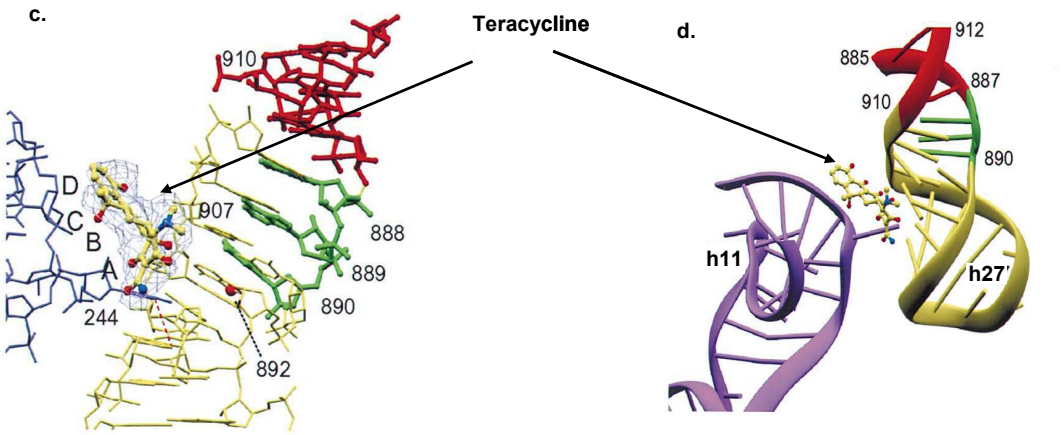

Figure 1.11 Molecular details of secondary Tc binding on the 30S subunit and its interaction with the 16S rRNA residues [69]

(c) Secondary Tc binding sites. h27 (switch region) residues are shown in yellow/green/red and h11 residues are shown in violet. A892 (red sphere) residue that interacts with N1 in Tc is shielded from dimethyl sulphate following Tc binding; (d) Summary of the secondary Tc binding sites indicating h11 and h27 elements that interact with Tc.

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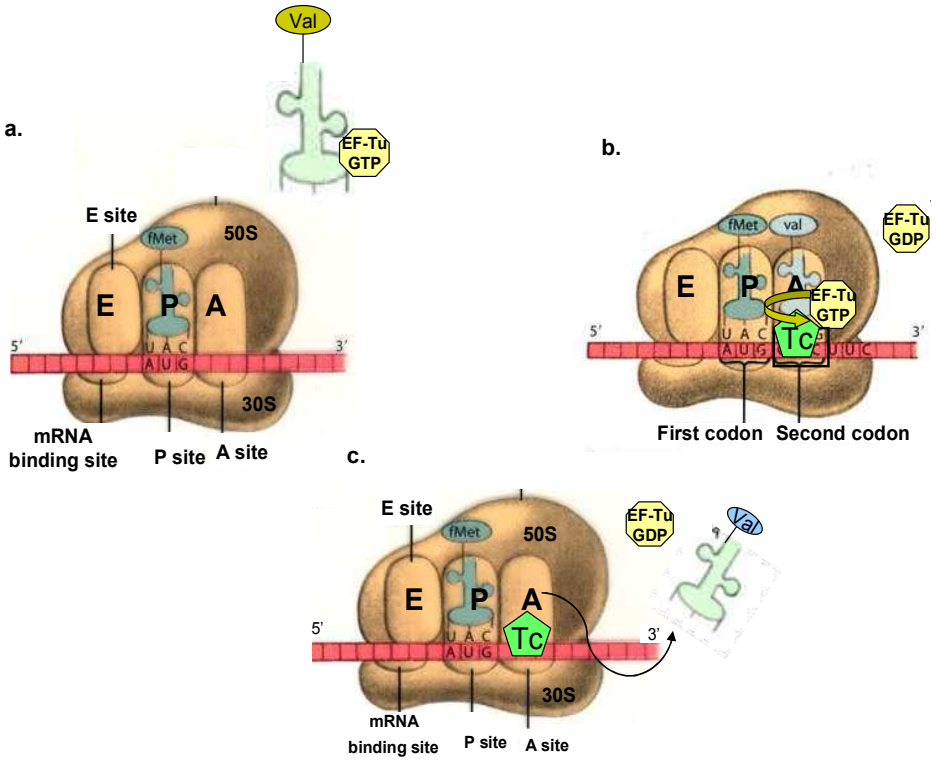

Figure 1.12 Binding of Tc inhibits the elongations step of protein synthesis.
[Modified from:
http://biology.unm.edu/ccouncil/Biology_124/Images/RNAtranslation.jpeg]

(a) Following translation initiation the 70S ribosome has a vacant A (aminoacyl site), Initiator tRNA occupies P(peptidyl site) and the E (exit site) remains vacant. The ternary complex (EF-Tu•GTP•aa-tRNA) occupies the vacant A-site of the 70S ribosome, (b)Tetracycline (Tc) binds to the 30S subunit of the 70S ribosome. Ternary complex binds to 30S in the presence of Tc. GTP ase activity of EF-Tu is activated following binding, which is then released as EF-Tu-GDP complex. The steric clash (indicated by arrow) between the aa tRNA and Tc inhibits accomodation of aa tRNA to A site, (c) In the presence of Tc, aa tRNA is released from the A site.The 70S ribosome has a vacant A site, protein elongation comes to a halt because amino acids cannot be added to the growing polypeptide chain.

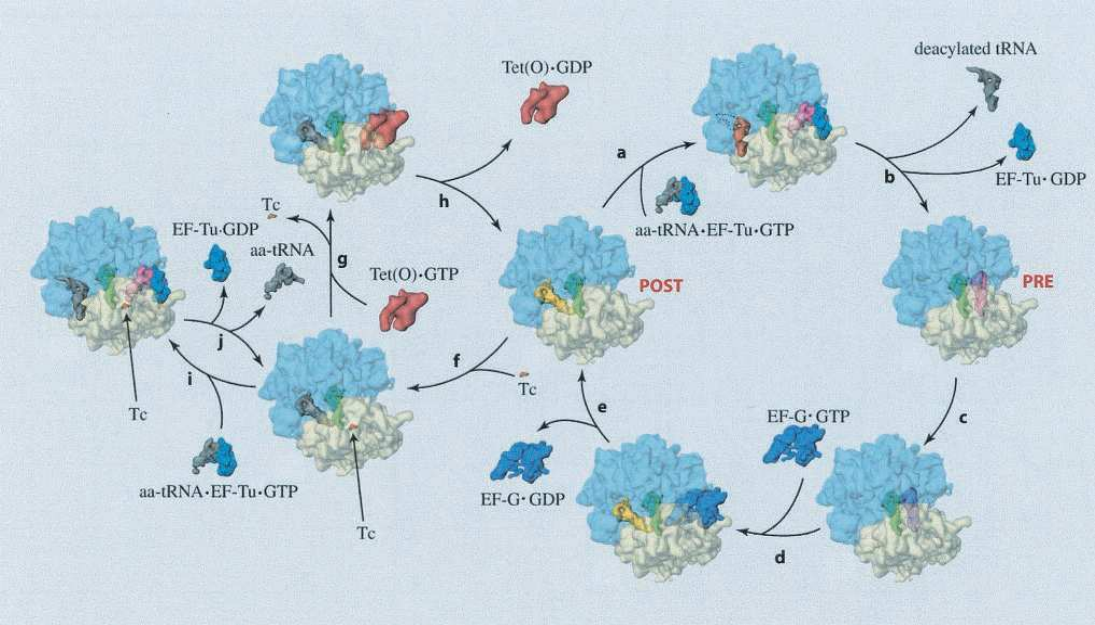

Figure 1.13 Model of the polypeptide chain elongation in the presence of Tc and Tet(O) [95, 107]

(a) The P-site bound peptidyl-tRNA (green) and the E-site bound tRNA (yellow) in a postranlocation 70S ribosome (30S subunit shown in yellow, 50S subunit shown in green). The EF-Tu•GTP•aa-tRNA (ternary complex) binds to the posttranslocation ribosome; (b) EF-Tu has a 70S-dependent GTPase activity. Following GTP hydrolysis EF-Tu•GDP complex dissociates from the 70S which is now in the pretranslocational state; (c) aa-tRNA (pink) occupies A site and peptidyl-tRNA (green) the P site. Peptide bond formation is activated by the peptidyl transferase activity of the 50S sub unit in the pretranslocational ribosome; (d) A-site is then occupied with a peptidyl-tRNA (purple), P site carries deacylated (without amino acids) tRNA. This state of the ribosome is the substrate for EF-G•GTP complex which binds and translocates the tRNAs from A and P sites into P (green) and E sites (yellow), respectively; (e). EF-G like EF-Tu has a 70S- dependent GTPase activity. EF-G•GDP complex dissociates from the 70S which brings the ribosome into the posttranslocational state; (f)–(h) represents the mode of action of Tc and Tet(O) during elongation of protein syntheis. Tc (orange) binds a posttranslocational ribosome at the A-site. Tc allows the binding of EF-Tu•GTP•aa-tRNA; (g) but prevents successful accommodation of aa-tRNA in the A-site following dissociation of EF-Tu•GDP. Tet(O) senses a Tc-stalled 70S and is recruited as a Tet(O)•GTP complex (red) to the 70S.Tet(O) has a 70S-dependent GTPase activity and chases Tc; (h) following GTP hydrolysis Tet(O)• GDP is released from the 70S, bringing it back into the normal posttranslocational state. EF-G binds to pretranslocation 70S (A-site and P-site occupied by tRNA) Tc and Tet(O) binds to posttranslocation 70S (A-site vacant, P-site occupied by tRNA).

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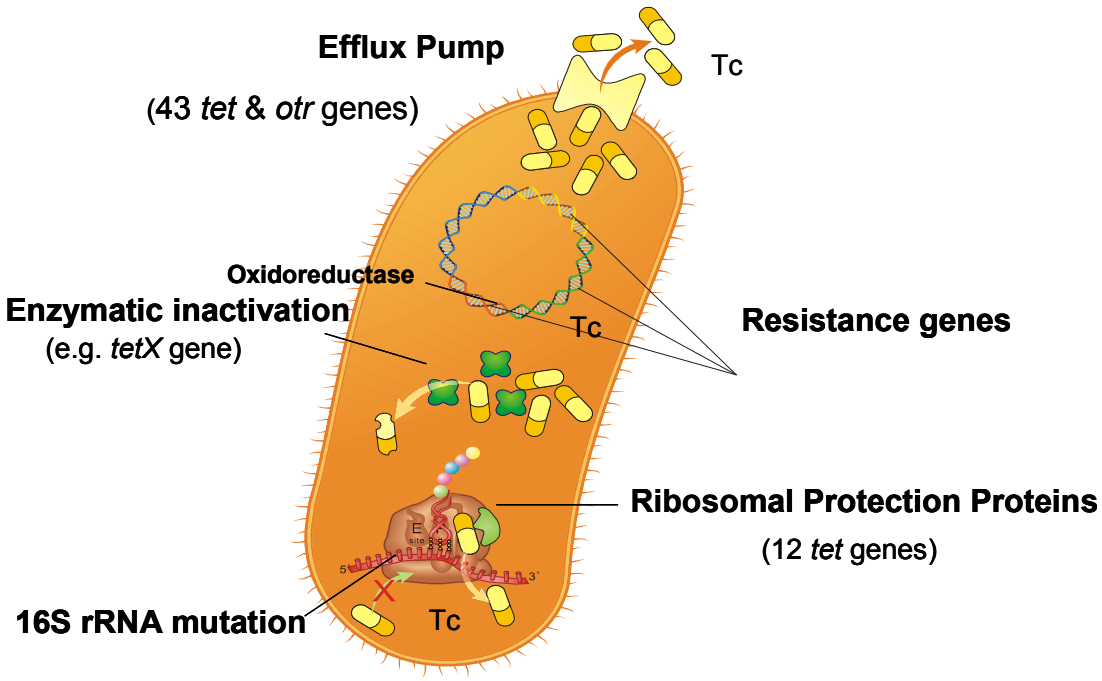

Figure 1.14 Mechanisms of tetracycline resistance in a bacterial cell

The most common determinants of Tc^R include the efflux pump and the ribosomal protection proteins. Enzymatic inactivation of tetracycline or tetracycline resistance via mutations in 16S rRNA is less common mechanism of gaining tetracycline resistance.

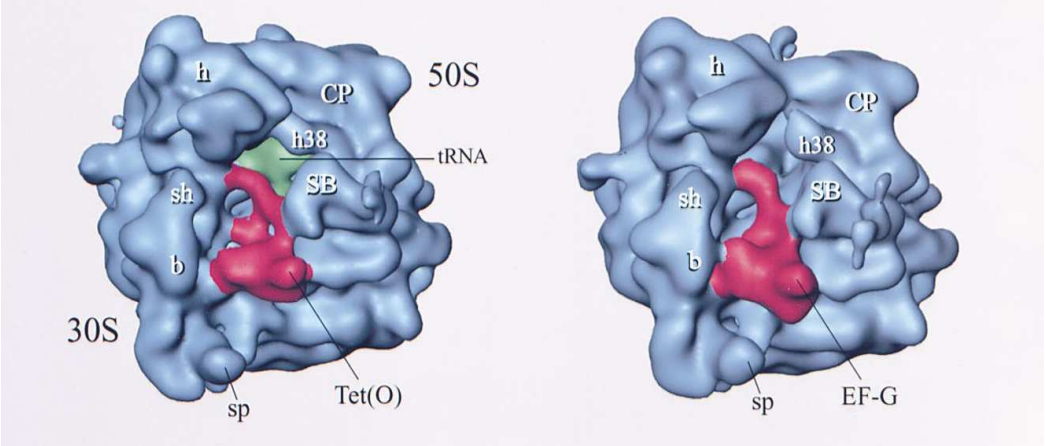

Figure 1.15 Cryo-EM reconstructions of Tet(O) and EF-G with *E. coli* 70S at 16 Å [107]

70S ribosome from *E. coli* in complex with (a) Tet(O)•GTPγS and fMet-tRNA in the P site and; (b) in complex with EF-G•GMPPCP. Both Tet(O) and EF-G have a similar shape and are seen to contact the 70S at almost similar sites.

Tet(O) and EF-G are shown in red, and the tRNA is shown in green. Landmarks, small subunit: h, head; b, body; sh, shoulder; and sp, spur. Large subunit: CP, central protuberance; SB, stalk base; and h38, helix 38 of 23S rRNA.

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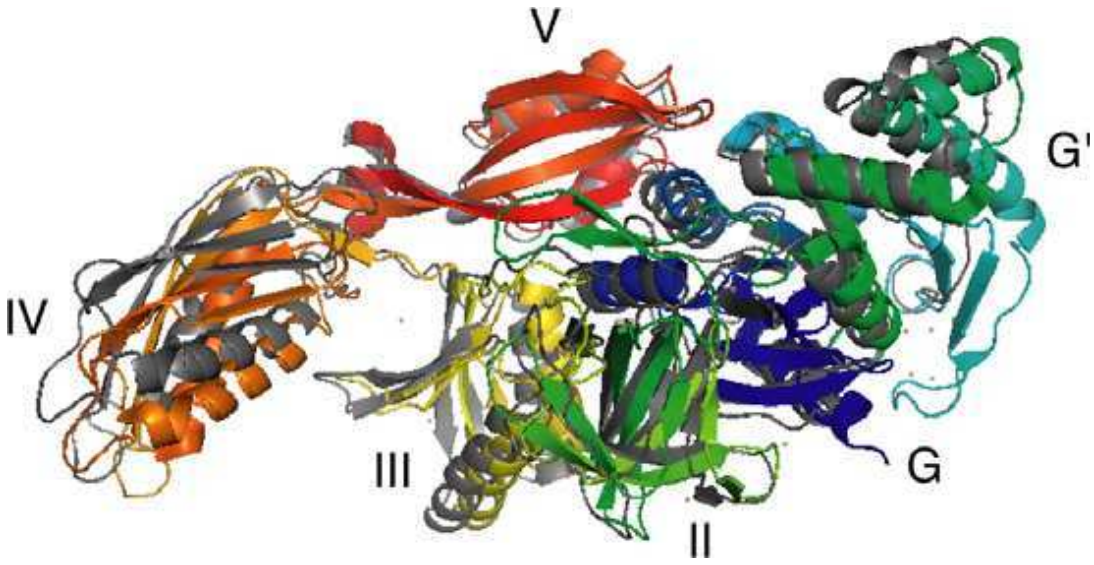

Figure 1.16 Homology-modeled structure of Tet(O) with EF-G [217]

The domains of *C. jejuni* Tet(O) are colour coded and superimposed on the known structure of *Thermus thermophilus* EF-G. Note that the tip of domain IV of Tet(O) (orange) does not overlap the tip of domain IV of EF-G (gray).

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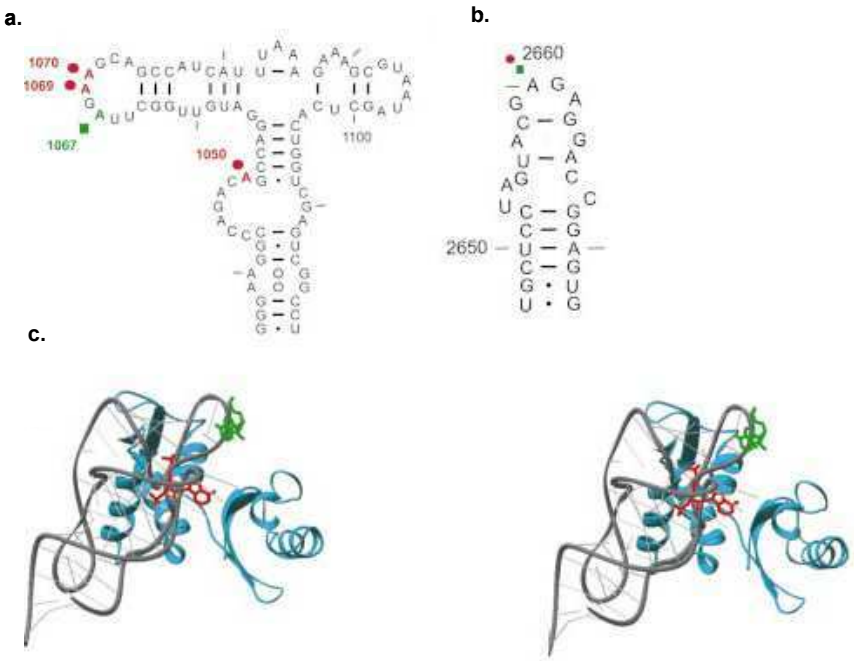

Figure 1.17 Bases of the 23S rRNAin the 50S subunit whose DMS reactivity is altered following the binding of Tet(O) and EF-G in the L11 region (a) and the α-sarcin loop (b) [178]

EF-G or Tet(O) binding alters DMS reactivity of bases marked in green squares and red circles respectively on secondary structure of the L11 region (H42/43/44) and the α-sarcin loop (H95); (c) Crystal structure of the L11 region indicating the locations of the Tet(O) and EF-G-associated base protections.

The 23S rRNA of the L11 region is shown as a gray ribbon, and the protein L11 is colored cyan. A1070 (red), which is concealed and protected by Tet(O), while the exposed A1067 (green) is protected by EF-G.

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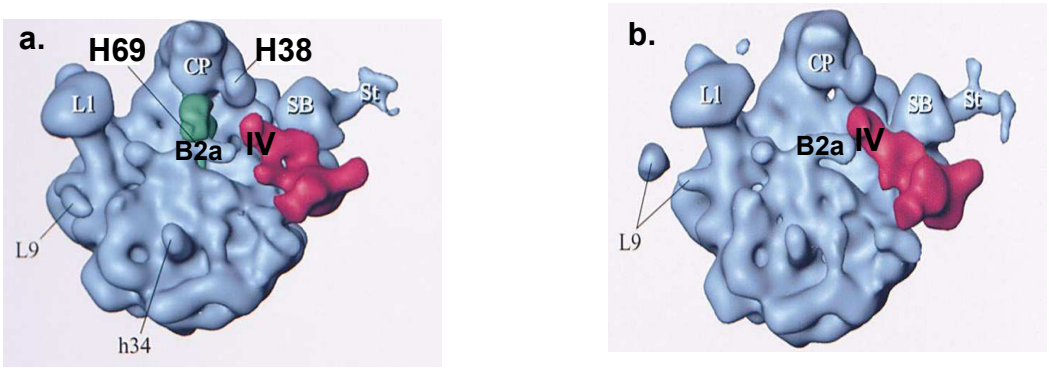

Figure 1.18 Cryo-EM of 50S Subunit of *E. coli* 70S indicating binding sites of (a) Tet(O) bound to fMet-tRNA•70S (b) EF-G bound to 70S in the presence of noncleavable GTP analog GMPPCP [107]

(a) The tip of the domain IV of Tet(O) (red) does not contact the bridge B2a (H69 of 23S rRNA) and does not overlap the anticodon arm of the A-site tRNA (Figure 1.18a); (b) the tip of the domain IV of EF-G(red) contacts bridge B2a and penetrates deep into the A-site and overlaps the anticodon arm of the tRNA (Figure 1.18b) which is critical for translocation.

P-site bound fMet-tRNA is shown in green. The position of L9 is indicated. C-terminal domain of L9 has different positions in the Tet(O) (a) and the EF-G map (b). Landmarks: CP, central protuberance;L1, L1 protuberance; SB, stalk base; St, extended stalk; h34, helix 34 of 23S rRNA;h38, helix 38 of 23S rRNA; and h69, helix 69 of 23S rRNA (bridge B2a).

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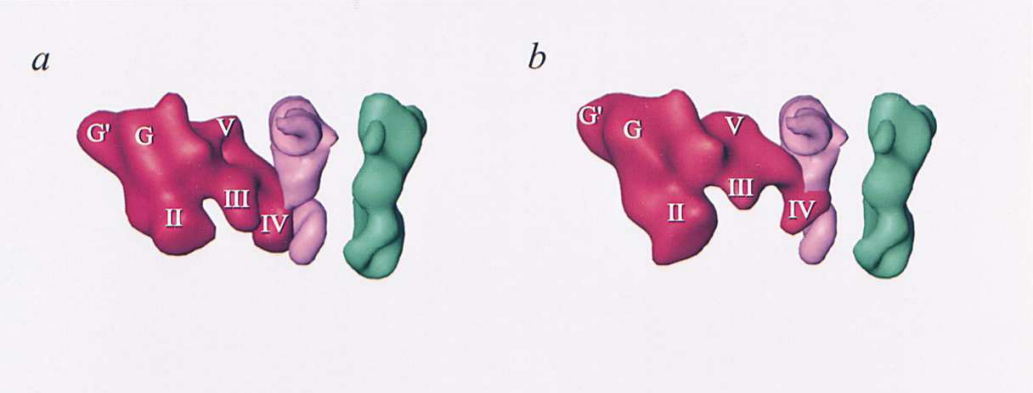

Figure 1.19 The contact of domain IV of Tet(O) and EF-G with the A-site tRNAs [107]

(a) The domain IV of Tet(O)•GTP complex (red) contacts the A-site tRNA (pink) but does not overlap the anticodon arm of the A-site tRNA; (b) The domain IV of EF-G•GTP complex (red) contacts and overlaps the anticodon arm of the A-site (pink).
P-site bound tRNA is shown in green. (Roman numerals II–V and the letter G mark the domains of EF-G and the homologous domains in Tet(O).

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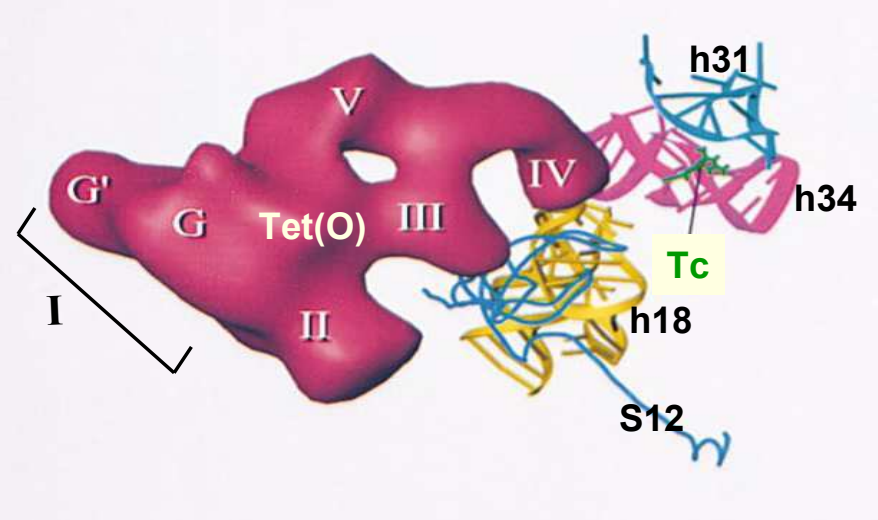

Figure 1.20 Comparison of the Tet(O) Binding Site with the Tc Binding Site [107]

The tip of the domain IV of Tet(O) (in red) closely approaches the primary Tc (green) binding site in the ribosome. Tc contacts helices 34 and 31 in its primary site. Tet(O) contacts h34,h18 and S12 protein of the 30S subunit. The domain I with GTP-ase activity lies remote from the domain IV.

Roman numerals II–V and the letter G mark the domains in Tet(O). The atomic model is color coded as follows: yellow, helix 18; cyan, helix31; pink, helix 34; and blue, protein S12. Tc is shown in green.

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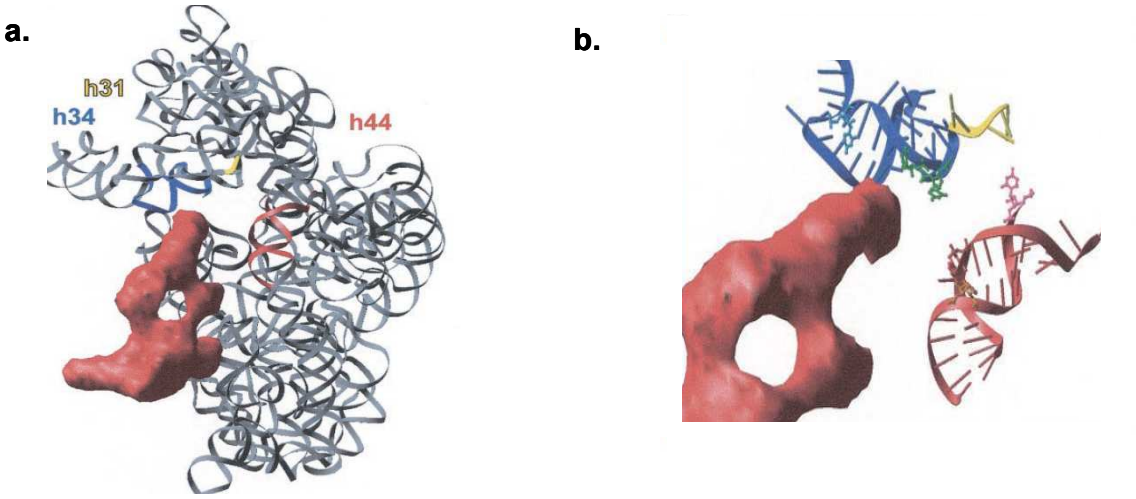

Figure 1.21 DMS modification of the 16S rRNA bases altered by the binding of Tet(O) to the A-site [95, 216]

The DMS accessibility of the bases that are altered upon Tc (U1052 and C1054, green), EF-G (A1408, orange; C1400, pink), or Tet(O) (C1214, blue; A1408, orange) binding are drawn in a ball and stick representation.

(a)Tet(O) (red density) bound to the 30S subunit (ribbon representation);
(b) Interaction of domain IV of Tet(O) (red density) with bases around the primary Tc binding site.
Helices 31 (nucleotides 964 to 968), 34 (nucleotides 1199 to 1217 and 1058 to 1046) and 44 (nucleotides 1400 to 1414 and 1486 to 1503) shown in yellow, blue, and red ribbons, respectively.

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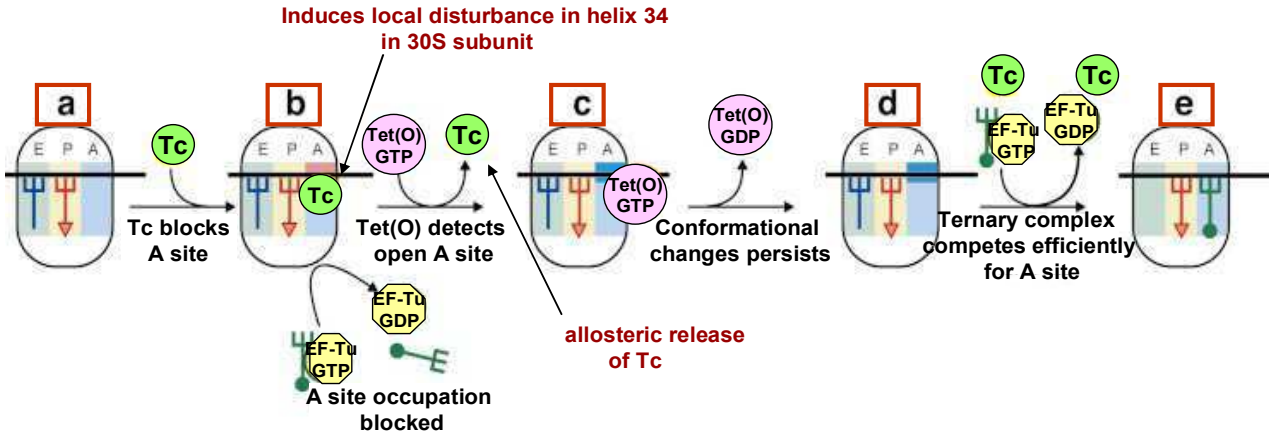

Figure 1.22 A model for Tet(O)-mediated Tc^R [178]

(a) Tc(green) binds an elongating 70S in the posttranslocation state at the A-site and iduces a conformational change (b) binding of Tc prevents the occupation of the A-site by aa-tRNA in the ternary complex (EF-Tu•GTP•aa-tRNA) (c) Tet(O) (pink) recognizes a Tc-stalled 70S ribosome by the virtue of a vacant A-site and binds to the posttranslocational 70S. Binding of Tet(O) induces a conformational change in the 70S that allosterically releases Tc from its primary binding site. The 70S-dependant GTPase activity of Tet(O) is activated, GTP is hydrolysed, Tet(O) •GDP complex dissociates from the 70S (d) the conformational change induced by Tet(O) in the 70S persists and (e) allows the favourable recruitment of ternary complex in the A-site instead of the released Tc.

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Chapter 2 Materials and Methods

2.1 Bacterial strains

Escherichia coli BL21 Star™ (DE3) (Invitrogen, Carlsbad, CA, USA) was used as the host strain to over express recombinant N terminally His_6-tagged $Tet(O)^{WT}$ and the different Tet(O) point mutants ($Tet(O)^{mutants}$) created by site-directed mutagenesis of the *tet(O)* gene (Table 2.1, Figure 2.1).

E. coli BL21 Star™ (DE3) strain is engineered to carry the bacteriophage T7 RNA polymerase which binds to the T7 promoter in the pET200/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) (see section 2.5.1) and transcribes the cloned downstream *tet(O)* gene.

The use of *E.coli* BL21(DE3) strains over other *E.coli* strains have a definite advantage for improved expression and yields of the heterologous protein. The T7-phage promoter is a strong promoter, therefore the rate of transcription of the heterologous mRNA from the T7-phage promoter is much faster than from a normal *E.coli* RNA polymerase promoter. This means transcription from the T7 promoter is not coupled to translation, leaving a pool of unprotected mRNA transcripts in the cell which are prone to degradation by endogenous RNases. The BL21(DE3) strains carry a mutation in the *rne131* gene encoding for RNase E, one of the principal enzymes involved with mRNA degradation in *E.coli*. This mutation improves the stability of mRNA transcripts and increases the protein production in this strain resulting in the accumulation of the desired protein at very high (40-50% of the total cell protein) concentrations [243]. The BL21 strain is engineered to carry the λ(DE3) lysogen with a T7-RNA polymerase gene integrated into the bacterial chromosome under the control of an IPTG inducible *lacUV5* promoter, that allows the regulated expression of the T7-RNA polymerase in the presence of IPTG only. The λ(DE3) lysogen is also designed to carry the *lacI* repressor gene and a portion of the *lacZ* gene. The *lac* construct is inserted into the *integrase* (*int*) gene so as to inactivate the gene and prevent the excision of the phage and hence lysis of the *E. coli* in the absence of a helper phage.

Addition of isopropyl β-D-thiogalactoside (IPTG) allows the expression of T7 RNA polymerase from *lacUV5* promoter [244].

The lacI repressor protein binds to the operator in the *lacUV5* promoter in the absence of IPTG that prevents the basal level expression of T7-RNA polymerase gene. Addition of the inducer IPTG allows its interaction with the lacI repressor, which induces a conformational change in the LacI protein allowing it to dissociate form the operator site. This allows expression of the T7-RNA polymerase from the *lacUV5* promoter, which can then bind T7 promoter in the pET200 expression vector and promote the transcription of the cloned downstream *tet(O)* gene. This reduces the basal expression of the heterologous *tet(O)* gene, which allows the pET200 vector to be stably maintained in the BL21(DE3) strains. This strain is also devoid of the *lon* and *ompT* proteases, which reduces the degradation of the heterologous proteins expressed in these strains[245-246] .

The pET200-*tet(O)*^{WT} or pET200-*tet(O)*^{mutants} were transformed and stably maintained in *E.coli* TOP10 (Invitrogen, Carlsbad, CA, USA) cells. TOP10 *E. coli* cells do not contain T7 RNA polymerase, hence the *tet(O)* gene cannot be expressed, and this avoids loss of the plasmid. Sometimes certain gene expression may be potentially toxic to the host cell in which case they want to get rid of the plasmid. Hence TOP10 cells are used for characterization of the construct as well as stable maintenance and propagation of recombinant plasmids [244].

E.coli MRE 600 (generously provided by Dr. R.P. Fahlman, Department of Biochemistry, University of Alberta) was used to prepare high quality 70S ribosomes because this strain lacks ribonuclease I and therefore displays almost no ribonuclease activity [247].

All *E.coli* strains were stored in -80°C in 50% glycerol Luria- Bertani (LB) broth (1 % Tryptone, 0.5% Yeast Extract, 0.05% NaCl) (BD Biosciences, Oakville, ON, Canada).

2.2 Reagents and equipments

All chemicals, reagents, disposables and equipments were purchased from Fisher Scientific (Ottawa, ON, Canada) unless specifically stated otherwise.

2.3 Media

E.coli (MRE 600, TOP10 and BL21 Star^{TM} (DE3)) were grown in LB broth or on LB-agar (LB broth + 2 g% (w/v) Select agar, Invitrogen, Carlsbad, CA, USA). TOP10 and BL21 Star^{TM} (DE3) cells transformed with pET200-*tet(O)*^{WT} or pET200- *tet(O)*^{mutants} were grown on LB broth or LB-agar supplemented with 50 μg/mL kanamycin (Sigma- Aldrich, Oakville, ON, Canada) (LB broth-Kan or LB agar-Kan). Kanamycin was added as a selective agent to select for the growth of the cells carrying recombinant pET200 plasmid that carries the kanamycin resistance cassette.

Super Optimal Broth with Catabolite repression (S.O.C.) containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose (Invitrogen, Carlsbad, CA, USA) was used for the recovery of chemically competent *E.coli* TOP10 and BL21 Star^{TM} (DE3) cells following heat shock and transformation with pET200- *tet(O)*^{WT} and pET200- *tet(O)*^{mutants} . To induce Tet(O) expression in transformed *E.coli* BL21 Star^{TM} (DE3) cells, 1M IPTG was added to a final concentration of 1 mM (in the LB agar-Kan plates) or to a final concentration of 0.5 mM (in LB broth-Kan) to induce the expression of T7 RNA polymerase.

2.4 Growth conditions for bacterial cultures

Frozen *E.coli* (MRE 600, TOP10 and BL21 Star^{TM} (DE3)) stocks were streaked on to LB-agar-Kan plates and incubated at 37°C for 14 h to obtain single colonies. Overnight seed cultures of *E. coli* were set up by inoculating a single colony of MRE 600, TOP10 or BL21 Star^{TM} (DE3) strains into 100 mL of 2 g% (w/v) LB broth or LB broth-Kan and incubated for 12-14 h at 37°C with shaking at 200 rpm (Forma Orbital Shaker, Thermo Electron Corporation, Gormley, ON, Canada). The overnight seed cultures were transferred into 1000 mL of 2 g%

(w/v) LB broth (for ribosome preparation) /or LB broth-Kan, to which 100 mL of Phosphate Buffer (17 mM KH_2PO_4- 17 mM K_2HPO_4-1% glycerol, pH 7.4) was added (for protein expression). In both cases cells were grown to mid-log phase (OD_{600} ~ 0.5-0.6) at 37°C with shaking at 200 rpm.

2.5 Construction of recombinant plasmids and transformation into *E.coli*

The *tet(O)*^{WT} gene carried in the pUA466 plasmid of the *Campylobacter jejuni* UA466 strain was first cloned into the pMS119EH plasmid [229]. The *tet(O)*^{WT} gene was subsequently cloned into the pET200/D-TOPO expression vector [248] by polymerase chain reaction (PCR) (EppendorfAG Thermocycler, Hamburg, Germany) using the primers shown in Table 2.2. The *tet(O)*^{WT} gene was then subjected to site-directed mutagenesis to generate a series of *tet (O)*^{mutant} genes (single alanine: *S508A*, *P509A*, *V510A*, *S511A*, *T512A*, double alanine: *S508-P509A* (*NT1*), *P509-V510A* (*NT2*), *V510A-S511A* (*NT3*), *S511A-T512A* (*NT4*), deletion mutants: *508ΔS*, *509ΔP*, *510ΔV*, *511ΔS*, *512ΔT*, and the polar/non polar mutants: *S508D*, *P509V*, *V510D*, *T512S*). The primers used in site-directed mutagenesis to create the different *tet(O)*^{mutant} genes are also listed in Table 2.2. *E.coli* TOP10 and *E. coli* BL21 Star^{TM} (DE3) strains were transformed with recombinant pET200-*tet(O)* by Dr. Nehal Thakor. Breanna Baranec, provided technical assistance in the preparation of the *tet(O)* deletion mutants (unpublished data). The *fusA* gene encoding the EF-G protein was cloned from the *E. coli fusA* gene (plasmid pET24b) by PCR. The primers for the amplification of *fusA* gene were are listed on Table 2.1.

2.5.1 Features of pET200/D-TOPO vector

The pET200/D-TOPO vector (Figure 2.2) is a linearized topoisomerase I-activated T7 expression plasmid which is used for directional cloning, high level expression, purification and detection of heterologous proteins in *E. coli.* The proteins are expressed in fusion with six histidine (6xHis/His_6) residues at their N-terminal end which allows the one step purification of the expressed recombinant Tet(O) protein on on a metal chelating resin such as a Ni^{2+}clomun. His_6- tags are

commonly used because it is easy to add them by the virtue of their small size and is therefore less likely to interfere with protein function and crystal packing. The vector is endowed with an Xpress™ epitope that allows detection of fusion proteins by Anti Xpress™ antibodies [244]. Enterokinases such as EKMax™ can act on the Enterokinase (EK) recognition site (Asp-Asp-Asp-Lys) and remove the N-terminal His_6-tag from the protein on the vector [244]. The vector carries the kanamycin resistance gene that allows successful selection of cells that are transformed with the pET200/D-TOPO vector. Kanamycin is resistant to degradation from the β-lactamase production in *E.coli* systems [244]. This allows kanamycin to be used as a stable marker on LB-agar plates that minimises contamination of plates with satellite colonies. The vector is equipped with a strong, inducible bacteriophage T7 promoter [244] that allows high level expression of the cloned downstream gene in the presence of a suitable inducer like IPTG [243]. The T7 expression system is ideal for expressing soluble, non toxic recombinant proteins in *E.coli*. The vector is furnished with pBR322 origin of replication that permits the replication of the vector and its maintenance in *E.coli.* The vector carries a *lacI* open reading frame (ORF) that encodes for a repressor protein which binds to the T7*lac* promoter to block basal level expression of the cloned gene in the absence of an inducer [244]. The *ROP* ORF interacts with pBR322 origin to facilitate low copy replication of the plasmid in *E.coli* [244] which prevents its loss and stable maintenance in the host cell. [243].

2.5.2 Site-directed mutagenesis to generate *tet(O)*^{mutants}

Site-directed mutagenesis was accomplished by overlap extension PCR. For each *tet(O)*^{mutant} forward and reverse primers were designed (Table 2.2) to carry the mutation and were completely complementary to each other. Primer pairs consisted of an outer flanking primer + a mutagenic primer as outlined (Table 2.1, Figure 2.3). Each primer set was used in a separate reaction but under the same PCR cycling conditions (Table 2.3) to generate two halves of the *tet(O)*^{mutant} gene. The two separately amplified products were then mixed so that they annealed in their region of complementarity and acted as primers to generate

the full length *tet(O)*^{mutant}. Platinum® *Pfx* (Invitrogen, Carlsbad, CA, USA) was used as the DNA polymerase in site-directed mutagenesis, as its proof reading ability is necessary to ensure the desired alteration in the *tet(O)* gene sequence. Platinum® *Pfx* is a high fidelity and highly sensitive DNA polymerase whose 3'-5' exonuclease proof reading activity eliminates mismatched base pairings and improves the chances of getting a relatively error free amplicon. An advantage of *Pfx* is that it contains Platinum® antibody that inhibits *Pfx* from exhibiting any polymerase or proof reading activity during the initial assembly and the initial denaturation step of PCR. This is crucial for significantly reducing mis-priming and production of non-specific amplifications and formation of primer-dimers or any artifacts during PCR. This endows *Pfx* with high accuracy and high specificity. A 50 μL PCR reaction mix contained 1.5X *Pfx* Amplification Buffer, 2 mM $MgSO_4$, 0.3 mM dNTP, 0.4 μM of each of forward and reverse primer, 20 ng template DNA and 2.5 U Platinum® *Pfx**.

*One unit *Pfx* is defined as the amount of polymerase required to catalyze the incorporation of 10 nmol of dNTPs into acid-insoluble/acid precipitable material in 30 minutes at 74°C.

2.5.3 Construction of recombinant plasmids pET200- *fusA*, pET200- *tet(O)*^{WT} and pET200- *tet(O)*^{mutants}

The principle of the TOPO cloning reaction is based on the mode of action of topoisomerase I of *Vacinia* virus [244]. Topoisomerase I binds double-stranded DNA and cleaves the phosphodiester bond following the 5'-CCCTT-3' sequence in one strand. The energy derived in the process of cleaving the phosphodiester bond is stored by the formation of a covalent phosphor-tyrosyl bond between the 3' phosphate of the cleaved strand and a tyrosyl (Tyr-274) of topoisomerase I. The reaction can be reversed when the phospho-tyrosyl bond is attacked by the 5'-hydroxyl group of the original strand thus releasing the topoisomerase I (Figure 2.4) [244].

The PCR amplified *tet(O)*^{WT}, *tet(O)*^{mutant} and *fusA* genes were inserted into the pET200/D-TOPO plasmid according to the manufacturer's protocol [244]. In the TOPO cloning system, PCR products carry a 4 nucleotide overhang (CACC) at their 5' end that allows the amplified PCR products to be directionally cloned into the TOPO cloning vector, which carries a complementary GTGG overhang in its 3'end (Figure 2.4) [244]. In order to achieve maximum efficiency (>90-95% colonies/reaction) in a TOPO cloning reaction, the molar ratio of *tet(O)* PCR product:TOPO vector was maintained at 0.5:1 [244]. TOPO cloning reactions (6 μL) contained 0.2 M NaCl, 0.01 M $MgCl_2$ salt solution, 10 ng fresh PCR amplicon and 20 ng pET200/D-TOPO vector. The reaction was incubated for 15 minutes at room temperature (RT) and then placed on ice.

2.5.4 Transformation of *E.coli* TOP10 and BL21 Star™ (DE3) cells with recombinant pET200/D-TOPO

E.coli TOP10 and BL21 Star™ (DE3) cells were transformed according to the manufacturer's protocol [244]. The manufacturer reports that when One Shot® chemically competent *E.coli* cells were transformed with 10 pg of pUC19 control plasmid and grown on LB-agar plates supplemented with 100 μg/ml ampicillin, the transformation efficiency of was greater than 1 x 10^9 cfu/μg plasmid DNA for TOP10 cells, and greater than 1 x 10^6 cfu/μg plasmid DNA for BL21 Star™ (DE3) cells [244].

$$Transformation\ efficiency = \frac{\#\ of\ cfu\ /\ mL}{Amount\ of\ DNA\ plated\ (\mu g/mL)}$$

Where: # of cfu (colony forming units) = a measure of viable cells where a single colony embodies a cluster of cells derived from a single progenitor cell

A 3 μL aliquot of the completed TOPO cloning reaction was added into separate vials of One Shot® TOP10/BL21 Star™ (DE3) chemically competent *E.coli*, mixed gently, and incubated on ice for 20 min. The cells were then heat shocked

for 30 seconds at 42°C without shaking, and then immediately transferred to ice. A 250 μL aliquot of RT S.O.C. medium was added to the cells which were allowed to recover at 37°C by shaking horizontally at 200 rpm for 1 hr. From each transformation, 100-200 μL was spread on a pre-warmed selective LB agar-Kan plate. The plates were incubated overnight at 37°C. Individual colonies were then inoculated into 5 mL LB broth-Kan, and incubated overnight as described in section 2.4.

The plasmid DNA was extracted using the QIAprep® Spin Miniprep Kit (50) (Qiagen Sciences, Maryland, USA). The isolated plasmid DNA (carrying the *tet(O)*^{WT} or *tet(O)*^{mutant} gene) was then sequenced at The Applied Genomic Centre core sequence facility at the University of Alberta.

2.6 Determination of Tc Susceptibility of *E.coli* BL21 Star™ (DE3) transformants

The *in vitro* agar dilution technique was used to assess the ability of the *C. jejuni tet(O)* gene to confer Tc susceptibility to *E. coli* BL21 Star™ (DE3) strains according to the previously reported protocol [217]. A single colony of each of *E. coli* BL21 Star™ (DE3)-pET200-*tet(O)*^{WT} / *tet(O)*^{mutants} was inoculated into 5 mL of LB broth-Kan and incubated overnight as described in section 2.4. From each overnight culture, 500 μL was transferred to fresh 5 mL aliquot of LB broth-Kan and the cells were grown to mid-log phase as described in section 2.4. Tc (Sigma-Aldrich, Oakville, ON, Canada) was added in serial two fold increasing concentrations (1-256 μg/mL) into LB broth-agar (according to the Clinical Laboratory Standard Institute (CLSI) guidelines). LB-agar-Tc plates were prepared and a 25 μl aliquot of 1M IPTG was spread onto them. From each culture 5 μL was spotted on to LB-agar-Tc-IPTG plates. The Tc minimal inhibitory concentration (MIC) reported was the lowest concentration of Tc that inhibited the growth of bacteria following 48 h incubation at 37°C. In *E. coli* cells are considered to be Tc resistant (reduced Tc susceptibility of *E. coli* transformed with *tet(O)*^{WT}/*tet(O)*^{mutants} above predefined limits set by the CLSI) if they fail to grow at a Tc MIC of 16 μg/mL or more. The *in vitro* breakpoints of resistance of

the agar dilution methods are crude, insensitive and often an imprecise measure of *in vivo* susceptibility of bacteria to antibiotics since they do not account for tissue penetration, dosage or target organs, and host response to infection outcome or non-antimicrobial effects [249].

E. coli BL21 Star^{TM} (DE3) - pET200-*fusA (fus A* encodes for EF-G) was used as a Tc^S control to confirm that Tc^R was not rendered by the pET200 vector and the *fusA)* gene.

Agar dilution was used instead of microbroth dilution because it was more convenient to grow *E. coli* BL21 strains carrying the *tet(O)*^{WT} and *tet(O)*^{mutant} genes on one plate for each concentration of Tc (0-256 μg/mL). It is easier to visualize colonies on an agar plate, particularly for slow growing *tet(O)*^{mutant} strains. It is also easier to detect contamination on agar plates versus broth culture.

2.7 Isolation of Tet(O) from *E. coli* BL21 Star^{TM} (DE3)

2.7.1 Overexpression of Tet(O)

The wild type and mutant His_6-Tet(O) fusion proteins were over expressed in *E.coli* BL21 Star^{TM} (DE3) strains (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified according to the previously reported protocol [248]. LB broth-Kan seed cultures were prepared with *E. coli* BL21 Star^{TM} (DE3) carrying the pET200-*tet(O)*^{WT}/*tet(O)*^{mutants} and incubated overnight as described in section 2.4. Each entire overnight seed culture was transferred to 1000 mL LB broth-Kan, and the cells were grown to mid log phase as described in section 2.4. The temperature was then reduced (to 30°C for Tet(O)^{WT} and 18°C for Tet(O)^{mutant}) to enhance Tet(O) protein solubility. IPTG was added to the broth to a final concentration of 1 mM to induce the over-expression of Tet(O) proteins and incubation was continued for 5 h. The cells were then harvested by centrifugation at 6,000 x g (Sorvall RC2-B, GSA rotor, DuPont, Canada) for 15 min, the supernatant was decanted and discarded. The cell pellets were re-suspended and pooled together in 100 mL Phosphate Buffer, and centrifuged at 6,000 x g for 15

min. The supernatant was decanted and discarded, and the pellets were stored at -80°C.

2.7.2 Lysis and solubilisation of Tet(O)

The frozen cell pellets (~16-20g) were re-suspended in 100 mL Binding Buffer (20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) -200 mM KCl- 1 % (v/v) glycerol-1 mM DTT (dithiothreitol), pH -7.4). The resuspended pellets were lysed in 2 mg/ml lysozyme (Sigma-Aldrich Oakville, ON, Canada) at RT for 30 min in the presence of 2 tablets of Complete^{TM} Ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Mississauga, ON,Canada) and 1500 U of DNase I (Invitrogen Life Technologies, Burlington, ON, Canada) and 3 mM $MgCl_2$. Lysozyme destroys the integrity of the bacterial cell wall, Complete^{TM} EDTA-free protease inhibitor is a proprietary mixture of a broad spectrum serine and cysteine protease inhibitors (but not metalloprotease inhibitor) and prevents the disintegration of bacterial protein, DNase I is a Mg^{2+} dependent endonuclease that by preferentially cleaving at phosphodiester linkages adjacent to pyrimidine nucleotides removes the unwanted single or double stranded bacterial DNA. The suspension was sonicated on ice for 90 seconds with five 10 second pulses, separated by 10 second pauses, to break open the bacterial cells (Sonic Dismembrator model 500). The sonication was done on ice to prevent heat denaturation of the protein. The suspension was then treated with 1.5 mL Triton-X 100 and stirred at 150 rpm for 30 min at room temperature. Triton-X 100 is a non-ionic surfactant used to increase the solubility of hydrophobic proteins like Tet(O). Following centrifugation at 6,000 x g in Sorvall rotor for 30 min, the supernatant containing the His_6-Tet(O) fusion protein was collected and passed through a 0.45 μm filter (Millipore, Toronto, ON, Canada).

2.7.3.1 Purification of His_6-Tet(O) Fusion Protein

His_6-$Tet(O)^{WT/mutant}$ proteins were purified by Immobilized Metal Affinity Chromatography (IMAC) on a His Trap High Performance (HP) Ni^{2+} column (GE

Healthcare, Uppsala, Sweden). A 5 ml HP Ni^{2+} column was equilibrated with 5 column volumes (cv) of Binding Buffer to wash away the unbound proteins. Following centrifugation, the supernatant, containing His_6–$Tet(O)^{WT}$/$Tet(O)^{mutants}$ was loaded onto the column using a 60 ml syringe. An increasing concentration of imidazole ranging from 25 to 500 mM, was used in a batch gradient method to replace and elute the His-tagged Tet(O) protein. Each eluate fraction was collected and then run on a 10% sodium dodecyl sulphate polyacylamide gel (SDS-PAGE) to visually detect the Tet(O) protein band.

2.7.3.2 SDS-PAGE

The isolated Tet(O) proteins were separated by their molecular weight and their purity was assessed using a 10% SDS-PAGE on a Mini-PROTEAN Tetra Electrophoresis System (Biorad, Missisauga, ON, Canada).

SDS is an anionic detergent that disrupts the secondary, tertiary and quarternary structure of proteins to produce linear polypeptide chains coated with negatively charged SDS molecules. SDS binds to hydrophobic regions of denatured protein chain in a constant ratio of 1.4 g SDS per g protein. The negative charge of SDS masks the charge of protein [250]. Hence polypeptide chains having the same MW and charge-to-mass ratio will have the same electrophoretic mobility. A linear relation exists between the log molecular weight and the electrophoretic mobility of SDS coated proteins [250].

The polyacrylamide gel is prepared by polymerization of acrylamide and the cross linking agent N,N'-methylene-bis-acrylamide controlled by the initiator catalyst system, ammonium persulfate- N,N,N',N'-tetramethylethylenediamine (TEMED) [250]. The resolving power and the molecular size range of a gel depend on the concentrations of acrylamide and bisacrylamide. The stacking gel has a lower acrylamide concentration so its pore size is larger which allows the protein molecules to migrate to the same level before they are separated based on their molecular weight in the resolving gel which has a higher acrylamide concentration and hence smaller pore sizes. The upper stacking and the lower resolving/running gel was prepared using the protocol in Table 2.4. The resolving

gel was allowed to set and then the stacking gel was carefully layered on the top of it. The buffers used to prepare the two gel layers have different ionic strengths and pH.

A 20 µL aliquot of each of the 25-500 mM Imidazole eluates were treated with equal volumes of 2x SDS Loading Buffer (0.125 M Tris-HCl- pH 6.8, 5 % SDS, 0.02% bromophenol blue, 25% glycerol, 0.2 M DTT), and boiled for 3-5 mins at 85°C. DTT aids protein denaturation by reducing all disulfide bonds. A 40 µL aliquot of each sample eluate and 6 µl of the PageRuler^{TM} Plus Prestained Protein Ladder (Fermentas, Burlinton, ON, Canada) were loaded into the wells of the stacking gel of the SDS-PAGE and run for 1 hr at 150 V in SDS-PAGE Running Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). The gel was then washed three times with MilliQ water for 10 mins each on a platform shaker, and then stained with Bio-Safe Coomassie G-250 stain (Bio-Rad, Mississauga, ON, Canada) for 20 mins to visualize the protein bands. The gel was destained with MilliQ water for 30 min to reduce background staining.

2.7.3.3 Concentration of the purified Tet(O) protein

The IMAC eluate containing the target protein (~ 25 mL) was concentrated to less than 2.0 mL using ultracentrifuge filter (Amicon Ultra-15 Centrifugal Filter Devices, 30K MWCO, Millipore, Toronto, ON, Canada) at 6000 x g for 20 min at 4°C and then, dialyzed overnight using Tube-O-Dialyzer (15K MWCO, G-Biosciences, Borkville, ON, Canada) in Binding Buffer. Dialysis permitted salting out via osmosis through the semi permeable membrane in the dialysing tubes which increased the solubility of the protein to ensure its storage stability [251]. The following day the dialyzed protein was mixed with equal an volume of Storage Buffer (20 mM HEPES- 200 mM KCl-20% (v/v) glycerol-1 mM DTT, pH -7.4), and stored in aliquots of 30-40 µl in -80°C.

2.8 Bradford protein assay

The Bradford assay [252] was used to determine the concentration of the purified Tet(O) proteins. This assay involves the binding of Coomassie Brilliant

Blue G-250 (G=green) dye to protein that results in a shift in its absorbance from 470 nm (free unbound dye) to 595 nm (protein-bound dye).

The Bradford dye can exist in three states:

Form	Absorbance	Colour
Cation	470 nm	red
Neutral	650 nm	green
Anion	595 nm	blue

When the dye binds to basic amino acid residues (arginine) or aromatic residues (tyrosine, tryptophan and phenylalanine), it is converted to a stable unprotonated blue form (A_{max} = 595 nm) [252] that is detected at 595 nm in the Bradford assay using a microplate reader.

According to the manufacturer's instructions (Quick Start ^{TM} Bradford Protein Assay, Biorad, Mississauga, ON, Canada), the 300 µl microplate assay protocol was used to determine the concentration of the Tet(O) proteins. A standard curve was generated by plotting A_{595} versus µg/mL standard protein solution of Bovine Serum Albumin (BSA).

The commercially available BSA protein standard (2 mg/mL) was diluted 1/20 with sterile MilliQ H_2O to obtain 100 µg/mL of BSA. This stock standard was then used to prepare the working standards of BSA (2, 4, 6, 8 and 10 µg/mL). The BSA standards were assayed in triplicate (Table 2.5). Purified concentrated Tet(O) preparations may vary widely in protein concentration. A 1/20 dilution was prepared for each Tet(O) preparation which was then serially diluted two fold to 1/1280 (Table 2.6). A 150 µL aliquot of the 1x Bradford dye reagent (prewarmed to room temperature) was then added to 150 µl of each BSA standard and to each of the serially diluted Tet(O) protein samples + the MilliQ H_2O blank. They were then mixed well, and incubated in RT for 5 minutes. The absorbance of the proteins standards and the samples were measured at 595 nm on a microplate spectrophotometer (XMark, Bio-Rad, Mississauga, ON, Canada) against MilliQ H_2O + Bradford reagent blank. The actual protein concentration (µM)* was estimated from the diluted protein sample concentrations (µg/mL) that

was detected using a standard curve obtained by linear regression analysis of A_{595} vs. concentrations of BSA standards.

$$\textit{Concentration of Tet(O) } (\mu M) = \frac{\textit{Tet(O) } (\mu g/mL)}{\textit{Tet(O) MW } (\mu g/\mu mol)} \quad X\ 1000^{*}$$

Where: MW of Tet(O) = 72562.62 μg/μmol

* 1000 converts Tet(O) protein concentration from μmol/mL to μmol/L.

2.9 Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of Tet(O)-associated Protein Bands

The LC-MS is a powerful analytical technique that combines the resolving power of high performance liquid chromatography (HPLC) with the mass detection specificity of mass spectrometry (MS). The sample components separated by liquid chromatography enter the mass spectrometer which then creates charged ions and detects them by computing the mass (m) to charge (z) ratio (m/z) of the particles as they travel through an electromagnetic field. The ability of mass spectrometry analyzing proteins is made possible through soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) that can converts biomolecules into ions. MALDI minimizes spectral complexity and produces singly charged ions of peptides and proteins. The sensitivity of a mass spectrometer is related to the mass analyzer where ion separation occurs [253]. Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) is a relatively novel technique in which a co-precipitate of an UV-light absorbing matrix and a biomolecule is irradiated by a nanosecond laser pulse. The unwanted fragmentation of the biomolecule is prevented through the absorption of most of the laser energy by the matrix. The ionized biomolecules are accelerated in an electric field and enter the flight tube where they separate according to their m/z ratio and reach the detector at different times [253].

In this study, the protein bands that co-eluted with Tet(O) at 80 mM Imidazole were precisely cut out from the SDS-PAGE (using new sterile blades

as close to bands as possible to minimise gel volumes) and collected in sterile 1.5 mL microfuge tubes. LC-MS analysis of the bands was carried out by the Institute for Biomolecular Design, Department of Biochemistry at the University of Alberta by the in-gel digestion protocol. Coomassie stained gels were washed with 50 mM ammonium bicarbonate buffer for 5 mins with shaking at RT. The liquid waste was discarded and the gel pieces were incubated in 70% acetonitrile in water for 15 min, following which the liquid was discarded. Washing was continued until the gel slice was completely colourless. The gel pieces were dehydrated by adding 100% acetonitrile and incubated for at least 5 min. The liquid was discarded into waste. The gel pieces were vacuum dried for 5 min. The gel fragments were swollen in 30 μL of 50 mM ammonium bicarbonate, 10 mM DTT and incubated between 55°C-60°C. The sample was then cooled to RT for 15 min. The sample was centrifuged and the supernatant was discarded. To the gel, 30 μL freshly prepared 100 mM iodoacetate and 50 mM ammonium bicarbonate was added and incubated in the dark at RT for 15 min and centrifuged. The supernatant was discarded. The gel was washed with 100 μL of 50 mM ammonium bicarbonate, vortexed and allowed to stand for 10 min. The supernatant was discarded. The gel was completely dehydrated by adding 100% acetonitrile and incubated for at least 10 min. The liquid was discarded into waste. The gel pieces were vacuum dried for 5 min. Trypsin (15 ng/μL of trypsin in 50 mM ammonium bicarbonate) was prepared to digest the gel. A 28 μL trypsin aliquot was added to the gel and incubated in ice for 10 min. More trypsin was added if the gel appeared dry and incubated on ice for another 5 min. A 10 μL of 25 mM ammonium bicarbonate was added to the tubes containing the gel and incubated at 37°C for 10 mins. Following digestion, 100 μL of 1% formic acid was added to the tubes, vortexed and shaken for 20 min. This extraction contains the more hydrophilic peptides. The 1% formic acid wash was removed and transferred to a new, washed, silanised Eppendorf. To the gel piece 200 μL of 50% acetonitrile containing 1% formic acid was added, vortexed and shaken for 20 min. This wash contained the more tryptic petides. This supernatant was added to the 1% formic acid extraction. The samples were speed vacuumed down to 40

μL (complete drying of the samples was avoided). The samples were stored at 4°C and finally analysed by MALDI-TOF MS.

2.10 Preparation of High Quality 70S Ribosomes

70S ribosomes were isolated from MRE 600 *E.coli* cells as previously described with some modifications [237].

2.10.1 Growth of *E.coli* MRE600 Cells

LB broth seed cultures were prepared with *E. coli* MRE600 and incubated overnight as described in section 2.4.

2.10.2 Pelleting and Storage of *E. coli* MRE600 cells

The overnight seed culture was transferred to 1 L LB broth and the cells were grown to the mid log phase as described in section 2.4. The mid-log phase culture was then cooled on an ice bath for 30 minutes to allow the formation of run off 70S ribosomes (70S ribosomes that do no exist as ploysomes). The cells were harvested at 4°C by centrifugation at 5000 x g (Sorvall RC2-B, GSA rotor) for 15 min. The supernatant was decanted and discarded. The cell pellets were resuspended and pooled together in 100 mL Phosphate Buffer, and centrifuged at 6,000 x g for 15 min at 4°C. The supernatant was decanted and discarded and the pellets were stored at -80°C.

2.10.3 Cell disruption and crude ribosome preparation

The frozen pellets were thawed on ice and suspended in 50 mL Lysis Buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 M NH_4Cl, 6 mM β-mercaptoethanol, 0.5 mM EDTA).The cells were homogenised at 25,000 psi using a dynamic high pressure homogeniser (Emulsiflex C3, Avestin Inc, Ottawa, ON, Canada). The initial cell sample was viscous and as the homogenization proceeded, the viscosity of the solution decreased as the particle size was reduced. The homogenate was then split into 2 Ti-45 rotor tubes and centrifuged at 61,334 x g (Ti-45 rotor, Beckman Coulter, Mississauga, ON, Canada) for 15 min at 4°C

to remove the cell debris. The supernatant was transferred into 2 fresh Ti-45 rotor tubes and centrifuged at 61,334 x g for 30 min at 4°C.The supernatant was carefully decanted into 4 Beckman Ti-70 centrifuge tubes and centrifuged at 111,921 x g (70 Ti rotor, Beckman Coulter, Mississauga, ON, Canada) for 14 h at 4°C to pellet crude 70S ribosomes.

2.10.4 Preparation of 10%-40% sucrose gradients

A 10% and 40% sucrose gradient solution was prepared in Buffer 1(50 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1M NH_4Cl, 6 mM β- Mercaptoethanol). 40% sucrose (19 ml) was layered carefully on the top of 10% sucrose (19 mL) in SW-27 centrifuge tubes. The top of the tubes (6 x 38 mL) were sealed with parafilm and the tubes were laid on the bench tops in horizontal position to allow gradients to form. After 4-5 hrs the tubes were reverted to vertical position and stored at 4°C overnight.

2.10.5 70S tight couple ribosome preparation

The supernantant from 2.10.3 was decanted and discarded. Buffer 1 (1.5 mL) was added to each pellet and stirred using a micro stir bar for ~ 4 h at 4°C until resuspended. The suspensions were then centrifuged at 5000 x g (Sorvall RC2-B, GSA rotor) for 5 min at 4°C. The supernatant was carefully layered on to 6 x 38 mL sucrose gradients, transferred into SW-27 rotor buckets, and centrifuged at 55, 070 x g (SW27 rotor, Beckman Coulter) for 13 h at 4°C. Gradient centrifugation allows the samples to travel through the gradient to the point where their density matches the density of the surrounding sucrose.

2.10.6 70S ribosome fractionation

The 70S ribosomes were fractionated by using a Brandel gradient fractionator (model BR-186) (2-mm-path-length flow cell) connected to a syringe pump (model SYR-101). Fractionation is a separation process in which a certain quantity of a mixture is divided up in a number of smaller fractions in which the composition of the sample changes according to a gradient. Fractions

are collected based on differences in a specific property of the individual components. The syringe was filled with 50% sucrose. Sucrose gradients from section 2.10.5 were transferred to the ISCO tube piercing system; the bottom of the tubes was pierced, the gradient was drawn using an in-line peristaltic pump at the slowest speed. Once the unloading initiated, the pump speed was increased to maximum the chart recorder speed was set to 150 (cm/h). The UV absorbance (260 nm) of the sample was monitored and recorded by the UA-6 ISCO UV/VIS Detector (Biostad, Saint-Julie, Quebec, Canada). Upon detection of the 70S peak, the fraction collection was initiated (Figure 2.5). The Mg^{2+} concentration of the collected fraction was adjusted to 10 mM (from 6 mM) to maintain the 70S tight coupled ribosome state. The 70S fraction was then transferred to 2 Ti-70 centrifuge tubes and then centrifuged at 72,551 x g (70 Ti rotor, Beckman Coulter) for 20 h at 4°C to pellet 70S ribosomes. Mg^{2+} is crucial for the bacterial ribosome to exist in a 70S state, i.e. the 30S and 50S remain associated to form 70S in the presence of Mg^{2+}.

2.10.7 Collection, quantitation and storage of 70S ribosomes

The supernatant was decanted carefully and discarded. The ribosome pellet was transparent and glassy. They were dissolved in 2 x 250 μL of Buffer 2 (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM DTT). At this step it was important to avoid the formation of air bubbles which could potentially oxidise and damage the proteins of the 70S. A 1:1000 dilution of the 70S ribosomes was made by adding 1 μL resuspended 70S to 999 μL Buffer 2.The absorbance of the prepared 70S ribosomes was determined at 260 nm using a spectrophotometer (Beckman DU530 spectrophotomter, Beckman Coulter, Mississauga, ON, Canada). The concentration (μM) of 70S ribosomes was calculated from their absorbance at 260 nm (A_{260}) using the following equation:

$$Concentration\ of\ 70S\ (\mu M) = \frac{A_{260}\ X\ 23\ X\ Dilution\ factor}{1000*}$$

Where: 23 = 23 pmoL/mL of RNA per absorbance unit at A_{260} [237]

*1/1000 converts pmol/mL to μmol/L

The dissolved ribosome pellets were then aliquoted, flash frozen in liquid nitrogen and stored in -80 °C.

2.11 Determination of Tet(O)-mediated Tc release by *in vitro* tetracycline binding assay

The ability of the purified His_6-$Tet(O)^{WT}$/$Tet(O)^{mutant}$ proteins to release 70S ribosome bound [^{3}H]-Tc was assessed by performing a Tc binding assay in the absence or presence of Tet(O) (Figure 2.6). The Tc binding assays were performed in binding buffer containing β-mercaptoethanol, which reduces the disulfide bonds formed between cysteine residues of proteins and allows them to exist as a monomer in solutions instead of disulfide linked dimmers or oligomers. It can also denature and inactivate ribonucleases. It can also act as a biological antioxidant by scavenging hydroxyl radicals. The wash buffer used for this assay contains polyamines such as spermine and spermidine that carry one and two secondary amine groups respectively. Their major function is to protect DNA and RNA against a highly reactive and unstable form of molecular oxygen called singlet oxygen ($^1O_2$*) [254] which preferentially attacks the guanine residues. Spermines and spermidines have also been implicated in protecting the structure and function of proteins against damage by Advanced Glycation End products (AGEs) [255]. Besides polyamines also prevent non specific binding of [^{3}H]-Tc to the filters and reduce the background radioactivity.

The binding assays were performed in 20 μL reaction volumes containing 0.5 μM 70S ribosomes, 50 μM GTP and increasing concentrations (1, 2.5,5,10,20,30, and 40 μM) of [^{3}H]-Tc (Perkin-Elmer, Boston, MA, USA) in the

absence or presence of 2 μM Tet(O). Binding Buffer (20 mM HEPES, 6 mM Magnesium acetate, 150 mM NH_4Cl and 4 mM β-mercaptoethanol, pH 7.4) was added to the binding assays and incubated for 20 minutes at 37°C. Reactions were terminated upon the addition of 1 ml of the wash buffer containing 20 mM HEPES, 6 mM Magnesium acetate, 150 mM NH_4Cl, 4 mM β-mercaptoethanol, 0.05 mM spermine and 2 mM spermidine, pH 7.4, and immediately vacuum-filtered through 0.45 μ nitrocellulose filters.

The filters were washed twice with 10 mL of ice cold wash buffer and 5 ml scintillation fluid (Cytoscint ES™, MP Biomedicals, Ottawa, ON, Canada) was added to each. The decay min^{-1}(dpm) of [^{3}H]-Tc bound to the 70S ribosomes was determined using a liquid scintillation counter (LS6500, Beckham Coulter, Mississauga, On, Canada). The scintillation fluid contains a fluor which when hit by a beta particle, is excited and emits a flash of light that can be detected by the scintillation counter. Ideally every beta emission results in a flash of light. The pmoles of [^{3}H]-Tc bound to 70S was directly calculated from dpm using the equation:

$$\#\ of\ pmol\ of\ [^3H]\text{-}Tc \bullet 70S = \frac{(dpm^{Total} - dpm^{Bkgd})}{dpm/pmol\ of\ [^3H]\text{-}Tc}$$

Where: dpm = decay per minute

dpm/pmol = specific activity of [^{3}H]-Tc

dpm^{Bkgd} = background levels of radioactivity which is a measure of the [^{3}H]-Tc (1-40 μM) that binds non-specifically to the filter.

The background was subtracted from the total counts (dpm) to obtain the radioactive counts of specific binding of [^{3}H]-Tc to the 70S ribosomes. The [^{3}H]-Tc dpm was then divided by the [^{3}H]-Tc specific activity to obtain the pmoles of [^{3}H]-Tc specifically bound to 70S (i.e. [^{3}H]-Tc•70S complex). This was plotted against increasing concentration of [^{3}H]-Tc (1-40 μM) by non linear regression.

The apparent dissociation constant, Kd (μM) represents the binding affinity of Tc to 70S ribosomes and was derived using the Michealis Menten equation:

$$Y = \frac{Bmax\ X\ [[^3H]\text{-}Tc]}{Kd + [[^3H]\text{-}Tc]}$$

which may be rearranged to solve for Kd:

$$Kd = \frac{[[^3H]\text{-}Tc]\ (Bmax\text{-}Y)}{Y}$$

Where: Y = # of pmol of 70S• [^{3}H]-Tc complex formed

$Bmax$ = maximum # of pmoles of [^{3}H]-Tc bound per 10 pmoles of 70S ribosome (0.5 μM/20μL reaction)

Kd = concentration of [^{3}H]-Tc when half the binding sites on 70S ribosomes are occupied by [^{3}H]-Tc in equilibrium

2.11.1 Determination of the percentage of 70S ribosome-bound Tc released by Tet(O)

The percentage of Tc released from 70S by Tet(O) was calculated by subtracting the number of pmoles of 70S• [^{3}H]-Tc complex formed in the absence of Tet(O) from that formed in the presence of Tet(O). This was then divided by the pmoles of 70S• [^{3}H]-Tc in the absence of Tet(O) for each concentration of [^{3}H]-Tc (0, 1, 2.5,5,10,20,30 and 40 μM) and multiplied by 100 to obtain the percentage of 70S bound [^{3}H]-Tc released by Tet(O) as shown below:

$$\%\ [^3H]\text{-}Tc\ released\ from\ 70S = \frac{X_{70S} - Y_{Tet(O)}}{X_{70S}}\ X\ 100$$

Where: X_{70} = # of pmoles of 70S• [^{3}H]-Tc complex formed when no Tet(O) is present

$Y_{Tet(O)}$ = # of pmoles of 70S• [^{3}H]-Tc complex formed in the presence of Tet(O)

The % Tc released was then plotted against increasing concentrations of [^{3}H]-Tc (1-40 μM) to obtain an exponential two phase decay curve.

2.11.2 Demonstrating Tet(O)-mediated Tc Release is Catalytic and not Stoichiometric

The binding assays were performed in 20 μL reaction volumes containing 0.5 μM 70S ribosomes, 50 μM GTP, 5 μM [^{3}H]-Tc in the absence or presence of increasing concentrations of purified His_6–Tet(O)^{WT} (0.25, 0.5,1, 1.5, 2.0 μM). The experiment was performed using 5 μM Tc because at concentrations above this Tc binds to the 70S at secondary sites, from which Tet(O) cannot release Tc. The reactions were performed in the same way as described in section 2.11.The pmoles of [^{3}H]-Tc bound to 70S was calculated using the equation described in section 2.11.

The percentage of [^{3}H]-Tc released from 70S at each concentration (0.25, 5, 1.0, 1.5 and 2.0 μM) of Tet(O)^{WT} were calculated by subtracting the number of pmoles 70S• [^{3}H]-Tc complex formed at each concentration of Tet(O)^{WT} from that formed in the absence of Tet(O)^{WT}, each of which was then divided by the specific activity of [^{3}H]-Tc and multiplied by 100 as shown below:

$$\#\ of\ pmol\ [^3H]\text{-}Tc\ released\ from\ 70S = \frac{M_{70S} - N_{Tet(O)m}}{dpm/pmol\ [^3H]\text{-}Tc} \quad X\ 100$$

Where: M_{70S} = # of pmoles of 70S•[^{3}H]-Tc complex formed in the absence of Tet(O)^{WT}

$N_{Tet(O)m}$ = # of pmoles of 70S•[^{3}H]-Tc complex formed in the presence of Tet(O)^{WT}, where *m* represents the different concentrations of Tet(O)

The percentage of [^{3}H]-Tc released from 70S was plotted against increasing concentration of Tet(O) (0.25-2 μM) (Graph Pad Prism 5.0, La Jolla, CA, USA).

2.12 Determination of kinetics of GTP hydrolysis by Tet(O)

A ribosome-dependant GTP hydrolysis is necessary for the turnover of Tet(O), i.e. for Tet(O) to be released from the ribosome so it can bind to more GTP and cause another round of Tc release. The Tet(O) mutants were unable to release 70S bound Tc as effectively as Tet(O)^{WT}. In order to determine if this impaired ability to release Tc was either due to:

(i) the inability of the Tet(O)^{mutants} to bind to 70S, or

(ii) the inability of the Tet(O)^{mutants} to be released after binding,

the GTP hydrolysis assays were performed using the protocol reported by Thakor *et al*. [248].

The GTP hydrolysis assays were performed in 20 μL reaction volumes containing 0.2 μM 70S ribosomes, 0.05 μM Tet(O)$^{WT/mutants}$,10-500 μM (10, 20, 50, 100, 200, 400, 500 μM) 0.3 μCi [γ-^{32}P]GTP (6000 Ci/mmol, Perkin-Elmer, Boston, MA, USA), non-radiolabelled GTP in reaction buffer (30 mM Tris-Cl pH 8.0, 80 mM NH_4Cl, 20 mM $MgCl_2$) and were incubated at 37°C. For each GTP concentration, three reactions were set up at different incubation times (10, 20 μM: 1, 2, 5 min; 50 μM: 2, 5, 10 min; 100, 200 μM: 5, 10, 20 min; 300, 400, 500 μM: 5, 15, 30 min). Three different incubation times were necessary as it was not known what level of GTPase activity was present and it was necessary to ensure activity (velocity) was not measured under conditions of GTP exhaustion. The Tet(O)$^{WT/mutants}$ were also incubated (for 30 min) without 70S ribosomes as controls to detect any GTP-hydrolysis in the absence of 70S. Following incubation, 1 μL of the sample was removed and quenched using 1 μL 30% (v/v) formic acid. Then 1 μL of each sample was loaded onto a water treated polyethyleneimine (PEI) cellulose thin layer chromatography (TLC) plate (Sigma-Aldrich, Oakville, ON, Canada) and developed in 4 M sodium formate (pH 3.5), followed by acetone rinse to allow the plate to dry quickly. The treated TLC plates were wrapped with SARAN plastic wrap, exposed overnight on a

phosphorimager plate (Fujifilm, Mississauga, ON, Canada) and scanned on FLA5100 imaging system (Fujifilm, Mississauga, ON, Canada). The images were analyzed using ImageQuant (GE Healthcare, Baie d'Urfe, Quebec, Canada) to obtain the signal volume (Figure 2.7).

The GTP hydrolysis velocity i.e. the μmoles of [γ-^{32}P]GTP hydrolyzed per μmole Tet(O)$^{WT/mutants}$ per second was calculated from the signal volume using the following equation:

$$Y = \frac{V_{[\gamma\text{-}^{32}P]Pi}}{V_{[\gamma\text{-}^{32}P]GTP} + V_{[\gamma\text{-}^{32}P]Pi}} \quad X \quad \frac{[\gamma\text{-}^{32}P]GTP\ (\mu M)}{[Tet(O)^{WT/mutants}]\ (\mu M)}$$

Where: Y = μM [γ-^{32}P]GTP hydrolyzed per μM Tet(O)

$V_{[\gamma\text{-}^{32}P]Pi}$ = signal volume of hydrolyzed [γ-^{32}P]GTP

$V_{[\gamma\text{-}^{32}P]GTP}$ = signal volume of unhydrolyzed [γ-^{32}P]GTP

$[[\gamma\text{-}^{32}P]GTP]$ = 10, 20, 50, 100, 200, 300, 400 or 500 μM

$[GTPases]$ = 0.05 μM Tet(O)

The Y for each concentration of [γ-^{32}P]GTP was plotted versus their corresponding incubation times (10, 20 μM: 1, 2, 5 min; 50 μM: 2, 5, 10 min; 100, 200 μM: 5, 10, 20 min; 300, 400, 500 μM: 5, 15, 30 min) for linear regression analysis. The slope (b), which represents the μM GTP hydrolyzed/min when Y= bX + a, R^2> 0.95, was then divided by 60 to obtain the velocity of GTPase reactions as μM.s^{-1}. The velocity was then plotted versus the increasing concentrations (10-500 μM) of [γ-^{32}P]GTP. The enzyme kinetic parameters (Vmax, Km, k_{cat}, k_{cat}/Km) were derived by non-linear regression analysis according to the following equation:

$$Vmax\ (\mu M \bullet s^{-1}) = \frac{V\ (Km + [[\gamma\text{-}^{32}P]GTP])}{[[\gamma\text{-}^{32}P]GTP]}$$

Where: *Vma x* = maximal Tet(O) GTPase velocity ($\mu M \bullet s^{-1}$)

V = velocity of Tet(O) GTPase reaction ($\mu M \bullet s^{-1}$)

[[γ–^{32}P]GTP] = substrate for Tet(O) GTPase (μM)

Km = μM [γ-^{32}P]GTP needed for half maximal GTP velocity

$$Km = \frac{[[\gamma\text{-}^{32}P]GTP]\ (Vmax - V)}{V}$$

Where: *Km* = μM [γ-^{32}P]GTP needed for half maximal GTP velocity

V = velocity of Tet(O) GTPase reaction ($\mu M \bullet s^{-1}$)

Vmax = maximal Tet(O) GTPase velocity ($\mu M \bullet s^{-1}$)

$$k_{cat}\ (s^{-1}) = \frac{Vmax}{[GTPase]}$$

Where: $k_{cat}\ (s^{-1})$ = turnover number per second, the number of [γ-^{32}P]GTP converted to GDP and [γ-^{32}P]Pi by Tet(O) GTPase per second when Tet(O) GTPase is fully saturated with [γ-^{32}P]GTP.

[GTPase] = 0.05 μM Tet(O)

$$Specificity\ constant\ (\mu M^{-1}.s^{-1}) = \frac{k_{cat}}{Km}$$

Where: specificity constant = catalytic efficiency of Tet(O).

2.13 Statistical Analysis

The results were expressed as mean ± standard error mean (SEM) of 2-17 experiments. Each experiment was done in triplicate. Linear regression analysis was performed using Microsoft excel (Microsoft, Redmond, WA, USA). Curve fitting (non-linear regression, exponential two phase decay curve) and statistical parameters were calculated and analyzed using GraphPad Prism 5.0 (La Jolla, CA, USA).

Table 2.1 The *tet(O)*^{mutants} generated from *tet(O)*^{WT}

tet(O) Mutants	From		To		Primer pairs to generate the mutant
	codon	amino acid	codon	amino acid	
Single Alanine					
S508A	AGT	Ser	GCA	Ala	OF-F + S508A-R, S508A-F + OF-R
P509A	CCT	Pro	GCA	Ala	OF-F + P509A-R, S508A-F + OF-R
V510A	GTA	Val	GCA	Ala	OF-F + V510V-R, S508A-F + OF-R
S511A	AGT	Ser	GCA	Ala	OF-F + S511A-R, S508A-F + OF-R
T512A	ACC	Thr	GCA	Ala	OF-F + T512A-R, S508A-F + OF-R
Double Alanine					
S508A-P509A	AGT,CCT	Ser, Pro	GCA,GCT	Ala, Ala	OF-F + S508A,P509A-R, S508A,P509A-R -F + OF-R
P509A-V510A	CCT,GTA	Pro, Val	GCA,GCT	Ala, Ala	OF-F + S508A,P509A-R, S508A,P509A-R -F + OF-R
V510A-S511A	GTA,AGT	Val, Ser	GCA,GCT	Ala, Ala	OF-F + S508A,P509A-R, S508A,P509A-R -F + OF-R
S511A-T512A	AGT,ACC	Ser, Thr	GCA,GCT	Ala, Ala	OF-F + S508A,P509A-R, S508A,P509A-R -F + OF-R
Deletion					
508ΔS	AGT	Ser	-	-	OF-F + 508ΔS-R, 508ΔS-F + OF-R
509ΔP	CCT	Pro	-	-	OF-F + 509ΔP-R, 509ΔP-F + OF-R
510ΔV	GTA	Val	-	-	OF-F + 510ΔV-R, 510ΔV-F + OF-R
511ΔS	AGT	Ser	-	-	OF-F + 511ΔS-R, 511ΔS-F + OF-R
512ΔT	ACC	Thr	-	-	OF-F + 512ΔT-R, 512ΔT-F + OF-R
Polar / Nonpolar					
Y507H	TAT	Tyr	CAT	His	OF-F + Y507H-R, Y507H -F + OF-R
S508D	AGT	Ser	GAC	Asp	OF-F + S508D-R, S508D -F + OF-R
P509V	CCT	Pro	GTA	Val	OF-F + P509V-R, P509V -F + OF-R
V510D	GTA	Val	GAC	Asp	OF-F + V510D-R, V510D -F + OF-R
T512S	ACC	Thr	AGT	Ser	OF-F + T512S-R, T512S -F + OF-R

Table 2.2 Primers used to generate *tet(O)*^{WT}, *tet(O)* ^{mutants}

Mutagenic Forward	**Primer Sequence (5'-3')**	**Mutagenic Reverse**	**Primer Sequence (5'-3')**
S508A-F	TTGTATTAT**GCA**CCTGTAAGTAC	S508A-R	GTACTTACAGG**TGC**ATAATACAA
P509A-F	TGTATTATAGT**GCA**GTAAGTAC	P509A-R	GGTACTTAC**TGC**ACTATAATACA
V510A-F	ATAGTCCT**GCA**AGTACCCCC	V510A-R	GGGGGTACT**TGC**AGGACTAT
S511A-F	AGTCCTGT**GCA**ACCCCCG	S511A-R	CGGGGGT**TGC**ACAGGACT
T512A-F	CCTGTAAGT**GCA**CCCGCAG	T512A-R	CTGCGGG**TGC**ACTTACAGG
S508A-P509A-F	TTGTATTAT**GCAGCT**GTAAGTAC	S508A-P509A-R	GTACTTAC**AGCTGC**ATAATACAA
P509A-V510A-F	GTATTATAGT**GCAGCT**AGTCC	P509A-V510A-R	GGTACT**AGCTGC**ACTATAATAC
V510A-S511A-F	TAGTCCT**GCAGCT**ACCCCC	V510A-S511A-R	GGGGGT**AGCTGC**AGGACTA
S511A-T512A-F	AGTCCTGTA**GCAGCT**CCCG	S511A-T512A-R	CGGG**AGCTGA**CTACAGGACT
508ΔS-F	GGATTGTATTATCCTGTAAGTAC	508ΔS-R	GTACTTACAGGATAATACAATCC
509ΔP-F	TTGTATTATAGTGTAAGTACCCC	509ΔP-R	GGGGTACTTACACTATAATACAA
510ΔV-F	ATAGTCCTAGTACCCCCGCA	510ΔV-R	TGCGGGGGTACTAGGACTAT
511ΔS-F	AGTCCTGTAACCCCCGCAT	511ΔS-R	CTGCGGGGGTTACAGGACT
512ΔT-F	TCCTGTAAGTCCCGCAGACT	512ΔT-R	AGTCTGCGGGACTTACAGGA
Y507H-F	GATTGTAT**CAT**AGTCCTGTAAGT	Y507H-R	ACTTACAGGACT**ATG**ATACAATC
S508D-F	TTGTATTAT**GAC**CCTGTAAGTAC	S508D-R	GTACTTACAGG**GTC**ATAATACAA
P509V-F	TTGTATTATAGT**GTT**GTAAGTACC	P509V-R	GGTACTTAC**AAC**ACTATAATACAA
V510D-F	TATAGTCCT**GAT**AGTACCCCC	V510D-R	GGGGGTACT**ATC**AGGACTATA
T512S-F	TCCTGTAAGT**AGT**CCCGCAG	T512S-R	CTGCGGG**ACT**ACTTACAGGA
Outer Flanking Forward	**Primer Sequence (5'-3')**	**Outer Flanking Reverse**	**Primer Sequence (5'-3')**
OF-F	CACCAAAATAATTAACTTAGGCAT	OF-R	**TTA**AGCTAACTTGTGAACATATGC
EF-G Forward Primer Sequence (5'-3')		**EF-G Reverse Primer Sequence (5'-3')**	
fusA-F	CACCGCTTCGTACAACACCCATC	*fusA-R*	**TTA**TTTACCACGGGCTTCAATTACG

CACC represents the recognition site for directional cloning by topoisomerase I; **TTA** represents the stop codon; bold underlined sequences represent the codon change produced by site-directed mutagenesis to generate point mutants of *tet(O)*

Table 2.3 PCR cycling conditions used in site-directed mutagenesis

Step	Temperature	Time	Number of cycles
Initial Denaturation	95°C	60 sec	1
Denaturation	95°C	45 sec	30
Annealing	60°C	45 sec	
Extension	68°C	90 sec	
Final Extension	68°C	10 mins	1

Table 2.4 Composition of 10% SDS-PAGE gel

Ingredients	10% Separating Gel	10% Stacking Gel
MilliQ water	3.6 mL	2.0 mL
40% Acrylamide/ Bis Solution 29: 1	1.9 mL	0.3 mL
1.5 M Tris-HCl, pH 8.8	1.9 mL	-
1.0 M Tris-HCl, pH 6.8	-	0.313 mL
10 % SDS	75 µL	25 µL
10% Ammonium persulfate (APS)	75 µL	25 µL
TEMED	4 µL	4 µL

Table 2.5 Preparation of BSA working standards for the Bradford protein assay

Working BSA Standards (μg/mL)	H_2O (μL)	Standard100μg/mL BSA (μL)
0	150	0
2	980	20
4	960	40
6	940	60
8	920	80
10	900	100

Table 2.6 Preparation of serial dilutions of unknown protein samples

Diluted Sample	Dilution	Sample (μL)	MilliQ H_2O(μL)	D1 (μL)	D2 (μL)	D3 (μL)	D4 (μL)	D5 (μL)	D6 (μL)
D1	1/20	15	285	-	-	-	-	-	-
D2	1/40	-	150	150	-	-	-	-	-
D3	1/80	-	150	-	150	-	-	-	-
D4	1/160	-	150	-	-	150	-	-	-
D5	1/320	-	150	-	-	-	150	-	-
D6	1/640	-	150	-	-	-	-	150	-
D7	1/1280	-	150	-	-	-	-	-	150

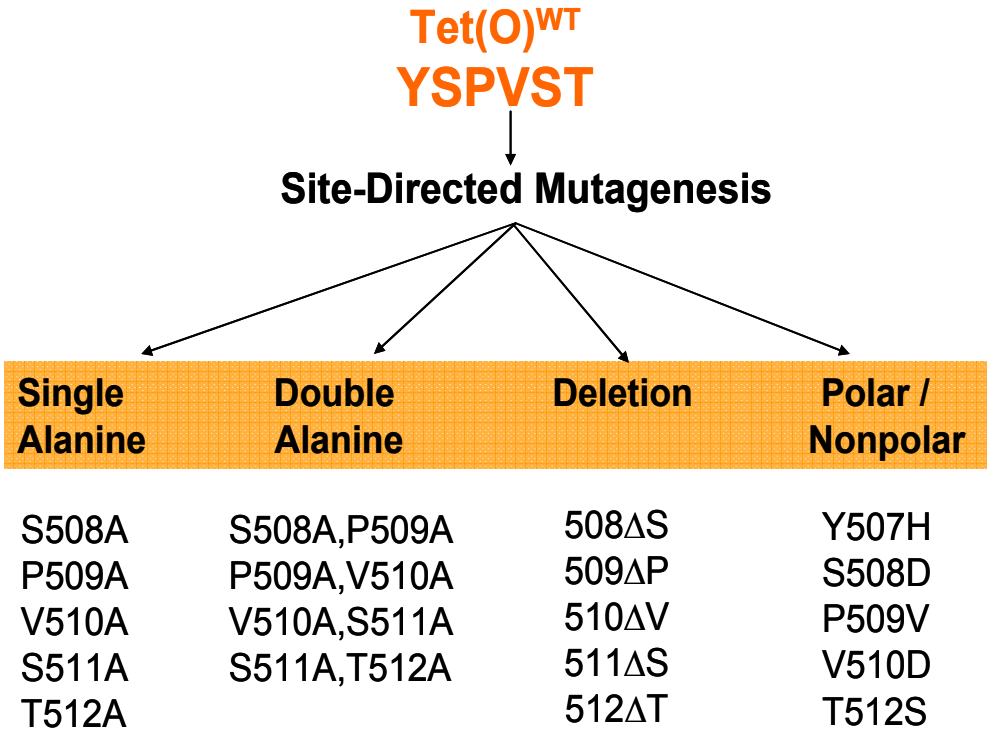

Figure 2.1 The study design

A series of Tet(O)^{mutants} (single alanine, double alanine, deletion and polar/ non-polar mutants) were generated by site-directed mutagenesis of the target amino acid residues: YSPVST, occupying positions 507-512 at the tip of the domain IV of Tet(O)^{WT}.

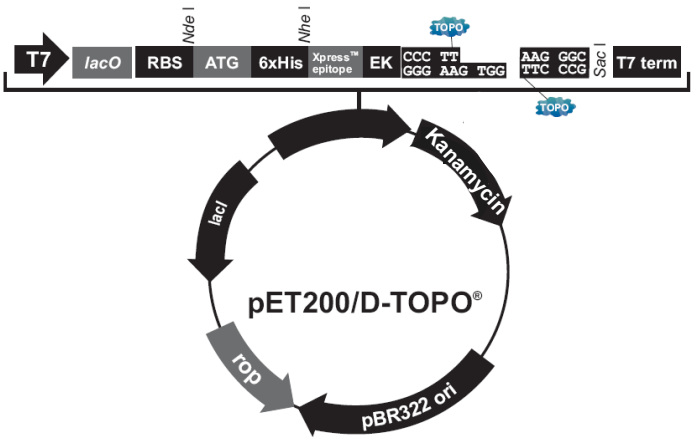

Fig 2.2: Features and map of pET200/D-TOPO vector. [modified from 244].

T7 promoter: binding site for T7 RNA polymerase that actively transcribes the downstream *tet(O)* gene in the presence of the inducer IPTG which in turn leads to overexpression of Tet(O) protein.

T7 promoter/priming site: For sequencing of the cloned gene from the sense strand.

lac operator(lacO): Site for binding of *lacI* repressor to minimise the basal expression of *tet(O.)*

Ribosome binding site: Shine Dalgarno sequence that allows 70S to bind to efficiently translate *tet(O.)*

ATG: start codon.

N-terminal 6x His tag: allows purification of fusion proteins using IMAC (Immobilized metal affinity chromatography), and detecting the fusion proteins using anti-HisG antibodies.

Xpress™ epitope (Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys): detection of fusion proteins with anti- Xpress™ antibodies.

Enterokinase (EK) recognition site (Asp-Asp-Asp-Asp-Lys):allows N-terminal His_6 –tags to be removed from fusion proteins using enterokinases such as EKmax™.

TOPO Cloning site (directional): allows directional cloning of the tet(O) PCR product into pET200 vector.

T7 reverse priming site: allows sequencing of the *tet(O)* gene from the antisense strand.

T7 transcription termination region: bacteriophage T7 transcription termination region to efficiently stop transcription.

Kanamycin resistance gene: allows selection of cells carrying pET200/D-TOPO vector.

pBR322 origin of replication(*ori*): allows the pET200 plasmid to replicate.

ROP ORF: interacts with pBR322 to maintain a low copy plasmid number in BL21
Star ™ (DE3).

lacI ORF: encodes lac repressor that binds to T7 lac operator to repress basal transcription of *tet(O)* in the absence of the inducer

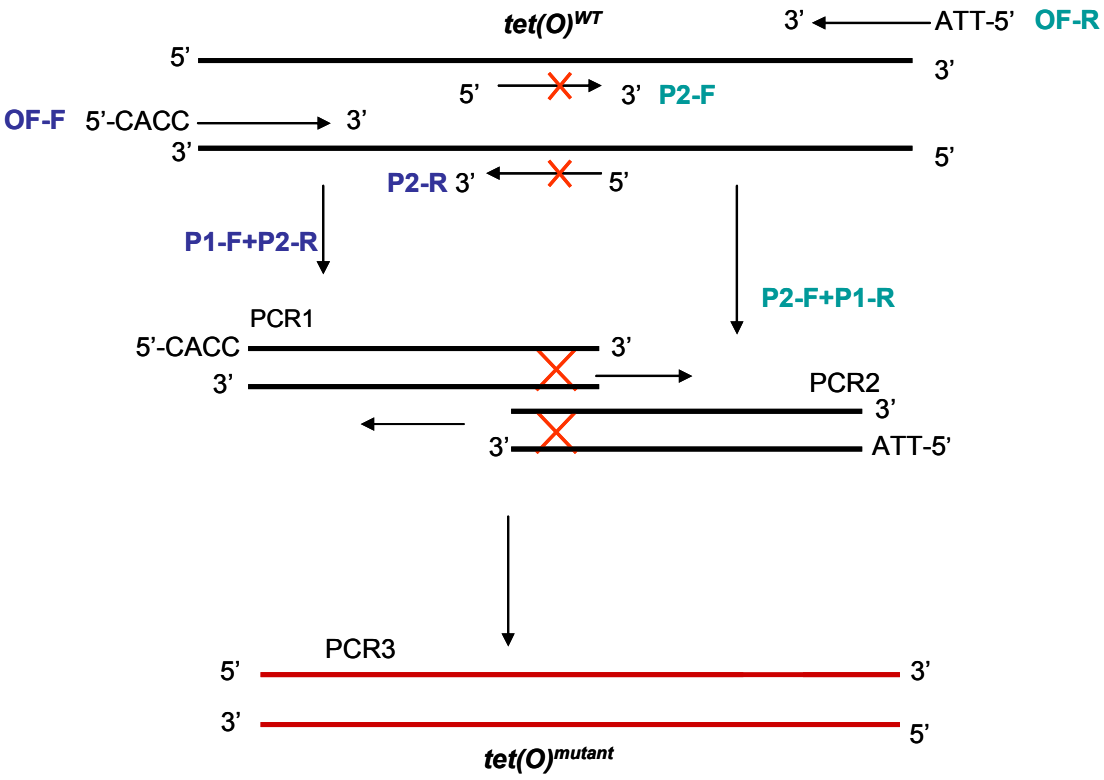

Figure 2.3: The principle of polymerase chain reaction using overlap extension to generate *tet(O)* point mutants

P2-F and P2-R are the mutagenic primers, OF-F and OF-R are outer flanking forward and reverse primers respectively.

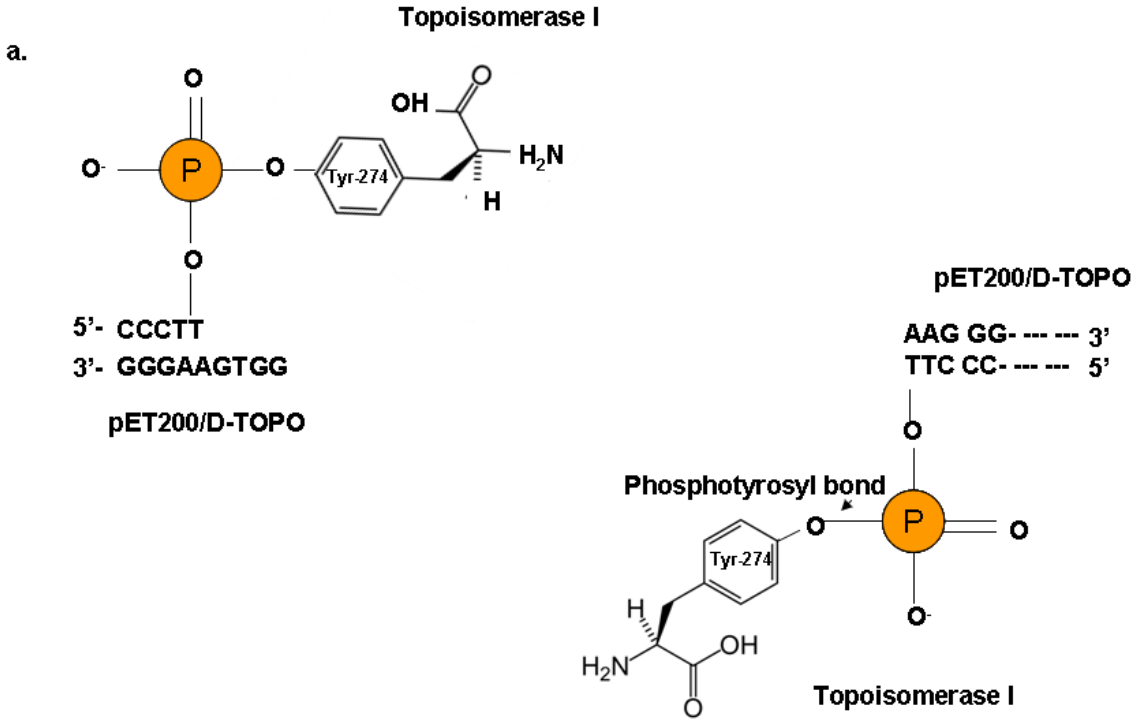

Figure 2.4: Mode of action of Topoisomerase I. [modified from 244]

(a)The topoisomerase-I activated pET200/D-TOPOvector.

.

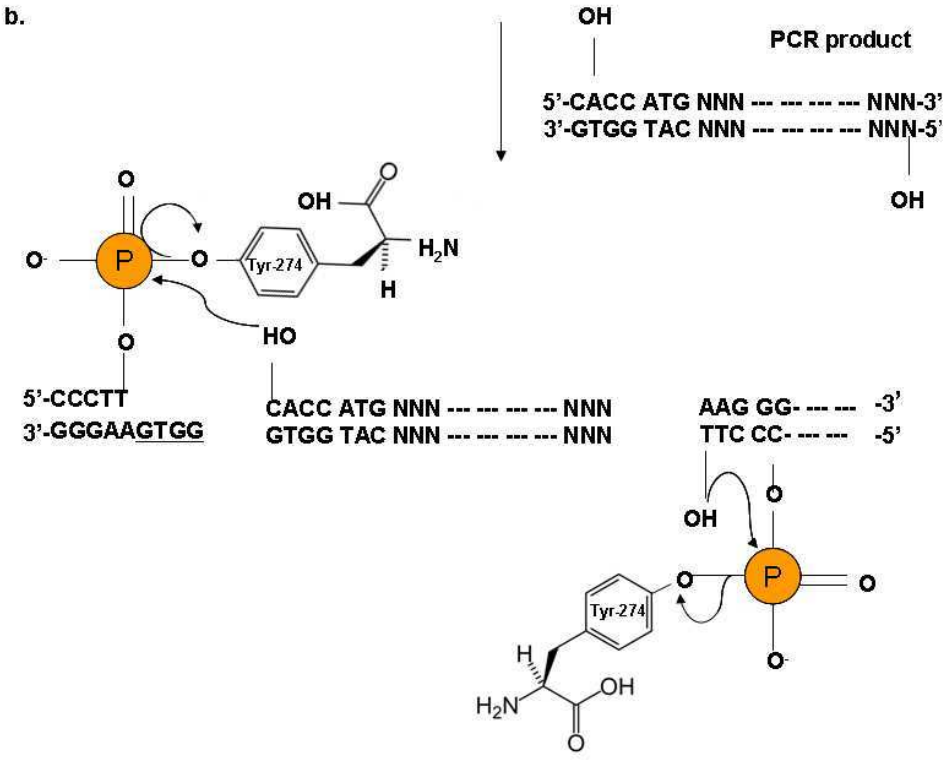

Figure 2.4: Mode of action of Topoisomerase I.[modified from 244].

(b) The addition of the blunt end PCR product (PCR product that has 5'-OH and 3'-OH groups). The 3'-phosphotyrosyl bond present in the pET200/D-TOPO vector is attacked by the 5'-OH group of the PCR product generated by overlap extension thus releasing the tyrosyl-274 residue of topoisomerase I.

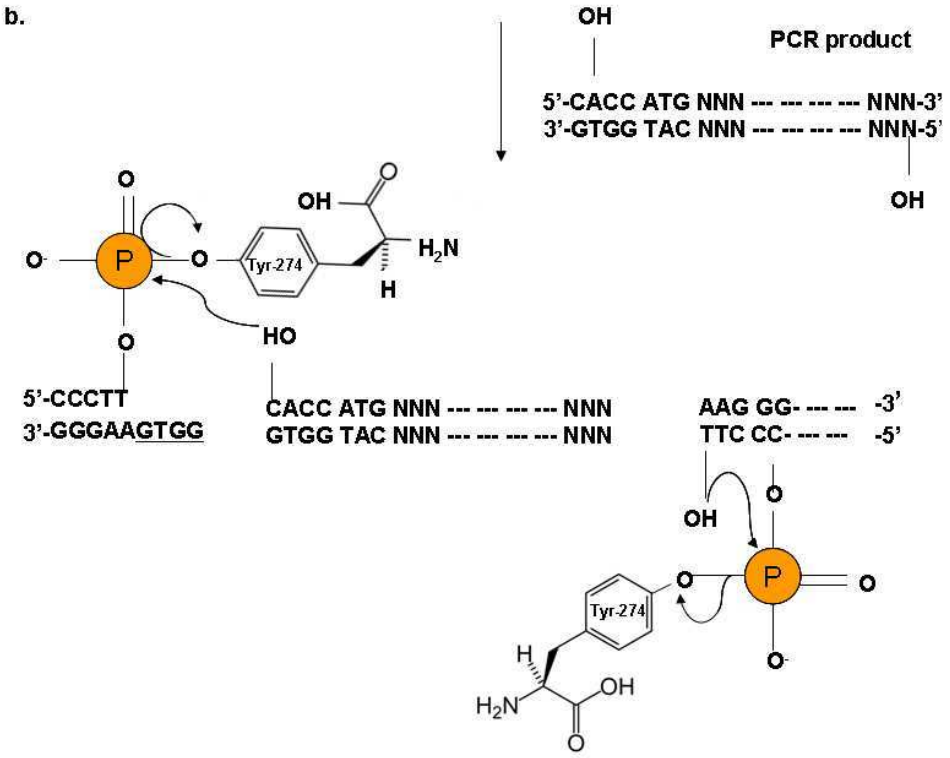

Figure 2.4: Mode of action of Topoisomerase I. [modified from 244].

(c) The amplicon carries a 5'- CACC-3' sequence, complementary to the 5'-GTGG-3' overhang in the pET200/D-TOPO vector. The GTGG overhang invades double-stranded DNA, displacing the bottom strand allowing the PCR product amplified by overlap extension to anneal into the pET200/D-TOPO vector.

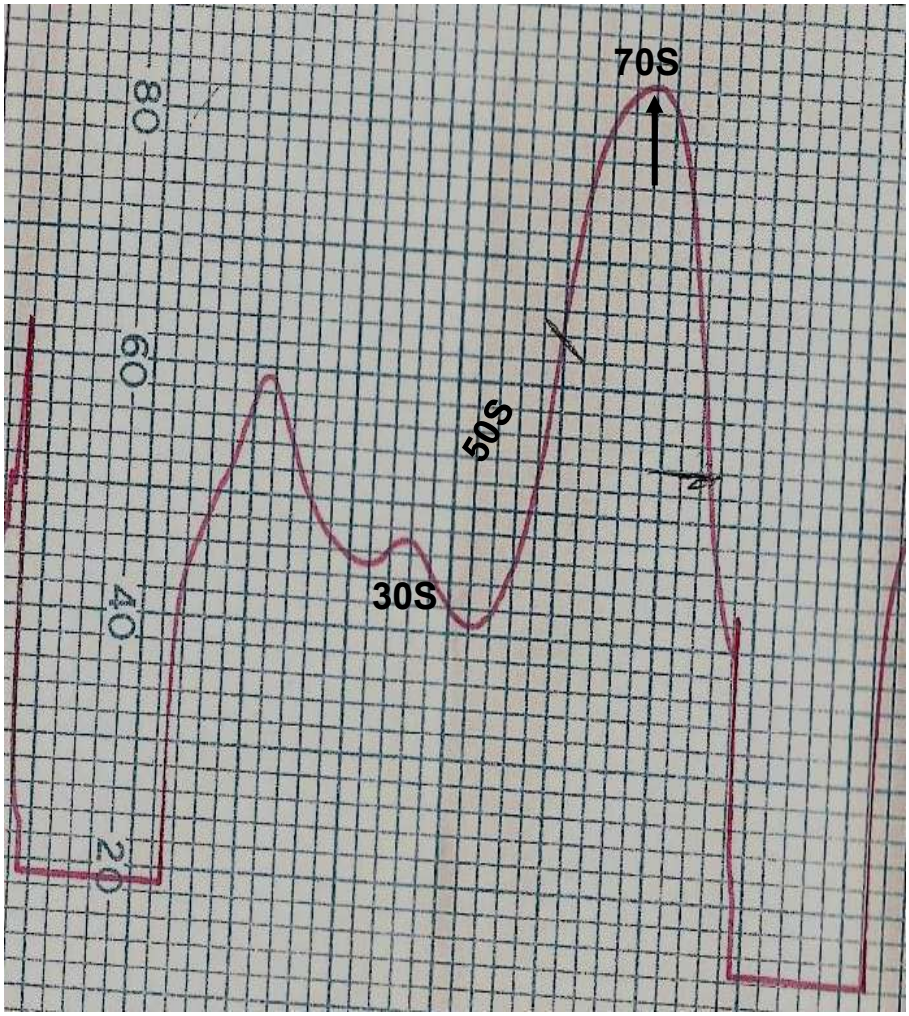

Figure 2.5 Tight coupled 70S ribosomes were detected under UV-detector at 260 nm and collected from a sucrose gradient

The collection started at the arrow to ensure only tight coupled 70S ribosome complexes were collected.

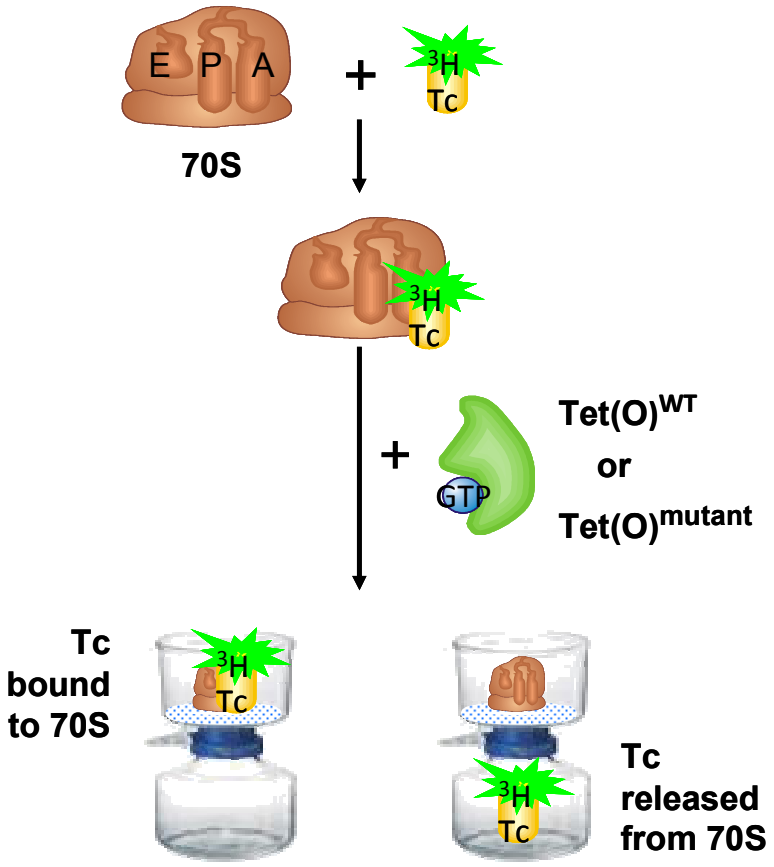

Figure 2.6 The principle of Tc binding assay

Incubation of [^{3}H]-Tc (yellow) with 70S ribosomes (brown) results in the binding of Tc to its primary site in the 70S. In the absence of Tet(O)^{WT} (green) the [^{3}H]-Tc•70S complex is trapped on the surface of the nitrocellulose filter (0.45 μ). Tet(O)^{WT} releases [^{3}H]-Tc from its primary binding site. The released [^{3}H]-Tc easily passes through the filter while the 70S stays bound to the filter. Tet(O)^{mutants} (green) carrying mutations in the residues hypothesized to be critical for Tc release are expected to be unable to release Tc, and the [^{3}H]-Tc•70S complex is expected to be trapped on the filter.

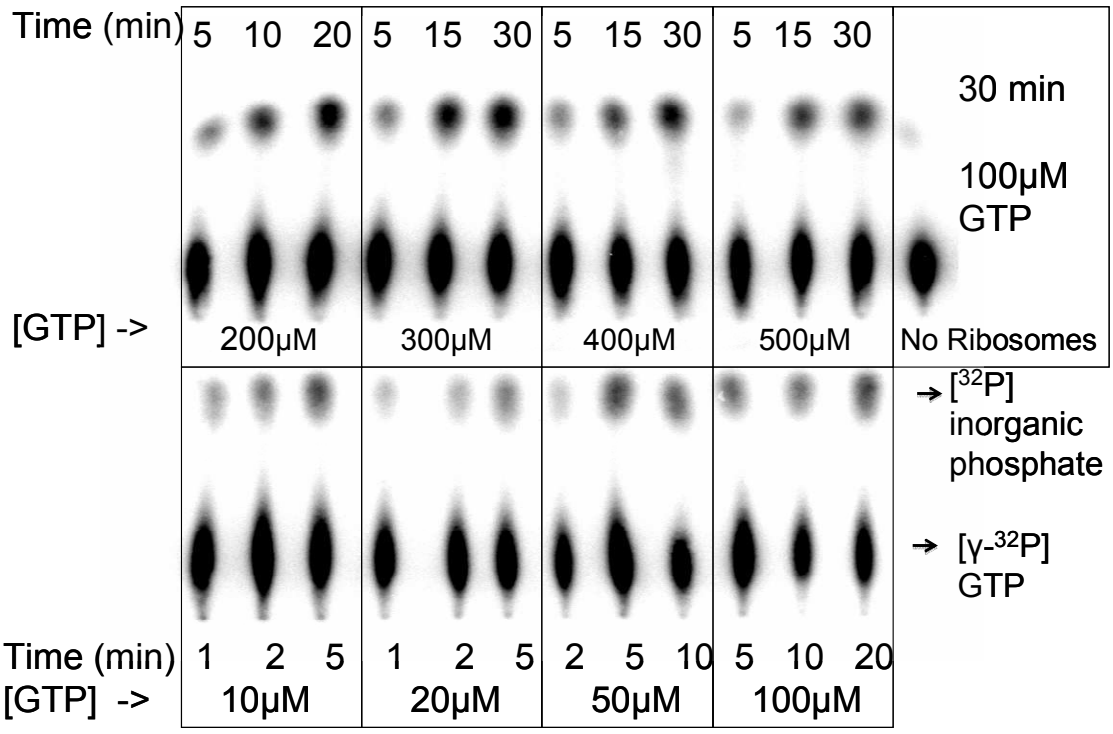

Figure 2.7 Storm840® acquired image of TLC Plate showing [γ-^{32}P] GTP hydrolysis by 511ΔS

Chapter 3 Results

3.1 Tc susceptibility phenotype conferred by *tet(O)*^{WT} and *tet(O)*^{mutants}

3.1.1. *C. jejuni tet(O)*^{WT} gene conferred Tc^{R} to *E.coli* BL21(DE3)

E.coli BL21 (DE3) strain transformed with the *fusA* gene (encoding for EF-G) produced a tetracycline susceptible (Tc^{S}) phenotype (MIC= 4 μg/mL, Figure 3.1, Table 3.1), confirming pET200/D-TOPO vector itself does not confer Tc^{R} to the *E.coli* BL21(DE3) strains. Transformation of the *E.coli* BL21(DE3) strain with the *C. jejuni tet(O)*^{WT} gene produced a Tc^{R} phenotype (MIC= 32 μg/mL, Figure 3.1, Table 3.1) as observed with an 8-fold increased Tc MIC relative to the BL21 strain transformed with *fusA* (MIC= 4 μg/mL). The Tc^{R} phenotype conferred by the *tet(O)*^{WT} in *E. coli* (MIC= 32 μg/mL), is comparable to the Tc^{R} phenotype of *C. jejuni* (MIC= 64 μg/mL) carrying *tet(O)*^{WT} [179]. The difference in the Tc MIC may be related to the minor differences in the 70S ribosomes of *E. coli* versus *C. jejuni*.

3.1.2 The Tet(O) single alanine mutants were Tc^{R}

The Tet(O) single alanine mutants, S508A, P509A, V510A, S511A and T512A have the same Tc^{R} phenotype (Figure 3.1, Table 3.1) as the Tet(O)^{WT}. The Tc MIC of S508A, V510A and S511A mutants (32 μg/mL) were the same as Tet(O)^{WT}, while that of P509A and T512A (64 μg/mL) increased 2-fold versus Tet(O)^{WT} (MIC= 32 μg/mL). Accordingly these mutants were not characterized any further, because their Tc susceptibility did not differ from the Tet(O)^{WT}.

3.1.3 Double alanine Tet(O) substitutions at positions 510-511 and 511-512 were Tc^{S}

Although the Tc MICs of the Tet(O) double alanine mutants, S508A-P509A (NT1) and P509V-V51A (NT2) (MIC= 16 μg/mL), were reduced 2-fold as compared to Tet(O)^{WT}, (Figure 3.1, Table 3.1), they retained the Tc^{R} phenotype.

The Tc MICs of the remaining double alanine mutants, V510A-S511A (NT3) and S511A-T512A (NT4) (MIC= 8 μg/mL), were reduced 4-fold relative to Tet(O)^{WT} (Figure 3.1, Table 3.1), indicating a Tc^{S} phenotype. This implies the double alanine substitutions at positions 510-512 affected Tet(O) function.

3.1.4 The Tet(O) deletion mutants were Tc^{S}

The Tc MICs of all of the Tet(O) deletion mutants, 508ΔS, 509ΔP, 510ΔV, 511ΔS, 512ΔT (MIC= 8 μg/mL) were reduced 4-fold relative to Tet(O)^{WT} (Figure 3.1, Table 3.1), indicating they are Tc^{S} and that individual deletion at any of the positions between 508-512 of Tet(O)^{WT} impaired Tet(O) function.

3.1.5 The Tet(O) polar/non-polar mutants were Tc^{S}

Although the Tc MIC of Y507H (MIC= 16 μg/mL) was reduced 2-fold relative to Tet(O)^{WT} (Figure 3.1, Table 3.1), it retained resistance to Tc. The Tc MIC of the non-polar mutant P509V (MIC= 8 μg/mL) was reduced 4-fold relative to Tet(O)^{WT}, while the Tc MIC of the remaining polar mutants (S508D, V510D and T512S) (MIC= 4 μg/mL) were reduced 8-fold relative to Tet(O)^{WT} (Figure 3.1, Table 3.1). Accordingly, the Tet(O)-polar/non-polar mutants between positions 508-512 were Tc^{S}. This indicates polar/non-polar substitutions at positions 508, 509, 510 and 512 of Tet(O) altered Tet(O) function.

3.2 Tet(O)^{WT} and the Tet(O)^{mutants} were successfully over expressed, isolated and purified

The recombinant Tet(O)^{WT} and Tet(O)^{mutant} proteins were overexpressed in their soluble form, by induction with IPTG at suboptimal growth temperature (30°C for WT and 18°C for mutants) and purified by IMAC (see section 2.7 for details). Tet(O)^{WT} and each of the Tet(O)^{mutant} proteins eluted at 100 mM imidazole and were run on SDS-PAGE to assess their purity (Figures 3.2a-3.2b). In most cases (with the exception of the double alanine mutant V510A-S511A (NT3)), the purity of the isolated proteins was greater than 90%. The Tet(O)^{WT} and mutant proteins were detected on the gel just above the 70 kDa marker in the

protein ladder, which corresponds to the expected Tet(O) MW of 72.5 kDa. Protein concentrations were determined by the Bradford assay (see section 2.8). A typical standard curve is shown in Figure 3.3.

3.2.1 Yield and purity of the Tet(O) proteins

Typically the yields of the Tet(O)^{mutant} proteins ranged from 0.9-3.5 mg/mL as compared with 4.0 mg/mL for Tet(O)^{WT} (Table 3.2). The low yield (0.54 mg/mL) obtained for His_6-S508D Tet(O)^{mutant} was due to an accidental loss of the supernatant prior to IMAC purification. The purity of all the isolated Tet(O)$^{WT/mutant}$ proteins was >90% with the exception of the double alanine mutant NT3 (V510A-S511A) (Figure 3.2a-b).

3.2.1.1 Protein bands co-eluted with NT3

NT3 co-eluted with several other proteins (between 27-55 kDa, Figure 3.2b) over each of the three occasions that it was purified. It is possible that other like hydrophobic proteins associated with NT3 which made it difficult to purify it using IMAC alone. The use of a second column such as hydrophobic interaction chromatography (HIC) on a resource phenyl (HIC-PHE) column, might have improved the purity of the protein, but these columns were unavailable. The proteins that co-eluted with NT3 were similar to the co-eluates observed with the 80 mM imidazole eluates of Tet(O)^{WT} and deletion mutants. These co-eluted proteins were identified by LC-MS (Figure 3.4, bands 4, 5, 6, 7and 8) and have no known role in Tc release. One protein was identified to be FKBP type petidyl prolyl isomerise (PPIase) which may assist in protein folding [256]. Rho and Cap proteins are common contaminants of His_6-fusion proteins eluted by Ni^{2+} column chromatography. The use of an impure NT3 preparation may impair the ability to assess its function.

3.2.1.2 Protein bands co-eluted with Tet(O)^{WT} and Tet(O) deletion mutants

The identity and function of a variety of proteins that co-elute with Tet(O)^{WT} and Tet(O) deletion mutants at 80 mM imidazole are summarized in

Table 3.3. Representative samples were taken from the 508ΔS, 509ΔP and 510ΔV deletion mutants as shown in Figure 3.4. The identified proteins were metabolic enzymes, stress proteins or ribosomal proteins associated with the large 50S subunit of the bacterial ribosome. None of the 50S subunit proteins co-eluted with Tet(O) are presumed to have a role in Tc release, since Tc exclusively binds and interacts with the components of the 30S subunit of the bacterial ribosome. Hence they were not characterized any further.

The only protein of concern was RNase E, and whether it could impact our studies with 70S ribosomes. This in fact was not the case. RNase E was identified as one of the contaminant proteins present in both 80 mM and 100 mM imidazole eluates of the 511ΔS mutant. RNase E, along with RNase G (Caf A protein), are known to play a critical role in the 5' maturation of the 16S rRNA which is a component of the 30S subunit of the bacterial ribosome [257]. RNase E preferentially cleaves regions of single-stranded RNA with a consensus sequence RAUUUW (R= A or G, W= A or U). In some cases the presence of a downstream hairpin has appeared to be important for maximal cleavage rates [258]. Fortunately, since the level of RNase E contamination was small (7-10%) and there was no single stranded RNA in the 70S preparations, RNase E would not likely interfere with the studies of the 511ΔS mutant and the Tc-bound 70S.

3.3 Tc Binding to the 70S Ribosomes in the presence of $Tet(O)^{WT}$ and Tc^{S} $Tet(O)^{mutants}$

The Tc binding assay was used to determine the amount of 70S-bound Tc released by the $Tet(O)^{WT}$ and/or the $Tet(O)^{mutants}$. Background levels of [^{3}H]-Tc determined at each [^{3}H]-Tc concentration revealed a linear increase in the non-specific binding of the [^{3}H]-Tc to the 0.45 micron nitrocellulose filter in the absence of the 70S ribosomes and the $Tet(O)^{WT/mutant}$ proteins (Figure 3.5). All the Tc binding assays were corrected for the non-specific binding of [^{3}H]-Tc to the nitrocellulose.

The pmoles of [^{3}H]-Tc bound to 70S ribosomes was plotted versus increasing concentrations (1-40 μM) of free [^{3}H]-Tc (Figures 3.6-3.9). The

apparent dissociation constant (Kd) was estimated from the graphs and reflected a measure of the binding affinity of Tc to its primary binding site on the 70S ribosome. In the absence of Tet(O)^{WT}, [^{3}H]-Tc bound the 70S ribosomes readily, to a binding maximum (Bmax) of 13.08 $\pm$ 0.68 pmoles, with an apparent binding affinity (Kd) of 6.89 $\pm$ 1.15 (Table 3.4).

3.3.1 Tet(O)^{WT} decreases the amount of Tc bound to the 70S

In the presence of the Tet(O)^{WT} protein, less [^{3}H]-Tc was bound to the primary site on the 70S ribosomes (Figure 3.6b, shown by the boxed, shaded area). The reduced Tc binding to the 70S was reflected by a ~2 fold increase in the apparent Kd (Table 3.4). This implies Tc has a lower binding affinity for the 70S in the presence of Tet(O)^{WT}, which corresponds to the release of Tc from its primary binding site. This suggests that, in the bacteria, protein synthesis would be restored in the presence of the Tet(O)^{WT}. The apparent Bmax of [^{3}H]-Tc to the 70S was similar in the absence and presence of Tet(O)^{WT} (Table 3.4). This is not an unexpected finding as Tc is known to have other lower affinity secondary binding sites on the 70S [69, 76]. Maximum Tc release by Tet(O)^{WT} occurs below 10 μM Tc (Figure 3.6b). Since Tet(O)^{WT} cannot release Tc from the secondary binding sites [216], the Bmax of Tc for the 70S in the presence of Tet(O)^{WT} gradually approaches the Bmax observed in the absence of Tet(O)^{WT}.

3.3.2 70S-bound Tc release is impaired by Tet(O) double alanine mutants

Tc^{S} Tet(O) double alanine mutants, V510A-S511A (NT3) and S511A-T512A (NT4), did not release Tc from 70S below 10 μM Tc (Figures 3.7a-b, Table 3.4). This implies that, in the bacteria, protein synthesis would not be restored in the presence of the double alanine mutants. Both NT3 and NT4 (S511A-T512A) mutants have the same Tc^{S} phenotype (Figure 3.1, Table 3.1), and similar Kd for Tc binding to the 70S (Table 3.4). The presence of other co-eluted proteins in the NT3 preparation (see section 3.2.1.1) did not appear to interfere with Tc release.

The apparent [^{3}H]-Tc Bmax in the presence of V510A-S511A, was similar to that observed in the absence or presence of Tet(O)^{WT}. In contrast, an increase in the apparent Bmax was observed in the presence of the S511A-T512A mutant relative to that observed in the absence or presence of Tet(O)^{WT} (Table 3.4). The reason for this is unclear as the resulting increased apparent Kd (Table 3.4) does not agree with the observed Tc^S phenotype (Figure 3.1, Table 3.1).

3.3.3 70S-bound Tc release is impaired by the Tet(O) deletion mutants

None of the Tet(O) deletion mutants could release Tc from the primary binding site (Figures 3.8 a-e) as indicated by the apparent Kd values that resemble the absence of Tet(O)^{WT} (except 511ΔS, Table 3.4). This is compatible with their Tc^S phenotype and implies that, in the bacteria, no restoration of protein synthesis would occur in the presence of these deletion mutants.

The apparent Bmax of [^{3}H]-Tc to the 70S in the presence of all the deletion mutants (except 511ΔS) was similar to that observed in the absence or presence Tet(O)^{WT} (Table 3.4). It is unclear why there is an increased apparent Bmax observed for 511ΔS because the increased apparent Kd of Tc for the 70S in the presence of 511ΔS (Table 3.4) does not agree with its observed Tc^S phenotype (Figure 3.1, Table 3.1).

3.3.4 70S-bound Tc release is impaired by the Tet(O) polar/non-polar mutants

The S508D mutant very weakly released [^{3}H]-Tc from its primary binding site on the 70S ribosome as compared to Tet(O)^{WT} (Figure 3.9a). Tc release was impaired in the presence of the P509V, V510D and T512S mutants (Figures 3.9b-d, Table 3.4) and resembled the absence of Tet(O)^{WT}. The decreased ability of these polar/non-polar mutants to release Tc is consistent with their Tc^S phenotype. This is agrees with the observed Tc^S phenotype (Figure 3.1, Table 3.1) and implies that, in the bacteria, no restoration of protein synthesis would occur in the presence of these polar/non-polar mutants.

The apparent Bmax of [^{3}H]-Tc to the 70S in the presence of P509V and V510D mutants were similar to the absence or presence of Tet(O)^{WT}. In contrast, the apparent Bmax of [^{3}H]-Tc to the 70S in the presence of the S508D and the T512S mutants was higher than in the presence or absence of the Tet(O)^{WT} (Table 3.4). It is unclear why there is an increased apparent Bmax observed for S508D and T512S mutants because the increased apparent Kd of Tc for the 70S in the presence of these mutants (Table 3.4) does not agree with their observed Tc^S phenotype (Figure 3.1, Table 3.1).

3.4 Tet(O)-mediated Tc release is catalytic and not stoichiometric

The pmoles of [^{3}H]-Tc bound to the 70S ribosomes was constant in the presence of increasing (0.25, 0.5,1, 1.5, 2.0 μM) concentrations of Tet(O) or in other words a fixed amount (20%) of 70S bound Tc was released per unit μM Tet(O) (Figure 3.10). The 80% of the 70S-bound Tc represents the secondary site binding which does not inhibit protein synthesis and cannot be released by Tet(O)^{WT}. Increasing concentrations of Tet(O)^{WT} does not result in an increase in the release of Tc from its primary binding site in the 70S suggesting Tet(O)-mediated Tc release is catalytic and not stoichiometric as reported by Trieber *et al.* [229].

3.5 GTP hydrolysis by Tet(O)^{WT} and Tet(O)^{mutants}

GTP hydrolysis assays were performed to indirectly assess the binding ability of the Tet(O)^{mutants} to 70S ribosomes. Tet(O)^{WT} protein has a 70S ribosome-dependant GTPase activity which is considered to be essential for Tc release. [γ-^{32}P]GTP hydrolysis (μM) per Tet(O) protein (μM) per unit time (sec^{-1}) was plotted versus increasing concentrations (10-500 μM) of free unhydrolyzed [γ-^{32}P]GTP (Figures 3.11a-b). The parameters Km, Kcat and Vmax were estimated from the graphs (See section 2.12 for details).

3.5.1 Kinetics of GTP hydrolysis by Tet(O)

Tet(O)^{WT} binding to the 70S ribosome was detected by the hydrolysis of [γ-^{32}P]GTP (Figures 3.11a-b, Table 3.5). All the Tet(O) deletion mutants were able to hydrolyze [γ-^{32}P]GTP suggesting they were able to bind to the 70S ribosomes. However, their affinity for GTP, GTP turnover number and their catalytic efficiency for GTP hydrolysis was reduced relative to the Tet(O)^{WT} (Figure 3.11b, Table 3.5). This implies they were not able to bind GTP, and hydrolyze it as efficiently as Tet(O)^{WT}, which in turn reduced their ability to release 70S bound Tc.

Table 3.1 Tc MIC for *E.coli* BL21(DE3) harbouring *tet(O)*^{WT} & *tet(O)*^{mutants}

Tet(O)	MIC of T_C(μg/mL)	Interpretation
fusA	4	Sensitive
WT	32	Resistant
Single Alanine mutants	32	
S508A	64	Resistant
P509A	32	Resistant
V510A	32	Resistant
S511A	32	Resistant
T512A	64	Resistant
Double Alanine Mutants		
S508A-P509A	16	Resistant
P509A-V510A	16	Resistant
V510A-S511A	8	Sensitive
S511A-T512A	8	Sensitive
Deletion mutants		
508ΔS	8	Resistant
509ΔP	8	Resistant
510ΔV	8	Resistant
511ΔS	8	Resistant
512ΔT	8	Resistant
Tet(O)-polar/non polar mutants		
Y507H	16	Resistant
S508D	4	Sensitive
P509V	8	Sensitive
V510D	4	Sensitive
T512S	4	Sensitive

Tc^R: MIC ≥16 μg/mL

Table 3.2 Yield of Tet(O)^{WT} and Tet(O)^{mutant} proteins

Tet(O)	Yield (mg/mL)
WT	4.0
508ΔS	3.4
509ΔP	2.0
510ΔV	2.2
511ΔV	1.2
512ΔT	3.5
V510A,S511A (NT3)	1.0
S511A,T512T (NT4)	1.6
S508D	0.5*
P509V	3.2
V510D	0.9
T512S	1.5

* accidental loss of the supernatant containing the protein prior to IMAC purification

Table 3.3 Identification of the protein bands associated with Tet(O)

Band number	Protein Identification	Protein Function
1	Chaperonin GroEL	- proper folding of misfolded proteins
	RNAse E (possible fragment)	- maturation of 9 S RNA to 5S rRNA
2	NAD Dehydrogenase	-oxidises a substrate by transferring one or more hydrides to an acceptor, using NAD+ as coenzyme
	Rho trans Terminator	-transcription terminator
	Gal ABC transporter	-ATP-binding cassette transporter is a transmembrane protein and a major class of cellular translocation machinery in all bacterial cells
3	Rho	transcription terminator
4	Respiratory NADH dehydrogenase cupric reductase	-reduces cupric ions to Cu(I) which mediates damage of the respiratory system by hyperoxides
	lac repressor	-represses lac operon
5	Cys B, monooxigenase + others	-member of Lys R family of proteins causing de-repression of Cysteine B regulon
6	P-loop kinase	-phosphorylates nucleotides, sugars, coenzyme precursors, adenosine 5'-phosphosulfate and polynucleotides
	Lys R	-activates gene expression of more than one loci negatively regulating their own expression
7	FKBP type PPIase	-inhibits calcineurin
	L1	**-interacts with EF-G to direct tRNA movement during translocation**
8	Catabolite Gene Activator Protein (Cap) DNA complex	positively controls activity of inducible operons like lac, gal and ara operon
9	**L15**	**-late large subunit assembly protein**
	ferric uptake regulator	-regulates iron homeostasis
	anti RNA polymerase sigma factor	-binds and inhibits its cognate sigma factor
10	UP12	-stress protein
	L9	**-ribosome assembly**
11	Lysozyme	-cleaves the bacterial cell wall
	UP12	-stress protein
12	**L28**	**-ribosome assembly and protein synthesis**
	L25	**-5SrRNA binding protein**
13	**L20**	**-ribosome assembly**
	UP12	-stress protein
	Ferric uptake regulator	-regulates iron homeostasis

Table 3.4 The binding of [^{3}H]-Tc to 70S ribosomes in the absence and presence of Tet(O)^{WT} and Tet(O)^{mutants}

Tet(O)	n = number of experiments	Kd (μM)	Bmax (pmoles)
-Tet(O) (70S)	17	6.9 ± 1.2	13.1 ± 0.7
WT	7	11.8 ± 2.8	12.5 ± 1.1
Deletion			
508ΔS	3	6.4 ± 2.7	10.6 ± 1.4
509ΔP	2	4.8 ± 2.3	11.0 ± 1.5
510ΔV	3	6.4 ± 2.3	13.9 ± 1.5
511ΔS	3	15.4 ± 3.6	19.4 ± 1.9
512ΔT	3	5.8 ± 1.8	11.8 ± 1.2
Double Alanine			
NT3	3	8.6 ± 1.9	14.4 ± 1.1
NT4	3	9.7 ± 3.8	16.6 ± 2.3
Polar/Non polar			
S508D	3	15.9 ± 5.1	18.3 ± 2.6
P509V	3	8.0 + 3.4	14.2 ± 1.9
V510D	2	6.5 ± 1.7	13.2 ± 1.0
T512S	2	13.0 ± 2.4	17.8 ± 1.3

The values represent mean ± SEM
Tet(O)^{WT} = Tc^R
Tet(O)^{mutant}= Tc^S

Table 3.5 Impaired GTP binding ability and catalytic efficiency of Tet(O) deletion mutants

Tet(O)	Km (μM)	k_{cat} (sec^{-1})	k_{cat}/Km (μM-1.sec^{-1})	k_{cat}/Km relative to WT
WT	80 ± 21	33 ± 3	0.41	
508ΔS	319 ± 264	35 ± 15	0.11	Reduced 4 fold
509ΔP	140 ± 69	27 ± 5	0.19	Reduced 2 fold
510ΔV	134 ± 34	19 ± 2	0.14	Reduced 3 fold
511ΔS	179 ± 35	10 ± 2	0.05	Reduced 8 fold
512ΔT	216 ± 74	18 ± 3	0.08	Reduced 5 fold

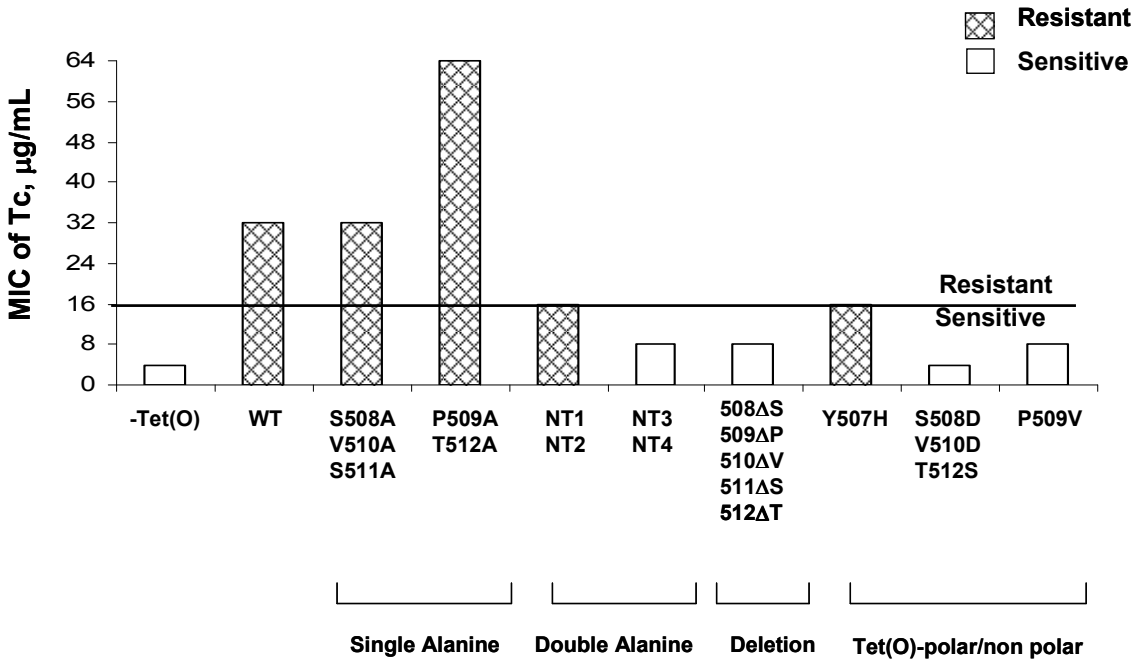

Figure 3.1 Tc MIC for *E.coli* BL21(DE3) harbouring *tet(O)* gene

Tc susceptibility is determined by growing *E.coli* BL21 strains (carrying *fusA, tet(O)*^{WT} or the *tet(O)*^{mutants}) in the presence of increasing Tc concentrations (0-256 µg/mL; in serial two fold dilutions) on sterile LB agar plates. The Minimal Inhibitory Concentration (MIC) is the lowest concentration of Tc that inhibits bacterial growth following incubation at 37°C for 48 h.
Tc^{R}: MIC $\geq$16 µg/mL.

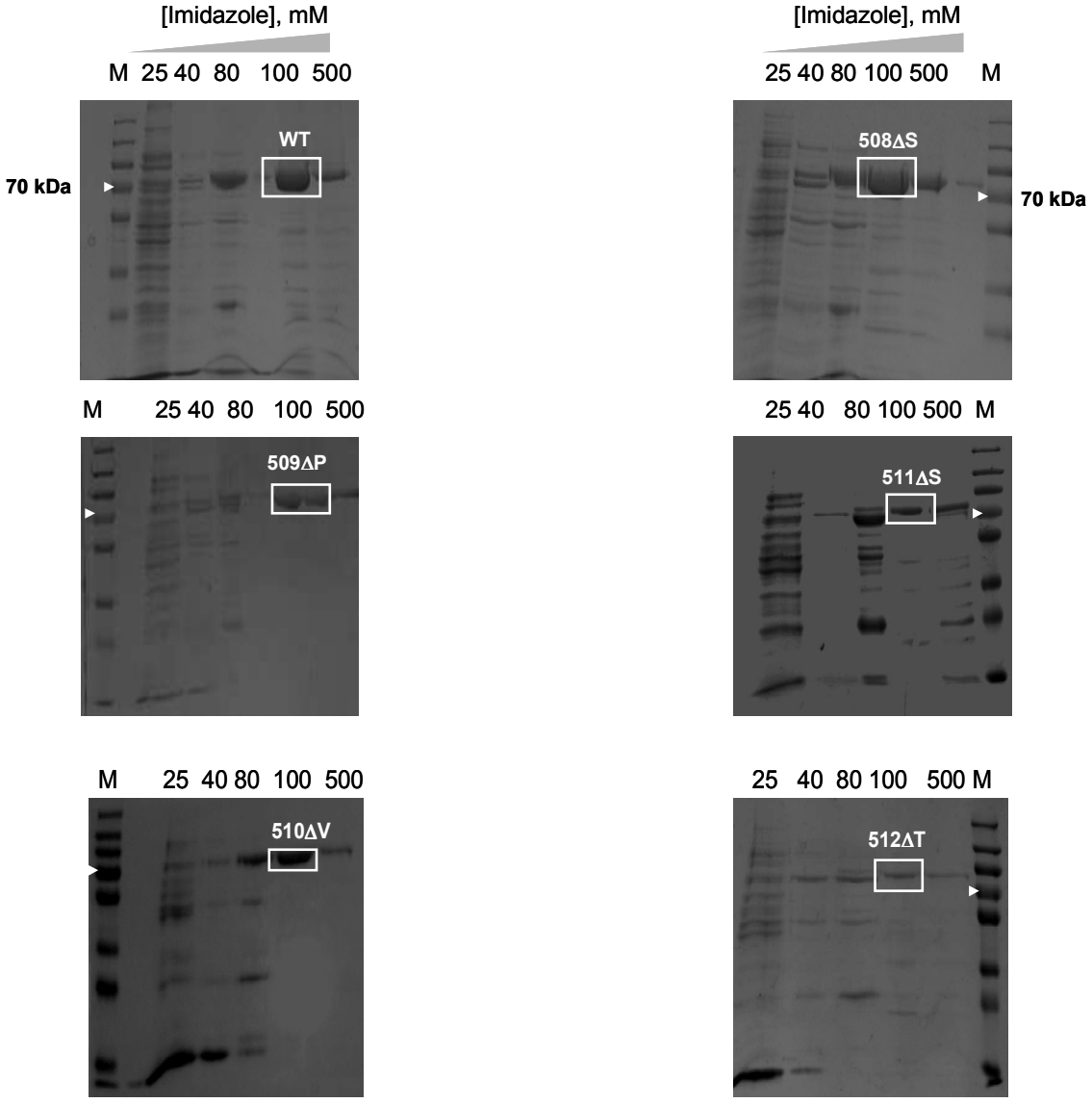

Figure 3.2a 10% SDS-PAGE of imidazole eluates of His_6-Tet(O)^{WT} and Tet(O)^{mutants} purified by immobilized metal affinity chromatography (IMAC) on a His trap HP Ni^{2+} column. 25-500 mM Imidazole was used in a batch gradient method. Tet(O)^{WT} and Tet(O) deletion mutants eluted at 100 mM imidazole. M represents the molecular weight marker (See section 2.7.3).

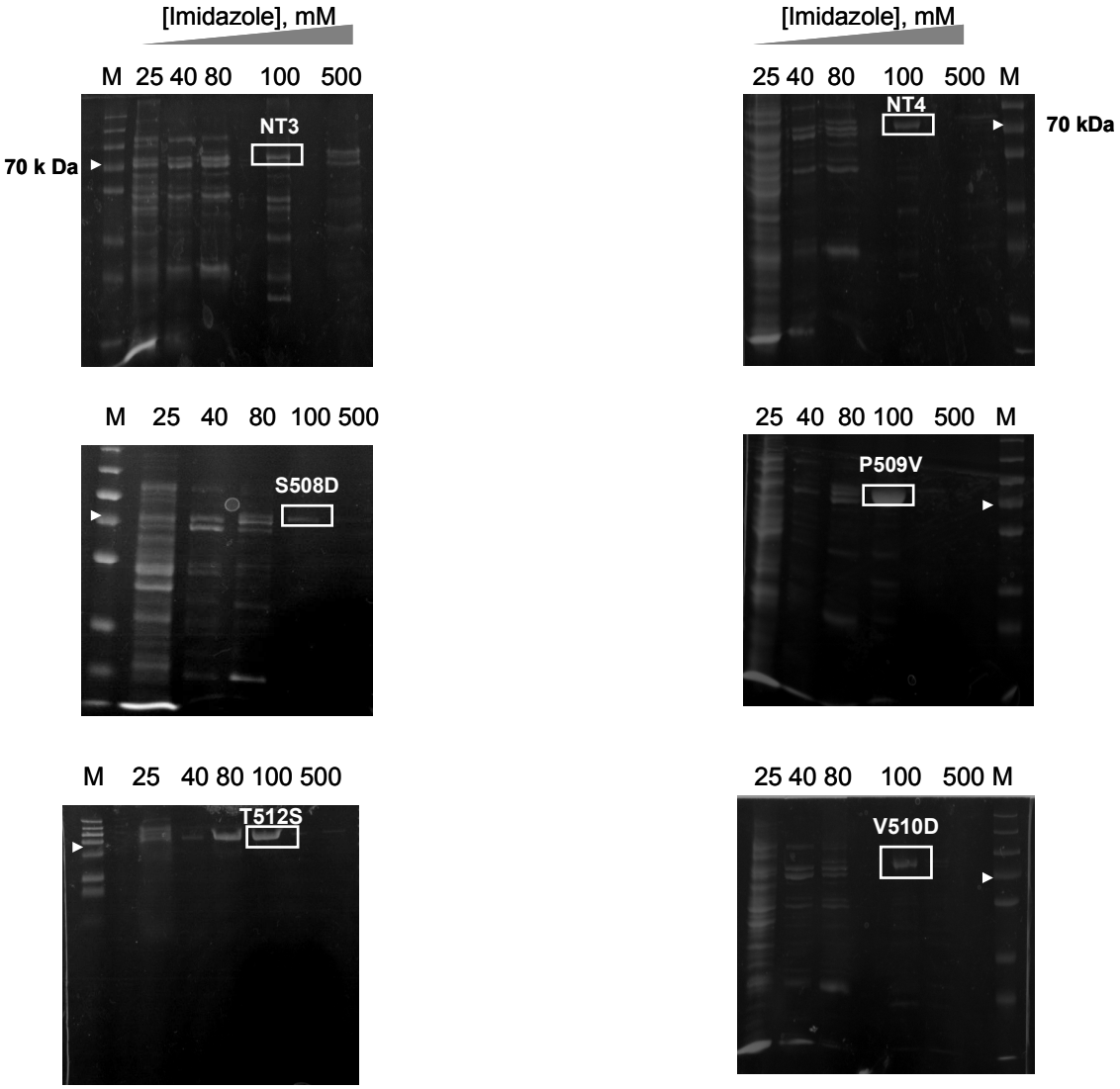

Figure 3.2b 10% SDS-PAGE of imidazole eluates of His_6-Tet(O)^{WT} and Tet(O)^{mutants} purified by immobilized metal affinity chromatography (IMAC) on a His trap HP Ni^{2+} column. 25-500 mM Imidazole was used in a batch gradient method. Tet(O)^{WT} and the Tet(O) double alanine and polar/non polar mutants eluted at 100 mM imidazole. M represents the molecular weight marker (See section 2.7.3).

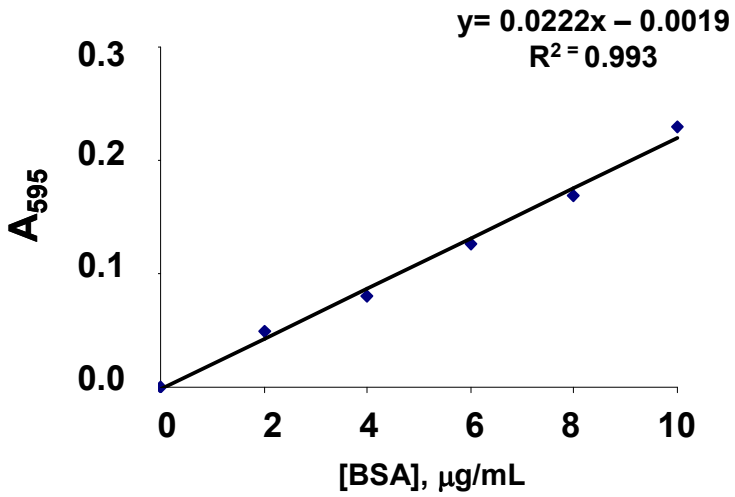

Figure 3.3 Standard Curve of Bradford Protein Assay

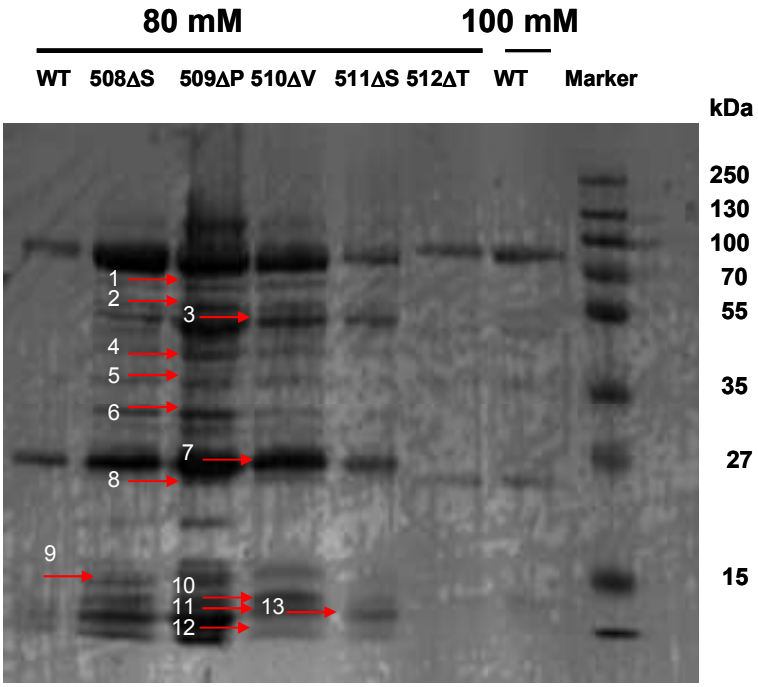

Figure 3.4 12.5% SDS-PAGE of the Ni^{2+}column 80mM Imidazole eluates for His_6-$Tet(O)^{WT}$ and Tet(O) deletion mutants. 100 mM purified $Tet(O)^{WT}$ was loaded as a control. The arrows indicate the bands that were cut out and analyzed by LC-MS (see section 2.9 for details).

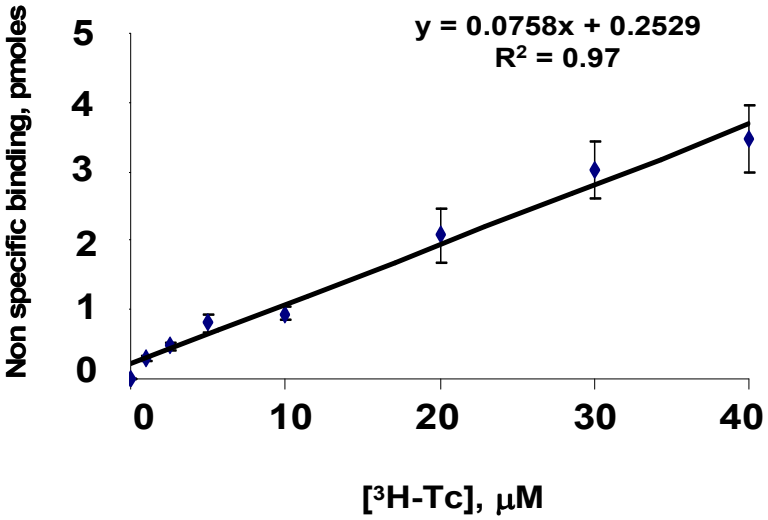

Figure 3.5 Non specific binding of [^{3}H]-Tc (1-40) μM to 0.45 μm nitrocellulose filters

n= 14 experiments, each experiment was done in triplicate.
The values represent mean ± SEM.

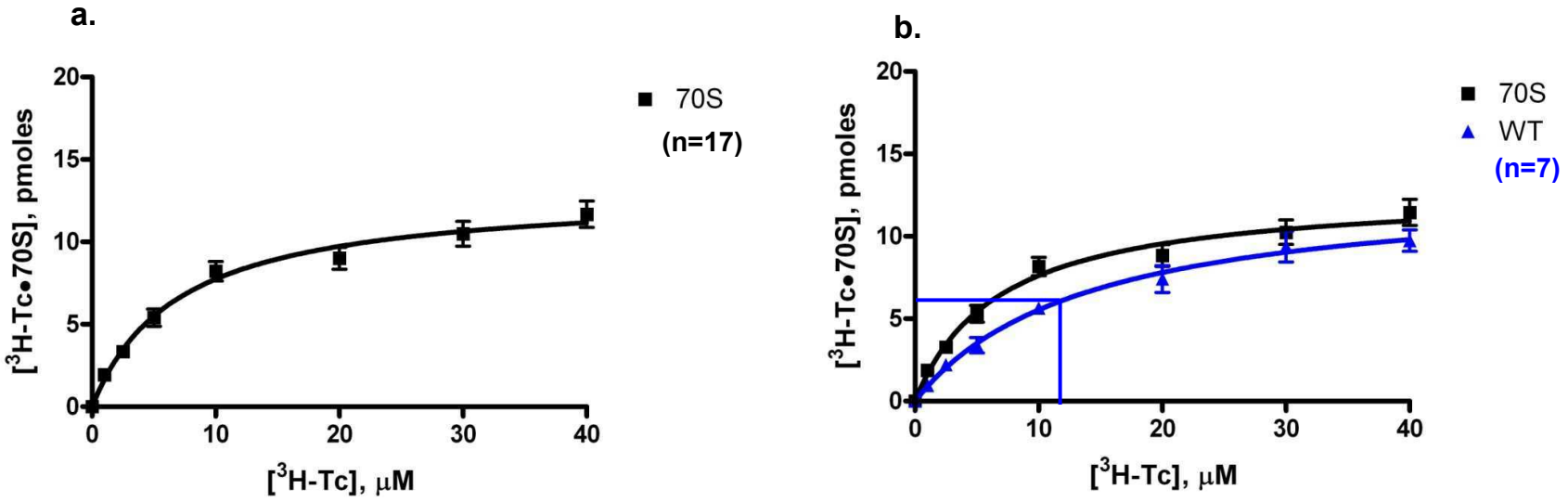

Figure 3.6 Binding of [^{3}H]-Tc to the 70S ribosomes in the absence and presence of Tet(O)^{WT}

n represents the number of experiments. Each experiment was done in triplicate. The values represent mean ± SEM. The reactions were performed in the presence of increasing concentrations (1-40) μM of [^{3}H]-Tc and 0.5 μM 70S ribosomes, (see section 2.11 for details); (a) in the absence of Tet(O)^{WT} (shown as a black solid line) or; (b) in the presence of 50 μM GTP and 2 μM Tet(O)^{WT} (shown as a solid blue line).The pmoles of [^{3}H]-Tc bound to the 70S ribosomes in the presence of the Tet(O)^{WT} is less than the absence of Tet(O)^{WT}. The boxed area represents the release of [^{3}H]-Tc from its primary binding site by Tet(O)^{WT}, which restores protein synthesis.

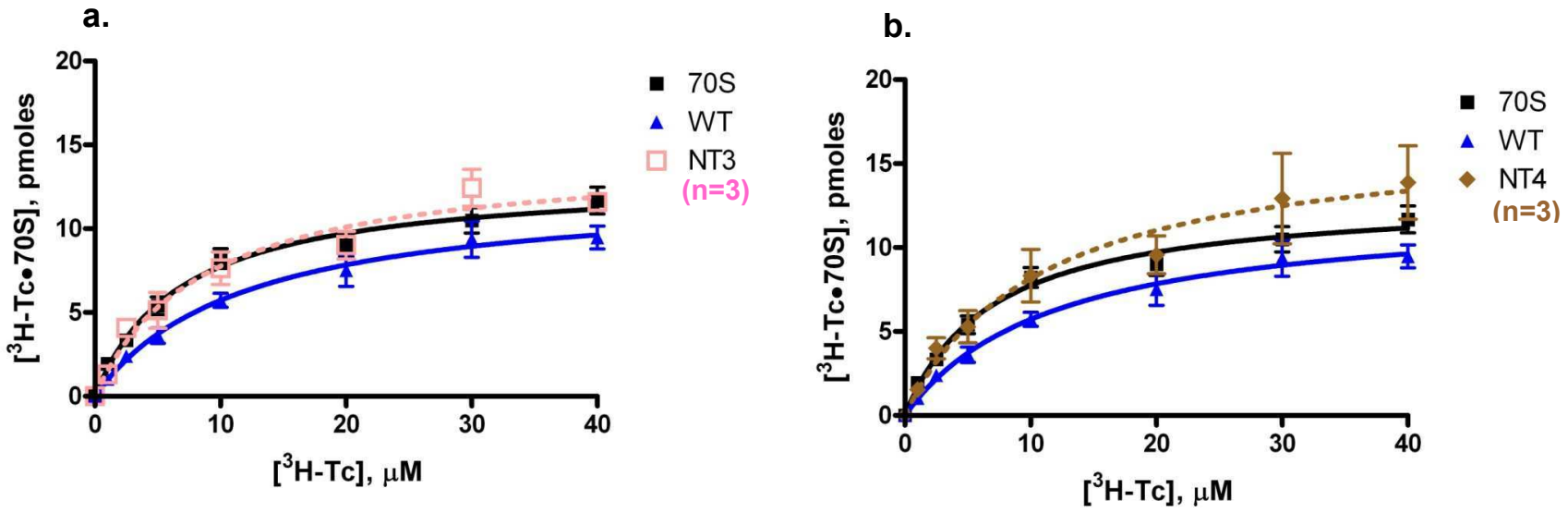

Figure 3.7 Impaired Binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the NT3 (V510A, S511A) and NT4 (S511A, T512A) double alanine mutants

n represents the number of experiments. Each experiment was done in triplicate. The values represent mean ± SEM. The reactions were performed in the presence of increasing concentrations (1-40) μM of [^{3}H]-Tc and in the presence of 2 μM of (a) NT3 shown as a pink dotted line and; (b) NT4 shown as a brown dotted line. The binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the NT3 and NT4 mutants were compared to the binding obtained in the absence of Tet(O)^{WT} (shown as a solid black line) and the presence of the Tet(O)^{WT} (shown as a solid blue line).

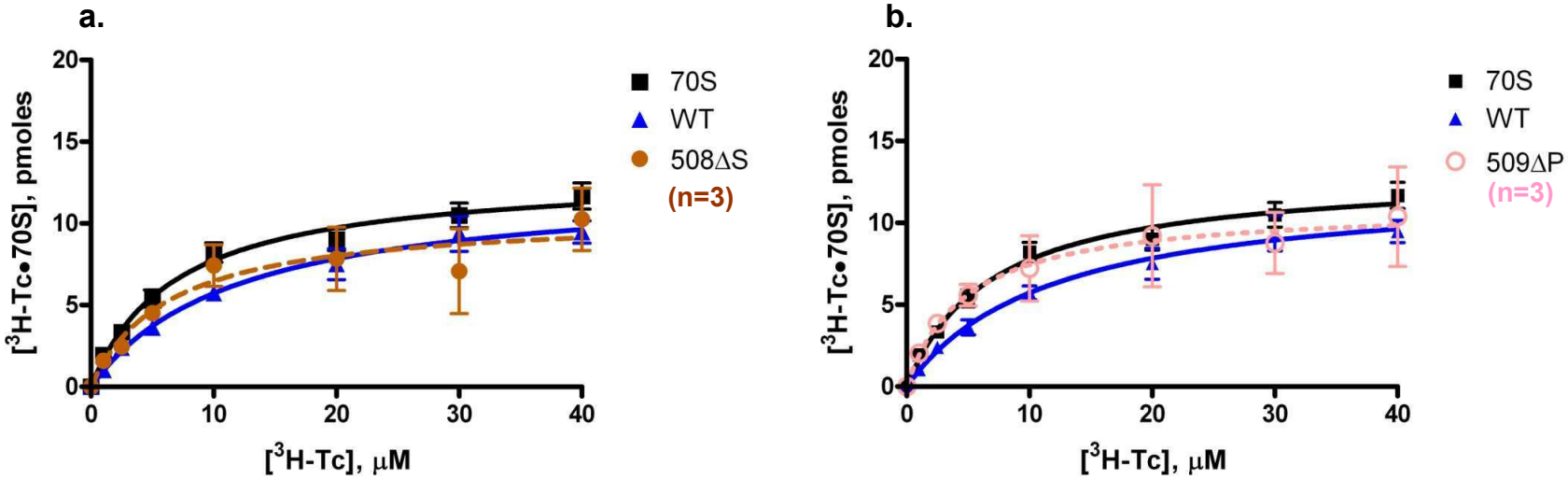

Figure 3.8 Impaired Binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the 509ΔP Tet(O)^{mutant}

n represents the number of experiments. Each experiment was done in triplicate. The values represent mean ± SEM. The reactions were performed in the presence of increasing concentrations (1-40) μM of [^{3}H]-Tc and in the presence of 2 μM of each of the Tet(O)^{mutants} (a) 508ΔS shown as a brown dotted line and; (b) 509ΔP shown as a pink dotted line. In each case binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the mutants was compared to the binding obtained in the absence of Tet(O)^{WT} (shown as a solid black line) and the presence of the Tet(O)^{WT} (shown as a solid blue line). The pmoles of [^{3}H]-Tc bound to the 70S is more than the Tet(O)^{WT} in the presence of 509ΔP. 508ΔS releases much less Tc relative to Tet(O)^{WT}.

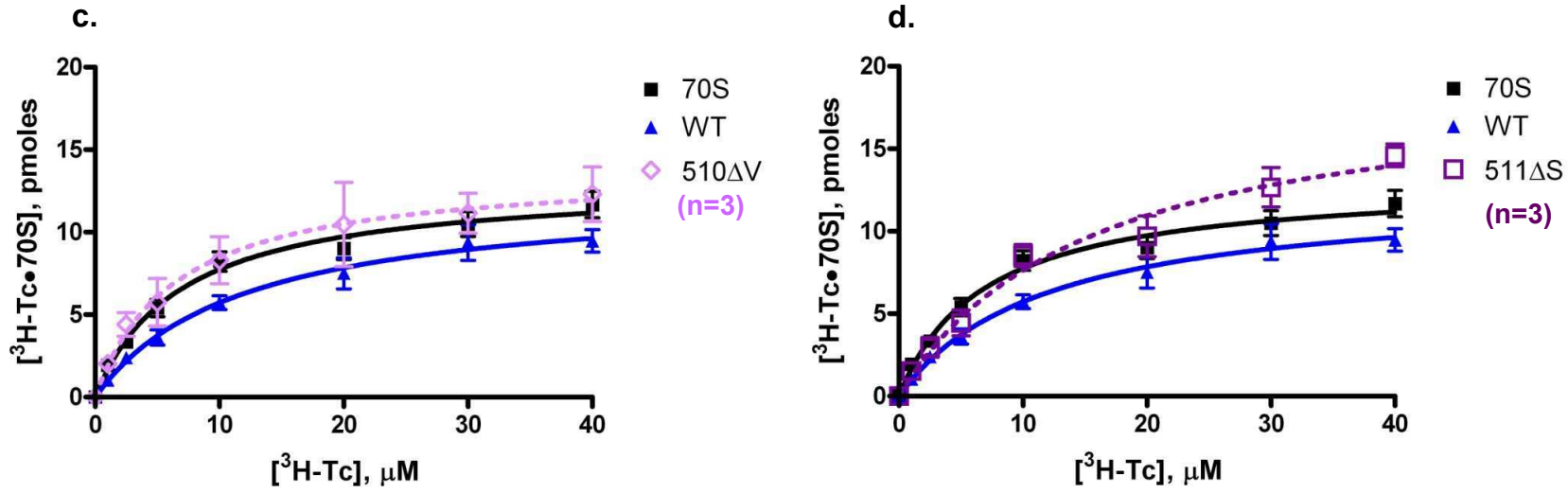

Figure 3.8 Impaired Binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the 510ΔV and 511ΔS Tet(O)^{mutants}

n represents the number of experiments. Each experiment was done in triplicate. The values represent mean ± SEM. The reactions were performed in the presence of increasing concentrations (1-40) μM of [^{3}H]-Tc and in the presence of 2 μM of each of the Tet(O)^{mutants} (c) 510ΔV shown as a pink dotted line and; (d) 511ΔS shown as a purple dotted line. In each case binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the mutants was compared to the binding obtained in the absence of Tet(O)^{WT} (shown as a solid black line) and the presence of the Tet(O)^{WT} (shown as a solid blue line). The pmoles of [^{3}H]-Tc bound to the 70S in the presence of each of 510ΔV and 511ΔS mutants is more compared to the Tet(O)^{WT}.

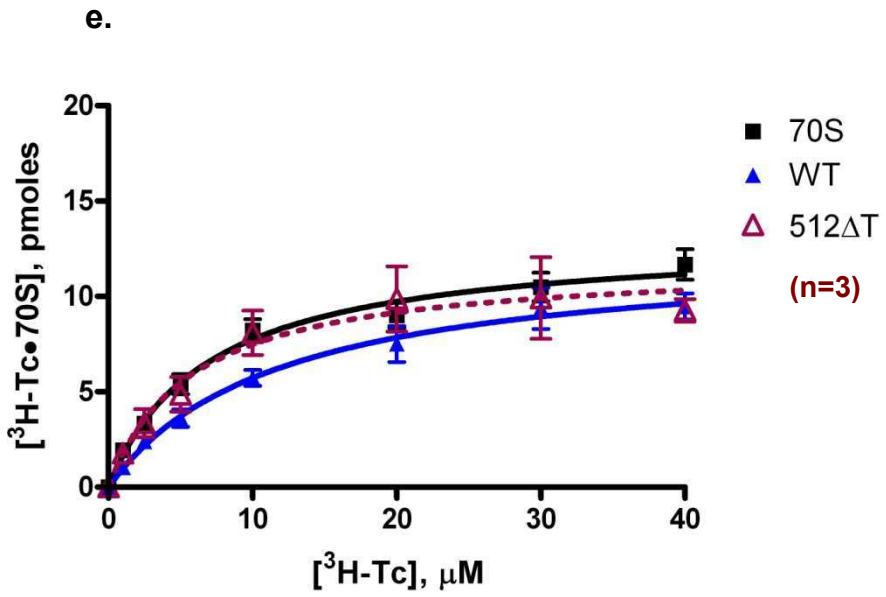

Figure 3.8 Impaired Binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the 512ΔT Tet(O)^{mutant}

n represents the number of experiments. Each experiment was done in triplicate. The values represent mean ± SEM. The reactions were performed in the presence of increasing concentrations (1-40) μM of [^{3}H]-Tc and in the presence of 2 μM of (e) 512ΔT shown as a maroon dotted line. The binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the 512ΔT mutant was compared to the binding obtained in the absence of Tet(O)^{WT} (shown as a solid black line) and the presence of the Tet(O)^{WT} (shown as a solid blue line). The pmoles of [^{3}H]-Tc bound to the 70S in the presence of 512ΔT mutant is more compared to the Tet(O)^{WT}.

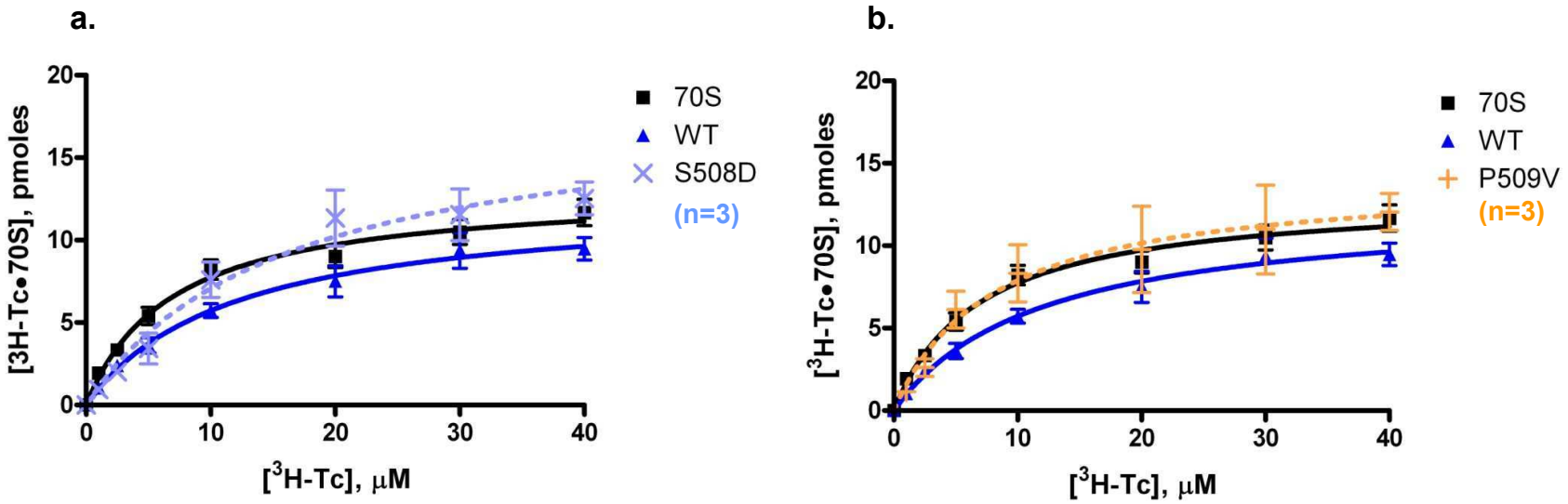

Figure 3.9 Impaired Binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the non polar P509V mutants

n represents the number of experiments. Each experiment was done in triplicate. The values represent mean ± SEM. The reactions were performed in the presence of increasing concentrations (1-40) μM of [^{3}H]-Tc and in the presence of 2 μM of (a) S508D shown as a light blue dotted line and; (b) P509V shown as an orange dotted line. The binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the S508D and P509V mutants were compared to the binding obtained in the absence of Tet(O)^{WT} (shown as a solid black line) and the presence of the Tet(O)^{WT} (shown as a solid blue line). The pmoles of [^{3}H]-Tc bound to the 70S in the presence of both the polar mutant S508D and the non polar mutant P509V is more compared to the Tet(O)^{WT}.

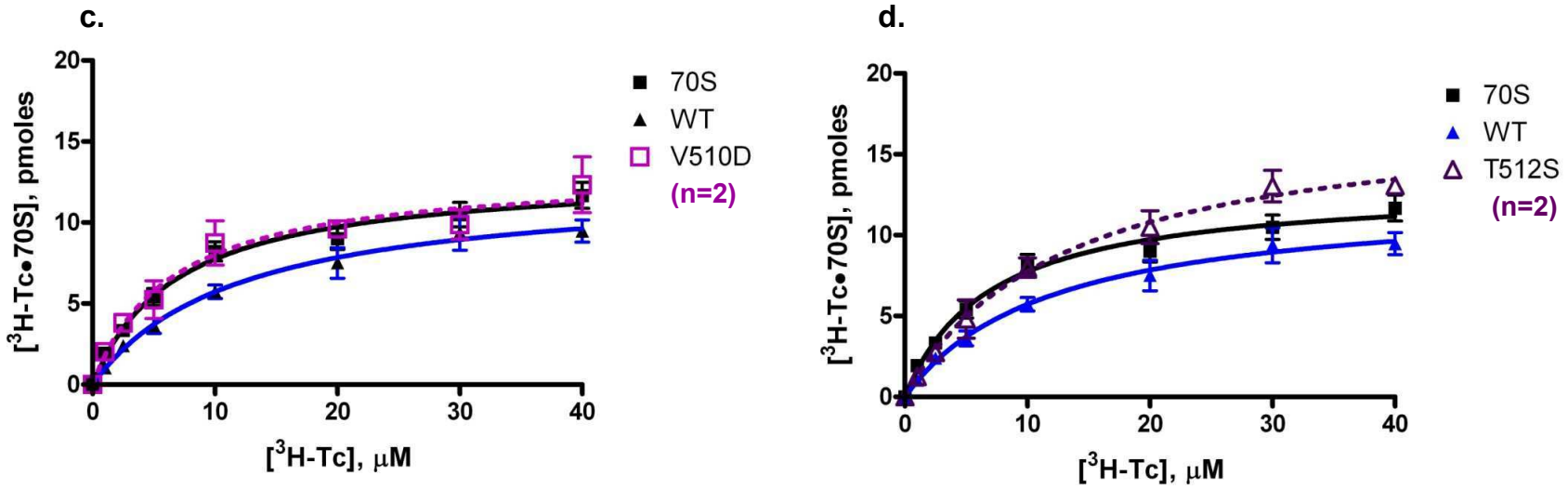

Figure 3.9 Impaired Binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the polar V510D and T512S mutants

n represents the number of experiments. Each experiment was done in triplicate. The values represent mean ± SEM. The reactions were performed in the presence of increasing concentrations (1-40) μM of [^{3}H]-Tc and in the presence of 2 μM of (c) V510D shown as a bright pink dotted line and; (d) T512S shown as a purple dotted line. The binding of [^{3}H]-Tc to the 70S ribosomes in the presence of the V510D and T512S mutants were compared to the binding obtained in the absence of Tet(O)^{WT} (shown as a solid black line) and the presence of the Tet(O)^{WT} (shown as a solid blue line). The pmoles of [^{3}H]-Tc bound to the 70S in the presence of both the polar mutant V510D and T512S is more compared to the Tet(O)^{WT}.

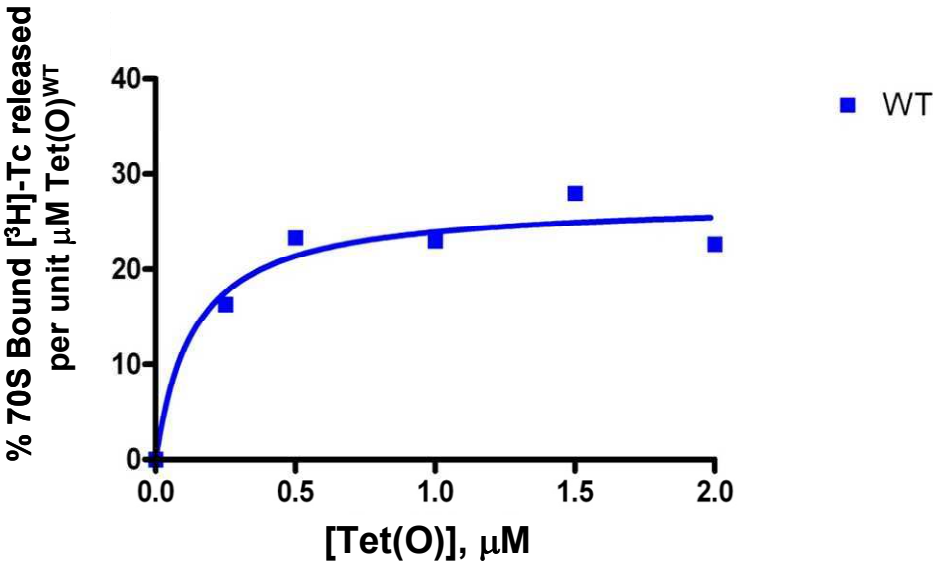

Figure 3.10 The Tet(O)^{WT} releases 70S bound [^{3}H]-Tc from its primary binding site catalytically and not stoichiometrically

n= 1 experiment, each concentration of Tet(O) (0.25, 0.50, 1.0, 1.5 and 2.0 μM) was tested in triplicates (see section 2. 11.2 for details). The reaction was performed in the presence of 0.5 μM 70S, 50 μM GTP and 5 μM [^{3}H]-Tc.

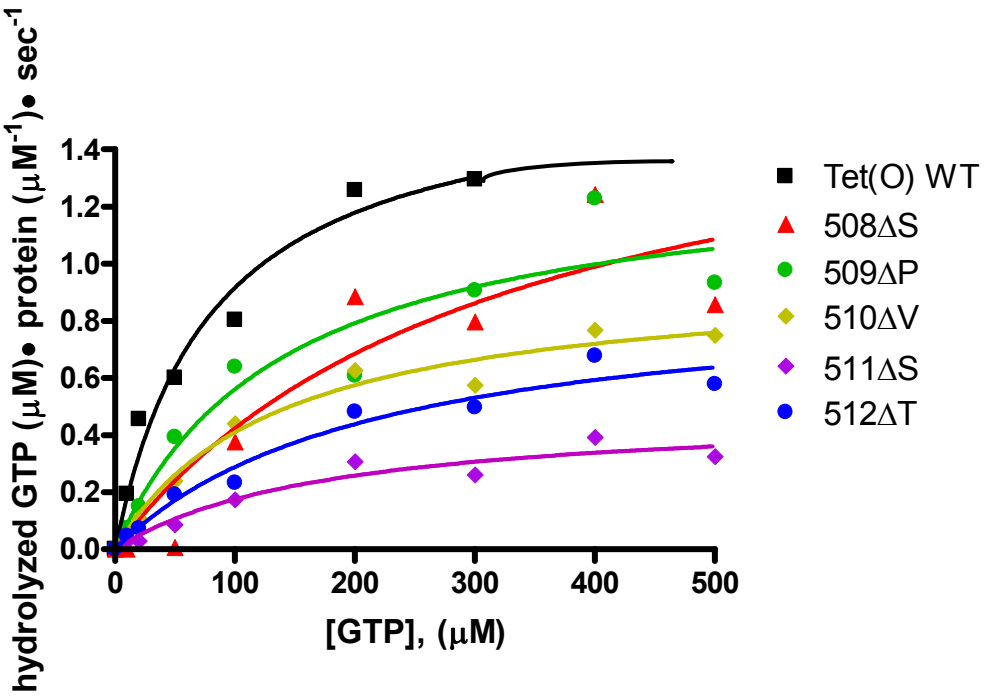

Figure 3.11 GTP hydrolysis by Tet(O)^{WT} and Tet(O) deletion mutants

n =1 experiment, values represent mean only.
The reactions were performed in the presence of increasing concentrations (10-500 μM) of [γ-^{32}P]-GTP, 0.2 μM 70S and in the presence of 0.05 μM of Tet(O)^{WT} and each of the deletion mutants. GTP hydrolysis assay performed with 508ΔS, 509ΔP and 510ΔV, 511ΔS and 512ΔT mutants. [γ-^{32}P]-GTP hydrolysis is compared with the Tet(O)^{WT}(shown as a solid black line). Mutants are shown in color.(see section 2.12 for details).

Chapter 4 Discussion

This thesis tested the hypothesis that the amino acid residues YSPVST at the tip of the domain IV of Tet(O) are critical for Tet(O)-mediated Tc release from the primary binding site in the 70S ribosomes. This hypothesis was tested by creating a series of Tet(O) deletion and substitution mutants of YSPVST and then testing them for their susceptibility to Tc and their ability to release 70S-bound Tc from the primary binding site. Understanding the precise role of the individual domains of Tet(O) in promoting Tc release is necessary to decipher the mechanism of Tet(O)-mediated Tc^R. Comprehension of the molecular mechanism of Tet(O)-mediated Tc^R may provide insight for the development of novel therapeutics and thereby offer new treatment for campylobacteriosis and other infections.

The *C. jejuni* Tet(O), one of the major and best characterized RPPs, shares ~51% amino acid sequence similarity with the elongation factor protein, EF-G [184] of *Thermus thermophilus*. Each of the EF-G and RPP families of proteins are found to carry a conserved set of amino acid residues at the distal tip of domain IV. The conserved amino acids, H(E/D)VDSS, that occupy position 583-588 at the distal tip of domain IV in EF-G are critical for translocation [233-234].

A previous study [217] assessed the functional importance of the conserved sequences at the distal tip of domain IV in EF-G (see sbove) versus Tet(O) (YSPVST, occupying positions 507-512). This study demonstrated YSPVST are essential but are alone not sufficient for Tc release. Chimeric proteins of *C. jejuni* Tet(O) and *E.coli* EF-G were generated by swapping the conserved loop sequences at the tip of the domain IV. The Tet(O)E chimera, containing the EF-G domain IV loop sequence (H(E/D)VDSS), was impaired in its ability to release 70S-bound Tc relative to $Tet(O)^{WT}$. This indicated that the residues YSPVST are required for Tc release. However the reverse chimera, EF-GT, carrying the domain IV loop sequence (YSPVST) of Tet(O), failed to release Tc, thereby implying YSPVST alone is not adequate for Tc release.

Domains III, IV and V were shown to be important for the translocation function of EF-G [234, 259-260]. Accordingly, domains III-V in Tet(O) were proposed to be important for the Tc release function of Tet(O). Two additional Tet(O)-EF-G chimeric proteins were studied. One chimeric protein carried domains III, IV and V of EF-G and domains I and II of Tet(O). The other Tet(O)-EF-G chimera carried domains IV and V of EF-G and domains I, II and III of Tet(O). Both the chimeric proteins had a decreased ability to release Tc relative to $Tet(O)^{WT}$, suggesting that domains III-V of Tet(O) did not have a direct role in Tc release.

This thesis is the first report that assessed which of the conserved amino acid residues (YSPVST) at the tip of the domain IV of Tet(O) are vital for Tet(O)-mediated Tc release. A series of single amino acid deletion and substitution mutants of Tet(O) created by site directed mutagenesis of YSPVST residues of $Tet(O)^{WT}$ were tested for their ability to release 70S-bound Tc (Figure 4.1a-d).

4.1 Screening of the Tc^S phenotype of the $Tet(O)^{mutants}$

An ideal way to determine whether the point mutations introduced in the domain IV loop of Tet(O) had an impact on its function was to determine the susceptibility of the $Tet(O)^{mutants}$ to Tc.

4.1.1 The *tet(O)* single alanine mutants were Tc^R

Alanine is an uncharged amino acid with a small methyl (-CH3) group and no other side chains past the β-atoms (Figure 4.2). Thus alanine can be easily incorporated into a polypeptide chain without altering the overall conformation of the polypeptide [261], and at the same time allows the identification of functional residues of the protein [262], in this case the identification of functional residues that are important for the interaction between Tet(O) and 70S ribosomes. All of the Tet(O) single alanine mutants were Tc^R like the $Tet(O)^{WT}$ (Figure 3.1, Table 3.1). This implies that substitution of the YSPVST residues sequentially with alanine, did not significantly impair the interactions of the $Tet(O)^{mutants}$ with the 70S ribosomes.

4.1.2 Double alanine *tet(O)* substitutions at positions 510-511 and 511-512 were Tc^S

Double alanine scanning was performed at positions 508-512, to determine whether double alanine substitution is a more sensitive indicator of subtle changes in the conformation of domain IV and therefore the interaction of Tet(O) with the 70S ribosomes. Two of the double alanine mutants, at positions 508-510 were Tc^R (Figure 3.1, Table 3.1). This suggests that positions 508-510 were not essential for Tc release.

However, the remaining two double alanine mutants at positions 510-512 were Tc^S (Figure 3.1, Table 3.1). This indicates positions 510-512 may play a role in Tc release, with 511 and 512 having a more critical role relative to 510.

4.1.3 The *tet(O)* deletion mutants were Tc^S

The YSPVST residues were individually deleted to produce a more drastic alteration in the conformation of domain IV of Tet(O) as compared with the alanine substitution mutants. All the Tet(O) deletion mutants were susceptible to Tc (Figure 3.1, Table 3.1). This indicates deletion of each of the amino acids at position 508-512, distorts the loop structure at the tip of the domain IV of Tet(O) which in turn impaired the ability of the deletion mutants to release Tc.

4.1.4 The *tet(O)* polar/non-polar mutants were Tc^S

The substitution of the YSPVST amino acids sequentially with the equivalent conserved amino acids in the distal tip of domain IV of the translocation factor EF-G, was the best way to determine whether any of the residues YSPVST in Tet(O) were exclusive for the Tc release and ribosome protection activity of Tet(O). All the substitution mutants (except Y507H) were sensitive to Tc (Figure 3.1, Table3.1), suggesting position 507 may not play a key role in Tc release. Thus alanine substitutions or deletion mutants at position 507 were not generated. The Tc^S phenotype of the remaining polar/non polar mutations at positions 508, 509, 510 and 512 suggest that they do play a role in Tc release.

4.2 Factors affecting the expression of Tet(O)

Two major factors have improved the expression of Tet(O) protein: the use of a superior expression system and the use of a low induction temperature to improve protein folding and solubility.

4.2.1 pET200 expression system

In order to functionally characterize the ability of the Tc^S $Tet(O)^{mutants}$ to release Tc by the *in vitro* Tc binding assay, it was important to purify the Tet(O) proteins in sufficient and pure amounts. This was achieved by using *E. coli* BL21(DE3) strains with bacteriophage T7-promoter based pET200 expression system and a suitable His_6 tag to simplify the purification of the His_6-Tet(O) fusion protein.

Thakor *et al*. [248] first successfully achieved an improved procedure for the high level overexpression, purification and isolation of $Tet(O)^{WT}$. Prior attempts to purify Tet(O) involved the use of *E. coli* strain MRE 600 that was transformed with prokaryotic expression vector pMS119EH carrying the under the IPTG inducible tac promoter [229], which were unable to express sufficient amounts of proteins. The expression of $Tet(O)^{WT}$ using the *E. coli* BL21(DE3) strains transformed with the bacteriophage T7-promoter based pET200 expression vector by Thakor *et al* 248] greatly improved the $Tet(O)^{WT}$ yield. The same expression system and *E. coli* strain were used for the successful purification of $Tet(O)^{WT}$ and $Tet(O)^{mutants}$ in this study.

4.2.2 Induction temperature

One of the drawbacks of using the inducible pET-T7 expression systems is that the accumulation of high amounts of heterologous mRNA may cause ribosome destruction and cell death [243]. The use of a strong T7 promoter and high IPTG concentration may lead to the accumulation of insoluble protein aggregates (inclusion bodies) in *E. coli*. Overexpression of the heterologous protein (upto 40-50% of the total cellular protein) can be overwhelming for the protein folding pathway of a cell, which in turn may increase the probability of

improper protein folding and the aggregation of biologically inactive proteins in inclusion bodies [263]. *In vitro* refolding of improperly folded proteins in inclusion bodies may be attempted but does not guarantee biologically active protein products [243].

An easy way to overcome protein misfolding is the use of low induction temperature (15-30°C) to improve protein solubility. Induction of recombinant green fluorescent protein (GFP) cloned and expressed using pET21-b vector in *E.coli* BL21(DE3) strains at 37°C produced 58.8% of the recombinant protein in the insoluble cell fraction [264]. A decrease in the induction temperature to 30°C and 15°C enhanced the solubility by 4.4% and 40.9% respectively [264]. Factors contributing to the greater percentage of soluble protein products below 37°C in *E. coli* include the use of suboptimal growth temperatures which allows the nascent proteins to have more time to fold correctly. Transferring cultures to lower temperatures induces the expression of chaperones and cold shock proteins, which not only maintain the proteins in soluble form but also assist in their folding, by binding to the hydrophobic regions of the proteins [263].

In the present study the His_6-$Tet(O)^{WT/mutants}$ were induced at suboptimal growth temperatures ($Tet(O)^{WT}$ at 30°C and $Tet(O)^{mutants}$ at 18°C). The low induction temperature improved the solubility of overexpressed $Tet(O)^{WT/mutant}$ proteins and resulted in a reasonable protein yield to permit further functional characterization.

4.3 Correlation between Tc susceptibility and Tc release

4.3.1 $Tet(O)^{WT}$

The ability of $Tet(O)^{WT}$ to release Tc supports what has been reported by others [248, 265] and validates this assay in my hands so that I could proceed to compare $Tet(O)^{mutants}$ to $Tet(O)^{WT}$. The nature of Tc release by $Tet(O)^{WT}$ is more readily understood when the data is presented as percentage of 70S bound Tc released by the $Tet(O)^{WT}$ versus increasing concentrations of Tc (Figure 4.3, Table 4.1, see section 2.11.1 for details). This graph illustrates that 50% of 70S-

bound Tc is released by Tet(O)^{WT} at 1 μM Tc which represents release from the primary binding site. At higher concentrations, more Tc binds to the secondary sites on the 70S ribosome. At Tc concentrations above 10 μM, Tet(O)^{WT} could only release 15-20% of the 70S-bound Tc because Tet(O)^{WT} is unable to release Tc from the secondary sites on the 70S ribosomes.

4.3.2 Tet(O)^{mutants}

The binding of Tc to 70S in the presence of several Tc^{S} Tet(O)^{mutants} mirrors Tc binding in the absence of any Tet(O) protein indicating they are unable to release Tc from its primary binding site. This includes the double alanine mutants, V510A-S511A (NT3) and 511A-T512A (NT4), the deletion mutants, 509ΔP, 510ΔV and 512ΔS, and the polar/non polar mutants, P509V, V510D and T512S. This suggests positions 509, 510 and 512, have a role in Tc release from 70S.

Three of the Tc^{S} mutants have an intermediate binding affinity between the absence or presence of Tet(O)^{WT}. These mutants: 508ΔS, 511ΔS and S508D all involve the loss of a serine residue suggesting the serine residue may play a role but is not essential for Tc release.

Unexpectedly the Tc Bmax in the presence of S511A-T512A, T512S, 511ΔS and S508D Tet(O)^{mutants} was greater than in the absence of any Tet(O) protein. This increased apparent Bmax may result from an increased binding of Tc to secondary sites on the 70S ribosomes, because there is only one primary Tc binding site per 70S. The increased apparent Bmax resulted in an increased apparent Kd. The Kd therefore did not just reflect alterations of Tc binding only to the primary site, but also the secondary binding sites on the 70S. Binding of these mutants to the 70S may alter the conformation of the 70S in a way that allows more Tc to bind 70S at random non-specific secondary sites. These mutants may actually behave like the EF-G whose ratchet like motion of domains IV and V relative to domains I, II and III induces a conformational change in the 70S. However, closer examination of the Tc binding curves revealed that these

Tet(O)^{mutants} bound more Tc at the primary binding site (1-5 μM Tc) as compared with Tet(O)^{WT} which does support their Tc^{S} phenotype.

4.3.3 Discrepancies in estimating Kd of Tc for 70S in the presence of Tet(O)

Although the purified Tet(O)^{WT} protein in the current study was able to release Tc, the absolute value of Kd of Tc for 70S in the presence of Tet(O)^{WT} reported in this thesis (Kd = 11.77 $\pm$ 2.35 μM) was lower than that reported by other researchers. This may arise due to a number of different reasons [Li, Thesis 2010].

4.3.3.1 Variations in the quality of the Tet(O) protein

The Tet(O)^{WT} protein used in the study by Trieber *et al.* [229] was cloned in pMS119EH plasmid and expressed in *E. coli* MRE600. Different plasmid-expression host systems, and different purification procedures used may influence the activity of the isolated Tet(O)^{WT} protein. This in turn can give rise to inconsistencies in the reported binding affinity values of Tc to the 70S in the presence of Tet(O)^{WT}. Studies by Thakor *et al.* [217, 248], involved the use of the pET200/D-TOPO vector for cloning and *E.coli* BL21(DE3) strains for expressing the His$_6$-Tet(O)^{WT} protein. A lower binding affinity (Kd= 20.4 μM) of Tc to the 70S in the presence of Tet(O)^{WT} was reported by Thakor *et al.* [248] as compared to Trieber *et al.* [229] (Kd =30 μM).

4.3.3.2 Correction for non-specific binding of Tc to the nitrocellulose filters

In the current study the plasmid and expression system used to purify Tet(O)^{WT} was identical to that reported by Thakor *et al*. [217, 248], but the Kd was found to be even lower (Kd= 11.77 $\pm$ 2.35 μM) as compared with that reported by Thakor *et al* [248]. This discrepancy arises from the fact that Thakor *et al.* [248] did not correct for non-specific binding of [^{3}H]-Tc to the nitrocellulose filters (personal communication from Thakor to Li.). There is a linear increase in non-specific [^{3}H]-Tc binding to the filter (Figure 3.5) with increasing [^{3}H]-Tc concentrations. Background levels of non-specific binding

represents about 15% of counts between 0-10 μM [^{3}H]-Tc concentrations and about 30% of counts between 20-40 μM [^{3}H]-Tc concentrations. No correction for background radioactivity can result in an overestimation of Kd values for 70S-bound Tc in the presence of Tet(O)^{WT}.

4.3.3 Multiple measurements of Kd

Another reason for the difference in the reported Tc Kd values in the study by Thakor *et al.* [248] likely arises from the difference in the number of times the experiments were performed. Thakor *et al*. [248] performed the study once in triplicate, while in this thesis, the mean Kd of Tc for the 70S in the presence of Tet(O)^{WT} represents an average of seven experiments, where each experiment was done in triplicate. Accordingly studies in this thesis were able to report a more reproducible estimate of Kd under the conditions tested.

4.3.4 Variability in 70S ribosome preparations

Variations in the preparations of 70S ribosomes may contribute to differences in maximal Tc binding (55%-85%) due to the presence of different ribosome states [236-237]. Although the concentration of Mg^{2+} was adjusted in the final stage of 70S ribosome isolation to maintain the 70S complexes, there is no independent method to confirm their correct conformation. More than one batch of ribosomes (Figure 4.4) was required to complete the experiments reported in this thesis. However, the Kd values of Tc binding to the 70S in the absence of Tet(O)^{WT} were reproducible for all the ribosome batches (6.89 $\pm$ 1.15 μM), and agree with the previously reported values of 3.4 μM [248] and 5 μM **[**229].

4.4 Do Tet(O)^{mutants} bind 70S ribosomes?

The Tc^S Tet(O)^{mutants} were impaired in their ability to release 70S-bound Tc as compared to Tet(O)^{WT} but it is not clear if this is due to an impaired ability of the Tet(O$^{)mutants}$ to interact with the 70S. It is unknown whether the Tet(O)^{mutants} were :

(i) unable to bind to 70S, or

(ii) bound 70S with lower affinity relative to Tet(O)^{WT}, or

(iii) bound and turned over less frequently than Tet(O)^{WT}, or

(iv) there was no turnover of the Tet(O)^{mutant} (i.e. once bound to 70S, the Tet(O)^{mutant} could not dissociate from the 70S).

Domain I of Tet(O) has a 70S-dependent GTPase activity which is remote from domain IV of Tet(O). The activity of this domain I GTPase is dependent on the association of Tet(O) with the 70S ribosome. GTP hydrolysis is required for Tet(O) to dissociate from the ribosome and hence GTPase activity may serve as an indirect measure of Tet(O) binding to 70S. Accordingly, the determination of the kinetics of GTP hydrolysis by Tet(O)^{mutants} may provide insight into whether they are capable of binding to and being released from the 70S ribosomes. The velocity of GTP hydrolysis, measured as the concentration of GTP hydrolyzed per second by per unit concentration of Tet(O) is expected to increase with time. The absence of GTP hydrolysis would suggest the Tet(O)^{mutants} do not interact with the 70S ribosomes.

All the Tet(O) deletion mutants were able to hydrolyze GTP, indicating that all Tet(O)^{mutants} were still able to interact with the 70S, which eliminates possibility (i) above. However, a review of the GTPase kinetic parameters (Table 3.5) suggested that all the Tet(O) deletion mutants were much less efficient in their ability to hydrolyze GTP as compared to Tet(O)^{WT}. All the deletion mutants had a higher Km for GTP than Tet(O)^{WT} which translates to a lower binding affinity consistent with possibility (ii) above. Unfortunately, the large error bar on the Km for GTP (319 $\pm$ 264) in the presence of 508ΔS made it difficult to clearly assess the ability of 508ΔS to bind and be released from the 70S. The rest of the deletion mutants demonstrated an apparent 2-8 fold reduction in their catalytic efficiency to hydrolyze GTP compared to Tet(O)^{WT} which is consistent with possibility (iii) above. This would suggest that mutations in domain IV, which is remote from domain I in Tet(O), likely altered the interaction of domain I with 70S and resulted in impaired GTP hydrolysis in the mutants. The impaired GTP-

hydrolysis could be responsible for the failure of the Tet(O)^{mutants} to invoke the conformational change in the 70S necessary to allosterically release Tc from its primary binding site, and at the same time prevent it from dissociating from the 70S ribosome itself. In order to test possibility (iv), it would be necessary to carry out GTP hydrolysis in the presence of increasing concentrations of Tet(O) but these studies must be the subject of future work.

4.5 Summary

The individual Tet(O)^{mutants} were grouped according to the nature of their mutation, Tc^{S} phenotype and ability to release Tc (Figure 4.5). Substituting 507Y with a basic H residue carrying a bulky polar side chain did not alter the interaction of Tet(O) with 70S, and therefore retained a Tc^{R} phenotype. This indicated 507Y was not critical for Tet(O)-mediated Tc release.

Double alanine substitution at positions 508-509 of Tet(O)^{WT} also retained a Tc^{R} phenotype. Deletion of 508S or its polar substitution (S508D) produced Tc^{S} Tet(O)^{mutants} with intermediate Tc release ability relative to the absence or presence of Tet(O)^{WT}. This data suggests either the 508S residue by itself may not play a critical role in Tc release, or its interaction with neighbouring residues may assist Tc release.

Double alanine substitutions around 509P (508-509, 509-510) produced a Tc^{R} phenotype. In contrast, non-polar substitution at position 509 (P509V) or its deletion produced a Tc^{S} mutant, which was unable to release Tc from the primary binding site on the 70S. This suggests 509P has a greater role in Tc release than 508S. Without structural information, it is unclear why no change in Tc^{R} phenotype was detected with the double alanine substitutions around 509P.

The Tc^{S} 511ΔS mutant had an intermediate ability to release Tc relative to Tet(O)$^{WT.}$ Double alanine substitution around 511S (V510A-S511A and S511A-T512A) produced Tc^{S} mutants, both of which were unable to release Tc. Polar/non polar substitutions with equivalent EF-G residues at 511S was unnecessary because the serine residue is conserved in both EF-G and Tet(O). This suggests that 511S plays an intermediate role in Tc release.

Deletion, double alanine or polar substitution at positions 510 and 512 produced Tc^S mutants all of which were unable to release Tc from its primary binding site in the 70S. These suggest 510V and 512T residues are critical for Tc release.

The results of the Tc binding assay suggests deletion or substitutions with residues at 509P, 510V, 511S and 512T most drastically impair Tet(O)-mediated Tc release. The changes in Tet(O) function are most likely due to the differences in the interaction of Tet(O) with 70S that arise from the alterations in the structure of the loop at the tip of domain IV of Tet(O).

Although these findings contribute to the knowledge as to which of the residues of Tet(O) protein are crucial for Tc release, further studies are required to clarify which residues in the 70S interact with Tet(O) in order to develop strategies to circumvent Tc^R.

4.6 Concluding Remarks

This thesis has provided a better understanding of the molecular mechanism of Tet(O)-mediated Tc^R through the identification of residues (509P, 510V, 511S, 512T) that play an essential role in Tc release from its primary binding site on the 70S. Studies of the GTPase activity of Tet(O) deletion mutants reinforces the previous unpublished data [265] that GTP hydrolysis is essential for Tc release. These findings have the potential to develop a new therapeutic agent to overcome Tet(O)-mediated Tc^R by interrupting the interaction of Tet(O) with 70S around domains I and IV.

Table 4.1: Tc binding to the 70S ribosomes in the absence and the presence of Tet(O)^{WT}

		- Tet(O)^{WT}			+Tet(O)^{WT}		
[^{3}H-Tc], μM	# of Tc molecules	Tc Bound to 1° site	Tc Bound to 2° sites	Unbound Tc	Tc released	Tc Bound to 2° sites	Unbound Tc
1	20	10	10	0	10	10	0
2.5	30	10	20	0	10	20	0
5	40	10	30	0	10	30	0
10	50	10	40	0	10	40	0
20	60	10	50	0	10	50	0
30	70	10	50 Bmax	10	10	50 Bmax	10
40	80	10	50	10	10	50	10

Let us assume that
0.5 μM 70S = 10 molecules of 70S
one 1° binding site/70S suggests there are 10, 1° binding sites in total
One to five 2° binding sites/70S suggests 10 to 50, 2° binding sites in total
2 μM Tet(O) = 40 molecules of Tet(O)

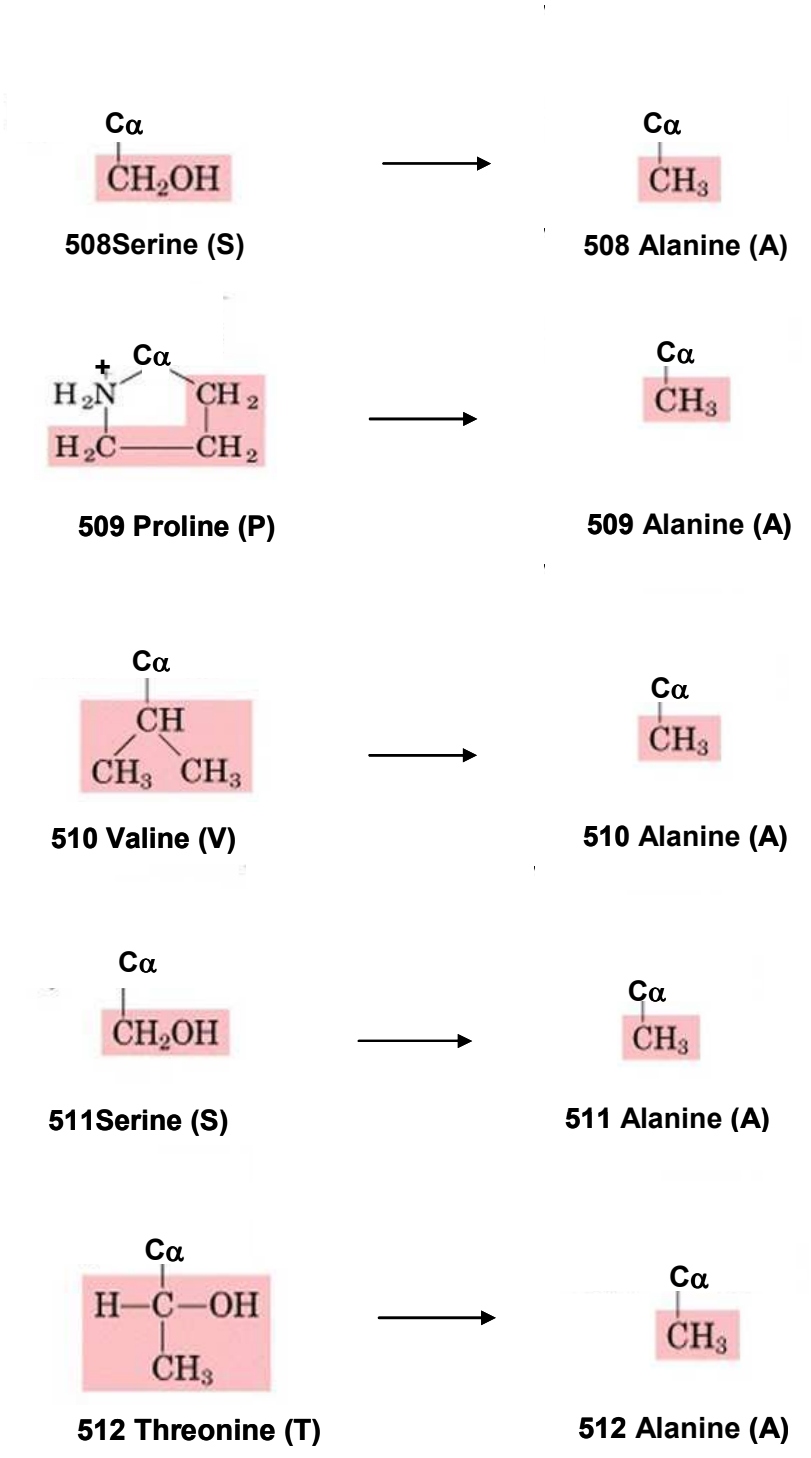

Figure 4.1a The single alanine Tet(O) point mutations generated between positions 508-512 at the loop of domain IV of Tet(O). The Cα carries an acidic –COOH group and a basic –NH_2 group. [Modified from http://lh3.ggpht.com/_Qa4RgMp9v88/Sm5Q-wT5SnI/AAAAAAAAAEk/0vj3pWgdLPg/ch5-amino-acids.jpg]

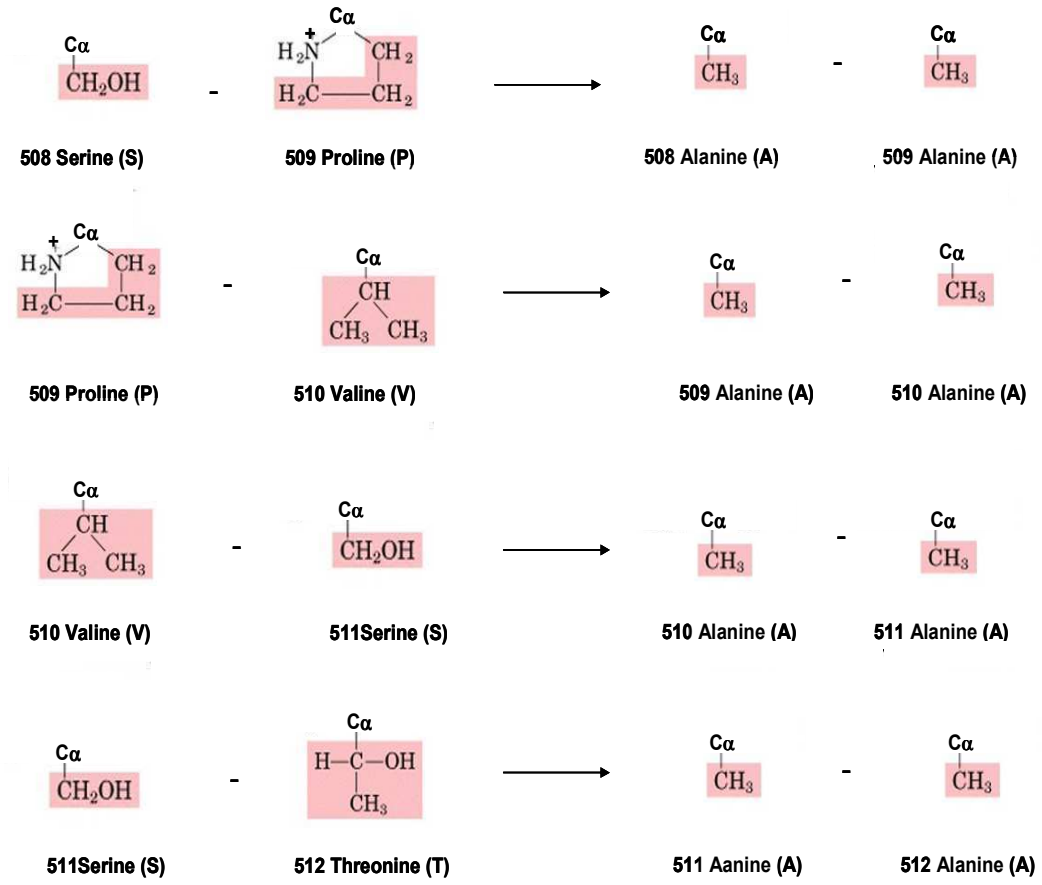

Figure 4.1b The double alanine Tet(O) mutations generated between positions 508-512 at the loop of domain IV of Tet(O). The Cα carries an acidic –COOH group and a basic $–NH_2$ group. [Modified from http://lh3.ggpht.com/_Qa4RgMp9v88/Sm5Q-wT5SnI/AAAAAAAAAEk/0vj3pWgdLPg/ch5-amino-acids.jpg]

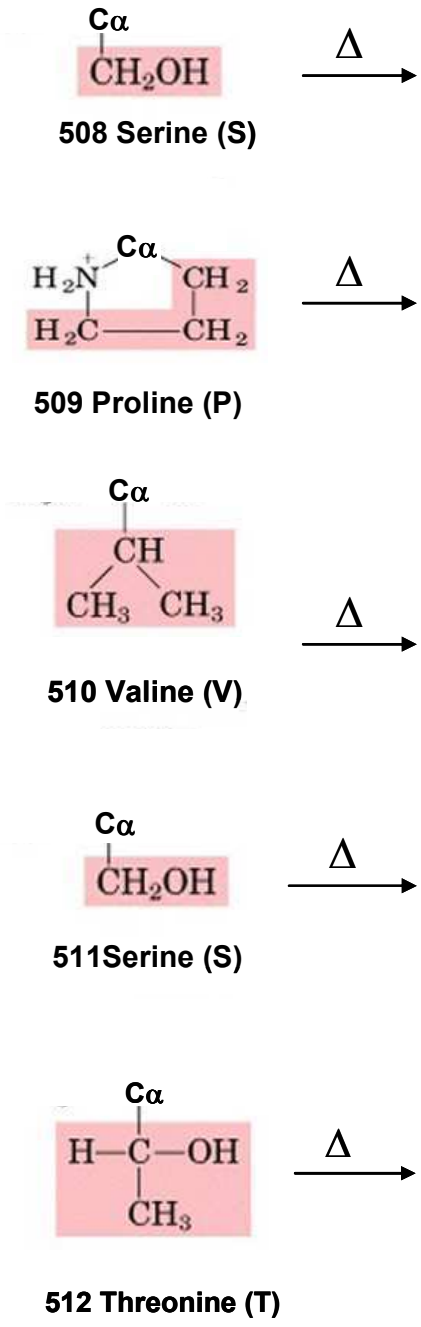

Figure 4.1c The deletion Tet(O) mutations generated between positions 508-512 at the loop of domain IV of Tet(O). The Cα carries an acidic –COOH group and a basic $–NH_2$ group. [Modified from http://lh3.ggpht.com/_Qa4RgMp9v88/Sm5Q-wT5SnI/AAAAAAAAAEk/0vj3pWgdLPg/ch5-amino-acids.jpg]

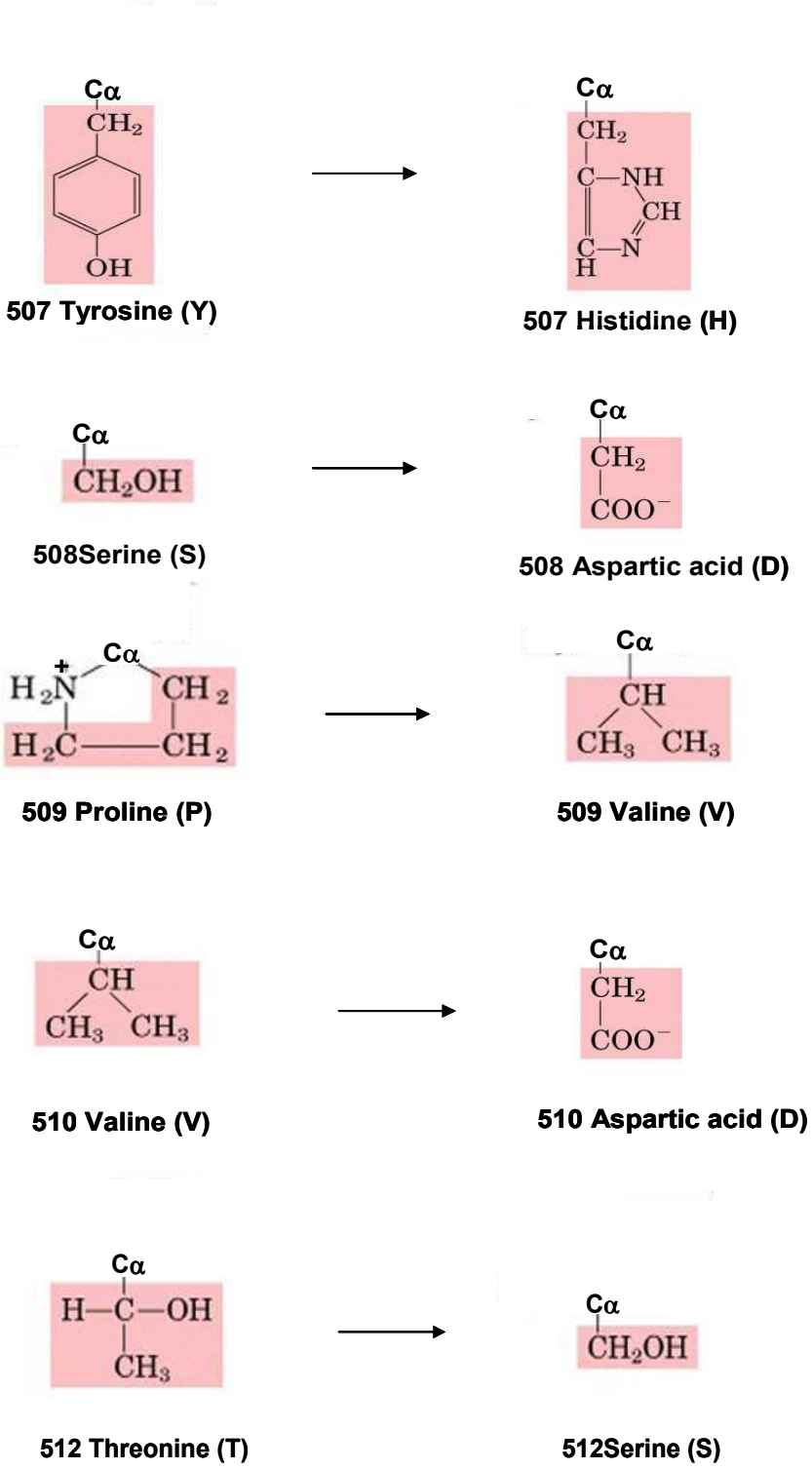

Figure 4.1d The polar/non-polar Tet(O) mutations generated between positions 507-512 at the loop of domain IV of Tet(O). The Cα carries an acidic –COOH group and a basic $–NH_2$ group. [Modified from http://lh3.ggpht.com/_Qa4RgMp9v88/Sm5Q-wT5SnI/AAAAAAAAAEk/0vj3pWgdLPg/ch5-amino-acids.jpg]

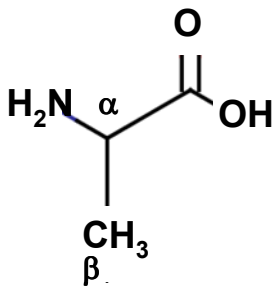

Figure 4.2 Chemical structure of L-alanine

The C_α carries the basic amino ($-NH_2$) and acidic carboxyl (-COOH) group. The non polar methyl ($-CH_3$) side chain remains bound to the C_α. The CH_3 group eliminates any side chain beyond the C_β.

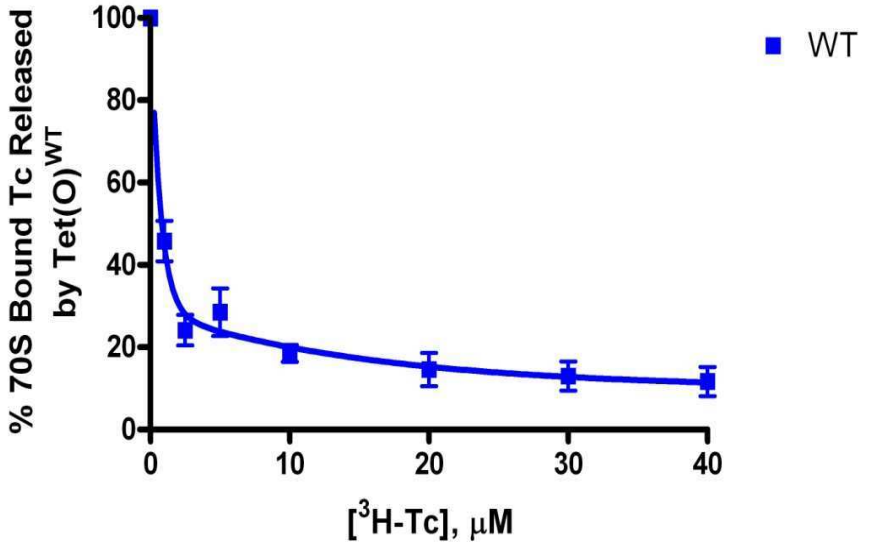

Figure 4.3 The percentage of 70S-bound Tc released by Tet(O)^{WT}

n= 7 experiments. Each experiment was done in triplicates. The values represent mean ± SEM. The exponential two phase decay equation shows how [^{3}H]-Tc release by Tet(O)^{WT} decreases with the increasing [^{3}H]-Tc concentration, indicating Tet(O)^{WT} fails to release Tc from its secondary binding site, which has no role in inhibiting protein synthesis (see Table 4.1, section 2.11.1 for details).

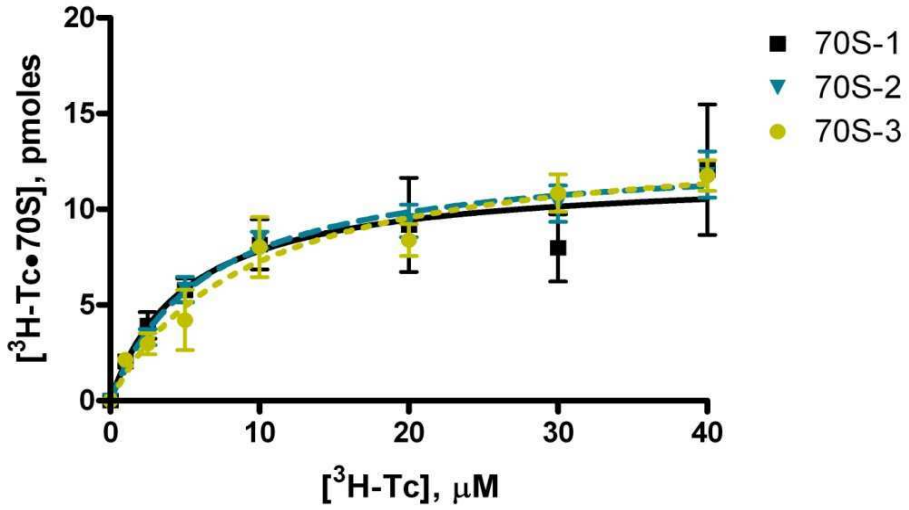

Figure 4.4 The three batches of 70S ribosomes used for performing the [^{3}H]-Tc binding assays

values represent mean ± SEM. All 3 batches of 70S had a consistent Tc binding ability which was responsible for the reproducibility of Tc binding to the 70S in the absence of Tet(O)^{WT}.

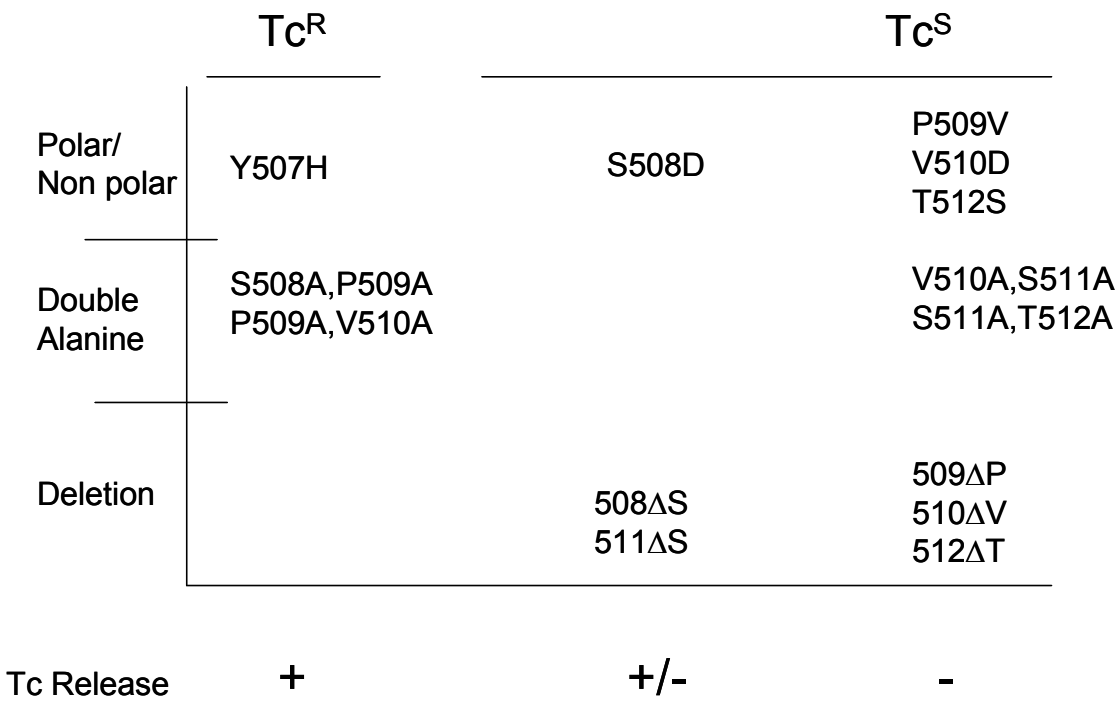

Figure 4.5 Residues 509P, 510V and 512S appear most critical for Tet(O)-mediated Tc release

Comparison of the Tc^S of $Tet(O)^{mutants}$ with their ability to release 70S-bound Tc from its primary binding site indicates 509P, 510V and 512T are most critical residues for Tc release.

Chapter 5 Future Directions

Although this thesis has identified the residues that play a role in Tet(O)-mediated Tc release, the order of the events that lead to Tc release are still not well understood. The current model [Connell *et al.* 2003] proposes that Tet(O) binds to the 70S, and the 70S-dependent GTP hydrolysis causes a conformational change in the 70S, which allosterically releases Tc from its primary binding site. It is also possible that GTP hydrolysis may invoke a conformational change in the Tet(O) to facilitate its dissociation from the 70S.

The Tc^S $Tet(O)^{mutants}$ were either unable to release Tc from the 70S or had an intermediate ability to cause Tc release. Preliminary data on the GTPase activity of the Tc^S Tet(O) deletion mutants suggests the mutants were still able to bind to 70S, although the nature of the binding affinity is unknown. The following proposed studies may provide more insight into the mechanism of Tet(O)-mediated Tc release.

5.1 Investigating the ability of the Tc^S Tet(O) double alanine and Tet(O) polar/non polar mutants to bind the 70S

The ability of the Tc^S Tet(O) double alanine and the polar/non polar mutants with reduced Tc release ability to bind the 70S should be confirmed by performing a GTP hydrolysis assay. This will determine:

(i) whether the double alanine and polar/non polar mutants were able to interact with 70S like the Tet(O) deletion mutants

(ii) whether the GTPase kinetic parameters were similar to $Tet(O)^{WT}$.

5.2 To determine the binding affinity of $Tet(O)^{WT}$ and $Tet(O)^{mutants}$ to the 70S ribosome

If all the Tc^S $Tet(O)^{mutants}$ are found to retain their ability to hydrolyze GTP (which is 70S-dependent), the cause of impaired Tc release is not due to their failure of the $Tet(O)^{mutants}$ to bind 70S. Studies of GTP hydrolysis in the presence of constant concentration of Tet(O) and increasing concentrations of 70S will allow the determination of the efficiency of the interaction of the $Tet(O)^{mutants}$

with 70S. Tet(O)^{WT} will be used as a control. Comparing the Km of the Tet(O)^{mutants}•GTP for the 70S will allow the indirect determination of the affinity of Tet(O)^{mutants}•GTP for the 70S relative to the Tet(O)^{WT}•GTP. It is expected that the Tet(O)^{mutants}•GTP will have a higher Km for the 70S compared to Tet(O)^{WT}•GTP, which would indicate that the Tet(O)^{mutants} do not bind the 70S as efficiently as Tet(O)^{WT}.

5.3 To investigate the release of 70S-bound Tet(O)^{mutants}

In order to determine whether 70S-bound Tc^{S} Tet(O)^{mutants} dissociate from the 70S, a GTP-hydrolysis assay may be performed in the presence of increasing concentrations of Tet(O). If GTP hydrolysis does not increase with the concentration of the Tet(O)^{mutants}, they are likely unable to release from 70S. Tet(O)^{WT} will be used as a positive control where GTP hydrolysis will increase with increasing Tet(O)^{WT} concentrations indicating that Tet(O)^{WT} can be released from 70S, allowing other Tet(O)^{WT} to bind 70S .

The above experiments (5.1-5.3) may determine whether the GTP hydrolysis activity of domain I of Tet(O) plays a role in catalyzing Tc release by domain IV. It is possible that domain I is required for the correct positioning of domain IV relative to 30S, an interaction that may be crucial for Tc release. There may be conformational changes that occur in domain I following GTP hydrolysis which are transmitted to domain IV resulting in: i) conformational changes in the 70S that allows Tc to be released allosterically from the primary binding site, and ii) Tet(O) to be released from the 70S as well.

5.4 Determination of the exact residues the domain IV of Tet(O)^{WT} and Tet(O)^{mutants} contact in the 70S ribosome

The main objective of this thesis was to understand more about the molecular mechanism of Tet(O)-mediated Tc release and circumvent the problem of Tc^{R} in bacteria. In the current study an attempt was made to identify if the amino acid residues in the tip of domain IV of Tet(O) were critical for Tet(O)-mediated Tc release. It turned out that all the Tet(O)^{mutants} that either carried a

deletion or substitution of the residues in the tip of the domain IV were impaired in their ability to release Tc. This suggests these residues have a critical role in Tc release. Cryo-EM studies of Tet(O)-GTPγS (a non cleavable GTP analogue) complexes at 16Å resolution by Spahn *et al*.[2001] has shown that the tip of the Domain IV of Tet(O) as a whole does not overlap with the A-site (where Tc binds) but only closely approaches it within 6 Å of the primary Tc binding site in the 70S. The tip of the domain IV of Tet(O) contacts residues 1051-1053:1208-1210 in the minor groove of h34 of 16S rRNA and primary Tc binding site maps to residues 1053-1056:1196-1200 in h34. Hence it was suggested that Tet(O) functions by catalyzing the removal of Tc, probably by stimulating a local conformational change in h34.

What is still unknown is which residues in the h34 of 16S rRNA are contacted by YSPVST at the tip of the domain IV of $Tet(O)^{WT}$, and if any side chain of these residues protrudes into the primary Tc binding site. Details of the chemical interaction at atomic resolution is possible through X-ray crystallography studies of 70S ribosome and Tet(O) functional complexes. To date, no successful crystals of Tet(O) have been prepared. Should this become possible, studies could be carried out to specifically identify and compare the interactions of the Tc^R $Tet(O)^{WT}$ and the Tc^S $Tet(O)^{mutants}$ with the 70S. This would facilitate the design of a Tc molecule with additional side chains which extend into the Tet(O) binding site and thus prevent Tet(O) from releasing Tc from the 70S.

5.5 Investigation of enhanced Tc^R phenotype

It is not clear why the Tet(O) single alanine substitution mutants P509A and T512A (Figure 3.1) had a greater Tc MIC relative to $Tet(O)^{WT}$. Investigation into this enhanced Tc^R phenotype (by *in vitro* Tc release assay) may provide further insight into the molecular mechanism of Tc^R.

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