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Interaction of the Tetracycline Resistance Protein Tet(O) with the Bacterial
Ribosome

by

Sean R.C. Connell ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfilment of the requirements for the degree of Doctor of Philosophy.

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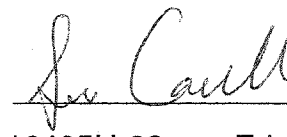**Name of Author:** Sean R.C. Connell

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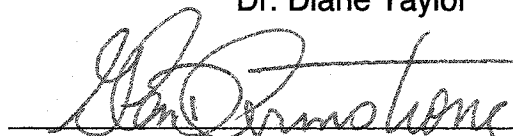
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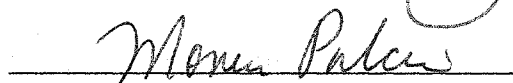
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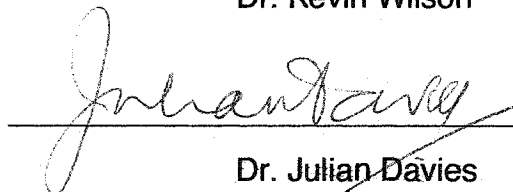
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Abstract

Ribosomal protection represents an important tactic for promoting resistance to tetracycline in the medically relevant *Campylobacter* species. This mechanism relies on a soluble protein called Tet(O), which actively promotes the release of tetracycline from its inhibitory target, the 70S ribosome. The ribosome is a large macromolecular complex of proteins and RNA that represents the central machinery of the protein synthesis pathway. In the current study we investigated the interaction of Tet(O) with the 70S ribosome and found that Tet(O) interacts with many of the ribosome's key functional centres. Using chemical probing we showed that Tet(O) interacts with helix 34 (C1214) and helix 44 (A1408) of the 16S rRNA. These interactions cluster within the decoding site such that C1214 is close to but does not overlap the primary tetracycline-binding site. Moreover A1408 is distinct from the Tet(O) binding site visualized by cryo-EM and is therefore indicative of a long-range rearrangement. In the 23S rRNA we identified interactions between Tet(O) and elements of the GTPase-associated region (Helices 42,43,44). This region has been implicated as an important determinant for elongation factor binding and activity and the results presented herein suggest it plays a similar role in Tet(O)-mediated tetracycline resistance. In terms of the mechanism of Tet(O) action we showed that it dislodges tetracycline from the primary tetracycline-binding site while ignoring tetracycline bound to the secondary site. Additionally, we demonstrated that the post-translocational state of the ribosome represents the functional state with which Tet(O) interacts and therefore shows at which step Tet(O) intercalates into

the elongation cycle. These results are incorporated into a model describing the overall functional cycle of Tet(O) and the molecular mechanism of tetracycline release.

Acknowledgments

I would like to thank Dr. Taylor for her unwavering support through the last couple years and Dr. Nierhaus for among many things instilling in me a great appreciation for scientific literature; there really is a whole world out there waiting to be discovered. To Dr. Trieber I would like to extend my gratitude for teaching me all about Tet(O), and generally helping me through this project. I would also like to extend my thanks to Uli Stelzl and Paola Fucini for painstakingly teaching me that controls are not a waste of time and to look closely to the details of an experiment. Also I would like to thank Norbert Polacek for showing me that RNA is actually pretty important. In addition to those I have thanked already I would also like to convey to the members of the Nierhaus and Taylor lab how much I appreciated their company and assistance over the years. Specifically I would like to thank Daniel Wilson, Uli Stelzl, Norbert Polacek, Trevor Lawley, Matt Gilmour, James Gunton and Dobryan Tracz for their friendship. To my family I would like to say that I could not have done this without you. And finally I would like to apologize to my friends and my family for the many times I neglected them in favour of work.

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List of Abbreviations

2-D	Two dimensional
3-D	Three dimensional
Å	Angstrom
A site	Aminoacyl-tRNA site
aa-tRNA	Aminoacyl-tRNA
ASL	Anticodon stem-loop
ATP	Adenosine 5' triphosphate
A-tRNA	A site bound tRNA
BipA	BPI inducible protein A
Cryo-EM	Cryo-electron microscopy
CTD	Carboxyl-terminal domain
C-terminal	Carboxyl-terminal
Da	Dalton
DMS	Dimethyl sulfate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E site	Exit site
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EF-G	Elongation factor G
EF-Tu	Elongation factor Tu
f.c.	Final concentration
fmet	N-formylmethionine
fmet-tRNA^{fmet}	formylmethionine tRNA^{fmet}
GAR	GTPase associated region
GDP	Guanosine 5' diphosphate
GMPPCP	β,γ-methyleneguanosine 5'triphophate
GMPPNP	Guanosine 5'-[β,γ-imido]triphosphate

GTP	Guanosine 5' triphosphate
GTPγS	Guanosine 5' –O-(3-thiotriphosphate)
HEPES	4-(2-Hydroxyethyl)piperazine-1-(2-ethanesulphonic acid)
His$_6$-tag	Six histidine tag
HRP	Horseradish peroxidase
IF1	Initiation factor 1
IF2	Initiation factor 2
IF3	Initiation factor 3
IPTG	Isopropylthiogalactoside
kb	Kilobase
K_D	Dissociation constant
kDa	Kilodaltons
LB	Luria-Bertani
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MCS	Multiple cloning site
MDa	Megadalton
mRNA	Messenger RNA
N-terminal	Amino-terminal
ORF	Open reading frame
P site	Peptidyl-tRNA site
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PEG	Polyethylene glycol
Phe-tRNA^{Phe}	Phenylalanine-tRNA^{Phe}
Poly(Phe)	Poly Phenylalanine
POST	Post-translocation
PRE	Pre-translocation

Abbreviations

P-tRNA	P site bound tRNA
RBS	Ribosome binding site
ref.	Reference
RF1	Release factor 1
RF2	Release factor 2
RF3	Release factor 3
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPP	Ribosomal protection protein
r-protein	Ribosomal protein
RRF	Ribosome recycling factor
rRNA	ribosomal ribonucleic acid
S	Svedberg
S100	Supernatant of a 100 000g centrifuge step
S30	Supernatant of a 30 000g centrifuge step
SDS	Sodium dodecyl sulphate
SDS PAGE	SDS polyacrylamide gel electrophoresis
Tc	Tetracycline
tRNA	Transfer RNA
UV	Ultraviolet
Wt	Wild type

Chapter 1

Tet(O), Tetracycline and Bacterial Protein Synthesis

1 Tet(O), Tetracycline, and Bacterial Protein Synthesis

In 1977, *Campylobacter* was implicated as a major cause of gastroenteritis in humans [1] and additionally, *C. jejuni* infections are associated with the subsequent development of Guillian-Barré syndrome [2]. *Campylobacter* infections primarily originate from the ingestion of contaminated meat products, usually poultry, or from contaminated water [3]. In the U.S., there are estimated to be 2.5 millions symptomatic cases of *C. jejuni* infections every year and accordingly, it is one of the most common bacterial pathogens observed in stool cultures of individuals inflicted with diarrhoeal illness (ref. [3] and references within). Clinical isolates of *C. jejuni* are showing a heightened occurrence of tetracycline resistance where, for example, in a Canadian study, the incidence of tetracycline resistance in isolates from 1985-86 was 19% whereas it was 56% in isolates from 1995-1997 [4]. In *Campylobacter,* tetracycline resistance is generally associated with large conjugative plasmids [5], however, an example of a chromosomally encoded tetracycline resistance determinant exists in *C. coli* [6]. The gene conferring resistance on these plasmids has been cloned and sequenced thus identifying a new class of tetracycline resistance determinant, the ribosomal protection protein (RPP) Tet(O) [7-9]. As the acronym implies, Tet(O), confers resistance to tetracycline directly at the level of protein synthesis [10] where it dislodges the drug from its target, the 70S ribosome, a central figure in protein synthesis [11]. As such, the study of Tet(O) reveals details not only about RPP-mediated tetracycline resistance but also about the mode of action of tetracycline, and the intricacies of protein synthesis.

1.1 Protein synthesis

1.1.1 General Introduction

The central dogma of molecular biology describes the ordered transfer of information from a storage form (DNA) to a functional form (protein) through an

RNA intermediate (although it should be noted that many exceptions or deviations to this model exist). In this model, protein synthesis represents the process of translating the genetic information, contained in the RNA intermediate, (mRNA) into a polypeptide. Translation occurs primarily within the scaffolding of the 70S ribosome where both the ribosome itself and auxiliary factors drive the reaction. During production of a single protein the ribosome progresses through three distinct phases: initiation, elongation, and termination (Figure 1-1).

In eubacteria, during the initiation phase of protein synthesis (Figure 1-1, red arrows) the 70S ribosome assembles from its 30S and 50S subunits on the mRNA, generally centering on an AUG start codon that is recognized by its cognate initiator tRNA, fMet-tRNA^{fmet} (reviewed in ref. [12]). This process is facilitated by three protein initiation factors, IF1, IF2, and IF3, which promote the proper selection of the start codon as well as its cognate initiator tRNA and the assembly of the 30S and 50S subunits into a 70S ribosome. Initiation primes the ribosome for the next phase of protein synthesis, elongation (Figure 1-1, green arrows). During the elongation phase incoming aa-tRNAs decode the mRNA and thus sequentially deliver the new amino acids that are incorporated into the growing polypeptide. It is this phase where tetracycline exerts its primary effects and therefore, will be discussed in more detail below (section 1.1.3). The elongation phase of protein synthesis continues until the ribosome reaches a stop codon on the mRNA which triggers the third and final phase, termination and recycling (Figure 1-1, blue arrows). During this phase the polypeptide is released from the ribosome in a reaction promoted by either RF1 or RF2 depending on the stop codon (reviewed in ref. [13]). After release of the polypeptide, RF3 stimulates the dissociation of RF1/2 [14] and subsequently the deacyl-tRNAs and mRNA are released while the ribosome is broken down into its constituent subunits through the action of RRF, IF3, and EF-G [15]. This recycles the ribosome so it can reinitiate another round of protein synthesis.

Figure 1-1: The 3 phases of protein synthesis

A complete cycle of protein synthesis is represented, showing the progression of the ribosome through three phases: initiation (red arrows), elongation (green arrows), and termination (blue arrows). See section 1.1.1 for a detailed description of the cycle. This figure was obtained from Knud Nierhaus and adapted.

Legend:
- 30S subunit
- 50S subunit
- Post-translational Ribosome
- Pre-translational Ribosome
- mRNA
- Deacyl-tRNA
- N-formylmethionyl-tRNA
- Aminoacyl-tRNA
- Peptidyl-tRNA
- Polypeptide
- EF-Tu·GTP·aa-tRNA
- EF-Tu·GDP
- Initiation
- Elongation
- Termination
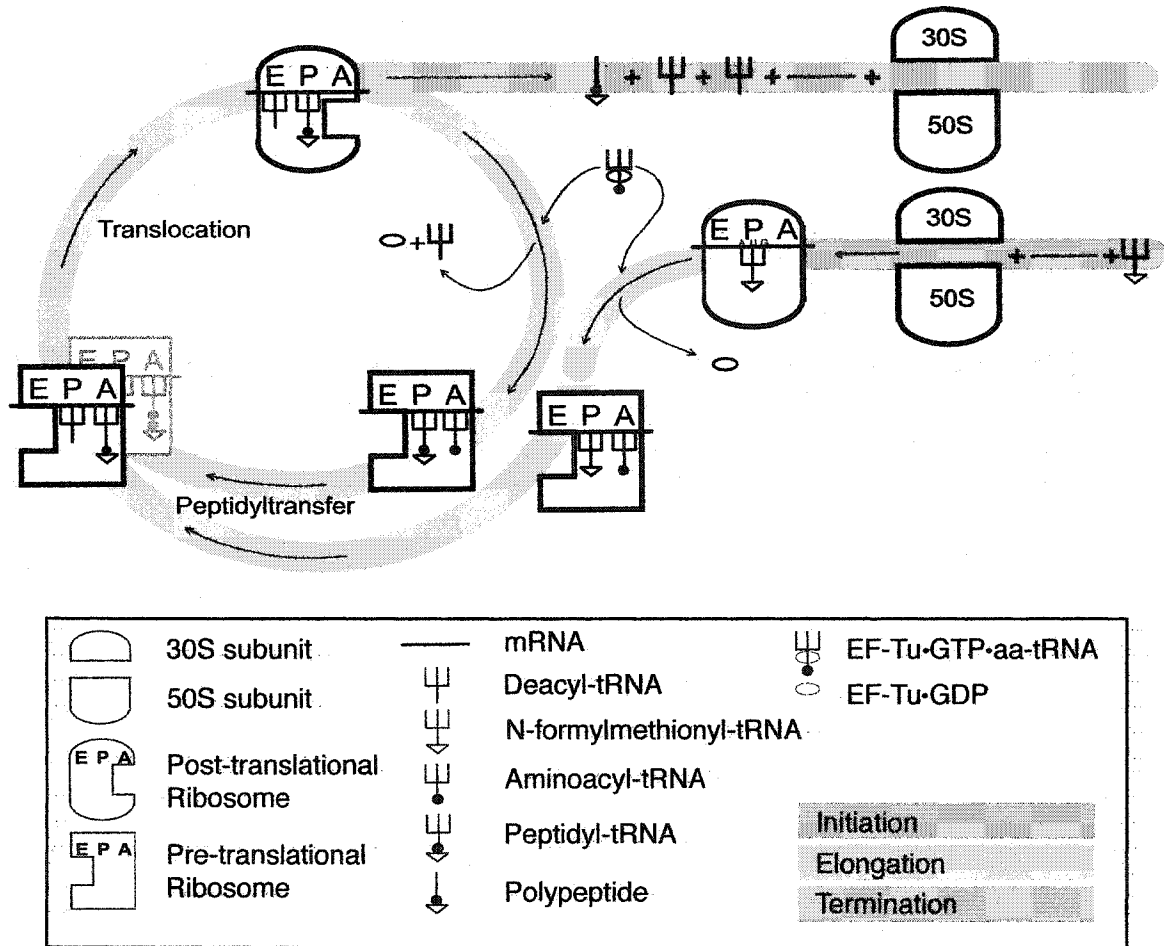
1.1.2 The 70S ribosome

As is obvious from the above description the ribosome plays a central role in all stages of protein synthesis. In eubacteria the 70S ribosome is a massive ~2.3 MDa complex comprised of roughly two-thirds RNA and one-third protein. The 70S ribosome can be broken down into two unequal components termed the 30S and 50S subunits and they can be readily distinguished both structurally and functionally. The 30S subunit (~0.85 MDa), the smaller of the two, is composed of a single RNA molecule termed the 16S rRNA (~1500 nt.; Figure 1-2[i]) and approximately 20 proteins (S1-S21). The 30S subunit is primarily associated with correctly decoding the mRNA. The 50S subunit (1.45 MDa) is the larger of the two and is accordingly composed of two rRNA molecules, the 5S and 23S rRNA (~120 nt. and ~2900 nt, respectively; Figure 1-3A-C), and approximately 33 proteins (L1-L33). The large subunit harbours the peptidyl transferase activity and as such is responsible for catalyzing peptide bond formation.

Our structural perception of the ribosome has evolved over the last several decades and has cumulated, recently, in atomic resolution structures depicting the 30S subunit from *Thermus thermophilus* (3-3.3 Å; Figure 1-4A upper panel) and the 50S subunit from *Haloarcula marismortui* (2.5 Å) and *Deinococcus radiodurans* (3.1 Å; Figure 1-4A lower panel) [16-19]. Within the ribosome the rRNA is highly structured existing mostly in helical elements [16, 17] formed by base-pairs (Figure 1-4B). Interestingly the crystal structures show that the A-minor motif[ii] [20, 21] is prevalent within the ribosome where it forms the basis for many helix-helix and loop-helix interactions [17, 21]. In Figure 1-4A it can be seen that the ribosomal proteins are generally concentrated on the cytosolic surface of both the 30S and 50S subunits such that the interface (the surface of

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[i] Unless otherwise indicated when positions within the rRNA are quoted in this work they are given according to the *E. coli* secondary structure as seen in Figures 1-2 and 1-3.
[ii] The A-minor motif describes an RNA-RNA interaction where the minor groove face of an unpaired adenosine inserts and interacts, to varying degrees, with the minor groove surface of two base-paired residues [20, 21].

Figure 1-2: Secondary structure of *E. coli* 16S rRNA

The secondary structure of *E. coli* 16S rRNA derived from comparative sequence alignments. This structure was obtained from the Comparative RNA Website [22].

Symbols Used In This Diagram:

G — C   - Canonical base pair (A-U, G-C)

G · U   - G-U base pair

G ○ A   - G-A base pair

U ● U   - Non-canonical base pair
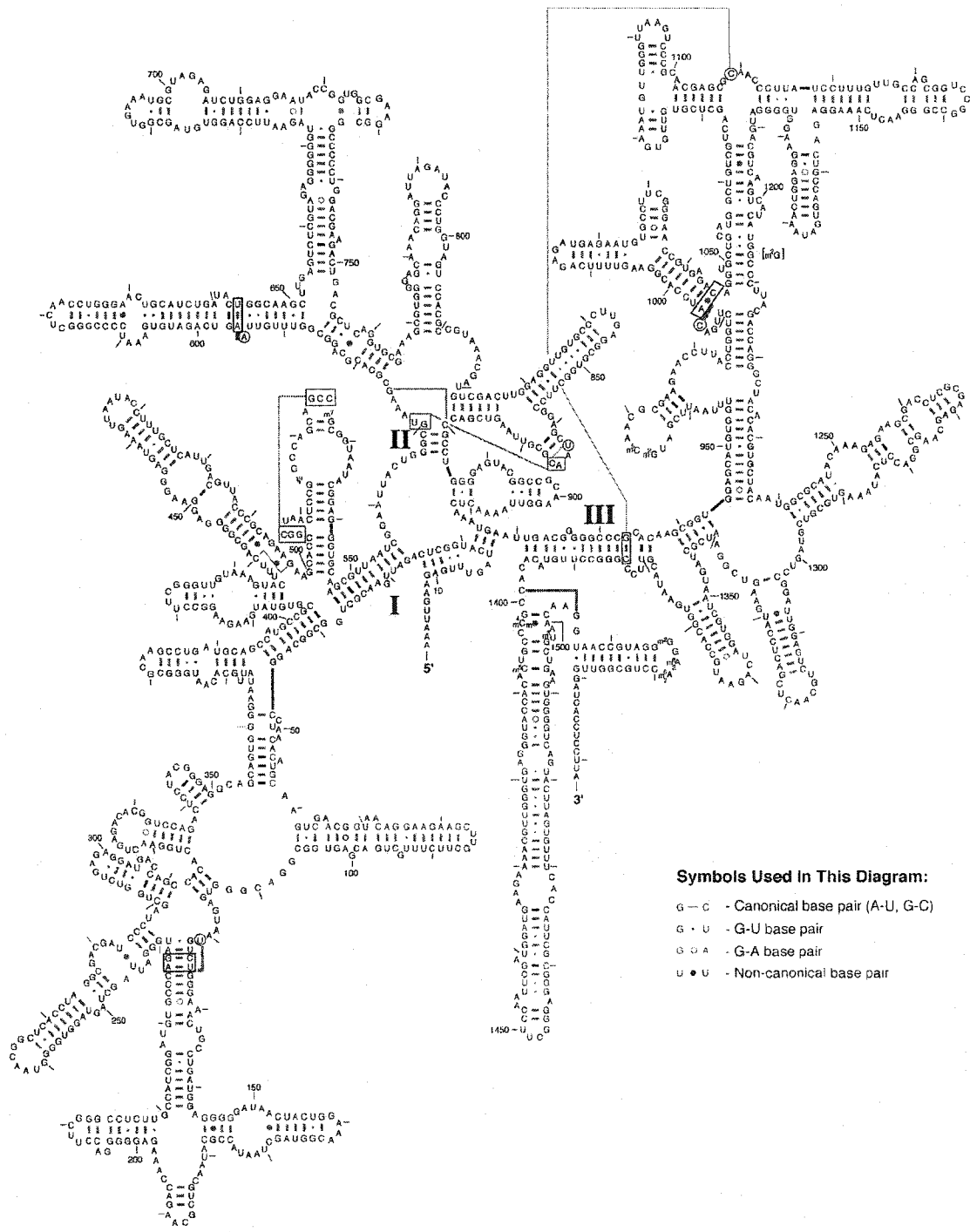
Figure 1-3A: Secondary structure of the 5' end of *E. coli* 23S rRNA

The secondary structure of the 5' end of *E. coli* 23S rRNA derived from comparative sequence alignments. This structure was obtained from the Comparative RNA Website [22].

Symbols Used In This Diagram:

a — c   - Canonical base pair (A-U, G-C)
G • U   - G-U base pair
G ○ A   - G-A base pair
U • U   - Non-canonical base pair
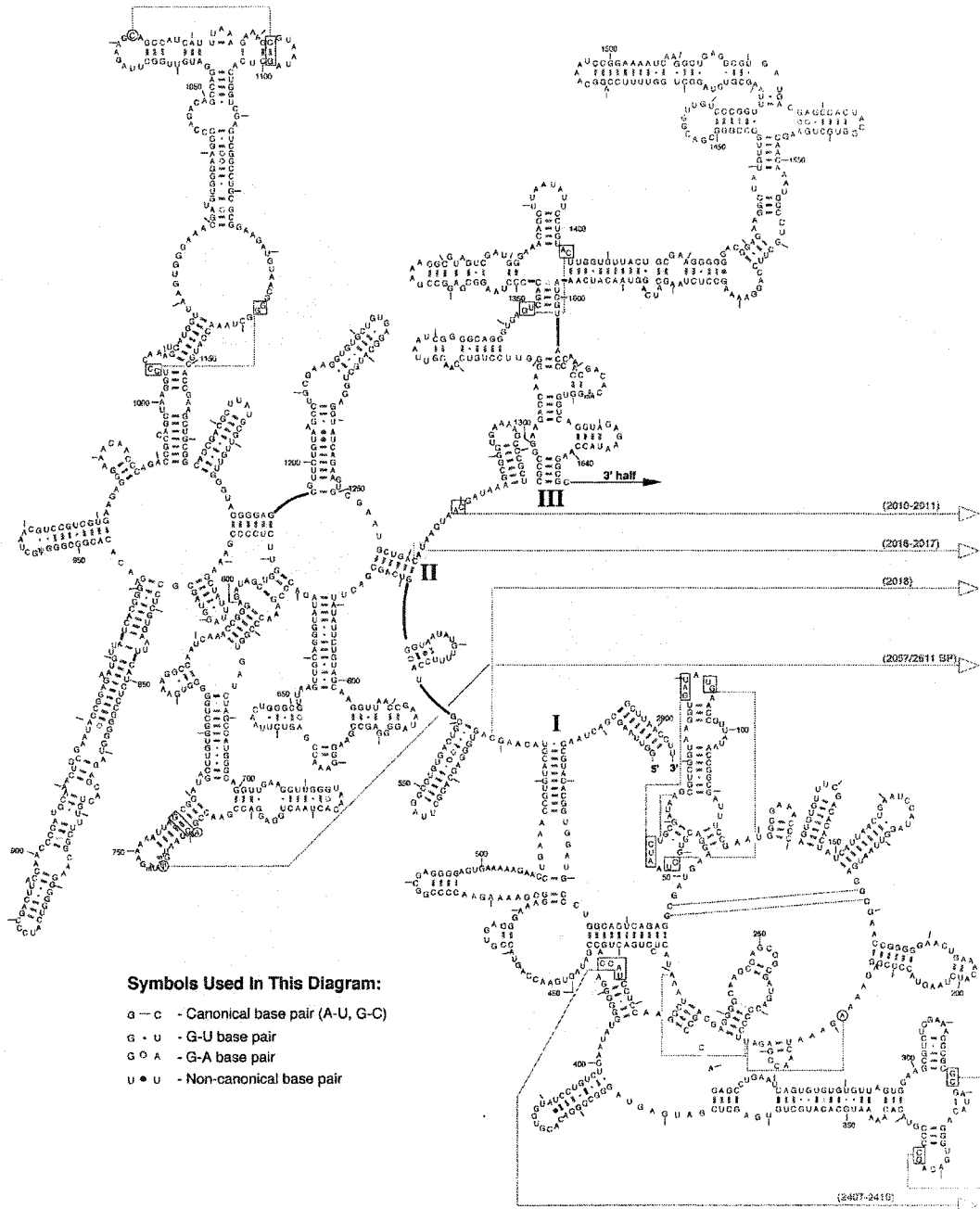
Figure 1-3B: Secondary structure of the 3' end of *E. coli* 23S rRNA

The secondary structure of the 3' end of *E. coli* 23S rRNA derived from comparative sequence alignments. This structure was obtained from the Comparative RNA Website [22].

Symbols Used In This Diagram:

G — C - Canonical base pair (A-U, G-C)
G • U - G-U base pair
G ○ A - G-A base pair
U ● U - Non-canonical base pair
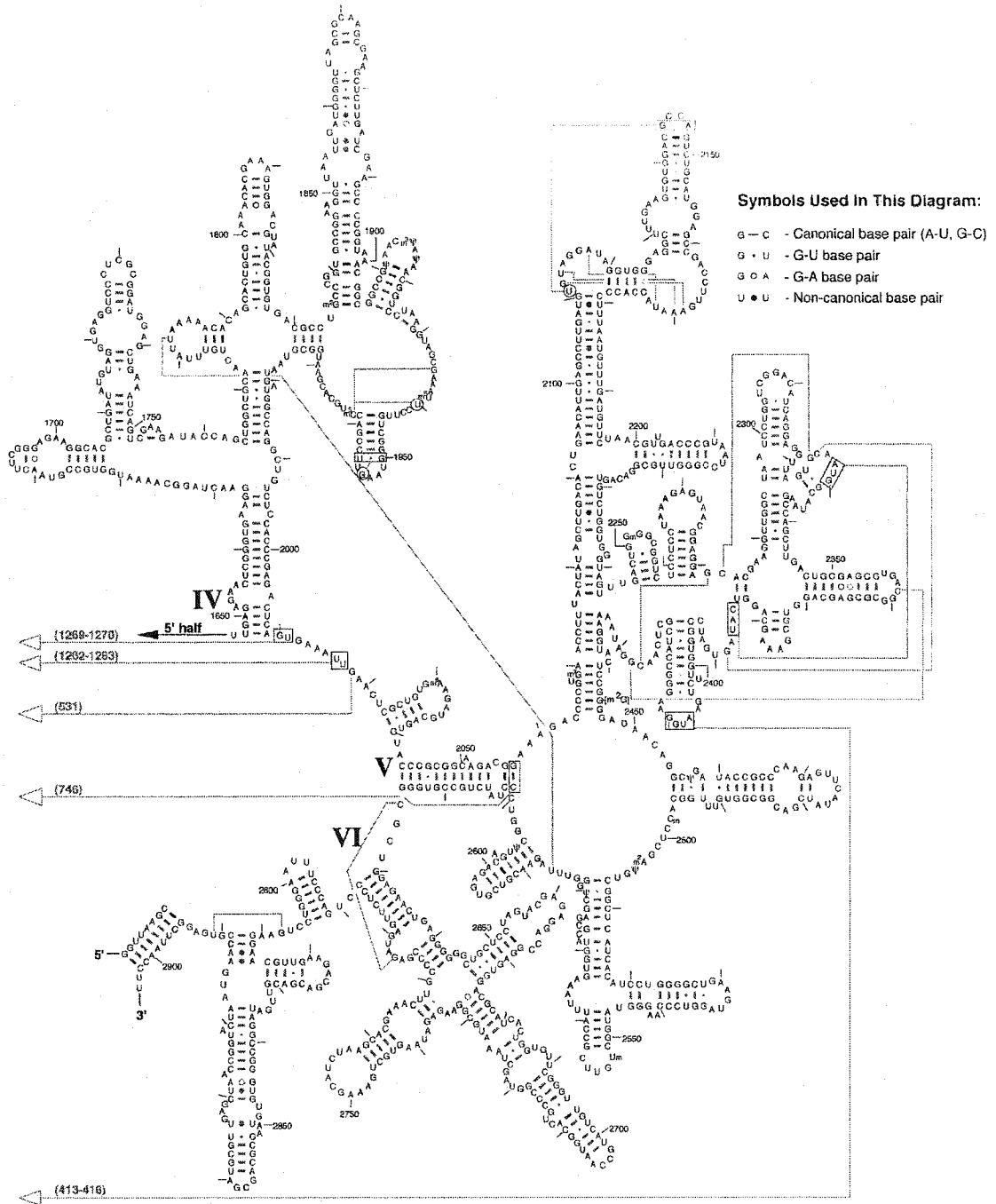
Figure 1-3C: Secondary structure of *E. coli* 5S rRNA

The secondary structure of *E. coli* 5S rRNA derived from comparative sequence alignments. This structure was obtained from the Comparative RNA Website [22].

3'   5'

```
120  U
     A ═ U
     C ═ G
     G ═ C
     G ═ C
     A ═ U
     C ═ G
     C ═ G
     G ═ C
     U · G  10
     C ═ G C C
   A           G
   A          U
   A    A    G U C C            C C C C
   G  GCGCGGUG    CACCUGA      C      A
             | | · | | | · |   | | | | | | |          U
     G ═ C GCGUGCCGC   GUGGACU          G
     G ═ C        A   A A A   A A    C      C
     A O G                        50  C A A G
     U ═ A
     G · U
     A O G
100  G ● G
   A       U
   G       A
     C ═ G
     G · U
     U · G
     A ═ U
     C ═ G
     C ═ G
     C ═ G
     C ═ G
     U C U
```

Figure 1-4: Properties of the 70S ribosome

(A) The crystal structures of the *T. thermophilus* 30S (upper) and *D. radiodurans* 50S (lower) subunits are illustrated from 4 roughly orthogonal views. Ribosomal protein S12, which is located on the interface surface of the 30S subunit, is coloured red, rRNA is blue and the other r-proteins are gold. Major landmarks are indicated on the figure where: b, beak; h, head; pt, platform; sp, spur; L1, L1 stalk; CP, central protuberance; st, L7/L12 stalk. The PDB coordinates are from 1FKA and 1LNR.

(B) A U:A (left) and C:T (right) base-pair is illustrated with the probable hydrogen bonds drawn as green-dashed lines. The atoms are numbered as indicated in the figure. The atoms are coloured such that C, cyan; N, blue; O, red, P, yellow.

(C) The ribosomal-protein S12 as it is found in the 30S subunit. The PDB coordinates are from 1FKA. These figures were prepared with SwissPDB viewer [23], VMD [24], and PovRay (www.povray.org).

A

interface   A site side   cytosolic   E site side

B

U:A base-pair   C:U base-pair

C

S12
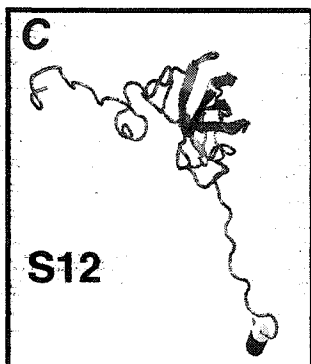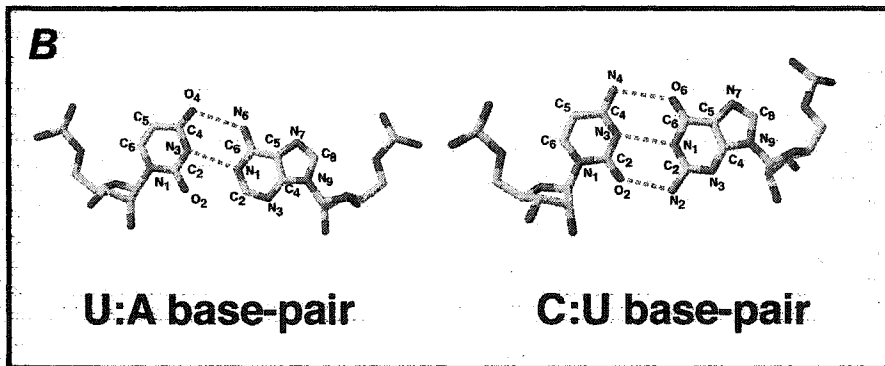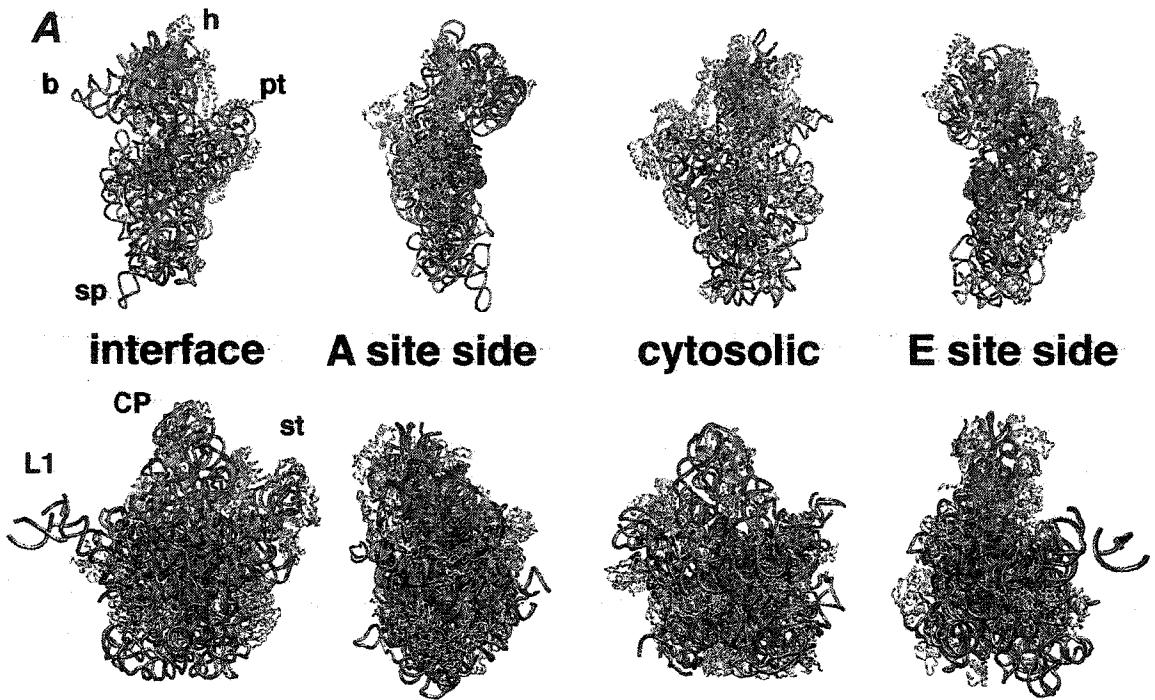
the subunits which contact each other) is largely devoid of proteins [16-18]. One notable exception to this statement is S12 (Figure 1-4A; red ribbon), which is bound to the interface side of the 30S subunit [17, 18]. The ribosomal proteins mediate many interactions between rRNA elements and have been likened to the mortar that holds the rRNA together [16]. Several ribosomal proteins, for example S12, are interesting in that they exist as a fusion of a compact folded domain and a relatively unstructured extended domain (Figure 1-4C; ref. [16-18]). The extended domain can penetrate deeply into the ribosome interior and mediate interactions between distant ribosomal elements [16, 25] possibly facilitating communication between different functional sites.

1.1.3 The elongation cycle

The elongation cycle[iii] is the central phase of protein synthesis (Figure 1-5, green arrow) and it is during this phase that the polypeptide is assembled from its constitutive amino acids. These amino acids are covalently linked to a specific tRNA to form an aminoacyl-tRNA (aa-tRNA) such that the tRNA can act as an 'adaptor' and carry the correct amino acid to the ribosome based on the complementarity of the codon on the mRNA and the anticodon on the tRNA. In doing so the tRNA passes through three distinct tRNA binding sites (Figure 1-5) on the ribosome, the A, P and E sites [27].

The first elongation phase after initiation begins with an fmet-tRNA[fmet] bound to the ribosomal P site. This is a special case that occurs only immediately after initiation, whereas in all subsequent cycles the ribosome begins the elongation phase with a peptidyl-tRNA bound to the P site (yellow site) and a deacylated tRNA at the E site (pink site) as depicted in Figure 1-5. In the first reaction of the elongation cycle (decoding, step 1a), a ternary complex consisting of EF-Tu, GTP, and aa-tRNA binds to the 70S ribosome. During decoding, the anticodon of the tRNA interacts with the mRNA in the 30S A site (green site)

[iii] Here I have presented a generalized version of the elongation cycle, and it should be noted that many models exist which differ in their intricacies and are described in ref. [26].

Figure 1-5: The ribosomal elongation cycle

A generalized 3-site model for the elongation cycle is shown. The 3 tRNA binding sites are the A site (green/blue), the P site (yellow), and E site (pink). The forth A/T site mentioned in the text (section 1.1.3) is seen in POST state ribosome after reaction 1a where the tRNA is bound to the decoding site on the 30S subunit (green) and to EF-Tu. See section 1.1.3 for a detailed description of the cycle. This figure was obtained from Knud Nierhaus and adapted.

EF-G +GDP + P$_i$

EF-G +GTP

3
(translocation)

POST

= +EF-Tu +GTP

1a
(decoding)

PRE

(peptidyl
transfer)
2

POST

(accomodation)

1b

+ EF-Tu +GDP + P$_i$

PRE
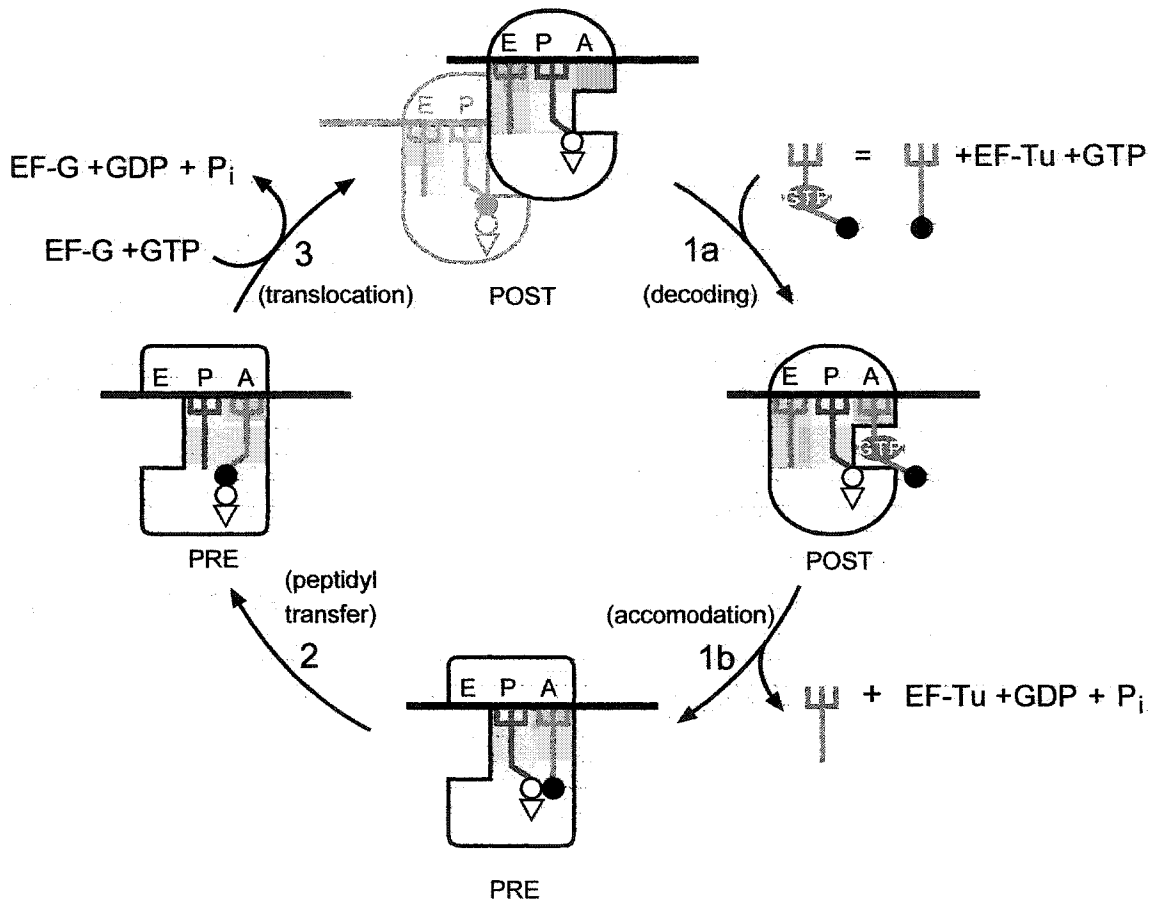
while the aminoacyl end of the tRNA remains bound to EF-Tu [28]. Because the tRNA is bound to both the ribosome and EF-Tu this tRNA binding site is often referred to as the forth tRNA binding site or the A/T site [28]. The ribosome judges the accuracy of the interaction between the tRNA and mRNA by sensing the correctness of the helix formed by the base-pairing of the codon and anticodon [29]. Correct decoding of the mRNA rapidly triggers GTP hydrolysis by EF-Tu and subsequently EF-Tu releases the aminoacyl end of the tRNA and EF-Tu falls off the ribosome (accommodation, step 1b; ref. [30]). After being released from EF-Tu the aminoacyl end of the tRNA accommodates in the 50S A site (light blue) and thus the tRNA is completely bound in the A site. It is this accommodation reaction (step 1b) that is apparently inhibited by tetracycline (see section 1.2.2.1). Accommodation of the aa-tRNA into the A site is coupled to the release of the deacylated-tRNA from the E site (step 1b; ref. [31]). This entire decoding reaction (step 1a-b), therefore, takes the ribosome from the POST state (post-translocational state) to a PRE state (pre-translocational state).

Accommodation of the tRNA into the A site, positions the aminoacyl-end of the tRNA in the peptidyl transferase centre (PTC). In the PTC the 3' end of the A- and P-tRNAs base-pair with the rRNA such that they are locked into place [32] and orientated correctly for the next step of elongation, peptide bond formation (step 2). During peptide bond formation, the α-amino group of the A site bound aa-tRNA attacks the carbonyl carbon linked to the 3'OH of the P site bound tRNA, resulting in the transfer of the P site bound polypeptide to the amino group of the aa-tRNA. Thus the aa-tRNA is converted to a peptidyl-tRNA and is bound to the A site immediately after the reaction [33]. The ribosome is proposed to play an active role in peptide bond formation such that A2451 is proposed to abstract a proton from the α-amino group thus facilitating the nucleophilic attack of the carbonyl carbon [32]. This idea led to the conclusion that 'the ribosome is a ribozyme' [34], although the exact catalytic role the rRNA plays is still being debated (reviewed recently in ref. [35]).

Peptide bond formation, therefore, results in the extension of the polypeptide by one amino acid. The addition of the next amino acid requires that the peptidyl tRNA bound to the A site be translocated to the P site, thus opening the A site for the new aa-tRNA. This reaction is depicted in Figure 1-5 (translocation; step 3), such that EF-G·GTP binds the ribosome and triggers the translocation of the peptidyl-tRNA and deacylated tRNA from the A and P sites to the P and E sites, respectively. In doing so, the ribosome is converted from a pre-translocational state to a post-translocational state and the A site is freed to accept a new aa-tRNA.

1.1.4 The elongation factors[iv]

Progression of the ribosome through the various stages of the elongation cycle (Figure 1-5) is promoted by protein factors called elongation factors or more specifically EF-G, EF-Tu, and EF-Ts in eubacteria. These factors unlike the r-proteins only transiently interact with the ribosome at specific steps in the elongation cycle and facilitate its movement through the various states. EF-Tu for example is responsible for promoting the accommodation of aa-tRNA into the A site (Figure 1-5; step 1a-b) while EF-G translocates the aa-tRNA from the A site to the P site (Figure 1-5; step 3). These elongation factors, in particular EF-G, exhibit extensive sequence homology with the RPPs, such as Tet(O)/(M) [36], and therefore will be discussed in detail below.

1.1.4.1 Domain structure of the elongation factors

EF-G has a 5-domain structure [37] while EF-Tu has 3 domains [38, 39]. The first two domains of both factors share a common fold, where the first domain, the G-domain, also displays strong structural similarity to p21^{ras} [37]. Accordingly, the elongation actors are both GTPases and previous analysis

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[iv] Portions of the section have been published as Wilson, D.N., Blaha, G., Connell, S.R., Ivanov, P.I., Jenke, H., Stelzl, U., Teraoka, Y., Nierhaus, K.H.. (2002) Protein synthesis at atomic resolution: Mechanistics of translation in the light of highly resolved structures for the ribosome. *Current Peptides and Protein Science* 3:1-53

demonstrated that they contain the four common GTP binding motifs (Figure 1-6A and Appendix 6.2; ref [40]). These motifs as well as the G5 motif in the ribosomal GTPases generally form the GTP binding/hydrolysis pocket [40]. In addition, the G3 motif (switch II region) is involved in promoting conformational changes in response to the GTP state of the factor, whereas the G2 motif is a component of the effector loop, a region proposed to interact with effector molecules [40]. As such the G2 motif is conserved only within specific families of the GTPases, i.e. the ribosomal GTPases [40].

Despite the conserved nature of the G-domain in the ribosomal GTPases, the G-domains cannot be readily exchanged. For example, when the region surrounding the G2 motif (effector loop) of EF-G was replaced with the corresponding structure in EF-Tu, the resulting hybrid protein was completely inactive as a translocase [41]. Similarly when as little as the first 30 amino acids from LepA was used to replace those of EF-G, the resulting hybrid was unable to complement a temperature sensitive EF-G [42]. Studies of this nature led the authors to conclude that the effector loop is responsible for mediating interactions with the ribosome, and possibly for coordinating the interaction of a factor with the ribosome in a specific functional state [41-43]. The subtle differences in the effector loop could, therefore, help to distinguish the function of the different GTPases by limiting or promoting their interaction with the ribosome at specific stages in its functional cycle. In this sense the differences between EF-G and Tet(O) in this region (Appendix 6.2) could be important for conferring tetracycline resistance.

Beyond domain II the similarity between EF-G and Tet(O) continues in contrast to that seen with EF-Tu, however, as described in the next section, domains III, IV and V of EF-G (and Tet(O)) may mimic the tRNA moiety of the ternary complex formed by EF-Tu·GTP·aa-tRNA [38]. The role of these domains is not clearly established but studies where single domains have been deleted or

Figure 1-6: Functional regions of the elongations factors

(A) The locations of the GTP-binding motifs [40] are shown on the structure of the G domain of EF-Tu bound to GMPPNP (IEFT; ref. [44]). The G1 motif (XOOOOGXXGXGKS) is yellow, the G2 motif (D-X_n-T) is green, the G3 motif (OJOODXAGJX) is blue, the G4 motif (OOOONKXD) is purple and the G5 motif (OOM(A/R/P)(G/T)SAL is red. The bound GMPPNP is coloured cyan. The motifs are designated as in ref. [40] such that the amino acids are indicated by their 1 letter code, X indicates any amino acid, O a hydrophobic amino acid, and J a hydrophilic amino acid.

(B) Domain IV of EFG (1EFG; ref. [45]) is illustrated with the residues forming the distal tip of the domain coloured blue (loop 1) and green (loop 2). Lys593 which corresponds to His715 in EF-2 - the site of diptheria toxin dependent ADP-ribosylation – is coloured red.
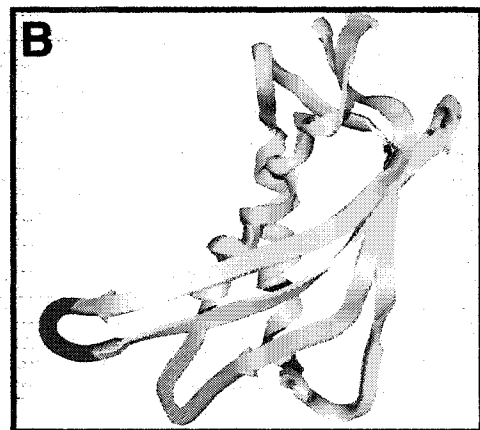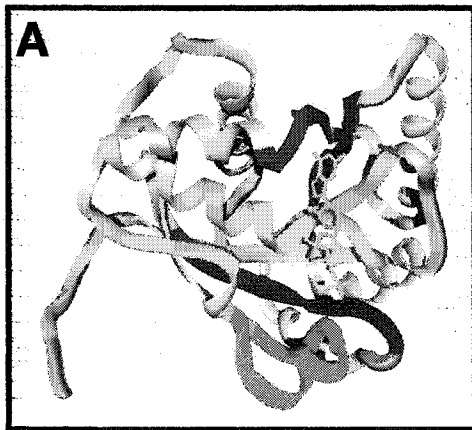
specific residues mutated suggest they have a role in translocation. Deletion of domain III, for example, in EF-G does not significantly affect the association of the factor with the ribosome but does reduce its GTPase and translocase activities [46]. The authors suggest that the interaction of domain III with the ribosome may be important for inducing ribosomal conformational changes, which lead to the stimulation of GTPase and translocase activities [46]. In this respect it is interesting that cryo-EM analysis suggests domain III of EF-G contacts S12 [47] and S12 is implicated in translocation [48].

Deletion studies on domain IV and V also show that these domains are important for promoting rapid translocation [49-51]. More specifically, the distal tip of domain IV (Figure 1-6B) appears to be an important determinant for promoting translocation as mutations in the loop formed by residues 573-579 in *T. thermophilus* (residues 582-587 in *E. coli*; Appendix 6.2) can severely inhibit or completely inactivate EF-G as a translocase [51, 52]. Furthermore EF-2, the eukaryotic homologue of EF-G, is inactivated by diphtheria toxin dependent ADP-ribosylation of His715 [53], which can be aligned to Lys593 in the tip of domain IV in *E. coli* EF-G (Appendix 6.2). Ævarsson *et al.* also pointed out the possible functional significance of the tip of domain IV when they observed that it has a highly unusual topology involving a "left-handed cross-over connection" between two parallel beta stands [37]. Interestingly, the residues in Tet(O) which correspond to those forming the distal tip of domain IV (Figure 1-6B; loops 1 and 2) as judged by sequence alignments are also conserved in the RPP family but distinct from those in EF-G, suggesting they could play a role in tetracycline release (Appendix 6.2).

1.1.4.2 Molecular mimicry in protein synthesis

Structures of the elongation factors in various complexes have been solved to atomic resolution by X-ray crystallography (reviewed in ref. [54]). When the crystal structure of the ternary complex (EF-Tu·GTP·Phe-tRNA) was solved, it was noticed that it was similar in overall shape to EF-G·GDP where domains

III, IV and V of EF-G mimic the tRNA (Figure 1-7; ref. [38]). This exciting observation was subsequently observed with other factors such as RRF[v] (Figure 1-7; ref. [56]) and, to lesser extent, eRF1 [57], two proteins involved in termination. Originally the similarity shared by EF-G and the ternary complex led to several ideas concerning the role of macromolecular mimicry in protein synthesis. The first of these hypotheses stated that when EF-G·GDP left the ribosome after translocating the tRNA, it imprinted the ribosome such that ternary complex binding was promoted due to the similarity in shape of the exiting EF-G·GDP and the incoming EF-Tu·GTP·Phe-tRNA [54, 58]. Macromolecular mimicry can also be interpreted such that the structural similarity of EF-G·GTP and the ternary complex allow both to stabilize an intermediate state of the ribosome between the PRE and POST states and thus promote the transition between the two [59]. In either case, molecular mimicry seems to exist because of constraints put on the factors by the ribosome such that they have to adopt a shape similar to a tRNA to interact functionally with the A site.

1.1.4.3 Factor binding site on the 70S ribosome

Inherent in the molecular mimicry hypothesis is the idea that the ribosomal factors interact at a similar site on the ribosome, and this is supported by several lines of evidence, including: (1) EF-Tu, IF-2 and Tet(M) all compete with EF-G for binding to the ribosome [60-62], (2) EF-Tu and EF-G have overlapping DMS footprints on the α-sarcin loop (H95) of the 23S rRNA [63], and this loop has been shown to be an important determinant for binding of both factors [64], (3) hydroxyl-radical probing experiments show that RF1 and EF-G are near similar rRNA elements [65, 66], and (4) cryo-EM reconstructions show EF-G, EF-Tu, and Tet(O) bind to similar regions on the ribosomes [47, 67-70]. These data define a

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[v] A recent report suggests that although RRF may structurally mimic the tRNA it does not interact with the ribosome in a similar manner as a tRNA and therefore may not be a functional mimic [55].

Figure 1-7: Molecular mimicry in translation

Molecular mimicry among several proteins involved in translation is shown. On the left the EF-Tu•tRNA complex represented with the tRNA moiety coloured yellow and with EF-Tu blue. In the centre EF-G is illustrated with domain III, IV, and V –the domains proposed to mimic the tRNA – coloured yellow and domains I and II coloured blue. On the right RRF, a tRNA mimic, is coloured yellow. The PDB files used to illustrate the structures are 1DD5 [56], 1FNM [71], and 1TTT [38]. These figures were prepared with SwissPDB viewer [23], and PovRay (www.povray.org).
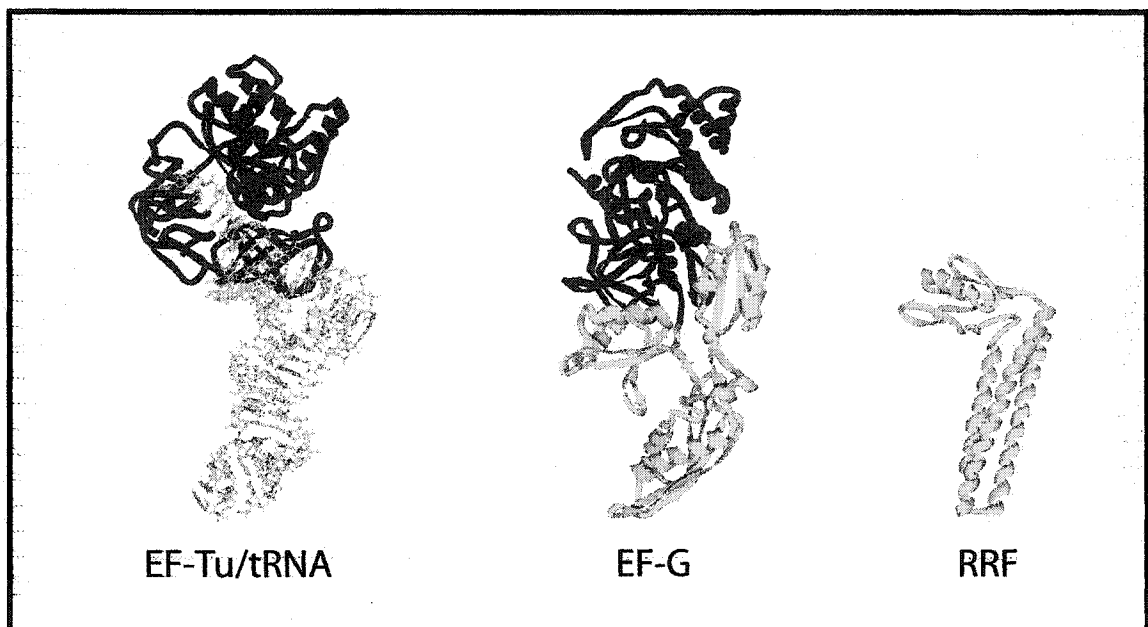
EF-Tu/tRNA   EF-G   RRF

common binding site located at the interface of the two subunits, beneath the L7/L12 stalk (Figure 1-12).

Although there are no high-resolution structures of ribosomal factors bound to the ribosome, with the exception of IF1 [72] and the C-terminal domain of IF3 [73], cryo-EM has been used to localize interactions between the factors and the ribosome by docking crystal structures into the cryo-EM density. Results of this nature, from the EF-G, EF-Tu and Tet(O) cryo-EM reconstructions [47, 69, 70], are summarized and compared in Table 1-1. Here it can seen that the conserved domains, the G-domain and domain II, also have similar ribosomal contacts, namely with H95[vi] and h5. In contrast, domain IV, which appears to be intimately involved in EF-G-dependent activity (section 1.1.4.1), has distinctly different ribosomal contacts when EF-G and Tet(O) are compared.

1.1.4.4 Mechanism of EF-G promoted translocation

In general the role of the last three domains in EF-G seems to involve the translocation reaction (section 1.1.4.1) where they are proposed to mimic the tRNA (section 1.1.4.2). As such they could insert into the A site and either actively push the A site bound tRNA to the P site, or simply occupy the A-site which was vacated through a ribosome-inherent translocation and prevent the tRNA from sliding back [50, 65]. In accordance with the role of domain IV in translocation, site-direct hydroxyl radical probing suggests that domain IV is located in the decoding site [65]. Additionally, cryo-EM reconstructions of both EF-G and EF-2 demonstrate that the tip of domain IV is located in the A site such that it overlaps with the A site bound tRNA (Figure 1-13), and contacts H69 a component of the major intersubunit bridge B2a [47, 74, 75].

[vi] Throughout this manuscript helix abbreviated with a lower case "h" refers to a helix within the 16S rRNA while an uppercase "H" refers to one within the 23S rRNA.

Table 1-1: EF-G, Tet(O), and EF-Tu interactions with the ribosome[A]

Domain	EF-G	Tet(O)	EF-Tu•GTP•aa-tRNA
G	H95	H95	H95
II	h5	h5	h5
III	S12	S12	
IV	**h69**	**h18/34**	n/a
V	h43/44	h43/44	n/a
tRNA	n/a	n/a	t-loop with h43; acceptor arm with S12;

[A] this table is adapted from ref. [47]
n/a, not applicable

In any case, translocation may involve a conformational change in EF-G since the introduction of a disulphide bond at the interface of the G domain and domain V, which should decrease flexibility, results in a loss of translocase activity [76]. A conformational change is also suggested by cryo-EM reconstructions since the density corresponding to EF-G does not accommodate the crystal structure unless domains III, IV, and V are allowed to rotate relative to domains G and II [75]. Although this conformational change has not been observed in the two crystal structures of EF-G obtained to date (nucleotide-free and GDP bound) a conformational change is observed when comparing EF-Tu crystal structures in the GDP and GTP (ternary complex) form. This rearrangement is associated with changes in the switch regions of the G domain and results in a 90° rotation (maximal movement of 40 Å) of domains 2 and 3 relative to the G domain [77].

1.2 The tetracyclines

Upon their introduction into medicine in 1948, tetracyclines were quickly accepted because they offered a broad spectrum of activity, being active against Gram positive and negative bacteria as well as Chlamydia, mycoplasmas, rickettsia and some protozoan parasites [78]. However, this may not represent the first human exposure to tetracyclines as consumption may extend back as far as 350-550 AD based on the 'serendipitous observation' of fluorescent staining in bones from Sudanese-Nubian civilizations reminiscent of the staining observed in present-day patients treated with tetracyclines [79]. The first member of the tetracycline family to be discovered and applied by modern medicine was chlorotetracycline (1948) and this was followed shortly, by the discovery of oxytetracycline (1953), tetracycline (1953) and finally demethylchlorotetracycline (1957) (reviewed in ref. [80]). These compounds are natural products produced by *Streptomyces* species while chemical modification of these compounds led to the development of semi-synthetic derivatives with improved pharmacokinetics

including, the medically relevant, methacycline (1965), doxycycline (1967), minocycline (1972) and glycylcyclines (1993) (reviewed in ref. [80, 81]).

The basic structure of a typical tetracycline is shown in Figure 1-8 where the central 4-ring structure is substituted with a variety of functional groups to produce the derivatives described above. In general chemical modification of the groups in the shaded region (Figure 1-8) leads to a loss of biological activity while modification at the other positions is allowed (reviewed in ref. [78, 83]). The tetracyclines in Figure 1-8, are all inhibitors of protein synthesis with bacteriostatic effects and are thus classified as typical tetracyclines in contrast to the atypical tetracyclines that exert bactericidal effects by disrupting cellular membranes [84, 85].

1.2.1 Inhibitory Action

Early *in vivo* studies showed that protein synthesis in *Staphylococcus aureus* was the most sensitive cellular process to the presence of tetracycline, suggesting that this pathway is the target of tetracycline's antimicrobial activity [82, 86]. More specifically, polysome breakdown experiments using metabolically active *Bacillus* protoplasts suggested that it is the elongation phase (section 1.1.3) of protein synthesis that is primarily inhibited [82, 87]. The precise step in the elongation phase was elucidated *in vitro* by several groups, who showed that tetracyclines block binding of aa-tRNA to the ribosome [88-91]. It can be seen in Figure 1-5 that blocking aa-tRNA binding (step 1a-b) would have a detrimental effect on the elongation cycle because this reaction brings in the new amino acid required for extension of the polypeptide.

Although tetracycline's role as an A site inhibitor represents its primary inhibitory effect, tetracycline has shown many secondary effects on protein synthesis. These effects include blocking P site binding and preventing the interaction of release factors with the termination codons (reviewed in ref. [82]).

Figure 1-8: Chemical structures of the tetracyclines

The basic chemical structure of tetracycline is drawn where the ring structure is substituted as indicated in the table to produce specific tetracycline derivatives. The shaded region, on the tetracycline structure, indicates positions that upon substitution result in a loss of biological activity. The compounds that are unshaded in the table represent the first-generation tetracyclines derived from *Streptomyces* species, whereas the compounds shaded in light grey are the second-generation semi-synthetic tetracycline derivatives and those shaded in dark grey are the third-generation glyclcyclines. This figure has been adapted from ref. [82].
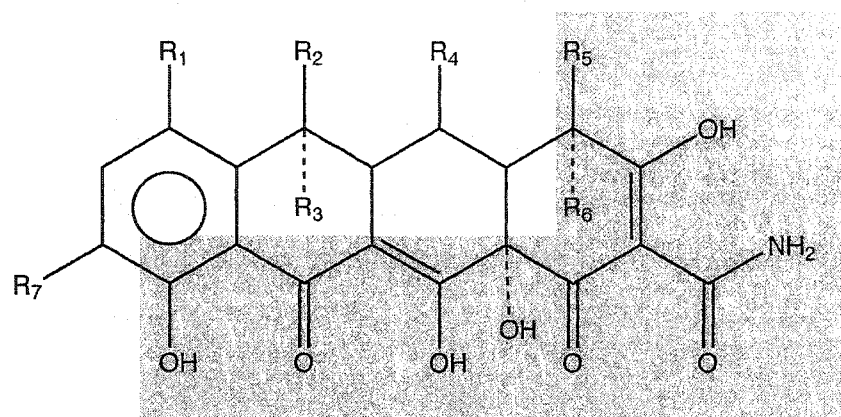
Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Tetracycline	H	OH	CH_3	H	H	$N(CH_3)_2$	H
Oxytetracycline	H	OH	CH_3	OH	H	$N(CH_3)_2$	H
Chlorotetracycline	Cl	OH	CH_3	H	H	$N(CH_3)_2$	H
Demethylchloro-tetracycline	Cl	OH	H	H	H	$N(CH_3)_2$	H
methacycline	H	CH_2	N/A	OH	H	$N(CH_3)_2$	H
doxycycline	H	H	CH_3	OH	H	$N(CH_3)_2$	H
minocycline	$N(CH_3)_2$	H	H	H	H	$N(CH_3)_2$	H
GAR-936	$N(CH_3)_2$	H	H	H	H	$N(CH_3)_2$	9-t-butyl-glycylamido

1.2.2 Interaction with the bacterial ribosome

Tetracycline binding to a single high affinity site (K_d=1-20 μM) on the 30S subunit is generally presumed to confer the inhibitory effect of the drug on A site occupation (ref. [92] and references within). This is significant, as tetracycline has been shown to interact with both the 50S and 30S ribosomal subunits where studies have shown that at high concentrations several hundred molecules of tetracycline can be bound to a single ribosome [93, 94].

In the simplest case, one would expect that this single inhibitory site is in close proximity to the ribosomal A site as it is aa-tRNA binding to this site that is inhibited by the drug. Several groups have attempted to define the tetracycline binding site using photo-affinity labelling [92, 95], chemical probing [96], single-protein omission reconstitution studies [97], UV-crosslinking [98] and most recently, X-ray crystallography [73, 99]. The results of these studies are summarized in Table 1-2 and are correlated with the crystallographically determined sites (described below).

Interpretation of the biochemical studies summarized in Table 1-2 are generally complicated by the fact that (1) tetracycline is known to be photo- and thermo-labile and therefore the observations in the experiments summarized in Table 1-2 may be due to degradation products and not native tetracycline [92], (2) multiple tetracycline binding sites on the ribosome make it difficult to know if the observations in the experiments are due to tetracycline binding the inhibitory site [92], and (3) particularly in the photo-labelling experiments, the photoproduct may move from the site of binding before becoming covalently attached or may preferentially photo-label from a site that is not the major binding site. Despite these problems several groups persisted and provided a significant body of evidence characterizing the tetracycline binding site, the most convincing being the studies on the crystal structures of *Thermus thermophilus* 30S subunits soaked with tetracycline (Figure 1-9A and B; ref [73, 99]).

Figure 1-9: Tetracycline binding sites

(A) The location of the tetracycline binding sites determined by Brodersen et al. are shown, where tetracycline bound in the primary site is red (surface representation) and tetracycline bound in the secondary site is orange [99]. The structure shown is derived from the 3.4 Å model (PBD accession is 1HNW).

(B) The location of the tetracycline binding sites determined by Pioletti et al. are shown, where tetracycline bound to the Tet-1 site is red, to the Tet-2 site is dark blue, the Tet-3 site is cyan, the Tet-4 site is green, the Tet-5 site is orange, and the Tet-6 site purple [73]. The structure shown is derived from the 4.5 Å model (PBD accession is 1I97). Tetracycline bound to the Tet-1 site is nearly identical to that in the primary site (for a comparison see panel C) whereas the Tet-5 and secondary site are quite distinct (see panel F for a comparison).

(C) The primary tetracycline binding site, formed by h34 (blue tube; 1196-1200:1053-1056) and the loop of h31 (yellow tube; 964-967), from the Brodersen and Pioletti structures have been superimposed to highlight the similarity of the tetracycline binding site. In this merged structure tetracycline bound to the primary site is dark grey and tetracycline bound to the Tet-1 site is light grey.

(D) The interactions, as proposed by Brodersen et al., between tetracycline and the rRNA of the primary site are illustrated [99]. Positions that can be substituted without loss of biological activity are shaded. This figure has been reproduced from ref. [99].

(E) The structure of tetracycline bound to the 30S subunit (1HNW; ref. [99]) has been merged with the structure of an anti-codon stem-loop (ASL) bound to the 30S A site (1IBM; ref. [29]) to illustrate the steric clash between an A site bound tRNA and tetracycline bound at the primary site. The ASL (blue molecule) is base pairing with a mRNA (yellow molecule) while tetracycline (purple surface) is bound to the primary site. In this position there is an obvious clash (indicated with arrow) of the tRNA (around A34), with tetracycline. Similar conclusions were made by Pioletti and Brodersen when they docked tRNA derived from the 70S(tRNA)₃ [100] with the tetracycline bound 30S subunit.

(F) The components of the secondary tetracycline binding site, H11 (mauve) and H27 (purple) have been superimposed to highlight the difference between the secondary [99] and Tet-5 site [73]. In this merged structure tetracycline bound to the secondary site is dark grey and that bound to the Tet-5 site is light grey. These figures were prepared with SwissPDB viewer [23], VMD [24], and PovRay (www.povray.org).

A Primary, Secondary

B Tet-4, Tet-6, Tet-3, Tet-1, Tet-2, Tet-5

C H31, H34, Tet-1, primary tc

D

E tRNA, tetracycline, mRNA

F secondary tc, H27, H11, Tet-5
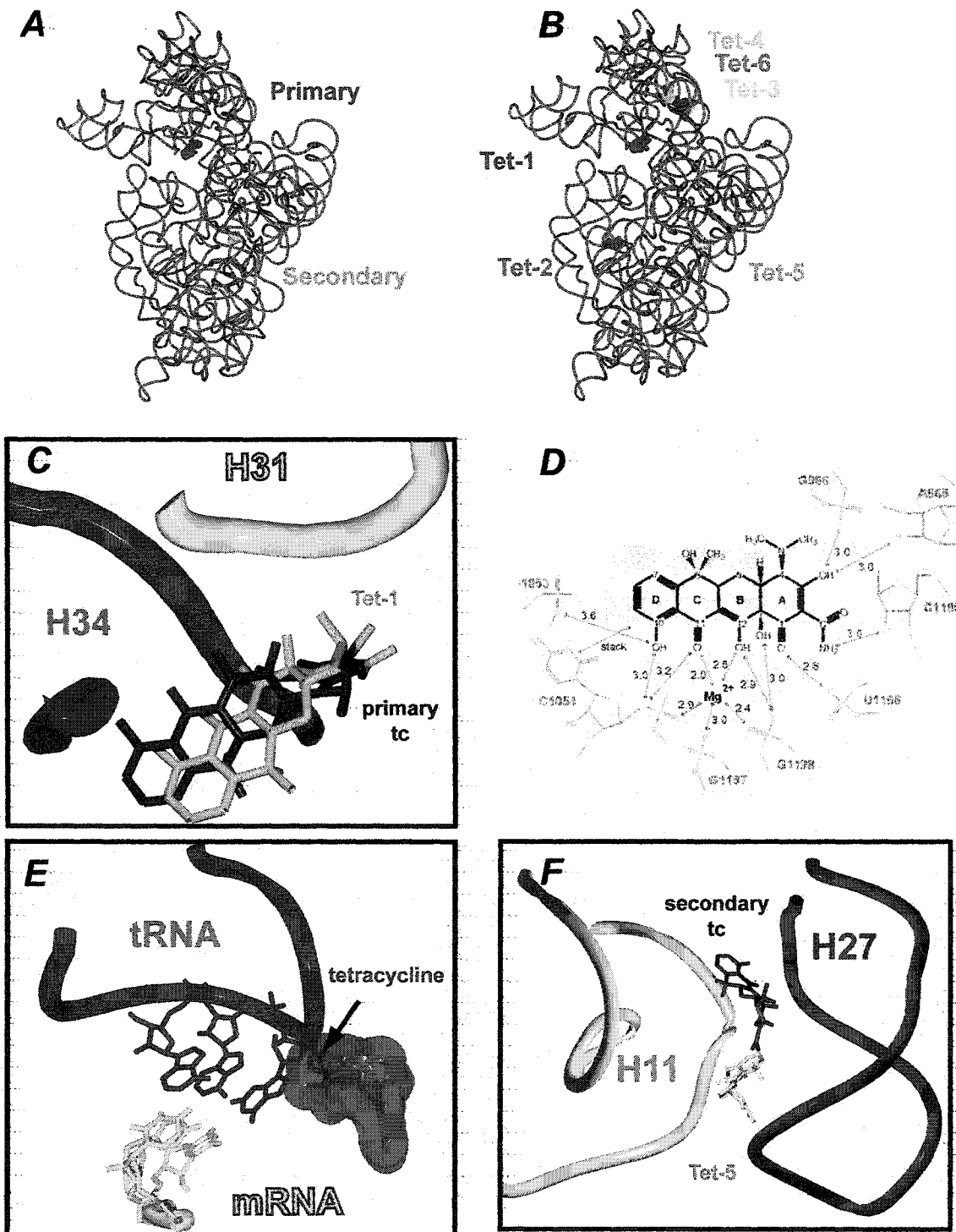
Table 1-2: Ribosomal components that interact with tetracycline

Technique	Reference	Component identified	Correlation to binding sites derived from X-ray crystallography
Photo-affinity labelling[B]	Goldman 1983	S7	Arg 4 and 5 of S7 are components of the Tet-6 site and could therefore be readily labelled [73]. The Tet-3 and –4 site may also label S7 assuming the photo-product can diffuse before incorporation (Figure 1-10C)
Chemical probing[C]	Moazed 1987	A892	A892:N1 is proposed to H-bond with Tc in the secondary site thus explaining the decreased DMS reactivity of this base (Figure 1-10B) [99]. The Tet-5 site does not directly interact with A892 but it is in close proximity and therefore may protect this base by an indirect conformational change [73].
		U1052	U1052 is close to the tet-1 site and primary site but does not make direct interactions or change conformation. It is important to note that U is not normally reactive with DMS in a manner that can be detected by reverse transcription at physiological pH [101]. Therefore the enhancement of U1052 may indicate tetracycline binding is subtly altering the chemical environment near the primary site [73, 99].
		C1054	C1054 actually is shifted by tetracycline binding to the primary site and tet-1. This conformational change may explain the increased DMS reactivity of C1054 in the presence of tetracycline [73, 99]
Photo-affinity labelling	Oehler 1997	G890	G890 is in close proximity to the secondary and Tet-5 sites may label G890 assuming the photo-product can diffuse before incorporation (Figure 1-10B)
		G693 G1300 G1338	G693, G1300, and G1338 are in close proximity to the Tet-3, -4, and 6 sites may be labelled assuming the photo-product can diffuse before incorporation (Figure 1-10C)
UV-cross linking[D]	Noah 1999	U244xG894	U244 and G894 are components of both the secondary and Tet-5 sites (Figure 1-10B) [73, 99] and therefore tetracycline binding could explain the disruption of this cross-link.
		C967xC1400	C967 is a component of the primary and Tet-1 site (Figure 1-10A) [73, 99] and therefore tetracycline binding could explain the disruption of this cross-link.
		C1402xC1501	C1402 and C1501 are close to the primary site and Tet-1 sites but tetracycline does not interact with either and therefore the enhancement of this cross-link may be do to tetracycline induced conformational changes [73, 99]

[A] this table is adapted from Pioletti *et al.* (2001).

[B] Photo-affinity labelling takes advantage of tetracycline's photo-reactive properties such that upon irradiation (~ 366 nm) tetracycline can react and become covalently linked to RNA and protein.

[C] chemical probing experiments monitor changes in the reactivity of the rRNA bases towards specific chemical probes. The chemical reactivity of an individual base can increase, remain the same, or decrease when a ligand is bound to the ribosome, thereby indicating sites of interaction (direct or indirect).

[D] upon UV-irradiation specific cross-links are formed between ribosomal components. Noah *et al.* monitored changes in the UV-dependent cross-links upon tetracycline addition which are interpreted much the same as the changes in chemical reactivity discussed above.

Tc, tetracycline.

1.2.2.1 The primary tetracycline binding site

The two independent ribosome/tetracycline crystal structures show tetracycline bound to either 2 [99] or 6 sites [73] on the 30S subunit (Figure 1-9A and B respectively). The most highly occupied binding site (red molecule; Figure 1-9A-B), the Tet-1 and primary site on the Pioletti and Brodersen structures, respectively, is located in the ribosomal A site. This site in the two structures is roughly equivalent (Figure 1-9C) such that tetracycline is bound by the irregular minor groove of h34 and the loop of h31 [73, 99]. In this binding pocket tetracycline primarily interacts via hydrogen bonds, or Mg^{++} mediated salt bridges, with the sugar-phosphate backbone of the rRNA[vii] (Figure 1-9D; ref. [73, 99]). Tetracycline binding to this site, as stated by the authors and shown in Figure 1-9E, could sterically interfere with A site occupation [73, 99]. Interestingly, Brodersen *et al.* point out that during the initial stages of aa-tRNA binding, when the aa-tRNA is still bound in the A/T site (section 1.1.3) tetracycline would not clash with the tRNA. This, Brodersen *et al.* state, would explain the observation that tetracycline does not inhibit the GTPase activity of EF-Tu but does inhibit subsequent A site occupation [91, 99].

The molecular mechanism of tetracycline action can easily be inferred from its location in the A site. Brodersen *et al.* hypothesize that, with tetracycline bound to the primary site, the ternary complex would be able to initiate decoding (Figure 1-5; step 1a), such that the interaction between the codon and the anticodon of the A/T bound aa-tRNA would be unaffected by the presence of the drug [99]. The subsequent step (Figure 1-5; step 1b) involving the release of the aa-tRNA from EF-Tu and its accommodation into the A site, however, would be inhibited, such that as the aa-tRNA rotates into the A site, the anticodon-loop of the tRNA would clash with tetracycline (Figure 1-9E; ref. [99]). Although the

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[vii] The reliance on sugar-phosphate backbone interactions, as opposed to base-specific interactions, could explain tetracycline's broad spectrum of activity despite the apparent lack of sequence conservation in the binding site. Furthermore this might explain the difficulty of obtaining 16S rRNA mutations conferring tetracycline resistance.

accommodation reaction is inhibited, EF-Tu-dependent GTP hydrolysis is not [91], and therefore Brodersen *et al.* speculate that a non-productive cycle of ternary complex binding, and GTP hydrolysis without A site occupation will ensue [99].

As summarized in Table 1-2, binding to this site is compatible with earlier chemical probing experiments showing that C1054 of the 16S rRNA experiences an increase in DMS reactivity [96] upon tetracycline binding because C1054 forms one end of the binding pocket and is slightly displaced during binding (Figure 1-10A; ref. [73, 99]). Additionally, a UV-dependent crosslink between C967 and C1400 of the 16S rRNA is disrupted by tetracycline binding [98] and C967 is located in the loop of H31, which forms part of the binding pocket (Figure 1-10A).

1.2.2.2 The secondary tetracycline binding site

In the Brodersen structure the site with the second highest occupancy, and henceforth called the secondary tetracycline binding site, is located in the so-called switch region [102] of the 30S subunit [99]. In the Pioletti structure [73] a similar site, Tet-5 (5th highest occupancy), is also observed but the exact nature of the binding site is somewhat different (Figure 1-9F). In the Brodersen structure the binding pocket is formed by h27 (residues 891-894:908-911) and h11 (residues 242-245; ref. [99]), whereas in the Pioletti structure the binding site is formed by h27 (residues 894-896) and h11 (residues 244-247; ref. [73]). Although not being in a position to directly interfere with tRNA binding, tetracycline bound to this site could exert its inhibitory effect by restricting the flexibility of h27. Helix 27 in *E. coli* has been suggested to switch between two base pairing conformations[viii], one that induces an error-prone, *ram*, state and one that induces a hyperaccurate, *restrictive*, state [102, 104]. In this case, it is

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[viii] Recent studies in *Saccharomyces cervevisiae* confirm that h27 is important for translational accuracy but do not support the claim that this helix switches between two base-pairing conformation in yeast [103].

possible that tetracycline bound to the secondary site may lock h27 in one of these two conformations and therefore affect decoding [73, 99]. In the crystal structures depicting tetracycline bound to the 30S subunit, h27 is in the *ram* configuration suggesting that it is this state, that is stabilized by tetracycline[ix] [73, 99]. Intuitively, the idea that tetracycline stabilizes a state where tRNA occupation should be relaxed – it is error-prone – does not fit with the idea that tetracycline inhibits tRNA binding. However, if during the course of A site occupation the ribosome switches between the *ram* and *restrictive* states than inhibition of this switch could prevent the overall accommodation reaction.

Tetracycline binding to the secondary site is compatible with data obtained from chemical probing experiments where tetracycline protected A892 (h27) from DMS modification, and cross-linking studies showing that tetracycline labelled G890 (h27) and inhibited a U244 (h11) x G894 (h27) cross-link (Figure 1-10B and Table 1-2; ref. [95, 98, 106]).

1.2.2.3 Additional tetracycline binding sites

The other 4 tetracycline-binding sites (tet-2, -3, -4, and –6) observed by Pioletti *et al.* are not so easily correlated with the inhibitory action of tetracycline but some do roughly correlate with earlier data from photo-labelling experiments (summarized in Figure 1-10C and Table 1-2). The authors propose [73], however, that these sites may have an effect on ribosome assembly[x], in particular the Tet-4 and –6 site which interact with S4 and S7, respectively, two proteins that initiate assembly of the 30S subunit [108].

[ix] It should be noted that in the crystal structures of the 30S subunit h27 is always in the so-called *ram* configuration [17, 18, 29, 72, 73, 99, 105].

[x] The ability of antibiotics to affect ribosome assembly is exemplified by erythromycin whose primary effect may involve preventing assembly of the 50S particle [107]

Figure 1-10: Comparison of tetracycline binding sites with biochemical data

(A) Tetracycline (purple) bound to the primary site is illustrated with h34 in blue, the loop of h31 in yellow and the top of h44 in red. U1052 and C1054 which where shown to experience a increase in DMS reactivity upon tetracycline binding [96] are shown as green wireframes. The C967xC1400 UV-dependent cross-link that is inhibited by tetracycline is represented as a dashed line between h31 and h44 [98]. The C1402xC1501 UV-dependent cross-link that is stimulated by tetracycline is represented as a dashed line between the two strands of h44 [98].

(B) Tetracycline (grey) bound to the secondary site is illustrated with h11 in mauve, and h27 in purple. A892, which is protected from DMS by the binding of tetracycline [96], is shown in green. This protection can be explained by the hydrogen bond (green dashed line) it forms with tetracycline though its N1 position. G890, which is labelled by tetracycline [95], is shown in yellow. The UV-dependent cross-link between U244 and G894 that is inhibited by tetracycline is shown as a black dashed line [98]. A hydrogen bond between tetracycline and U244, which may explain the different effects of tetracycline derivatives on the protection of A892 from DMS is marked with an asterisk[xi].

(C) The labelling of S7 (blue tube) [92] and G693, G1300, and G1338 (red spacefill) by tetracycline are correlated with tetracycline bound to the Tet-3, Tet-4, and Tet-6 sites (green spacefill). These figures were prepared with SwissPDB viewer [23], VMD [24], and PovRay (www.povray.org).
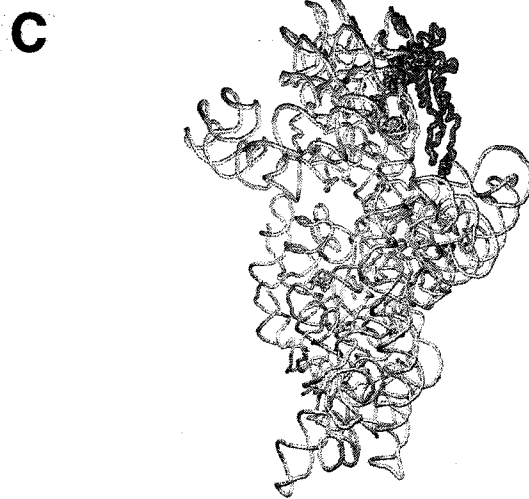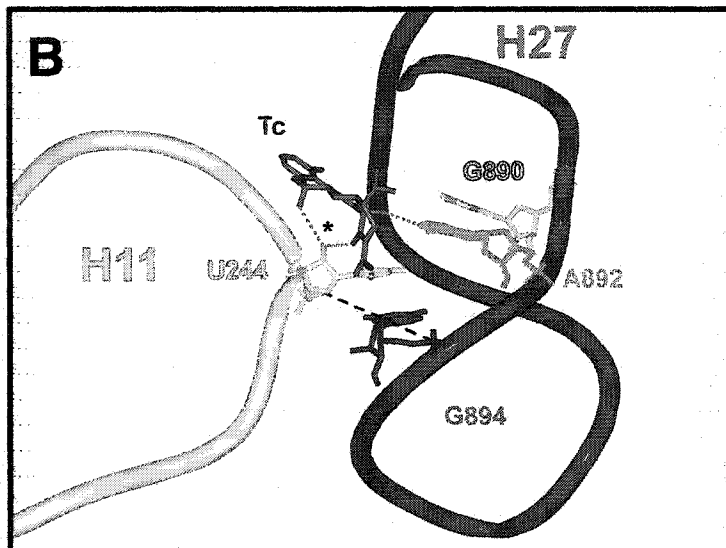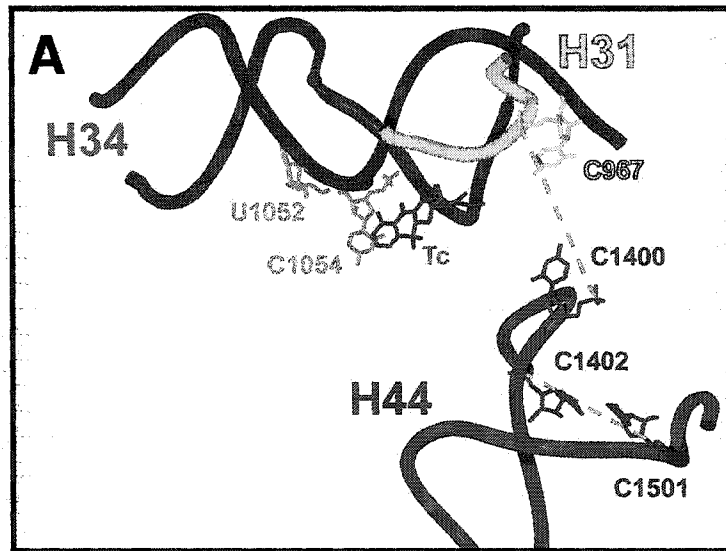
1.2.2.4 The inhibitory binding site

As mentioned above, tetracycline inhibition of elongation is proposed to originate from binding to a single site [109], which therefore presents the problem of determining which of these crystallographically determined sites is the biologically relevant inhibitory site. There is no direct evidence but several lines of indirect evidence suggest that the primary tetracycline binding site represents the inhibitory site:

(1) The primary binding site is the most highly occupied site in both structures [73, 99] fitting with the idea that the high-affinity site is the inhibitory site [93, 94, 109].

(2) The primary binding site is closely associated with the site of action of tetracycline and can explain the drug's inhibitory action (see section 1.2.2.1).

(3) Interactions within the primary binding site are mediated by a Mg^{++} ion which is known to be important for tetracycline binding [99, 110].

(4) 16S rRNA mutations seen in *Helicobacter pylori* [111, 112] and *Propionibacterium acnes* [113] that confer resistance to tetracycline are in close proximity to the primary binding site (see section 1.2.3.4; Figure 1-11A and B).

(5) The face of tetracycline that interacts with the rRNA in this site is that where substitutions result in a loss of biological activity (see section 1.2 and Figure 1-9D; ref. [99]).

(6) Tetracycline derivatives that bind the ribosome and inhibit protein synthesis all enhance the DMS reactivity of C1054 and U1052 in the 16S rRNA (associated with the primary tetracycline binding site; Figure 1-10A) while only a subset protect A892[xi] (associated with the

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[xi] Incidentally Chopra *et al.* suggest that the ability to interact with A892 might depend on the presence of a hydroxyl group at carbon 6 (Figure 1-9D). This is based on the fact that a hydroxyl group is present in chlorotetracycline and tetracycline, which protect A892 but absent in

secondary tetracycline binding site; Figure 1-10B) from DMS modification [84]. However, some atypical tetracyclines, which have no effect on protein synthesis, also enhance DMS modification of C1054 and U1052 [84].

(7) Tet(O), a protein conferring resistance to tetracycline, dislodges the drug from the primary binding site but not the secondary binding site when binding is followed by monitoring the DMS reactivity of A892, U1052, and C1054 (see section 3.2.3 and ref. [115]).

1.2.3 Resistance mechanisms

Resistance to tetracycline in bacteria has increased dramatically and has largely led to a decrease in the clinical effectiveness of most tetracyclines [78]. The factors leading to the increased incidence of resistance are not understood but the rise could be attributed to several sources. First tetracyclines are widely used in human and veterinary medicine because it one of the cheapest antibiotics while offering a broad spectrum of activity [78]. Secondly tetracyclines are used extensively in aquaculture and agriculture to control infections and additionally are used in sub-therapeutic levels as animal growth promoters [78]. Tetracycline resistance is usually associated with acquisition of one or more of the *tet* determinants described below and many of these *tet* genes are found on mobile genetic elements that can readily transfer between different genera, possibly explaining their wide dispersal [78].

1.2.3.1 Efflux-mediated tetracycline resistance

Efflux based mechanisms represent one of the most numerous tetracycline resistance determinants and are found in Gram-positive[xii] and Gram-negative[xiii]

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minocycline and doxycycline, which have no effect on A892 [114]. Accordingly tetracycline bound to the secondary binding site is proposed to interact via a hydrogen bond with U244 through this hydroxyl group [99].

[xii] The *tet*(Z), *tet* (K), *tet* (L), *otr*(B), and *tcr*3 determinants are found in Gram-positive bacteria [78]

[xiii] The *tet*(A), *tet* (B), *tet* (C), *tet* (D), *tet* (E), *tet* (G), *tet* (K), *tet* (L), *tet* (H), *tet* (I), *tet* (J), *tet* (Z) *tet* (30) and *tet* (31) determinants are found in Gram-negative bacteria [78].

Figure 1-11: rRNA mutations involved in tetracycline resistance

(A) The G1058 mutation (red wireframe) in h34 (blue) and **(B)** mutations is h31 (bases 965-967, yellow wireframe) that confer resistance to tetracycline are shown in relation to tetracycline bound to the primary binding site (purple wireframe). These figures were prepared with SwissPDB viewer [23], and PovRay (www.povray.org).
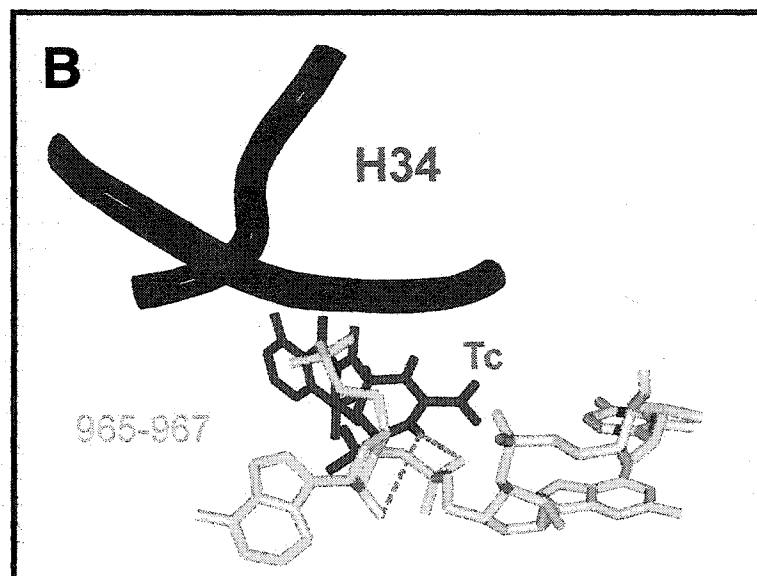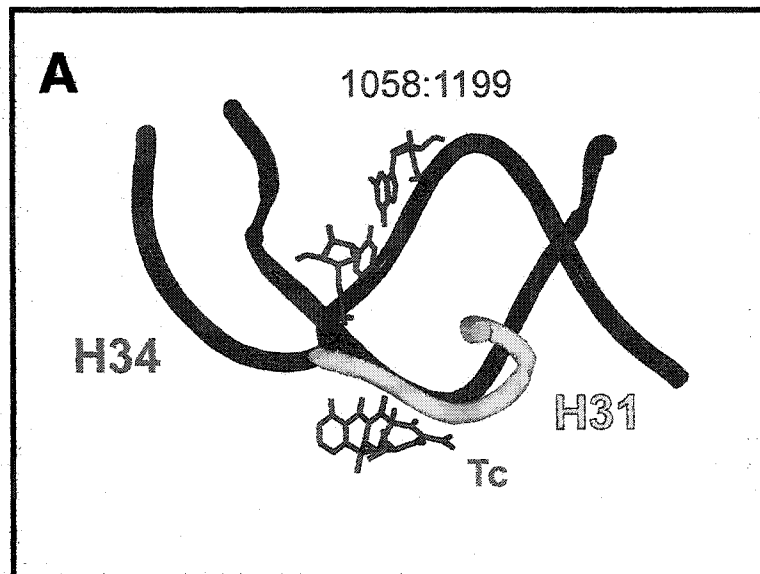
bacteria [78]. The efflux determinants, with the exception of Tet(B), can be phenotypically distinguished from the ribosomal protection determinants (section 1.2.3.2) because they confer resistance to tetracycline but not to minocycline unlike the ribosomal protection determinant which are active against both [78]. Efflux mechanisms operate on the principle that in order for tetracycline to inhibit bacterial growth it must concentrate within the cell and as a result, they prevent this intracellular accumulation. Accordingly, the efflux determinants encode a membrane protein, which actively pumps tetracycline out of the cell by coupling the export of tetracycline with the import of a proton (reviewed in ref. [116]). In general, the Gram-negative determinants, exemplified by Tet(A)-(C), encode a 43 kDa inner membrane protein which is believed to form multimers [116]. These proteins are proposed to consist of 12 membrane-spanning α-helixes which form 2 functional domains, the α- and β-domains [116]. The β-domain seems to have evolved from the α-domain through a gene duplication event [116]. The Gram-positive efflux determinants, Tet(K) and (L), differ from that described above such that they appear to be comprised of 14 membrane-spanning α-helixes forming 2 functional domains [116]. The first domain, α, appears related to the α-domain of the Gram-negative determinants based on sequence similarity, but the second domain, β, seems to have a unique origin [116].

1.2.3.2 Ribosomal protection protein-mediated tetracycline resistance

The ribosomal protection determinants[xiv] are also widely distributed with tet(M), the most highly dispersed determinant, being found in 8 Gram-negative genera and 18 Gram-positive genera [78]. The ribosomal protection proteins (RPPs) will be discussed in more detail below (section 1.3) but generally consist of a soluble cytoplasmic protein that dislodges tetracycline from its binding site on the ribosome thus preventing it from inhibiting protein synthesis [10, 11, 117-119].

[xiv] The ribosomal protection determinants include tet(M), tet(O), tet(S), tet(W), tet(Q), tet(T), otr(A) and tetP(B) [78].

1.2.3.3 Enzymatic inactivation of tetracycline

The tet(X) determinant is found on two *Bacteroides* transposons. Sequence analysis has determined that similarities exists between Tet(X) and NADPH-binding proteins [120]. Accordingly, Tet(X) enzymatically inactivates tetracycline in the presence of oxygen and NADPH [120-122]. This oxygen requirement presents an interesting puzzle because the natural host of Tet(X), *Bacteroides fragilis,* is an obligate anaerobe. Not surprisingly then Tet(X) does not confer resistance in its natural host, although it does so in aerobically grown *E. coli* cultures [123]. In this respect it is important to note that in *Bacteroides,* Tet(X) is found linked to the erythromycin resistance determinant *ermF* and this may be an important factor for maintenance of this element in *Bacteroides.*

1.2.3.4 Ribosomal mutations conferring tetracycline resistance

Recent studies have identified 16S rRNA mutations that confer tetracycline resistance [111, 113]. In *Propionibacteria* resistance arises from a G1058C mutation [113] which Brodersen *et al.* proposed would allosterically alter the primary tetracycline binding site by disrupting the G1058:U1199 base-pair (Figure 1-11A; ref. [99]). In another study a triple mutation in the loop of h31 (AGA965-967TTC) was found to confer resistance in *Helicobacter pylori* [111]. Subsequently the same h31 mutation was found in another tetracycline-resistant *H. pylori* strain in the Netherlands [112]. Helix 31 is a component of the primary tetracycline-binding site (Figure 1-11B) and it interacts with tetracycline through its sugar-phosphate backbone [73, 99]. Because the interaction is mediated by the backbone it is unlikely that resistance is directly due to sequence variation introduced by the mutation but rather the mutation, most likely, changes the architecture of the binding site. Trieber and Taylor postulated that this results in a decreased affinity for the ribosome allowing tRNA to effectively compete with tetracycline for A site occupation [11].

1.2.3.5 Miscellaneous / unknown resistance mechanisms

Tet 34 was isolated from tetracycline resistant Vibrio species found in the intestinal contents of an aqua-farm reared yellowtail fish [124]. Sequence comparisons show similarity between Tet 34 and xanthine guanine phosphoribosyltransferase (XPRT), which is involved in purine nucleotide salvage metabolism [124]. This prompted the authors to suggest that Tet 34 is involved in GTP production and reverses the effects of tetracycline by promoting an increase in the amount of free GTP which in turn would increase the amount of ternary complex (EF-Tu•GTP•aa-tRNA) thus facilitating A site occupation by aa-tRNA rather than tetracycline [124].

Chopra et al. also noted that Tet(U) and Otr(C) confer tetracycline resistance through unknown mechanisms because Tet(U) shows little sequence similarity to either efflux- or RPP-based resistance mechanisms while otr(C) has yet to be sequenced [78].

1.3 Ribosomal Protection Proteins

The ribosomal protection proteins (RPPs), as mentioned in Section 1.2.3.2, mediate tetracycline resistance by chasing the bound drug from its inhibitory site on the 70S ribosome. Tet(M) and Tet(O) are the best studied ribosomal RPPs and they share ~75% amino acid identity with each other and greater than ~40% amino acid identity with the more distantly related RPPs such as OtrA and TetB(P)[xv] [125, 126].

1.3.1 Isolation of tet(O)

Tetracycline resistance in Campylobacter jejuni is associated with the presence of large self-transmissible plasmids such as pUA466 [127, 128]. Initially the identity of the resistance determinant on these plasmids was elusive as

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[xv] The high degree of similarity between the RPPs suggests they operate in a similar manner and this assumption is made through this manuscript unless clear discrepancies have been demonstrated.

Southern blot experiments demonstrated that the then-known *tet*A-D determinants found in *Enterobacteriaceae* were not present on these plasmids [129]. The tetracycline resistance determinant could, however, be localized to a *Hind*III fragment which conferred tetracycline resistance when cloned into pUC8 and transformed into *E. coli* [128][xvi]. Subsequently Southern blot analysis showed that this determinant was similar to the Gram-positive *Streptococcal tet*(M) determinant [128]. Furthermore, a probe derived from the *Hinc*II fragment of pUA466 was able to hybridize (under stringent conditions) to plasmids derived from tetracycline resistant *C. jejuni* and *C. coli* but not to plasmids isolated from tetracycline sensitive strains [6]. In contrast, a probe derived from *tet*(M) would only hybridize under moderately stringent conditions to the plasmids associated with tetracycline resistance [6]. These results indicate that, in *Campylobacter*, tetracycline resistance is mediated by a single determinant and this determinant although similar to the *Streptococcal tet*(M) is distinct and therefore it was designated *tet*(O).

The *tet*(O) determinant derived from *C. jejuni* and *C. coli* was subsequently sequenced and a 1911-bp and 1917-bp ORF, respectively, was identified [8, 9]. The predicted product of these genes, Tet(O), is an ~72 kDa protein and shows 76-77% amino acid identity with Tet(M) [8, 9]. Two promoters are found upstream of this gene – designated P1 and P2 – where transcription initiation was mapped to the P1 promoter in *C. jejuni* and to both the P1 and P2 promoters in *E. coli* [130].

1.3.2 Origin of *tet*(O)

In the natural producer of oxytetracycline *Streptomyces rimosus*, tetracycline resistance is conferred by *otr*A, a RPP-like determinant [126]. Interestingly OtrA, like the other RPPs, shows sequence similarity to EF-G

[xvi] In *Campylobacter jejuni* pUA466 conferred resistance with an MIC of 64 μg/ml, which is identical to the MIC of *E. coli* transformed with the *Hind*III fragment, indicating there are no barriers to expression of Tet(O) in *E. coli* [128].

suggesting that a gene duplication event, where EF-G was used as the scaffolding to evolve a tetracycline resistance protein, led to the development of the RPPs. In any case, it appears that *otr*A was disseminated throughout the eubacteria by lateral gene transfer events [126]. The spread of the RPP between different bacterial species is substantiated by the fact that many RPP determinants are located on mobile genetic elements which would facilitate their spread (reviewed in ref. [78]). Furthermore, many of the individual determinants are found in several species of both the Gram-negative and Gram-positive type [78]. In the case of *tet*(O), codon usage is more similar to that in Gram-positive bacteria [9] and the G+C content of *tet*(O) is 40% [8, 9], much higher than the 30.6% G+C content of *C. jejuni* chromosomal DNA [131]. Also indicative of *tet*(O) originating from a Gram-positive source is the fact that the putative ribosome binding site for the *tet*(O) gene shows more complementarity to that of Gram-positive species than Gram-negative species [9, 132].

1.3.3 Regulation of RPP expression

In many cases the *tet* genes appear to be regulated, and this is especially true for many of the Gram-negative efflux determinants where, upstream of the efflux gene, an ORF encoding a repressor protein is often found (reviewed in ref. [78, 133]). This repressor protein, in the absence of tetracycline, inhibits transcription of the efflux gene by binding to the promoter region, but in the presence of tetracycline this repressor dissociates and transcription ensues (reviewed in ref. [133]). Accordingly, prior growth of bacteria, containing these resistance determinants, in sub-inhibitory concentrations of tetracycline results in an induction of transcription of the *tet* gene and subsequent production of the Tet protein [78, 133].

Experiments looking at induction of tetracycline resistance in the case of the ribosomal protection proteins show mixed results[xvii]. When the induction experiments used Tet(M) the authors reported that there is an increase in resistance[xviii] [117], an increase in Tet(M) expression[xviii] [117] and an increase in *tet*(M) transcripts[xix] [134]. In contrast, induction experiments with *tet*(O) cloned into *E. coli* [7], into *C. jejuni* [129] or with *tet*(O) produced in an *E. coli*-based *in vitro* translation system [7] show that prior exposure to sub-inhibitory levels of tetracycline has no effect on induction of high-level resistance.

Su *et al.* observed that the Tet(M) ORF is preceded by a small ORF and several sets of inverted repeats, some resembling a factor-independent terminator [135]. These qualities are reminiscent of the *trp* operon, which is regulated by transcriptional attenuation, and therefore Su *et al.* have proposed a similar mechanism for regulation of Tet(M) expression [135]. Although, as mentioned above, Tet(O) expression is constitutive and not inducible, there is a high degree of conservation between Tet(O) and Tet(M) in this upstream region [130]. Furthermore, deletions in the conserved upstream region of Tet(O) result in a decrease in the tetracycline MIC; deletion of the region upstream of the P1 promoter decreased the MIC from 80 μg/ml to 14 μg/mL in *E. coli* JM107 [130]. Trans-complementation of this deleted region does not restore the wild type MIC [130] suggesting that a diffusible product of this region is not involved in

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[xvii] Although not explaining the confusing results some of the discrepancies could arise from the fact that many induction experiments rely on following bacterial growth after tetracycline challenge by monitoring the A_{600}. In a report by Hash *et al.*, the authors state that *S. aureus* continues to grow after tetracycline challenge, but do not divide and are therefore not viable [86]. In this case the A_{600} of a culture inhibited by tetracycline would still increase even though it is, for all intents and purposes, not viable.

[xviii] Although this paper is often cited as showing that tetracycline resistance is induced, the actual effect is very small and only reproducible 50% of the time as stated by the author [117]. Additionally, this paper is also quoted as saying that Tet(M) expression increases but this can only be concluded indirectly from *in vitro* experiments showing that extracts prepared from cells grown in tetracycline are more resistant to tetracycline, and even then, controls are lacking.

[xix] The northern blot showing an increase in Tet(M) transcripts [134] is not well controlled and has problems with specificity and/or degradation, and is therefore not conclusive evidence. Additionally, the authors claim that prior exposure to tetracycline increases the MIC is not well documented [134].

tetracycline resistance. Further studies into the role of this upstream region in Tet(O) and the mechanism of transcriptional attenuation in Tet(M) may prove to be an exciting avenue of research.

1.3.4 General mechanism of the RPPs

Early studies on tetracycline resistance demonstrated that in many cases resistance is due to a reduced accumulation of the drug, but this explanation is not consistent with the Tet(M) or Tet(O) mechanism, as cells harbouring these determinants contained as much tetracycline as sensitive cells [10, 117]. Instead *tet*(M) was shown to confer resistance by making the protein biosynthetic machinery immune to the effects of tetracycline [117]. The resistance phenotype appeared to be conferred by a soluble factor, presumably Tet(O)/(M), in the cytoplasmic cell extract and not inherent to the ribosomes themselves [10, 118]. This was demonstrated by the fact that salt-washed[xx] ribosomes, isolated from resistant cells, were inhibited by tetracycline in *in vitro* protein synthesis assays, but sensitive ribosomes could be converted to resistant ribosomes by the addition of the 'wash'[xx] from resistant ribosomes [118]. Interestingly, ribosomes purified from resistant cells that were not salt-washed were also resistant in *in vitro* assays [10, 118] indicating that Tet(O) can associate with the ribosome. The amount of Tet(O) that associates and co-purifies is, however, sub-stoichiometric as it was not detected by RP-HPLC or SDS-PAGE analysis of purified ribosomes [10]. Accordingly, ribosomes purified from resistant cells bound tetracycline at identical levels to ribosomes from sensitive cells indicating that they were not inherently resistant[xxi] [10]. These results indicated that Tet(O)/(M) is a soluble protein and its continued presence is needed to maintain tetracycline resistance.

[xx] The salt-wash will strip away weakly associated molecules, i.e. proteins like elongation factors, from the ribosome. In this case these factors will be contained in the 'wash'.

[xxi] This result is probably over-interpreted since the authors have shown that their preparations of purified ribosomes (not salt washed see Methods in Enzymology 20:391) contain trace amounts Tet(O), namely because they are resistant in *in vitro* assays. In hindsight, had GTP been present in the reaction, tetracycline would have probably been released.

This suggested that Tet(O)/(M) is acting catalytically [10] and is not modifying the ribosome in any way such that it becomes permanently resistant. Instead it appears that Tet(O)/(M) transiently interacts with the ribosome and alleviates tetracycline inhibition of protein synthesis.

More specific indications of RPP activity were obtained when purified preparations of Tet(O) and Tet(M) became available [118, 136]. These studies nicely confirmed the results above such that the addition of purified Tet(O)/(M) to a tetracycline-inhibited poly(Phe) system could restore activity [11, 118]. In fact, Tet(O) could shift the IC_{50} of the poly(Phe) system from 100 μM tetracycline to over 500 μM [11]. Burdett was also able to show that tRNA binding to the A site, which is normally inhibited by tetracycline is, in fact, protected in the presence of Tet(M) [119]. This effect that can be attributed to the fact that Tet(O)/(M) can dislodge previously bound tetracycline from the ribosome [11, 119] and, in so doing, increase the apparent dissociation constant (K_D) of tetracycline binding to the ribosome from 5 μM to 30 μM.

Together, these results suggest that Tet(O)-mediates tetracycline resistance by chasing bound drug from the ribosome. This alleviates the inhibitory effect tetracycline has on protein synthesis or, more specifically, it allows tRNA to bind to the ribosome and elongation to continue.

1.3.5 Similarities between RPP and elongation factors

Further inferences about the mechanism of RPP action can be derived from the fact that the RPPs all display sequence similarity to the elongation factors EF-G and EF-Tu [36]. This sequence similarity is concentrated mostly in the N-terminal region of the proteins but in the case of the RPPs and EF-G the similarity, to a lesser extent, continues throughout the entire length of the proteins (Appendix 6.2; ref. [10]). Sanchez-Pescador *et al.* interpreted this to indicate that the RPPs are functioning as tetracycline resistant elongation factors [36], however, Burdett showed that Tet(M) cannot substitute for the elongation factors in *in vivo* or *in vitro* assays [118, 119].

Despite the fact that Tet(M) cannot substitute for the elongation factors, it has been demonstrated that they do display similar properties. For example, Dantley *et al.* showed that Tet(M) and EF-G compete for a similar site on the ribosome [61]. Additionally, the experiments of Dantley *et al.* demonstrate that a component of this common site, is the L11 region on the 50S subunit. This derives from the fact that thiostrepton, which binds this region and purportedly locks in it a conformation unfavourable for EF-G binding [60], also inhibits the binding of Tet(M) [61].

Manavathu *et al.* also expanded on the similarities of the elongation factors and the RPPs by pointing out that the conserved sequences in the N-terminal domain of the proteins include the GTP binding motifs found in many GTPases (Appendix 6.2; ref. [10, 40]). Building on this, it was subsequently shown that purified Tet(O)/(M) binds and hydrolyzes GTP and this reaction is strongly stimulated by the presence of 70S ribosomes [118, 136]. This ribosome-dependent GTPase activity is similar to that possessed by the elongation factors, where the rate of the reaction more closely resembles that of EF-G such that both hydrolyze ~60 pmols of GTP / min / pmol of protein [119]. This ribosome-stimulated GTPase activity is 18-20 times higher than endogenous activity of the factor [119]. This GTPase activity is not only a consequence of Tet(O) possibly evolving from the elongation factors but, it has functional significance as mutations in the G4 motif (Appendix 6.2) of Tet(O) lead to a decrease in its *in vivo* activity [137]. In this study, it was shown that replacement of Asn-128 - which purportedly H-bonds with the O_6 position of the guanine moiety - with amino acids harbouring long or cyclic side chains decreased the MIC 16-32-fold [137]. In contrast substitution with polar amino acids of equal or smaller size led only to a 4-fold decrease in the MIC [137].

These results indicate that, although sharing many properties (i.e. GTPase activity, ribosomal interactions, GTP binding interactions) with the elongation factors, the RPPs do not function as tetracycline-resistant elongation factors.

Instead, it is likely the RPPs were built on an elongation factor scaffolding and have maintained many of the elongation factors' properties, which the RPP found useful for mediating tetracycline release. For example, the elongation factors' ability to bind the ribosome can easily be exploited by the RPPs because the inhibitory target of tetracycline is the ribosome. This theme of adapting a new function on the framework of an existing protein is common in evolution.

1.3.6 The role of GTP-hydrolysis in RPP activity

In addition to a similar GTPase activity, the role of GTP hydrolysis in EF-G-promoted translocation and Tet(O)-promoted tetracycline release may be similar. Trieber *et al.* showed that Tet(O)-dependent release of tetracycline is dependent on Tet(O) being bound to GTP or an analogue of GTP [11]. Interestingly, non-hydrolysable analogues of GTP, such as GTPγS and GMPPNP, can promote Tet(O)-dependent release of tetracycline from the ribosomes when Tet(O) is used in excess but not when it is present in limiting amounts [11]. This indicates that GTP hydrolysis is not required for triggering the release of tetracycline but rather is important for recycling Tet(O) so it can act catalytically as would be necessary when it is present in limiting amounts [11]. EF-G has a similar property such that it can promote translocation when bound to GTP in multiple turnover experiments and when bound to non-hydrolysable GTP analogues in single turnover experiments [138]. In the case of EF-G, this led to the conclusion that GTP hydrolysis does not drive the reaction but rather, that the binding of EF-G·GTP to the ribosome promotes the reaction (reviewed in ref. [139]). This property is best explained thermodynamically by Spirin who proposes that binding of EF-G·GTP to the ribosome stabilizes a ribosomal transition state resulting in an increase in free energy [139]. By stabilizing the transition state the ribosome is "unlocked", allowing the tRNA to translocate from the A and P sites to the P and E site, however, the translocation reaction does not compensate for the increase in free energy associated with the EF-G·GTP·ribosome complex [139]. As a result, release of the factor requires an exogenic reaction, namely

GTP hydrolysis, where the liberated energy of the phosphate bound is dissipated solely as heat [139].

At this point it is necessary to note that this interpretation is controversial and that kinetic experiments have come to exactly the opposite conclusion, namely that the energy from GTP hydrolysis is converted to mechanical energy to drive translocation [50]. In the kinetic studies the authors acknowledge that a non-hydrolysable analogue can promote the translocation, as mentioned above, but at a rate much slower than native GTP [50]. This interpretation describing the role of GTP hydrolysis in EF-G function is more compatible with the results of Burdett where she observes that Tet(M) promotes the release of tetracycline only when GTP is present and not with non-hydrolysable GTP analogues [119] unlike the situation with Tet(O) [11]. It is unlikely that Tet(M) and Tet(O) function through two completely different reaction mechanisms but instead it is more likely that small differences in the reaction conditions have led to the conflicting results and, as in the case of EF-G, further work is necessary to clarify the situation.

1.3.7 Structural Studies of the mechanism of Tet(O)

The mechanism of Tet(O)-mediated release has been studied using cryo-EM. In these studies Tet(O)•GTPγS was complexed with a 70S ribosome programmed with MF-mRNA and deacyl-tRNA^{fmet} in the P site [47]. The final 16 Å 3-D reconstruction of this complex can be seen in Figure 1-12A (left) where a reconstruction of an EF-G•GMPPNP•70S complex (right) is shown for comparison [47, 75]. True to the idea that the RPPs evolved on a EF-G scaffolding, the density attributed to Tet(O) in the cryo-EM reconstruction has an overall shape similar to that of EF-G (compare red densities in Figure 1-12A-C; ref. [47]). This similarity in shape allowed the domain structure of Tet(O) to be tentively assigned (Figure 1-13, left) by comparison with the EF-G density (right) and X-ray crystal structures [37, 45]. Also evident in the reconstruction is that Tet(O) and EF-G are binding to a common site (Figure 12; ref. [47]). This common site is located at the interface of the ribosomal subunit on the A site side

Figure 1-12: Cryo-EM reconstructions of Tet(O) and EF-G ribosomal complexes

(A) Tet(O) and EF-G (red density; left and right panels, respectively) are bound to the 70S ribosome.

(B) The 2 factors (as in A) are shown bound to the 50S subunit such that the 30S subunit has been cut away and viewed from the interface side. The density of the P site bound tRNA^{fmet} (green) is now visible.

(C) The 2 factors (as in A) are shown bound to the 30S subunit such that the 50S subunit has been cut away and the 30S subunit is viewed from the interface side. Ribosomal elements previously shown to be involved in tetracycline binding (see Table 1-2) are indicated. These figures have been reproduced from ref. [47].

h, head; CP, central protuberance; h38, helix 38 of 23S rRNA; SB, stalk base; sp, spur; sh, shoulder; b, beak; st, stalk; pt, platform.
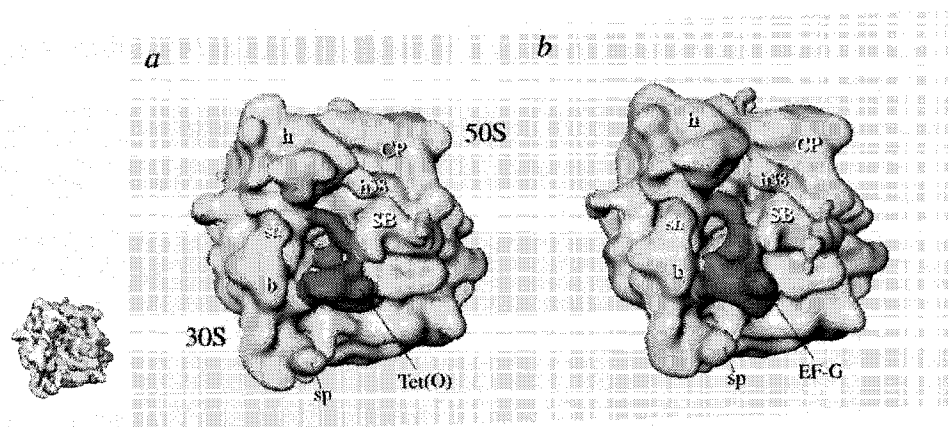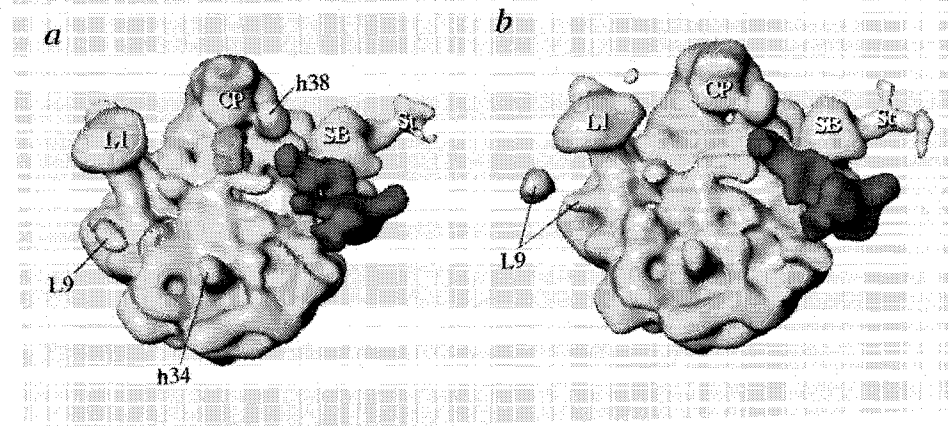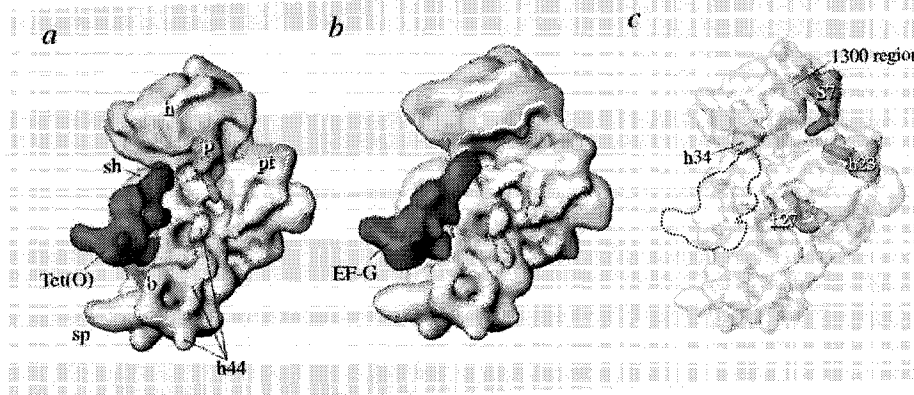
Figure 1-13: The relation of Tet(O) to the tRNA

Tet(O) (left) and EF-G (right) are shown relative to the A (purple) and P (green) site bound tRNA. The A-site bound tRNA was not present in the Tet(O) complex but instead was modelled using prior cryo-EM data [144]. This figure has been reproduced from ref. [47].
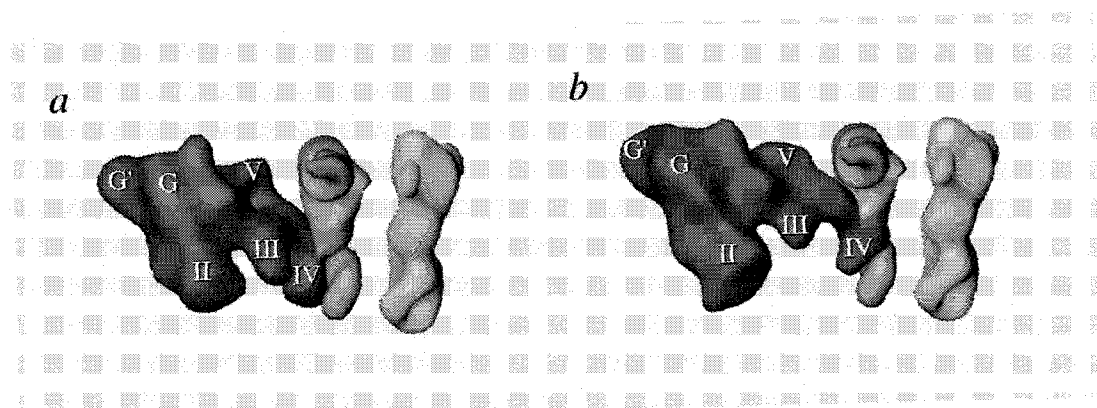
at the base of the L7/L12 stalk (Figure 1-12B; ref. [47]). This binding site is in agreement with the work of Dantley *et al.* implicating the L11 region in Tet(M) binding [61] since L11 and its associated rRNA comprise the stalk base [19].

EF-G·GMPPNP binding to the ribosome provokes drastic changes in the ribosomal architecture, for example, the L7/L12 stalk is extended (Figure 1-12B; ref. [140]), the ribosomal subunits rotate 6° with respect to one another [69], the L1 region moves, and L9 adopts an altered conformation (Figure 1-12B (right); ref. [47]). Tet(O), on the other hand, does not have such dramatic effects. When it is bound in the presence of GTPγS, it only produces changes in the ribosomal stalk (Figure 1-12B, left). These differences between Tet(O) and EF-G are understandable as the conformational changes are allegedly associated with the translocase activity of EF-G, a function Tet(O) lacks. In contrast, the extension of the stalk both in the presence of EF-G and Tet(O) is understandable as this extension is generally attributed to loss of flexibility in the stalk region upon factor/tRNA binding to the ribosome [140, 141].

In the cryo-EM study, Spahn *et al.* exploited the recent crystal structures of the ribosomal subunits [16-18] by docking them into the cryo-EM derived density map of the Tet(O)·70S complex to localize sites of interaction [47]. The sites of interaction between Tet(O) and 50S and 30S subunits are seen in Figure 1-12B-C and are summarized in Table 1-1. The most obvious conclusion from Table 1-1 is that the majority of the interactions are between Tet(O) and the rRNA [47]. The only exception being a single interaction between domain III of Tet(O) and the r-protein S12 [47].

Furthermore, a comparison of the EF-G and Tet(O) ribosomal contacts indicates that they differ primarily in the vicinity of domain IV (Table 1-1) where EF-G contacts H69 [47, 74] and Tet(O) interacts with h18/34 of the 16S rRNA [47]. This is significant as domain IV in EF-G has been implicated as an important determinant for promoting translocation of the tRNAs (see section 1.1.4.1). In this case, the difference in the position of domain IV of Tet(O) and EF-G may be what

ultimately distinguishes them with respect to their activities. In Figure 1-13, a density corresponding to A site bound tRNA (purple) is positioned with respect to the densities of a P site bound tRNA (green) and either Tet(O) or EF-G (red density; A and B, respectively). This model indicates that domain IV of EF-G more intimately overlaps with the A site bound tRNA, an idea that is consistent with the role of EF-G in translocation. In contrast, the interaction of domain IV of Tet(O) and h34 of the 30S subunit is consistent with its role in tetracycline release because, as mentioned in section 1.2.2.1 and illustrated in Figure 1-14, h34 is a component of the primary tetracycline binding site [73, 99]. It can be seen in Figure 1-14 that the density attributed to Tet(O) does not directly overlap tetracycline bound to this site. Accordingly, Spahn *et al.* propose that Tet(O) contacts the base of h34 which leads to a local disturbance in the conformation of this helix disrupting the primary binding site [47]. In other words, the molecular mechanism of Tet(O) action appears to include a conformational change within the decoding site such that Tet(O) acts allosterically to dislodge tetracycline from the ribosome. However, direct displacement of tetracycline cannot be completely ruled out as the tip of domain IV approaches within 6 Å[xxii] of the primary tetracycline binding site, a distance that can easily be spanned by unresolved elements of Tet(O).

1.4 Research Objectives

The aim of this work is to study the interaction of Tet(O) with the bacterial ribosome and identify ribosomal elements or functional centres that interact with Tet(O). The ribosome and its interactions with various factors have been extensively studied over the last 50 years (reviewed recently in ref. [143]). Identification of ribosomal contacts may highlight commonalities between Tet(O)

[xxii] For comparison a C-H covalent bond has a length of ~1 Å and a hydrogen bond a length of ~ 2 Å [142].

Figure 1-14: Tet(O) and the primary tetracycline binding site

Tet(O) (red density) is shown relative to the primary tetracycline binding site (1HNW; ref. [99]). Tetracycline (Tc) bound to the primary site is coloured green, h31 is blue, h34 is pink, h18 is yellow and S12 is a blue tube. The domains of Tet(O) are labelled according to those of EF-G. This figure has been reproduced from ref. [47].
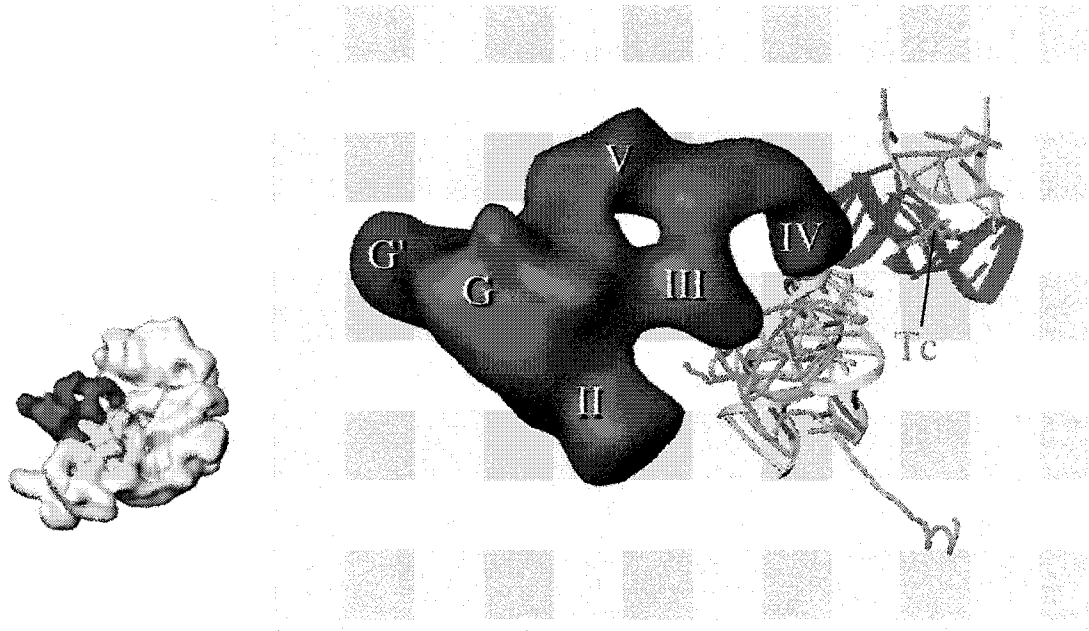
and previously studied factors, thus providing insight into the mechanism of Tet(O)-mediated tetracycline release. The interactions we identify may be important for Tet(O)-mediated tetracycline resistance where, in particular, they could be involved in the three major activities of Tet(O):

1) Ribosome stimulated GTPase activity

2) Ribosome binding activity

3) Ribosomal protection activity (removal of tetracycline)

Towards this aim Tet(O) and other components of the cellular translation pathway were purified for subsequent use *in vitro* assays (Chapter 2). These *in vitro* assays included binding Tet(O) to the 70S ribosome and identifying rRNA elements that interact with Tet(O) using chemical probing (Chapter 3 and 4). Following a similar methodology, the functional state of the ribosomal elongation cycle with which Tet(O) interacts is also identified (Chapter 4). The importance of some of these interactions for Tet(O) activity are confirmed using antibiotics which target the identified ribosomal elements (Chapter 4). Many of the identified interactions are associated with well-studied functional centres, namely the decoding centre and the GTPase-associated region and furthermore, the identified interactions are in good agreement with data derived from cryo-EM reconstructions showing Tet(O) bound to the ribosome. In Chapter 5 the data from all these sources are integrated into a final model describing the functional cycle of tetracycline and Tet(O) where possible molecular mechanisms for Tet(O)-mediated tetracycline resistance are discussed.

Chapter 2

Initial Purification and Characterization of Tet(O) and 70S Ribosomes

2 Purification and Characterization of Tet(O) and 70S Ribosomes

2.1 Introduction

Tet(O) is a ribosomal protection protein which confers resistance to the antibiotic tetracycline [132]. This determinant is generally associated with the presence of large self-transmissible plasmids in *Campylobacter* species, however, it has also reportedly been found associated with the chromosome in a single case [6]. Tetracycline is primarily a protein synthesis inhibitor and accordingly binds the ribosome, a central element in translation [82]. The binding of tetracycline to the ribosome impairs A site occupation by blocking incoming aminoacyl tRNAs, effectively preventing polypeptide elongation [88-91]. Tet(O) and the other RPPs appear to reverse the inhibitory effects of tetracycline by chasing the drug from its ribosomal binding site and allowing protein synthesis to continue [11, 119].

Currently we know that the RPPs have three characteristic activities. Firstly, they are able to hydrolyse GTP to produce GDP and inorganic phosphate [118, 136]. Interestingly, the GTPase activity of the RPPs is strongly stimulated by 70S ribosomes and accordingly they are grouped with the ribosomal GTPases [118]. The second characteristic activity of the RPPs is their ability to promote the release of bound tetracycline from the ribosome thereby protecting the ribosome from the inhibitory effects of the drug [11, 119]. This activity is henceforth referred to as the ribosomal protection activity (RP activity). It can be readily monitored by measuring tetracycline binding to the ribosome in the presence of a RPP using a filter-binding assay [11]. Or alternatively, by following the ability of the RPP to protect an *in vitro* protein synthesis assay, such as a poly(Phe) assay, from the inhibitory effects of tetracycline [11, 119]. Finally the RPPs display a ribosome binding activity which has been followed using size exclusion chromatography to

separate the ribosome bound RPP from the unbound RPP [11, 61]. With respect to this last activity the ribosomal binding site of Tet(O) has been characterized using cryo-EM, showing that it binds at the interface of the two ribosomal subunits below the L7/L12 stalk [47].

Much of the data describing RPP action has been derived from *in vitro* assays and accordingly both Tet(M) and Tet(O) have been purified to facilitate these assays [11, 61, 118, 136]. Here we cloned and purified Tet(O) with a N-terminal His_6-tag. As a consequence of cloning *tet*(O) we discover several deviations in its sequence as compared to that deposited in GenBank [8]. Additionally, the purified Tet(O) is characterized with respect to its GTPase and RP activities confirming that it is indeed active. Similarly 70S ribosomes are purified and characterized to confirm their activity. This initial characterization establishes the conditions needed to prepare Tet(O)•ribosome complexes in Chapters 3 and 4. Furthermore, this characterization provides useful insights that may facilitate the study of Tet(O)•ribosome interactions by structural methods.

2.2 Results

2.2.1 Cloning of Tet(O)

Previously *tet*(O) had been cloned into pMS119EH without a His_6-tag (pMSTetO) and with a C-terminal His_6-tag (pMSTetOHC), however, studies have shown that Tet(O)HC behaves differently from its homologue Tet(M) in *in vitro* systems [11, 119]. More specifically, Tet(M) was 2 times more inhibitory to poly(Phe) synthesis than Tet(O) and this could be due to the presence of a C–terminal His_6-tag on Tet(O)[i] [11, 119]. For this reason we cloned Tet(O) with a N-terminal His_6-tag such that it was over-expressed from pMS119EH [146] under control of the IPTG-inducible P_{Tac} promoter (Figure 2-1). The cloning strategy is

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[i] The CTD of EF-G has been suggested to be important for binding to the ribosome [145]. In this sense the C-terminal tag on Tet(O) could hinder its interaction with the ribosome and therefore it would appear to be less inhibitory to protein synthesis.

Figure 2-1: Cloning strategy for pMSTetOHN

(A) The primers used to amplify *tet*(O), Cat8 and Nch2 (Table 6-3), add the sequence encoding a 5' *Eco*RI site and a 3' *Bam*HI site (blue). Additionally, Cat8 adds a 5' sequence encoding a His_6-tag (orange) that is located between the start codon (green) and the second codon of the Tet(O) ORF (AAA; red). The A (purple) between the ATG and *Eco*RI site maintains correct spacing between the start codon and the ribosome binding site (RBS).

(B) The *tet*(O) construct shown in A is cloned into the *Eco*RI and *Bam*HI sites of pMS119EH to generate pMSTetOHN10 as shown. The CDS of *tet*(O) (blue) is shown relative to that of *bla* (red; Amp^r), LacI^q (cyan; Lac repressor) and the IPTG-inducible promoter (black arrow). The restriction enzyme recognition sites comprising the multiple cloning site (MCS) are also shown.

A

B
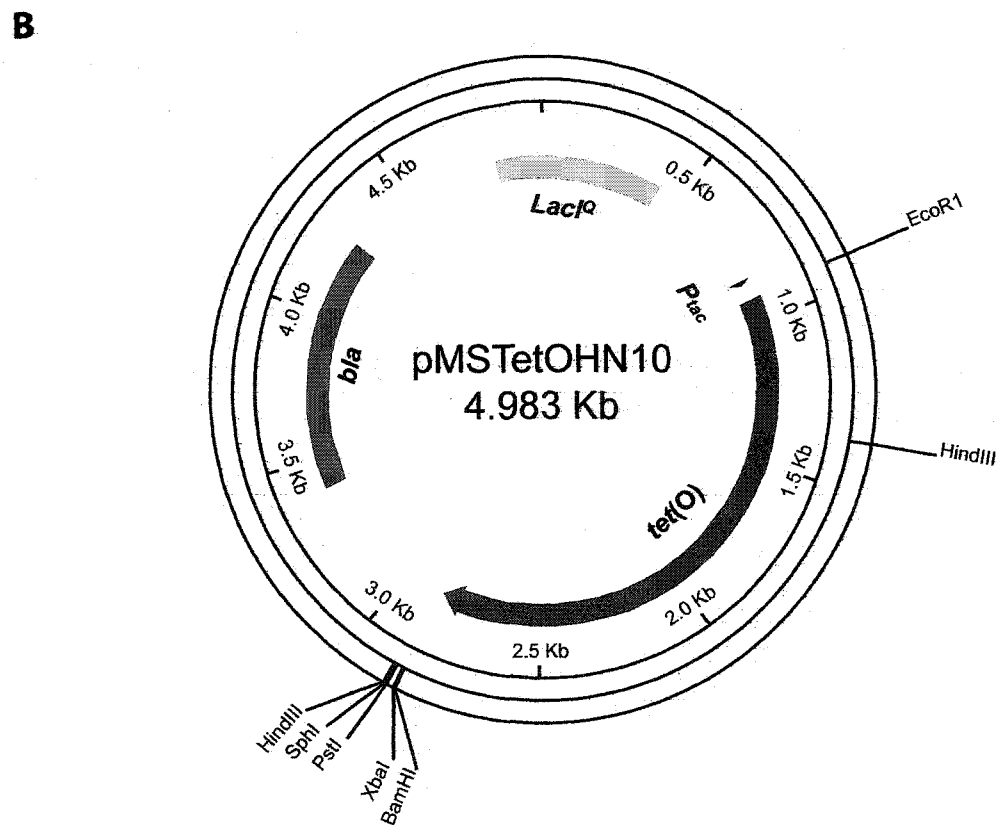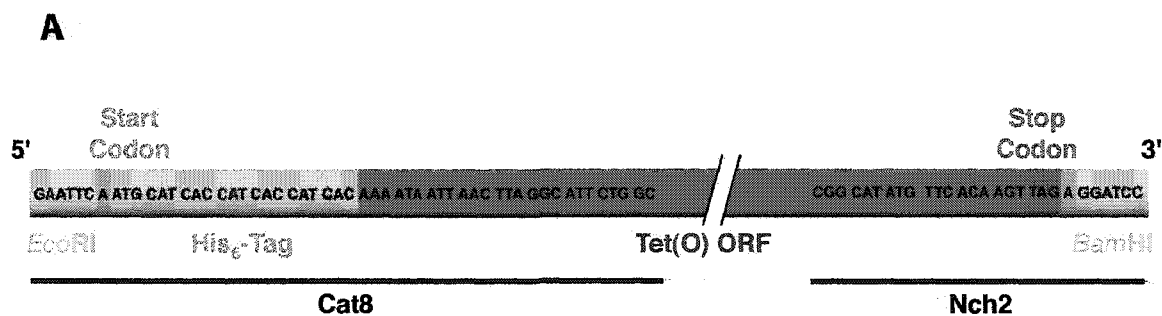
shown in Figure 2-1 and described in Experimental Procedures. In the resulting construct a sequence encoding an N-terminal His$_6$-tag was inserted between the natural start codon (ATG) and the codon encoding the second amino (AAA) acid (Figure 2-1). This construct when cloned into pMS119EH is designated pMSTetOHN10.

2.2.2 Sequence analysis of *tet*(O)

2.2.2.1 Sequence analysis of pMSTetOHN10

The plasmid, pMSTetOHN10, was isolated [147] and manually sequenced using the primers listed in Table 6-3. Sequence analysis confirmed that the His$_6$-tag was correctly fused to the N-terminus of the protein but identified a single point mutation, C1772A, (Figure 2-2A) resulting in a Thr591Asn substitution in Tet(O)[ii]. This point mutation was identified in all previous attempts to clone *tet*(O) (pMSTetOHN6 and 8) with an N-terminal His$_6$-tag. Furthermore, a comparison with the Tet(O) sequence from *C. coli* indicated that in this strain Tet(O) naturally had the Thr591Asn mutation [9], and *in vivo* activity assays indicated that Tet(O)HN10 was as active as the "wild type" Tet(O) previously cloned into pMS119EH[iii] (Table 2-1; ref. [11]). For these reasons we assumed the Thr591Asn mutation was silent and proceeded to purify and characterize Tet(O)HN10 as described in section 2.2.3.

2.2.2.2 Sequence analysis of pUOA2E1 and pUOA2

The fact that the C1772A mutation was observed in several independent attempts to clone *tet*(O) by PCR (section 2.2.2.1) strongly suggests that it was not due to a random error during replication of the template and, in fact, can indicate that this mutation may be carried on the template, pUOA2E1, used in the

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[ii] All numbering is given relative to the *tet*(O) sequence in GenBank (M18896) and seen in Appendix 6.1 where the 'A' of the start codon ATG or initiator Met is designated 1.
[iii] Subsequent sequence analysis confirmed, however, that the 'wild type' Tet(O), also carries the Thr591Asn mutation.

Figure 2-2: Sequence analysis of *tet*(O)

(A) A chromatogram showing the C1772A substitution (indicated with red box) in pMSTetOHN10.

(B) The chromatograms compare the sequence around 1906 (indicated with red box) to highlight the deletion of C1906 in pMSTet(O)HN10 with respect to pUOA2E1.

(C) The 3' sequences of several *tet*(O) genes are aligned and compared to illustrate the C1772A substitution found in all sequenced constructs except for that derived from GenBank (M18896). Additionally C1906 is deleted (Δ1906) in the GenBank sequence and in *tet*(O) constructs cloned based on the GenBank sequence (pMSTetOHN10 and pMSTetOHC).

(D) The C-terminal amino acid sequences derived from translating the *tet*(O) genes in C are aligned and compared. The C1772A substitution results in the Thr591Asn mutation, while the Δ1906 mutation causes a frame shift.

(E) The C-terminal amino acid sequences of a representative group of RPP determinants are aligned and compared. The Tet(O) C-terminal sequence derived from GenBank (M18896) is distinct from the other Tet(O) determinants while that derived from pUOA2 is identical.

A

pMSTetOHN10 C1772->A

ACAGGA[A]CGATTT

80   90

B

pUOA2E1 C1906

TTC[C]ACAAGT

pMSTetOHN10 ΔC1906

TTC[-]ACA--G

C

1757   C1772A   1826

```
M18896      TTCAAGAATACAGGA[C]CGATTTAACTTATTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGG
pUOA2       TTCAAGAATACAGGA[A]CGATTTAACTTATTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGG
pUOA2E1     TTCAAGAATACAGGA[A]CGATTTAACTTATTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGG
pMSTetOHN10 TTCAAGAATACAGGA[A]CGATTTAACTTATTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGG
pMSTetOHC   TTCAAGAATACAGGA[A]CGATTTAACTTATTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGG
```

1827   1896

```
M18896      ATACCAGCCAGCTATTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGG
pUOA2       ATACCAGCCAGCTATTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGG
pUOA2E1     ATACCAGCCAGCTATTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGG
pMSTetOHN10 ATACCAGCCAGCTATTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGG
pMSTetOHC   ATACCAGCCAGCTATTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGG
```

1897   ΔC1906   1914

```
M18896      CATATGTTC.ACAAGTTAGCTTAA.................
pUOA2       CATATGTTCCACAAGTTAGCTTAA.................
pUOA2E1     CATATGTTCCACAAGTTAGCTTAA.................
pMSTetOHN10 CATATGTTC.ACAAGTTAG......................
pMSTetOHC   CATATGTTC.ACAAGTGCACATCACCATCACCATCACTAG
```

D

T591N   Frame-shift

```
M18896      QEYR[T]DLTYFTNGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFTS.......
pUOA2       QEYR[N]DLTYFTNGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFHKLA.....
pUOA2E1     QEYR[N]DLTYFTNGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFHKLA.....
pMSTetOHN10 QEYR[T]DLTYFTNGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFTS.......
pMSTetOHC   QEYR[T]DLTYFTNGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFTSAHHHHHH
```

587   637

E

```
TETM_NEIME  PARCIQEMRSDLTFFTGRSVCLTELKGYHVTTGEPVCQPRRPNSRIDKVRYMFNKIT................
TETM_STAAU  PARCIQDYRNDLTFFTGLSVCLAELKGYQVTTGEPVCQTRRLNSRIDKVRYMFNKIT................
TETM_STRPN  PARCIQEYRSDLTFFTGRSVCLTELKGYHVTTGEPVCQPRRPNSRIDKVRYMFNKIT................
TETM_UREUR  PARCIQEIRSDLTFFTGRSVCLTELKGYHVTTGEPVCQPRRPNSRIDKVRYMFNKIT................
TETO_CAMCO  PARCIQEYRNDLTNFTGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFHKLA................
TETO_M18896 PARCIQSYRTDLTYFTGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFTS.................
TETO_pUOA2  PARCIQEYRNDLTYFTGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFHKLA................
TETO_STRMU  PARCIQEYRNDLTYFTGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFHKLA................
TETO_STRPN  PARCIQSYRNDLTYFTGQGVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFHKLA................
TETQ_PREIN  FYNTSKDYASEVSSIEKGLGVFMVKPCGTQITKDD.YSDNIRMNER.DRILFMFQESNVIKIMERSGNFYKAIQ
TETQ_PRERU  PLNTSKDYASEVSSIEKGLGIFMVRPCGTQITKDG.YSDNIRMNEK.DRILFMFQEKSMSLK...........
TETS_LACLA  PARCIQEYRNSLTFFTGRSVCLTELKGYQVTNIKSAFQPRRPFNFEIDKVRHMFNKINLH.............
TETS_LISMO  PARCIQEYRNSLTFFTGRSVCLTELKGYQVTNIKSAFQPRRPNFRIDKVRHMFNKINLH.............
TETW_BUTFI  PARCIQAVRTDLAFYTGRSVCLTELKGYQAAVGQPVIQPRRPNSRLDKVRHMFQKVM...............
```

582   637

Table 2-1: *In vivo* activity of TetOHN10

Strain	Tetracycline Concentration μg/ml									MIC[A]
	0	2	4	8	16	32	64	128	256	
JM109/pMS119EH	+[B]	+	+	-[B]	-	-	-	-	-	8
JM109/pMSTetO[C]	+	+	+	+	+	+	+	+	+/-	>256
JM109/pMSTetOHC[C]	+	+	+	+	+	+	+	+	-	256
JM109/pMSTetOHN10	+	+	+	+	+	+	+	+	+/-	>256

[A] MIC is the minimum inhibitory concentration, or the lowest concentration of antibiotic needed to prevent bacterial growth. It should be noted that these results were repeated recently and indicated that all the Tet(O) clones have an MIC of 64 μg/mL (Lisa Nonaka; personal communication). The differences with the reported results could be due to the different methods used, i.e. inoculum size and media used for growth.

[B] A '+' symbol indicated growth on the plate, whereas the '-' symbol indicates no growth.

[C] Dr. C.A. Trieber provided the pMSTetO and pMSTetOHC constructs.

PCR reaction. For this reason pUOA2E1 [130] was sequenced. This analysis demonstrated that the C1772A mutation is carried on pUOA2E1 (Figure 2-2C). Surprisingly it also indicated the sequence of tet(O) on pUOA2E1 differed from the GenBank sequence (M18896), such that a C was inserted at position 1906 (Figure 2-2B and C). As indicated in Figure 2-2D, this insertion results in a frame-shift in the translated protein affecting only the last 2 residues of the protein.

The deposited GenBank sequence was not derived from pUOA2E1, but instead corresponds to tet(O) carried on the HindIII fragment (6.9-kb) of pUA466 [8]. At the time UA466 was not available and for this reason sequence analysis was performed using the plasmid pUOA2 which harbours a ~5.1 kb fragment from pUA466 and confers tetracycline resistance [7]. This analysis revealed that pUOA2 carries the C1772A mutation and the C1906 insertion (Figure 2-2C).

These results indicate that Tet(O) from C. jejuni, like Tet(O) from C. coli [9], is probably naturally found with an Asn in position 591. Similarly the frame-shift that results from the insertion of a C at position 1906, changes the C-terminal sequence such that it matches exactly the other Tet(O) determinants (Figure 2-2E). With this sequence change the nature of the C-terminal end of Tet(O) is also more similar to the Tet(M) determinants and the strongly conserved Lys637 is now maintained in Tet(O) from C. jejuni (Figure 2-2E). The sequence of the Tet(O) gene and protein derived from pUOA2 is shown in Appendix 6.1. It is also important to note that the primers used to PCR amplify tet(O) for cloning the His$_6$-tagged derivatives (section 2.2.1) overlap the site of the insertion and therefore they produce a tet(O) clone with a 3' sequence identical to that in GenBank (Figure 2-2B and C)[iv].

[iv] tet(O) with the 'correct' 3' sequence has been cloned recently and confers the same MIC as the previously cloned pMSTetO, pMSTetOHC and pMSTetOHN10 (Lisa Nonaka, personal communication)

2.2.3 Purification and characterization of Tet(O)

2.2.3.1 Over-expression and purification of Tet(O)

In order to study the interaction of Tet(O) with the bacterial ribosome in *in vitro* studies as presented in Chapters 3 and 4, it was necessary to purify Tet(O). Tet(O)HN and Tet(O)HC were over-expressed in *E. coli* (see Experimental Procedures) but as noted previously the over-expression was not easily observed in a Coomassie stained gel [11]. Since Tet(O)HN and Tet(O)HC are present in such low amounts in the bacterial lysate we purified them from 8 L cultures using Ni^{++}-affinity chromatography as described in Experimental Procedures. Figure 2-3A shows the steps in the purification of Tet(O)HC by Ni^{2+}-affinity chromatography as followed on a Coomassie stained gel whereas Figure 2-3B shows the same for Tet(O)HN but with an immuno-blot. The cell extract is applied to the Ni^{2+}-affinity column and subsequently washed with buffer containing 5, 100^{v} and 300 mM imidazole (see Experimental Procedures). The buffers containing 5 and 50 mM imidazole wash the matrix, and release only small amounts of the bound Tet(O)HN (Figure 2-3B). At 300 mM imidazole Tet(O)HC and HN are efficiently displaced from the matrix and released from the column (Figure 2-3). The eluate from the Ni^{2+}-affinity column has a high salt and imidazole concentration, and therefore the buffer was exchanged using a G-75 Sephadex column. Subsequently, this protein solution was concentrated to 7-10 mg/mL. This resulted in the production of 10.4 mg of Tet(O)HC and 2.8 mg of Tet(O)HN. Immuno-blot analysis (Experimental Procedures) using a rabbit anti-Tet(O) antibody [136] confirmed that the major product in the purified protein preparation was Tet(O) (Figure 2-3). Additionally MALDI-TOF MS analysis of the

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^{v} A 100 mM imidazole wash is used for purification of Tet(O)HC, but a 50 mM imidazole wash is used to purify Tet(O)HN. I think the 50 mM wash may be too strong in the case of the Tet(O)HN purification because as seen in Figure 2-3B a significant amount of protein is eluted in the 50 mM wash.

Figure 2-3: Purification of Tet(O)

(A) A Coomassie stained gel representing the various stages of Tet(O)HC purification. The molecular weight marker is shown in the first lane. The cell extract is the supernatant of the 100000g centrifuge step (Experimental Procedures). The flow-through represents the eluate from the column as the cell extract is applied. The 5-300 mM imidazole lanes represent the column elute as these solutions are applied. The lane marked 'Final' contains the purified protein solution after the buffer was exchanged using the G-75 column and the solution was concentrated.

(B) An immuno-blot representing the various stages of Tet(O)HN purification. The lanes are the same as indicated in **A**, but additionally the last lane contains previously purified Tet(O)HC.

A

Lanes: Ladder, Cell Extract, Flow-through, 5 mM Imidazole, 100 mM Imidazole, 300 mM Imidazole, Final

Markers: 241, 147, 99, 69, 57, 43, 29

Tet(O)HC

B

Lanes: Cell Extract, Flow-through, 5 mM Imidazole, 50 mM Imidazole, 300 mM Imidazole, Final, Tet(O) std.

Markers: 241, 147, 99, 69, 57, 43, 29

Tet(O)HN

primary Ab = α-Tet(O) (rabbit)
secondary Ab = α-rabbit-HRP (goat)
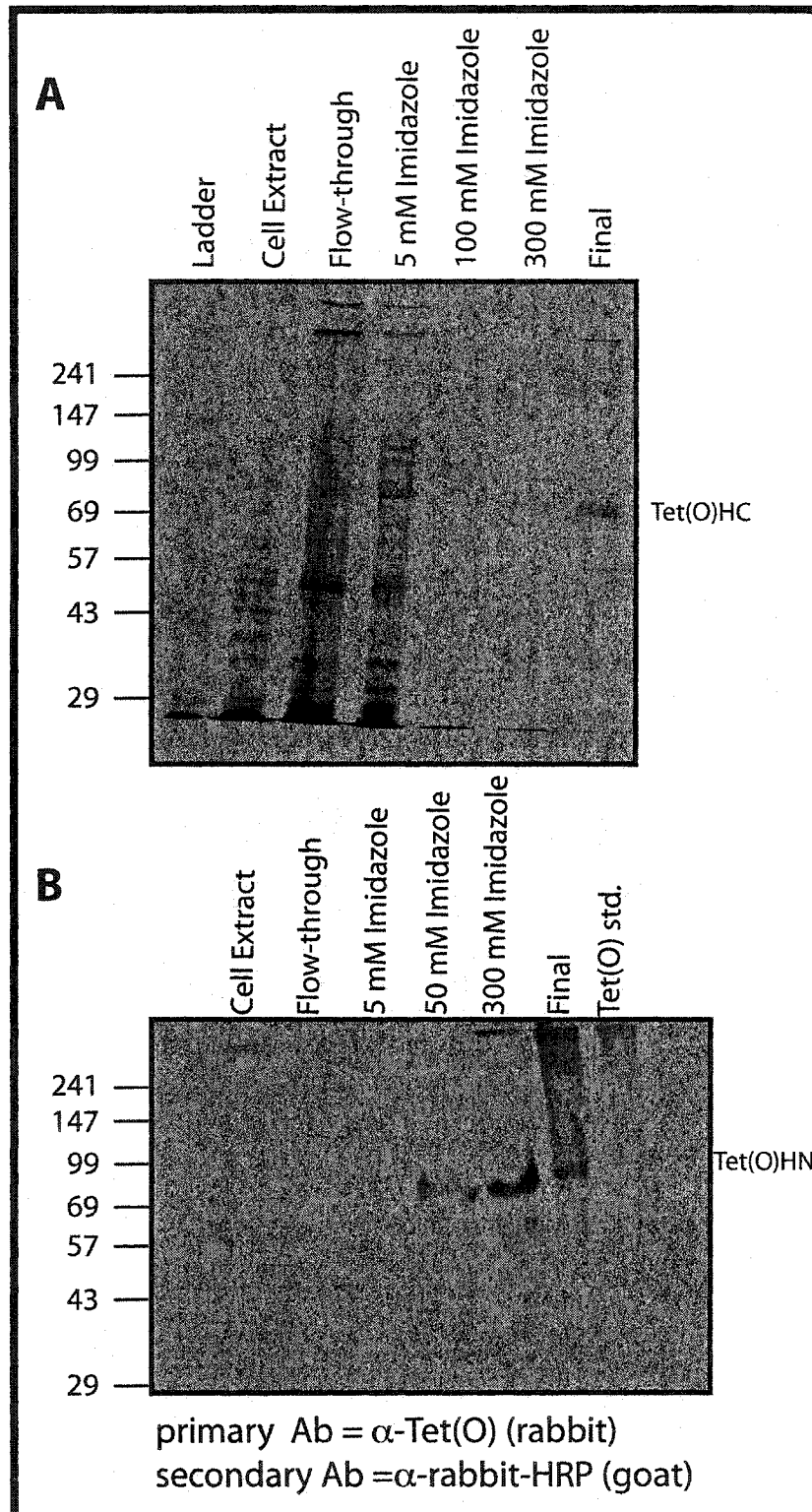
purified protein solution showed a broad peak in the 73 kDa range, consistent with the predicted mass of 73124 kDa for Tet(O)HN (Institute for Biomolecular Design: Mass Spectrometry Facility).

2.2.3.2 Over-expression of Tet(O) using alternative expression systems

EF-G is expressed readily from systems utilizing both the pQE70 (Qiagen) and pET (Invitrogen) vector series (personal observation; see Figure 2-4A). For this reason, tet(O) was cloned into pET14b and pQE70 (Figure 2-5). Subsequently, the XL1/pQE70TetOHN clones and the BL21(DE3) pET14bTetOHN clones were screened for high-level expression[vi], however, none of these showed dramatic over-expression similar to that seen with EF-G (Figure 2-4B; data not shown for the pET14b clones). Automated sequence analysis of two clones, pET14bTetOHN13 and 14, showed that tet(O) was correctly cloned into the vector and theoretically over-expression should be possible.

A closer look at the codon usage in tet(O) revealed that 14 out of the 36 Arg codons in tet(O) corresponded to the rare - in E. coli – AGA and AGG codons. For comparison, E. coli EF-G has no rare Arg codons but contains 36 arginine residues. It, therefore, could be possible that the inability to express Tet(O) at high levels is due to the presence of these rare codons[vii]. For this reason we tried to express Tet(O) in the Rosetta strains (Table 6-1). The Rosetta strains harbour the pRARE plasmid that carries the tRNA genes decoding the "problematic rarely used codons encoding Arg, Ile, Gly, Leu and Pro" (inNovations, June 2001) and therefore these strains attempt to compensate for the use of these codons by increasing the cellular concentration of their cognate tRNA. Unfortunately expression of Tet(O)HN from the pET14b vector system was

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[vi] EF-G over-expression in pQE70 is readily visible in a Coomassie stained gel and I was only looking for significant improvements over the existing system (pMS119) so I only screened for expression by coomassie stain.

[vii] The presence of these rare codons could be a consequence of the fact that the RPP are likely derived from a Gram-positive source (section 1.3.2).

Figure 2-4: Over-expression of Tet(O) using pQE70 and pET14b

(**A**) EF-G (indicated with arrow) is over-expressed in *E. coli* XL1-Blue harbouring the plasmid pQE70-EFG. Lane 1 contains molecular weight marker (Roth), lane 2 the un-induced sample, and lanes 3-5 induced sample at times 1, 2, and 3 hours. The samples are induced at OD_{600} of 0.4 with 1 mM IPTG. The gel photograph was provided by D. Wilson (MPIMG, Germany).

(**B**) The gel shows a similar attempt to express Tet(O) (indicated with arrow) in *E. coli* XL1-Blue harbouring pQE70-TetO. Lane 1 contains purified Tet(O), lane 2 is an un-induced pQE70TetOHN clone, and lanes 3-7 contain pQE70TetOHN clones 3-7 induced with IPTG. The samples are induced at approximately an OD_{600} of 0.4 with 1 mM IPTG.

(**C**) The gels show the expression of TetOHN from pET14bTetOHN13 and 14 in the strains BL21(DE3)plysS, Rosetta(DE3) and Rosetta(DE3)plysS. Strains harbouring pLysS express T7 lysozyme to suppress 'leaky' expression of the T7 RNA polymerase and therefore reduce un-induced expression of Tet(O). The contents of the lanes are indicated in the figure where: V indicates the strain harbours only the vector (pET14b), and 13 and 14 that the strain carries either pET14bTetOHN13 or 14, respectively.

A

Mol.Wt.Marker / no IPTG / IPTG;T=1 hr / IPTG;T=2 hr / IPTG;T=3 hr

← EF-G

XL-1/pQE70EF-G

B

Tet(O) std. / no IPTG / clone 3, IPTG / clone 4, IPTG / clone 5, IPTG / clone 6, IPTG / clone 7, IPTG

← Tet(O)

XL-1/pQE70TetOHN

C

Mol.Wt.Marker / V,-IPTG / V,+IPTG / 13,-IPTG / 13,+IPTG / 14,-IPTG / 14,+IPTG / V,-IPTG / V,+IPTG / Tet(O) std.

Rosetta   Rosetta(pLysS)

Mol.Wt.Marker / 13,-IPTG / 13,+IPTG / 14,-IPTG / 14,+IPTG / 13,-IPTG / 13,+IPTG / 14,-IPTG / 14,+IPTG / Tet(O) std.

Rosetta(pLysS)   BL21(pLysS)

Figure 2-5: pET14bTetOHN and pQE70Tet(O)HN

(A) *tet*(O) was cloned into pET14b using a 5' *Nco*I site and a 3' *Bam*H1 site that were added by PCR (primers sc009 and sc010; Table 6-3). The Tet(O) ORF is coloured blue and that of β-lactamase is red.

(B) *tet*(O) was cloned into pQE70 using a 5' *Sph*I site and a 3' *Bam*H1 site that were added by PCR (primers sc011 and sc010; Table 6-3). The Tet(O) ORF is coloured blue and that of β-lactamase is red.

A

pET14bTetOHN
6.544 kb

BamH I
Xho I
Nde I

bla

tet(O)

Nco I
Xba I
Bgl II

0.0 Kb
1.0 Kb
2.0 Kb
3.0 Kb
4.0 Kb
5.0 Kb
6.0 Kb

B

pQE70TetOHN
5.361 kb

Sph I

bla

tet(O)

BamH I
Bgl II

0.0 Kb
1.0 Kb
2.0 Kb
3.0 Kb
4.0 Kb
5.0 Kb
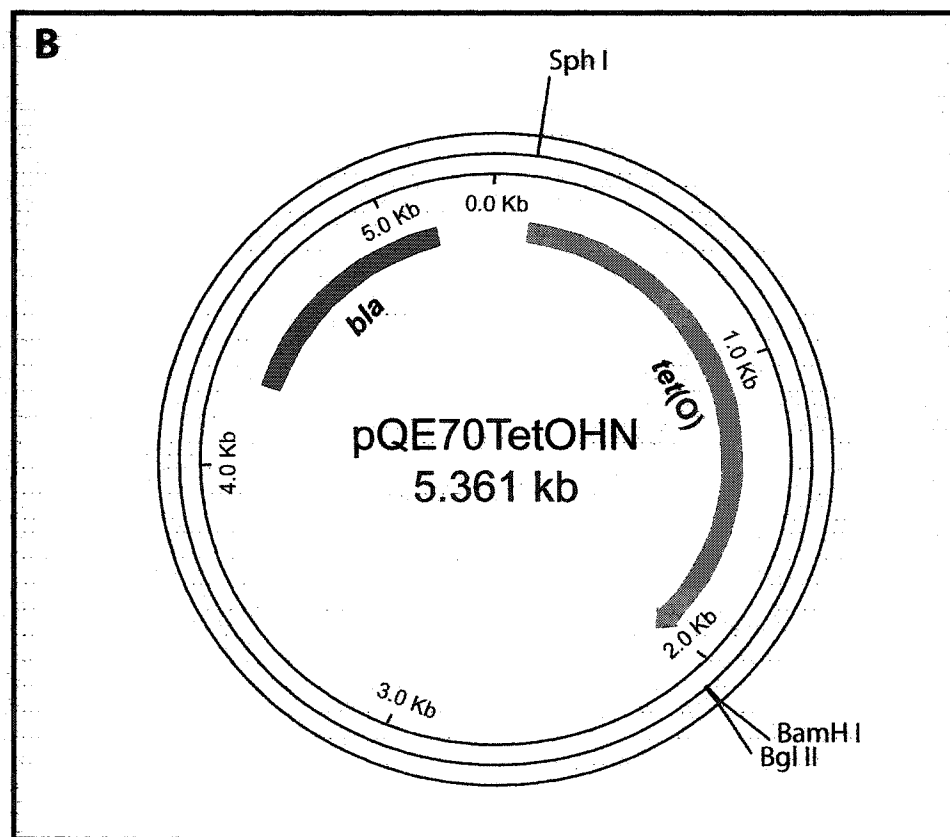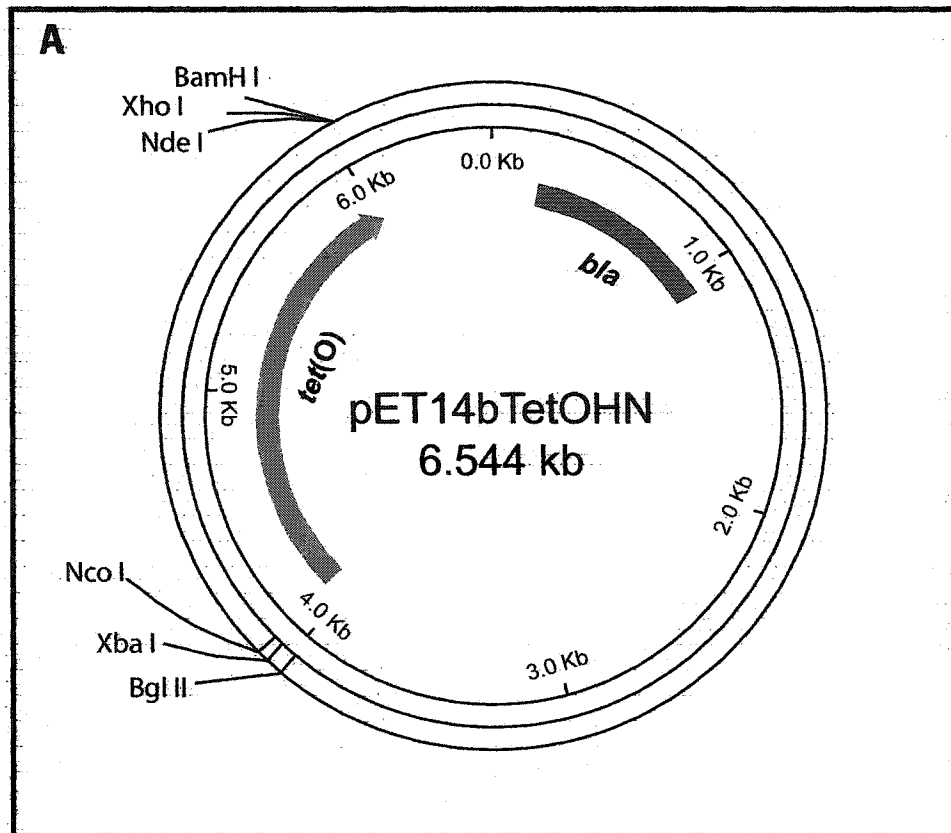
not significantly enhanced in these strains (Figure 2-4C). The Novagen pET System Manual also indicates that plasmid stability and consequently protein expression can be negatively affected by allowing the seed culture to enter stationary phase. Unfortunately, this observation did not improve Tet(O) expression (data not shown).

These experiments failed to find the root cause of the apparent inability to express Tet(O) at levels comparable to that of EF-G. It is possible that Tet(O), being a ribosome-associated protein, is inhibitory to protein synthesis and therefore its over-expression is detrimental to the cell.

2.2.3.3 In vitro GTPase activity of purified Tet(O)HN[viii]

Tet(O) is a G-protein much like the elongation factors EF-G and EF-Tu and, as such, it binds and hydrolyses GTP in a manner that is stimulated by 70S ribosomes [136]. As shown in Figure 2-6A this GTPase activity is confirmed for the purified Tet(O)HN10. On its own, Tet(O)HN10 has a very low GTPase activity; it hydrolyses only 1 pmol of GTP after 20 min. In comparison the purified Tet(O)HN in the presence of 70S ribosomes hydrolyses 70 pmols of GTP in 20 min. Importantly, the stimulation is specific to ribosomes in the sense that a homopolymeric RNA like poly(U) will not stimulate the GTPase activity. Under the conditions used in Figure 2-6A – 0.3 μM Tet(O), 0.3 μM 70S ribosomes, 20 μM GTP - the GTPase activity begins to saturate after about 15 min.

When directly compared to EF-G under the same conditions (Figure 2-6B) Tet(O) displays a similar overall ribosome stimulated GTPase activity; ~190 pmols of GTP hydrolysed per pmol of Tet(O) in 15 min. In the case of both EF-G and Tet(O) this represents a greater than 20-fold increase over the ribosome independent or intrinsic GTPase activity of the factor. An interesting aspect of the

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[viii] Tet(O)HN is used exclusively in Chapters 3 and 4 and therefore it is the subject of most of the characterization presented here.

Figure 2-6: *In vitro* RPP activity of Tet(O)

(A) The apparent intrinsic and ribosome stimulated GTPase activity of Tet(O) is plotted relative to the time in minutes. The reaction contains as indicated in the legend 0.3 µM Tet(O)HN10, 0.3 µM 70S ribosomes, 20 µM [^{32}P]-GTP, and/or 10 µg poly(U). The background GTPase activity of the 70S ribosomes and buffer have been subtracted.

(B) The GTPase activities of EF-G (dark bars) and Tet(O)HN10 (light bars) are compared. As indicated in the figure the reactions contain 0.3 µM Tet(O)HN10, 0.3 µM 70S ribosomes, 165 µM [^{32}P]-GTP, and/or 10 µM fusidic acid. The background GTPase activity of the 70S ribosomes and buffer have been subtracted.

(C) The inhibition of poly(Phe) synthesis over a range of tetracycline concentrations is shown. The activity of the system is given in pmols of incorporated phenylalanine per pmol of 70S ribosome present in the system. The assay used is as described in Experiment Procedures for the 4.5 mM poly(Phe) system.

(D) The ability of Tet(O) to protect a tetracycline inhibited poly(Phe) synthesis is shown. Tetracycline is either absent (O) or present at 25 μM (●) or 250 μM (Δ) whereas the amount of Tet(O) present is given relative to pmols of 70S ribosomes used (3.33 pmols). 100% activity corresponds to the activity of the system in the absence of tetracycline (257 pmol Phe / ribosome).

A

pmols GTP hydrolysed

80
60
40
20
0

0   10   20

Time (min)

- ● Tet(O)
- ○ Tet(O) + 70S
- △ Tet(O) + Poly(U)

B

pmols GTP hydrolysed per pmol protein

200
150
100
50
0

Intrinsic   Ribosome stimulated   Ribosome stimulated (+fusidic acid)

- Tet(O)
- EF-G

C

pmols Phe per pmol ribosome

200
150
100
50
0

0   200   400   600

Tetracycline conc. (mM)

D

% Activity

100%
80%
60%
40%
20%

0   1   2

Molar Ratio of Tet(O):70S ribosomes
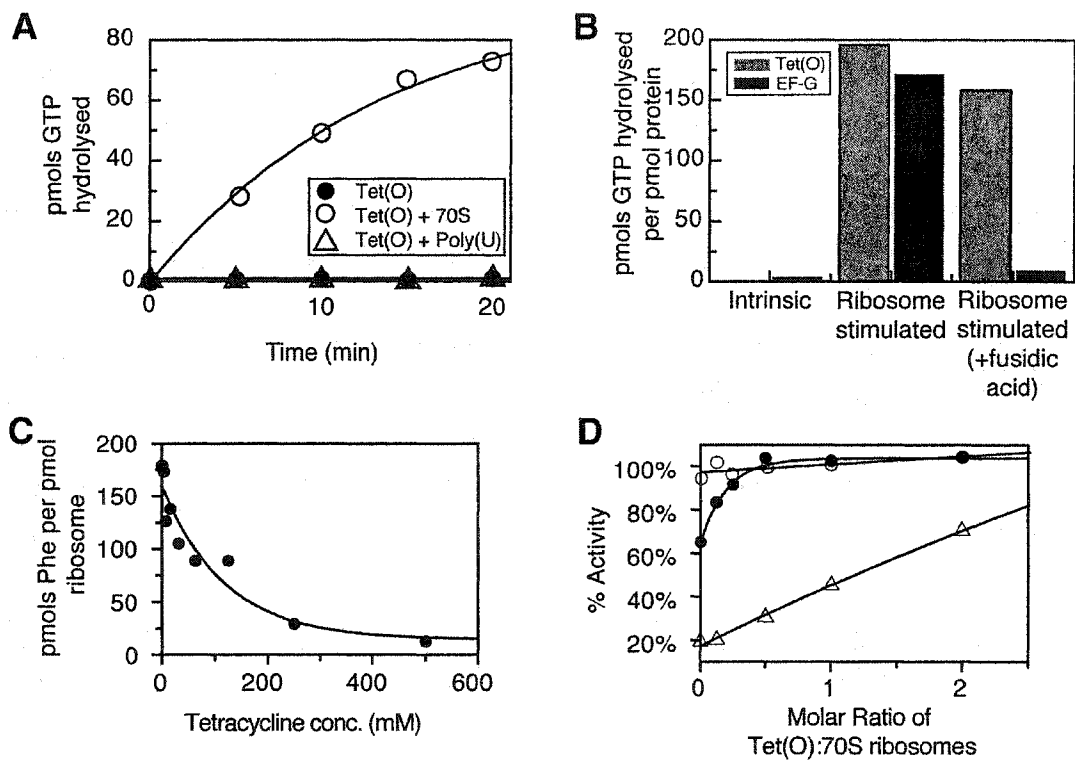
Tet(O) GTPase activity is that it is largely resistant to the effects of fusidic acid, an antibiotic that is able to bind EF-G preventing it from being released from the ribosome after GTP hydrolysis [148]. This has the effect of decreasing the overall GTPase activity in experiments where EF-G turns over multiple times. As seen in Figure 2-6B the ribosome-stimulated GTPase activity of EF-G is decreased more than 10-fold in the presence of 10 μM fusidic acid. In contrast the ribosome-stimulated GTPase activity of Tet(O) is only marginally effected (approximately an ~20 % decrease in GTPase activity) by the drug (Figure 2-6B). This is similar to the study done by Burdett which found that Tet(M) was also resistant to the effects of fusidic acid [119]. A direct comparison of the GTPase activities presented here and those done previously [118, 119, 136] is difficult as the specific reaction conditions are not consistent throughout. However the fact that Tet(O)HN was shown to display a GTPase activity similar to EF-G is important as the same was shown for un-tagged Tet(M) and EF-G [118]. In this sense these results suggest that Tet(O)HN remained active throughout the purification procedure, with respect to its GTPase activity, and that fusion of a N-terminal His$_6$-tag to Tet(O) does not significantly affect the overall apparent GTPase activity of the protein when compared to EF-G.

2.2.3.4 RP activity of Tet(O)HN10 in an in vitro poly(Phe) assay

The activity of the over-expressed and purified N-terminal His$_6$-tagged Tet(O) was assayed by measuring the ability of the protein to restore activity to a tetracycline-inhibited poly(Phe) system. The poly(Phe) synthesis system used (Experimental Procedures) is optimized with respect to rate – statistically catalyzing the formation of about 200 Phe-Phe peptide bonds per ribosome in 10 min (extent of reaction) – and therefore provides a more sensitive system for investigating RPP activity than was previously available [11, 118, 119]. Figure 2-6C shows that the system is increasingly retarded in its ability to produce poly(Phe) as the tetracycline concentration rises. The ability of Tet(O) to relieve this inhibition was tested at 25 μM and 250 μM tetracycline which reduces the

activity of the system to 65% and 20%, respectively (Figure 2-6C). At the lower tetracycline concentration, Tet(O) readily allowed protein synthesis when present at half the concentration of the ribosome, while in the presence of 250 μM tetracycline, a 2-fold molar excess of Tet(O) restored 70% activity to the system (Figure 2-6D). These results indicate that Tet(O) with a N-terminal His_6-tag is active as a ribosomal protection protein behaving similar to that previously seen with the C-terminally His_6-tagged Tet(O) [11]. Interestingly, Tet(O)HN does not interfere when added in the absence of tetracycline (Figure 2-6D) unlike that previously observed with Tet(M) [119] and Tet(O)HC [11]. However, because Tet(O)HC and Tet(M) were not compared directly with Tet(O)HN it is unclear if this lack of inhibition is due to the nature of the system used (4.5 mM poly(Phe); Experimental Procedures) or due to differences in the proteins themselves. The ability of Tet(O) to restore activity to such an efficient poly(Phe) synthesis system is indicative of Tet(O)'s proficiency.

2.2.4 Structural studies on Tet(O)

2.2.4.1 Analysis of cryo-EM maps

A cryo-EM reconstruction (16 Å) of Tet(O) bound to the ribosome in the presence of GTPγS was presented by Spahn et al. (section 1.3.7; ref. [47]). This reconstruction, when combined with high-resolution structures of a 30S subunit bound by tetracycline [99] suggests that Tet(O) does not directly overlap the primary tetracycline binding site [47]. This prompted Spahn et al. to suggest that Tet(O) promotes tetracycline release by an allosteric mechanism [47]. However, they note that they cannot completely discount the possibility that unresolved elements of Tet(O) are actually spanning the 6 Å distance which represents the closest approach of the bound tetracycline and Tet(O) density [47]. This possibility may have merit as the analysis[ix] in Table 2-2 shows that it is quite

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[ix] This analysis was carried out on a cryo-EM derived electron density map corresponding to the isolated Tet(O) density (provided by C.M.T. Spahn) and I was assisted by Dr. B. Hazes . Dr. B.

possible unresolved elements of Tet(O) do exist. The analysis in Table 2-2 shows that the volume of the density attributed to Tet(O) in the cryo-EM map only represents a fraction of the predicted volume of Tet(O). For example the density attributed to Tet(O) when viewed at a threshold of 2σ (Figure 2-7A) only has a volume that would account for a 55% of the predicted volume of Tet(O) based on it molecular mass (Table 2-2). Although the cryo-EM reconstruction [47] and the chemical probing experiments (Chapter 3 and ref. [115]) present convincing evidence that Tet(O) binds at a location distinct from the tetracycline binding site, the results presented here suggest it may be important to confirm the allosteric nature of Tet(O)-mediated release (see section 5.3.1). However, it is important to note that this analysis should be viewed with scepticism as first it involves many assumptions, i.e. the expected volume of the protein is calculated using the assumed average density of a protein [149]. In addition, subtleties of the cryo-EM density map may be ignored as here the map is treated as if derived from X-ray crystallography.

2.2.4.2 Role of S12 in RPP mediated tetracycline resistance.

The interactions between the ribosome and Tet(O) are mediated in a large part by the rRNA, such that a single contact with S12 represents the only protein-protein interaction [47]. As seen in Figure 2-7B, subsequent analysis of the Tet(O)•S12 contact shows that it is associated with highly conserved residues (74-76, *E. coli* numbering; see Appendix 6.2). There are several interesting aspects of this interaction:

(I) As mentioned in section 1.1.2, S12 is one of the rare proteins that line the interface of the subunits and this fact suggests that it may play an important role in ribosome activity.

(II) As seen in the alignment in Appendix 6.2 and in the structure of S12 (Figure 2-7B), $Q_{74}EH_{76}$ of this loop are highly conserved. The only other

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Hazes also wrote the program (Appendix 6.4.4) to count the number of pixels in this electron density map with a specified intensity (see Table 2-2).

residues that are similarly conserved and exposed to the solvent are those comprising the loop formed by amino acids 43-50 (Appendix 6.2) and they are intimately associated with decoding [29].

(III) The side chains of the residues $Q_{74}EH_{76}$ are projecting into the inter-subunit space such that they would be available to interact with proteins such as Tet(O) bound to the ribosome.

(IV) At the Ribosome meeting in Queenstown, New Zealand (2002) Dr. Agrawal presented a model in which EF-G also appeared to be contacting S12 at a similar position. (Note I have been unable to obtain the model to validate this observation)

In this respect, the conservation of $Q_{74}EH_{76}$ in S12 may result from the fact that they form the basis of an important interaction with several ribosome interacting proteins (i.e. EF-G and Tet(O)).

2.2.4.3 Initial screen for Tet(O) crystallization conditions

The first step in obtaining high-resolution structural data of a protein structure by X-ray crystallography is crystallization of the protein. We attempted to find crystallization conditions for Tet(O) using the sparse matrix screens, Crystal Screen and Crystal Screen II (Hampton Research; Experimental Procedures). In our initial screen we observed immediate precipitation or precipitation after only 1 day in greater than 50% of the conditions tested (98 different conditions). This suggests that the initial concentration of protein used (f.c. in drop is 2.3 mg/ml) was too high, and if repeated the protein concentration should be decreased by a factor of two and/or the incubation temperature decreased. However, some small crystals were obtained in this trial under the following conditions: 5% isopropanol, 2 M ammonium sulphate and 2% PEG 400, 0.1 M Na HEPES pH 7.5, 2 M ammonium sulphate (20°C, 1 year). An example of these crystals can be seen in Figure 2-8, they are small (0.03 x 0.05 mm), and irregular in shape (pseudo-crystal), but are bifringent under polarized light. The

Figure 2-7: Analysis of Tet(O) cryo-EM-derived electron densities

(A) The cryo-EM derived density of Tet(O) (red; ref. [47]) is represented at various thresholds (1-6σ). The density is shown relative to elements of the decoding centre as presented by Spahn *et al.* [47] where h34 is blue, h31 is yellow and h44 is red. Additionally, C1214 and A1408 which have been shown to interact with Tet(O) (Chapter 3) are coloured cyan and green, respectively. C1054, which forms one end of the tetracycline-binding pocket, is coloured purple.

(B) The interaction of Tet(O) (red wire-mesh) with the ribosomal protein S12 (blue ribbon) is shown as seen in the cryo-EM reconstruction of Tet(O) bound to the ribosome [47]. The residues $Q_{74}EH_{76}$ that are closely associated with the Tet(O) density are coloured red while the loop of S12 that has been associated with the decoding reaction [29] is coloured yellow.

These figures were prepared with SwissPDB viewer [23], and PovRay (www.povray.org).
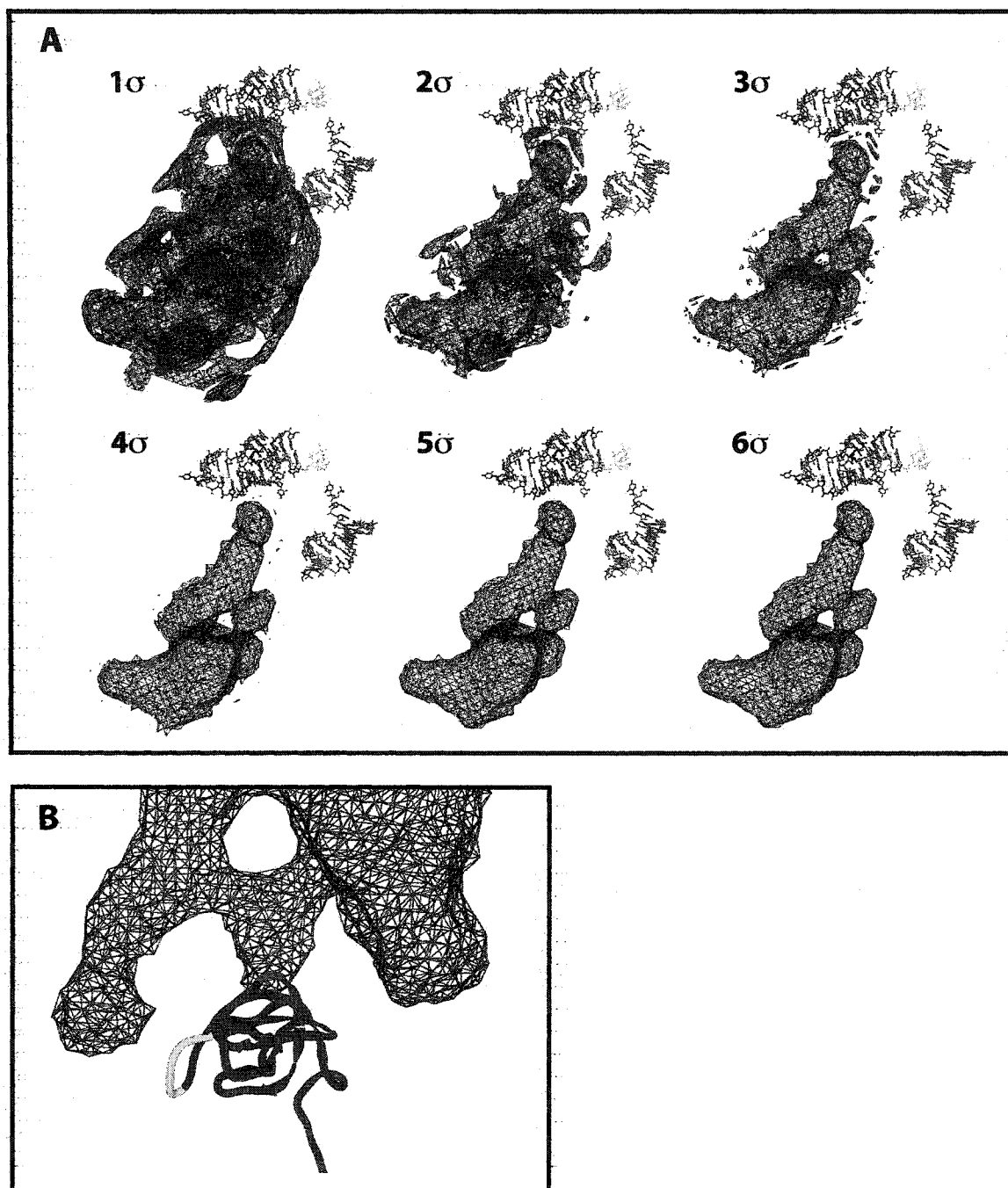
Table 2-2: Analysis of Cryo-EM derived electron density maps

Threshold[A]	no. of pixels[B]	Volume[C] ($\mathring{A}^3$)	Estimated mass[D] (g)	Fraction of Tet(O) represented[E] (%)
1σ	2029	51035	6.94×10^{-20}	63%
2σ	1768	44470	6.05×10^{-20}	55%
3σ	835	21002	2.86×10^{-20}	26%

[A] The threshold indicates the lower value of the pixel intensity that is counted. For example at 1σ all pixels with a intensity greater than the mean plus 1 standard deviation are counted and included in the column 'no. of pixels'. The cryo-EM map studied here was provided by Christian Spahn and corresponds only to the Tet(O) density.

[B] The number of pixels used to calculate the volume of the Tet(O) density.

[C] The total volume of the pixels attributed to the Tet(O) density. One pixel equals 25.153 $\mathring{A}^3$.

[D] The mass is estimated using the calculated volume and the average density of a protein (1.36 g/cm^3; [149]) The indicated mass corresponds to the mass of a single molecule.

[E] The fraction of Tet(O) represented by the density is calculated by comparing the estimated mass and the predicted mass for the Tet(O) protein (1.19 x 10^{-19} g).

Figure 2-8: Tet(O) crystallization trials

The crystals observed in the hanging drop above a reservoir containing 5% isopropanol, 2 M ammonium sulphate.
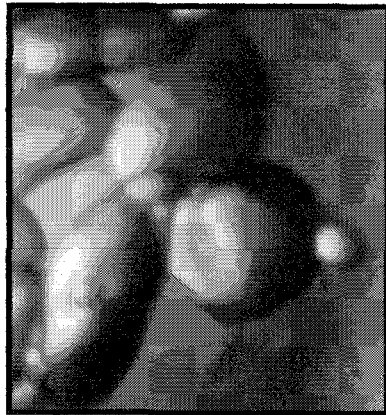
crystals were also very fragile and were destroyed upon handling and for this reason they were never analysed by X-ray diffraction.

2.2.5 Purification 70S ribosomes

Like Tet(O), isolation of purified 70S ribosomes is necessary for the *in vitro* assays in Chapters 3 and 4. Since re-associated ribosomes are superior for establishing functional complexes [150] we elected to purify ribosomes by this method rather than isolate tight-coupled 70S ribosomes[x]. Additionally, because the intactness of the rRNA is crucial for the DMS probing experiments (Chapters 3 and 4) where the rRNA is used as a template in a reverse transcription reaction, extreme caution was taken to minimize RNA degradation.

The methods used to purify the ribosomal subunits and subsequently re-associate them into 70S ribosomes are detailed in Experimental Procedures. The subunits were isolated though a 6-40% sucrose gradient in dissociation buffer which readily separates the two subunits based on their density (Figure 2-9A). The large peak in the light portion of the sucrose gradient is only observed when the S30 is loaded to the gradient and not when crude 70S ribosomes are loaded as described in the protocol of Bommer *et al.* [151]. The content of the peaks corresponding to the 30S and 50S subunits and the integrity of the rRNA comprising these subunits was checked by agarose gel electrophoresis (Figure 2-9B), demonstrating that the peaks attributed to 30S and 50S subunits in the sucrose gradient do in fact contain molecules that migrate similar to that of the 16S and 23S rRNA in 30S and 50S subunits.

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[x] Re-associated ribosomes are purified by isolating ribosomal subunits and subsequently re-associating them into 70S ribosomes. This contrasts the isolation of tight-coupled ribosomes where 70S ribosomes are purified directly from the cell extract. We have observed that the re-associated ribosomes show a higher tRNA binding ability and are therefore preferred for producing a defined population of functional complexes.

Figure 2-9: Purification of 70S ribosomes

(A) The elution profile of the 30S and 50S subunits from a 6-40% sucrose gradient (in $H_{20}M_1N_{200}SH_4$ buffer). The elution of the ribosomal subunits is followed by monitoring the absorbance at 275 nm.

(B) An agarose gel showing the rRNA derived from the subunits obtained from the sucrose gradient like that illustrated in A. The bands corresponding to the 23S and 16S rRNA are indicated with arrows. The 30S, 50S and 70S standards loaded on to the gel correspond to particles obtained previously by Norbert Polacek [152] while the 30S (std. T3 and T4) were provided by Gregor Blaha. The 30S and 50S lanes labelled zonal 1 and 2 correspond to different preparations obtained in this study.

(C) The elution profile of the re-associated 70S ribosomes from a 6-40% sucrose gradient (in $H_{20}M_{20}K_{30}SH_4$ buffer). The elution of the ribosomal subunits is followed by monitoring the absorbance at 275 nm.

(D) An agarose gel showing the rRNA derived from the re-associated 70S ribosomes (r/s 70S (1-3)) obtained from the sucrose gradients like that illustrated in C. The bands corresponding to the 23S and 16S rRNA are indicated with arrows. The 30S, 50S and 70S standards loaded on to the gel correspond to particles obtained previously by Norbert Polacek [152].

(E) The scan profiles of RNA tube gels (denaturing PAGE; Experimental Procedures) containing either re-associated 70S ribosomes derived in this study (blue), or control re-associated 70S ribosomes (red), 50S subunits (green), or 30S subunits (purple).

(F) A 4-15% SDS-PAGE gel (Coomassie stain) showing the protein content of the isolated 30S, 50S, and 70S ribosomal particles as compared to the standard particles. The red arrows highlight protein bands that are present in some samples but not in others (see text).
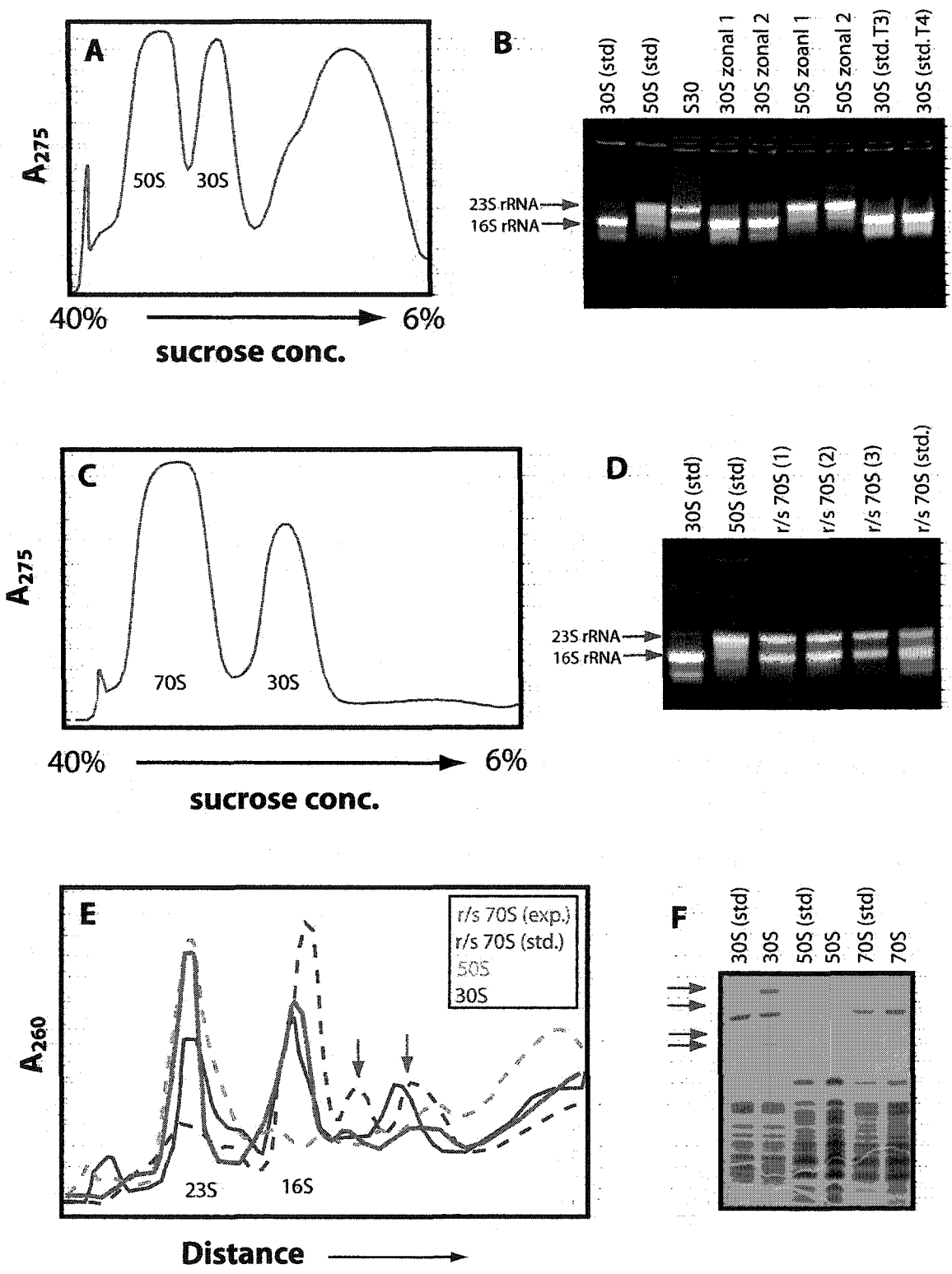
Subsequently 30S and 50S subunits with similar rRNA quality were re-associated (Experimental Procedures) and the resulting 70S ribosomes were isolated through a 6-40% sucrose gradient (Figure 2-9C). Again the rRNA integrity was confirmed as well as the protein content of the ribosomes (Figure 2-9D-F). The rRNA in the re-associated 70S appears to be largely intact when analysed on both an agarose gel (Figure 2-9D) and on a denaturing RNA tube gel (Figure 2-9E). When compared with the previously re-associated 70S ribosomes, used as a standard[xi], the rRNA in the re-associated 70S ribosomes appears to be more intact as evidenced by a decrease in the 'smearing' on the agarose gel (Figure 2-9D) and the lack of peaks smaller than the 16S rRNA in the A_{260} profile of the denaturing RNA tube gel (Figure 2-9E). When the isolated re-associated ribosomes and subunits were analysed by SDS-PAGE gel (coomassie stain) and compared to standard ribosomal particles it can be seen that there are more non-ribosomal proteins present (indicated with arrows in Figure 2-9F). These proteins are more evident in the subunits than in the re-associated 70S ribosomes (Figure 2-9F). The difference in protein content of the re-associated ribosomes isolated here and those isolated previously may reflect the different procedure with which they were isolated. The standard ribosomes were purified by first isolating crude 70S ribosomes from the cell extract and these crude 70S ribosomes were subsequently disassociated into subunits over a sucrose gradient. Here we isolated the subunits directly from the cell extract without first isolating the crude 70S ribosomes. The extra step of isolating the crude 70S may help to separate these additional proteins.

The activity of the purified ribosomes was assessed in a poly(Phe) assay, a P-site binding assay, and a translocation assay. Figure 2-10A shows that the isolated re-associated 70S ribosomes are active at promoting poly(Phe) synthesis similar to or slightly better than the standard ribosomes (compare 317 Phe incorporated per 70S ribosome to 269 Phe incorporated per 70S ribosome).

[xi] Throughout this section the purified ribosomes will be compared against standard ribosomes, which represent a previously obtained preparation of re-associated 70S ribosomes.

Figure 2-10: Characterization of re-associated 70S ribosomes

(A) A comparison of the ability of purified and standard re-associated ribosomes to participate in poly(Phe) synthesis. The results presented represent the average of two experiments where the error bars indicate the maximum deviation from the mean.

(B) A comparison of AcPhe-tRNA binding to the isolated re-associated 70S ribosomes (r/s 70S) or standard re-associated 70S ribosomes (70S std.) in the presence of MF-mRNA. tRNA binding was assayed as described previously [150] where the reaction contained 0.4 μM ribosomes, 2.8 μM MF-mRNA, and 0-1.2 μM AcPhe-tRNA^{Phe} in $H_{20}M_6N_{150}SH_4Spd_2Sm_{0.05}$ buffer.

(C) A comparison of the AcPhe-tRNA binding levels and puromycin reactivity in PRE and POST translocational complexes prepared with the isolated re-associated 70S ribosomes (r/s 70S) and standard re-associated 70S ribosomes (70S std). The complexes were prepared and assayed as indicated in Experimental Procedures.

(D) The binding of tetracycline to purified 30S, 50S and 70S ribosomal particles is shown. The binding reaction (Experimental Procedures) contained 2 μM ribosomes or ribosomal subunits and either 0, 0.5, 1, 2.5, 5, 10, or 50 μM ^{3}H-tetracycline. The results presented are the average of 3 experiments.

(E) The ability of Tet(O) to promote tetracycline release from E. coli and T. thermophilus 30S subunits or 70S ribosomes is shown. The binding reactions contain 10 μM tetracycline, 1 mM GMPPNP, 2 μM ribosomes and Tet(O) as indicated in the figure.
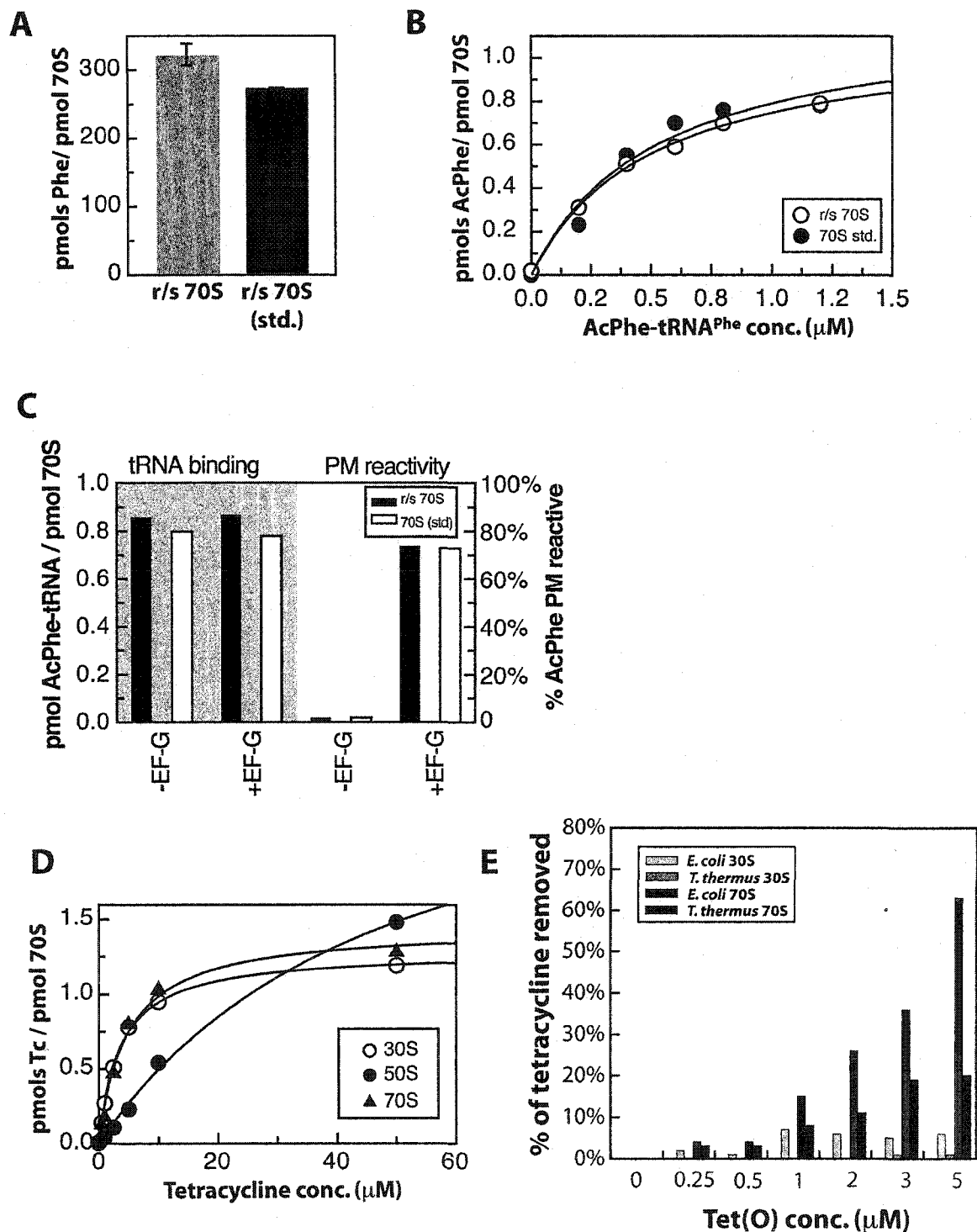
Similarly, AcPhe-tRNA^{Phe} binding to the P-site is roughly equivalent in the isolated and standard ribosomes (Figure 2-10B). In both cases, on average 0.7-0.8 AcPhe-tRNA^{Phe} were bound per ribosome when the tRNA was present at a 2-fold molar excess over the ribosomes. The ability of the ribosomes to partake in EF-G-dependent translocation was also followed. In Figure 2-10C a pre-translocational complex (PRE) was established where, on average, the purified ribosomes bound approximately 0.8 AcPhe-tRNA^{Phe} per 70S ribosome; a value that is consistent with the standard ribosomes. These complexes were then incubated in the presence or absence of EF-G and GTP, such that if EF-G and the ribosomes were active, together they would promote the translocation of the tRNA. As seen in Figure 2-10C the purified re-associated ribosomes in the presence of EF-G do undergo translocation as evidenced by the increase in the puromycin reactivity of AcPhe-tRNA^{Phe}; a similar increase is seen with the standard ribosomes. In the absence of EF-G the puromycin reactivity of the AcPhe remains at less than 1% indicating that the complexes are stable and do not undergo spontaneous translocation.

These results indicate that the isolated ribosomes are active, to a similar degree as the previously purified, 'standard', ribosomes. Furthermore, as the ribosomes readily bind tRNA and undergo translocation only in the presence of EF-G they are ideal for establishing defined functional complexes as needed in Chapters 3 and 4.

2.2.6 Tetracycline release

2.2.6.1 Tet(O)-mediated tetracycline release from T. thermophilus ribosomes.

In order to establish the feasibility of co-crystallizing Tet(O) and *T. thermophilus* ribosomes for eventual structure determination, we assayed the ability of Tet(O) to mediate tetracycline release from these ribosomes. Figure 2-10E compares the ability of Tet(O) to promote tetracycline release from *E. coli*

and *T. thermophilus* 30S subunits and 70S ribosomes[xii]. Tetracycline was bound to these ribosomes in a reaction containing 10 μM tetracycline; conditions which should fill primarily the high affinity tetracycline binding site (K_d approximately equal to 4 μM; see Figure 2-10D). Addition of Tet(O)•GMPPNP to this reaction triggers release of tetracycline from the 70S ribosomes, however release from the *E. coli* ribosomes is more pronounced than that from the *T. thermophilus* ribosomes. In fact, approximately three times more tetracycline is released from the *E. coli* ribosomes when Tet(O) is present at 5 μM. Additionally, it can be seen in Figure 2-10E that Tet(O) is unable to remove tetracycline from the 30S subunits. These results indicate that either Tet(O) does not interact with the 30S subunit or, if it does interact, it is such that it cannot promote tetracycline release. Furthermore, Tet(O) interacts better with *E. coli* ribosomes than it does with *T. thermophilus* ribosomes. This could be a consequence of a different binding affinity or of a reduced ability to catalyse tetracycline release from *T. thermophilus* ribosomes. These factors probably played a role in previous experiments done in collaboration with the group of Dr. François Franceschi where attempts were made to crystallize Tet(O) with the *T. thermophilus* 30S subunit. In these experiments 30S crystals were analysed by X-ray diffraction but they were found to contain no Tet(O) (data not shown).

2.2.6.2 Role of h27 in Tet(O)-mediated tetracycline release

Tet(O) is believed to function by inducing conformational rearrangements in the ribosome leading to tetracycline release [47]. Mutations that affect the base pairing of h27 have been suggested to alter the 30S subunit structure, in particular the structure of the decoding site [102, 104]. It is possible that Tet(O) might similarly interact with h27 to induce changes in the decoding site which result in tetracycline release. In this respect the mutants – 912G and 912G/885U - isolated by Lodmell *et al.* that allegedly stabilize h27 in either a *restrictive* (hyper

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[xii] Release from 50S subunits was not assayed as the inhibitory tetracycline-binding site is believed to be located on the 30S subunit.

accurate) or *ram* (error-prone) conformation, respectively [102], may have differential effects on Tet(O). For example, if Tet(O) depends on the switch from a *ram* to *restrictive* conformation, than mutations that stabilize the *ram* conformation may inhibit Tet(O) activity. This possibility was examined using ribosomes isolated from *E. coli* harbouring only a single rRNA operon carried on a plasmid that contained the desired mutations (Table 6-1 and ref. [104, 153])[xiii]. The ribosomes isolated from strains harbouring wt. 16S rRNA and 16S rRNA carrying either the 912G or 912G/885U mutations showed roughly equivalent tetracycline binding at low concentrations (Figure 2-11A).

The ability of Tet(O) to promote tetracycline release of the high-affinity site was investigated by binding tetracycline at low concentrations to the ribosome and subsequently triggering its release using Tet(O). As seen in Figure 2-11B, tetracycline release was roughly equivalent using all mutants, such that approximately 1.5 μM Tet(O) (a 1.5 molar excess over ribosomes) could remove 50% of the bound drug. It should be noted that tetracycline release is being measured under single turnover conditions since we are adding Tet(O) in the presence of a non-hydrolysable GTP analogue. In this sense we are likely only to observe differences if the mutants have profound effects; it could be possible to extend the sensitivity of the assay by using GTP such that Tet(O) must turnover efficiently to continuously prevent tetracycline binding. These results do indicate, though, that Tet(O) most likely does not depend on changes in the configuration of h27 for promoting tetracycline release.

[xiii] Although it would have been desirable to screen for Tet(O) activity in these strains by transforming them with a compatible *tet*(O)-harbouring plasmid, this was not feasible as the strains already carry a tetracycline resistance gene.

Figure 2-11: Effect of h27 mutations on Tet(O) activity

(A) The binding of tetracycline to 70S ribosomes harbouring mutations in h27 is shown. The binding reactions contain 1 μM 70S ribosomes and 0-50 μM tetracycline.

(B) The ability of Tet(O) to promote release of tetracycline from 70S ribosomes harbouring mutations in h27 is shown. The binding reactions contain 1 μM 70S ribosomes, 10 μM tetracycline, 1 mM GMPPNP, and either 0, 0.25, 0.5, 1, 2, 3, or 5 μM Tet(O).
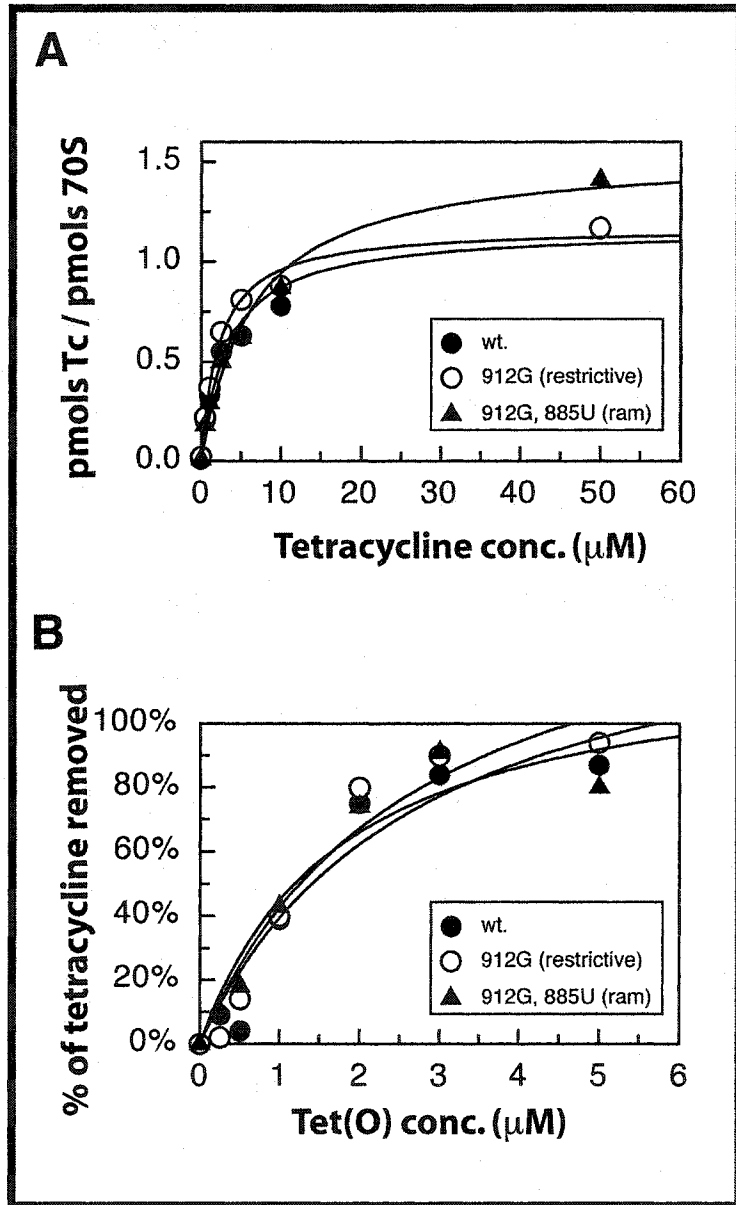
2.3 Discussion

In this work both 70S ribosomes and N-terminally His$_6$-tagged Tet(O) have been purified. Characterization of the purified components indicates that they are active and therefore their use in subsequent *in vitro* assays (see Chapters 3 and 4) is justified. With respect to Tet(O)HN10 we have shown that it is active *in vivo* such that under the conditions assayed it confers resistance to *E. coli* similar to native Tet(O) where strains harbouring pMSTetO and pMSTetOHN10 have an MIC of at least 256 µg/mL (Table 2-1). Similarly, *in vitro* studies show that after purification Tet(O)HN10 displays a similar activity as EF-G, a trait that was previously observed for Tet(M) [118]. Interestingly the GTPase activity of Tet(O) is largely resistant to fusidic acid, unlike that of EF-G which is strongly inhibited by the drug.

As mentioned above, fusidic acid is proposed to bind EF-G, preventing it from being released from the ribosome [148]. Interestingly though, fusidic acid appears to bind EF-G only when it is on the ribosome suggesting that it recognizes a specific conformation of EF-G that is only obtained in the ribosome bound state. In this respect, the inability of fusidic acid to affect Tet(O) could illustrate that Tet(O) does not assume a similar conformation on the ribosome despite the structural similarity of EF-G and Tet(O) (section 1.3.7) which could play a role in different activities of the two proteins. Alternatively, Burdett notes that Tet(M) naturally harbours many of the mutations that confer fusidic acid resistance in EF-G [119]. Thus fusidic acid resistance has important consequences for Tet(O) studies because when EF-G is bound to the ribosome, for studies such as cryo-EM analysis where high occupancy is desired, it is often locked in a stable complex with the ribosome using fusidic acid. However, in the case of Tet(O), this is obviously not possible due to its fusidic acid resistance and instead, one must use non-hydrolysable GTP analogues to stabilize a Tet(O)/ribosome complex.

In addition to being an active GTPase, Tet(O)HN also displays RP activity such that it can protect a poly(Phe) assay from the inhibitory effects of tetracycline and dislodge bound tetracycline from the ribosome. The ability of low molar ratios of Tet(O)HN10 to restore nearly 100% activity to a poly(Phe) system in the presence of 100 μM tetracycline is roughly comparable to the observation that cells harbouring pMSTetOHN10 can grow on a LB plate containing 65-130 μM tetracycline (32-64 μg/mL tetracycline)[xiv]. Furthermore, it is also interesting to note that in the studies presented here (Figure 2-6D) there was little indication that Tet(O)HN was inhibitory to poly(Phe) synthesis; although the studies were by no means exhaustive. In these studies when Tet(O)HN was present at 2 times the ribosome concentration no significant effect on the synthesis of poly(Phe) was observed; in a similar experiment Tet(M) (present at 1.5 times the ribosome concentration) showed a 40% inhibitory effect on protein synthesis [119].

With respect to the activities presented here, it is especially important to note that they are representative of a derivative of Tet(O) where the C-terminus differs from the 'wild type' sequence (Figure 2-2). However we are reasonably confident that this C-terminal mutation does not have a significant effect on the activity of Tet(O) under laboratory conditions because: (1) changing the C-terminal of Tet(O) by fusing it to a C-terminal His$_6$-tag had no profound effects on its *in vivo* activity (Table 2-1 and ref. [11]), (2) the C-terminal, with the exception of Lys637, is not strongly conserved throughout the entire family of RPPs (Figure 2-2E) and, most importantly (3) when *tet*(O) is cloned with a C-terminal sequence, as observed in pUOA2, it has an *in vivo* activity which is the same as that of the C-terminal 'mutants' of *tet*(O) (MIC = 64 μg/mL; Lisa Nonaka, personal communication).

With respect to the re-associated 70S ribosomes, we have shown that they are also active, at least in comparison to a previously purified preparation.

[xiv] This comparison is admittedly oversimplified, as tetracycline does concentrate within the cell, and additionally Tet(O) is likely present in the cell at a much lower concentration then that used in the assay.

Characterization of the ribosomes also helped to ascertain the basic conditions needed to establish functional complexes (pre- and post-translocational states), bind tetracycline specifically to the high affinity site, and maximize tetracycline release by Tet(O). For example, in Figure 2-10C it is demonstrated that highly defined PRE and POST complexes can be established. These complexes have a high tRNA occupancy (>80%) and tRNA binding to the A and P sites in the PRE and POST complex is defined as measured by puromycin reactivity [152]. In addition, we show that tetracycline binds to the re-associated 70S ribosomes with a K_d of approximately 4 μM (Figure 2-10D), a value that agrees with those previously obtained for the high affinity site (2-20 μM; ref [92]). This suggests that, by using a low concentration of tetracycline, the high-affinity tetracycline-binding site can be selectivity occupied.

The tetracycline release assay used in Figure 2-10E can also be used to indirectly follow Tet(O) binding if it is bound in the presence of an non-hydrolysable GTP analogue like GMPPNP. For example, when 10 μM tetracycline is added to the ribosomes near stoichiometric binding is achieved in the absence of Tet(O) (Figure 2-10D). However when increasing amounts of Tet(O) are added, tetracycline binding decreases. As a non-hydrolysable GTP analogue is used, and this allegedly locks Tet(O) on the ribosome in a stable complex [11], one can assume that the decrease in tetracycline binding is correlated to the amount of Tet(O) bound. Therefore, the results in Figure 2-10E suggest that in order to achieve a relatively high occupancy (~70%) of Tet(O) on the *E. coli* 70S ribosomes a large molar excess of Tet(O) over ribosomes is required. In this respect, when Tet(O)•70S complexes are prepared in Chapter 3 and 4 for chemical probing studies, where a high occupancy is preferable, Tet(O) was bound to the ribosomes in excess.

The initial characterization of Tet(O) revealed some important properties of Tet(O) that may be significant for future structural studies. In the screen for crystallization conditions presented in section 2.2.4.3 it was observed that Tet(O)

readily formed a precipitate in our trials suggesting that, in future studies, the concentration of Tet(O) should be lowered. Furthermore, we observed formation of pseudo-crystals (unconfirmed) under two conditions (section 2. 2.2.4.3) and these may serve as a starting point for a more detailed screen to improve the crystal quality. In section 2.2.6 Tet(O) binding was followed indirectly by monitoring tetracycline release (discussed above). In terms of structural studies, these results indicate it is better to co-crystallise or soak Tet(O) into 70S ribosomes rather than 30S subunits[xv]. In addition, *E. coli* 70S ribosomes may be a better substrate for Tet(O) than *T. thermophilus* ribosomes which should be considered when preparing complexes for crystallography, although it is important to note that until now high-resolution structures of *E. coli* ribosomes have not been forthcoming.

As well as attempting to study the interaction of Tet(O) with the ribosome using X-ray crystallography, additional examination of the previously obtained cryo-EM-derived maps of Tet(O) bound to the 70S ribosome [47] yielded two interesting results. Firstly, the possible role of conserved residues in S12 in Tet(O) activity and secondly, the possibility that Tet(O)-mediated tetracycline release is a consequence of a direct interaction. In section 2.2.4.1, it was observed that Tet(O) makes contact with S12 at a site comprised of highly conserved residues ($Q_{74}EH_{76}$). The conservation of these residues suggests that the interaction may be of functional importance for Tet(O), either as a binding determinant or for promoting conformational changes leading to tetracycline release. These possibilities are discussed in more detail in Section 5.2 in light of results obtained in Chapters 3 and 4. As for the possibility that tetracycline may be released by a direct interaction with Tet(O) the idea was presented by Spahn *et al.* when it was stated that unresolved elements may mediate a direct interaction [47]. This possibility is substantiated by the analysis in Table 2-2

[xv] It should be noted, however, that to date, high-resolution structures (2.8-3.3 Å; ref [16-19]) have only resulted from subunit crystals whereas those from 70S ribosomes are in the 5.5 Å range [154].

which suggests that some elements of Tet(O) may be lost in the noise of the cryo-EM map. It also should be noted that when interpreting the maps to determine if a direct interaction between Tet(O) and tetracycline exists, the high-resolution structure of the tetracycline-binding site utilized an isolated 30S subunit and the subunit does change conformation when bound to the 50S subunit [99, 100]. Additionally, tetracycline binding itself may affect the conformation of the ribosome [98, 109, 155] and no tetracycline is present in the Tet(O)·70S complex. However, it should be stated that when we prepared 70S·Tc complexes, which were analyzed by Dr. Spahn using cryo-EM, no significant changes were observed in the reconstruction (C.M.T. Spahn, personal communication). The contribution of these conformational changes would therefore not be accounted for when the high-resolution structures are docked into the cryo-EM map and could have subtle effects on the relation of Tet(O) and the tetracycline binding site.

2.4 Experimental Procedures

2.4.1 Materials

Purified His_6-tagged EF-G from *E. coli* was provided by Ulrich Stelzl and Edda Einfeldt. The re-associated 70S ribosomes used as a standard in these experiments were provided by Norbert Polacek and Gregor Blaha. The 70S ribosomes from *T. thermophilus* were provided by Dr. Franceschi (MPIMG; Berlin, Germany). The AcPhe-tRNA[Phe] was prepared as described previously [156] and the MF-mRNA was prepared by Detlev Kamp. All other materials were purchased from commercial suppliers.

2.4.2 DNA methods

All DNA-based methods were preformed according to standard protocols [147] unless otherwise indicated. In some cases plasmid purification kits (Qiagen), PCR clean-up kits (Qiagen), and Gel extraction kits (Qiagen) were also used according to the manufacture's instructions.

For PCR cloning into pMS119 a mixture of *Taq* and *Pfu* (10:1) was used where the reaction included 0.2 mM dNTPS, 1 μM forward primer, 1 μM reverse primer, 80 ng template DNA, 5 U polymerase mixture in 50 mM TrisHCl pH 9.2, 16 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$. For cloning into pET14b and pQE70 the High-Fidelity Expand PCR kit (Roche) was used according to the manufacture's instructions.

DNA sequencing was done manually using the Thermo Sequenase [33P] Radiolabeled Terminator Sequencing kit (Amersham Biosciences) according to the manufacture's instructions. Automated sequencing was performed at the Molecular Biology Service Unit (Dept. Biological Sciences; University of Alberta), the Service Group at the MPI for Molecular Genetics (Berlin, Germany) or by Agowa (Berlin, Germany). When automated sequencing was used, the reactions

were prepared using the BigDye Terminator kit (ABI) and the reactions were done either by the sequencing service or by Trinh Ngo.

2.4.3 Cloning of *tet*(O) into pMS119

The Tet(O)-harbouring plasmid, pUOA2E1 [130] was isolated from the strain DT2726 (*E. coli* W3110 / pUOA2E1) using standard methods [147]. The DNA primers Cat8 and Nch2 (Table 6-3) were used to amplify *tet(O)* from pUOA2E1 as described in section 2.4.2. The PCR product was then digested with *Eco*R1 and *Bam*H1 and ligated into the similarly digested pMS119 following standard methods [147]. The ligation was then transformed into $RbCl_2$-competent *E. coli* JM109 [147] and the resulting transformants screened by PCR using the primers Cat8 and Nch2 [147]. Of the 12 colonies screened, 2 were found to contain *tet*(O) and designated JM109/pMSTetOHN10 and JM109/pMSTetOHN11.

2.4.4 Cloning *tet*(O) into pQE70 and pET14b

tet(O) from pMSTetO [11] was amplified by PCR (see Table 6-3 for primers) for cloning into pET14b and pQE70. The PCR products were purified (PCR Purification Kit; Qiagen), digested with appropriate enzymes and ligated [147] into similarly digested pET14b or pQE70. The ligation mixture was transformed into *E. coli* XL1 and the resulting ampicillin-resistant transformants were screened by PCR for the presence of *tet*(O) [147]. Plasmids from the *tet*(O)-containing pET14b clones were isolated (Plasmid Midi kit; Qiagen) and transformed into *E. coli* BL21(DE3) whereas the *tet*(O)-containing pQE70 clones where kept in XL1-Blue for over-expression studies.

2.4.5 Over-expression and purification of Tet(O)

C-terminally His_6-tagged Tet(O)HC, was over-expressed using the strain MRE600/pTetOH [11], and purified by Ni^{2+}-affinity chromatography as described previously [11]. N-terminally His_6-tagged Tet(O)HN was purified from

JM109/pMSTetOHN10 as previously described [11] with the following modifications. (1) After application of the Tet(O)HN expressing cell lysate to a Ni^{2+} column (Chelating Fast-Flow Sepharose; Amersham Biosciences), it was washed with 50 mM imidazole and eluted with 300 mM imidazole. (2) The buffer was exchanged after the Ni^{2+} column for 50 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 10% glycerol and 0.5 mM DTT using a G-75 Sephadex column (Amersham Biosciences) and subsequently concentrated in a Centriprep-30 concentrator (Amicon). Protein concentrations were determined using a Bradford assay with BSA standards.

2.4.6 Tet(O) immuno-blot

Proteins were transferred to nitrocellulose using the BioRad Mini Trans-Blot electrophoretic transfer cell. The transfer was performed at 100V (~250 mA), for 1 hour at 4°C in 25 mM Tris pH 8.3, 192 mM glycine, and 20% methanol. The blot is then rinsed with PBS-T (PBS (Sigma) + 0.5% Tween) and subsequently blocked for 1 hour in PBS-T+10% skim milk powder. Next, the blot was rinsed two times with PBS-T+5% skim milk powder and then incubated 30-60 min with the primary antibody (rabbit α-Tet(O) diluted 1:2000 in PBS-T+5% skim milk powder; ref. [136]). The primary antibody was rinsed away with two 10 min washes of PBS-T, after which the blot was incubated 15 min in the presence of HRP-conjugated secondary antibody (goat α-rabbit (Sigma) diluted 1:5000 in PBS-T+5% skim milk powder). The blot was then washed for 10 min (2 times) with PBS-T. Detection of the Tet(O)/antibody complex was accomplished using the ECL Western Blotting System (Amersham Biosciences) according to the manufacture's instructions.

2.4.7 Crystallization Screen

Crystallization conditions were screened using a sparse matrix approach and the hanging drop vapour diffusion method. The precipitates used in the sparse matrix screen were exactly as described for the Crystal Screen and

Crystal Screen II kits (Hampton Research). The hanging drops were set up in 24-well Linbro plates (Hampton Research), where 0.5 mL of the precipitant was added to each well. The drops were established on 22 mm cover slips (Berlart Products) by mixing 2 μL of the protein solution (TetOHC, prep 26.09.98 in 50 mM Tris pH 7.5, 10 mM MgCl$_2$, 1 mM DTT, 40% glycerol) with 2 μL of the precipitant from the reservoir. This cover slip was then inverted over the well on the Linbro plate such that it was sealed along the edge with vacuum grease (Dow Corning). The plates were subsequently stored at 20°C and scored for crystal growth/precipitation after 1 day, 5 days, 2 weeks, 1 month and 1 year.

2.4.8 Isolation of re-associated 70S ribosomes

The re-associated 70S ribosomes were isolated by washing 295 g of *E. coli* CAN/20-E12 [157] with 1 L of 10 mM Tris acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, 1 mM DTT ($T_{10}M_{14}K_{60}DTT_1$) and subsequently these cells were recovered with a 5000g centrifuge step (GS3, 5500 rpm, 1 hr). The resulting pellet (243 g) was re-suspended in 400 mL $T_{10}M_{14}K_{60}DTT_1$ + 0.72 mM β-mercaptoethanol. The cells were disrupted by passage through a microfluidizer at 75 psi. The cell debris was removed by 2 consecutive centrifuge steps (30000g ; GSA, 12 000 rpm, 45 min). This S30 cell extract was divided into 30 mL fractions with an absorbance of 300 A_{260}/mL and stored at –80°C. To isolate the subunits, a 30 mL S30 fraction (9000 A_{260}) was loaded onto a 6-40% sucrose gradient (in $H_{20}M_1N_{200}SH_4$ buffer; 20 mM HEPES [pH 7.6], 1 mM magnesium acetate, 200 mM ammonium chloride, and 4 mM β-mercaptoethanol) established in a Ti-15 zonal rotor (Beckman). The gradient was run at 25000 rpm for 16 hrs at 4-5°C. Subsequently the gradient was pumped out from the zonal rotor and fractionated while monitoring the absorbance of the eluate at ~275 nm, thus generating an elution profile as seen in Figure 2-9A. The fractions corresponding to the 30S and 50S peaks in the elution profile were pooled (30S and 50S separately) and the subunits sedimented with a 95000 g centrifuge step (45Ti, 35 000 rpm, 24 hrs, 4°C). The subunits were subsequently re-suspended

in $H_{20}M_6N_{30}SH_4$ buffer (20 mM HEPES [pH 7.6], 6 mM magnesium acetate, 30 mM ammonium acetate, and 4 mM β-mercaptoethanol) to a final concentration of approximately 500 A_{260}/mL^{xvi} and stored at −80°C. Afterwards, the subunits were re-associated such that a 40 mL re-association reaction contained 2000 A_{260} of 50S and 30S (therefore 30S:50S is 2:1) in $H_{20}M_{20}K_{30}SH_4$ buffer (20 mM HEPES [pH7.5], 20 mM magnesium acetate, 30 mM potassium chloride, and 4 mM β-mercaptoethanol). This reaction was incubated for 50 min at 40°C with gentle shaking. Next, it was loaded onto a 6-40% sucrose gradient (in $H_{20}M_{20}K_{30}SH_4$ buffer) established in a Ti-15 zonal rotor. The gradient was centrifuged at 20000 rpm for 16 hrs at 4-5°C. Subsequently, the gradient was eluted from the zonal rotor and fractionated while monitoring the absorbance of the eluate at ~275 nm generating an elution profile as seen in Figure 2-9C. The fractions corresponding to the 70S peak in the elution profile were pooled and the 70S ribosomes sedimented with a 45000 g centrifuge step (Ti45, 24 000 rpm, 27 hrs, 4°C). The 70S ribosomes were subsequently re-suspended in $H_{20}M_{20}K_{30}SH_4$ buffer to a final concentration of approximately 500 A_{260}/mL and incubated an additional 30 min at 40°C. After the incubation, the ribosomes were dialyzed into $H_{20}M_6N_{30}SH_4$ buffer (Three 20 min dialysis steps against 100 volumes $H_{20}M_6N_{30}SH_4$ buffer; 3500 MWCO; Spectrum). The ribosomes were then divided into 50 μL aliquots and stored at −80°C.

2.4.9 RNA agarose gels

The rRNA was run on a 2% TAE agarose gel [147]. Generally, about 0.05-0.15 A_{260} of rRNA (in 15-20 μL DNA loading buffer [147]) was loaded per lane and it was heated at 70°C for 5 min prior to loading. The gel and running buffer both contain ethidium bromide (0.5 μg/mL).

[xvi] Ribosome and ribosomal subunit concentrations are expressed as absorbance units (at 260 nm) per mL (A_{260}/mL). Note that: 1 A_{260} of 70S ribosomes = 24 pmols; 1 A_{260} of 50S subunits = 36 pmols; and 1 A_{260} of 30S subunits = 72 pmols.

2.4.10   RNA tube gels

RNA tube gels consisted of a 3.1% acrylamide (37.5:1) gel (prepared in TBE [147] + 0.18% SDS) contained in a glass tube (5 x 140 mm) capped on one end with dialysis membrane (MWCO = 3500 Da). To prepare the sample, 0.5-1 A_{260} of RNA (in 40 μL water + 1% SDS) was heated to 70°C for 2 min and then subsequently mixed with 10 μL 60% (w/v) sucrose + 0.1% bromophenol blue. The sample was kept on ice until it was loaded onto the gel. A current of 0.5 mAmp / tube gel was applied to the gels for 15 min before loading. After loading, a current of 0.5 mAmps / tube gel was applied for 1 hour after which the current was increased to 1.5 mAmps / tube gel for 4.5 hours. The gels were then transferred from the glass tube to a quartz cuvette and scanned throughout their length using a Beckman DU-70 spectrophotometer.

2.4.11   Isolation of 70S ribosomes with h27 mutants

70S ribosomes were isolated from *E. coli* AVS69009 pSTL102, AVS69009 pSTL912G, or AVS69009 pSTL102 (Table 6-1; ref. [104]). In these strains, all chromosomal rRNA genes have been removed such that they harbour a single rRNA operon on a plasmid [153]. 800 mL of LB + ampicillin (100 μg/mL) was inoculated with a 20 mL overnight starter culture (for each strain), and grown at 37°C until the absorbance at 560 nm was 0.4-0.6. The cells were harvested by centrifugation (GSA rotor, 7000 rpm, 4°C, 15min) and subsequently washed in 50 mL $H_{20}M_6N_{30}SH_4$ buffer. This cell suspension was centrifuged again at 7000 rpm, at 4°C, for 15 min in a GSA rotor and the resulting cell pellet was transferred to a mortar. Aluminium oxide (Alcoa A-305, Serva Feinbiochemica; 2x the mass of the cell pellet) was added to the mortar and the pellet was ground using a pestle for 8 min. Next, 5 mL $H_{20}M_6N_{30}SH_4$ buffer was added and the cells were ground for another 5 min. The cell paste was transferred to SA-600 centrifuge tubes, using 10 mL $H_{20}M_6N_{30}SH_4$ buffer to completely remove the cell paste from the mortar. The resulting cell suspension was centrifuged in a SA-600 rotor at 10500 rpm, for

10 min, at 4°C. The supernatant was subsequently centrifuged in a SA-600 rotor at 15000 rpm for 60min at 4°C. The supernatant (S30) was transferred to 60Ti tubes and centrifuged at 28000 rpm for 17hrs at 4°C. Afterwards, the supernatant was removed, the tubes were cleaned with $H_{20}M_6N_{30}SH_4$ buffer, and the pellet was re-suspended in $H_{20}M_6N_{30}SH_4$ buffer yielding approximately 150 A_{260} of crude 70S ribosomes per gram of starting material. To isolate tight-coupled 70S particles, the crude 70S ribosomes (50-100 A_{260}) were loaded onto a 10%-30% sucrose gradient (in $H_{20}M_6N_{30}SH_4$ buffer) and centrifuged in a SW27/28 rotor (18000 rpm, 17 hr, 4°C). This gradient was eluted and fractionated while monitoring the absorbance at 260 nm. The fractions containing 70S ribosomes were pooled and the ribosomes were isolated by centrifuging in a 60Ti rotor at 37000 rpm for 20hrs at 4°C. Again, the supernatant was removed, the tubes were cleaned with $H_{20}M_6N_{30}SH_4$ buffer, and the pellet was re-suspended in $H_{20}M_6N_{30}SH_4$ buffer such that recovery was about 50% of the crude 70S input. The isolated tight-coupled 70S ribosomes were divided into aliquots and stored at –80°C.

2.4.12   Poly(Phe) synthesis

The 4.5 mM system is a modified version of the 6 mM poly(Phe) system (ref. [151]; modifications provided by Yoshika Teraoka, Berlin; personal communication). In this system, the final reaction contained 20 mM HEPES-KOH [pH 7.5], 4.5 mM magnesium acetate, 150 mM ammonium acetate, 4 mM β-mercaptoethanol, 2 mM spermidine, 0.05 mM spermine, 0.22 μM 70S ribosomes 1.78 μg/μL poly(U), 0.46 mM Phe (10 dpm/pmol), 2.67 μM tRNA[Phe], 3 mM ATP, 1.5 mM GTP, 5 mM acetyl-phosphate, and tRNA free S100.

2.4.13   Preparation of functional complexes

Construction of pre- and post-translocational complexes followed the procedure described by Blaha et al. [150], which was based on the work of Watanabe [158]. Deacyl-tRNA[fmet] was bound to the ribosomal P site in the

presence of MF-mRNA (a 46 nucleotide messenger RNA (mRNA) with an AUG (Met) and a UUC (Phe) codon in the centre [159]). This was accomplished by incubating 1 nmol re-associated 70S ribosomes with 5 nmols MF-mRNA and 2 nmols deacyl-tRNA^{fmet} in 625 μl $H_{20}M_6N_{150}SH_4Spd_2Sm_{0.05}$ buffer (20 mM HEPES-KOH [pH 7.5], 6 mM magnesium acetate, 150 mM ammonium acetate, 4 mM β-mercaptoethanol, 2 mM spermidine and 0.05 mM spermine) and incubating the reaction for 15 min at 37°C. The A site was then filled with AcPhe-tRNA^{phe} to yield a pre-translocational state complex by adding 1.5 nmols [^{14}C]AcPhe-tRNA^{phe} (diluted in 625 μL $H_{20}M_6N_{150}SH_4Spd_2Sm_{0.05}$ buffer) and the incubation was continued at 37°C for 30 min. At this point, the reaction mixture was split in two and to 1 aliquot 0.05 nmols EF-G and GTP (in 187.5 μL $H_{20}M_6N_{150}SH_4Spd_2Sm_{0.05}$ buffer) was added. To the second aliquot EF-G was omitted but GTP (in 187.5 μL $H_{20}M_6N_{150}SH_4Spd_2Sm_{0.05}$ buffer) was added. EF-G in the presence of GTP will promote the translocation of the tRNA in the A and P sites into the P and E site and thereby generate a post-translocational state. The translocation reaction was allowed to proceed for 10 min at 37°C after which the level and location of tRNA binding, before and after centrifugation, was assayed using nitrocellulose binding assays and puromycin reactivity assays [150].

2.4.14   Tetracycline binding and Tet(O)-mediated tetracycline release

Tetracycline binding and Tet(O)-mediated tetracycline release was determined using a modification of the tetracycline binding assay described earlier [11]. To measure tetracycline binding, [^{3}H]-tetracycline (concentrations are indicated in figure legend) was incubated in the presence of 70S ribosomes or ribosomal subunits (1-2 μM) in $H_{20}M_{4.5}N_{150}SH_4Spd_2Sm_{0.05}$ buffer for 10 min at 37°C. The binding reaction was then vacuum filtered through a 0.45 μM nitrocellulose filter and washed twice with 2 mL $H_{20}M_{4.5}N_{150}SH_4$ buffer to remove the free [^{3}H]-tetracycline. The ribosome-bound [^{3}H]-tetracycline was then

quantified by scintillation counting. To measure Tet(O)-promoted tetracycline release a mixture containing 1-2 μM 70S ribosomes (or 30S subunits), 10 μM [^{3}H]-tetracycline (NEN 100 dpm/pmol), 0-10 μM purified Tet(O), and 1 mM GMPPNP (Roche) in $H_{20}M_{4.5}N_{150}SH_4Spd_2Sm_{0.05}$ buffer was incubated for 20 min at 37°C. The binding reaction was washed through a 0.45 μM nitrocellulose filter and quantitated as described for the tetracycline binding assay.

2.4.15   Tet(O) and EF-G GTPase Activity

The GTPase assays were done as described previously [160] with some modifications. The reactions were set up as to maintain $H_{20}M_6N_{150}SH_4Spd_2Sm_{0.05}$ buffer conditions and the final ribosome, protein and GTP concentration were as indicated in the appropriate figure legend. The reaction was incubated at 37°C for the indicated time and then stopped by transferring 80% of the reaction into the stop solution (500 μL 2-butanol[xvii] (H_2O saturated) 200 μL 0.5 M H_2SO_4 + 1.5 mM NaH_2PO_4 and 50 μL 200 mM $MoNaO_4 \cdot 2H_2O$) and briefly mixed. After stopping the reaction it was vortexed for 1 min and then centrifuged at 16000g for 10 min. After centrifugation, 150 μL of the 2-butanol layer (determine total volume of layer with pipette) was transferred to 5 ml Ready Value (Beckman) and quantitatied by scintillation counting. The resulting counts were corrected for the volume of the organic layer counted and for the extraction of 80% of the reaction volume. The pmols of $\gamma^{32}P$ hydrolyzed was then determined from the specific activity of the GTP solution.

[xvii] The use of 2-butanol is very important if one substitutes n-butanol, for example, the reproducibility of the extraction is compromised.

Chapter 3

The tetracycline resistance protein, Tet(O), alters the conformation of the ribosomal decoding centre[1]

[1] A version of this work has been published as:

3   The tetracycline resistance protein, Tet(O), alters the conformation of the ribosomal decoding centre

3.1   Introduction

The widespread use of tetracycline for over 50 years in both medicine and as an animal growth promoter has increased the occurrence of tetracycline resistance in microbial organisms (reviewed in ref. [78]). Efflux and ribosomal protection are by far the most common resistance determinants and are found widely in both Gram-positive and -negative species [78]. The dissemination of these resistance determinants is largely dependent on horizontal gene transfer events [78]. Efflux mechanisms limit the accumulation of toxic tetracycline levels in the cytoplasm. In contrast, the ribosomal protection proteins (RPPs) interact directly with the target of tetracycline, the ribosome, to promote release of the bound drug. Two less common resistance mechanisms are chemical modification of tetracycline [122] and mutations in the 16S rRNA genes (seen in *Helicobacter pylori* and *Propionibacterium acnes* [111, 113]).

The role and interaction of the RPPs with the 70S ribosome during tetracycline release has been studied primarily using the RPP determinants, Tet(O) and Tet(M). These specific determinants exemplify the RPPs but this class of proteins also includes Tet(Q), Tet(S), Tet(W) and OtrA. Tet(O) and Tet(M) are generally found on mobile genetic elements, which explains their wide dissemination throughout the eubacteria [78]. The RPPs appear to be derived from Gram-positive species possibly evolving from OtrA [9, 132], which is found in the natural producer of oxytetracycline, *Streptomyces rimosus* [126]. Sequence comparisons show that the amino-terminal GTP binding domains (G-domain) of the RPPs are very similar to the G-domains of other ribosome binding proteins such as the elongation factors and, in the case of EF-G, this sequence similarity exists throughout the length of the protein, albeit to a lesser degree [10, 36, 118].

This similarity extends to the 3-dimensional structure as a 16 Å resolution cryo-EM reconstruction demonstrated that Tet(O) and EF-G have an overall similar shape [47]. The similarity of the RPP's G-domain to that of the elongation factors is exemplified by the fact that a mutation which changes EF-Tu from a GTPase to a XTPase [161, 162] can also be made in Tet(O) on the basis of sequence alignments alone (see section 4.2.3). Furthermore, like the elongation factors, the RPPs require GTP to function [119, 137] and Tet(M) has been shown to compete with EF-G for binding to the ribosome [61]. However, the RPP is not functionally equivalent to the elongation factors *in vivo* or in *in vitro* assays [118, 119].

Crystallographic studies revealed several tetracycline binding sites on the 30S ribosomal subunit [73, 99]. The most highly occupied (primary) site is located such that it sterically interferes with accommodation of the tRNA in the A-site [73, 99]. This agrees well with early studies showing that tetracycline functions as an inhibitor of aminoacyl-tRNA binding to the A-site [88-90]. In the primary binding site, tetracycline interacts with the irregular minor grove of helix 34 (h34; rRNA residues 1196-1200:1053-1056) and the loop of helix 31 (h31; rRNA residues 964-967) [73, 99]. Upon tetracycline binding, Brodersen *et al.* state that the ribosome appears to be competent for the initial stages of ternary complex (EF-Tu·GTP·aa-tRNA) binding, namely decoding and GTP hydrolysis [91, 99]. The subsequent accommodation step of the tRNA into the 50S A-site, however, is effectively blocked preventing the extension of the nascent chain. The ribosome may then be locked in a non-productive and energetically expensive cycle of ternary complex binding and release [99]. The RPPs would be expected to interfere with this unproductive cycle by binding to the tetracycline-blocked ribosome, triggering the release of tetracycline and returning it to the elongation cycle [47].

Visualization of Tet(O) on the *E. coli* 70S ribosome (16 Å resolution) showed that Tet(O) binds to the ribosome in a similar fashion to the elongation factors [47]. In this position, Tet(O) approaches the 70S ribosome from the A-site

side and binds in the intersubunit space contacting the 50S subunit near the base of L7/L12 stalk in the vicinity of both the α-sarcin/ricin loop (H95) and thiostrepton/L11 binding site (H43/44) [47]. On the 30S subunit, Tet(O) contacts the ribosomal protein S12, and the 16S rRNA at h5 and h34 of the decoding site [47]. Despite the general similarity between the interaction of Tet(O) and EF-G with the ribosome, Tet(O) is unable to invoke any of the gross conformational changes seen with EF-G [69] with the exception of the extension of the L7/L12 stalk [47]. When the high-resolution x-ray structures depicting a tetracycline-ribosome complex [99] are combined with the cryo-EM maps of the 70S·Tet(O) complex, Tet(O) appears to approach, but does not overlap the primary tetracycline binding site [47]. Domain IV of Tet(O) instead contacts the base of h34 [47].

In this study, we investigated the interaction of Tet(O) with the 16S rRNA component of the 70S ribosome by chemical probing. This method monitors the chemical accessibility of the rRNA bases and is therefore sensitive to subtle architectural changes, which might go unnoticed by cryo-EM or be constrained by crystal packing forces in the high resolution x-ray crystallography structures. We identified specific sites of Tet(O) interaction with the 16S rRNA that are suggestive of both a close contact and of long-range conformational rearrangements. These interactions may form the basis of Tet(O)-mediated tetracycline resistance.

3.2 Results

3.2.1 Tet(O)·GMPPNP binding to the 70S ribosome

To establish the conditions needed to bind Tet(O) to the ribosome or ribosomal subunits, we measured the binding of tetracycline when Tet(O) was added in the presence of GMPPNP. It was shown previously that Tet(O), in the presence of GTP or a non-hydrolysable GTP analogue (GMPPNP or GTPγS), was able to remove tetracycline from the 70S ribosome, but only in the presence

of GTP was Tet(O) able to act in multiple turnover experiments [11]. When the non-hydrolysable analogues were used, an excess of Tet(O) over ribosomes was required to remove tetracycline, suggesting that without GTP hydrolysis Tet(O) lost its ability to recycle and was locked in a stable complex with the ribosome [11]. Thus in the presence of non-hydrolysable GTP analogues, the binding of Tet(O) is irreversible, tetracycline is prevented from rebinding and therefore Tet(O) binding can be measured indirectly by following tetracycline release. In addition, since tetracycline binds similarly to both 30S subunits and 70S ribosomes, we also tested the ability of Tet(O) to remove tetracycline from the 30S subunit.

The 70S ribosomes or 30S subunits were incubated with tetracycline to bind the drug and subsequently, Tet(O) and GMPPNP were added to the mixture and the incubation continued. As the concentration of Tet(O) increases with respect to the ribosome concentration, the relative tetracycline binding to the 70S ribosomes decreases until reaching a minimum when Tet(O) was present in a 3 molar excess over ribosomes (Figure 3-1). Increasing the Tet(O) concentration above this point did not further the removal of tetracycline, suggesting that tetracycline was occupying sites that were not accessible to be released by Tet(O).

Because it has been suggested that the inhibitory tetracycline binding site is located on the 30S subunit [73, 92, 99], we investigated the ability of Tet(O) to act on the 30S subunit alone. In the concentration range where Tet(O) was able to fully remove tetracycline from 70S ribosomes, it had no effect on tetracycline binding to 30S subunits (Figure 3-1), suggesting that Tet(O) is unable to form a functional and stable interaction with the 30S subunit alone.

3.2.2 Interaction of Tet(O) with the 16S rRNA

Contact between Tet(O) and the ribosome is generally mediated by the rRNA with the exception of a single contact between domain III of Tet(O) and the

Figure 3-1: Tet(O) mediated release of tetracycline

The relative binding of [^{3}H]-tetracycline to either 2 μM 30S ribosomal subunits (grey bars) or 2 μM 70S ribosomes (solid bars) in the presence of increasing amounts of Tet(O) is shown. Tetracycline is bound to ribosomal particles that are then incubated with Tet(O)·GMPPNP thereby releasing the tetracycline, which can be followed using a nitrocellulose filter binding assay (see Experimental Procedures). Tetracycline binding is reported relative to the amount of tetracycline bound in the absence of Tet(O), which corresponds to 0.7 pmol tetracycline per pmol 30S subunit and 0.8 pmol tetracycline per pmol 70S ribosome. The error bars shown correspond to one standard deviation in the reported mean.
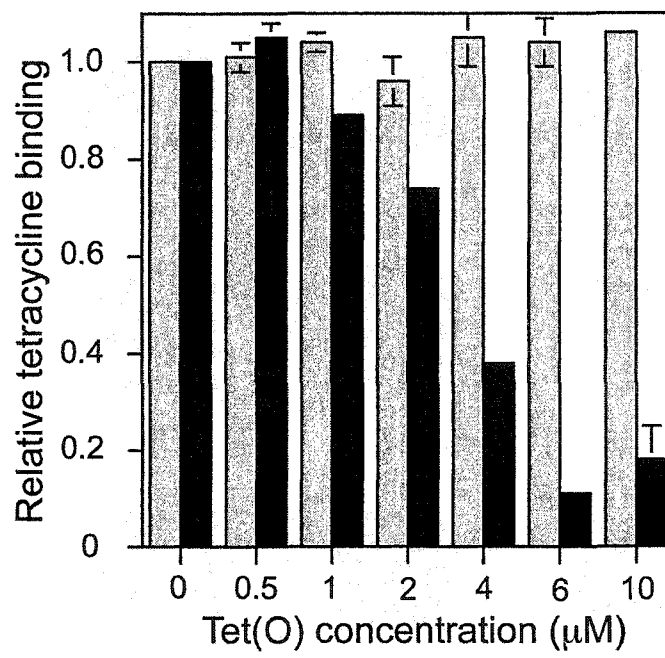
ribosomal protein S12 [47]. To study the interaction of Tet(O) with the 30S ribosomal subunit, the target of tetracycline, we monitored the change in DMS reactivity of the 16S rRNA in response to Tet(O) binding. For these experiments Tet(O), EF-G and tetracycline were bound to 70S•poly(U)•AcPhe-tRNA^{phe} complexes in the presence of GMPPNP. In this complex AcPhe-tRNA^{phe} is bound in an mRNA dependent manner to the P-site. After preparation, the complexes were treated with DMS, which modifies adenosine at its N1 position and cytosine at its N3 position (Figure 1-4B) depending on their accessibility and pKa values. Using primer extension analysis we identified two Tet(O)-dependent changes in DMS reactivity, one at the base of h34 and one within h44 which, surprisingly, is not structurally associated with tetracycline binding [73, 99]. No other bases in the 16S rRNA experienced changes in DMS reactivity upon Tet(O) binding. More than 90% of the 16S rRNA was scanned, with the exception of the initial 20 bases at the 5' end, and 100 bases at the 3' end (Figure 3-2A).

Typical gels illustrating the pattern of reverse transcription stops in h34 and h44 are shown in Figure 3-2B and C. In h44, Tet(O) enhances DMS modification of A1408 which is reflected in Figure 3-2B, lanes 2 and 4, as a marked increase in the amount of product formed by blockage of the reverse transcriptase at the 3' base, C1409, when compared to lane 1. Figure 3-2C (lanes 2 and 4) shows a clear difference in the pattern of reverse transcription stops, where at the base of h34, at the junction of h32, 33 and 34, Tet(O) protects C1214 from DMS modification. In Table 3-1 the intensity of the bands that undergo changes in accessibility upon Tet(O) binding have been quantitated (see Experimental Procedures) and expressed as a ratio compared to the corresponding band in the unbound 70S•poly(U)•AcPhe-tRNA^{phe} complex.

Interestingly, the nature of the GTP nucleotide, used to form the Tet(O)•GTP•Ribosome complex, affects the changes in DMS modification of C1214 and A1408. In the above studies Tet(O) is bound to the ribosome in the

Figure 3-2: Effects of Tet(O), tetracycline and EF-G on the DMS modification pattern of the 16S rRNA.

(A) The 16S rRNA screened for changes in DMS reactivity by primer extension is shown in red.

(B+C) PhosphorImager scans from the primer extension analysis of h44 (B) and h34 (C) are shown. Positions A1408, C1400 and C1214 that experience changes in DMS reactivity are marked with arrows. Changes in the DMS accessibility of a base due to binding of Tet(O), EF-G or tetracycline can be ascertained by comparing the intensity of a band in lane 1 (modified complex alone) with the intensity of a band in lanes 2 through 6. Reverse transcription stops that are independent of the DMS and therefore not considered in this analysis are highlighted by comparison of lane 1 and lane K, which contains the product of a reverse transcription reaction using unmodified rRNA. The position of the reverse transcription stops in the rRNA can be read from the sequencing lanes G, A, T and C. In panels B and C the template rRNA was isolated from DMS modified 70S·AcPhe-tRNA·poly(U) complex alone (lane 1) or in the presence of Tet(O) (lane 2), tetracycline (lane 3), Tet(O) and tetracycline (lane 4), and EF-G (lane 5).

(D) A PhosphorImager scan from the primer extension analysis of positions 1150-1420 of the 16S rRNA. The template rRNA was isolated from DMS modified 70S·AcPhe-tRNA·poly(U) complex in the presence of Tet(O)·GTP (lane 1), heat inactivated Tet(O)·GNPPNP (lane 2), and Tet(O)·GNPPNP (lane 3). The position of the reverse transcription stops in the rRNA can be read from the sequencing lanes G, A, T and C.
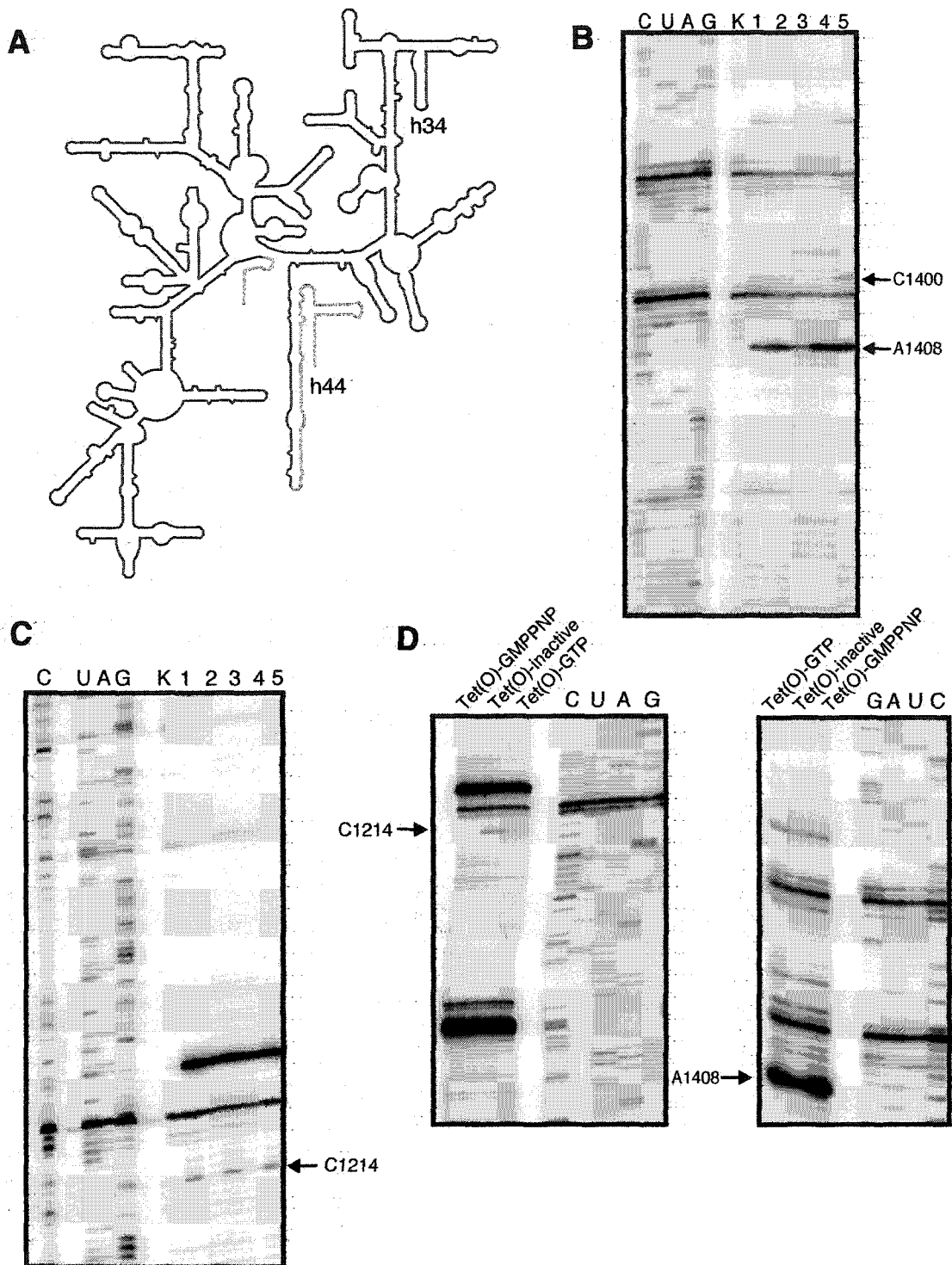
Table 3-1: Tet(O) and EF-G dependent alterations in 16S rRNA DMS reactivity

Position	Relative DMS modification[a]				
	70S	Tet(O)	Tc[d]	Tet(O) + Tc[d]	EF-G
C1214[b]	1.00	0.18 (±0.03)	1.07(±0.13)	0.39(±0.05)	0.97 (±0.12)
C1400[c]	1.00	0.97 (±0.18)	1.06 (±0.09)	1.34 (±0.32)	2.56 (±1.12)
A1408[c]	1.00	1.93 (±0.09)	1.13 (±0.13)	2.37 (±0.58)	1.94 (±0.66)

[a] relative modification refers to the ratio of the product of a reverse transcription stop at a specific base in the sample lane to that in the 70S·AcPhe-tRNA·poly(U) lane.

[b] values reported for alterations in C1214 reactivity represent the average and standard deviation of six primer extension experiments from two independent complexes.

[c] values reported for alterations in C1400 and A1408 reactivity represent the average and standard deviation of four primer extension experiments from two independent complexes.

[d] the tetracycline (Tc) was added to a concentration of 250 μM.

presence of GMPPNP, a non-hydrolysable GTP analogue, resulting in the protection of C1214 and the enhancement of A1408 (Figure 3-2B and C). However, if the non-hydrolysable GTP analogue is replaced with GTP than the C1214 protection is lost but the A1408 enhancement remains (Figure3-2D). This could be explained by the fact that, in the presence of GTP, Tet(O) acts catalytically and therefore is not constantly on the ribosome. This therefore results in the loss of the C1214 protection which is likely dependent on Tet(O) binding. In terms of the A1408 enhancement, this could indicate that the Tet(O) induced changes in h44 persist after Tet(O) leaves the ribosome and as such have a longer half-life than Tet(O) binding.

Tet(O) was bound to the 70S·poly(U)·AcPhe-tRNA^{phe} complex both in the presence and absence of 250 μM tetracycline. Comparison of lanes 2 and 4 in Figure 3-2 (B and C) clearly shows that the presence of tetracycline does not have a gross affect on the Tet(O)-dependent protection of C1214 and enhancement of A1408 toward DMS modification. Quantification of C1214 (Table 3-1) indicates that its relative protection is decreased in the presence of 250 μM tetracycline possibly as a consequence of a competition between Tet(O) and tetracycline. This trend though is not observed in the case of the enhancement of A1408 to DMS modification (Table 3-1). It, however, could be obscured by the high variability in the relative enhancement of A1408 in the presence of Tet(O) and tetracycline. Interestingly, EF-G also appears to enhance the reactivity of C1400 and A1408 to DMS (Figure 3-2B, lane 5; Table 3-1). The increase in DMS reactivity of A1408 and C1400 in the presence of EF-G has not been previously reported in the literature to our knowledge but has been observed in a 70S·EF-G·fusidic acid complex (Dr. K. Wilson, personal communication).

The bases that undergo changes in DMS reactivity in a Tet(O)-dependent manner are located in the decoding site, a location where possibly mRNA or tRNA could influence the accessibility of DMS. Tet(O), tetracycline, and EF-G had no affect on the level of [^{14}C]AcPhe-tRNA^{phe} binding to the ribosome

compared to the 70S•poly(U)•AcPhe-tRNA[phe] complex alone (data not shown), thus eliminating the possibility that differences in the tRNA occupation contributed to alterations in the DMS modification described below. To determine if the reported changes in DMS reactivity were dependent only on the protein, we bound Tet(O) to empty ribosomes and ribosomes complexed with tRNA and/or poly(U) mRNA. In all cases, the characteristic C1214 protection and A1408 enhancement were clearly visible (Figure 3-3A and B), indicating that they are a consequence of the interaction between Tet(O) and the ribosome. In addition, heat inactivation (95°C, 10 min) of Tet(O), before addition to the 70S ribosome eliminated both the protection and the enhancement, demonstrating that they are due to the protein and not an unknown component of the buffer system (data not shown).

3.2.3 Influence of Tet(O) on tetracycline-dependent alterations in DMS modification.

The interaction of tetracycline with 16S rRNA has been studied previously by chemical probing where it was shown to protect A892 and enhance C1054 and U1052 towards DMS modification [96]. In this study, these three sites of interaction were confirmed (Figure 3-4A; lane 3) such that 250 μM tetracycline was able to decrease the modification of A892 by a factor of two and increase the modification of C1054 and U1052 by roughly the same degree (Table 3-2). As stated above DMS usually modifies only adenosine and cytidine at neutral pH in a manner that can be detected by primer extension analysis, thus the detection of DMS-modified uridine is uncommon but observed [96, 101] and may reflect that U1052 is in an unusual chemical environment. When bound to the 70S•poly(U)•AcPhe-tRNA[phe] complex in the absence of tetracycline (Figure 3-4A; lane 2), Tet(O) had no significant effect on DMS modification of the bases implicated in drug binding. This suggests that Tet(O) does not interact with the

Figure 3-3: Effect of mRNA and tRNA on the interaction of Tet(O) with the decoding centre

(A+B) PhosphorImager scans show the primer extension analysis of h44 (A) and h34 (B) when Tet(O) is bound to the 70S ribosome in conjunction with various combinations of mRNA and tRNA. Positions A1408, and C1214 that experience changes in DMS reactivity are marked with arrows. In panels A and B the DMS modified template rRNA was isolated from 70S ribosomes alone (lane 1) or in the presence of Tet(O) (lane 2), Tet(O) + AcPhe-tRNA (lane 3), Tet(O) + poly(U) (lane 4), Tet(O) + AcPhe-tRNA + poly(U) (lane 5), and EF-G + AcPhe-tRNA + poly(U) (lane 6). Dideoxy sequencing reactions are labelled G, A, U, and C. The unmodified control lane is indicated with a K. Quantification of the gel reveals that the relative DMS reactivity of C1214 decreases in lane 2 (0.43), lane 3 (0.52), lane 4 (0.42), and lane 5 (0.66), while the relative DMS reactivity of A1408 increases in lane 2 (1.83), lane 3 (1.94), lane 4 (1.85), lane 5 (1.73) and lane 6 (2.16). The number in brackets indicates the relative DMS reactivity and refers to the ratio of the product of a reverse transcription stop at a specific base in the sample lane (C1214 or A1408) to that in the 70S lane. The values reported for alterations in C1214 and A1408 reactivity represent the average of two experiments.

A

G A U C   6 5 4 3 2 1 K

B

G A U C 6 5 4 3 2 1 K

C1214→
←A1408
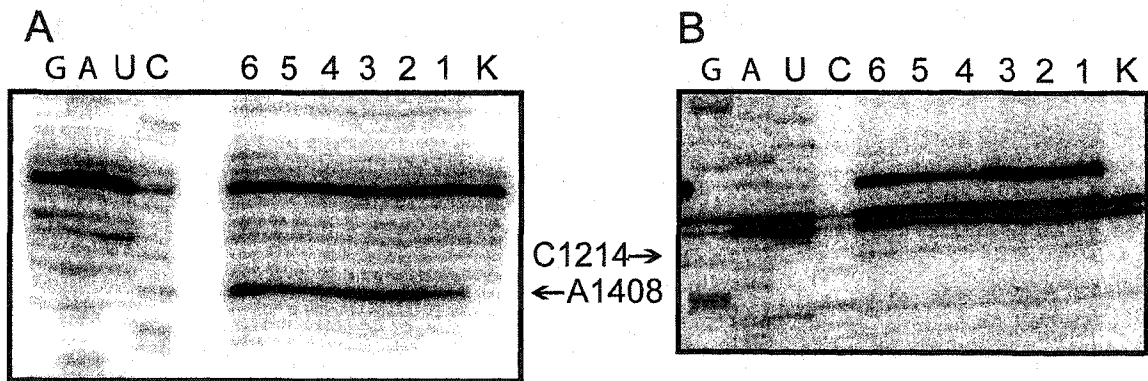
Figure 3-4: Effect of Tet(O) on tetracycline-dependent alterations in DMS modification

(A) PhosphorImager scans show the effect of Tet(O) on the DMS reactivity of 16S rRNA bases (A892, U1052 and C1054) implicated in tetracycline binding. Dideoxy sequencing reactions are labelled G, A, U, and C. The unmodified control lane is indicated with a K. The template rRNA was isolated from DMS modified 70S•AcPhe-tRNA•poly(U) complex alone (lane 1) or in the presence of Tet(O) (lane 2), 250 µM tetracycline (lane 3),and Tet(O) + 250 µM tetracycline (lane 4).

(B+C) PhosphorImager scans show the effect of Tet(O) on A892, U1052 and C1054 when tetracycline is present in a range of concentrations (2.5-250 µM). Dideoxy sequencing reactions are labelled G, A, U, and C. The unmodified control lane is indicated with a K. In panels **B** and **C** the template rRNA was isolated from DMS modified 70S•AcPhe-tRNA•poly(U) complexes alone (lane 1) or in the presence of to Tet(O) (lane 2). Additionally as indicated in panels **B** and **C** the rRNA was isolated from a 70S•AcPhe-tRNA•poly(U) complex with increasing concentrations of tetracycline (2.5, 10, 25, 100, 250 µM) in the presence and absence of Tet(O).

(D + E) Results from the quantification of the gels in panels **B** and **C** are plotted to demonstrate the influence of Tet(O) on the tetracycline-dependent DMS modification of C1054 and A892. The relative modification of C1054 (panel **D**) and A892 (panel **E**) in the presence (●) and absence (Δ) of Tet(O) is indicated at various tetracycline concentrations. Quantification of the U1052 band in panel **C** yielded similar results as seen with C1054. The plotted values represent the mean of two reverse transcription experiments with the error bars corresponding to ±1 standard deviation.
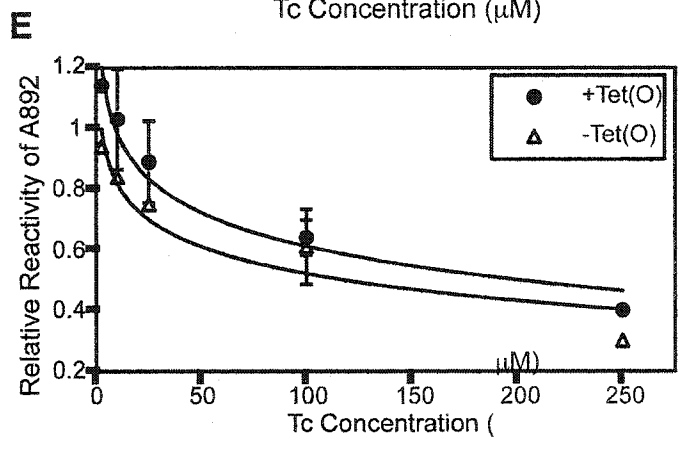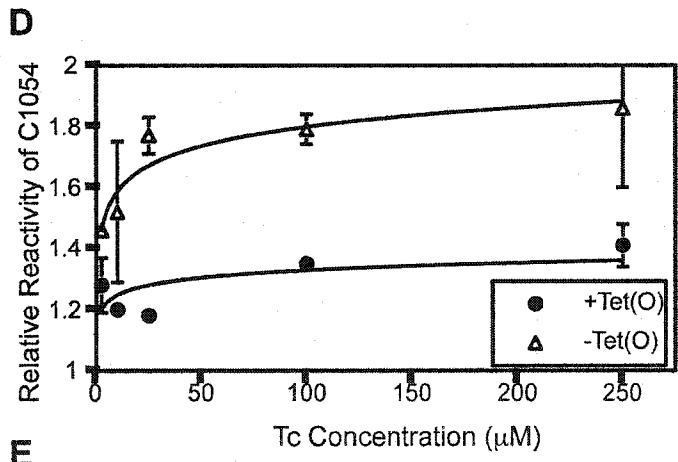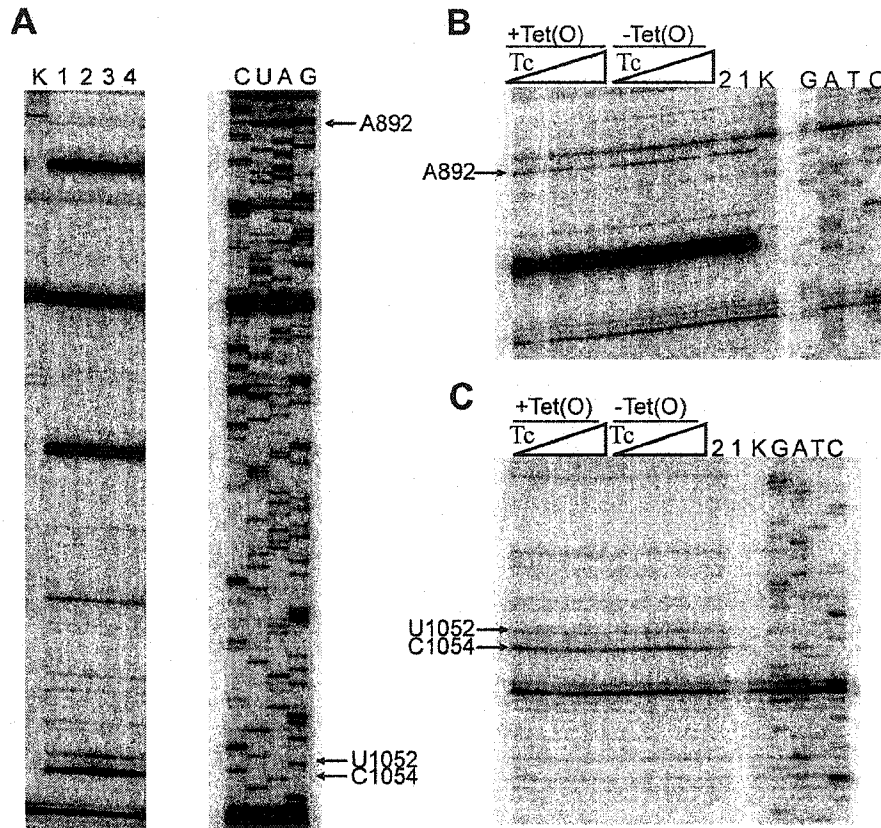
Table 3-2: Effect of Tet(O) on tetracycline-dependent alterations in DMS modification

Position	Relative DMS modification[a]			
	complex	Tet(O)	Tc[c]	Tet(O) + Tc[c]
U1052[b]	1.00	1.11 (±0.16)	2.08 (±0.23)	1.68 (±0.38)
C1054[b]	1.00	1.11(±0.14)	2.17 (±0.30)	1.73 (±0.26)
A892[b]	1.00	0.9 (±0.14)	0.50 (±0.07)	0.53 (±0.10)

[a] relative modification refers to the ratio of the product of a reverse transcription stop at a specific base in the sample lane to that in the 70S·AcPhe-tRNA·poly(U) lane.
[b] values reported for alterations in U1052, C1054 and A892 reactivity represent the average and standard deviation of two to three primer extension experiments.
[c] the tetracycline (Tc) was added to a concentration of 250 μM.

sites of tetracycline binding directly in a manner that can be detected by DMS probing or that it does not interact with these sites in the absence of tetracycline. When Tet(O) is bound in the presence of 250 μM tetracycline and compared to a control complex with only tetracycline bound (Figure 3-4A; lanes 3 and 4) a weak but reproducible protection of C1054 by Tet(O) was observed. This decrease in DMS reactivity was observed in all cases but quantification of the data was not conclusive when the relative modification of C1054 was averaged and the error considered (Table 3-2). In Figure 3-4B and C the effect of Tet(O) on tetracycline-dependent alterations in DMS reactivity over a range of drug concentrations (2.5-250 μM) was investigated and the results plotted in Figure 3-4D and E. At lower concentrations of tetracycline the effect of Tet(O) on the DMS modification of C1054 is more apparent. In Figure 3-4C, C1054 and U1052 show visibly enhanced modification when tetracycline is present in concentrations greater than 25 μM while at the same concentrations but in the presence of Tet(O) the enhancement is clearly reduced. This indicates that Tet(O) is responsible for the decrease in DMS reactivity of C1054 and U1052 in the presence of tetracycline. Unlike C1054 the protection of A892 from DMS modification is largely unaffected by Tet(O) (Figure 3-4B). At concentrations above 100 μM the protection of A892 is observed both in the presence and absence of Tet(O). In Figure 3-4D and E where the relative modification of A892 and C1054 has been plotted as a function of the tetracycline concentration it can be seen that over the range of tetracycline concentrations Tet(O) inhibits the tetracycline induced increase of DMS modification at C1054 while having no effect on A892. The low level modification of C1054 in the presence of Tet(O) could be a consequence of incomplete occupation of the ribosome with Tet(O). This effect is shown in Figure 3-1, where in the presence of excess Tet(O), some ribosomes are still bound by tetracycline. In this case modification of C1054, to a small degree, would occur regardless of the presence of Tet(O) as seen in Figure 3-4D. These results

suggest that Tet(O) prevents the interaction of tetracycline with C1054 while ignoring tetracycline bound to A892.

3.3 Discussion

In many pathogenic bacteria tetracycline resistance is becoming increasingly common and in the case of the intestinal pathogen, *Campylobacter jejuni*, tetracycline resistance is conferred by a ribosomal protection protein (RPP) protein called Tet(O). Recent cryo-EM analysis of a 70S•Tet(O)•GTPγS complex indicates that Tet(O) binds to the elongation factor binding site on the 70S ribosome but does not overlap the primary tetracycline binding site [47]. Using DMS probing, we have identified two sites of interaction between Tet(O) and the 16S rRNA; nucleotides C1214 and A1408 (Figure 3-5A). The contacts with the 30S subunit alone are not enough to promote binding and/or a functional interaction with the 30S subunit, as we have shown that Tet(O) cannot release tetracycline from the 30S subunit but only from the intact 70S ribosome (Figure 3-1). Additionally, by following the tetracycline-dependent changes in DMS reactivity, we have shown that Tet(O) prevents the interaction of the drug with C1054 and U1052 but not A892 (Figure 3-4).

Recent structural studies suggest that these changes in DMS accessibility of C1054 and A892 correspond to tetracycline binding to two discrete sites on the 30S subunit; the primary (h34 and h31) and secondary (h27 and h11) sites [73, 99]. In the secondary binding site tetracycline interacts directly with A892:N1, which explains the protection of this position whereas in the primary binding site a slight shift of C1054 can explain the increase in DMS modification of this base [73, 99]. The enhanced modification of C1054 and U1052 at low concentrations of tetracycline (Figure 3-4D) is in agreement with the dissociation constant (K_d= 2-20 μM) for the single, high affinity, inhibitory tetracycline binding site ([92] and references within). The protection of A892 both in the presence and absence of Tet(O) suggests that Tet(O)'s function is not the removal of tetracycline from the

Figure 3-5: rRNA bases that are altered in DMS modification by the binding of Tet(O) cluster around the decoding centre.

(A) The *E. coli* 16S rRNA secondary structure [22] is shown with the tetracycline-dependent changes in DMS modification (U1052, C1054, A892 [96]) marked in green, the Tet(O) specific C1214 protection in cyan, the EF-G dependent enhancement (C1400) in violet and the A1408 enhancement which is common to both Tet(O) and EF-G in orange.

(B) Tet(O) (red density; [47]) bound to the 30S subunit [17] (PDB ID code 1FJF) in the same orientations as seen in panel **C**. h31, 34 and 44 are coloured yellow blue and red, respectively, while the remaining rRNA is a grey ribbon.

(C) The interaction of domain IV of Tet(O) (red density) with the region around the primary tetracycline binding site. Helices 31(964-968), 34 (1199-1217; 1058-1046) and 44 (1400-1414; 1486-1503) are represented as yellow, blue and red ribbons. The bases that experience changes in DMS accessibility upon tetracycline, EF-G or Tet(O) binding are drawn in a ball and stick representation (colour as in panel A) and additionally A1493 is also similarly represented in red.
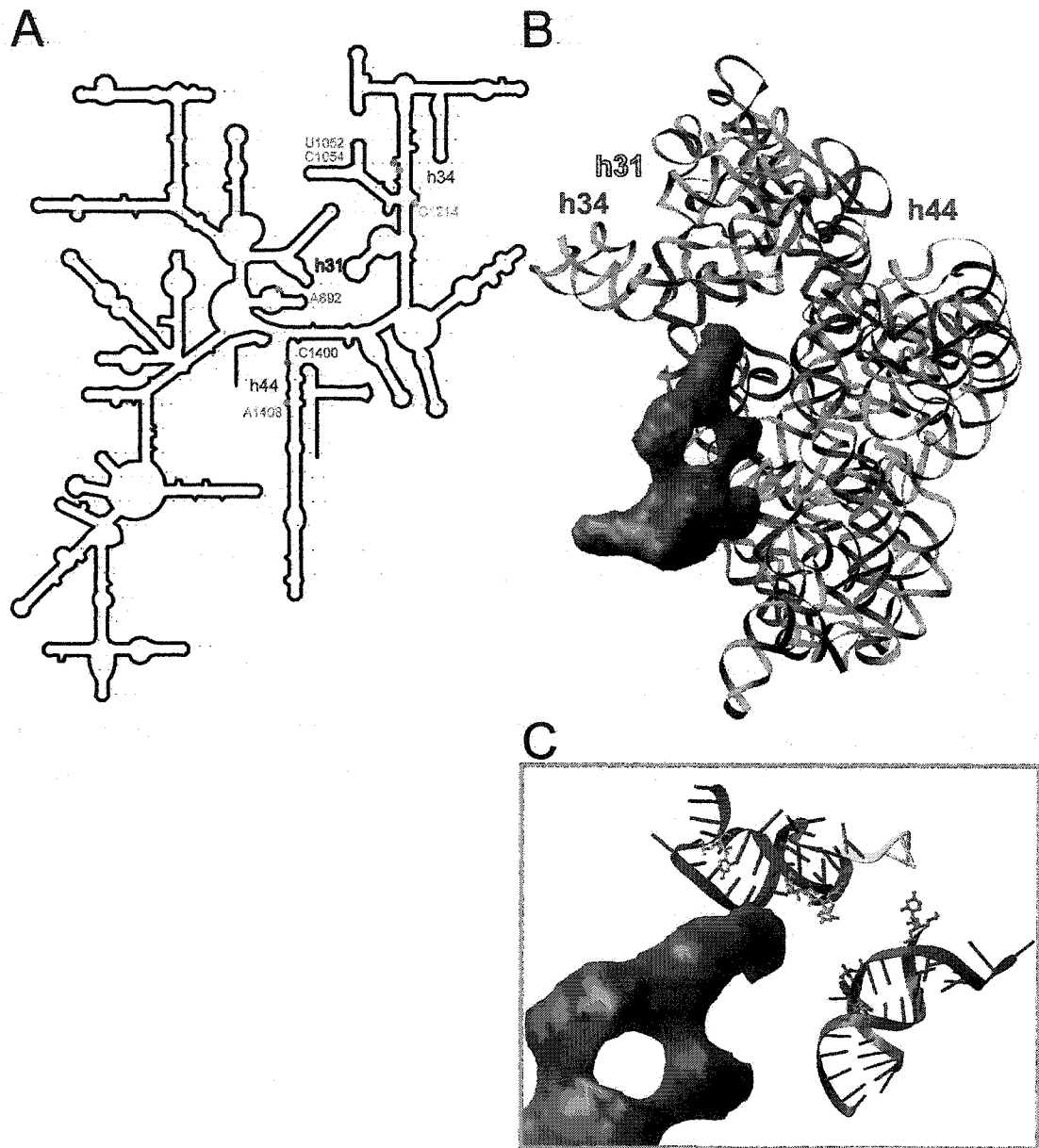
secondary binding site but rather to release tetracycline specifically from the primary binding site. This and the clustering of the Tet(O)-dependent alterations in DMS reactivity near the A-site suggest the primary tetracycline binding site is the inhibitory site and the site which Tet(O) serves to clear (Figure 3-5C). It should be noted, however, the functional significance the other four tetracycline binding sites observed by Pioletti *et al.* [73] cannot be assessed as we were not able to follow their release by DMS probing although they are not correlated with the sites that experience changes in DMS reactivity upon Tet(O) binding.

The localization of a Tet(O) dependent protection in the base of h34 (Figure 3-5A) is in strong agreement with the model of Tet(O) binding demonstrated by cryo-EM [47] and is indicative of a close association of Tet(O) with C1214. The protection of a base from chemical modification indicates that the base is being shielded directly or that it undergoes a conformational change resulting in decreased accessibility. Figure 3-5C illustrates that the cryo-EM derived density of Tet(O) [47] (red) is in close proximity to C1214 (cyan wire frame) and could be interacting directly via unresolved elements or alternatively indirectly though other contacts in h34 (blue ribbon). This is in contrast to the A1408 (orange wire frame) enhancement in h44 (red ribbon) because an enhancement results entirely from a conformational change that increases the chemical accessibility of a base. In this case, Tet(O) could generate a rearrangement in h44 without close contact but rather through indirect interactions possibly using ribosomal protein S12 as a intermediary. In accordance with this notion, the core of S12 makes contact with the backbone of h44 around residues 1491 and 1492 [25] and cryo-EM reconstructions show that Tet(O) does not approach h44 (Figure 3-5C; red ribbon) but does contact S12 [47].

Of the three sites shown on the 16S rRNA secondary structure in Figure 3-5A, that undergo alterations in DMS reactivity upon EF-G or Tet(O) binding, the C1400 enhancement (violet circle) is characteristic of EF-G, the A1408

enhancement (orange circle) is common to both whereas only the C1214 protection (blue circle) is unique to Tet(O), suggesting the importance of the h34 interaction in Tet(O) activity. Although a C1214 interaction seems to be unique in the literature to Tet(O), the role of helix 34 in ribosomal activity is quite well documented. Mutational analysis of h34 has shown that it is involved in maintenance of translational fidelity particularly in stop codon decoding and frameshifting [163]. The importance of this helix is confirmed by the fact that it is targeted by two antibiotics, tetracycline [73, 99] and spectinomycin [105], which block A-site occupation and inhibit translocation, respectively. A role for h34 in translocation has also been suggested recently by chemical probing experiments showing C1054, A1201 and C1203 are protected when EF-G is bound to a pre-translocational complex stalled with thiostrepton [164]. The EF-G-dependent DMS protections reported here and by Matassova *et al.* probably differ because of the nature of the ribosomal complex used. Matassova *et al.* propose that the protections induced by EF-G are indirect and are a consequence of a conformational change in h34 that destabilizes the interaction between h34 and the A-site bound tRNA thus facilitating translocation [164]. This dynamic nature of h34 is in agreement with that envisioned for the role of h34 in tetracycline resistance, where the direct interaction of Tet(O) with the base of h34 would induce a local disturbance in h34 disrupting the binding pocket of tetracycline.

Unlike C1214, which seems to have a unique interaction with Tet(O), A1408 has been shown to undergo changes in DMS accessibility when the ribosome is bound to various factors. The binding of the IF1 [165], EF-G, (this study), Tet(O) (this study) and the translocation inhibitor hygromycin [96] all enhance the reactivity of A1408 whereas A-site bound tRNA [166] and aminoglycoside antibiotics like paromomycin [96] protect it. In some part, these changes can be explained by the dynamic nature of the internal loop of h44 containing A1408. In this loop A1492 and A1493, the latter of which is based paired with A1408 [167], flip out of h44 and insert into the minor groove of the helix formed by the

interaction of the codon (on the mRNA) and anticodon (on the tRNA) during decoding [29]. Analysis of cryo-EM maps of EF-G bound to the ribosome suggests that domain IV of EF-G is contacting h44 and that it distorts the upper half of this helix during the course of translocation [168] possibly leading to the enhancement of DMS modification of C1400 and A1408.

The different effects of GTP and a non-hydrolysable GTP analogue (GMPPNP) on the DMS modification of C1214 and A1408 could be explained by the fact that in the presence of GTP Tet(O) acts catalytically and therefore is not locked on the ribosome as it would be with GMPPNP. This therefore results in the loss of the C1214 protection which is likely dependent on Tet(O) binding. In terms of the persistence of the A1408 enhancement, this could indicate that the Tet(O) induced changes in h44 endure after Tet(O) leaves the ribosome and, as such, have a longer half-life than Tet(O) binding.

The common effect of Tet(O) and EF-G on A1408 of h44 could result from the fact that Tet(O) is evolutionarily derived from EF-G and the interaction of Tet(O) with h44 may simply be a consequence of their common ancestry. On the other hand, tetracycline seems to influence h44 such that its binding disrupts a C967-C1400 UV-induced cross-link and enhances a C1402-C1501 UV-induced cross-link [98]. Additional evidence that tetracycline may alter h44 derives from the fact that both streptomycin and tetracycline are able to block the cleavage between nucleotide 1493 and 1494 by colicin E3 [155]. In this sense, the interaction of Tet(O) with h44 might not be an evolutionary relic but rather could be important in either the release of tetracycline directly or by counteracting effects induced by tetracycline in h44. Additionally the fact that A1408 is enhanced in the presence of Tet(O)•GTP could indicate, as mentioned above, that Tet(O) induced changes in h44 persist after Tet(O) leaves the ribosome and therefore may consequently be involved in preventing tetracycline from rebinding. This, in effect, would prolong the resistant phenotype of the ribosome beyond the

time span when Tet(O) is bound and may enhance the ability of ternary complex to compete with tetracycline for A site occupation.

Our results show that Tet(O) interacts with the base of h34, releases tetracycline from the primary binding pocket and makes further long-range conformational changes in h44, altering the decoding site. The localization of these contacts and/or conformational changes to regions implicated in tetracycline binding and activity suggest they contribute to Tet(O) mediated resistance to tetracycline.

3.4 Experimental Procedures

Re-associated *E. coli* 70S ribosomes from *E. coli* strain CAN/20-E12 [157] were prepared is described in section 2.4.8. Preparation of AcPhe-tRNA^{Phe} followed previously established methods [156].

3.4.1 Determination of Tet(O) dependent tetracycline release

The ability of Tet(O) to dislodge tetracycline from the ribosome was determined using a modification of the tetracycline binding assay described earlier [11]. Tetracycline was bound to 70S ribosomes or 30S subunits in a mixture containing 2 μM 70S ribosomes (or 30S subunits), 10 μM [^{3}H]-tetracycline (NEN; Guelph, Canada; 100 dpm/pmol), 0-10 μM purified Tet(O), and 50 μM GMPPNP (Roche; Laval, Canada) in 12.5 μL binding buffer (20 mM HEPES-KOH [pH 7.5], 6 mM magnesium acetate, 150 mM ammonium acetate, 4 mM β-mercaptoethanol, 2 mM spermidine and 0.05 mM spermine) and incubated 10 min at 37°C. The binding reaction was then vacuum filtered through a 0.45 μM nitrocellulose filter and washed twice with 2 ml binding buffer to remove the free [^{3}H]-tetracycline. The ribosome-bound [^{3}H]-tetracycline was then quantified by scintillation counting.

3.4.2 Preparation and modification of complexes

The protein factors were complexed with re-associated 70S ribosomes programmed with poly(U) mRNA (Amersham Biosciences) and P-site bound AcPhe-tRNA^{Phe}. Initially, 50 pmols of re-associated 70S were incubated for 15 min at 37°C with 40 pmols of AcPhe-tRNA^{Phe}, and 50 μg of poly(U) in 50 μL of N10 buffer (20 mM HEPES-KOH [pH 7.5], 10 mM magnesium acetate, 100 mM ammonium aceate, and 4 mM β-mercaptoethanol) to fill the P-site with tRNA. Subsequently, 200 pmols of purified protein factor (EF-G or Tet(O)) and 5 nmols of GMPPNP in 50 μL of N10 buffer were added to the programmed 70S ribosomes and incubated for a further 30 min at 37°C. Tet(O) was added at a 4:1 ratio (protein to ribosome), because prior experiments showed that this was sufficient to achieve a high occupancy of Tet(O) on the ribosome (Figure 3-1). After the 37°C incubation, 5 μL of the complexes were taken for nitrocellulose filter binding tests to determine the level of tRNA binding in the complex, which was unaffected by the presence of EF-G or Tet(O) (0.7-0.8 [^{14}C]AcPhe-tRNA^{Phe} per 70S ribosome). The remaining 95 μL of the 70S ribosome complexes were chemically modified by adding 1.9 μL of DMS (dimethyl sulfate) diluted (1:5) in ethanol (or only ethanol to the unmodified control) and incubated for a further 10 minutes. The modification reaction was stopped by adding 25 μL of DMS stop buffer (1 M Tris-HCl [pH 7.5], 1 M β-mercaptoethanol, 0.1 M EDTA) and 300 μL 95% ethanol. The rRNA was precipitated and resuspended in 200 μL TE/SDS (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5% SDS, 5 mM EDTA) and phenol extracted three times (1 volume), followed by three chloroform extractions (1 volume). Finally, the rRNA was ethanol precipitated and resuspended in water to a final concentration of 0.1 μg/μL.

3.4.3 Primer Extension Analysis

DMS modification of adenosine and cytosine stops cDNA synthesis by reverse transcriptase one base before the site of modification allowing for the

localization of modification by primer extension experiments. Primer extension analysis of the modified isolated rRNA was performed as described previously [169] using the primers listed in Table 6-4. Briefly, 0.6 pmol of the appropriate primer, [^{32}P]-labelled at 5' end, was hybridized to approximately 1 pmol of rRNA, which served as the template for primer extension. The extension reaction was carried out using 0.4 units AMV reverse transcriptase (Roche) for 45 min at 42°C in a buffer containing 122.5 mM Tris-HCl [pH 8.4], 11 mM MgCl$_2$, 15 mM KCl, 11 mM DTT, 250 μM dNTPs. For sequencing reactions, dideoxynucleotides were added to a concentration of 5 μM. The cDNA products of the primer extension reaction were ethanol precipitated and loaded on a 6% polyacrylamide gel. Gels were scanned using a Molecular Dynamics PhosphorImager and quantified using the ImageQuant software package (Molecular Dynamics). Changes in the DMS modification profile were identified by visual inspection and, using an area profile of each lane normalized according to bands corresponding to DMS independent stops. Bands that displayed visible changes in the PhosphorImager scans were quantified by integrating the area under the peak in the normalized area profile. Reported alterations were visible in multiple primer extension experiments performed on independent complexes, where the values reported correspond to the average and standard deviation of all experiments.

Chapter 4

Analysis of Tet(O)-mediated tetracycline resistance: Interaction with the ribosomal elongation cycle[1].

[1] A version of this work has been submitted to EMBO Journal as:

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4 Analysis of Tet(O)-mediated tetracycline resistance: Interaction with the ribosomal elongation cycle.

4.1 *Introduction*

Tetracycline resistance in the intestinal pathogen, *Campylobacter jejuni*, can be conferred by a soluble protein factor called Tet(O) [10]. Tet(O) is part of a larger group of proteins called Ribosomal Protection Proteins (RPP), which includes Tet(M), Tet(Q), Tet(S), Tet(T), Tet(W) and OtrA [78]. RPPs share extensive sequence homology with ribosome-binding proteins involved in protein synthesis [36] and, based on the presence of conserved motifs, can be grouped within the translation factor superfamily of GTPases [170]. As such, Tet(O) interacts directly with the target of tetracycline, the 70S ribosome, and promotes the release of tetracycline in a GTP dependent manner [11, 119].

Tetracycline inhibits protein synthesis where it specifically blocks the elongation cycle by preventing incoming aminoacyl-tRNA (aa-tRNA) from binding to the ribosomal A site. Recently X-ray crystallographic studies of a tetracycline-ribosome complex revealed a tetracycline binding site that is positioned so as to sterically interfere with aa-tRNA binding [73, 99]. Accordingly, recent dimethyl sulfate (DMS) probing experiments demonstrated that Tet(O) removes tetracycline bound specifically to this site [115], consistent with its role as the single inhibitory binding site [109].

Current models based on cryo-EM reconstructions propose that Tet(O) does not directly interfere with tetracycline binding but rather acts allosterically distorting the tetracycline binding site, and releasing the bound drug [47]. The proposed conformational change resulting in tetracycline release probably involves helix 34 (h34) of the 16S rRNA as (1) h34 forms an integral part of the primary tetracycline binding site [73, 99], (2) cryo-EM reconstructions show that domain IV of Tet(O) contacts the base of h34 [47] and (3) Tet(O) protects

nucleotides at the base of h34 from chemical modification by DMS [115]. It should be noted, however, that the interaction of Tet(O) is not limited to h34. Cryo-EM data suggests that it also makes contact with the ribosomal protein S12 and helices h5/18 of the small ribosomal subunit while on the large subunit it contacts helices H43/44/95 [47].

In spite of this rather detailed knowledge, several basic aspects of the mechanism of Tet(O) action have still to be elucidated. In this paper we first determine at which point Tet(O) is intercalating into the elongation cycle, showing that Tet(O) preferentially interacts with the post-translocational ribosome. Furthermore, we analyze the interaction of Tet(O) with the GTPase-associated region (GAR) on the ribosome, finding that Tet(O) displays a distinctive interaction with the L11 region and is capable of inducing conformational changes within the ribosome. The interaction of tetracycline with the ribosome has also been also studied, revealing that binding is accompanied by a rate-limiting step which follows first order reaction kinetics. These results are incorporated into a model that describes the entire cycle of Tet(O)-mediated tetracycline resistance.

4.2 Results

4.2.1 Chemical probing of Tet(O) functional complexes

Prior studies showed that there is likely a direct interaction between Tet(O) and the small ribosomal subunit that is important for triggering the release of tetracycline and is revealed as a characteristic Tet(O)-dependent protection from DMS modification at the base of helix 34 (C1214) in the 16S rRNA (section 3.2.2 and ref. [115]). Here we use this protection as a marker to monitor the interaction of Tet(O) with the ribosome in various functional states. Tet(O) was bound to pre- and post-translocational (PRE and POST) ribosomal complexes (see Figure 4-1A

Figure 4-1: Tet(O) interacts with the POST state

(A) A schematic representation of the pre and post-translocational states is shown. In the PRE complex deacyl-tRNA^{fmet} (black tRNA) is located in the P site and AcPhe-tRNA^{Phe} (grey tRNA) is located in the A site, whereas in the POST state the tRNAs have been translocated to the E and P sites, respectively.

(B) PhosphorImager scans show the DMS reactivity of 16S rRNA isolated from pre- and post-translocational complexes. A magnified view of the region around C1214 (indicated with an arrow) is shown and the lanes are numbered exactly as in the upper gel. The dideoxy sequencing lanes are indicated with G, A, T, and C, whereas the components in the other reactions are indicated in the table above the gel (70S, empty ribosomes; PRE, pre-translocational state ribosomes; Post, post-translocational state ribosomes). The gel shown is representative of that seen in 4 primer extension experiments made from 2 independent complexes. The intensity of the bands was normalized using the band indicated (*).

(C) The amount of AcPhe-tRNA^{Phe} bound to the pre- and post-translocational states in the presence (black bars) and absence of Tet(O) (grey bars) is shown. AcPhe-tRNA^{Phe} binding is given as the ratio of pmols [^{14}C]AcPhe-tRNA^{Phe} bound per pmol of 70S ribosomes.

(D) The puromycin reactivity of the complexes used in the chemical probing experiments is indicated, where in addition EF-G was added to PRE and POST complexes to illustrate the change in puromycin reactivity that accompanies translocation of the tRNAs. The grey bars represent PRE state complexes and the black bars are POST state complexes, where EF-G and Tet(O) are added as indicated on the x-axis. The puromycin reactivity is given relative to the puromycin reactivity of the POST complex, which corresponds to 0.6 pmols of PM+ AcPhe per pmol 70S ribosome, and is given as percent.

(E) The elution of GTPγS from the Sephacryl S300 spun column when incubated with Tet(O)HN and either empty ribosomes (●), POST state ribosomes (Δ), or PRE state ribosomes (○) is shown. Tet(O)HN was bound to the functional complexes (Experimental Procedures) and the complex isolated essentially as described previously [11], where the binding reaction contained 1.6 μM Tet(O)HN, 1.6 μM ribosomal complex and 25 μM [^{35}S]GTPγS as indicated. The DPM values plotted have been normalized based on the amount of ribosomes eluted in the first fraction. Additionally the background values derived from the amount of GTPγS that elutes with either Tet(O) or ribosomes alone have been subtracted. The results plotted are the representative of two experiments, where the duplicate determinations deviate from the mean by <11%.
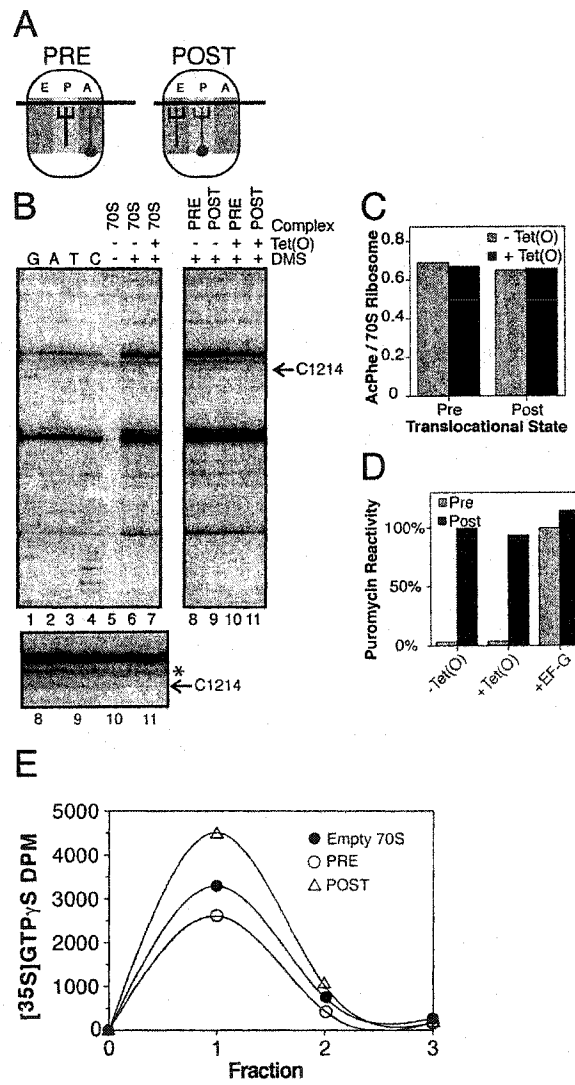
and Experimental Procedures) in the presence of a non-hydrolysable GTP analogue (GMPPNP) which allows for formation of a stable 70S•Tet(O)•GMPPNP complex [11]. Subsequently, these complexes were treated with DMS, which modifies adenosine and cytidine at their N1 and N3 position (Figure 1-4B), respectively, depending on their chemical environment, in a manner that can be readily detected by primer extension analysis [171]. The results of a typical primer extension experiment are shown in Figure 4-1B. C1214 in the POST state ribosome is strongly protected from DMS modification in the presence of Tet(O) (Figure 4-1B; compare lanes 9 and 11) while that of the PRE state ribosome is only weakly affected by Tet(O) (Figure 4-1B; compare lanes 8 and 10). When several gels are quantified, C1214 in the POST/Tet(O) complex (Figure 4-1B; lane 11) is 51±13% as reactive as it is in the POST complex alone (Figure 4-1B; lane 9). In contrast, when Tet(O) is incubated with ribosomes in the PRE state (Figure 4-1B; lane 10) the reactivity of C1214 is relatively unchanged as it is 98±26% as reactive as in the PRE state ribosome alone (Figure 4-1B; lane 8). This shows that Tet(O) interacts with the POST state ribosome shielding C1214:N3 from methylation, but does not interact correspondingly with the PRE state ribosome. Thus, it appears that Tet(O) interacts specifically with the post-translocational ribosome, a state that resembles a tetracycline-blocked ribosome with an open A site.

In order to confirm that the PRE and POST complexes are not altered by the presence of Tet(O), the effect of Tet(O) on the level of tRNA binding and on the puromycin reactivity of the AcPhe-tRNA^{Phe} was ascertained (Figure 4-1C and D). In Figure 4-1C it can be seen that the presence of Tet(O) has no effect on the level of AcPhe-tRNA^{Phe} binding in both the PRE and POST complexes. Furthermore, Tet(O) is unable to alter the location of the tRNA and, accordingly, has no effect on the puromycin reactivity of the PRE complex unlike EF-G (Figure 4-1D). These results indicate that we are indeed detecting the interaction of Tet(O) with defined PRE and POST complexes and that the change in C1214

DMS reactivity is due exclusively to the action of Tet(O) rather than to changes in tRNA occupation or location. Moreover, the fact that Tet(O) does not translocate the tRNA or interact with the PRE complex clearly explains why Tet(M), a RPP like Tet(O), cannot substitute for EF-G *in vitro* or *in vivo* [118, 119] despite their overall sequence similarity.

4.2.2 Tet(O) binding to PRE and POST complexes

In the above DMS probing experiments we made the assumption that the Tet(O)-dependent protection of C1214 from DMS modification is an indication of Tet(O) binding to the ribosome. To confirm this assumption, we directly measured Tet(O) binding to PRE and POST complexes by following their co-elution from a size-exclusion column. When Tet(O) and GTPγS were applied to the column in the presence of either empty ribosomes, a PRE complex, or a POST complex, we observed that 1.4-1.7 times more GTPγS is co-eluted with the POST complex as compared to empty ribosomes or to the PRE complex (compare the Δ with the • and o in Figure 4-1E), indicating that Tet(O) binds preferentially to the POST state. The occupancy of the ribosomal complex in the first fraction (Figure 4-1E) as judged by the co-elution of GTPγS and 70S ribosomes corresponds to 0.59, 0.81, and 0.47 pmols Tet(O) per pmol 70S ribosome in the empty, POST, and PRE complexes, respectively. In this case, it is especially significant that the POST/Tet(O) complex displayed a 35% higher occupancy compared to the empty ribosome/Tet(O) complex because this corresponds to the fraction of ribosomes in a defined POST state (see Experimental Procedures). This implies that in the sample of POST state ribosomes, the 30% that are in a defined POST state are completely occupied by Tet(O) because the amount of Tet(O) used is not saturating (1:1 Tet(O) to ribosome ratio). By the same reasoning the similar binding of Tet(O) to pre-translocational ribosomes and empty ribosomes suggests that Tet(O) is only binding to the empty ribosome fraction in the PRE sample. These results support

the chemical probing experiments above and establish that Tet(O) preferentially interacts with the post-translocational ribosome and in this sense the absence of a Tet(O)-dependent footprint on C1214 in the pre-translocational state probably results from a lack of Tet(O) binding.

4.2.3 Construction of XTP-dependent mutant of Tet(O)[i]

Previous studies employing a xanthosine triphosphate (XTP)-dependent mutant of EF-Tu have been especially useful for studying the interaction of the elongation factors by following their (G/X)TPase activity [161, 162, 172]. With a similar aim, we constructed an XTP-dependent mutant of Tet(O) using a D131N mutation homologous to the D138N mutation in EF-Tu that abolished the affinity of protein for guanosine diphosphate (GDP) while increasing its affinity for xanthosine diphosphate (XDP) [161, 162]. Accordingly, *in vitro* studies showed that Tet(O)D131N has very low ribosome-stimulated GTPase activity, compared to that of wild type Tet(O) (Figure 4-2A). The low GTPase activity of Tet(O)D131N indicates that the protein has lost its ability to hydrolyze GTP efficiently. In line with this observation, Tet(O)D131N was unable to confer tetracycline resistance *in vivo* (Table 4-1), since GTPase activity is essential for Tet(O) [137] and XTP is not present within *E. coli* [161].

In order to confirm that this mutant is in fact active and XTP-dependent we examined the ability of Tet(O)D131N to promote poly(Phe) synthesis in the presence of tetracycline (Figure 4-2B). When 240 μM tetracycline was added to the poly(Phe) producing system the activity dropped to 40% (compare black and white bars in Figure 4-2B) and this was reversed by the addition of wild type Tet(O) (Figure 4-2B; grey bar). In contrast, when Tet(O)D131N was added to the tetracycline-inhibited system in the absence of XTP (Figure 4-2B; white cross-hatched bar) there was only a slight change in activity, however, activity was completely restored when Tet(O)D131N and XTP are added together (Figure 4-

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[i] The Tet(O)D131N mutant was constructed by Dr. C.A. Trieber.

Figure 4-2: Tet(O)D131N stimulates EF-Tu dependent GTPase activity

(A) The intrinsic GTPase activity (grey bars) and ribosome-stimulated GTPase activity (black bars) of Tet(O), Tet(O)D131N, and heat-inactivated Tet(O) (95°C, 10 min) are shown. 100% GTPase activity corresponds to the ribosome-stimulated GTPase activity of wild-type Tet(O) (2800 pmols GTP hydrolyzed per pmol Tet(O) at 37°C over 10 min).

(B) The ability of Tet(O) and Tet(O)D131N to rescue a tetracycline-inhibited poly(Phe) synthesizing system is shown. The activity of the uninhibited system (24 pmols [^{14}C]Phe incorporated per ribosome in 60 sec at 37°C) is indicated with the black bar. The composition of the other reactions is indicated below the graph and the activity of these reactions is given relative to the uninhibited system. The assay used was modified version of that described previously [151].

(C) The effect of Tet(O)D131N on the ribosome-stimulated GTPase activity of the binary (EF-Tu•GTP; shaded bars) or ternary (EF-Tu•Phe-tRNA•GTP; black bars) complex is shown. The reported GTPase activities are given relative to the ribosome-stimulated GTPase activity of the binary and ternary complexes (0.6 and 2.7 pmols GTP hydrolyzed in 5 min at 37°C, respectively).
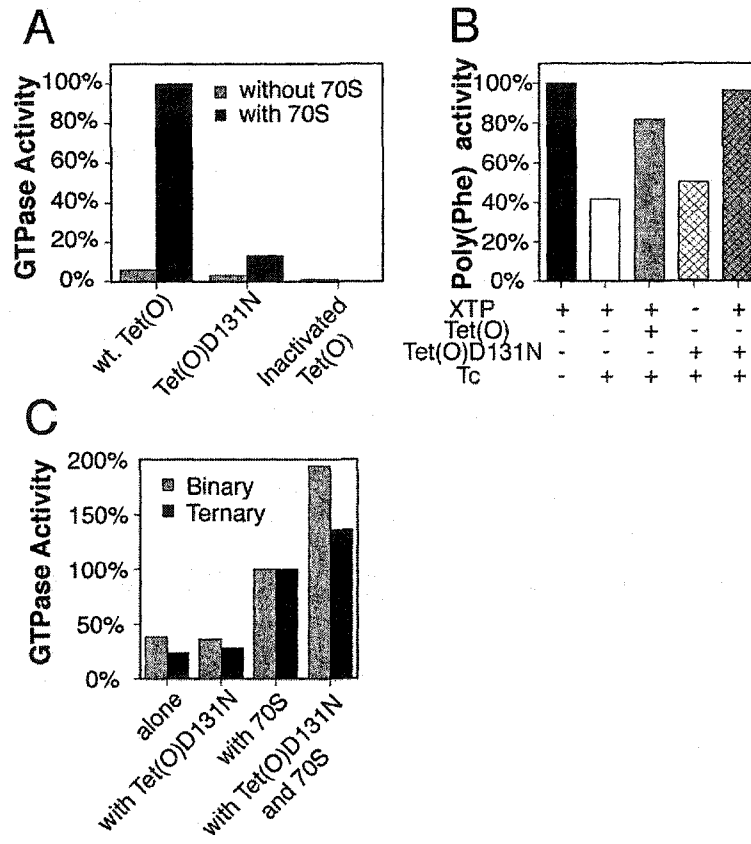
Table 4-1: *In vivo* activity of Tet(O)D131N[A]

Strain	Plasmid	MIC (μg/mL)
JM109	pMS119EH	4
JM109	pMSTet(O)	128
JM109	pMSTet(O)HC	64
JM109	pMSTet(O)D131NHC	4

[A] The MICs were determined as described previously [11]. This data was kindly provided by Dr. C.A. Trieber.

2B; grey cross-hatched bar). Note that the poly(phe) synthesis system contains GTP indicating that Tet(O)D131N is no longer active in the presence of GTP and absence of XTP. Together these results indicate that the $Asp_{131} \rightarrow Asn$ mutation in the nucleoside binding motif ($\underline{N}_{128}K\underline{ID}_{131}$) of Tet(O) greatly reduces the GTPase activity of Tet(O) while making it dependent on XTP for activity. The functional equivalence of Asp-138 in EF-Tu and Asp-131 in Tet(O) suggests that the two have a similar GTP binding pocket. Additionally, as previously described for EF-Tu [161], Asp-131 of Tet(O) probably interacts with the 2-amino group of guanine, whereas in Tet(O)D131N the Asn residue favours the interaction with the 2-carbonyl group of xanthosine.

4.2.4 Interplay of Tet(O)D131N with EF-Tu in the presence of ribosomes

The two ribosomal elongation factors, EF-G and EF-Tu, stimulate each other's GTPase activity in the presence of empty ribosomes [173]. Does Tet(O) as a derivative of EF-G do the same? To clearly measure a possible change in the GTPase activity of EF-Tu in the presence of Tet(O) and 70S ribosomes, the Tet(O)D131N mutant was used because the ribosome-stimulated GTPase activity of wild type Tet(O) is much larger than that of EF-Tu; the D131N mutant will consume XTP, so the measured GTPase will be exclusive to EF-Tu. The GTPase activity of EF-Tu was studied using isolated binary (EF-Tu•GTP) or ternary (EF-Tu•Phe-tRNA•GTP) complexes on their own or in the presence of 70S ribosomes and/or Tet(O)D131N plus XTP. In Figure 4-2C, it can be seen that on their own, the binary and ternary complexes have a low GTPase activity, and the activity is unchanged by the addition of Tet(O)D131N. This indicates that there is no direct interaction, between the two, that affects the GTPase activity when free in solution. In contrast, when either the binary or ternary complex are mixed with 70S ribosomes, there is an approximately 3-fold increase in the GTPase activity (Figure 4-2C). This ribosome-stimulated GTPase activity is enhanced a further 1.4-1.9 times by the addition of Tet(O)D131N to the ternary

and binary complex, respectively (Figure 4-2C). The increase in ribosome-stimulated EF-Tu GTPase activity by Tet(O)D131N suggests that Tet(O) invokes a conformational change in the ribosome that persists after it has left the ribosome thus leading to a stimulation in EF-Tu's ribosome-dependent GTPase activity, similar to what was suggested for the synergy between EF-G and EF-Tu [173]. It is unlikely that the increase in GTPase activity is due to residual GTPase activity in the Tet(O)D131N mutant, as the concentration of free GTP in the solution is low; and what does dissociate from the isolated EF-Tu would have to compete with the large excess of free XTP for binding to Tet(O). Furthermore, the same amount of Tet(O)D131N was present in the reactions containing either binary or ternary complex, but the extent of GTP hydrolysis was different in these reactions indicating that GTP hydrolysis was not caused by the residual GTPase activity of Tet(O)D131N.

4.2.5 Defining the interaction between Tet(O) and the ribosomal GTPase-associated centre

The stimulatory effect of Tet(O) on EF-Tu may be mediated by the GTPase-associated region (GAR) on the 50S ribosomal subunit. Furthermore, since elements of the GAR, such as the L11 region and α-sarcin loop have been proposed to coordinate elongation factor binding [174-177], they could play a role in the selective binding of Tet(O) to the POST state ribosome.

DMS probing and primer extension analysis of the rRNA elements in the GAR shows that Tet(O) interacts with the L11 region, namely H42/43/44 (Figure 4-3A). In this region Tet(O) shows a unique interaction compared to that already described for EF-G [63]. Like EF-G, Tet(O) protects bases in the loop of H43 from DMS modification but the specific bases differ somewhat. Tet(O) reduces the DMS reactivity of A1070 to 75% ($\pm$4%) of its respective reactivity in the empty

Figure 4-3: Interaction of Tet(O) with the GAR

(A) The PhosphorImager scans show the primer extension analysis of H42/43/44 where the template used was unmodified rRNA (lane 4) or DMS modified rRNA from 70S ribosomes (lane 3), 70S ribosomes + AcPhe-tRNA^{Phe} + Tet(O) (lane 5), 70S ribosomes + AcPhe-tRNA^{Phe}+EF-G (lane 6) or 70S ribosomes + AcPhe-tRNA^{Phe} (lane 7). The dideoxy sequencing reactions are labelled G and C. Positions corresponding to DMS independent stops in the 23S rRNA and thus omitted from our analysis are revealed in lane 4. Primer extension was done using an oligo-nucleotide complementary to positions 1120-1136 of the 23S rRNA. Positions that experience changes in DMS reactivity are marked with arrows. This gel of the L11 region is representative of three primer extension experiments done on independent complexes.

(B+C)The effect of an increasing concentration of thiostrepton (B) and α-sarcin (C) on the GTPase activity of Tet(O) (●) and EF-G (Δ) is shown.
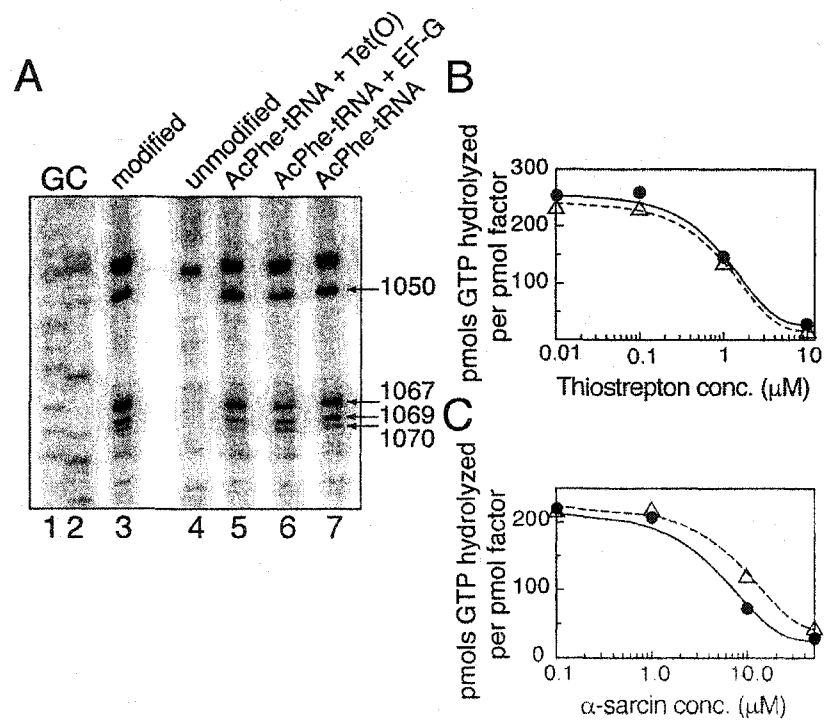
ribosome while reducing that of A1069 to 84% (±7%), in contrast, EF-G decreases the DMS reactivity of A1067 to 79% (±5%) (Figure 4-3A, compare bands indicated in lanes 3, 5 and 6). Tet(O) also differs from EF-G in that it interacts with H42 where it enhances the accessibility of A1050 such that its DMS reactivity increases to 136% (±10%) (Figure 4-3A, lane 5). Furthermore, the presence of AcPhe-tRNA^{Phe} had no effect on the DMS reactivity of the rRNA scanned (Figure 4-3A, lane 7) and therefore the alterations in DMS reactivity observed above are due exclusively to the added protein. Additionally, scanning by reverse-transcription detected changes in the DMS modification pattern within H95 where the Tet(O)•70S complex shows a pattern very similar to that already described for the EF-G•70S ribosome complex [63]. More specifically, Tet(O) like EF-G protects A2660 from DMS modification (data not shown). These results illustrate that the interaction with the GAR, although similar to that of the EF-G [63], is unique to Tet(O), a feature that could be important for coordinating the interaction of Tet(O) with the elongating ribosome or responsible for Tet(O)'s inability to promote translocation.

To establish that the interaction with the L11 region has functional significance for Tet(O), we investigated the effect of thiostrepton and α-sarcin on Tet(O) activity. These compounds were selected because they target the L11 region and α-sarcin loop, respectively, and are inhibitors of EF-G's GTPase activity presumably by preventing EF-G from stably interacting with the ribosome [60, 64, 178-180]. As demonstrated in Figure 4-3B, thiostrepton blocked the uncoupled ribosome-stimulated GTPase of Tet(O) in a similar fashion to that of EF-G such that both proteins lost 50% of their activity when thiostrepton was present at 1 μM. Thiostrepton sensitivity has also been observed with Tet(M), a homologue of Tet(O) sharing 76% amino acid identity, and was shown to result from the disruption of the Tet(M)/ribosome interaction [61], suggesting that the interaction with the L11 region detected by DMS probing is an important determinant in Tet(O)/(M) binding. Moreover, as 70S ribosomes are treated with

increasing amounts of α-sarcin, a ribotoxin that clips the phosphodiester backbone in H95 3' to G2661 [64, 181], they become increasingly defective in stimulating the GTPase activity of both factors (Figure 4-3C). In general, the GTPase activities of Tet(O) and EF-G were similarly sensitive to α-sarcin, where 50% of their activity was lost at 7 and 12 μM α-sarcin, respectively. The sensitivity of Tet(O)'s ribosome-dependent GTPase activity to thiostrepton and α-sarcin suggests that the interactions between Tet(O) and the 23S rRNA (H95 and H42/43/44) detected above are important for Tet(O) activity and may regulate its binding similar to that seen with EF-G [60, 64].

4.2.6 Tetracycline binding to 70S ribosome

Tet(O) and tetracycline both seemingly affect h44 in the 16S rRNA through long-range interactions [115, 155]. To investigate the possibility that tetracycline binding is accompanied by a conformational change, we calculated the activation energy for tetracycline binding. The kinetics of tetracycline binding to 70S ribosomes were followed at various temperatures for times up to 60 min, but as seen in Figure 4-4A, the binding saturated after approximately 180 sec. As the binding kinetics in Figure 4-4A showed a clear temperature dependence they were processed to derive a value for the activation energy (Ea) for tetracycline binding (see Experimental Procedures). This involved the assumption that the binding reaction is second order with a rate-limiting first order step such as a conformational change and this rate-limiting step is measured in filter binding assays. If this assumption is true then the reaction should follow Equation 1 (Experimental Procedures). As seen in Figure 4-4B, this assumption indeed holds true as the plot of $\ln((dpm_{max}-dpm_t)/dpm_{max})$ versus time yielded straight lines, where the slope corresponds to the initial apparent rate constant. As illustrated in Figure 4-4C, the temperature dependence of the rate constants was

Figure 4-4 Kinetics of tetracycline binding:

(A) The reaction kinetics for tetracycline binding to the ribosome are shown at $4^\circ C$ (O), $10^\circ C$ (▲), $18^\circ C$ (●) and $29^\circ C$ (△).

(B) The data from the binding kinetics described in panel **A** are processed according to Equation 1 (Experimental Procedures) and plotted as $\ln((dpm_{max}-dpm_t)/dpm_{max})$ versus time. The slopes of the straight lines give the apparent rate constants: $k_4 = 0.0088$ sec^{-1} ($4^\circ C$), $k_{10} = 0.020$ sec^{-1} ($10^\circ C$), $k_{18} = 0.038$ sec^{-1} ($18^\circ C$), and $k_{29} = 0.0748$ sec^{-1} ($29^\circ C$).

(C) The rate apparent constants derived in panel **B** are plotted according to the Arrhenius equation (Equation 2. Experimental Procedures) and the resulting activation energy amounts to 58 kJ mol^{-1}.

(D) The effects of 375 μM tetracycline on the occupation of the ribosomal P site by $[^{14}C]AcPhe-tRNA^{Phe}$ (white bars), on the occupation of the E site by $[^{32}P]tRNA$ (grey bars) and on the puromycin reactivity of the P site bound $[^{14}C]AcPhe-tRNA^{Phe}$ (black bars). The tRNA occupation is given as the amount of tRNA bound per ribosome and the puromycin reactivity represents the percentage of P site-bound tRNA that is puromycin reactive.
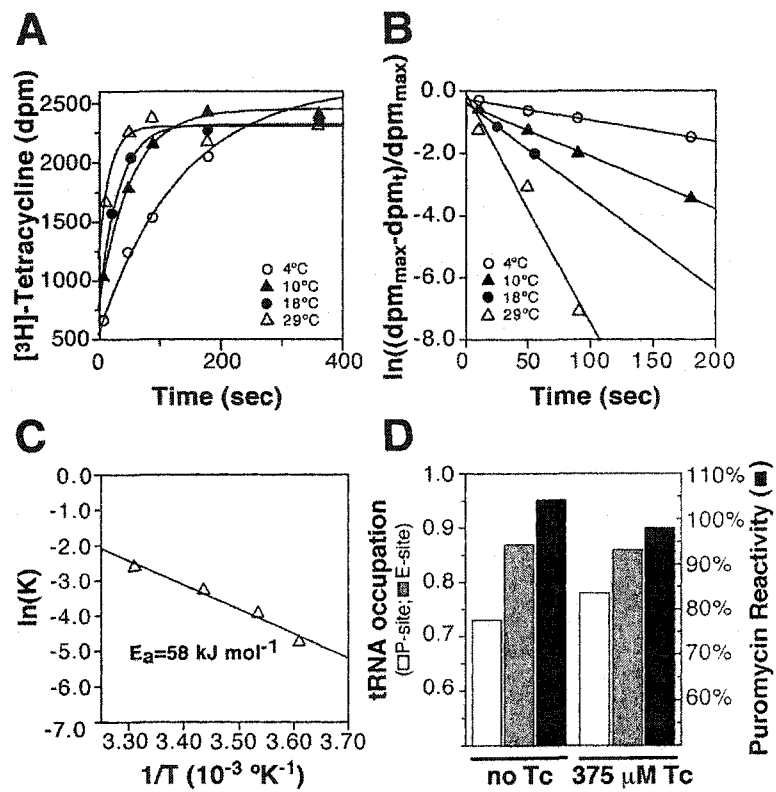
then determined using the Arrhenius equation (Equation 2 in Experimental Procedures) and an E_a of 58 kJ mol^{-1} was derived from the slope of the resulting line.

The binding of aa-tRNA to the A site of a post-translocational ribosome triggers the release of deacylated-tRNA from the E site [31]. Tetracycline, like the tRNA, binds to the decoding component of the ribosomal A site [73, 99], therefore we investigated the possibility that the activation energy associated with tetracycline binding is used to similarly release the E site bound tRNA. For this reason a POST complex was constructed in the presence of MF-mRNA, with a deacylated [^{32}P]tRNA^{fmet} at the E site and an Ac[^{14}C]Phe-tRNA at the P site. As clearly seen in Figure 4-4D, tetracycline has no effect on [^{32}P]-tRNA occupation of the E site (grey bars). Additionally, tetracycline has no effect on the AcPhe-tRNA^{Phe} bound to the P site (white bars), nor on its puromycin reactivity (black bars), indicating that tetracycline is not shifting the tRNA between various binding sites. These results indicate that, although tetracycline does bind to the A site, it does not mimic the tRNA in its ability to trigger tRNA release from the E site.

4.3 Discussion

Here evidence is presented showing that Tet(O) interacts with the post-translocational ribosome and thus demonstrates, for the first time, the step at which the ribosomal protection proteins interact with the elongation cycle. Additionally, the observation made by cryo-EM [47] that Tet(O) interacts with the GAR region on the 50S subunit is substantiated and extended by identifying the specific bases in the rRNA whose local environment changes upon Tet(O) binding. Moreover, the presented evidence suggests that Tet(O) is able to alter the conformation of the ribosome. These results broaden our knowledge of Tet(O) action, which was previously limited only to the step of actual tetracycline release and here we present a model detailing the complete cycle of Tet(O)-mediated tetracycline resistance.

Chemical probing and binding experiments clearly demonstrate that Tet(O) interacts and binds to the post-translocational ribosome. The interaction of Tet(O) with C1214 near the primary tetracycline binding site [73, 99], in the post-translocational ribosome, is consistent with the role of Tet(O) in conferring tetracycline resistance, since tetracycline arrests the elongation cycle in the post-translocational state by blocking A site occupation. It is interesting to note that Tet(O) has been footprinted on several ribosomal complexes - empty 70S, 70S with AcPhe-tRNA^{Phe} in the P site, and POST complexes - all of which have an empty A site and it is only the PRE complex, with an occupied A site, that fails to be engaged by Tet(O) (this study and ref. [115]). Cryo-EM reconstructions [47] do, in fact, suggest that domain IV of Tet(O) and the A site bound tRNA are in close proximity. Regulation based on A site occupation is logical since tetracycline's role is to block A-site occupation and, as such, the prolonged pause of the ribosome with an open A site could provide the kinetic opportunity for Tet(O) to interact with the ribosome. Additionally, a tetracycline-associated conformational change [98, 109, 155] could also serve as a recognition determinant to promote Tet(O) binding and subsequent tetracycline release.

In this study, the α-sarcin loop and the L11 region are identified as important determinants in Tet(O) activity by showing that Tet(O) alters the DMS reactivity of specific rRNA bases in these regions and that compounds (thiostrepton and α-sarcin), which target these regions, inhibit the ribosome-dependent GTPase activity of Tet(O). These same regions are important for the binding of EF-G to the ribosome [60, 64, 178], suggesting that Tet(O) and EF-G depend on the same ribosomal elements for binding. However, the specifics of this interaction are clearly different as illustrated in Figure 4-5A and B where the sites of interaction in H42/43/44 differ between EF-G and Tet(O). The unique interaction of Tet(O) is probably dictated by the fact that the L11 region is proposed to undergo conformational changes during elongation [174] and,

Figure 4-5: Tet(O)-dependent changes in 23S rRNA DMS reactivity

(A + B) Positions whose DMS reactivity changes in response to EF-G (green squares) or Tet(O) (red circles) binding are marked on the secondary structure [22] of the L11 region (A; H42/43/44; nt. 1036-1119) and the α-sarcin loop (B; H95; nt. 2647-2673). The protection of A1069 by Tet(O) is weak but was consistently observed in several reverse transcription experiments.

(C) The EF-G and Tet(O)-dependent protections illustrated in A are modelled on the 3-D structure of the L11 region (Protein Data Bank accession code: 1MMS; ref. [174]). The 23S rRNA of the L11 region (1051-1108) is shown as a grey ribbon and the protein L11 is coloured cyan. A1070 which is concealed and protected by Tet(O) is coloured red, while the exposed A1067 is protected by EF-G and coloured green. The structure was illustrated using Ribbons [182].

therefore, since Tet(O) interacts at a different step than EF-G it must recognize an altered conformation.

When the sites that experience changes in DMS reactivity upon EF-G or Tet(O) binding are viewed on the 3-dimensional structure [174] of the L11 region as in Figure 4-5C, A1067:N1 (protected by EF-G; green wireframe) seems to be exposed to the elongation factor, whereas A1070:N1 (protected by Tet(O); red wireframe) is less accessible. The change in the chemical accessibility of A1070, being in a confined location, could be direct where a conformational change in L11 or the rRNA allows Tet(O) access to this base or indirect where a change in L11 or the rRNA leads to the protection of this base. In any case, a conformational change is involved and evidence of this conformational change is also illustrated by the enhanced of DMS modification of A1050 upon Tet(O)·GMPPNP binding, which could reflect the movement of the L11 region around a 'hinge' that brings it closer to the SRL [141]. Interestingly, cryo-EM models [47] indicate that domain V in Tet(O) is closer to the N-terminal domain (NTD) of L11 compared to EF-G, possibly leading to the difference in their interaction.

Further evidence for a Tet(O)-dependent change in the GAR of the 50S subunit derives from the fact that Tet(O) is able to stimulate the ribosome dependent-GTPase activity of EF-Tu (Figure 4-2C). Cryo-EM reconstructions [47, 67, 70], and chemical probing experiments (ref. [63] and this study) show that EF-Tu and Tet(O) occupy overlapping sites on the ribosome, and therefore cannot occupy the ribosome simultaneously. In this respect, the stimulation of EF-Tu's GTPase activity is indicative that Tet(O) stabilizes a conformation of the GAR such that upon interaction with a population of empty ribosomes, it leaves the ribosomes in a configuration that is favourable for the interaction of EF-Tu. This feature would be important *in vivo* because after Tet(O) removes tetracycline the ternary complex (EF-Tu·GTP·aa-tRNA) must bind efficiently for protein synthesis to continue.

It follows that Tet(O) may also enhance the ability of the ternary complex to compete with tetracycline for the A site, by invoking conformational changes that disrupt the subsequent interaction of tetracycline with the A site. This interpretation is supported by two lines of evidence, (1) Tet(O) dislodges tetracycline by disrupting the conformation of its binding site [47] and (2) the enhancement of A1408 in h44 to DMS modification by Tet(O), which is an indication of rearrangements in the decoding site [115], remains after Tet(O) leaves the ribosome since this enhancement is also observed when GTP is used instead of a non-hydrolysable GTP-analogue (section 3.2.2).

Additionally biochemical evidence exists to suggest that tetracycline also induces structural changes in the ribosome [98, 109, 155]. DMS probing experiments support at least a local conformational change, since the modification of bases U1052 and C1054 of 16S rRNA were enhanced in the presence of the tetracycline [96, 115] and a change in the position of C1054 is also visualized by X-ray crystallography [73, 99]. In this sense the activation energy associated with tetracycline binding (E_a=58 kJ mol^{-1}) could be involved in promoting these changes. In fact activation energies of the magnitude derived for tetracycline binding often "involve entropy effects due to structural rearrangements" in biological systems, although gross-conformational changes in proteins characteristically have larger values of 90 to 120 kJ mol^{-1} [183].

Figure 4-6 shows a model derived from current and previous studies on RPPs. Initially (step a) tetracycline binds to the POST state ribosome, induces conformational changes without releasing the E site bound tRNA and blocks the ternary complex from occupying the A site (step b). Tet(O) then recognizes this blocked ribosome by virtue of its open A site, by its prolonged pausing in the POST state, and possibly by the tetracycline-induced conformational change. The interaction of Tet(O) with the ribosome triggers the release of tetracycline prior to GTP hydrolysis [11] and induces rearrangements in the A site as evidenced by changes in the DMS reactivity of A1408 of the 16S rRNA (ref.

[115]; *step c*). Tet(O) then hydrolyses the bound GTP and leaves the ribosome with the GAR in a configuration compatible with EF-Tu binding. A shift in the conformation of the GAR is supported by the change in DMS reactivity of A1070 upon Tet(O) binding to the ribosome and the stimulation of the GTPase activity of EF-Tu. We hypothesize that the conformational changes in the A site remain after Tet(O) release (*step d*) enhancing the ability of ternary complex to compete with tetracycline in the subsequent round of A site occupation (*step e*). This model presents the mechanism of Tet(O)-mediated resistance in the context of the overall elongation cycle such that further experimentation may now be done to validate the kinetic aspects of Tet(O) action.

Figure 4-6: A model for Tet(O)-mediated tetracycline resistance

(A) The binding of tetracycline to the elongating ribosome (*a*) is accompanied by a conformational change in the decoding site but not the release of the E-site bound tRNA (*b*; indicated by the blue to red colour change). The binding of tetracycline blocks subsequent EF-Tu•GTP•aa-tRNA-dependent occupation of the A site by sterically interfering with the accommodation of the aa-tRNA [73, 99]. Tet(O) binds to the tetracycline blocked ribosome (*c*) and in doing so triggers the release of the bound tetracycline by changing the conformation of the decoding site, or more specifically h34 and h44 [47, 115]. Next the GTPase activity of Tet(O) is activated and it is released from the ribosome, leaving the decoding site in a conformation (*d* indicated with dark blue) which disfavours tetracycline binding allowing ternary complex to compete efficiently for the A site (*e*).
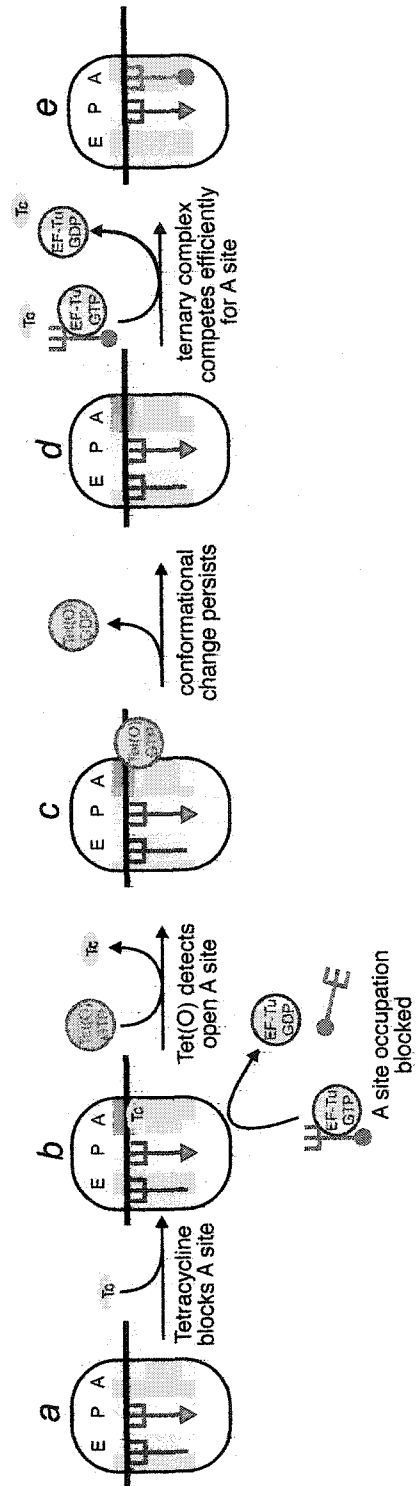
4.4 Experimental Procedures

Purified histidine-tagged EF-G and EF-Tu from *E. coli* were provided by Ulrich Stelzl. The AcPhe-tRNA^{Phe} was prepared as described previously [156] and the MF-mRNA prepared by Detlev Kamp. All other materials were purchased from commercial suppliers.

4.4.1 Construction and purification Tet(O)D131N

tet(O) was amplified from pUOA2E1 [130] by PCR as previously described [11] and cloned into the phagemid pTZ19R [184]. Using the Sculptor *in vitro* mutagenesis system (Amersham Biosciences), Asp$_{131}$ was changed to Asn. The mutation was confirmed by sequencing and a 0.7 kb EcoRI-Eco47III fragment of the *tet(O)* gene containing the mutation was subcloned into pMS119-Tet(O)-H [11] yielding pMS119-Tet(O)D131N. The protein was purified as described previously [11]. This work was done by Dr. C.A.Trieber.

4.4.2 Preparation of defined ribosomal complexes

Pre- and post-translocational complexes used in Figure 4-1B-D were made as described previously [150]. The PRE complex consisted of re-associated 70S ribosomes (section 2.4.8) programmed with MF-mRNA, a deacyl-tRNA^{fmet} in the P site and Ac[^{14}C]Phe-tRNA^{Phe} in the A site and were subsequently translocated by EF-G to yield the POST complexes. The PRE and POST complexes were sedimented through a 10% sucrose cushion in a TLA 100.3 rotor (76000g, 17 hr, 4°C) to remove EF-G and exchange the buffer for H$_{20}$M$_{10}$N$_{100}$SH$_4$ (20 mM HEPES-KOH [pH 7.5], 10 mM magnesium acetate, 100 mM ammonium acetate, and 4 mM β-mercaptoethanol). The homogeneity of the complex can be illustrated by the ratio of the puromycin reactive tRNA in the PRE state to that in the POST state (PM+$_{pre}$/PM+$_{post}$; ref. [152]). These PRE and POST

complexes contained 0.7 AcPhe-tRNA[Phe] per 70S and the homogeneity of the complex was 97% as determined by the puromycin reactivity.

PRE and POST complexes used in Figure 4-1E were constructed as described above, however, they employed tight-coupled 70S ribosomes [151] rather than re-associated 70S ribosomes and, as such, had lower tRNA binding (0.3 AcPhe-tRNA[Phe] per 70S ribosome, with 97% homogeneity) and were maintained in $H_{20}M_6N_{150}SH_4Spd_2Sp_{0.05}$.

For the DMS probing experiments in Figure 4-3A, AcPhe-tRNA[Phe] was bound to the P site of tight-coupled 70S ribosomes in the presence of poly(U) mRNA (0.7 AcPhe-tRNA[Phe] per 70S ribosome) and were maintained in $H_{20}M_6N_{150}SH_4Spd_2Sp_{0.05}$ buffer [150].

For E-site binding in the presence of tetracycline, the POST complex was prepared such that 0.62 μM [^{32}P]tRNA[fMet], 0.46 μM Ac[^{14}C]Phe-tRNA[Phe], were bound to MF-mRNA programmed 70S ribosomes (0.31 μM) in the presence of EF-G·GTP under $H_{20}M_6N_{150}SH_4Spd_2Sp_{0.05}$ buffer conditions. Where indicated tetracycline was present at 370 μM. tRNA binding was monitored in nitrocellulose filter binding assays and puromycin reactivity assays [150].

4.4.3 DMS modification of Tet(O)·ribosomal complexes

Tet(O) was bound to the ribosomal complexes by incubating 100 pmols of purified Tet(O) with 2.5 nmols of GMPPNP, and 25 pmols of the desired ribosomal complex in 50 μL of $H_{20}M_{10}N_{100}SH_4$ buffer (experiments described in Figure 4-1) or $H_{20}M_6N_{150}SH_4Spd_2Sp_{0.05}$ buffer (experiments described in Figure 4-3) for 15 min at 37°C. Subsequently, the complexes were chemically modified, analyzed by primer extension analysis, and quantified as described in section 3.4.2-3 and 6.4.3. The values reported in the text for changes in DMS reactivity correspond to the ratio of the intensity of a band corresponding to a DMS-dependent stop in a sample lane (i.e. Tet(O) + 70S ribosomes) compared to that in a control lane (i.e. 70S ribosomes).

4.4.4 Isolation of EF-Tu·GTP complex

GTP was exchanged for GDP bound to EF-Tu as previously described [173] with some modifications. Briefly, this reaction is done in $H_{20}M_6N_{150}SH_4Spd_2Sp_{0.05}$ buffer with 50 μM [^{32}P]γGTP, 1 mM PEP, 0.1 mg/ml pyruvate kinase and with 2 μM EF-Tu. This reaction is incubated at 37°C for 10 min. Next, the EF-Tu·GTP complex was isolated by loading the binding reaction on a G-50 spun column (Boehringer Mannheim) and centrifuged 2 min at 1100xg. The EF-Tu·GTP complex is collected in the first fraction.

4.4.5 GTPase activity

The GTPase assays were as described previously [160] except that reactions were set up to maintain $H_{20}M_6N_{150}SH_4Spd_2Sp_{0.05}$ buffer conditions with a final ribosome concentration of 0.2 μM, a final protein concentration of 0.2 μM, and nucleotide (XTP or [^{32}P]γGTP) concentration of 50 μM.

4.4.6 Tetracycline binding

Tetracycline binding was measured using [^{3}H]-tetracycline in a nitrocellulose binding assay [11]. The activation energy of the binding reaction was determined as described [185] using the Arrhenius equation. This involved the underlying assumption that tetracycline binding requires a rate limiting conformational change and thus follows pseudo-first order reaction kinetics such that:

$$(1) \qquad \ln\left(\frac{dpm_{max} - dpm_t}{dpm_{max}}\right) = -kt$$

where dpm_{max} is the maximal amount of [^{3}H]-tetracycline (measured in dpm) retained on the filter and dpm_t amount of [^{3}H]-tetracycline (measured in dpm) retained on the filter at the time t. The apparent rate constants, k, are then derived from this plot and their temperature dependence determined according to the Arrhenius equation:

(2)
$$\ln(k) = \frac{-E_a}{RT} + \ln(A)$$

where A is the frequency factor, E_a the activation energy, R the universal gas constant (8.314 J·(mol·K)$^{-1}$) and T the absolute temperature. The slope of a line resulting from a plot of ln(k) *vs.* 1/T will be equal to the (E_a/R).

Chapter 5

Discussion

5 Discussion

Ribosomal protection represents an important tactic for promoting resistance to tetracyclines in the medically relevant *Camplylobacter* species. As such, understanding the RPPs mode of action may facilitate the rational design of drugs to circumvent resistance mediated by the RPPs. Additionally because of the RPPs interaction with the ribosome and similarity to the ribosomal elongation factors, insight into their activity can answer basic questions about ribosomal functions and the mechanism of protein synthesis. In the current study we identified ribosomal elements that may play a role in Tet(O) activity and describe a model (Figure 5-1) detailing the mechanism of Tet(O)-mediated tetracycline resistance in molecular detail.

5.1 *Interaction of Tet(O) with the 70S bacterial ribosome*

Using chemical probing it was demonstrated that Tet(O) interacts with both subunits of the ribosome. The interaction between Tet(O) and the small subunit was studied extensively in Chapter 3 where the entire 16S rRNA was screened and the sites of interaction were localized to h34 (C1214) and h44 (A1408), components of the decoding centre. When screening the interactions in the 50S subunit we utilized the cryo-EM data which suggested Tet(O) interacts with the rRNA of the L11 region and the α-sarcin loop [47]. For this reason chemical probing experiments in Chapter 4 focused on these regions and identified contacts within H43 (A1069, A1070) and H95 (A2660), components of the GTPase-associated region (GAR). In general the results from the chemical probing experiments agree with those obtained from the cryo-EM reconstructions [47], however two differences exist. Firstly cryo-EM predicted an interaction with h5 and h18 of the 16S rRNA [47], but this was not observed by DMS probing. Secondly DMS probing demonstrated an interaction with h44 of the 16S rRNA

Figure 5-1: The functional cycles of Tet(O) and tetracycline

The pathway of Tet(O)-mediated tetracycline release is illustrated using cryo-EM reconstructions of ribosomes in various functional states. The natural elongation cycle is represented by reactions **a-e** and is described in detail in section 1.1.3. In the presence of tetracycline the ribosome allegedly enters a non-productive cycle illustrated by reactions **i-j** [99]. In this cycle ternary complex repeatedly tries to bind aa-tRNA to the A site but fails. Tet(O) is able to rescue the ribosome from this non-productive cycle by chasing tetracycline from its binding site on the 30S subunit (reaction **g**). After promoting the release of tetracycline, Tet(O) hydrolysis its bound GTP and disassociates from the ribosome, thereby returning the ribosome to the elongation cycle (reaction **h**). This figure is reproduced from ref. [47].
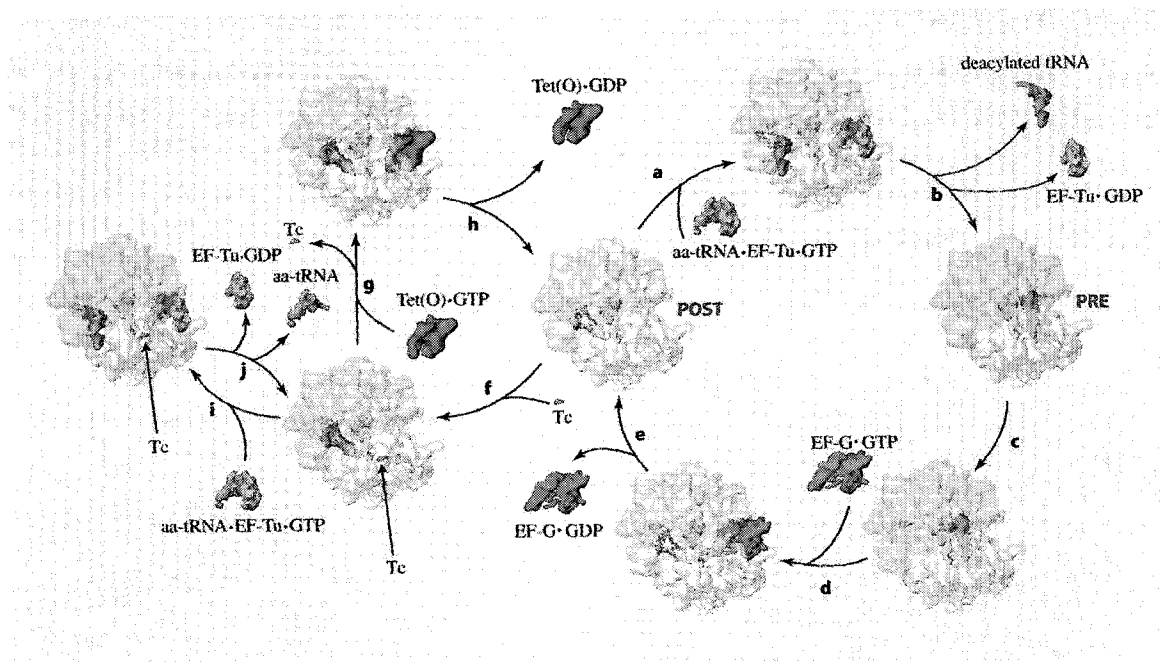
but this cannot be explained by the cryo-EM reconstructions. These discrepancies can, however, be resolved by understanding the inherent limitations of the two techniques. For example, the failure of DMS probing to find interaction within h5 and h18 could lie in the fact that DMS is a specific probe in that it can only be used to monitor A and Cs in a specific chemical environment and therefore some interactions may go undetected. Similarly the interaction in h44 detected by DMS probing may reflect a subtle long-range interaction of Tet(O) and thus could be beyond the threshold to allow detection in the relatively low-resolution cryo-EM map. In this sense the two techniques used to study the interaction of Tet(O) with the ribosome, cryo-EM [47] and chemical probing (Chapters 3 and 4) complement one another. Cryo-EM can provide the overall general picture, such that it can predict interactions, which can be studied further by more sensitive techniques like chemical probing. Additionally the results provided by chemical probing are much more informative when combined with structural results obtained from cryo-EM, as seen in Figure 3-5.

It appears likely that the interaction with the GAR is important for the activity of Tet(O) as well as regulating the interaction of Tet(O) with the ribosome (Chapter 4). The conformation of the L11 region, for example, is proposed to change throughout the elongation cycle and to regulate binding of the elongation factors [60, 174]. In this respect it is appropriate that Tet(O) interacts differently with the L11 region than EF-G as demonstrated by chemical probing (section 4.2.5) because they both bind different functional states. For example EF-G•GTP must bind to the PRE state so that it can promote translocation whereas Tet(O) should interact with the tetracycline-blocked POST state to relieve the drug's inhibitory effects on protein synthesis. This being said, it is important to realize that there are also similarities between the interaction of EF-G and Tet(O) with the GAR. Some similarities are demonstrated by the fact that antibiotics that bind the GAR, such as thiostrepton and α-sarcin, and affect the GTPase activity of ribosomal factors also affect the activity of Tet(O) (section 4.2.5). Thiostrepton, for example, is proposed to bind in a pocket formed by the r-protein L11 and

H42/43/44 of the 23S rRNA [174], and consequently positions these elements so as to alter the binding of the various ribosomal GTPases [60, 174]. In the case of EF-G, thiostrepton is suspected to lock the L11 region in a conformation unfavourable for EF-G binding such that all ribosomal interaction is lost thus resulting in a decrease in GTPase activity [60]. In contrast, the interaction between the ribosome and IF2 is only weakened by thiostrepton and this is alleged to allow IF2 to turnover faster and accordingly it experiences an increase in GTPase activity [60]. In this respect, since thiostrepton inhibits the GTPase activity of Tet(O), it appears that that Tet(O) interacts with the ribosome in a manner similar to EF-G (section 4.2.5), which indicates that the thiostrepton-dependent conformation of the L11 region is incompatible with Tet(O) binding. This conclusion is supported by the work of Dantley *et al.* which demonstrated that thiostrepton blocks the binding of Tet(M) to the ribosome [61]. Similarly α-sarcin, a ribotoxin that clips the rRNA backbone in the aptly named sarcin/ricin loop of the 23S rRNA, also inhibits all factor-dependent ribosomal functions [64]. It is reported that cleavage of this loop in *E. coli* ribosomes prevents binding of the elongation factors EF-G and EF-Tu [64], and accordingly this should result in a decrease in the GTPase activity of the factors. This is indeed the case as treatment of ribosomes with α-sarcin before addition of Tet(O) or EF-G knocks-out the GTPase activity of these factors (section 4.2.5). These results suggest that the GAR may represent an important binding determinant for Tet(O) and as suggested for other ribosomal GTPases its conformation may coordinate the binding of Tet(O) with the specific functional state of the ribosome.

On the small ribosomal subunit Tet(O) interacts near the decoding site and dislodges tetracycline specifically from the primary tetracycline binding site while ignoring tetracycline bound to the secondary site (section 3.2.3). Accordingly Tet(O) interacts near the primary binding site such that it protects C1214 from DMS modification (section 3.2.2). The protection of a base can indicate that it is directly shielded by a bound ligand and this conclusion is supported by the fact that the Tet(O) binding site observed by cryo-EM approaches C1214 (Figure 2-

7). In this sense it appears that Tet(O) contacts the base of h34 (C1214) and does not directly interact with the tetracycline binding site as we observed no changes in its DMS modification profile. It should be noted that this does not preclude the possibility that Tet(O) interacts directly with the primary site, it may simply interact in a manner that does not alter the DMS reactivity of the constituent rRNA. In this respect, the fact that we observed U1052 and C1054 change DMS reactivity in the presence of tetracycline and also in the presence of both tetracycline and Tet(O) but not with Tet(O) alone, could indicate that Tet(O) interacts directly with the primary site but only when it has been altered by binding tetracycline. However, currently it is preferable to interpret the data such that Tet(O) is not interacting directly with the primary site but rather that it affects the reactivity of U1052 and C1054 indirectly by triggering the release of tetracycline, thus restoring the primary binding site to its original conformation. This interpretation is in agreement with cryo-EM findings that suggest Tet(O) does not directly interact with the primary binding site [47].

The close association of C1214 with the Tet(O) binding site [47] contrasts with the enhancement of A1408. As illustrated in Figure 2-7 and presented by Spahn *et al.* [47] Tet(O) does not approach A1408 in h44. Since an enhancement of chemical modification is clearly indicative of a conformational change, and since Tet(O) does not interact with h44, this result can be taken to indicate that Tet(O) is inducing long-range conformational changes. These changes could be mediated by S12. As mentioned in section 1.1.2, S12 is remarkable for several reasons; first it is one of the few proteins located at the interface of the subunits and secondly it has an extended topology and as such contacts many elements of the 30S subunit. Notably, S12 is in close proximity to the top of h44 and also appears to interact with Tet(O) [47]. Furthermore as indicated in Table 1-1 both EF-G and Tet(O) interact with S12 and this would explain why both enhance A1408 (h44) to DMS modification.

The observed interactions are suggestive of both close contacts and of long-range conformational rearrangements within regions of functional

importance to the ribosome. As such these interactions may form the basis of Tet(O)-mediated tetracycline resistance as discussed below.

5.2 Mechanism of Tet(O)-mediated tetracycline resistance

The model in Figure 5-1 was originally presented by Spahn *et al.* and in the current study we present experimental evidence that confirms many aspects of this pathway. In the absence of tetracycline, the 70S ribosome progresses through the various states of the elongation cycle (reactions **a-e** in Figure 5-1). Progression through these states is driven by two elongation factors such that EF-Tu promotes the occupation of the A site by incoming aminoacyl-tRNA (reactions **a-b**) and EF-G subsequently triggers the translocation of the tRNA (reactions **d-e**; see section 1.1.3 for a detailed description). In the presence of tetracycline, however, the ordered progression though the elongation cycle is interrupted most likely after reaction **e** when the ribosome is in the POST translocational state. It can be inferred that the elongation cycle is blocked in this state as tetracycline serves to inhibit reactions **a-b** [11, 88-91] and therefore the ribosome would not be able to progress out of the POST state.

The binding of tetracycline to the ribosome (reaction **f**) appears to be accompanied by a structural rearrangement. This rearrangement is not observed in the crystal structure of tetracycline bound to the ribosome [73, 99] but can be inferred from several biochemical experiments [98, 109, 155, 186]. In these experiments Dahlberg *et al.* report, for example, that tetracycline is able to inhibit the cleavage of the rRNA by colicin E3. The site of cleavage is located in h44, and as such is distinct from the tetracycline binding sites indicating that tetracycline has long-range effects on the ribosomal architecture. Noah *et al.* [98] also presented evidence that tetracycline affects h44 as they observed that a UV-dependent cross-link between C1402 and C1501 – two bases located at the top of h44 – is enhanced by the presence of tetracycline. Again these bases are distinct from the observed tetracycline binding sites. Finally in section 4.2.6 it is demonstrated that when tetracycline binds to the ribosome, the reaction displays

a temperature dependence, indicating that binding is accompanied by a conformational change (Ea = 58 kJ mol^{-1}). The role of this alleged conformational change is not well understood, however the change might simply be required to move the ribosome into a configuration compatible with stable tetracycline binding. Additionally as h44 is a component of the decoding site it may have a role in the inhibitory action of tetracycline. However, models presented in the papers of Brodersen and Pioletti [73, 99] suggest that tetracycline exerts it effects simply through a steric clash with an accommodating tRNA such that its binding is blocked, which presumably makes a conformational change unnecessary.

As mentioned, tetracycline binding to the ribosome prevents occupation of the A-site by incoming aminoacyl-tRNA. Brodersen *et al.* [99] suggest that the initial stages of aminoacyl-tRNA binding would progress unhindered by tetracycline (reaction **i**) but the second step would be blocked (compare reaction **j** in the presence of tetracycline and reaction **b** in the absence) and therefore the A site can not be occupied when tetracycline is bound. Furthermore they propose that the non-productive cycle formed by reactions **i** and **j** would deplete the cellular energy reserves due to GTP hydrolysis by EF-Tu [99]. In the presence of Tet(O) this non productive cycle would be averted as Tet(O) would bind the tetracycline blocked ribosome, release tetracycline and return the ribosome to the elongation cycle (reactions **g** and **h**). The mechanism by which Tet(O) distinguishes the tetracycline-blocked ribosome has not been conclusively established but in section 4.3 it is suggested that it could involve two mechanisms. Firstly a tetracycline-induced conformational change in the ribosome may promote Tet(O) binding. Secondly tetracycline blocks the ribosome in a state with an open A site. A ribosome in this condition seems to be the substrate for Tet(O) because as presented in section 4.3 Tet(O) cannot bind a ribosome with an occupied A site. Obviously an active ribosome would also exist in a state with an open A site once per elongation cycle, however, the prolong pause in this state induced by tetracycline binding could give Tet(O) the

opportunity to act and thus distinguish the tetracycline-blocked ribosome from a translating ribosome.

After Tet(O) has bound the tetracycline-blocked ribosome, it must free it from tetracycline (reaction g in Figure 5-1). Trieber *et al.* [11] demonstrated that the binding of Tet(O) in the GTP state is sufficient to trigger the release of tetracycline. This indicates that Tet(O) may bind and directly displace tetracycline or alternatively, as suggested by the available evidence, Tet(O) binding may indirectly trigger the release of tetracycline by contacting the base of h34 [47, 115]. This would cause a disturbance in h34 which would be propagated to the tetracycline-binding site resulting in the release of tetracycline. The proposed conformational change resulting in tetracycline release probably involves h34 as (1) h34 forms an integral part of the primary tetracycline binding site [73, 99], (2) cryo-EM reconstructions show that domain IV of Tet(O) contacts the base of h34 [47] and (3) Tet(O) protects C1214 at the base of h34 from chemical modification by DMS (section 3.2.2).

It is likely that Tet(O) specifically triggers release of tetracycline from the primary tetracycline binding site [73, 99]. This is supported by DMS probing experiments (section 3.2.3) which demonstrate that the tetracycline-dependent changes in DMS modification associated with the primary binding site are abolished in the presence of Tet(O) but those associated with the secondary site are largely unaffected. Furthermore, similar DMS probing experiments (section 3.2.3) where the ribosomes are exposed to increasing amounts of tetracycline and subsequently probed with DMS indicate that tetracycline binds with higher affinity to the primary site. Accordingly this suggests that the primary site is the inhibitory site when interpreted with the finding that binding to a single high affinity site confers the inhibitory effects of tetracycline (ref. [92] and references within).

Tet(O) also interacts with the 16S rRNA (A1408 of h44) at sites distinct from the primary tetracycline binding site and the Tet(O) binding site as observed by cryo-EM (section 3.2.2). The reasons for these long-range rearrangements are

not well understood but may reflect that (1) Tet(O) is reversing tetracycline induced rearrangements in h44 (discussed above), (2) that the effect on h44 is a consequence of Tet(O) being derived from EF-G and may or may not be related to Tet(O) activity, or (3) may indicate that Tet(O) is inducing an altered conformation in the ribosome in order to prevent tetracycline rebinding and/or promote ternary complex binding.

The enhancement of A1408, although not likely mediated by a direct contact between h44 and Tet(O), could be mediated by S12 (discussed above). With respect to the role of S12 in ribosomal functions, it is implicated in both decoding (reviewed in ref. [187]) and translocation [48]. In the case of the experiments implicating S12 in translocation, modification of S12 stimulates factor-independent translocation [48]. In this sense S12 may be an important regulator for "unlocking" and "locking" the ribosome in various functional states. It may be then that the interaction of EF-G with S12 "unlocks" the ribosome and allows the ribosome the conformational flexibility to catalyze the translocation of tRNA. This flexibility of course could be related to both h34 and h44, which have been implicated in translocation [164, 168]. Similarly the interaction of Tet(O) with S12 may "unlock" the ribosome and afford it the flexibility needed for Tet(O) to distort h34 and trigger the release of tetracycline. In this sense the enhancement of A1408 could indicate the change to an "unlocked" state and may not be directly involved in Tet(O) activity. Alternatively as proposed in section 4.3 and Figure 4-6 this rearrangement in h44 might persist after Tet(O) leaves the ribosome, promoting ternary complex binding rather than tetracycline rebinding and thus contribute to tetracycline resistance directly. The fact that this enhancement is observed when Tet(O) is added to the ribosome in the presence of GTP (in this case Tet(O) would turnover and not be locked in a stable complex as it would be if a non-hydrolysable analogue were used) suggests that the

rearrangement leading to the enhancement has a long half-life[i]. In this sense the rearrangements might reverse those purportedly induced by tetracycline in h44 [98, 155]. These tetracycline-induced conformational changes may be related to moving the ribosome into a state that has a high affinity for tetracycline and therefore their reversal may slow rebinding after Tet(O)-promoted release and allow the ternary complex to compete effectively. Additionally, or possibly alternatively, this rearrangement may actively promote ternary complex binding, an idea that is consistent with the fact that A1408 is a component of the decoding site.

After release of tetracycline, Tet(O) hydrolyses GTP and subsequently is released from the ribosome [11]. This returns the ribosome to its natural elongation cycle such that it resumes where it left off before being interrupted by tetracycline. We hope that this model will form the foundation for subsequent studies which probe further into the mechanism of Tet(O)-mediated tetracycline release and the promotion of ternary complex binding in contrast to tetracycline rebinding.

5.3 Future directions

5.3.1 Direct *vs.* indirect competition between Tet(O) and tetracycline

Our current working model for Tet(O)-promoted tetracycline release dictates that Tet(O) acts allosterically to remove tetracycline from the primary tetracycline binding site [47]. This is purported to be mediated by a contact between the base of h34 and domain IV of Tet(O). This contact distorts h34 and consequently the primary tetracycline-binding site leading to the release of tetracycline. Support for this theory derives from the fact that in the cryo-EM reconstruction, the density attributed to Tet(O) does not overlap the primary tetracycline binding site [47] and similarly DMS probing shows that Tet(O) footprints the rRNA at sites distinct from

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[i] In should be noted this is not conclusive evidence as Tet(O) may just be turning over very slowly with respect to the DMS modification reaction so that it appears that it is always on the ribosome, or alternatively turning over so fast it also appears to be on the ribosome continuously.

the tetracycline binding site [115]. This evidence supports the idea that the tetracycline release is allosteric in nature but does not conclusively prove it.

A kinetic study describing the competition of Tet(O) and tetracycline may however be able to establish if these two ribosomal ligands are direct or indirect competitors. This study would involve binding tetracycline to the ribosome in the presence of various Tet(O) concentrations and following the kinetics of tetracycline binding/release. Tetracycline binding in these experiments could be followed by a filter binding assay, however the rapid binding of the drug may complicate kinetic measurements (note at lower temperatures it might be possible to obtain clearly resolved time points). Alternatively tetracycline binding could be followed using its natural fluorescence, which is reported to increase upon binding to the ribosome [188]. This approach would have several advantages over filter binding assays, such as (1) binding could be followed in real time, (2) the approach is an equilibrium method unlike filter binding, and (3) the potentially disruptive effect of the filter on the ribosome tetracycline interaction could be ignored. It is important to note that I have tried this experiment in collaboration with Dr. Raymond Turner (University of Calgary) without success. In our studies the photo-degradation of tetracycline seemed to overshadow any increase in tetracycline fluorescence promoted by ribosome binding. However I have subsequently found a report suggesting that 6-demethylchlortetracycline is better suited to fluorescence-based studies as it is much less photo-labile [94].

5.3.2 Interaction with S12

Mutations in S12 were shown to affect the level of tetracycline resistance mediated by Tet(O) [189]. As judged by the cryo-EM reconstruction of Tet(O) bound to the ribosome [47], residues (43-50) in the loop of S12, which were shown to be important for Tet(O) activity [189], do not directly interact directly with Tet(O) but most likely interact with the A site bound tRNA as indicated in the crystal structure of a 30S subunit with an A site bound ASL [29]. However, the

cryo-EM reconstructions suggest that Tet(O) interacts directly with residues 74-76 of S12 (section 2.2.4.2). In this respect it is unfortunate that a high-resolution structure of Tet(O) does not exist so that we can determine which residues on Tet(O) are interacting with these conserved residues on S12. In spite of this we might be able to ascertain the identity of these residues using the cryo-EM derived models of EF-G bound to the ribosome. When these models [69, 168] are freely available one can confirm that EF-G does, in fact, also interact with conserved residues (74-76) of S12 as proposed in section 2.2.4.2 and subsequently identify the interacting region of EF-G. It is possible that these residues identified in EF-G will be conserved in Tet(O) based on the evolutionary linkage of the two proteins and maybe in other ribosomal proteins which possess a domain homologous to domain III of EF-G. Identification of a motif mediating the interaction with S12 could then allow mutation of these residues in Tet(O) to study the role of S12 in tetracycline resistance. Possible outcomes could be that the interaction with S12 is absolutely required for ribosome binding or for tetracycline release in which case the mutations would knock out Tet(O) activity. Alternatively the interaction with S12 may promote the reversal of tetracycline's long range effects in h44 (section 3.3) or promote the efficient binding of ternary complex after Tet(O)-promoted tetracycline release (section 4.3). In any case purification of the mutant proteins and subsequent analysis in *in vitro* assays could elucidate the role of the S12/Tet(O) interaction.

5.3.3 Identification of functionally important regions in Tet(O)

With the exception of the G-domain, which has been studied somewhat by mutational analysis [137, 190], there is very little information describing the roles of the other domains in Tet(O). Some information can be gleaned from studies on EF-G where corresponding residues in Tet(O) can be identified by sequence alignments. For example residues which form the distal loop of domain IV in EF-G (Figure 1-6B) and are allegedly involved in translocation can be aligned to residues 506-511 in Tet(O) (Appendix 6.2). Based on cryo-EM reconstructions

the distal tip of domain IV in Tet(O) would approach h34 near the primary tetracycline binding site and therefore may be involved in Tet(O) mediated tetracycline release.

In any case to further the study of the role of the various Tet(O) domains in its activity we could   select mutants of Tet(O) which can compensate for conditions known to limit the effectiveness of Tet(O). Examples of such conditions could include:

(A) Growth in the presence of higher than normal tetracycline concentrations.

(B) Growth in the presence of tetracycline derivatives that bind similarly to the ribosome as tetracycline but yet escape RPP-mediated resistance, ie glycylcyclines [78].

(C) Growth within strains harbouring *miaA* or S12 mutations which are known to decrease the effectiveness of Tet(O) [189].

In these studies mutations in Tet(O) yielding a gain in function under various conditions could be promoted through the use of error-prone PCR or passage through mutator *E. coli* strains coupled with DNA shuffling techniques [191, 192]. After selection of the mutants they could be over-expressed and purified to facilitate their characterization in *in vitro* studies thus localizing the effects of the mutations to changes in specific Tet(O)-dependent activities, namely binding, GTPase, or RP activity.

5.4 Concluding remarks

This study has made several important contributions to the study of Tet(O), tetracycline and bacterial protein synthesis. The evidence presented here agrees with the idea that the ribosome contains a common factor-binding site and that it is used by several of the ribosomal GTPases. However, the ribosomal GTPases may not interact identically with the elements of the factor-binding site and this

may form the basis of a regulation mechanism that restricts their access to only the ribosomal state with which they are required to interact. Additionally we present evidence that supports the notion that h34 and h44 are flexible rRNA elements and that their movement may be associated with the action of ribosomal factors such as EF-G and Tet(O). The mechanism of tetracycline action has also been explored and fairly conclusive evidence presented that the primary tetracycline-binding site is in fact the inhibitory site and the target of Tet(O). With respect to the mechanism of Tet(O), we identify direct and indirect interactions with both the small and large subunit which may play a role in tetracycline resistance. Furthermore the sites of these interactions substantiate the claim that Tet(O) dislodges tetracycline from the ribosome through an allosteric type mechanism.

Chapter 6

Appendices

6 Appendices

6.1 Tet(O) gene and protein sequences

6.1.1 tet(O)_pUOA2 CDS

```
1     ATGAAAATAA TTAACTTAGG CATTCTGGCT CACGTTGACG CAGGAAAGAC AACATTAACG
61    GAAAGTTTAT TGTATACCAG TGGTGCAATT GCAGAACTAG GGAGCGTAGA TGAAGGCACA
121   ACAAGGACAG ATACAATGAA TTTGGAGCGT CAAAGGGGAA TCACTATCCA GACAGCAGTG
181   ACATCTTTTC AGTGGGAGGA TGTAAAAGTC AACATTATAG ATACGCCAGG CCATATGGAT
241   TTTTTGGCGG AAGTATACCG TTCTTTATCC GTATTAGACG GAGCAGTATT ATTAGTTTCT
301   GCAAAGGATG GCATACAGGC ACAGACCCGT ATACTGTTTC ATGCACTACA GATAATGAAG
361   ATTCCGACAA TTTTTTTCAT CAATAAAATT GACCAAGAGG GGATTGATTT GCCAATGGTA
421   TATCGGGAAA TGAAAGCAAA GCTTTCTTCG GAAATTATAG TGAAGCAAAA GGTTGGGCAG
481   CATCCCCATA TAAATGTAAC GGACAATGAC GATATGGAAC AGTGGGATGC GGTAATTATG
541   GGAAACGATG AACTATTAGA GAAATATATG TCAGGGAAAC CGTTTAAAAT GTCAGAACTG
601   GAACAGGAAG AAAACAGGAG ATTCCAAAAC GGAACGTTAT TTCCCGTTTA TCACGGAAGC
661   GCTAAAAACA ATCTGGGGAC TCGGCAGCTT ATAGAAGTAA TTGCCAGTAA ATTTTATTCA
721   TCAACGCCTG AAGGTCAATC TGAACTATGC GGGCAGGTTT TTAAGATTGA ATATTCAGAG
781   AAAAGGCGGC GTTTTGTTTA TGTGCGTATA TATAGCGGAA CATTGCATTT GAGGGATGTT
841   ATTAGAATAT CTGAAAAAGA GAAAATAAAA ATCACAGAGA TGTATGTTCC GACAAACGGT
901   GAATTATATT CATCCGATAC AGCCTGCTCT GGTGATATTG TAATTTTACC AAATGATGTT
961   TTGCAGCTAA ACAGTATTTT GGGGAACGAA ATACTGTTGC CGCAGAGAAA ATTTATTGAA
1021  AATCCTCTCC CTATGATCCA AACAACGATT GCAGTAAAGA AATCTGAACA GCGGGAAATA
1081  TTGCTTGGGG CACTTACAGA AATTTCAGAT TGCGACCCTC TTTTAAAATA TTATGTGGAT
1141  ACTACAACGC ATGAGATTAT ACTTTCTTTT TTGGGGAATG TGCAGATGGA AGTCATTTGT
1201  GCCATCCTTG AGGAAAAATA TCATGTGGAG GCAGAAATAA AAGAGCCTAC TGTTATATAT
1261  ATGGAAAGAC CGCTTAGAAA AGCAGAATAT ACCATCCACA TAGAAGTCCC GCCAAATCCT
1321  TTCTGGGCTT CTGTCGGGTT GTCCATAGAG CCGCTCCCTA TTGGAAGCGG AGTGCAGTAT
1381  GAAAGCAGAG TTTCACTTGG ATATTTAAAT CAATCGTTCC AAAATGCGGT TATGGAGGGG
1441  GTTCTTTATG GCTGCGAGCA GGGGCTGTAT GGATGGAAAG TGACAGACTG TAAAATCTGT
1501  TTTGAATATG GATTGTATTA TAGTCCTGTA AGTACCCCCG CAGACTTTCG GCTGCTTTCC
1561  CCTATCGTAT TGGAGCAGGC TTTAAAAAAA GCAGGGACAG AACTATTAGA GCCATATCTC
1621  CACTTTGAAA TTTATGCACC GCAGGAATAT CTCTCACGGG CGTATCATGA TGCTCCAAGG
1681  TATTGTGCAG ATATTGTAAG TACTCAGATA AAGAATGACG AGGTCATTCT GAAAGGAGAA
1741  ATCCCTGCTA GATGTATTCA AGAATACAGG AACGATTTAA CTTATTTCAC AAATGGGCAG
1801  GGAGTCTGCT TGACAGAGTT AAAAGGATAC CAGCCAGCTA TTGGTAAATT TATTTGCCAA
1861  CCCCGCCGCC CGAATAGCCG TATAGATAAG GTTCGGCATA TGTTCCACAA GTTAGCTTAA
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6.1.2 Tet(O)_pUOA2 ORF

```
1     MKIINLGILA HVDAGKTTLT ESLLYTSGAI AELGSVDEGT TRTDTMNLER QRGITIQTAV
61    TSFQWEDVKV NIIDTPGHMD FLAEVYRSLS VLDGAVLLVS AKDGIQAQTR ILFHALQIMK
121   IPTIFFINKI DQEGIDLPMV YREMKAKLSS EIIVKQKVGQ HPHINVTDND DMEQWDAVIM
181   GNDELLEKYM SGKPFKMSEL EQEENRRFQN GTLFPVYHGS AKNNLGTRQL IEVIASKFYS
241   STPEGQSELC GQVFKIEYSE KRRRFVYVRI YSGTLHLRDV IRISEKEKIK ITEMYVPTNG
301   ELYSSDTACS GDIVILPNDV LQLNSILGNE ILLPQRKFIE NPLPMIQTTI AVKKSEQREI
361   LLGALTEISD CDPLLKYYVD TTTHEIILSF LGNVQMEVIC AILEEKYHVE AEIKEPTVIY
421   MERPLRKAEY TIHIEVPPNP FWASVGLSIE PLPIGSGVQY ESRVSLGYLN QSFQNAVMEG
481   VLYGCEQGLY GWKVTDCKIC FEYGLYYSPV STPADFRLLS PIVLEQALKK AGTELLEPYL
541   HFEIYAPQEY LSRAYHDAPR YCADIVSTQI KNDEVILKGE IPARCIQEYR NDLTYFTNGQ
601   GVCLTELKGY QPAIGKFICQ PRRPNSRIDK VRHMFHKLA
```

6.2 Alignments

The first alignment compares EF-G and the RPPs. The domain structure of EF-G based on the X-ray crystal structure [45] and that proposed for Tet(O) based on sequence alignments is indicated with a thick bar below the alignment. The G-domain is represented by the red bar, the G' domain is orange, domain II is yellow, domain III is green, domain IV is blue and domain V is purple. The GTP binding motifs [40] are indicated with a green line above the alignment whereas loop 1 and 2, which form the distal tip of domain IV, are indicated with blue and green lines, respectively. The alignment was generated with Clustal W, and illustrated using GeneDoc where residues that are similar in all sequences are coloured black, residues a that are similar in greater than 75% of the sequences are coloured dark grey and those conserved in greater than 50% of the sequences are coloured light grey.

In the second alignment the amino acid sequence of several eubacterial S12 proteins are compared. The alignment was generated with ClustalW and coloured such that residues that are 100% conserved are coloured red.

G1 G2

```
EFG AQUAE  : ---MAREVP--IE...KTYK.E.HE.AATM.W.PQE...GI.T.TV.T.ACY.TRNG : 76
EFG THETH  : ---MAVKVEYDLK...RIEHK.E.HE.AATM.E.EQ....GI.TA.V..CF.K--- : 75
EFG ECOLI  : ----ARTTP--IA.YR...VNHK.E.HD.AATM.W.EQ.Q.GI.T...A..AF.SGMA : 75
EFG SALTY  : ----ARTTP--IA.YR...VNHK.E.HD.AATM.W.EQ.Q.GI.T...A..AF.SGMA : 75
EFG HAEIN  : ----ARTTP--IE.YR...VSHK.E.HD.AATM.W.EQ.Q.GI.T...A..AF.SGMS : 75
EFG HELPY  : ----ARKTP--LN...VSHK.E.HD.AATM.W.EQ.Q.GI.T...A..CF.K--- : 72
EFG MYCPN  : ---MARTVD--LINF...RIHK.E.THD.RSVM.W.EQ........A..VS.K--- : 73
EFG UREPA  : ------MSKE--LKLF...KNHK.E.THD.AATM.N.AQ....GI.T....A..YAK.K--- : 71
EFG STRPY  : ----AREFS--LA.IR...KIHK.E.THE.ASQM.W.EQ.Q.GI.T...A..AQ.D--- : 72
EFG RICCN  : ---MSKINK--LEF...KSHK.E.HE.GATM.W.EQ.Q.GI.T...A..CR.D--- : 73
EFG MYCTU  : MAQKDVLTD--LS...INYK.E.HD.AATM.W.EQ.Q.GI.T...A..TF.K--- : 76
TETM NEIME : -----------M...GAITE.QS.DK.TTR.NTL.L...GI.T.Q.GI..FQ.E--- : 66
TETM UREUR : -----------M...GAITE.QS.DK.TTR.NTL.L...GI.T.Q.GI..FQ.E--- : 66
TETM STRPN : -----------M...GAITE.QS.DK.TTR.NTL.L...GI.T.Q.GI..FQ.E--- : 66
TETM STAAU : -----------M...GAITE.QS.DK.TTR.NTL.L...GI.T.Q.GI..FQ.E--- : 66
TETS LACLA : -------MEEIKL...RIKE.QS.DS.CTK.TT.FL....GI.T.Q.II..FQRE--- : 71
TETS LISMO : -----------M...RAIKE.QS.DS.TKT.T....GI.T.Q.II..FQRE--- : 66
TETO STRMU : -----------M...AIAEP.QS.DR.CTR.T.NL....GI.T.Q.VI..FQ.E--- : 66
TETO STRPN : -----------M...AIAEP.QS.DK.TTR.T.NL....GI.T.Q.VI..FQ.E--- : 66
TETO CAMCO : -----------M...AIAEP.QS.DK.TTR.T.NL....GI.T.Q.VI..FQ.E--- : 66
TETO CAMJE : -----------M...AIAE..QS.DE.TTR.T.NL....GI.T.Q.VI..FQ.E--- : 66
TETQ PREIN : -----------MN...ATEKCER.QN.DI.IT.S.DI....GI.RAST..II.N--- : 66
TETQ PRERU : -----------MN...ATEKCER.QN.DI.IT.S.DI....GI.RAST..II.N--- : 66
```

G3 G4

```
EFG AQUAE  : ---ERYQ...SV...VE..C...EAN.RWAD..PQ...E.....MK.R.LG..FYR.KE : 154
EFG THETH  : ----DH...TI...FDSSQ..PQ.SET.RQAE.YK.R.A.ANKMEKT.ADLWI.IRT : 152
EFG ECOLI  : KQYEPH...TI.VE...YC.VG..PQ.SET.RQAN.YK.R.A..NKPM.ANFLK.VNQ : 156
EFG SALTY  : KQYEPH...TI.VE...YC.VG..PQ.SET.RQAN.YK.R.A..NKRM.ANFLK.VGQ : 156
EFG HAEIN  : QQFFQR...TV.VE...YC.VG..PQ.SET.RQAN.YEV.R.A..NKMRT.ANFLR.VEQ : 156
EFG HELPY  : ----DHQ...TI.VE...FCSVG..PQ.SET.RQAN.YG.R.VI..NK.RIFA.FYN.ENQ : 149
EFG MYCPN  : ----NCS...TV.VE...VLD.MN..PQ.ET.RQAS.YEV.N.II...MKT.ANFERSVQS : 150
EFG UREPA  : ----GHS...TV.VE...LDGQN..PQ.ET.RQAT.YN.R.V..NKEKT.A.FYYS1ZT : 148
EFG STRPY  : ----GH...TI.VE...TLDSQS..PQ.ET.RQATEYC.R.V.AN..KI.A.FLYSVQT : 149
EFG RICCN  : ----DKI...TI.VE...FDGVA..PQ.ET.RQAD.YN.R.C..N.RM.A.FYRCVEM : 150
EFG MYCTU  : ----DNQ...TV.VE...FDGKE..PQ.EQ.RQAD.YD.R.C..NKI.A.FYFSVRT : 153
TETM NEIME : ----NT...LA.VY...IS.KD..A.RI.HALR.MG..TI...NKI.QN.LST.YQD : 143
TETM UREUR : ----NT...LA.VY...IS.KD..A.RI.HALR.MG..TI...NKI.CN.LST.YQD : 143
TETM STRPN : ----NT...LA.VY...IS.KD..A.RI.HALR.MG..TI...NKI.CN.LST.YQD : 143
TETM STAAU : ----NT...LA.VY...IS.KDF..A.RI.HALR.MG..TI...NKI.CN.LST.YQD : 143
TETS LACLA : ----NV...LADVY...IS.KD..S.RI.HALR.MN..II...NKI.CN.LPD.YQD : 148
TETS LISMO : ----NV...LADVY...IS.KD..A.RI.HALR.MN..II...NKI.CN.LPI.YQD : 143
TETO STRMU : ----DV...LA.VY...VS.KD..A.RI.HALQTMK..TI...NKI.QES.LPM.YRE : 143
TETO STRPN : ----DV...LA.VY...VS.KD..A.RI.HALQTMK..TI...NKI.QES.LPM.YQE : 143
TETO CAMCO : ----DV...LA.VY...VS.KD..A.RI.HALQIMK..TI...NKI.QES.LPM.YQE : 143
TETO CAMJE : ----QV...LA.VY...VS.KD..A.RI.HALQIMK..TI...NKI.QES.LPM.YRE : 143
TETQ PREIN : ----GV...IA.FK...LS.KE..A.RL.NTLQ.LQ..TI...NKI.RDG.VERR.YLD : 143
TETQ PRERU : ----GV...IA.FK...LS.KE..A.RL.STLQ.LQ..TI...NKI.RAE.VZER.YMD : 143
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EFG AQUAE  : EE..TIKP.A.QIP.GAEDQFEGV.D.MEMKAIRWLEETL-GAKYEVVDIPPEYQEKAQEWREKMI.T.V.T.DE... : 234
EFG THETH  : QE..GARP.V.QLP.GREDTFSGI.D.LRMKAYTYGNDL-GTDIREI-PIPEEYLDQAREYHEKLV.VAADE.EN.L. : 231
EFG ECOLI  : T..GANP.P.QLA.GAEEHFTGV.D.VKMKAINWNDAD-QGVTFEYEDIPADMVELANEWHQNLI.SAA.ASEE.. : 236
EFG SALTY  : T..GANP.P.QLA.GAEEGFTGV.D.VKMKAINWNDAD-QGVTFEYEDIPADMQDLANEWHQNLI.SAA.ASEE.. : 236
EFG HAEIN  : T..GANA.P.QLP.GAEENFTGV.D.IKMKAINWNEAD-QGMTFTYEEVPANMQADCEEWRQNLV.IAAA.ASEE.. : 236
EFG HELPY  : L..KANP.P.NIP.GAEDTFIGV.D.VQMKAIVWNNET-MGAKYDVEEIPSDLLEKAKEYREKLV.A.A.C.EA.. : 229
EFG MYCPN  : QQ..GVKA.P.QLF.GAENDFNGI.D.EEKVYFFDGGK--EEKAEEKPIIPDQFKDQVKQMPAHLVE.E.ANF.DQ.AD. : 229
EFG UREPA  : N..GVKATA.QIP.GAEADFVGS.D.EMKAYIYDGKA--DEEYKIEDIPADYLTKAQVMRSQMIDD.AVF.DE.. : 227
EFG STRPY  : HD..QANAHP.QLP.GAEDDFRGI.D.IKMKAEIYTNDL--GTDILEEDIPEEYLEQAQEYREKLI.A.ART.ED.M. : 228
EFG RICCN  : D..GAKP.V.QLP.GIEENFKGI.D.IKMKAVIWKDEA-LGAEYFEEDIPADMKDKAEEYRAKLLDM.V.L.DH.. : 230
EFG MYCTU  : GE..GANA.P.QLF.GAEADFEGV.D.VEMNAKVWRGETKLGETYDTVEIPADLAEQAEEYRTKLLEV.AES.EH.. : 234
TETM NEIME : E..S-AE.V.KQK.E----LYPN.C.TNFT------------------------------ESEQWDT.IEG.DD.. : 189
TETM UREUR : E..S-AE.V.KQK.E----LYPN.C.TNFT------------------------------ESEQWDT.IEG.DD.. : 189
TETM STRPN : E..S-AE.V.KQK.E----LYPN.C.TNFT------------------------------ESEQWDT.IEG.DD.. : 189
TETM STAAU : E..S-AE.V.KQK.E----LYPN.C.TNFT------------------------------ESEQWDT.IEG.DD.. : 189
TETS LACLA : D..S-DD.I.KQT.N----LNLKPY.IDYT------------------------------RPEQWET.IVG.DY.. : 194
TETS LISMO : S-DD..KQT.N----LNLKPY.IDYT------------------------------EPEQWET.IVG.DY.. : 189
TETO STRMU : A..S-SE.I.KQK.G----QHPH.N.TDND------------------------------DMEQWDT.IMG.DE.. : 189
TETO STRPN : A..S-SE.I.KQK.G----QHPH.N.TDND------------------------------DMEQWDA.IMG.DE.. : 189
TETO CAMCO : A..S-SE.I.KQK.G----QHPH.N.TDND------------------------------DMEQWDA.IMG.DE.. : 189
TETO CAMJE : A..S-SE.I.KQK.G----QHPH.N.TDND------------------------------DMEQWDA.IMG.DE.. : 189
TETQ PREIN : TN.S-QD.LFMQT.VDG--LVYP.CSQTYI------------------------------KSEYKEF.CNH.DN.. : 191
TETQ PRERU : TN.G-QD.LFMQT.VDG--SVYP.CSQTYI------------------------------KEEYKEF.CNH.DD.. : 191
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EFG AQUAE  : E QE----  SID  RKALRKATIER---K V LC   FKNK  QPL DA LDYLP PIDLPPVKGTNPKT-GEEEVRH : 306
EFG THETH  : E EE----PTEE  VAAIRKGTIDL--K T VFLG  LK K  QL  CA VDYLP PLDIPPIKGTTPE--GEVVEIH : 302
EFG ECOLI  : G EE---- TEA  KGALRQRVLNN---E IL TC   FKNK  QAM DA IDYLP PVDVPAINGILDDGKDTPAERH : 309
EFG SALTY  : G EE---- TEE  KQALRQRVLAN---E II TC   FKNK  QAM DA IDYLP PVDVPAINGILDDGKDTPAERH : 309
EFG HAEIN  : G ED---- TEE  KSALRQRVLAN---E IL TC   FKNG  QAM DA VEYLPAP DIPAIKGINPD--ETEGERH : 307
EFG HELPY  : G EE---- SIE  KKGIKAGCI M---S V LC   SFKN S  QTI DA IDYLPAPTEVVDIKGIDPKT-EEEVFVK : 301
EFG MYCPN  : E KE---- SIAD  KRC RKGVIGC---QF VLC   FK K  KL  DA VDFLP PVDVPQAKAYG-EDGNEVLISA : 301
EFG UREPA  : S EE---- SHED  KKC RKGVIST--E  VLC   FKN   KKL LA VDFLP PIDVPPIKGVD-DHGN IEYHN : 299
EFG STRPY  : E EE---- TND  IAGIRKATINV--EF VLC   FKN   QIA DA IAYLP PLDIPAIKGVNPDTDAEEERPA : 301
EFG RICCN  : S EE---- TAE  KRL RKGTISA---AF VLC   FKNK  QPL DA VDFLP PIDIGIVKGMEVSTGEEKDFPI : 303
EFG MYCTU  : G ED---- TVD  KGAIRKLTIAS---E VLC   FK R  QPM DA VDYLP PLDVPPAIGHAPAKEDEEVVRK : 307
TETM NEIME : S KS---- EAL  EQEESIRFHNC---S  VYHG  S KN  DNL EV TNKFY STHRGP---------------- : 246
TETM UREUR : S KS---- EAL  EQEESIRFHNC---S  VYHG  S KN G DNL EV TNKFY STHRGP---------------- : 246
TETM STRPN : S KS---- EAL  EQEESIRFHNC---S  VYHG  S KIN  DNL EV TNKFY STHRGQ---------------- : 246
TETM STAAU : S KS---- EAL  EQEESIRFQNC---S  VYHG  S KSI  DNL EV TNKFY STHRGP---------------- : 246
TETS LACLA : TI KT---- NIA  EKEENE IQSC---S  VYHG  S KN  KQL EV ISKLF PTQLNS--------------- : 251
TETS LISMO : TI KT---- NIA  EKEENERIQSC---S  VYHG  S KN  KQL EV ITSKLF PTQLNS--------------- : 246
TETO STRMU : S KP----FKMS  EQEENRRFQNG---T  VYHG  S KNN  RQL EV ASKFY STPEGQ---------------- : 246
TETO STRPN : S KP----FKMS  EQEENRRFQNG---T  VYHG  S KNN  RQL EV ASKFY STPEGQ---------------- : 246
TETO CAMCO : S KP----FKMS  EQEENRRFQNG---T  VYHG  S KNN  RQL EV ASKFY STPEGQ---------------- : 246
TETO CAMJE : S KP----FKMS  EQEENRRFQNG---T  VYHG  S KNN  RQL EV ASKFY STPEGQ---------------- : 246
TETQ PREIN : ADSE---- SPADYWNT IDLVAKA---K  VLHG  S MFN N NEL DA SS-FILPPESVS----------- : 247
TETQ PRERU : ADSE---- SPADYWNT IALVAKA---K  VLHG  S MFN N NEL DA TS-FILPPASVS----------- : 247


EFG AQUAE  : PSDDEPFCAYA  MSDPYAGQ  Y RV  GI KAGSY YNAT DEKQ AGR LLMHANSREE QQVSA D CA VGLDA- : 386
EFG THETH  : PDPNGP AALA  MADPYVG  TE RV  T TSGSY YN T G KEA AR LRMKANHREE EELKA  GA VGLKET : 383
EFG ECOLI  : ASDDEPFSALA  ATD FVGN  T FRV  GV NSGDT LM V AARE FGR VQMHANKREE KEVRA G AAAIGLKDV : 390
EFG SALTY  : ASDDEPFSALA  ATD FVGN  T FRV  GV NSGDT LN V TARE FGR VQMHANKREE KEVRA G AAAIGLKDV : 390
EFG HAEIN  : ASDEEPFSSLA  ATD FVGN  T FRV  GV NSGDT LN V RE FGR IVQMHANKREE KEVRA  AAAIGLKDV : 388
EFG HELPY  : SSDDGEFAGLA  MTDPFVGQ  T FRV RCK ESGGSY YN T D KE CGR LKMHSNKRED KEVYA D CAFVGLKDT : 382
EFG MYCPN  : SDD-APFVGLA  VATD FVG  G RV  VK SGSY KNVR N KE SR VKMHAQNRNE EEIRA  CA IGLKDT : 381
EFG UREPA  : DPN-EPFAALA  MTDPFVG  T RV  GK DKGTY YNAT D KE SR VKMHSNNRDE DSISA D CA IGLKDT : 379
EFG STRPY  : SDE-EPFAALA  IMTDPFVG  T FRV  GV NSGGSY YMN S G RE GR LQMHANSRQE ETVYA  AAAVGLKDT : 381
EFG RICCN  : SVT-EPFAALA  MNDPFVGS  I RI  GK TSGTT IN V N RE GR LLMHANNRED KEASA D VA AGLKDT : 383
EFG MYCTU  : ATTDEPFAALA  ATH FFG  IV RV  T ESGSGQ INAT G KE GK FQMHSNKENP DRASA H YA IGLKDT : 388
TETM NEIME : ----SELCGNV  I YTKKRQ  AY RI  GV HLRDS RV E E -I  TE YTSINGELCK DRAYS  VI QN-EFL : 321
TETM UREUR : ----SELCGNV  I YTKKRQ  AY RI  GV HLRDS RV E E -I  TE YTSINGELCK DRAYS  VI QN-EFL : 321
TETM STRPN : ----SELCGNV KI YTKKRQ  AY RI  GV HLRDS RV E E -I  TE YTSINGELCK DRAYS  VI QN-EFL : 321
TETM STAAU : ----SELCGNV KI YTKKRQ  AY RI  GV HLRDS RV E E -I  TE YTSINGELCK DRAYS  VI QN-EFL : 321
TETS LACLA : ----DKLCGNV  I YSDDGQ  VY RI  GT HLRDS NI E E -I  TE YTSINGELRQ DKAEP  II KN-ELL : 326
TETS LISMO : ----DKLCGNV  I YSDDGQ  VI RI  GT HLRDS NI E E -I  TE YTSINGELRQ DKAEP  II KN-ELL : 321
TETO STRMU : ----SELC OV KI YSEKRR FV  RI  GT HLRDV RI E E -I  TF CVPTNGELYSSDTACS VI PN-DVL : 321
TETO STRPN : ----SELC OV KI YSEKRR FV  RI  GT HLRDV KI E E -I  TF CVPTNGELYSSDTACS VI PN-DVL : 321
TETO CAMCO : ----SELC OV  I YSEKRR FV  RI  GT HLRDV RI E E -I  TE CVPTNGELYSSDTACS VI PN-DVL : 321
TETO CAMJE : ----SELC OV  I YSEKRR FV  RI  GT HLRDV RI E E -I  TE YVPTNGELYSSDTACS VI PN-DVL : 321
TETQ PREIN : ----NRLSAYI  EHDPKGH RSE II DGS RLRDI RINDSE FI KN KTIYQGRKIN DEVGAN AL EDMEDF : 324
TETQ PRERU : ----NRLSAYI  EHDPKGH RSE II DGS RLRDV RINDSE FI KN KTIYQGREIN DEVGAN AL EDIEDF : 324


EFG AQUAE  : ATGDT C-DEKHP- II EK  F D  SMA  KTKKDQ  SQV NKFMK  F ATT PE G  HG  H  V : 465
EFG THETH  : ITGDT V-GEDAPRVILES  V E  DVA  KTKADQ  SQ AR AEK  H VSTHPE G T SG C H  V : 463
EFG ECOLI  : TTGDT C-DPDAP- I ILR  F E  SIA  KTKADQ  GL GR AKE  SF VWT EE   AG  H D V : 469
EFG SALTY  : TTGDT C-DPENP- I ILR  F E  SIA  KTKADQ  GL GR AK   SF VWT EE   AG  H D V : 469
EFG HAEIN  : TTGDT C-AIDAP- II LR  F E  SVA  KTKADQ  GL GR AQE  SF VHT EE   SG  H D V : 467
EFG HELPY  : LTGDT C-DEKNA-VVLR  F E  HIA  KTKADQ  GV GK AEE  SF VMTQEE   GC  H   V : 461
EFG MYCPN  : TTGET V-DDKID- QLEA  FAQ  SLA  KTKADQ  SI SK AEE  T T PE G   AG  H D V : 460
EFG UREPA  : TTGDT C-DEKKP-VILE  FAE  SL   KTKADQ  SI SK AEE  T T EE   AG  H D V : 458
EFG STRPY  : TTGDS T-DEKAK-VILES  V E  LA  KSKADQD GV GK AEE  F VET VE G   AG  H D V : 460
EFG RICCN  : TTGDT S-DIDQQ-VILR  F E  LA  KSTAD   GL SR AAE  F VST YE G   KG  H  I : 462
EFG MYCTU  : TTGDT S-DPNQQ- IVLES  F D  VA  KTKSDQ  SLS A AEE  T VHL SE G   GG  H   V : 467
TETM NEIME : KLNSV ---GDTKLLPQRKK  N H  RTT  SKPEQR MLD LE SDS LL  YYV GTH  SF  K Q  S : 400
TETM UREUR : KLNSV ---GDTKLLPQRKF  N H  ITT  SKPEQR MLD LE SDS LL  YYV SAH  SF  K Q  S : 400
TETM STRPN : KLNSV ---GDTKLLPQRER  N L  TT  SKPQQR MLD LE SDS LL  YYV SAH  SF  K Q  S : 400
TETM STAAU : KLNSV ---GDTKLLPQRKK  N H  TT  SKPEQR MLD LE SDS LL  YYV STH  SF  K Q  S : 400
TETS LACLA : KLNNV ---GDKKRLPHRE I  N L  TT  CKSVQR LD FE SDS LL QYY  TV   SF  K Q  TC : 405
TETS LISMO : KLNNV ---GDKKRLPHRE I  N L  TT  CKSVQR LD FE SDS LL QYY  TV   SF  K Q  TC : 400
TETO STRMU : QLNSI ---GNEILLPQRKF  N L  TT VKKSEQR ILG TE SDG LL  YYV TT   SF  N Q  C : 400
TETO STRPN : QLNSI ---GNEMLLPQRKF  N L  TT VKKSEQR ILG TE SDG LL  YYV TT   SF  N Q  C : 400
TETO CAMCO : QLNSI ---GNEILLPQRKF  N L  TT VKKSEQR ILG TE SDC LL  YYV TT   SF  N Q  C : 400
TETO CAMJE : QLNSI ---GNEILLPQRKF  N L  TT VKKSEQR ILG TE SDC LL  YYV TT   SF  N Q  C : 400
TETQ PREIN : RIGDY ---GTKPCLIQG--  SHQH AL KSEQ R CRSEERSKVIS NT WIE SLSFSI SYSD LE SLY LTQKE  Q : 401
TETQ PRERU : RIGMY ---GAKPCLIQG--  SHQH AL KCS R NKPEERSKVIS NT WIE SLSFSI SYSD LE SLY LTQKE  Q : 401
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Loop 1

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EFG AQUAE  : DR KRE GI VN GK Q A  TIR KAI-GEGKFIKQTGGRGQ GHAI   E  PR AG-----------F IDD HG V  : 536
EFG THETH  : DR KRE K DAN GK Q A   TIT PVD-VEGKFIRQTGGRGQ GH K  E  PR ---------S F VNA VG V  : 534
EFG ECOLI  : DR KRE N  AN GK Q A  TIRQKVTDVEGK AKQSGGRGQ GH V D Y  EP SN------PK Y IND KG V  : 544
EFG SALTY  : DR KRE N  AN GK Q A  AIRAKVTDIEGK AKQSGGRGQ GH V D Y  EP SN------PK Y IND KG V  : 544
EFG HAEIN  : DR KRE K  AN GK Q S  TIRTRVNDVEGK AKQSGGRGQ GH V D Y  DPEG--------P Y VNE KG V  : 540
EFG HELPY  : DR KRE K  AE GQ Q A  TIRSSVS-KEHKYAKQSGGRGQ   K E KEP S---------Y VNE SG V  : 532
EFG MYCPN  : DR RRE K  VN GA C S  TFN ESE-VEGKYIKQSGGRGQ GH K RFE NKD---------K F VDK VG R  : 530
EFG UREPA  : DR RRE N  VN GA Q SY  TFTEVAD-AEGKYIKQSGGRGQ GH W KFE NHD---------K F VDN IVG K  : 528
EFG STRPY  : DR KRE K  AN GA C S  TFRASTQ-ARGFFKRQSGGKGQ GD W EFT NEE -----------K F ENA VG V  : 531
EFG RICCN  : DR RRE K  AN GA Q A  TIT VCE-IDYTHKKQSGGGQ AR K IFE  KEVKDLKDEDKNKIFV ESK IG A V  : 542
EFG MYCTU  : DR RRS K  AN GK Q A  TIK LVQNVEYTHKKQTGGSGQ AK I N  FTG-------EEGATY ESK TG R  : 541
TETM NEIME : AI  QEK H  IE KE T I M RPL NAE--YTIHIEVPNP-P AS C S SP PI-----------S M ASS SI V  : 469
TETM UREUR : AL  QEK H  IE KE T I M RPL NAE--YTIHIEVPPNP-P AS G S SP PI-----------S M ESS SLG L  : 469
TETM STRPN : AL  QRN H  IE KE T I M RPL KAE--YTIHIEVPNP-P AS G S SP PI-----------S M ESS SI V  : 469
TETM STAAU : AI  QES H  IE KE T I M RPL NAE--YTIHIEVPNP-P AS C S SP PI-----------S M ESS SI V  : 469
TETS LACLA : TL  QER H  IETRK T I M RPL KSE--FTIDIEVPNP-P AS G S TP PI-----------S I ESI SL V  : 474
TETS LISMO : TL  QEK H  IETRK T I M RPL KSE--FTIDIEVPNP-P AS G S TP PI-----------S I EGI SL V  : 469
TETO STRMU : AI  QEK H  AE KE T I M RPL KAE--YTIHIEVPNP-P AS G E EP PI-----------S V ESR SL V  : 469
TETO STRPN : AI  QEK H  AE KE T I M RPL KAE--YTIHIEVPNP-P AS G L EP PI-----------S V ESR SI V  : 469
TETO CAMCO : A  EEK H  AE KE T I M RPL KAE--YTIHIEVPPNP-P AS G S EP LI-----------S V ESR SI V  : 469
TETO CAMJE : AI  EEK H  AE KE T I M REL KAE--YTIHIEVPNP-P LS G L EP PI-----------S V ESR SL V  : 469
TETQ PREIN : TL  EER S KVHFDEIKT I M RPV RVN--KIIQIEVPNP-V AT G T EP PI-----------T L IESD SY V  : 470
TETQ PRERU : TL  EER S KVHFDEIKT I M RPI KVN--KIIQIEVPNP-V AT G T EP PL---------A L IESD SY V  : 470
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Loop 2

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EFG AQUAE  : PKE IPS EK  KEAM N I A P V V RLFD S HEVD DIA Q AGS AFKD A KADPV LE I KV ET K  : 617
EFG THETH  : PKE IP  QDK  EEAM S P I P V I  TLYD S HEVD EMA  AGS AIK  VQKGDPV LE I RV TT    : 615
EFG ECOLI  : PGE IP  DK  QEQLKA P A P V MG RLHF S HDVD SELA  AAS AFK GF KAKPV LE I KV ET    : 625
EFG SALTY  : PGE IP  DK  QEQLKS P A P V LG RLHF S HDVD SELA  AAS AFK GF KAKPV LE I KV ET    : 625
EFG HAEIN  : PGE IP  DK  QEQLKS P A P V LG RLHF S HDVD SELA  AAS AFKA FSKANPV LL E I KV ET P  : 621
EFG HELPY  : PKE IP  DK  QEAM N V A P V F  TLYD S HDVD SEMA  AGS AFK  S AANPV LL E I KV EV    : 613
EFG MYCPN  : PRE IKP  QA  ENAMAS P A P I I ATLFD S HEVD EMA  AAS ALK  G VCSPV LL E P AI TV    : 611
EFG UREPA  : PKE IKE EN  IEALTS P A QTI V ATIFD S HDVD GMA  AAS AFK  A VCKPV LL E P SVD TT DD  : 609
EFG STRPY  : PRE IP  EK  IESMAN V A P V V AKLYD S HDVD SETA  AAS ALK  A SAQPA LL E P LVT TA D  : 612
EFG RICCN  : PKE IPG EK  NNIR TV A P I F ATLVD A HDVD VLA E AAKAAFR GMPKGNPK LL E I QV IT D  : 623
EFG MYCTU  : PRE IPS DA AQDAM Y V A P V L  TLLD A HEVD SEMA  AGSQVLKK AALAQPV LL E P AV VTT D  : 622
TETM NEIME : NQS   MF  RYGC Q  Y N T C CFKY L YSPV PAD  LAP VLE VL KAGTE LS Y SFK YT    : 549
TETM UREUR : NQS   MF  RYGC Q  Y N TEC CFKY L YSPV PAD  LAP VLE VL KAGTE LE Y SFK YA    : 549
TETM STRPN : NQS   MF  RYGC Q  Y N T C CFKY L YSPV PAD  LAP VLE VL KAGTE LE Y STK YA    : 549
TETM STAAU : NQS   MF  RYGC Q  Y N T C CFKY L YSPV PA   LT  VLE F  KAGTE LL Y SFK YA    : 549
TETS LACLA : NQS   MF  RYGC Q  Y K T C CFKY L YSPV PAD  LAP VLE F  KSGTE LL Y SF YV    : 554
TETS LISMO : NQS   MF  RYGC Q  Y K T C CFKY L YSPV PAD  LAP VLE F  KSGTE LL Y SF YV    : 549
TETO STRMU : NQS N MF  LYGC Q  Y K T C CFEY L YSPV PAD  LSP VLE I  KAGTE LE Y HT YA    : 549
TETO STRPN : NQS N MF  LYGC Q  Y K T C CFEY L YSPV PAD  LSP VLE L  KAGTE LL Y HF YA    : 549
TETO CAMCO : NQS N MF  LYGC Q  Y K T C CFEY L YSPV PAD  LSP VLE L  KAGTE LE Y HF YA    : 549
TETO CAMJE : NQS QN MF  LYGC Q  Y K T C CFEY L YSPV PAD  LSP VLE L  KAGTE LE Y HF YA A  : 549
TETQ PREIN : NHS  N FF  RMSC SG  N E T L  TFTQAF YSPV PAD  QLTPYVFRL LQQSGVD LL E M QI A  : 550
TETQ PRERU : NHS A FF  RMSC SG  N E T L  TFTQAF YSPV PAD  QLTPYVFRL LQQSGVD LL E CF QI V  : 550
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EFG AQUAE  :  GDV G LNSRRGK GM N-KG-VIT KAF LAR FG ATT RSI C GTF MKFSH DEVPQQIAFKIIGERMAG : 696
EFG THETH  :  GDV G LNARRGQ GM PRGN--AQ RAF LAE FG AT  RSKP RGS  MFFDH QEVPKQVQEKLIKGQ---- : 691
EFG ECOLI  : NTGDV G LS RRGM KGQ SEVT-GVK H-AE LSE FG ATQ RSI K RASY MEFLK DEAPSNVAQ-AVIEARGK : 703
EFG SALTY  : NTGDV G LS RRGM KGQ SEVT-GVK H-AE LSE FG ATQ RSI K RASY MEFLK DDAPNNVAQ-AVIEARGK : 703
EFG HAEIN  :  GDV G LS RRAM NGQ ANEF-VVK Y-AE LSE FG AT  RSQ  RASYSMEPLK AEAPTSVAA-AVIEARKK : 699
EFG HELPY  :  GDV G LN RRGQ NSMDD-RL-GLK NAF LVE FG STD RSA C RGTYSM FDH GEVPSNIAK-EIVEKRKG : 691
EFG MYCPN  : FGDT G ISSRRGI EGT Q-RD-NVQ KAK LKE FG AT  RSI Q RGNY MQFSH AETPKSVVNZIIATK-- : 688
EFG UREPA  : FGTV G IS RRGV EGQ Q-RG-NAQA KAK LSE FG AID RSN C RGQY MZFSH AQAPKSVTEZVMVARAKK : 688
EFG STRPY  : N GDV HVTARRGR DGM A-HG-NSQ RAY AF FG AT  RSK C NGT  MVFDH DEVPKSVQFEIITKKNKGE : 691
EFG RICCN  :  GDI G LNSRRGQ QSMDP-RG-NAQ TAN LAE FG VNT RSL  RAQFSMIFSH DQVPSQVADIIFAKK--- : 699
EFG MYCTU  :  GDV G LNSRRGQ QAM E-RA-GAR RAF LSE FG VGI RSK Q RANYSMVFDS SEVPANVSKEIIAKATGE : 701
TETM NEIME :  SRAYN AP YCAN VDT LKNN--EV SG  ARC QE RSI FF N RSVC TELKG HVTTGEPVCQPRRPNSRI : 628
TETM UREUR :  SRAYN AP YCAN VDT LKNN--FV SG  ARC QF RSL TFF N RSVC TELKG HVTTGEPVCQPRRPNSRI : 628
TETM STRPN :  SRAYT AP YCAN VDT LKNN--EV SG  ARC QE RSI TFF N RSVC TELKG HVTTGEPVCQPRRLNSPI : 628
TETM STAAU :  GRAYN AP YCAN VNT LKNN--EV IG  ARC QD RNI TFF N LSVC AELKG QVTTGEPVCQTRRLNSPI : 628
TETS LACLA :  SRAYN AS YCAN LNTKLKGN--EV IG  ARC QE RNS TFF N RSVC TELKG QVTNIKSAFQPRRPNNRI : 633
TETS LISMO :  SRAYN AS YCAN LNTKLKGN--EV IG  ARC QE RNS TFF N RSVC TELKG QVTNIKSAFQPRRPNNRI : 628
TETO STRMU :  SRAYH AP YCAD VST IKND--EV KG  ARC QE RNI TYF N QGVC TELKG QPAIGKFICQPRRPNSRI : 628
TETO STRPN :  SRAYH AP YCAD VST VKND--EV KG  ARC QE RNI TYF N QGVC TELKG QPAIGKFICQPRRPNSRI : 628
TETO CAMCO :  SRAYH AP YCAD VST IKND--EV KG  ARC QE RNI TNS N QGVC TELKG QPAIGKFICQPRRPNSRI : 628
TETO CAMJE :  SRAYH AP YCAD VST IKND--EV KG  ARC QE RNI TYF N QGVC TELKG QPAIGKFICQPRRPNSRI : 628
TETQ PREIN : ASSKAIT LQ MMSE EDISCNNE--WCH KGK YNTSKD ASE SSY KGLGVE VKPC QITKGDYSDNIR--MNEK : 627
TETQ PRERU : ASSKAIT LQ MMSE EDISCNNE--WCH KGK LNTSKD ASE SSY KGLGIF VKPC QITKDGYSDNIR--MNEK : 627
```

```
EFG AQUAE   : KSS------------------------ : 699
EFG THETH   : ------------------------ : -
EFG ECOLI   : ------------------------ : -
EFG SALTY   : ------------------------ : -
EFG HAEIN   : ------------------------ : -
EFG HELPY   : ------------------------ : -
EFG MYCPN   : ------------------------ : -
EFG UREPA   : ------------------------ : -
EFG STRPY   : ------------------------ : -
EFG RICCN   : ------------------------ : -
EFG MYCTU   : ------------------------ : -
TETM NEIME  : DKVRYMFNKIT--------------- : 639
TETM UREUR  : DKVRYMFNKIT--------------- : 639
TETM STRPN  : DKVRYMFNKIT--------------- : 639
TETM STAAU  : DKVRYMFNKIT--------------- : 639
TETS LACLA  : DKVRHMFNKINLH------------- : 646
TETS LISMO  : DKVRHMFNKINLH------------- : 641
TETO STRMU  : DKVRHMFHKLA--------------- : 639
TETO STRPN  : DKVRHMFHKLA--------------- : 639
TETO CAMCO  : DKVRHMFHKLA--------------- : 639
TETO CAMJE  : DKVRHMFTS----------------- : 637
TETQ PREIN  : DKILFMFQKSNVIKIMERSCNFYKAIQ : 654
TETQ_PRERU  : DKLLFMFQKSMSLK------------ : 641
```

6.3 Materials

Table 6-1: E. coli strains used in this study

E. coli Strains	Description	Selection Markers	Reference/ Source
CAN/20_12E	RNAse-deficient E. coli strain used for ribosome isolation	none	ref. [157]
MRE600	RNAse-deficient E. coli strain used for ribosome isolation	none	
BL21 (DE3)	General purpose expression host for expression using T7 promoter	none	Novagen
BL21(DE3)pLysS	High-stringency expression host for expression using T7 promoter	Chloramphenicol	Novagen
Rosetta(DE3)	Expression host for expression of genes with rare codons utilizing a T7 promoter	Chloramphenicol	Novagen
Rosetta(DE3)pLysS	High-stringency expression host for expression of genes with rare codons utilizing a T7 promoter	Chloramphenicol	Novagen
AVS6009 pSTL102	Carries chromosomal deletions of all 7 rRNA operons such that the only rRNA is derived from a plasmid-borne copy	Ampicillin Spectinomycin Erythromycin Tetracycline	ref. [104]
AVS6009 pSTL912G	Carries chromosomal deletions of all 7 rRNA operons such that the only rRNA is derived from a plasmid-borne copy. The plasmid-borne copy has a 912G mutation in the 16S rRNA	Ampicillin Spectinomycin Erythromycin Tetracycline	ref. [104]
AVS6009 pSTL912G/885U	Carries chromosomal deletions of all 7 rRNA operons such that the only rRNA is derived from a plasmid-borne copy. The plasmid-borne copy has a 912G and 885U mutation in the 16S rRNA	Ampicillin Spectinomycin Erythromycin Tetracycline	ref. [104]

Table 6-2: Plasmids used in this study

Vector	Description	Selection Marker	Reference/ source
pET14b	T7-based expression vector	Ampicillin	Novagen
pMS119EH	IPTG-inducible expression vector	Ampicillin	Ref. [146]
pMSTetO	Over-expression Tet(O). Note the 3' sequence of this construct may not be wild type (see section 2.2.2)	Ampicillin Tetracycline	Ref. [11]
pMSTetOD131NHC	Over-expression of C-terminally His-tagged XTPase mutant of Tet(O). Note the 3' sequence of this construct may not be wild type (see section 2.2.2)	Ampicillin	Section 4.4.1
pMSTetOHC	Over-expression of C-terminally His-tagged Tet(O). Note the 3' sequence of this construct may not be wild type (see section 2.2.2)	Ampicillin Tetracycline	Ref. [11]
pMSTetOHN10	Over-expression of N-terminally His-tagged Tet(O). Note the 3' sequence of this construct may not be wild type (see section 2.2.2)	Ampicillin Tetracycline	Section 2.2.1
pMSTetOHN6	Over-expression of N-terminally His-tagged Tet(O). Harbours R346L mutation.	Ampicillin Tetracycline	Section 2.2.1
pMSTetOHN8	Over-expression of N-terminally His-tagged Tet(O). Harbours R346L mutation.	Ampicillin Tetracycline	Section 2.2.1
pQE70	IPTG-inducible expression vector	Ampicillin	Qiagen
pUOA2	A pUC8 derivative harbouring a 5 kb fragment of pUA466 that confers resistance to tetracycline.	Ampicillin Tetracycline	Ref. [7]
pUOA2E1	A pUC8 derivative harbouring a 5 kb fragment of pUA466 that confers resistance to tetracycline.	Ampicillin Tetracycline	Ref. [130]

Table 6-3: Primers used to clone and sequence Tet(O)

Primer	Sequence (5'→3')	Application
Cat8	TATATGGAATTCAATGCATCACCATCACCATCAC AAAATAATTAACTTAGGCATTCTGGC	*tet*(O) cloning into pMS119 (EcoRI)
M13 forward	CGCCAGGGTTTTCCCAGTCACGAC	sequencing *tet*(O) flanking regions
M13(-48)	AGCGGATAACAATTTCACACAGGA	sequencing *tet*(O) flanking regions
Nch2	TATATGGATCCTCTAACTTGTGAACATATGCCG	*tet*(O) cloning into pMS119 (BamHI)
sc009	CATGCCATGGGCCATCACCATCACCATCACAAAA TAATTAACTTAGG	*tet*(O) cloning into pET14b (NcoI)
sc010	CGCGGATCCGCGTTACTAACTTGTGAACATATG	*tet*(O) cloning into pET14b and pQE70 (BamHI)
sc011	ACATGCATGCAGCATCACCATCACCATCACAAAA TAATTAACTTAGG	*tet*(O) cloning into pQE70 (SphI)
sean1	TGTCGCTATAGATAGACAGATG	Tn1000 sequencing
sean2	TCTATATGGAGGAAGCGT	Tn1000 sequencing
sean3	GTACGTTTTCGTTCCATTG	Tn1000 sequencing
sean4	TGGCGGAAGTATACCGTTC	*tet*(O) sequencing
sean5	ACTGCTCCGTCTAATACG	*tet*(O) sequencing
sean6	CAGAACTGGAACAGGAAG	*tet*(O) sequencing
sean7	ATCCGATACAGCCTGCTCTG	*tet*(O) sequencing
sean8	AAGCAGAATATACCATCCAC	*tet*(O) sequencing
sean9	ATGCACCGCAGGAATATC	*tet*(O) sequencing
sean10	TAGCTGGATCCTCTAATATATAACAGAGTAGGCTC	cloning *tet*(O) C-terminal truncations
sean11	TAGCTGGATCCTCTAACTATAATACAATCCATATTC	cloning *tet*(O) C-terminal truncations
sean12	TAGCTGGATCCTCTAAAATACTGTTTAGCTGCAA	cloning *tet*(O) C-terminal truncations
sean23	GCT TGG AGA AAG TCC GGG	sequencing *tet*(O) flanking regions
sean24	GCAGGATTTCCCCCTGCC	sequencing *tet*(O) flanking regions
sean25	TGCTCCATTCCGTGCAAG	sequencing *tet*(O) flanking regions
sean26	TTCTTCGAACGCTATTGAC	sequencing *tet*(O) flanking regions
sean27	TTAGGCGAACTAGCGACCC	sequencing *tet*(O) flanking regions
sean28	GCTCCATACTCTCCTTTCC	sequencing *tet*(O) flanking regions

Table 6-4: Primers used for primer extension analysis of 16S rRNA

Name	Sequence (5'→3')	Target [A]	Source [B]
S1	AGTCTGGACCGTGTCTC	*E.coli* 16S rRNA (323)	1
S2	CGCATTTCACCGCTACA	*E.coli* 16S rRNA (683)	1
S3	GACAGCCATGCAGCACC	*E.coli* 16S rRNA (1046)	1t
S4	ACGGGCGGTGTGTAC	*E.coli* 16S rRNA (1391)	1
SN	TAAGCGCCCTCCCGAATT	*E.coli* 16S rRNA (1447)	2
S7	CCAACCGCAGGAACCCCTACGG	*E.coli* 16S rRNA (1508)	1
S8	GTTCCCCTACGGTTACCTT	*E.coli* 16S rRNA (1501)	1
SW2	GCCGTTACCCCACCTACT	*E.coli* 16S rRNA (250)	3
SW4	CTTTACGCCCAGTAATT	*E.coli* 16S rRNA (559)	3
SW5	CGAAGGCACATTCTCAT	*E.coli* 16S rRNA (1016)	3
SW6	CTCTCGCGAGGTCGCTT	*E.coli* 16S rRNA (1256)	3
S4a	CAACATTTCACAACACG	*E.coli* 16S rRNA (1071)	4
S4b	GTTGCGGGACTTAACCC	*E.coli* 16S rRNA (1093)	4

[A] the number in brackets indicate the 1st nucleotide after the site where the primer anneals.

[B] The source of the primer sequence is indicated with numbers corresponding to:

1) Moazed, D., Stern, S., and Noller, H.F. (1986) Rapid chemical probing of conformation in 16 S ribosomal RNA and 30 S ribosomal subunits using primer extension. *J Mol Biol* **187**, 399-416.

2) Norbert Polacek (Personal communication). Center for Pharmaceutical Biotechnology M/C 870, University of Illinois, 900 S. Ashland Ave., Chicago, IL 60607, USA.

3) Ericson, G., Chevli, K., and Wollenzien, P. (1989) Structure of synthetic unmethylated 16S ribosomal RNA as purified RNA and in reconstituted 30S ribosomal subunits. *Biochemistry* **28**, 6446-6454.

4) this study

Table 6-5: Primers used for primer extension analysis of 23 S rRNA

Name	Sequence (5'→3')	Target [A]	Source [B]
R1	CTCGCCGCTACTGGGGG	*E.coli* 23S rRNA (235)	1
R2	TTCCCTCACGGTACTGG	*E.coli* 23S rRNA (454)	1
R2.5	GTGCTCCCACTGCTTGT	*E.coli* 23S rRNA (525)	2
R3	GATCACCGGGTTTCGGG	*E.coli* 23S rRNA (670)	1
R4	GGATGACCCCCTTGCCG	*E.coli* 23S rRNA (872)	1
R5	CGACTCGACCAGTGAGC	*E.coli* 23S rRNA (1098)	1
R5.5	CGTTACATCTTCCGCGC	*E.coli* 23S rRNA (1119)	2
R6	CGGCCTCGCCTTAGGGG	*E.coli* 23S rRNA (1347)	1
R7	TGTCGGTTTGGGGTACG	*E.coli* 23S rRNA (1599)	1
R8	TAACCTTCCGGCACCGG	*E.coli* 23S rRNA (1836)	1
R9	CCGTTATAGTTACGGCC	*E.coli* 23S rRNA (1905)	1
R10	TTCCGTCTTGCCGCGGG	*E.coli* 23S rRNA (2042)	1
R10.5	ATCCTACACATCAAGGC	*E.coli* 23S rRNA (2101)	2
R10.75	TTACGGGTCTTCGTTAG	*E.coli* 23S rRNA (2195)	2
R11	ACCTTCGTGCTCCTCCG	*E.coli* 23S rRNA (2274)	1
R12	GCCGACATCGAGGTGCC	*E.coli* 23S rRNA (2493)	1
R12.5	GGACCGAACTGTCTC	*E.coli* 23S rRNA (2596)	2
R13	ACTAGGAGCAGCCCCCC	*E.coli* 23S rRNA (2639)	1
R14	AAGGTTAAGCCTCACGG	*E.coli* 23S rRNA (2887)	1
R14a	GAGAACTCATCTCGGGG	*E.coli* 23S rRNA (2771)	3
R14b	TCCGCATTTAGCTACCG	*E.coli* 23S rRNA (2717)	3
R14c	ATGACAACCCGAACACC	*E.coli* 23S rRNA (2685)	3

[A] the number in brackets indicate the 1st nucleotide after the site where the primer anneals.

[B] The source of the primer sequence is indicated with numbers corresponding to:

1) Moazed, D., Stern, S., and Noller, H.F. (1986) Rapid chemical probing of conformation in 16 S ribosomal RNA and 30 S ribosomal subunits using primer extension. *J Mol Biol* **187**, 399-416.

2) Norbert Polacek (Personal communication). Center for Pharmaceutical Biotechnology M/C 870, University of Illinois, 900 S. Ashland Ave., Chicago, IL 60607, USA.

3) this study

6.4 Analysis of cryo-EM maps

The following program was written by Dr. B. Hazes to count the number of pixels in the electron density file (ccp4 format) with a value greater than the desired cutoff level.

```
        parameter (NSECT=125*125)
        real pixels(NSECT)

        open(unit=1, file='newtemp')

        write(*,*)'Give cutoff level'
        read(*,*)cutoff

        count=0
        kount=0
        sum=0.0
        sumsq=0.0
100 continue
        read(1,*,end=999)pixels
        do i=1,NSECT
             if(pixels(i) .ne. 0.0)then
             if(pixels(i) .gt. cutoff)then
                   count=count+1
                   sum=sum+pixels(i)
                   sumsq=sumsq+pixels(i)**2
             end if
        enddo
    goto 100
999 continue
    ave=sum/count
    stdev=sqrt((sumsq-ave*sum)/(count-1))

    write(*,*) 'Average: ',ave
    write(*,*) 'St. Dev: ',stdev
    write(*,*) 'Pixels : ',count

    end
```

Chapter 7

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7 Bibliography

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