

***Thermus Thermophilus* as a Model System for the Study of Ribosomal Antibiotic Resistance**

Steven T. Gregory

Department of Cell and Molecular Biology, College of the Environment and Life Science
The University of Rhode Island
Kingston, RI 02881 USA
and

Department of Molecular Biology, Cell Biology and Biochemistry, Brown University
Providence, RI 02912 USA

Email: stgregory@uri.edu

Abstract. Ribosomes are the intracellular ribonucleoprotein machines responsible for the translation of mRNA sequence into protein sequence. As an essential cell component, the ribosome is the target of numerous antibiotics that bind to critical functional sites to impair protein synthesis. Mutations causing resistance to antibiotics arise in antibiotic binding sites, and an understanding of the basis of resistance will be an essential component of efforts to develop new antibiotics by rational drug design. We have identified a number of antibiotic-resistance mutations in ribosomal genes of the thermophilic bacterium *Thermus thermophilus*. This species offers two primary advantages for examining the structural basis of antibiotic-resistance, in particular, its potential for genetic manipulation and the suitability of its ribosomes for analysis by X-ray crystallography. Mutations we have identified in this organism are in many instances identical to those found in other bacterial species, including important pathogens, a result of the extreme conservation of ribosome functional sites. Here I summarize the advantages of this organism as a model system to study antibiotic-resistance mechanisms at the molecular level.

1. Introduction: *Thermus thermophilus* as a Model Organism

Members of the *Thermus* genus are all extremely thermophilic bacteria belonging to the *Deinococcus-Thermus* phylum, one of the most deeply branching clades in the bacterial domain [1]. The type species of the genus *Thermus* was discovered in 1969 and named *Thermus aquaticus* [2]. Although *T. thermophilus* was discovered a year earlier, its taxonomic affiliation was not properly described until 1974 [3]. *T. thermophilus* has become one of the most widely used thermophiles due to several distinct advantages it provides as a model organism [4]. Several species have been found to be naturally transformable with genomic or plasmid DNA [5]. Together with the development of plasmid vectors and antibiotic-resistance genes functional at high temperatures, this provides the potential for genetic manipulations including the construction of gene knockouts [reviewed in 4].



T. thermophilus is easily cultivated, growing aerobically with an optimum growth temperature between 65 and 72 °C, depending on the strain. These temperatures are within the usable range for agar as a solidifying agent in culture media, and cultivation of *T. thermophilus* requires no specialized laboratory equipment. It forms colonies on agar plates, allowing genetic selections and colony purification. Our laboratory has employed three different strains of *T. thermophilus*. Strains HB8 (the type strain of the species) and HB27 were isolated from terrestrial hot springs in Japan [3], while strain IB-21 was isolated from a submarine hot spring off the coast of Iceland in 1986 [6]. The genome sequence of HB27 has been published [7], while the HB8 sequence is available through public databases. We have successfully isolated antibiotic-resistant mutants of each of these strains. *T. thermophilus* is sensitive to a wide range of antibiotics, allowing the isolation of mutants with phenotypes corresponding to those found among many important bacterial pathogens. We have also noted that genetic crosses by transformation between these strains occurs readily, attesting to their close relationship and allowing mutations to be transferred to various backgrounds.

Importantly, ribosomes from *T. thermophilus* form crystals that diffract X-rays to high resolution, allowing their three-dimensional structure to be determined [reviewed in 8]. Figure 1 shows the structure of the *T. thermophilus* 70S ribosome. Further, structures of antibiotic-ribosome complexes have been determined, thereby establishing the precise mode of ribosome-antibiotic interaction [for instance, 9, 10]. Importantly, these results are consistent with similar structural findings using mesophilic ribosomes [11, 12], thus validating the use of this thermophile as a model system. Such results aid in predicting possible mechanisms of resistance. More importantly, they provide a framework for the interpretation of structures of mutant ribosomes determined experimentally.

2. Antibiotic-resistance Mutations in Ribosomes of *T. thermophilus*.

For genetic studies of ribosomes, *T. thermophilus* has the attribute of having only two copies of each rRNA gene [7]. Deletion of one of these to produce strains having single rRNA gene copies allows the isolation of recessive mutations. One potential concern regarding the use of this species to investigate antibiotic-resistance mutations is the very thermophilic nature that is responsible for the facility with which its ribosomes form ordered crystals. As a result, some laboratories will choose to acquire high-resolution structural data using ribosomes from pathogenic species [for example, 13].

Several factors combine to argue in favor of *T. thermophilus* as a model system, however. First and foremost, antibiotics bind very specifically to their binding sites and do so with ribosomes from diverse clades within bacterial phylogeny. These binding sites are highly conserved in sequence; this conservation is due to the functionally critical nature of these sites, which is why antibiotics bind to them in the first place. Thus, the conserved binding of antibiotics to ribosomes from across the bacterial domain provides a strong case that ribosome sequence conservation reflects an underlying conservation of three-dimensional structure. This conclusion is supported by structures of ribosomes from several sources. Finally, as I will describe below, we have found that similar or identical mutations produce similar or identical phenotypes, which in turn implies that they produce similar or identical changes in ribosome structure to prevent binding. Thus, it can be argued that information from structural studies of antibiotic-resistant thermophilic ribosomes is sufficiently robust to interpret resistance mutations found in mesophilic bacterial pathogens.

2.1 Aminoglycoside-resistance Mutations.

Aminoglycosides, including streptomycin, kanamycin, paromomycin, neomycin, hygromycin B, and many others, act by binding at or near the decoding site on the 30S subunit, and in doing so, induce translational errors [reviewed in 14]. While paromomycin (and the structurally related drugs kanamycin and neomycin) and hygromycin B bind at distinct sites within 16S rRNA helix 44, streptomycin has a more complex binding site that consists of two 16S rRNA pseudoknots and 30S subunit ribosomal protein

S12. Consistent with these drugs binding to non-overlapping sites, mutations conferring resistance to these two antibiotic groups arise at distinct places in the ribosome structure. Streptomycin-resistance mutations in *T. thermophilus* result in amino acid substitutions in S12 [15, 16] or in base substitutions in the central pseudoknot of 16S rRNA [16]. Resistance to kanamycin is conferred by an A1408G substitution while hygromycin B resistance is conferred by base substitutions at the U1406-U1495 base pair [17].

X-ray crystal structures of 30S subunits bearing base substitutions in the central pseudoknot of 16S rRNA show clear changes in position due to disruption of the U13-A915-U20 base triple or the Type II G21-A914 base pair [18]. However, streptomycin contacts the central pseudoknot via interactions with the sugar-phosphate backbone, and makes no direct interaction with rRNA bases, as shown in Figure 2. In crystal structures of mutant ribosomes, the backbone trajectory remains very close to that of wild-type ribosomes, defying a straightforward explanation for resistance. Indeed, these mutant ribosomes are able to bind streptomycin at high concentrations. One possible interpretation of the structural data is that disruption of pseudoknot base-base hydrogen bonding increases backbone conformational dynamics, thereby reducing streptomycin binding affinity.

An interesting variation on the streptomycin-resistance phenotype is streptomycin dependence. Streptomycin-dependent mutants are incapable of growth in the absence of the drug. Given the capacity of streptomycin and other aminoglycosides to induce miscoding, it is perhaps not surprising that streptomycin-resistance mutations generally cause the ribosome to become hyper-accurate. Streptomycin dependence has been described as having an extreme form of hyperaccuracy [14]. Dependence mutations can arise either as amino acid substitutions in ribosomal protein S12, or as base substitutions in 16S rRNA. Crystal structures of the former are quite revealing, as they show a severe distortion of the 16S rRNA helix 44 decoding site, such that the ribosome's ability to monitor the geometry of codon recognition would be grievously if not critically impaired [19]. However, either binding of streptomycin or the inclusion of a suppressing mutation were found to restore the decoding site structure to a near-native conformation. In light of these observations, the notion of streptomycin dependence as a form of extreme hyperaccuracy would appear to be an oversimplification.

2.2 Thiostrepton-resistance Mutations.

Thiostrepton and related peptide antibiotics bind to a cleft between 50S ribosomal protein L11 and its 23S rRNA binding site. Its mode of action is to block the EF-G-dependent translocation step of protein synthesis [14]. *T. thermophilus* is exquisitely sensitive to thiostrepton, and mutants are readily selected with both amino acid substitutions in L11 and base substitutions in 23S rRNA occurring [20]. Some of these are located directly in the thiostrepton binding site, while several others occur at the hinge between the two domains of L11. Curiously, each of the L11 amino acid substitutions occurs at a proline residue. As prolines constrain polypeptide conformation, such substitutions may act by increasing the flexibility of various parts of L11 as they impinge upon the thiostrepton binding site. Thus, changes in conformational dynamics within L11 may potentially impact the binding affinity of thiostrepton to mutant ribosomes. In contrast, the sites of base substitutions in 23S rRNA, A1067 or A1095, are located at contact sites for thiostrepton, allowing a straightforward explanation for resistance.

2.3 Capreomycin-resistance Mutations.

Capreomycin and viomycin are examples of tuberactinomycin antibiotics [14] that bind to the interface between the 30S and 50S subunits, and in so doing, interfere with intersubunit rotations that occur during the translocation step of protein synthesis [21]. In our initial selections for capreomycin-resistant mutants of *T. thermophilus*, we identified only base substitutions in helix 44 of 16S rRNA, specifically A1408G, C1409G, and G1491A, mutations previously associated with aminoglycoside resistance [16]. This finding is consistent with aminoglycosides and tuberactinomycins having overlapping binding sites. In a

subsequent study, we identified mutations affecting 23S rRNA helix 69, including the base substitutions A1913U and mU1915G and the single base deletion Δ mU1915 [22]. These two rRNA structures, despite being on different subunits, interact directly with one another as part of an intersubunit bridge and together constitute the tuberactinomycin binding site [21]. Distortion of the drug binding site seems to be sufficient to cause resistance, although firmer conclusions await high-resolution structures of mutant ribosomes.

2.4 Chloramphenicol and Macrolides.

The peptidyltransferase active site (also known as the peptidyltransferase center, or PTC) consists exclusively of nucleotides of 23S rRNA in the 50S ribosomal subunit. The PTC is the most frequently targeted functional site of the ribosome, and multiple structurally unrelated classes of antibiotics bind to it [14]. Among these are macrolides (such as erythromycin, tylosin, and azithromycin, among many others), lincosamides (including lincomycin and clindamycin), streptogramins (virginiamycin, pristinamycin), chloramphenicol, hygromycin A, and sparsomycin.

Among the very first *T. thermophilus* antibiotic-resistant mutants we identified was an erythromycin-resistant mutant having the single base substitution A2058G of 23S rRNA [23]. Erythromycin is a macrolide that interferes with polypeptide chain elongation into the exit channel [14]. This mutation has been identified in a number of organisms, and is usually found to confer cross-resistance to lincosamides and streptogramin B antibiotics. Chloramphenicol by contrast is a true peptidyltransferase inhibitor, binding directly to the active site on the 50S ribosomal subunit and inhibiting the catalytic step of peptide bond formation. Selection for chloramphenicol-resistant mutants of *T. thermophilus* produced the largest variety of base substitutions, including A2030G, G2061A, A2062G, G2447A, C2452U, A2453G, U2500C, A2503G, U2504G, U2504C, U2504A, and G2505A, consistent with the highly complex three-dimensional organization of the PTC [17]. Because numerous antibiotics bind to the PTC, mutations conferring resistance to one antibiotic often confer resistance to one or more other antibiotics. This was indeed found for mutants in our study, as revealed by cross-resistance tests. Furthermore, base substitutions in the PTC occur at functionally critical residues, and can have significant to severe effects on growth and peptidyltransferase catalytic activity [24].

Given the large number of antibiotics that bind to the PTC, and the correspondingly large number of potential resistance mutations that can arise within it, one interesting question is how combinations of multiple mutations might impact resistance phenotypes. For instance, selection on multiple antibiotics, either sequentially or in combination, could conceivably give rise to multiple mutations in the PTC. Complex cross-resistance patterns could emerge, which is information that could prove of benefit in designing new drug combination therapies. How such mutation combinations might phenotypically interact is completely unknown and worthy of future investigation.

3. Conclusions

T. thermophilus in many ways provides an ideal model system for investigating antibiotic-resistance mechanisms. Its facile genetics combined with the capacity of its ribosomes to crystallize allow for detailed investigations into the precise mechanism by which mutations in the ribosome interfere with antibiotic action. Given that mutations in this organism are most often identical to those found in pathogenic species, *T. thermophilus* provides an excellent model for establishing basic principles that are applicable to medically important organisms. While the solution of pathogen ribosome structures may yield insights into specific organisms, the benefit of such insights would need to outweigh the potential hazards of growing large-scale cultures of antibiotic-resistant pathogens for ribosome purification. Given the remarkable conservation of ribosome structure, particularly in the functional centers that comprise antibiotic binding sites, the use of a model system as a proxy for pathogenic organisms may continue to prove advantageous.

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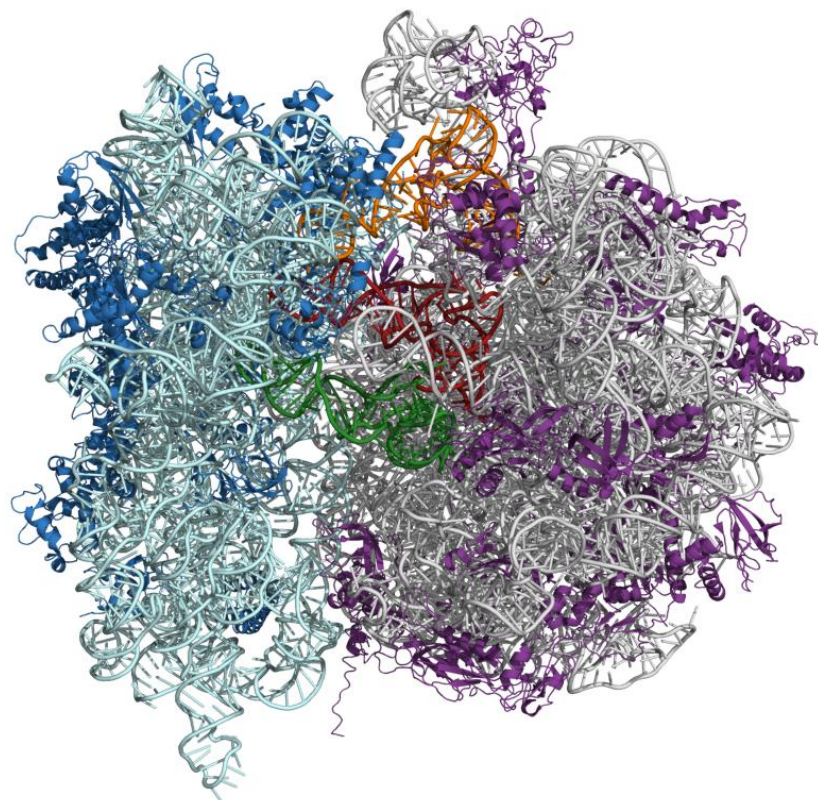


Figure 1. The crystal structure of the *T. thermophilus* 70S ribosome (pdb entries 2wdk and 2wdl; ref 25). The 30S subunit is to the left, with 16S rRNA colored palecyan and small subunit proteins colored skyblue. The 50S subunit is to the right, with 23S rRNA and 5S rRNA colored grey and large subunit proteins colored purple. Three tRNAs are located at the subunit interface, with the A-site tRNA colored green, P-site tRNA colored red, and E-site tRNA colored orange. These tRNAs mark the functional centers of the ribosome, also the binding sites for numerous antibiotics.

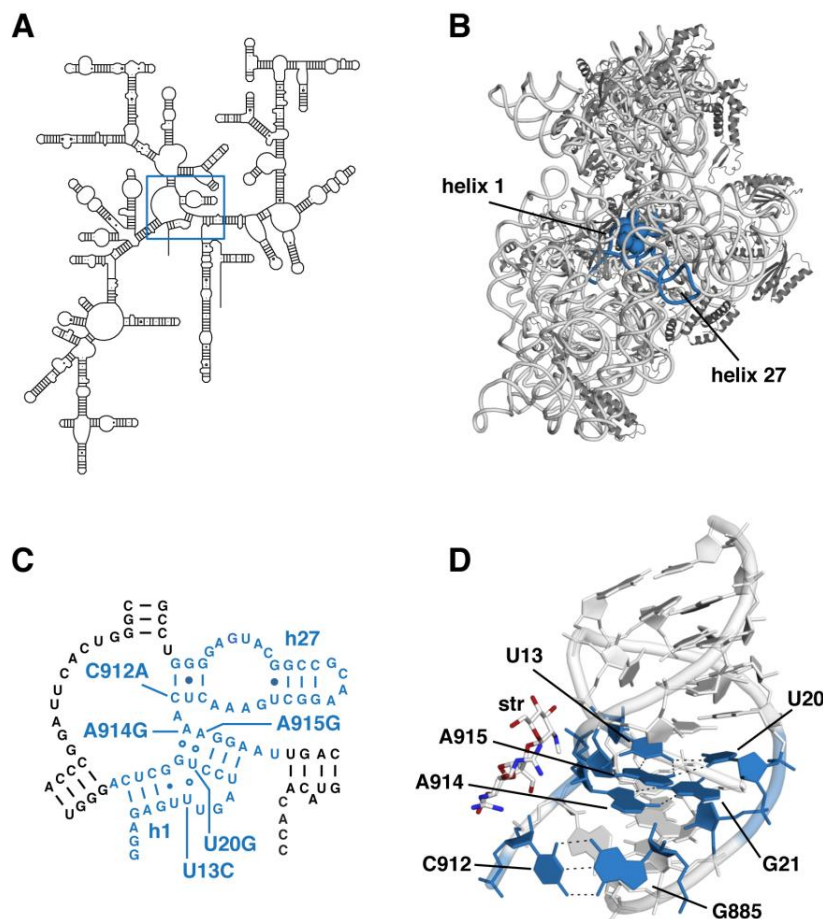


Figure 2. Streptomycin-resistance mutations in 16S rRNA, an example of antibiotic-resistance mutations found in *T. thermophilus*. (A) Secondary structure model of 16S rRNA, with the central pseudoknot region enclosed by a blue box. (B) Crystal structure for the 30S ribosomal subunit, with 16S rRNA helices 1 and 27 shown in blue, and streptomycin shown as blue spheres (pdb entry 4DR3; ref 26). (C) Sites of single base substitutions in the secondary structure model of 16S rRNA. (D) Close-up view of the central pseudoknot, showing hydrogen bonding interactions affected by resistance mutations in blue. Note that streptomycin makes contacts with the pseudoknot exclusively via the backbone and makes no direct contacts with bases.