Structures of the *Escherichia coli* ribosome with antibiotics bound near the peptidyl transferase center explain spectra of drug action

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**Antibiotics are small organic molecules synthesized by fungi and bacteria that can inhibit the growth of other microorganisms (1). The ribosome is a major target of antibiotics, which affect nearly all steps of protein synthesis (2). The peptidyl transferase center (PTC) of the ribosome is inhibited by a chemically diverse group of compounds including lincosamides and phenicols (Fig. 1A–C) (3). Despite the chemical dissimilarities of these compounds they share overlapping mechanisms of inhibition by preventing proper orientation of tRNA in the PTC and interfering with peptide bond formation. Another important group of drugs, macrolides and their modern ketolide derivatives, bind in the exit tunnel of the large ribosomal subunit and inhibit extrusion of the nascent peptide, leading to peptidyl tRNA drop-off (Fig. 1B and C) (4–6).

Bacterial pathogens have become resistant to antibiotics that inhibit protein synthesis over decades of clinical use of these compounds. One of the major mechanisms of resistance is based on altering ribosomal RNA (rRNA) in the drug binding site. For example, resistance to the macrolide erythromycin is often mediated by mutations of nucleotide A2058 in 23S rRNA located in the site of drug action or by chemical modification of this nucleotide by Erm methyltransferase, a gene that is often acquired by bacterial pathogens (Fig. 1B) (7, 8). Remarkably, similar mutations often provide resistance to multiple protein synthesis inhibitors that bind to the overlapping sites in ribosome. For example, mutations of 23S rRNA nucleotides 2058 or 2059 can provide resistance to macrolides, lincosamides, streptogramin B, or ketolides, yielding the so called MLSB phenotype. Mutation of nucleotide 2057 can provide resistance to the MLSB phenotype plus chloramphenicol (Fig. 1B) (9, 10). Other mutations (at positions 2452, 752, and 2611) described in several species can confer resistance to varying subsets of these compounds (Fig. 1B) (9, 10).

**Structural studies have greatly advanced our understanding of the inhibitory mechanisms of antibiotics that bind in the PTC or exit tunnel. However, uncertainty concerning the interactions of these compounds with rRNA and resistance phenotypes persist, due to many significant disagreements between the reports for antibiotics bound to either the *Deinococcus radiodurans* or *Haloarcula marismortui* 50S ribosomal subunits (11). These differences include the conformation of the macrolide ring, the conformation of the alkyl-aryl arm of telithromycin, the orientation of the pyrrolidinyl moiety of clindamycin and the two nonoverlapping binding sites observed for chloramphenicol (6, 12–15). Notably, neither *H. marismortui* nor *D. radiodurans* are closely related to pathogenic bacteria.

To address the differences that persist between the *H. marismortui* and *D. radiodurans* structural data, we solved structures of four antibiotics bound to the *Escherichia coli* ribosome: erythromycin, telithromycin, clindamycin and chloramphenicol, at resolutions of ∼3.3 Å–3.4 Å (Table S1). Because the *E. coli* ribosome has rRNAs sequences similar to bacterial species of medical interest, these data give a more accurate picture of the interactions between antibiotics and the large ribosomal subunit of pathogenic bacteria. Together with biochemical data probing the interactions of antibiotics with the PTC and exit tunnel, the present structures reveal how rRNA sequence differences contribute to the spectrum of activity for antibiotics and offer new clues as to why these compounds do not inhibit cytoplasmic eukaryotic ribosomes.

**Results**

**The Structure of Erythromycin Bound to the *E. coli* Ribosome.** The original macrolide antibiotic in clinical use since the 1950s, erythromycin is composed of a 14-membered macrolactone ring, with carbohydrates at positions 3 and 5 (Fig. 1A). The desosamine sugar at position 5, which contains a dimethyl amine that is crucial for binding to the ribosome, makes contact with A2058 (3OFD, 3OFC, 3OFB, 3OFA, 3OFB, 3OFC, 3OFD (70S ribosome in complex with chloramphenicol)].

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**Data deposition:** The coordinates for the structural models have been deposited in the Protein Data Bank, [www.pdb.org](http://www.pdb.org) [PDB ID codes 3OF0, 3OF1, 3OF2, 3OFQ (70S ribosome in complex with erythromycin), 3OAE, 3HAV, 3OAG, 3OAF (70S ribosome in complex with telithromycin), 3OFX, 3OBF, 3OFY, 3OFG (70S ribosome in complex with clindamycin), and 3OFA, 3OFB, 3OFC, 3OFD (70S ribosome in complex with chloramphenicol)].

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Fig. 1. Binding sites for antibiotics in the PTC and peptide exit tunnel. (A) The chemical structures of erythromycin (a macrolide), telithromycin (a keto- lide), chloramphenicol (a phenyl propanoside), and clindamycin (a lincosa- mide) are shown. (B) An overview of the antibiotic binding sites within the 50S subunit. Erythromycin (green), telithromycin (pink), clindamycin (purple), and chloramphenicol (orange) are shown as stick models. Ribbons denote the sugar phosphate backbone of 23S rRNA (gray) with nucleotides of interest colored light blue, the acceptor ends of A-site tRNA (yellow) and P-site tRNA (red). The location of the peptide exit tunnel is labeled “exit,” and an icon indicating the point of view is shown on the right. (C) The secondary structure of the 3′ region of 23S rRNA showing elements of the PTC and the adjacent peptide exit tunnel. Nucleotides that are divergent between *E. coli* and *H. marismortui* are shown in red. *D. radiodurans* diverges from *E. coli* at the 2057-2611 base pair and at nucleotides 752 and 2586. Ribosomal RNA helices emanating from this region are marked with dotted lines.

in 23S rRNA, the most commonly mutated nucleotide in resistant bacteria. The 14-atom macrolactone ring of erythromycin serves as the scaffold for several semisynthetic compounds in clinical use, with various appendages attached to the ring. The macrolactone ring was initially reported in two different conformations, “folded-in” and “folded-out” when bound to *D. radiodurans* and *H. marismortui* 50S subunits, respectively (12). However, the existence of the folded-in conformation for erythromycin when bound to the ribosome has been questioned because a putatively lower energy folded-out conformation of erythromycin exists in the crystal structure of the free compound (6).

In the structure of erythromycin bound to the *E. coli* 70S ribosome, we observed difference electron density for the drug in excellent agreement with its position bound to the *H. marismortui* 50S ribosomal subunit containing a G2058A mutation (6) and with its conformation in the crystal structure of the free compound (Fig. S1) (16). A slight movement of the antibiotic relative to its position when bound to the G2058A mutant *H. marismortui* 50S subunit is visible, possibly due to a movement of rRNA helix H73 and the adjacent nucleotides in the *E. coli* ribosome, relative to their position in the *H. marismortui* 50S subunit (Fig. S1B). In spite of this spatial translocation, the drug maintains its contacts with A2058, which involves a hydrogen bond between the desosamine hydroxyl and the N1 atom of A2058, and tight packing of the hydrophobic face of the lactone ring against nucleotides 2611 and 2057 in the peptide exit tunnel wall. Notably, *H. marismortui* contains a G-C base pair C2057-G2611 in the opposite polarity of nucleotides 2611 and 2057.

**A Key Moiety of Telithromycin Forms Species-Specific Contacts to rRNA**. Telithromycin belongs to the family of ketolide antibiotics that represent the newest generation of macrolides. In telithromycin, a carbonyl group replaces the C3 cladinose sugar (Fig. 1A), which in macrolides is necessary for ribosome stalling and regulating the induction of resistance genes (17). Similar to other clinically relevant ketolides, telithromycin contains an alkyl-aryl arm attached to a carbamate heterocycle that involves the C11 and C12 positions of the ketolide macrophore. This moiety increases the affinity of the ketolide scaffold for the ribosome by several hundred-fold, demonstrating that it is an important pharmacophore (18). The alkyl-aryl arm of telithromycin was seen in two distinct conformations in prior ribosome crystal structures. It was folded back over the macrolactone ring when bound to the G2058A *H. marismortui* 50S subunit, or interacting with rRNA further down the peptide exit tunnel when bound to the *D. radiodurans* 50S subunit (Fig. 2) (6, 13). Importantly, neither structure can easily explain telithromycin resistance mutants at residue U2609, telithromycin protection of A752 in RNA footprinting...
experiments, or the fact that deletion of A752 or mutations in its vicinity lead to resistance (9, 19–22).

In the E. coli ribosome, in contrast to H. marismortui or D. radiodurans, nucleotides A752 and U2609 form a base pair that bridges domains II and V in 23S rRNA. Notably, in the structure of telithromycin bound to the E. coli ribosome, the alkyl-aryl arm stacks on the A752-U2609 base pair, a conformation not observed in prior structures (Fig. 2 and Fig. S24). By contrast, the position of the macroactone ring remains in essentially the same conformation observed when telithromycin is bound to the G2058A H. marismortui 50S subunit (Fig. 2) (6, 13). The A752-U2609 base pair, which exists in E. coli and many other eu bacteria but not in H. marismortui or D. radiodurans (Fig. S2B), provides a surface for the entire face of the alkyl-aryl arm to engage in a stacking interaction that likely favors drug binding.

Stacking of the alkyl-aryl arm of telithromycin on the A752-U2609 base pair would also likely lead to protection of A752 from chemical probes by stabilizing the base pair interaction. To test this model, we probed E. coli ribosomes in solution by RNA footprinting for telithromycin protections of this base pair. We also examined Halobacterium halobium (a close relative of H. marismortui), D. radiodurans and Staphylococcus aureus ribosomes. Binding of telithromycin to E. coli or S. aureus ribosomes protected A752 from chemical modification (Fig. 3). By contrast, binding of telithromycin to D. radiodurans and H. halobium ribosomes did not protect the corresponding nucleotide, although both compounds were bound in all cases (Fig. 3). Given the sequence conservation of the A752-U2609 base pair among many eu bacteria, the structural and biochemical data obtained here suggest that the interactions of telithromycin with the E. coli ribosome likely reflect those that occur when telithromycin binds the ribosomes of medically relevant eubacterial species. Furthermore, the interaction between the alkyl-aryl arm and the A752-U2609 base pair helps to explain why deletion of A752 or mutations of U2609 provide resistance to telithromycin (9, 20, 22).

Clindamycin Sterically Clashes with A-site tRNA. Clindamycin is a semisynthetic derivative of the lincomycin class of compounds used clinically to treat gram-positive bacterial infections. Structural models of clindamycin bound to both the G2058A H. marismortui and D. radiodurans 50S ribosomal subunits (6, 12) agreed roughly in the placement of the galactose sugar of clindamycin but differed in the positioning of the propyl pyrrolidinyl moiety of the antibiotic, the portion that is hypothesized to interfere with A-site tRNA positioning (6). In the models of clindamycin bound to the H. marismortui and D. radiodurans 50S subunits, the pyrrolidinyl propyl group is rotated by 180° in one structure relative to the other (Fig. 4A). Structural studies of the ribosome containing acetylated A-site and P-site tRNAs, or oligonucleotide mimics of the tRNAs, show that the pyrrolidinyl propyl group, as modeled in the H. marismortui complex, would interfere with the positioning of A-site aminoacyl-tRNA (23). Unbiased difference electron density maps for clindamycin bound to the E. coli ribosome show density for the propyl group consistent with its position in the H. marismortui structure (Fig. 4A and B). Thus, clindamycin and other lincosamides likely interfere with A-site aminoacyl-tRNA binding, as proposed (6).

The location of the galactose ring, which forms numerous hydrogen bonds with A2058, A2059, G2505, and A2503 in both the G2058A H. marismortui and D. radiodurans 50S subunits, overlaps with the binding site of the desosamine sugar of macrolides and ketolides, explaining why lincosamides share resistance mutations with these antibiotic families. In the structure of the E. coli ribosome with clindamycin bound, difference electron density for the galactose ring agrees with the prior structural models for placement of the sugar (Fig. 4). The interactions of clindamycin with 23S rRNA nucleotides A2058 and A2503 in E. coli are identical to those observed with the corresponding H. marismortui nucleotides. However, nucleotides 2504–2507 are shifted relative to their positions in the G2058A H. marismortui 50S subunit, leading to alternative interactions with clindamycin (Fig. 4C). The shift probably is due to the presence of A2055 in H. marismortui versus C2055 in E. coli, which stacks against U2504 in the structure. This stacking interaction, together with differences in the base pair partners of G2505 and U2609, alters the conformations that nucleotides in this region adopt in E. coli ribosomes. In the unbiased difference electron density map, a strong positive peak indicates a shift of G2505 and U2506 toward the drug molecule, with U2506 packing perpendicular to the propyl group of clindamycin. Additional packing with C2452 leads to desolvation of the hydrophobic propyl group (Fig. S3). The altered position of these nucleotides in E. coli ribosomes relative to their position in H. marismortui 50S subunits allows the bridging amine of clindamycin to hydrogen bond to the ribose O4′ of G2505, while preserving contacts with A2058, A2059 and A2503 (Fig. 4C and D). Thus, a comparison of the structures of clindamycin bound to the G2058A H. marismortui and E. coli ribosomes suggests that clindamycin forms a similar number of hydrogen bonds to rRNA in the E. coli structure, with increased van der Waals contacts with the ribosome.

Chloramphenicol Positioning In and Near the 50S Subunit A-site. Prior structural studies identified one binding site for chloramphenicol in the D. radiodurans 50S subunit and a second distinct site in the H. marismortui 50S subunit (12, 15). The differing positions of chloramphenicol are probably due to the fact that wild type H. marismortui is resistant to chloramphenicol, possibly due to the low affinity of the drug for its primary binding site (10). This
intrinsic tolerance of archaea to chloramphenicol is likely due to rRNA sequence differences between archaea and bacteria; i.e., in nucleotides C2055 of H73, and U2609, and C2610 adjacent to the base of rRNA helix H73 (Fig. 1C).

In the structure of chloramphenicol bound to the E. coli ribosome, we did not observe any difference electron density in the chloramphenicol binding site identified in the H. marismortui 50S subunit (24). By contrast, clear positive difference electron density is located at the site of chloramphenicol binding reported in the D. radiodurans 50S subunit, but this density is not entirely consistent with the reported orientation of chloramphenicol (Fig. 5A and B) (12). In the E. coli ribosome complex, the nitrobenzene moiety of chloramphenicol overlaps with the position that would be occupied by the pyrrolidinyl propyl group of clindamycin (Figs. 1B, 4C, and 5B). When compared to the structure of clindamycin bound to the E. coli ribosome, the base of U2506 is rotated such that the hydrophilic nitro group in chloramphenicol points into solvent. The nitrobenzene ring of chloramphenicol is stacked on C2452 (Fig. 5B) and positions one chlorine atom of chloramphenicol in a similar position to the chlorine in clindamycin (Fig. 4C). The other chlorine atom in chloramphenicol is in a position to contact the exocyclic amine of A2062, a residue that when mutated results in chloramphenicol resistance (10). Finally, the amine of chloramphenicol hydrogen bonds to the nonbridging phosphate oxygen of G2505 (Fig. 5B).

Because rRNA sequences in the region of chloramphenicol binding are conserved in both E. coli and D. radiodurans, sequence divergence cannot explain the different orientations of the antibiotic. Unless the second shell rRNA residues dramatically affect chloramphenicol binding, there is likely one principal mode of interaction between chloramphenicol and euabacterial ribosomes (25). The unbiased difference electron density in the E. coli structure determination provides a much clearer view of the chloramphenicol orientation than is provided by the electron density used to model chloramphenicol bound to the D. radiodurans 50S subunit (compare Fig. 5A with Fig. 1C of ref. 12). Furthermore, the structural model of chloramphenicol bound to the E. coli ribosome is at higher resolution and with a lower Rfree value (25%) when compared to the D. radiodurans model (Rfree = 32%) (Table S1). Finally, the interactions between chloramphenicol and rRNA in the orientation in the present model are more chemically favorable due to additional stacking interactions that should facilitate drug binding. Notably, in the present structural model of chloramphenicol bound to the E. coli 70S ribosome, the nitrobenzene ring stacks on C2452 in the same orientation that the methoxyphenyl ring of the related compound anisomycin stacks on C2452 when bound to the H. marismortui 50S subunit (26).
Discussion

The rRNA of the PTC is highly conserved throughout the 3 domains of life, explaining why many antibiotics that bind in or near the PTC can inhibit a wide range of species. However, many antibiotics that bind in this region are selective. For example, erythromycin does not bind to eukaryotic or archaeal ribosomes except at extremely high concentrations. The identity of nucleotide 2058, adenosine in eubacteria, guanosine in eukaryotes and archaea, is thought to be a major contributor to this phenomenon (6). Conversely, wild type eubacteria are resistant to anisomycin whereas archaea and eukaryotes are sensitive (26, 27).

Our data reveal several rRNA residues that can account for the selectivity of antibiotic action. In the E. coli ribosome, 23S rRNA residues A752 and U2609 form a base pair that interacts with a key element of telithromycin, its extended 11,12 alkyl-aryl arm (Fig. 3A and B). Our data suggest that disruption of this base pair would decrease the affinity of telithromycin for the ribosome, consistent with mutation U2609C or deletion of A752 that lead to low level telithromycin resistance (20, 22). In D. radiodurans and H. marismortui, this base pair does not exist, leading to a different conformation of the alkyl-aryl side chain (6, 13) and likely decreasing telithromycin affinity for the ribosome (Fig. 4).

Nucleotide C2055 in eubacteria is an adenosine in archaea and eukaryotes (Table S2) (28), and also seems to serve as an important determinant for the spectrum of action of A-site inhibitors such as chloramphenicol and clindamycin, as well as other antibiotics (29, 30). Our structures reveal that alteration of A2055, which stacks on U2504 in the H. marismortui 50S subunit structures, to C2055 in E. coli leads to a displacement of U2504 along with G2505, U2506, and C2507 (Fig. 1B and 4C). Thus the sequence difference at C2055 results in a change in the position of four universally conserved PTC nucleotides (Table S2). The structures of the E. coli ribosome show that chloramphenicol and clindamycin both interact with these nucleotides. G2505 hydrogen bonds to clindamycin whereas U2506 desolvates the propyl group of clindamycin (Fig. 4 C and D). G2505 forms hydrogen bonds to chloramphenicol, interactions that are not possible in H. marismortui, given the conformation of this residue in H. marismortui ribosomal subunits (Fig. 4B). These structural data explain why archaeal and eukaryotic ribosomes bind lincomycin and chloramphenicol poorly. In fact, difference electron density for chloramphenicol poorly. In fact, difference electron density for chloramphenicol and clindamycin was not observed in the eubacterial binding site even when H. marismortui ribosomal subunits are soaked in millimolar concentrations of the compound (15). In addition, the mutation C2055A in eubacterial ribosomes increases the minimal inhibitory concentration (MIC) of chloramphenicol and clindamycin (31). Furthermore, the C2055A mutation in H. smithii ribosomes leads to resistance to pleuromutilin antibiotics, which also bind the eubacterial PTC (32).

The proposed role of 23S rRNA nucleotide 2055 as an antibiotic resistance spectrum determinant through its interaction with A-site clef nucleotides 2504 and 2506–2507 suggest that mutations of these residues should also appear in mutants resistant to chloramphenicol and lincomycin. Despite the sensitive location of these nucleotides in the PTC and their universal conservation, mutations of nucleotide 2504 confer resistance to chloramphenicol (31, 33) and increase the MIC of clindamycin (31). The lack of posttranscriptional modification (pseudouridylation) at this position renders bacteria hypersensitive to several peptidyl transferase inhibitors (34).

Mutation of G2058A in archaeal ribosomes leads to a ~10²-fold improvement in macrolide binding to H marismortui or H. halobium 50S subunits, suggesting that the identity of a single nucleotide could explain the insensitivity of eukaryal and eukaryotic ribosomes toward macrolides (6, 35). However, in S. cerevisiae ribosomes carrying the G2058A mutation in their ribosomal RNA were still not inhibited by erythromycin, suggesting additional phylogenetic differences contribute to resistance (36). Taken together with phylogenetic data, the present structural and biochemical data suggest that, in addition to position 2058, divergence of rRNA at positions 752, 2609, 2610, and 2055 also contribute to the species specificity of PTC and peptide elongation inhibitors. The availability of high-resolution structures of the ribosome and ribosomal subunits from archaea and divergent bacteria will now make it possible to probe these and other sequence determinants of PTC and peptide elongation inhibitors.

Experimental Procedures

Ribosome Purification, Crystallization, and Antibiotic Soaking Experiments. Ribosomes were purified from MRE600 E. coli cells as described previously (37). Ribosome crystals were grown as described previously (38). For antibiotic soaking experiments, the cryoprotection buffer was supplemented with 48 μM of telithromycin or 100 μM of either erythromycin, chloramphenicol or clindamycin. Erythromycin, chloramphenicol and clindamycin were obtained from Sigma. Telithromycin was a gift from Cempra Pharmaceuticals. The telithromycin stock solution in 50% acetic acid was diluted 800-fold in cryoprotection buffer for soaks. Erythromycin and chloramphenicol stock solutions were made in ethanol, and diluted 100-fold to working concentrations in cryoprotection buffer. Clindamycin was dissolved in DMSO and diluted 100x in cryoprotection buffer for soaking experiments. In all four cases incubation of the crystals with antibiotic took place overnight. Crystals were then flash frozen in liquid nitrogen.

X-ray Diffraction Experiments, Model Building, and Figure Preparation. X-ray diffraction data were measured at beamlines 8.3.1 and 12.3.1 of the Advanced Light Source, Lawrence Berkeley National Laboratory, using 0.1–0.3 °C oscillations at 100 K and recorded on an ADSC Q315 detector. X-ray reflections were reduced and scaled using HKL2000. Difference electron density (Fa-Fc) maps were calculated using the phenix.refine module of the PHENIX software suite (39). Antibiotics were positioned based on the unbiased difference electron density obtained in each experiment. If necessary, changes in the coordinates for RNA nucleotides dictated by the difference electron density were made using Coot (40) and phenix.refine was used for positional and atomic displacement parameter (ADP) refinement of the model. The crystals contain two copies of the ribosome in the asymmetric unit, with molecule 1 (PDB coordinates 3OAT, 3OFR, 3OFC, 3OFZ for the 50S subunit in complex with telithromycin, erythromycin, chloramphenicol, and clindamycin, respectively) exhibiting lower ADP values and clearer electron density than molecule 2. Whereas difference electron density for antibiotics was observed in both molecule 1 and molecule 2 of the crystal in each experiment, coordinates for antibiotics were only modeled into molecule 1. Ribosomal RNA sequence alignments were made using MUSCLE (41) or obtained from the Comparative RNA Website (28). Coordinate superpositions were performed in PyMOL using the Cα atoms of domain V of 23S rRNA, excluding regions where insertions or deletions occur. Figures were made using PyMOL (42).

Preparation of Ribosomes and Ribosomal Subunits for RNA Probing. Ribosomes and large ribosomal subunits were isolated from E. coli (strain J1M109) or S. aureus (strain RN4220) using standard protocols described in ref. 43. Large ribosomal subunits prepared from D. radiodurans were a gift of Francheschi and Skripkin (Rib-X, Inc). To increase affinity of macrolides for the archaeal large ribosomal subunits, G2058 in the 23S rRNA gene (E. coli numbering) of the single ribosomal operon of H. halobium was mutated to A. This mutation was introduced together with the selective anisomycin resistance mutation
C2452U using the procedure described in ref. 44. Haloarchaeal ribosomes were isolated as previously described (45).

Chemical Probing of the Interaction of Erythromycin and Telithromycin with Large Ribosomal Subunits. Chemical probing of interactions of erythromycin and telithromycin with bacterial and haloarchaeal large ribosomal subunits was carried out using procedures described in ref. 46 and in ref. 47 with ribosomes and drugs present at 200 nM and 50 μM, respectively. Ribosomal subunits, vacant or complexed with antibiotics, were modified with dimethylsulfate and the distribution of modified residues was analyzed by primer extension.

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