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Antibiotics at the Ribosomal Exit Tunnel – Selected Structural Aspects

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22.1
Introduction

Ribosomes, which are the target of many antibiotics [1, 2] possess spectacular architecture and inherent mobility, allowing their smooth performance in decoding the genetic information as well as in the formation of the peptide bond and the elongation of the newly synthesized proteins. The site for peptide bond formation (peptidyl transferase center, PTC, is located within a highly conserved pseudosymmetrical region [3, 4] that connects all of the remote ribosomal features involved in its functions, and seems to be a remnant of an ancient RNA machine for chemical bonding [5–10].The elaborate structure of this region and its dynamic properties place the aminoacylated and peptidyl tRNAs in the stereochemistry required for formation of peptide bonds, for substrate-mediated catalysis, and for substrate translocation, namely, for all activities enabling nascent chain elongation.

Adjacent to the PTC is the entrance to an elongated tunnel (Figure 22.1), a universal multifunctional feature of the ribosome, along which the nascent proteins progress until they emerge out of the ribosome. The existence of an internal ribosomal tunnel was proposed first in the 1960s, based on biochemical experiments indicating ribosomal masking of the last to be formed segments of the nascent chains [11, 12]. Nevertheless, at that time and during the following two decades it was widely assumed that growing nascent proteins ''travel'' on the ribosome's surface and are not degraded because they adopt compact helix conformations. In fact, doubts about the mere existence and the universality of the ribosomal tunnel were publicly expressed [13] and studies aimed at supporting this assumption were carried out [14] even after its initial visualization by three-dimensional image reconstructions at rather low resolution in eukaryotic and prokaryotic ribosomes, namely, 60 and 25 Å, respectively [15, 16]. This tunnel was rediscovered by cryo electron microscopy [17, 18] and finally verified when it was clearly observed in the first high-resolution crystal structures of the large ribosomal subunit [19, 20].

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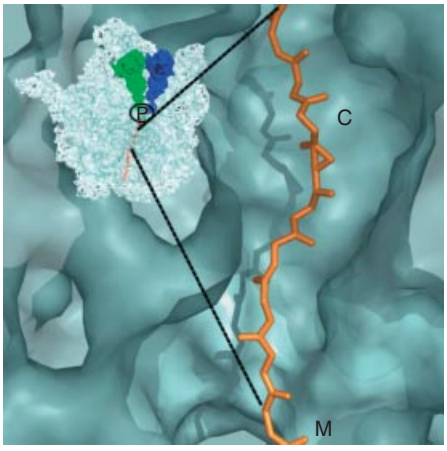

Figure 22.1 The universal nascent protein exit tunnel. Located in the large ribosomal subunit (top left) and extends from the site for peptide bond formation, PTC (P) to the other side of the subunit, the tunnel (highlighted by a modeled polyalanine chain) has a nonuniform shape (seen clearly in the zoomed region). This uneven shape contains a relatively wide crevice (C) alongside a narrow constriction, where members of the antibiotic family macrolides bind (M). A- and P-site tRNAs are shown in blue and green, respectively.

22.2 The Multifunctional Tunnel

Despite its considerable dimensions (about 100 Å in length and up to 25 Å width), uneven shape, and the existence of a wide crevice alongside narrow constrictions (Figure 22.1), tunnel involvement in the fate of the nascent chains was hard to conceive. Therefore, it was originally suggested to be a passive conduit, having a Teflon-like character with no chemical properties capable of facilitating its interactions with the progressively growing nascent chains [21]. However, further studies clearly indicated the significance of the tunnel and its intricate chemical nature and diverse functional roles, such as participation in nascent chain progression and its compaction are currently emerging. Evidence of nascent proteins/tunnel interactions have accumulated (for a review, see e.g., [22, 23]), some of which indicate extensive involvement in translation arrest and cellular signaling. It is conceivable that the interactions of the nascent chains with the tunnel alter the rate of translation elongation and, in extreme cases, lead to translation arrest. Thus, peptide monitoring and discriminating properties can be exploited for optimizing protein targeting and gene expression by small molecules such as tryptophan, arginine, and *S*-adenosyl-methionine [24–29].

The tunnel walls are lined predominantly by ribosomal RNA. The tips of ribosomal proteins L4, L22, and L23 are non-RNA tunnel wall components that are likely to control the tunnel gating and/or trafficking. While protein L23 resides

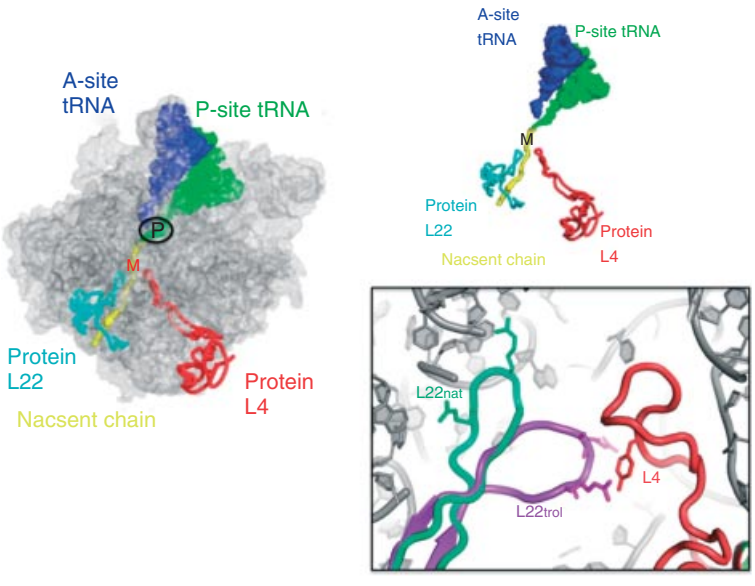

Figure 22.2 Proteins L22 and L4. Left: the positions of ribosomal proteins L4 and L22 in the large ribosomal subunit, shown above the "background," which is the entire large ribosomal subunit (D50S). M is the approximate position of the macrolide binding pocket. Note the proximity of it to the tips of L4 and L22, which, together with L22 elongated shape, allows its indirect involvement in antibiotic resistance as well as its direct participation in elongation arrest and transmission of cellular signals. Top right: same as in the left, but without the large ribosomal subunit. Bottom right: the possible interactions between L4 and L22 in its swung orientation [36]. Only the hairpin tips of the elongated proteins L4 and L22, which reside at the tunnel walls, are shown.

at the tunnel opening and in eubacteria possesses an extended internal loop that appears to have sufficient mobility for controlling the emergence of newly born proteins [30], the hairpin tips of the elongated proteins L4 and L22 reside in proximity to the constrictions of the tunnel wall (Figure 22.2) [20, 31–35] and are involved, mainly indirectly, in antibiotics binding and resistance as well as in nascent chain elongation arrest [36, 37].

Elongation arrest and its mutual impact on cellular processes have gained increased interest in recent years. The discovery of regulatory short nascent peptides that can promote stalling of the macrolide-bound ribosome stimulated studies on sequence-specific interactions of antibiotics with the nascent peptide [38–41] as well as on short peptides that expel macrolide antibiotics from the ribosome while being formed [42–46]; the disparity in the level of macrolides inhibition observed in these cell-free systems has also been investigated [47–50]. Furthermore, it was proposed that in some cases nascent proteins contain arrest segments that may assume specific folds within the tunnel, capable of preventing nascent protein progression along it. It is also conceivable that such semifolded segments could inhibit peptide bond formation or hinder tRNA translocation. Alternatively, arrest

can occur as a consequence of conformational alterations in the tunnel walls that are caused by semifolded segments of the nascent proteins [51, 52].

Strikingly, recent studies indicated that the nascent chains may act as cellular sensors while progressing through the tunnel for regulating membrane protein biogenesis [23, 53, 54]. Indications of possible active tunnel participation in initial nascent chain compaction, leading to semifolded chain segments were accumulated by electron microscopy and single molecule studies (e.g., [54–59]). In addition, indications of distinct conformations, including helical segments of the nascent polypeptide chains, were recently reported within several regions of the ribosomal exit tunnel that have been implicated in nascent chain–ribosome interaction (e.g., [60–63]). Furthermore, crystallographic analysis identified a crevice located at the tunnel wall, where cotranslational initial folding may occur [64]. The currently available observations imply direct interactions between specific residues of the nascent peptide with distinct locations in the ribosomal tunnel in prokaryotes and eukaryotes. These findings indicate that protein L22 appears to have dual functions: it acts as a cellular sensor as well as a progression barrier of the nascent peptide. The C- and N-termini of protein L22, located at the outer surface of the ribosomal particle within the vicinity of the tunnel opening (Figure 22.2), can sense cellular signals and transmit them into the ribosome through the tunnel so that the nascent protein exit tunnel together with intraribosomal regulation processes seem to be responsible for cell–ribosome signaling mechanisms and govern the fate of nascent proteins expression. Furthermore, as revealed in the crystal structure of the large ribosomal subunit in complex with the macrolide antibiotic troleandomycin (see subsequent text and in [36]), the tip of the L22 hair pin, similar to the consequences of troleandomycin binding, is capable of swinging across the tunnel, thereby hampering nascent protein progression, and thus can act as a tunnel gate. In support of this proposition is the finding that the arrest caused by the SecM arrest sequence is bypassed by mutations in the L22 hairpin tip region as well as in the 23S rRNA nucleotides [65] that were mapped to interact with L22 in its swung conformation [36].

22.3
A Binding Pocket within the Multifunctional Tunnel

Simultaneously with the emergence of the first high-resolution structures of the ribosome, the protein exit tunnel was shown to provide the binding pocket of the prominent antibiotics family, the macrolides [34]. Erythromycin, the "mother" of this clinically important antibiotic family [50], was introduced into clinical practice in 1952. It possesses strong bacteriostatic activity against a broad range of gram-positive and several gram-negative pathogens [66]. The location of the erythromycin binding pocket, as in the crystal structures of *Deinococcus radiodurans* 50S/erythromycin complex, can facilitate the obstruction of the tunnel and hamper the progression of the nascent proteins. This agrees with many biochemical experiments [67–69] that showed that erythromycin inhibits, to various extents,

the progression of nascent proteins through the exit tunnel. Indeed, the antibiotic binding to their pocket narrows the tunnel radically, and therefore should hinder the progression of the nascent peptide [34].

A major issue concerning the clinical usefulness of ribosomal antibiotics is their selectivity, namely, their capacity to discriminate between the ribosomes of the eubacterial pathogens and those of eukaryotes. Although prokaryotic and eukaryotic ribosomes differ in size (~2.4 and 4 MDa, respectively), their functional regions, which are the targets for the antibiotics, are highly conserved [70]. Therefore, the imperative distinction between eubacterial pathogens and mammals, the key to antibiotic usefulness, is achieved generally, albeit not exclusively, by subtle structural difference within the antibiotic binding pockets of the prokaryotic and eukaryotic ribosomes. A striking example of discrimination between pathogens and humans is the huge influence played by the minute difference at position 2058 of the rRNA, where the bacterial adenine is replaced by a guanine in eukaryotes. Indeed, this small difference was found to govern the binding of macrolides.

Investigations on the binding modes of the macrolides allowed the identification of the chemical parameters determining the mechanism of action of the various members of this family of antibiotics [31, 32, 34–36, 71–73]. Structural studies deciphered the parameters influencing and fine tuning antibiotic binding [73], revealed the inherent flexibility of tunnel wall components that facilitates remotely acquired antibiotics resistance (see preceding text and in [36]), and shed light on the passage of a distinct subset of polypeptides.

22.4
Remotely Acquired Resistance

Four decades ago resistance to erythromycin was detected in mutants of laboratory strains of *E. coli*, in which proteins L22 and L4 underwent minor modifications. These proteins are located in the vicinity of the macrolide binding pocket [34] and are involved in erythromycin resistance [74], in spite of not belonging to the pocket. Minute sequence alterations in the tip of the hairpin of protein L22 and/or in protein L4, in proximity to the swung L22 (Figure 22.2), were shown to confer erythromycin resistance, without preventing erythromycin binding [75, 76]. Analysis of the structures of an L22-resistant mutant showed that this mutation triggered significant displacements of the RNA components of the tunnel walls (Wekselman *et al.*, work in progress). These rearrangements seem to cause tunnel broadening, so that it can host erythromycin while allowing the progression of nascent polypeptide chain. Interestingly, the influence of L22 conformation on the shape of the tunnel wall was detected also by electron microscopy [77, 78]. Finally, it is interesting to note that remotely acquired resistance seems to be the mechanism for acquiring resistance to antibiotics targeting the PTC, including the pleuromutilins [79].

22.5
Resistance Warfare

Despite the initial, overwhelmingly positive clinical results obtained with erythromycin, this antibiotic was found to be rather sensitive to acidity and hence less suitable for treating stomach infections. These, and similar shortcomings, stimulated the design of semisynthetic antibiotics, such as clarithromycin, roxithromycin, and clindamycin. These also led to the design of new compounds meant to combat with antibiotic resistance that developed about a decade after the beginning of the clinical use of the antibiotic. Indeed, in several cases, enhanced chemical stability, higher inhibition activity (namely, lowering drug concentration), a wider coverage against various pathogens and binding to erythromycin-resistant strains were achieved by the modified macrolides, such as the second-generation azalides, such as, azithromycin [80] and the third-generation ketolides, such as telithromycin [81–83].

An interesting example is azithromycin, one of the world's best selling antibiotics that was designed in the 1980s by researchers at PLIVA, Croatia, with the aim of combating resistance. Its main ring is a 15-membered derivative of erythromycin, obtained by inserting a methyl-substituted nitrogen atom into the 14-membered macrolactone ring (Figure 22.3). Azithromycin is potent against several resistant

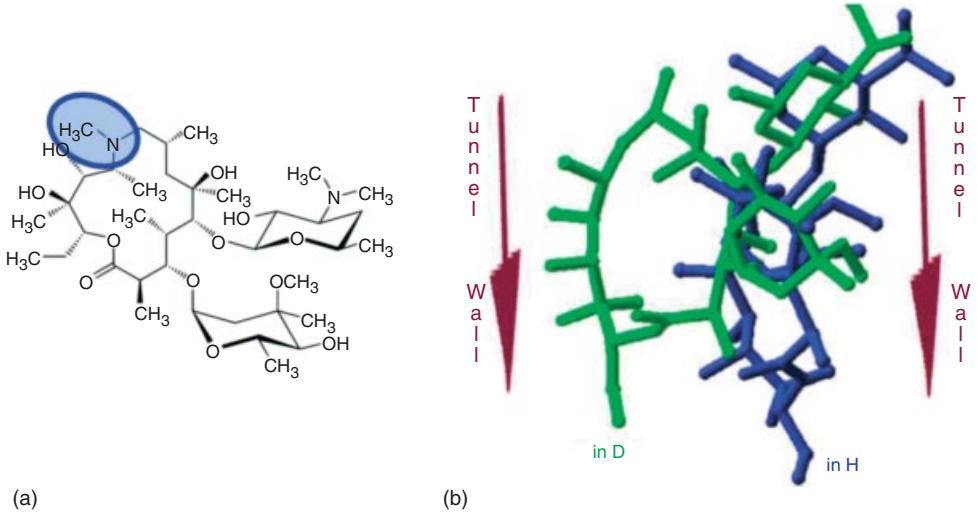

Figure 22.3 Azithromycin. (a) The chemical structure of the macrolide second-generation azithromycin that binds and inhibits erythromycin-resistant strains. This is a 15-membered macrolactone ring, derived from erythromycin by the insertion of a methyl-substituted nitrogen atom (in light blue) into the 14-membered macrolactone ring of erythromycin. (b) The modes of azithromycin binding: across the tunnel to D50S (in green) [72] and along the tunnel in H50S (in blue) in which 2058 is guanine, as in eukaryotes, showing the difference between azithromycin binding to pathogens (D50S) and patient (H50S) models and indicating the consequence in therapeutic effectiveness.

strains, including those with the potentially hazardous A to G mutation at position 2058, as this substitution may result in binding to eukaryotic ribosomes that carry G at this position. However, comparison between the azithromycin binding mode to ribosomes that can serve as pathogen models, namely, of *D. radiodurans* [72] with the binding to a eukaryotic model, namely, the large ribosomal subunit from the *Haloarcula marismortui*, H50S [33] showed clearly that mere binding of an antibiotic compound to the ribosome is not sufficient for obtaining efficient therapeutical effectiveness and indicated that other structural elements of the binding pocket are important for inhibitory activity. Similar observations were made by mutagenesis in the yeast *Saccharomyces cerevisiae* at a position equivalent to *Escherichia coli* A2058, which allows erythromycin binding but does not confer erythromycin susceptibility [76].

22.6 Synergism

Additional attempts aimed at controlling resistance include the development of synergetic antibiotics. An example is the very potent antibiotic called *Synercid*®, a combination of the two streptogramins, dalfopristin and quinupristin, each of which is a rather weak drug, but together they block the PTC as well as most of the tunnel while preventing each other from leaving their binding pockets (Figure 22.4). The impressive synergetic effect of this family can be understood

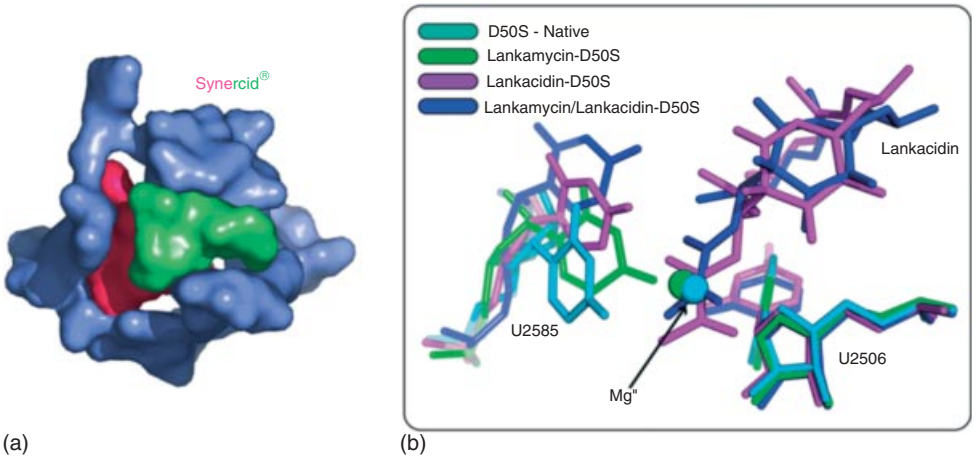

Figure 22.4 Synergism. (a) The two Synercid® components bound to the PTC and tunnel entrance and block them as well as preventing each other from leaving their binding pockets [86]. The tunnel wall is shown in blue. (b) The binding mode of the two components of the lankacidin–lankamycin to D50S [85] and the alterations in the orientation of the very flexible nucleotide U2585 that occur upon binding of the components of this pair.

by examining the mechanism of action of this antibiotic. The two components of the synergetic pair of Synercid bind to the PTC and to the tunnel entrance and displace A2062 and U2585 (Figure 22.4) [84, 85]. Thus, the inhibition is based not only on blocking the tunnel and the entrance to it but also on a dramatic alteration in the orientations of two highly flexible nucleotides, A2062 located at the entrance of the tunnel and U2585, a principal participant in peptide bond formation [3, 84–86].

A similar pair, produced by *Streptomyces rochei*, composed of lankacidin and lankamycin, is expected to be a potential synergistic drug although currently, this pair shows only a modest inhibitory effect on cell growth as well as on cell-free translation. Remarkably, lankamycin binds readily to preformed lankacidin-bound large ribosomal subunits, whereas erythromycin, which has a very similar structure (Figure 22.5 and on the book's cover), disrupts lankacidin binding. The molecular basis for this unexpected difference has been identified [85] and it is likely that it can be exploited for increasing the inhibitory effect of this pair.

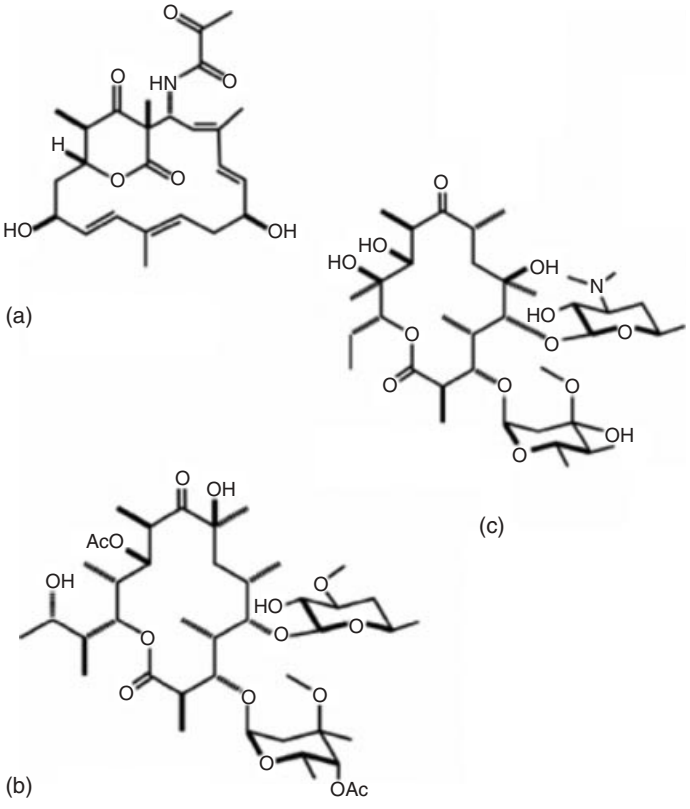

Figure 22.5 The chemical formula of (a) lankacidin C, (b) lankamycin, and (c) erythromycin.

22.7
Pathogen and "Patients" Models

High-resolution structures have provided many clues pertinent to antibiotic drug development. As most eubacteria utilize similar structural principles for antibiotic selectivity and resistance, it is expected that the factors allowing for selectivity should provide powerful tools to understand many of the mechanisms exploited for acquiring resistance. Indeed, the lessons learned from ribosome crystallography concerning combating resistance to antibiotics targeting the ribosome have led to new ideas for antibiotic improvement. However, it should not be forgotten that all of these insights are based on structures of ribosomes from eubacteria that were found to mimic pathogens under clinical-like conditions (e.g., *D. radiodurans, E. coli*, and *Thermus thermophilus*), as so far no ribosomes from genuine pathogens have been crystallized. Consequently, the current observations provided useful clues about common traits, such as modes of actions, details of binding interactions, rationalizations of resistance mechanisms, and the bases for synergism.

Although the currently available structural information is valuable, it seems to be still insufficient for the acute medical challenges. This is because (i) significant variability was detected between binding modes of drugs of the same family (e.g., [36, 71, 87, 88]); (ii) binding pockets contain species specific unique chemical properties that seem to confer resistance; and (iii) in several cases remote interactions are responsible for certain induced fit binding abilities. These enable species discrimination [79], which does not exist within the highly conserved antibiotics binding pockets, and may vary between pathogenic and nonpathogenic bacteria. Combined with the identification of deleterious mutations in rRNA, there is considerable justification to explore ribosomes from the actual pathogenic strains.

The large ribosomal subunit from *H. marismortui* (H50S) can be considered among the currently known high-resolution eubacterial structures that represent suitable models of pathogenic bacteria. Furthermore, in light of the properties that this archaea shares with eukaryotes, in some instances its ribosomes may be considered as a suitable model for patients.

Another example of different binding modes, similar to that of azithromycin binding (Figure 22.3), is observed in crystals of ribosomal complexes with the ketolide telithromycin (Figure 22.6). Thus, even when the nucleotide at the discrimination position for macrolides and ketolides was modified from G2058 (in native H50S) to A2058, as in eubacteria, significant differences were observed in the modes of telithromycin binding to these compared to D50S and to *T. thermophilus* and *E. coli* ribosomes. Importantly, all of the differences in the modes of binding could be rationalized structurally by stacking interactions with tunnel wall components situated in slightly different positions in the various structures (Figure 22.6); this highlights the significant species specificity existing in antibiotic susceptibility and sheds light on the clinical diversity between different pathogens. Importantly, although all macrolides bind to the same binding pocket in a similar manner, some differences in the exact binding modes, which can be explained

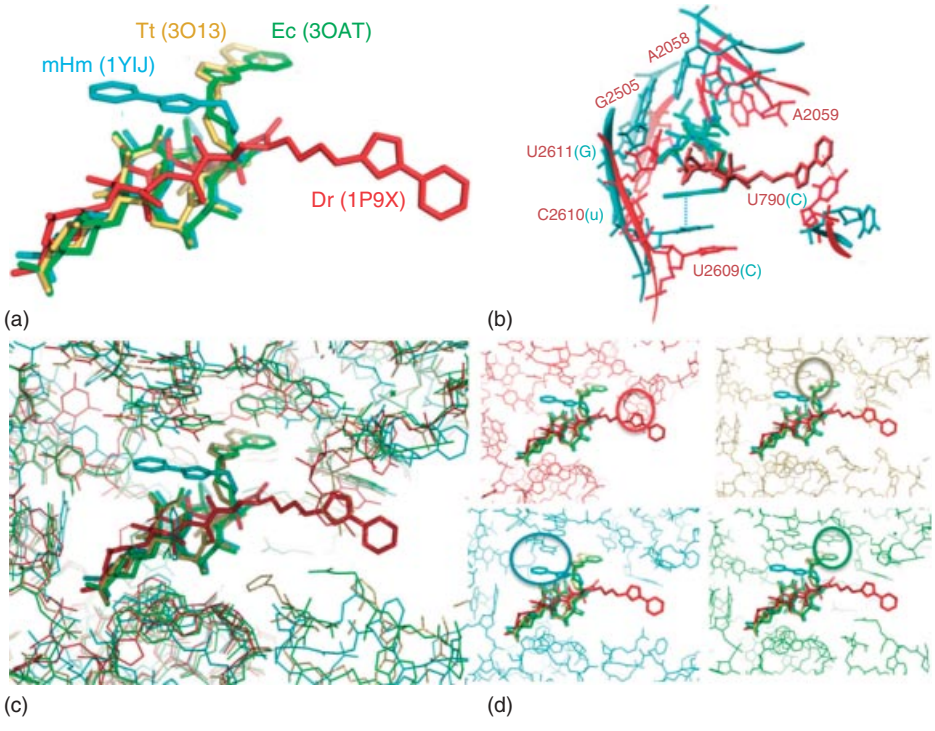

Figure 22.6 Species specificity revealed by the binding modes of the ketolide telithromycin, as observed in crystals of its complexes with various ribosomal particles. In all: color code for the orientations of telithromycin as well as the binding pockets: red: D50S (also called Dr), beige: T70S (also called Tt), green: E70S (also called Ec), cyan: mH50S (also called mHm, namely, a mutant of H50S in which G2058 was replaced by A2058 in order to enable telithromycin binding). (a) All four telithromycin orientations superposed on each other. Note that the macrolactone ring occupies the same position, whereas the long aliphatic arm is extremely flexible, and stretches to different directions, dictated by stacking interactions with the pocket's components. (b) Showing the orientations in the two extreme situations: in D50S and in mH50S, together with the various components of the binding pockets and indicating the stacking by broken lines. (c) All structures within their pockets. (d) The four panels show each of the binding modes within its pocket, with the circle indicating the stacking interactions.

chemically, were identified not only between the various members of this family but also between two erythromycin/D50S complexes [34, 88].

It should not be forgotten that the crystallographic information has shed light on mechanisms for antibiotic function and resistance, although the crystal structures were obtained under conditions barely mimicking the relevant pathogen–host relationships. Thus, *T. thermophilus* grows normally at temperatures that cause disintegration of the antibiotics (namely, $>75\,^{\circ}C$); the entire ribosome from *E. coli* was crystallized without mRNA and tRNA substrates, thus representing an artificial functional state: the archaeon *H. marismortui* grows at elevated temperatures

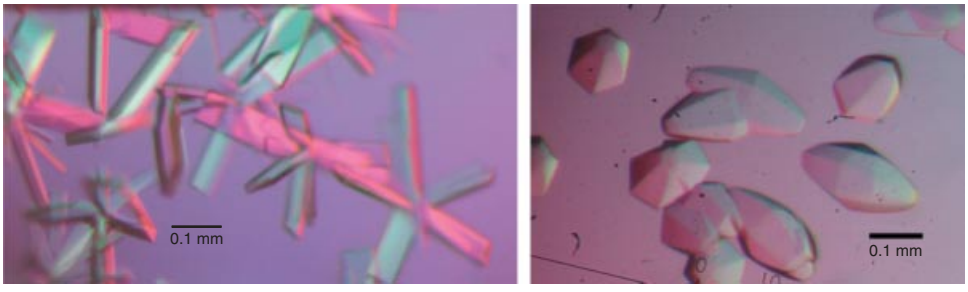

Figure 22.7 Crystals of ribosomes form pathogens. (a) Crystals of the large ribosomal subunits from *Staphylococcus aureus*. (b) Crystals of the small ribosomal subunits from *Mycobacterium smegmatis*, the diagnostic pathogen model for *Mycobacterium tuberculosis*.

in ~3 M KCl, conditions that obviously cannot exist within human or animal cells, and contains features representing eukaryotes and eubacteria, and *D. radiodurans* grows significantly slower than typical bacteria.

In light of this, it is clear that structural information obtained from ribosomes of genuine pathogens should reveal crucial parameters that can be useful for combating resistance. Attempts in this direction are currently under way (Figure 22.7).

22.8 Conclusion and Future Considerations

The rapid increase in antibiotic resistance among pathogenic bacterial strains poses a significant health threat. Hence, improvement of existing antibiotics and the design of advanced drugs are urgently needed. Attempts to overcome antibiotic resistance and increase their selectivity are currently going on, exploiting several strategies including the insertion of moieties that should compensate for the lost interactions of the resistant strains, designing and/or improving natural synergetic pairs, creation of novel compounds possessing inhibitory properties of various levels of potency, and reviving ''forgotten'' antibiotics families.

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University, SRS, Daresbury, SSRL/Stanford University, ESRF/EMBL, Grenoble, BL26/PF/KEK, Japan, and 19ID&23ID/APS/Argonne National Laboratory.

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