23S rRNA base pair 2057–2611 determines ketolide susceptibility and fitness cost of the macrolide resistance mutation 2058A→G

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The 23S rRNA A2058G alteration mediates macrolide, lincosamide, and streptogramin B resistance in the bacterial domain and determines the selectivity of macrolide antibiotics for eubacterial ribosomes, as opposed to eukaryotic ribosomes. However, this mutation is associated with a disparate resistance phenotype: It confers high-level resistance to ketolides in mycobacteria but only marginally affects ketolide susceptibility in streptococci. We used site-directed mutagenesis of nucleotides within domain V of 23S rRNA to study the molecular basis for this disparity. We show that mutational alteration of the polymorphic 2057–2611 base pair from A-U to G-C in isogenic mutants of *Mycobacterium smegmatis* significantly affects susceptibility to ketolides but does not influence susceptibility to other macrolide antibiotics. In addition, we provide evidence that the 2057–2611 polymorphism determines the fitness cost of the 23S rRNA A2058G resistance mutation. Supported by structural analysis, our results indicate that polymorphic nucleotides mediate the disparate phenotype of genotypically identical resistance mutations and provide an explanation for the large species differences in the epidemiology of defined drug resistance mutations.

antibiotics | ketolides | ribosomes | structure | conformation

Many groups of clinically useful antibiotics prevent the synthesis of new proteins by interacting with the bacterial ribosome (1). A prominent region targeted by ribosomal drugs is located around the peptide bond formation site (the peptidyl transferase center) and the entrance of the nascent protein exit tunnel on the large (50S) ribosomal subunit. The upper region of the tunnel provides the binding site for macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics (2–6). The primary inhibitory effect of the lincosamide drugs is to inhibit the formation of peptide bonds; the main inhibitory effect of macrolides is to block the passage of the newly synthesized peptide chain through the exit tunnel (2).

Macrolides have a common core structure formed by a lactone ring of different sizes (7). Ketolides are the latest derivatives developed from the macrolide erythromycin to improve antimicrobial activity. They are equipped with 3-keto and 6-methoxy groups that improve acid stability and allow drug binding without evoking resistance mediated by inducible *erm* genes (reviewed in ref. 8). Additional features are a cyclic carbamate at C11/C12 and an extended arm that provides additional interactions with domain II of 23S rRNA (4, 5, 9–11): an alkyl-aryl extension bound to the cyclic carbamate in the case of telithromycin and a quinolylallyl group tethered to the O-6 position in the case of ABT-773.

Resistance to macrolides occurs predominantly by modification of the drug-binding site and/or by drug efflux. Target modification may be the result of two different mechanisms: (*i*) modification in trans conferred by *erm* genes, which methylate the adenine of 23S rRNA position 2058 and (*ii*) modification in cis, including mutational alterations of 23S rRNA and/or ribosomal proteins L4/L22 (reviewed in refs. 12 and 13). Mutations in 23S rRNA conferring macrolide resistance have been described for clinical strains of *Mycobacterium intracellulare* (14), *Mycobacterium avium* (15, 16), *Mycobacterium kansasii* (17), *Mycobacterium chelonae*, *Mycobacterium abscessus* (18), *Helicobacter pylori* (19–21), *Brachyspira* (*Serpulina*) *hyodysenteriae* (22), *Propionibacterium* spp. (23), and *Streptococcus pneumoniae* (24). These mutations have been mapped to the macrolide-binding pocket in the ribosomal tunnel, specifically 23S rRNA nucleotides 2057, 2058, and 2059 in the *Escherichia coli* numbering system.

Nucleotides 2058 and 2059 act as key contact sites for macrolide binding (2, 25). Site-directed mutagenesis has been used to analyze in detail the role of 23S rRNA nucleotides 2058 and 2059 in drug-target interaction; these studies also alluded to the importance of distinct conformations of the lactone ring (26). Nucleotide 2057 is involved in base-pairing interaction with nucleotide 2611; disruption of the 2057–2611 base pair was found to confer resistance to macrolide antibiotics (27). It was thus assumed that alteration of residue 2057 should lead to a disruption of the rRNA structure at the end of the stem preceding the single-stranded portion of the peptidyl transferase region containing A2058 and A2059, resulting in conformational alterations (see Fig. 1). Consequently, the general consensus was that it is not nucleotide specificity but proper base-pairing that determines the role of 2057–2611 in macrolide binding (27).

Inspection of mutational macrolide resistance reveals an interesting paradox with respect to the phenotype conferred by the 2058A→G alteration. The 2058A→G alteration unanimously mediates high-level resistance to 14- and 15-membered macrolides in bacteria (13). Yet, the same mutational alteration results in significant resistance to the ketolide telithromycin in *Mycobacterium smegmatis* (26), but only little affects telithromycin susceptibility in *S. pneumoniae* (24, 28–30).

Resistance to antibiotics frequently reduces the fitness of bacteria in the absence of antibiotics, and chromosomal drug resistance-conferring mutations may or may not carry a biological cost (31, 32). Evidence has been provided that the biological cost of a genotypically identical resistance mutation may be different in different microorganisms, although the molecular basis for this finding is unclear. Thus, the A2058G alteration is a no-cost resistance mutation in mycobacteria (33) but carries a substantial cost in *H. pylori* (34). Because the fitness cost of a resistance determinant is the primary parameter that determines its frequency (35), species-specific differences in the biological cost associated with a defined resistance determinant are likely to influence its epidemiology. The most frequent mutational alteration in clinical strains of macrolide-resistant mycobacteria seems to affect both A2058 and A2059 (16,

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Abbreviations: MLS_B, macrolide, lincosamide, and streptogramin B; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration.

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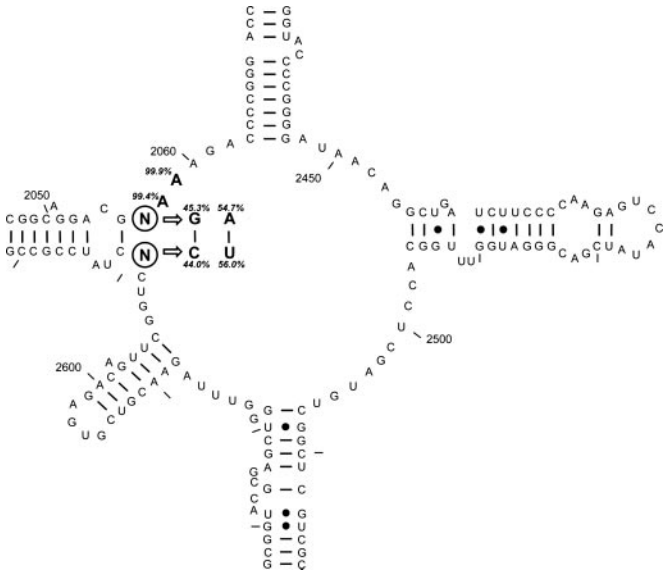

Fig. 1. Secondary structure of domain V of 23S rRNA. The rRNA sequence from *M. smegmatis* is used as a reference sequence. For selected nucleotides, the sequence polymorphism is indicated; numbering is according to *E. coli*.

18). In contrast, the 2059A→G alteration is by far the most frequent 23S rRNA macrolide resistance mutation found in clinical isolates of *S. pneumoniae* (24).

Here, we used site-directed mutagenesis in rRNA to study the basis for these puzzling findings. Using a strategy that results in homogenous populations of mutant ribosomes, we provide evidence that the polymorphic 2057–2611 base pair in 23S rRNA determines both ketolide susceptibility and fitness cost of the 23S rRNA 2058A→G alteration.

Materials and Methods

Bacterial Strains and Mutagenesis. Initial cloning and propagation of plasmids were done in *E. coli* strain XL-I blue (Stratagene). Cultures of *E. coli* were grown in Luria–Bertani (LB) medium containing either ampicilium (120 μg/ml) or hygromycin B (100 μg/ml). Strains of *M. smegmatis* were cultured in LB medium containing Tween 80 (0.05%) on LB-agar plates and on plates supplemented with the appropriate antibiotic for selection of transformants.

Selective plating was used to isolate spontaneous macrolide-resistant mutants, followed by PCR-mediated cycle sequence determination of domain V of the 23S rRNA gene to identify the resistance mutation. For generation of recombinant *M. smegmatis* mutants, partial 23S rRNA gene fragments were generated by PCR-mediated mutagenesis as described in ref. 26 and introduced into plasmid pMV361ΔKan-Gm (36); all primers and plasmids are listed in the supporting information, which is published on the PNAS web site. After transformation of the plasmids into *M. smegmatis* mc^{2}155 SMR5Δ*rrnB* (36), RecA-mediated gene conversion was used to transfer the mutation into the chromosomal rRNA gene (37). Drug-resistant mutants were colony-purified and subjected to 23S rRNA sequence determination by *Taq* cycle sequencing using fluorescent-labeled nucleotides (Applied Biosystems). See the supporting information for a list of mutants, oligonucleotides, and plasmids that were used.

Growth experiments were done in a microtiter plate in a total volume of 150 μl. A preculture was grown to an OD_{600} of ≈1.0 and then diluted to inoculate the plates with an OD_{600} of 0.025. The measurements were done in a PowerWave X5 ELISA plate reader (Bio-Tek, Burlington, VT). For 48 h, the OD_{600} was measured every 10 min.

Determinations of minimal inhibitory concentrations (MICs) were performed in a microtiter plate format as described in ref. 36. In brief, freshly grown cultures were diluted to an absorbance (A_{600}) of 0.025 in Luria–Bertani medium and incubated in the presence of 2-fold serial dilutions of the following drugs: azithromycin (Pfizer), clarithromycin (Abbott), telithromycin (Aventis Pharma SA, Antony, France), and erythromycin, tylosin, josamycin, and spiramycin (all from Sigma). Stock solutions (20–50 mg/ml) were made of tylosin and clarithromycin (lactobionate salt) in water, telithromycin in DMSO, and all other drugs in ethanol. The MIC is defined as the drug concentration at which the growth of the cultures was completely inhibited after an incubation time of 72 h, corresponding to 24 generations. For induction of *erm* (38), cultures of *M. smegmatis* were grown to saturation and incubated in the presence of subinhibitory concentrations of the indicated drug (0.15 μg/ml clarithromycin or 0.08 μg/ml telithromycin) for 20 h before processing for MIC testing.

Determination of minimal bactericidal concentrations (MBCs) was performed as follows. Starting from MIC assays, we sampled aliquots from those wells that showed growth inhibition and plated them on drug-free solid agar for a further 72 h. The MBC is defined as killing 99.9% of the original inoculum used for the growth-inhibition studies. Starting with a freshly grown culture of 5×10^5 cells per ml for inoculation [corresponding to an absorbance (A_{600}) of 0.025], the MBC corresponds to the drug concentration that results in $<5 \times 10^2$ viable bacterial cells per ml.

Structural Interpretations. Structural analysis was performed based on the crystal structures of the large ribosomal subunit from *Deinococcus radiodurans* and its complexes with antibiotics using the Protein Data Bank ID codes 1NKW, 1JZX, 1PGX, and 1NWX. Figures were prepared by using PYMOL (http://pymol.sourceforge.net).

Results

One of the two *rrn* operons in *M. smegmatis* has been inactivated to produce a strain with a single functional rRNA operon. This strain, *M. smegmatis rrn*$^-$, is the only Gram-positive bacterium for which procedures for genetic manipulation of rRNA are well defined and yield a homogeneous population of mutant ribosomes. Using this strain, single, dual, and triple substitutions

Table 1. Determination of MICs in μg/ml for A2058G mutants of *M. smegmatis* and induction of *erm* (38)

Strain	Telithromycin	Clarithromycin	Tylosin
M. smegmatis WT	0.25–0.5	0.5	8
M. smegmatis WT (pretreatment with clarithromycin)	>32	>512	n.d.
M. smegmatis WT (pretreatment with telithromycin)	16	128	n.d.
M. smegmatis A2058G	64–128	>512	32
M. smegmatis A2058G (pretreatment with clarithromycin)	64–128	>512	n.d.
M. smegmatis A2058G (pretreatment with telithromycin)	64–128	>512	n.d.

n.d., not done.

were created to result in mutants *M. smegmatis* A2058G, *M. smegmatis* A2058G/A2057G, and *M. smegmatis* A2058G/A2057G/U2611C.

MICs for WT and mutant 2058G *M. smegmatis* strains were determined for the 14-membered macrolide clarithromycin, the ketolide telithromycin, and the 16-membered macrolide tylosin. The 2058G alteration conferred little resistance to tylosin (relative resistance = 4) but led to high-level resistance toward clarithromycin (relative resistance > 1,000) and telithromycin (relative resistance = 256); see Table 1.

To study a possible involvement of the inducible *erm* (38) methylase in susceptibility to telithromycin, the following experiments were performed. (*i*) Cells were pretreated with subinhibitory concentrations of clarithromycin to induce the methylase (38) and (*ii*) cells were pretreated with subinhibitory concentrations of telithromycin to test for *erm* inducibility by telithromycin. Pretreatment of WT cells with subinhibitory concentrations of clarithromycin or telithromycin resulted in significant drug resistance, indicating induction of the *erm* methylase under these conditions. Induction of *erm* did not affect resistance to telithromycin in A2058G mutant ribosomes (see Table 1).

Next we investigated the drug susceptibility of A2058G, A2058G/A2057G, and A2058G/A2057G/U2611C *M. smegmatis* mutants to a range of macrolides, including the 14-membered macrolides erythromycin and clarithromycin; the 15-membered macrolide azithromycin; the 16-membered macrolides tylosin, spiramycin, and josamycin; and the ketolide telithromycin. (For drug structures see Fig. 2.) In addition to minimal inhibitory concentrations, we determined MBCs (see Table 2). Both MICs and MBCs were affected by the A2058G alteration, resulting in virtual loss of ribosomal susceptibility to ketolides as well as to 14- and 15-membered macrolides. As observed previously, the A2058G mutants retain considerable susceptibility to 16-membered macrolides (26). Drug susceptibility of the double mutant A2058G/A2057G was not significantly different from that of the A2058G mutant, although a slight increase in ribosomal resistance was noted. Thus, within the background of an A2058G alteration, disruption of proper Watson–Crick 2057–2611 base-pairing does not greatly influence drug binding.

Introduction of the U2611C alteration resulted in the triple mutant A2058G/A2057G/U2611C. However, the expected resto-

Table 2. Determination of MICs and MBCs in μg/ml in *M. smegmatis* mutants

Drug	2058A 2057A 2611U	2058A→G 2057A 2611U	2058A→G 2057A→G 2611U	2058A→G 2057A→G 2611U→C
Telithromycin				
MIC	0.25–0.5	64–128	128–256	4–8
MBC	8	>512	>512	64–128
Erythromycin				
MIC	16	>512	>512	>512
MBC	256	>512	n.d.	>512
Clarithromycin				
MIC	0.5	>512	>512	>512
MBC	8	>512	n.d.	>512
Azithromycin				
MIC	8	>512	>512	>512
MBC	32	>512	n.d.	>512
Tylosin				
MIC	8	32	32–64	32
MBC	64	256	n.d.	256
Spiramycin				
MIC	16	128–256	256	128–256
MBC	256	512	n.d.	512
Josamycin				
MIC	2	16	32	16
MBC	32	64	n.d.	64

n.d., not done.

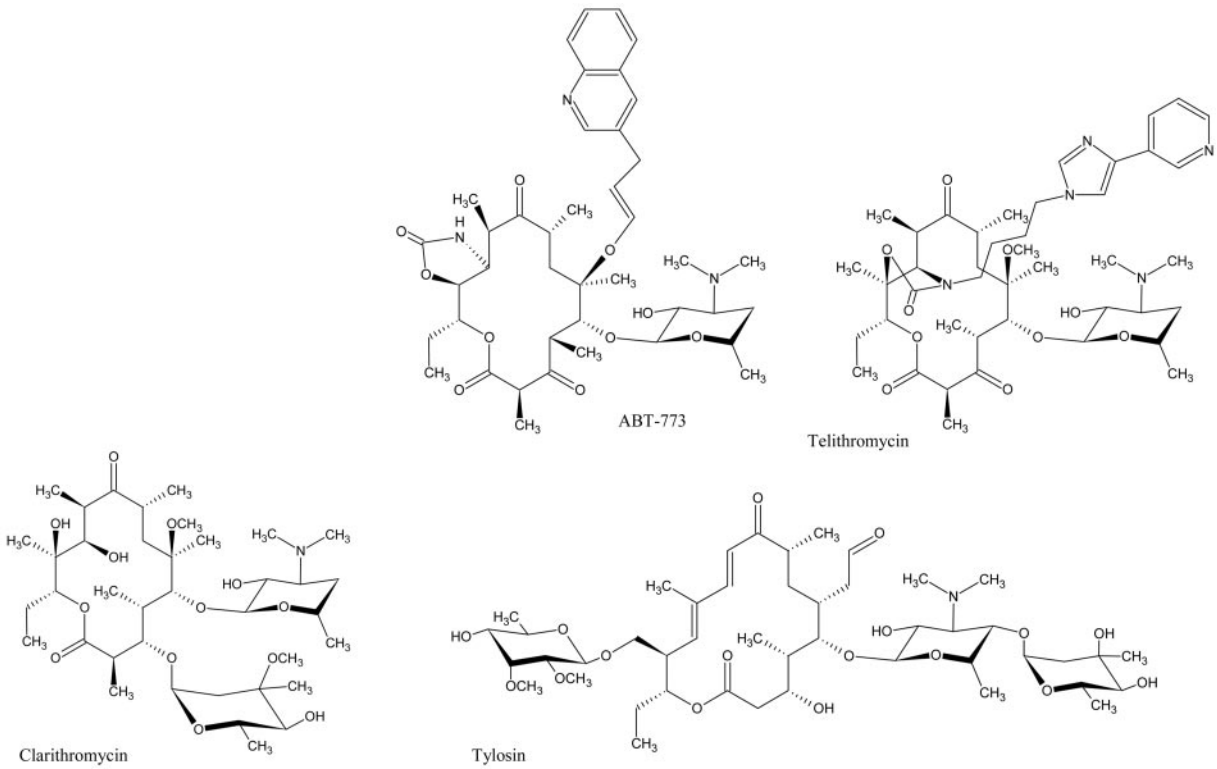

Fig. 2. Chemical structures of the studied macrolides and ketolides.

Table 3. Relative resistance to telithromycin in different microorganisms carrying the A2058G alteration

Strain	Genotype	Relative resistance*
S. pneumoniae†	G2058/G2057/C2611	12–16
E. coli‡	G2058/G2057/C2611	20–40
M. smegmatis	G2058/G2057/C2611	16–32
M. smegmatis	G2058/A2057/U2611	>256

*Defined as ratio of MIC of mutant cells to MIC of WT cells.
†Data from refs. 23, 28, and 29.
‡Data from ref. 42.

ration of proper base-pairing between 2057 and 2611 by a G-C base pair resulted in a disparate resistance phenotype. Ribosomal susceptibility to 14-, 15-, and 16-membered macrolides was identical to that of the A2058G mutant with the A2057–U2611 base pair. However, introduction of the A2057G–U2611C alteration restored susceptibility to the ketolide telithromycin. Compared with the 2058G mutant, the MIC of the triple A2058G/A2057G/U2611C mutant decreased by 16-fold. (Induction of *erm* methylase by pretreatment with clarithromycin did not measurably affect telithromycin susceptibility; data not shown.) As shown in Table 3, this level of telithromycin susceptibility in the triple mutant is comparable to the low level of telithromycin resistance observed in A2058G mutants of *S. pneumoniae* and *E. coli*, which both naturally carry a G0457–C2611 base pair (see Fig. 1). From these data, we conclude that the natural sequence polymorphism at 23S rRNA positions 2057–2611 determines telithromycin susceptibility of ribosomes carrying the MLS_B A2058G resistance mutation.

We next determined the effect of the 2057–2611 base pair on the biological cost of the A2058G resistance mutation. As described in ref. 39, a 2058G alteration does not affect cell growth to any measurable degree in *M. smegmatis*. Introduction of the A2057G–U2611C alteration into the A2058G mutant, however, resulted in a significant growth disadvantage (see Table 4).

Discussion

Substitution of 23S rRNA position A2058 has been reported to confer varied phenotypes in different bacterial groups, with respect to both drug resistance and biological cost (13). Here, we assessed the importance of key nucleotides in domain V of 23S rRNA for ketolide and macrolide binding. Independent of the type of Watson–Crick base pair at 23S rRNA positions 2057–2611, an A-to-G substitution at nucleotide 2058 conferred high-level resistance to 14- and 15-membered macrolides and little resistance to 16-membered macrolides. In contrast, ribosomal susceptibility to ketolides in A2058G mutants depended largely on the natural sequence polymorphism of the 2057–2611 base pair (i.e., the presence of G-C vs. A-U). Although ketolides are thought to bind to the ribosome without tripping the inducible *erm* resistance (8) and *erm* methylases specifically methylate adenines but not guanines (40), we experimentally ruled out the possibility that *erm* (38) contributes to telithromycin susceptibility in the 2058G mutants. Surprisingly, we noted that *erm* (38) is induced by telithromycin.

E. coli is not an ideal model to study drug-target interactions for macrolide antibiotics for a number of reasons. (*i*) Because of the multiplicity of *rrn* operons, mutagenized rRNA genes are frequently expressed from plasmid-encoded *rrn* operons, resulting in a merodiploid strain with a heterogeneous population of ribosomes. Although this concern has been removed by more refined genetic techniques that resulted in inactivation of all of the chromosomal *rrn* operons and ensured that rRNA is produced only from the plasmid-encoded rRNA operons (41), the resulting *E. coli* strain TA531 shows severe growth defects [e.g., a doubling of the generation time (42)]. (*ii*) The Gram-negative outer membrane acts as a barrier to most MLS_B drugs, requiring the use of the permeant *E. coli* derivative A519 (43). The changes in the *E. coli* strains do have their biological costs in terms of cell growth rates, and it has not been possible to combine the virtues of strains TA531 and A519 into a single strain (42). The experimental system chosen in this study is not compromised by any of the above disadvantages. Thus, (*i*) the single rRNA allelic *M. smegmatis* rrn^- shows the same generation time as the parental WT organism (44) and (*ii*) as a Gram-positive microorganism, *M. smegmatis* has no outer membrane and is permeable to macrolide antibiotics.

To explain species-specific differences in the frequency of defined macrolide resistance mutations, it has been hypothesized that compared with *S. pneumoniae*, other microorganisms may be better equipped to accommodate the 2058G resistance mutation by means of a compensatory mutation at another site that alleviates the biological cost of the A2058G mutation (24). Rather than invoking secondary site compensatory mutations (32), our data indicate that the natural sequence polymorphism within the macrolide-binding pocket affects the fitness cost of a drug resistance mutation. Although introduction of the A2058G resistance mutation in WT *M. smegmatis* with a A2057–U2611 base pair did not affect the growth rate, the subsequent alteration of the 2057–2611 base pair to G-C conferred a significant growth disadvantage to the A2058G-resistant mutant. Although it is difficult to extrapolate these findings to other species, these results provide a reasonable explanation of why the 2058G alteration is underrepresented in clinical strains of macrolide-resistant *S. pneumoniae* with resistance due to 23S rRNA gene mutations. This interpretation of the role of the 2057–2611 base pair in determining the biological cost of the

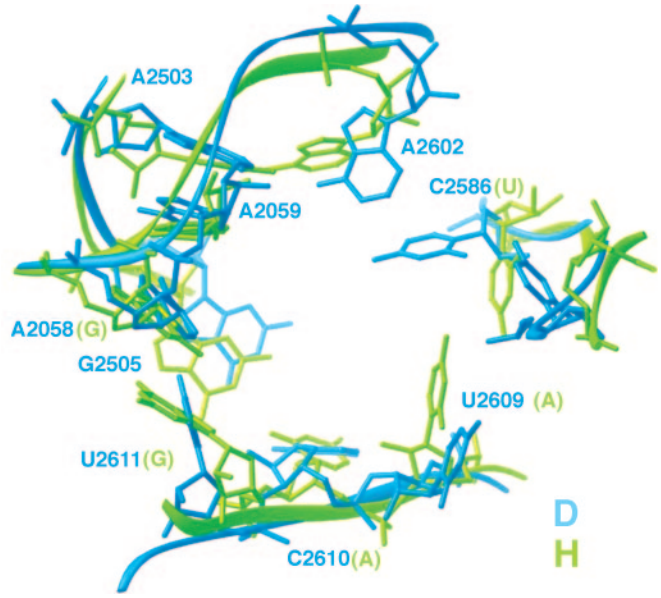

Fig. 3. The macrolide-binding pocket in *Deinococcus* 50S (cyan) and *Haloarcola* 50S (green). The differences in nucleotide type and orientation are highlighted. (Green letters in parentheses refer to the type of the nucleotide in *Haloarcola*, if different from that of *Deinococcus*.) Note the significant variability of 2611.

Table 4. Growth of strains

Strain	Generation time, h
M. smegmatis	3.1 ± 0.1
M. smegmatis 2058G	3.1 ± 0.3
M. smegmatis 2058G/2057G/2611C	4.1 ± 0.2

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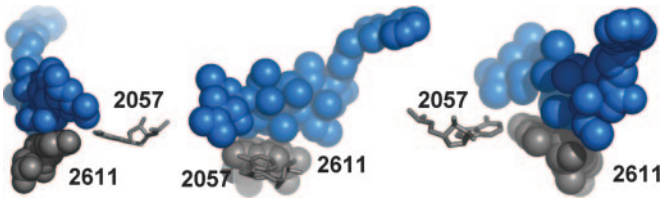

Fig. 4. Three different views obtained by two successive rotations of ≈90° each showing the stacking interactions between telithromycin (space filling in blue) and the base of U2611 (space filling in gray). In all views, A2057 is also shown (in gray atoms) to illuminate the relative position of the two participants in the 2611–2057 base pair.

A2058G alteration is supported by two further findings: (*i*) the resistance mutation has a high fitness cost in *H. pylori*, which carries a G2057–C2611 base pair (34) and (*ii*) in stationary phase cultures of *E. coli* that are maintained in the absence of the drug, A2058G mutant ribosomes are distinctly less stable than WT ribosomes (45).

The macrolide-binding pocket (Fig. 3) is located at the upper part of the ribosomal peptide exit tunnel, at a distance from the peptidyl transferase center that allows the accommodation of polypeptides of ≈5–6 residues. It is formed mainly by 23S rRNA domain V nucleotides, among which A2058 and A2059 play a prominent role in binding, selectivity, and resistance. Both 16-membered macrolides and ketolides make additional contacts with domain II of 23S rRNA, albeit with different substituents (4, 5, 46). The adenine bases at positions 2058 and 2059 are highly conserved in bacteria, whereas the bases at position 2057 and 2611 exhibit a significant degree of polymorphism: A-U vs. G-C. The residue 2057 pairs with 2611 in the secondary diagram of 23S rRNA (47) as well as in the three-dimensional structure of large ribosomal subunits (48, 49).

Previous mutational data indicated that proper base-pairing between 2057–2611 may be more important for binding of 14-membered macrolides than for 16-membered macrolides; e.g., a 2611C→G alteration in *S. pneumoniae* with a C2611–G2057 base pair leads to significant resistance toward erythromycin and clarithromycin but marginally affects susceptibility to spiramycin (29). This disparity is reminiscent of the importance of the distinctly different conformations of 14-membered and 16-membered lactone rings in interaction with mutant 2058A→G ribosomes (26). The observation that ketolides retain activity against MLS_B-resistant pneumococci has been attributed to two features: (*i*) the weak ability of ketolides to act as inducers of macrolide resistance conferred by inducible *erm* genes (8) and (*ii*) the interaction with domain II, which results in higher binding affinity (9–11).

The finding that the type of base pair at rRNA positions 2057–2611 (i.e., A-U vs. G-C) determines ketolide activity in mutant 2058A→G ribosomes was initially unexpected and surprising. The nature of the Watson–Crick base pair at 23S rRNA positions 2057–2611 does not greatly affect susceptibility of 2058G ribosomes to 14-, 15- and 16-membered macrolides but has a profound effect on susceptibility to the ketolide telithromycin. These findings not only explain the susceptibility of 2058G mutant *S. pneumoniae* ribosomes to telithromycin but also allude to the selectivity of ketolides for the bacterial ribosome, as opposed to the eukaryotic ribosome (50), the latter characterized by nucleotides G2058, A2057, and U2611.

Nucleotide 2611, which is a constituent of the macrolide-binding pocket (Fig. 3), is base-paired with A2057, a nucleotide that is hardly involved in macrolide or ketolide binding. In the two known structures of complexes of *D. radiodurans* 50S with ketolides, namely ABT-773 and telithromycin, U2611 is involved in interactions with them in slightly different fashions. In particular, the lactone ring of telithromycin makes intensive stacking interactions with U2611 (ref. 5 and Fig. 4), whereas for ABT-773, the main interactions with 2611 are through its desosamine sugar (4). It seems that this subtle, albeit significant, difference results from the conformational changes in the macrolide-binding pocket (especially in the region 2606–2611) induced by ABT-773 binding, supporting the suggestion of a possible conformational alteration of the macrolide-binding pocket in response to the chemical nature of the antibiotic compound (25).

An additional evidence for the high variability in the conformation of residue 2611 has been obtained by comparing its orientation in *D. radiodurans* 50S, where 2611 is a uridine, with that in the large

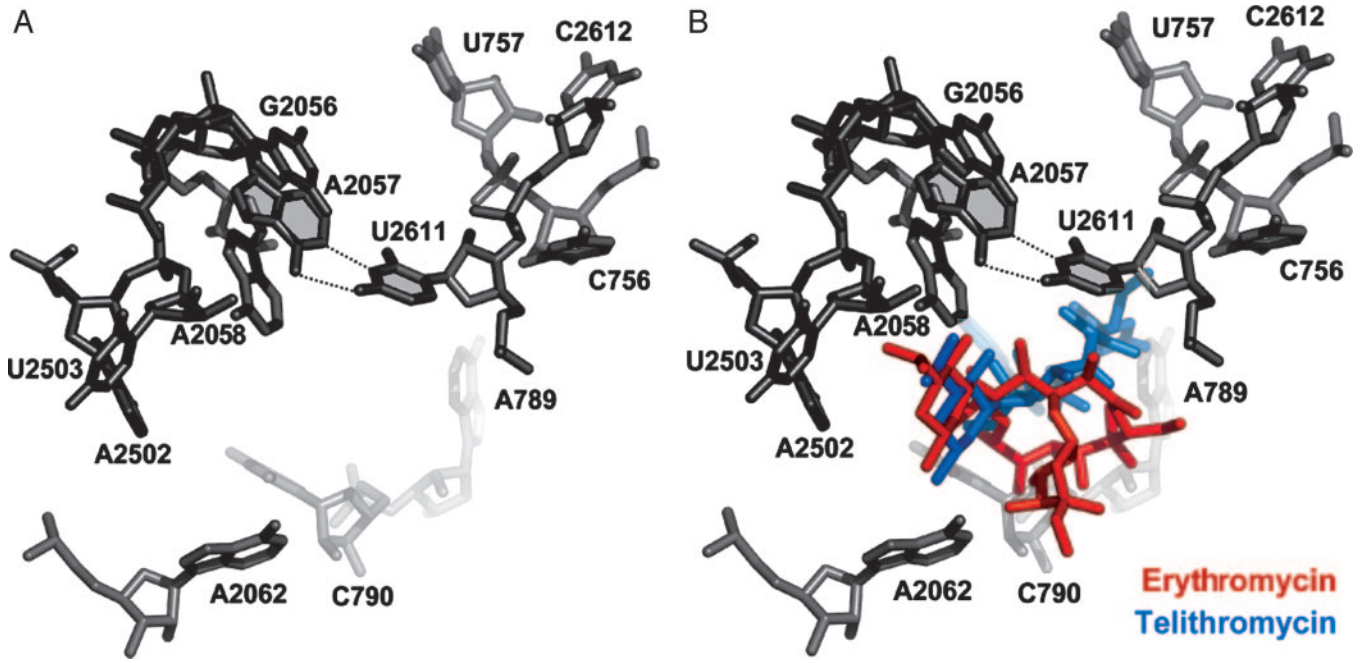

Fig. 5. A view into the macrolide-binding pocket (in gray) highlighting the specific geometry of the 2611–2057 base pair. H bonds are shown as dotted lines. (*A*) Empty pocket. (*B*) The positions of erythromycin (in red) and telithromycin (in blue) are superposed.

subunit from *Haloarcula marismortui*, where 2611 is a guanine (Fig. 3). Clearly, a U-to-C mutation should have a smaller effect on 2611 conformation, compared with a U-to-G replacement. Nevertheless, the inherent flexibility at this position seems to be sufficient for significant conformational alterations, even for the natural sequence polymorphism as present in the bacterial domain.

In the WT *D. radiodurans* 50S structure, the base-pairing contacts of U2611 with A2057 create a geometry that deviates significantly from that of the canonical Watson–Crick base pair (Fig. 5). Such geometry should allow U2611 to be more flexible than in normal base-pairing. The double mutation 2611U→C and 2057A→G introduces an additional base-pairing contact, which should be sufficient to minimize the conformational space of 2611 and consequently cause conformational rearrangements of the entire base pair. The new base-pair geometry imposed by the additional H bond should have a marginal or no effect on macrolide binding, because, unlike ketolides, the macrolides hardly interact with U2611 (Fig. 5). However, owing to the intimate contacts between the ketolides and U2611 (Fig. 4), any deviation in the orientation of the 2611 base should affect telithromycin binding.

Compared with eubacteria with a 2058A, in the archaeon *H. marismortui*, the macrolides are positioned at a larger distance to the 2058–2059 side of the macrolide-binding pocket (46). Assuming that a G in position 2058 will affect binding of telithromycin in a similar manner, it is conceivable that its macrolactone ring position will prevent the favorable interactions with U2611 or lead to a clash between them. Thus, in addition to losing some of its interactions with 2058, telithromycin binding would suffer from the loss of its contacts with U2611. This interpretation is supported by the experimental findings, which demonstrate that the single mutation A2058G confers significant resistance to telithromycin, which can be alleviated by the triple mutation A2058G/A2057G/U2611C. Thus, it seems that in its location within a macrolide-binding pocket with a 2058G, the telithromycin macrolactone ring cannot interact well with U2611 but should be able to interact with C2611, which is expected to be positioned differently from U2611, in an orientation determined by its base-pairing with G2057. These interactions may partially compensate for the loss of contacts with 2058 caused by the A-to-G alteration and consequently decrease ribosomal drug resistance, as observed by susceptibility testing.

The function of the rRNA has remained highly preserved in different organisms throughout evolution (51) and this preservation is reflected in the high conservation of the rRNA secondary (47) and tertiary structures (48, 49, 52). It is thus commonly assumed that observations made for the rRNA of one species of bacterium can invariably be extrapolated to other species (e.g., ref. 48). Together with recent results (33, 36, 53), our data support the view that this perception is oversimplified and testify that the subtle structural differences observed in different organisms (49, 52), even in highly conserved regions (25, 54), have a functional correlate.

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