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Genetic Basis for Natural and Acquired Resistance to the Diarylquinoline R207910 in Mycobacteria

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The *atpE* gene encoding the subunit *c* of the ATP synthase of *Mycobacterium tuberculosis*, the target of the new diarylquinoline drug R207910, has been sequenced from in vitro mutants resistant to the drug. The previously reported mutation A63P and a new mutation, I66M, were found. The genetic diversity of *atpE* in 13 mycobacterial species was also investigated, revealing that the region involved in resistance to R207910 is conserved, except in *Mycobacterium xenopi* in which the highly conserved residue Ala63 is replaced by Met, a modification that may be associated with the natural resistance of *M. xenopi* to R207910.

R207910 (also known as TMC207) is the lead compound of a series of recently discovered diarylquinolines (DARQs) (1). This new drug, exquisitely active against a broad range of mycobacteria, may significantly improve the treatment of tuberculosis. As it inhibits a new target, R207910 is active against both drug-sensitive and drug-resistant isolates of *Mycobacterium tuberculosis* (1).

The initial identification of the target of R207910 relied on sequence analysis of a single mutant of *M. tuberculosis* and two mutants of the fast-growing organism *Mycobacterium smegmatis* that were resistant to R207910 and harbored two mutations (D32V and A63P, respectively) in the subunit c of ATP synthase encoded by the *atpE* gene (1). This enzyme contains two structural domains, F0 and F1 (8). F0 includes 1 subunit a, 2 subunits b, and 9 to 12 subunits c made of two α-helices connected by a short loop and arranged in a symmetrical disk. The two mutations, A63P and D32V, affect the α-helices in subunit c and are located in the vicinity of the key glutamic acid residue (E61) involved in proton transport. Because these previous studies were conducted on a limited number of mutants and mycobacterial species, we have undertaken the investigation of *atpE* from new *M. tuberculosis* in vitro mutants and from

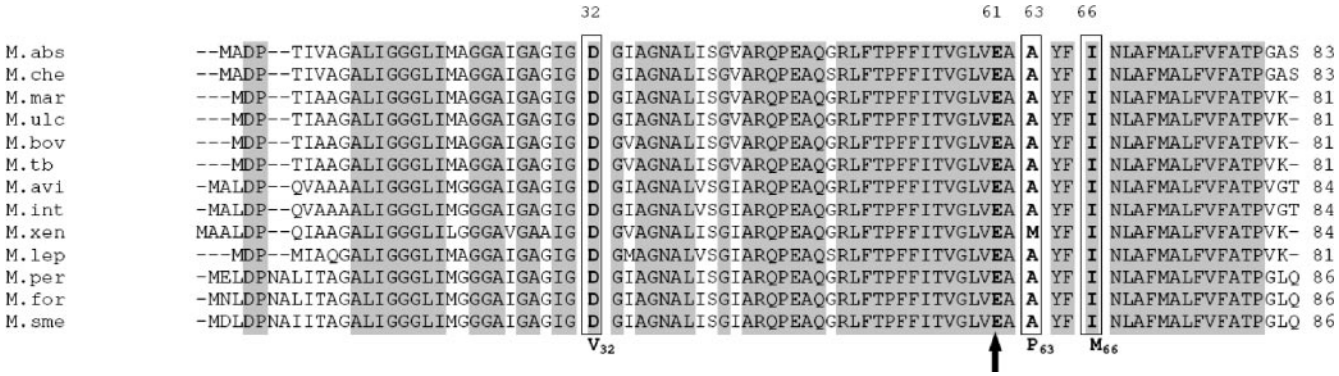

FIG. 1. Multiple sequence alignment for subunit c proteins from 13 mycobacterial species. Species abbreviations: M.abs, *M. abscessus* (GenBank accession no. DQ306899); M.che, *M. chelonae* (GenBank accession no. DQ306894); M.mar, *M. marinum* (GenBank accession no. DQ306898); M.ulc, *M. ulcerans* (GenBank accession no. DQ306897); M.bov, *M. bovis* (GenBank accession no. DQ306895); Mtb, *M. tuberculosis* H37Rv (Swiss-Prot accession no. Q10598); M.avi, *M. avium* (GenBank accession no. DQ378275); M.int, *M. intracellulare* (GenBank accession no. DQ378276); M.xen, *M. xenopi* (GenBank accession no. DQ306893); M.lep, *M. leprae* (GenBank accession no. DQ306896); M.per, *M. peregrinum* (GenBank accession no. DQ378277); M.for, *M. fortuitum* (GenBank accession no. DQ378278); M.sme, *M. smegmatis* (GenBank accession no. DQ306892). The proton-binding glutamic acid is indicated by an arrow. Gray shading shows conserved amino acids. The mutated positions found in the drug-resistant strains of *M. tuberculosis* (positions 63 and 66) and *M. smegmatis* (position 32) are boxed. The mutated residues are indicated below the boxes.

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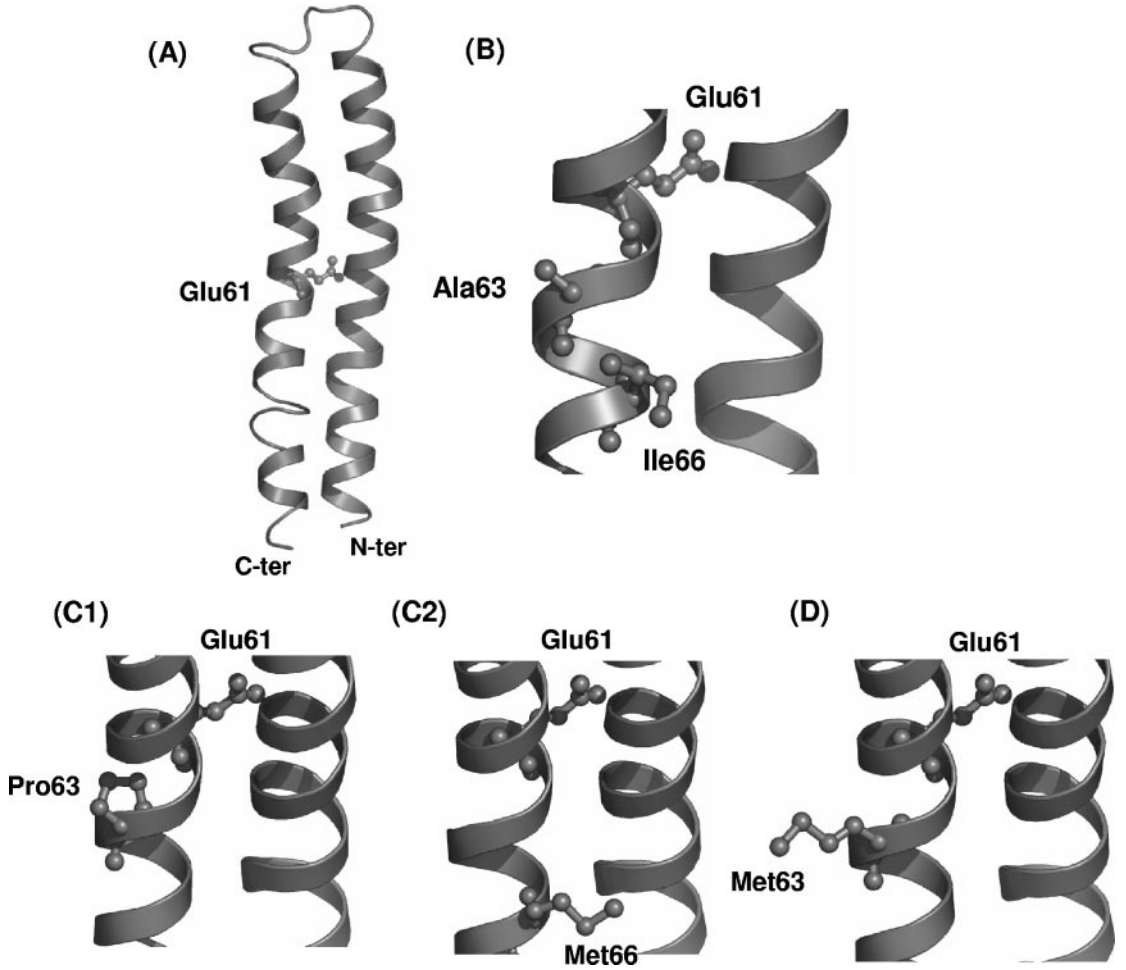

FIG. 2. Three-dimensional models of the subunit c from *M. tuberculosis* H37Rv (A, B, and C) and *M. xenopi* (D). (A) Ribbon representation of the monomeric subunit c from *M. tuberculosis* H37Rv (residues 1 to 75). The side chain of residue Glu61 is shown. (B) Closer view of the Glu61 region. The two residues Ala63 and Ile66 found in AtpE of strain H37Rv susceptible to R207910 are shown. (C and D) The same view showing the two mutated residues Pro63 (C1) and Met66 (C2) identified in the H37Rv mutants resistant to R207910 and the Met63 (D) found specifically in the subunit c from *M. xenopi*, respectively.

various mycobacterial species. Here, we report on the isolation of new *M. tuberculosis* in vitro mutants resistant to R207910 and the characterization of the corresponding subunit c sequences. We also describe the heterogeneity of this protein in the *Mycobacterium* genus, which was evaluated by sequencing the *atpE* gene from 13 different mycobacterial species.

The isolation of the mutants resistant to R207910 was carried out by spreading 100 μl of a culture of *M. tuberculosis* H37Rv (10^8 to 10^{10} CFU/ml) onto 7H11 plus OADC (oleic acid, albumin, dextrose, and catalase [Serlabo, Bonneuil sur Marne, France]) agar containing from 0.03 μg/ml (the MIC for H37Rv) to 0.5 μg/ml of R207910. After 3 to 6 weeks of incubation at 37°C, seven mutants of *M. tuberculosis* H37Rv resistant to R207910 were selected on R207910 at concentrations of 0.12 μg/ml for the mutants BK18, BK19, and BK21; 0.25 μg/ml for BK13, BK14, and BK15; and 0.5 μg/ml for BK11.

The *atpE* gene, as well as the 78-bp upstream and 121-bp downstream regions, were amplified using the degenerate primers atpBS [5′TGTA(**CT**)TTCAGCCA(**AG**)GC(**GC**)ATG G3′] and atpFAS [5′CCGTT(**GC**)GG(**AGT**)A(**GCT**)GAGGA AGTTG 3′] (Eurogentec, Belgium) (boldface indicates degenerate bases in the primers), designed from the sequences of the *atpB* and *atpF* genes located upstream and downstream of *atpE* in the ATP synthase operon. Using these two primers, we first confirmed the amino acid conservation of subunit c in *M. tuberculosis* by sequencing *atpE* in 20 nonrelated susceptible clinical isolates (data not shown). Using the same primers, we determined and compared the sequences obtained from the resistant mutants and found in two of them the presence of the mutation A63P previously described in the strain BK12 resistant to R207910 (1). Strikingly, in the five other mutants, the Ala residue found at position 63 in *M. tuberculosis* H37Rv was conserved while Ile66 was found to be replaced by a methionine (I66M) (Fig. 1).

The impact of these mutations was investigated by building a model structure of the monomeric subunit c of *M. tuberculosis* H37Rv using the homology-modeling server SWISS-MODEL (5–7) with the three-dimensional structure of subunit c from *E. coli* as a template (Protein Data Bank accession no. 1A91) (4). As shown on Fig. 2, Ala63 and Ile66, which were

TABLE 1. Percentages of identity worked out from pairwise alignments of *atpE* genes from 13 mycobacterial species

Species	% Identity to:												
	M. tuberculosis	*M. ulcerans*	*M. abscessus*	*M. leprae*	*M. bovis*	*M. marinum*	*M. xenopi*	*M. chelonae*	*M. smegmatis*	*M. avium*	*M. intracellulare*	*M. fortuitum*	*M. peregrinum*
M. tuberculosis	100	91	84	84	100	92	78	83	82	86	86	79	81
M. ulcerans		100	83	83	91	99	80	84	84	84	84	77	79
M. abscessus			100	77	84	85	77	96	82	84	85	77	78
M. leprae				100	84	83	74	76	75	80	80	73	75
M. bovis					100	92	78	83	82	86	86	79	81
M. marinum						100	81	84	86	85	85	80	82
M. xenopi							100	77	74	76	77	75	76
M. chelonae								100	80	85	86	77	78
M. smegmatis									100	83	83	94	93
M. avium										100	96	81	82
M. intracellulare											100	81	82
M. fortuitum												100	95
M. peregrinum													100

found to be modified in the two mutants, are positioned in the vicinity (5.7 Å and 9.4 Å, respectively) of the essential residue Glu61, the carboxyl side chain of which permits the proton transfer required for the creation of ATP (2) (Fig. 2A and B). A63P directly affects the α-helix in the Glu61 region (Fig. 2C1), whereas I66M is more distant but introduces significant steric hindrance at the surface of the α-helix in the same area (Fig. 2C2). It can be hypothesized that the two mutations Ala63Pro and Ile66Met, which occur in a region critical for the ATP synthase activity, affect the interactions between R207910 and the c subunits in a region of the ATP synthase where the diarylquinoline could bind.

The genetic diversity of *atpE* was investigated by amplifying with atpBS and atpFAS the *atpE* genes from 13 mycobacterial

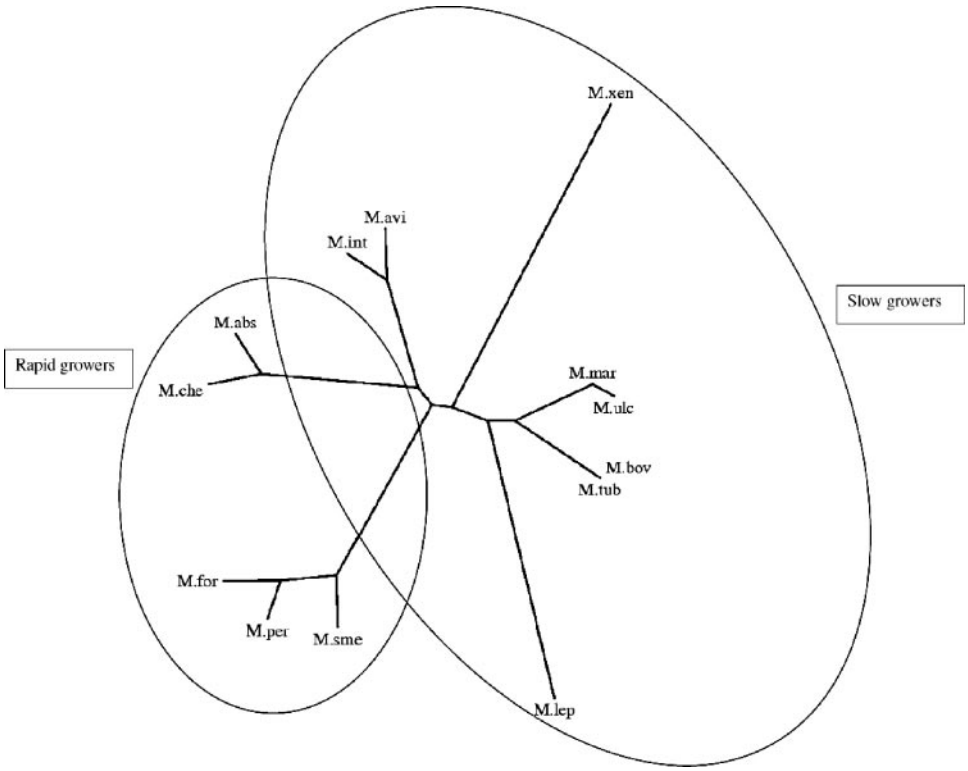

FIG. 3. Phylogenetic tree of *atpE* genes from 13 mycobacterial species. The tree was created using PHYLIP (3). Branch lengths correspond to the number of nucleotides exchanges of the *atpE* genes. Species abbreviations: M.abs, *M. abscessus* (GenBank accession no. DQ306899); M.che, *M. chelonae* (GenBank accession no. DQ306894); M.mar, *M. marinum* (GenBank accession no. DQ306898); M.ulc, *M. ulcerans* (GenBank accession no. DQ306897); M.bov, *M. bovis* (GenBank accession no. DQ306895); Mtub, *M. tuberculosis* H37Rv (Swiss-Prot accession no. Q10598); M.avi, *M. avium* (GenBank accession no. DQ378275); M.int, *M. intracellulare* (GenBank accession no. DQ378276); M.xen, *M. xenopi* (GenBank accession no. DQ306893); M.lep, *M. leprae* (GenBank accession no. DQ306896); M.per, *M. peregrinum* (GenBank accession no. DQ378277); M.for, *M. fortuitum* (GenBank accession no. DQ378278); M.sme, *M. smegmatis* (GenBank accession no. DQ306892).

species (Table 1). The degree of nucleotide identity found between these genes is high, the lowest identity being 73% (Table 1). The phylogenetic tree represented on Fig. 3 indicates that the *atpE* sequences can be grouped in seven distinct clusters corresponding well to those established from 16S rRNA sequencing (9) and clearly differentiates the slow growers from the rapid growers. The amino acid sequence alignment shown in Fig. 1 confirms that the degree of identity at the protein level is very high, from 90% for *Mycobacterium xenopi* to 100% for *Mycobacterium bovis* compared to *M. tuberculosis* H37Rv. The amino acid variations are scattered throughout the polypeptide sequence, with a higher level of divergence at the N- and C-terminus extremities. Interestingly, residues D32, A63, and I66, are highly conserved in the species included in this study, except in *M. xenopi*, for which residue 63 is neither an alanine, as found in the susceptible strain of H37Rv, nor a proline, as found in the mutant strains of H37Rv resistant to R207910, but a methionine (Fig. 1). We confirmed the presence of this methionine at position 63 by sequencing *atpE* in seven nonrelated strains of *M. xenopi*. The presence of this specific residue at a position clearly involved in resistance to R207910 in *M. tuberculosis* may be associated with the high MIC of R207910 (4 μg/ml) observed for *M. xenopi*, which is a species regarded as naturally resistant to the drug (1). Accordingly, in the model of the subunit c of *M. xenopi* (Fig. 2D), the bulky side chain of Met63 lies in the vicinity of Glu61, supporting the hypothesis that the role played by Met63 in the natural resistance of *M. xenopi* to R207910 could be similar to the one played by the two other mutations, A63P and I66M, found in the in vitro *M. tuberculosis* mutants.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *atpE* genes from the mycobacteria *M. xenopi*, *M. ulcerans*, *M. marinum*, *M. bovis*, *M. leprae*, *M. abscessus*, *M. chelonae*, *M. smegmatis*, *M. avium*, *M. intracellulare*, *M. fortuitum*, and *M. peregrinum* are DQ306893, DQ306897, DQ306898, DQ306895, DQ306896, DQ306899, DQ306894, DQ306892, DQ378275, DQ378276, DQ378278, and DQ378277, respectively.

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