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Different Rifampin Sensitivities of *Escherichia coli* and *Mycobacterium tuberculosis* RNA Polymerases Are Not Explained by the Difference in the β-Subunit Rifampin Regions I and II

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***Mycobacterium tuberculosis* RNA polymerase is 1,000-fold more sensitive to rifampin than *Escherichia coli* RNA polymerase. Chimeric *E. coli* RNA polymerase in which the β-subunit segment encompassing rifampin regions I and II (amino acids [aa] 463 through 590) was replaced with the corresponding region from *M. tuberculosis* (aa 382 through 509) did not show an increased sensitivity to the antibiotic. Thus, the difference in amino acid sequence between the rifampin regions I and II of the two species does not account for the difference in rifampin sensitivity of the two polymerases.**

Rifampin (RIF) is a broad-spectrum antibiotic that is used in the therapy of many infectious diseases, in particular, tuberculosis. The cellular target of RIF is RNA polymerase (RNAP) (7). RIF blocks the initiation of transcription by preventing the synthesis of RNAs larger than dinucleotides. At the same time, RIF has no effect on the formation of the first phosphodiester bond and does not inhibit RNA elongation. These facts led to the proposal that RIF sterically blocks the path of the nascent RNA during initiation (5, 9, 15, 25).

An analysis of the three-dimensional structure of *Thermus aquaticus* RNAP in a complex with RIF revealed that the antibiotic binds near the RNAP active site at a protein pocket formed by the β subunit (2). RIF overlaps with the position of the third RNA nucleotide in the elongation complex (11). These data strongly supported the initial hypothesis on the steric mechanism of RIF action.

All known mutations leading to RIF resistance in bacteria (Rif^r mutations) map in four regions in the RNAP β subunit. In total, 23 positions of the β subunit have been implicated in RIF resistance in different bacteria (Fig. 1) (2, 3, 8, 10, 12–14, 17, 18, 20, 22, 23). The majority of the mutations are found in the β-subunit regions I and II (positions 505 to 537 and 562 to 572 in *Escherichia coli* numbering); in addition, there are two mutations at the 687 (region III) and 146 positions of the β subunit. Most mutations found in clinical isolates of *Mycobacterium tuberculosis* are localized in three positions of the β-subunit region I (Fig. 1), while no mutations in regions II and III were described (18, 21, 24).

All amino acids changed by Rif^r mutations are spatially grouped around the RIF binding pocket (2, 26). Twelve amino acid residues of RNAP are involved in direct interactions with RIF, and substitutions of 11 of them were shown to lead to RIF resistance (Fig. 1). Twelve additional amino acids changed by Rif^r mutations surround the RIF pocket but do not make direct contact with the antibiotic. The effect of these substitutions is likely to come from local changes of the structure of the RIF pocket weakening the binding of the antibiotic.

RNAPs of various bacteria possess different levels of RIF sensitivity. *M. tuberculosis* RNAP was shown to be about 1,000-fold more sensitive to RIF than the RNAP from *E. coli*. It also formed much more stable complexes with the antibiotic (6). The formation of stable RIF-RNAP complexes probably explains the bactericidal activity of RIF and may be one of the factors explaining the efficacy of RIF in the tuberculosis therapy. It was reasonable to suppose that the different RIF sensitivities of *E. coli* and *M. tuberculosis* RNAPs result from the differences in the β-subunit regions involved in the binding of RIF. To test this hypothesis, we constructed two chimeric *E. coli* RNAPs in which either the RIF region I alone (amino acids [aa] 505 through 537) or a larger β-subunit segment encompassing RIF regions I and II and a portion of flanking sequences (aa 463 through 590) were replaced with the corresponding sequences from *M. tuberculosis* (Mtu[I] RNAP and Mtu[I-II] RNAP, respectively) (Fig. 1).

The mutant *rpoB* genes encoding the chimeric β subunits were generated by standard PCR mutagenesis methods. To produce the Mtu[I] gene, recognition sites of two restriction endonucleases, EcoRI and SmaI, were introduced by two-stage PCRs at both sides of the RIF region I (codons 504 and 539) in the plasmid containing *E. coli rpoB* (pMKSe2) (22). In each case, the first PCR was done with a reverse mutagenic primer containing the sequence of the restriction site (5′-CGAAGAATTCTTTCACTGCTGC and 5′-GCACGTTCCCGGGTCAGACC for EcoRI and SmaI sites [underlined], respectively) and direct primer 5′-GCTGGCTAAGCTGAGCC beginning at codon 321 of the *rpoB* gene. This product was used as a megaprimer in the second PCRs, with reverse primer 5′-GGAGAGCGCAGCTTCACCC beginning at codon 863. The re-

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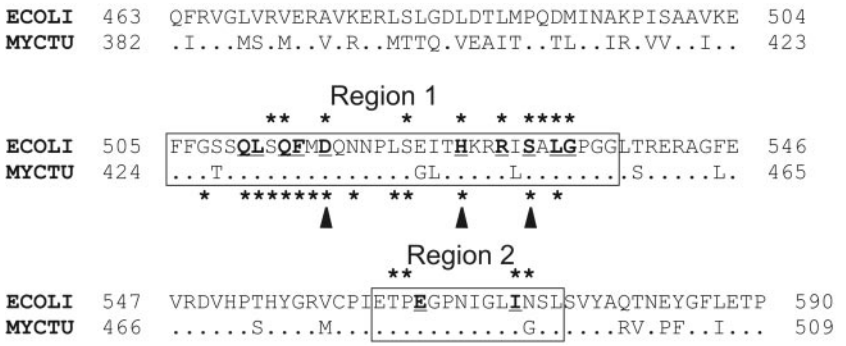

FIG. 1. Alignment of the β-subunit RIF regions of *E. coli* (ECOLI) and *M. tuberculosis* (MYCTU) RNAPs. Dots in the sequence of *M. tuberculosis* represent amino acids that are identical to those of *E. coli*. Numbers on both sides of the sequences indicate the positions of amino acids starting from the N terminus of the protein. The first and the second RIF regions are boxed. Positions of the Rif^r mutations are marked with asterisks; mutations in *E. coli* and *M. tuberculosis* RNAPs are shown above and below the alignment, respectively. Amino acids that directly interact with RIF in the structure of *T. aquaticus* RNAP (2) are bold and underlined. Three positions of the first RIF region which most frequently mutate in *M. tuberculosis* are indicated by arrowheads under the sequence.

sulting PCR product was treated with ClaI and BspEI and cloned into the same sites of the *E. coli rpoB* gene (located at codons 433 and 846). Then, the *M. tuberculosis rpoB* segment comprising RIF region I (codons 424 to 556) was amplified from the I376 cosmid with primers containing EcoRI and SmaI sites (5′-CAAGGAATTCTTCGGCACCAGCCAGC and 5′-CGCTCCCGGGTCAGACCGCCTGGCCCCAGC for the EcoRI site and the SmaI site [underlined], respectively) and cloned into the *E. coli* plasmid.

The Mtu[I-II] *rpoB* gene was generated in essentially the same way. In the first stage, the recognition sites of MfeI and MluI restriction endonucleases were introduced by two-stage PCRs at codons 463 and 590 of the *rpoB* gene in the pMKSe2 plasmid by using reverse mutagenic primers 5′-GGC CAACGCGCAATTGGTTTTCCGC and 5′-CACTTTACGA TACGCGTTCTCAGGG (with the MfeI site and the MluI site, respectively, underlined). Then, the corresponding *M. tuberculosis rpoB* segment (codons 382 through 509) was amplified with primers 5′-GCTGATCCAAAACCAATTGCGCG TCGGC and 5′-CACCTTGCGGTACGCGTTTTCGATG (with the MfeI site and the MluI site, respectively, underlined) and cloned into the MfeI and MluI sites of the *E. coli rpoB* gene.

The RNA polymerases containing the chimeric β subunits were reconstituted in vitro from individual subunits (1). Two control RNAPs were reconstituted in parallel. The first one was wild-type RIF-sensitive (Rif^s) RNAP, and the second contained a β subunit with a point Rif^r mutation at position 531 (S531F). The transcription activities and RIF sensitivities of RNAPs were studied with an in vitro transcription test using a DNA template containing a T7 A1 promoter followed by a λ tR2 terminator (19). The transcription of this template results in the synthesis of two major products corresponding to a full-length 130-nucleotide RNA transcribed to the end of the DNA fragment and a shorter 106-nucleotide product terminated at the tR2 sequence. The reaction mixture contained reconstituted core RNAP, σ subunit, a promoter DNA fragment (50 nM), and a mixture of nucleotides (25 μM concentrations of ATP, CTP, and GTP and 5 μM of UTP with the addition of [α-^{32}P]UTP). RIF was added 3 min prior to the addition of the nucleotides. Transcription was performed for 10 min at 37°C. The measured specific activities of the Rif^r, Mtu[I], and Mtu[I-II] RNAPs relative to the Rif^s enzyme were 105, 75, and 45%. Most probably, the lower activity of the Mtu[I-II] enzyme is explained by some defects in RNAP reconstitution caused by the mutation. At the same time, the mutation did not have a dramatic effect on the interactions of RNAP with promoters, elongation, and termination of RNA synthesis (Fig. 2 and data not shown).

In agreement with published data, the activity of the wild-type Rif^s RNAP was completely inhibited at 2 μg of RIF/ml, while the activity of the control Rif^r RNAP was not affected at this concentration of antibiotic (Fig. 2). Contrary to expectations, neither chimeric RNAP possessed an increased RIF sensitivity in comparison with the *E. coli* Rif^s RNAP. Moreover, the Mtu[I-II] chimera happened to be even more RIF resistant than the wild-type *E. coli* Rif^s enzyme (Fig. 2, compare lanes 4 and 16).

The sequences forming the RIF pocket are highly conserved among bacteria; the first and the second RIF regions of *M. tuberculosis* differ from the *E. coli* sequence at only five posi-

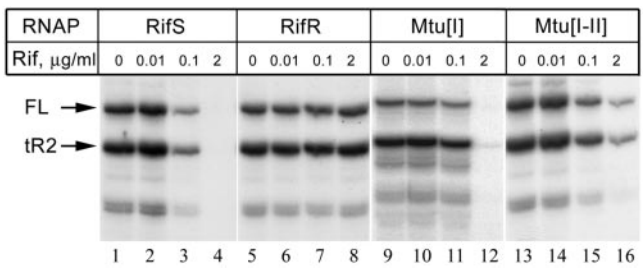

FIG. 2. The effect of RIF on the activity of RNAPs. The concentrations of RNAP in the reaction were 10 nM in the cases of the Rif^s (RifS), Rif^r (RifR), and Mtu[I] RNAPs and 20 nM in the case of the Mtu[I-II] enzyme. Full-length and terminated RNA transcripts are marked FL and tR2, respectively.

tions (Fig. 1). In addition, the substituted segment of the *E. coli* β subunit (aa 463 through 590) differs from the sequence of *M. tuberculosis* at 4 positions between the two RIF regions and at 27 positions at both sides of the segment (these parts of the protein are weakly conserved and do not contain any known Rif^r mutations) (Fig. 1). Our results demonstrate that all these differences do not account for the different RIF sensitivities of *E. coli* and *M. tuberculosis* RNAPs. At the same time, the changes in the nonconserved regions surrounding the RIF pocket are apparently responsible for the increased RIF resistance of the Mtu[I-II] chimeric RNAP.

It should be noted that none of the five amino acids that differ in the RIF regions of the two RNAPs makes direct contacts with the antibiotic in the complex of RIF with *T. aquaticus* RNAP (2). Only one of these amino acids (position 573 in *E. coli*) was shown to be replaced by the Rif^r mutation (Fig. 1). The low conservation of these positions and the absence of Rif^r mutations support the idea that these amino acids are not involved in RIF binding. In contrast, all 12 amino acids that directly interact with RIF (including positions in *M. tuberculosis* which are most frequently changed by Rif^r mutations) are identical in *E. coli* and *M. tuberculosis* RNAPs (Fig. 1). Thus, the increased RIF sensitivity of *M. tuberculosis* RNAP must be explained by the differences in other RNAP regions which may indirectly result in structural changes of the RIF pocket increasing the affinity of RNAP to the antibiotic.

Previously, RNAP from thermophilic *T. aquaticus* strains was shown to be about 100-fold less sensitive to RIF than *E. coli* RNAP (2, 4). As in the case with the polymerase from *M. tuberculosis*, all 12 amino acids of *T. aquaticus* RNAP that directly interact with RIF are identical to those in the *E. coli* enzyme. This fact led to the proposal that the increased RIF resistance of *T. aquaticus* RNAP also results from changes in the protein regions outside of the RIF binding pocket (16). These data clearly illustrate that the sensitivities of enzymes (and in particular, RNAP) to antibiotics are determined not only by the protein regions that are directly involved in antibiotic binding but also by other parts of the protein that can indirectly affect the structure of the binding site. Most probably, the structure of the RIF pocket may be affected by the nearby protein regions, in particular, the N-terminal region of the β subunit, where a single Rif^r mutation (position 146 in *E. coli*) has been found (8, 13). *M. tuberculosis* RNAP differs from the *E. coli* enzyme at the adjacent position 145 (where Ile is substituted for Val). This substitution may somehow improve RIF binding to *M. tuberculosis* RNAP. Another possibility is that the different RIF sensitivities of the *E. coli* and *M. tuberculosis* enzymes are explained by multiple amino acid changes dispersed over several regions of RNAP. The identification of these regions is an important subject for further studies.

It should be noted that no mutations in the rifampin regions of the β subunit have been found in 5 to 10% of RIF-resistant clinical isolates of *M. tuberculosis*. It was proposed that in these cases, Rif^r mutations may be present in other regions of the β subunit or in other subunits of *M. tuberculosis* RNAP (8, 18, 24). Our results indicate that these mutations may be localized in the RNAP regions responsible for its high affinity to RIF. Thus, new molecular mechanisms underlying the RIF resistance in *M. tuberculosis* and other bacteria may be discovered in the future. These considerations must be taken into account when developing new strategies for antituberculosis therapy.

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