

## Characterization of Mutations in the *rpoB* Gene That Confer Rifampin Resistance in *Staphylococcus aureus*

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Mutations in the rifampin resistance-determining (Rif) regions of the *rpoB* gene of *Staphylococcus aureus* mutants obtained during therapy or in vitro were analyzed by gene amplification and sequencing. Each of the resistant clinical isolates, including five nonrelated clones and two strains isolated from the same patient, and of the 10 in vitro mutants had a single base pair change that resulted in an amino acid substitution in the  $\beta$  subunit of RNA polymerase. Eight mutational changes at seven positions were found in cluster I of the central Rif region. Certain substitutions (His481/Tyr and Asp471/Tyr [*S. aureus* coordinates]) were present in several mutants. Substitutions Gln468/Arg, His481/Tyr, and Arg484/His, which conferred high-level rifampin resistance, were identical or in the same codon as those described in other bacterial genera, whereas Asp550/Gly has not been reported previously. Substitutions at codon 477 conferred high- or low-level resistance, depending on the nature of the new amino acid. The levels of resistance of in vivo and one-step in vitro mutants carrying identical mutations were similar, suggesting that no other resistance mechanism was present in the clinical isolates. On the basis of these data and the population distribution of more than 4,000 clinical *S. aureus* isolates, we propose  $\leq 0.5$  and  $\geq 8$   $\mu\text{g/ml}$  as new breakpoints for the clinical categorization of this species relative to rifampin.

*Staphylococcus* is the bacterial genus most frequently responsible for infections of prosthetic devices, osteomyelitis, and endocarditis (11, 15, 23). During the last 10 years, the proportion of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates in hospitals has reached nearly 29% in the United States (19) and 40% in Europe (27). Most MRSA isolates are resistant to multiple antibiotics (4, 16) and more than 50% are resistant to rifampin, whereas 1% of methicillin-susceptible *S. aureus* (MSSA) isolates are resistant to rifampin (27). Vancomycin is the therapy of choice against *S. aureus* when  $\beta$ -lactams are inappropriate. However, because of poor tissue diffusion and moderate bactericidal activity (2), vancomycin is often combined with rifampin for deep-seated infections (6, 10). Fluoroquinolones in association with rifampin also represent an alternative strategy for the prevention of the emergence of resistant mutants in the treatment of serious MSSA infections (7).

Rifampin acts by interacting specifically with the  $\beta$  subunit of the bacterial RNA polymerase encoded by the *rpoB* gene (1). Rifampin resistance in *Escherichia coli* (13, 22) and *S. aureus* is due to alterations in the target leading to a reduced affinity of the enzyme for the antibiotic (17). Alignment of the predicted amino acid sequence of the *S. aureus* RNA polymerase  $\beta$  subunit with those of other bacterial genera identified conserved domains in all the sequences (1). One of them, between amino acids 486 and 717 (*E. coli* coordinates), includes the loci responsible for rifampin resistance in *E. coli* (13), *Mycobacterium tuberculosis* (24), *Streptococcus pneumoniae* (9), and *Neisseria meningitidis* (5). We have thus amplified and sequenced portions of *rpoB* from rifampin-susceptible and -resistant *S. aureus* isolates from the same patient. These portions correspond to the mutated regions in rifampin-resistant mutants of the previously studied bacterial genera. Mutants ob-

tained in vitro were also studied. Attempts to correlate the various levels of rifampin resistance and the position of the *rpoB* mutations were made.

### MATERIALS AND METHODS

**Bacterial strains.** Rifampin-susceptible and -resistant matched clinical strains of MSSA (three pairs and three strains from the same patient) or MRSA (two pairs) were used in the study. Each pair and the three strains from the same patient were isolated between 1992 and 1997 before and during therapy with rifampin either alone or in combination (Table 1). Plasmid-free *S. aureus* RN4220, which was susceptible to antibiotics, was used for the in vitro selection of rifampin-resistant mutants (14).

**Isolation of rifampin-resistant mutants of *S. aureus* RN4220 and of clinical isolate MSSA BM4368.** Approximately  $10^8$  CFU of exponentially growing bacteria was plated onto Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) containing concentrations of rifampin (provided by Merrell-Dow Research, Milan, Italy) of 0.016 to 64  $\mu\text{g/ml}$ . After 24 h of incubation at 37°C, the numbers of colonies on agar with rifampin concentrations of  $\geq 0.032$   $\mu\text{g/ml}$  were counted and the mutation frequencies were determined relative to the total count of viable organisms plated.

**Antibiotic susceptibility testing.** *S. aureus* strains were screened for rifampin resistance by the disk-agar diffusion method (Sanofi Diagnostics Pasteur), and resistance was confirmed by determination of the MICs by dilution in Mueller-Hinton agar (18) with an inoculum of  $10^4$  CFU per spot.

**Analysis of total DNA by pulsed-field gel electrophoresis.** Contour-clamped homogeneous electric field electrophoresis of *Sma*I restriction endonuclease (United States Biochemicals, Cleveland, Ohio) digests of genomic DNA was performed with a CHEF-DR II system (Bio-Rad Laboratories, Nazareth, Belgium) as described previously (3). Strains were assigned to the same macrorestriction genotype when they shared electrophoretic restriction patterns that differed by three or fewer fragments (26) and displayed a coefficient of similarity (CS) equal to or greater than 0.85 (8). The CS was calculated as follows:  $\text{CS} = 2 \times \text{number of matching bands} / \text{total number of bands in both strains}$ .

**Detection of mutations in the *rpoB* gene.** Total DNA from *S. aureus* was purified (29) and was used as a template for amplification by PCR. Two portions of the *rpoB* gene from *S. aureus* were amplified: a 702-bp fragment from nucleotide positions 441 to 673 (*S. aureus* coordinates) corresponding to the so-called rifampin resistance-determining (Rif) region in the center of the *E. coli rpoB* gene (13) and a 158-bp fragment from nucleotide positions 94 to 144 in which a substitution (Val143/Phe) conferring rifampin resistance has also been reported in *E. coli* (22). The 20-mer oligodeoxyribonucleotides used as primers were F3 (5'-AGTCTATCACACCTCAACAA) and F4 (5'-TAATAGCCGACACAGAA TCA) for the larger fragment and D1 (5'-GTGTAAAAGTGCCTAATC) and D2 (5'-ATAAACGGATGGTGAACGAA) for the smaller fragment. Amplification was carried out in a 100- $\mu\text{l}$  volume containing 40 pmol of each oligonucleotide primer, each 2'-deoxynucleoside 5'-triphosphate at a concentra-

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TABLE 1. Origin and properties of the *S. aureus* strains

Strain	Date of isolation (mo/yr)	Origin	Patient's therapy	Resistance phenotype <sup>a</sup>	Rifampin MIC (μg/ml)
BM4626	4/1992	Bone	Rifampin plus pefloxacin	Tc	0.008
BM4627	4/1992			Pef Rif Tc	128–256
BM4364	2/1996	Bone	Vancomycin, followed by rifampin plus pristinamycin	Em <sub>c</sub> Fos Fus Km Met Pc Pef Tm	0.008
BM4364-R	3/1996			Em <sub>c</sub> Fos Fus Km Met Pc Pef Rif Tm	256
BM4365	6/1996	Blood	Rifampin	Pc	0.008
BM4365-R	10/1996			Pc Rif	256
BM4366	1/1997	Skin	Rifampin	Pc Em <sub>c</sub>	0.008
BM4366-R	1/1997			Pc Em <sub>c</sub> Rif	128–256
BM4367	3/1997	Urine	Rifampin plus vancomycin	Em <sub>c</sub> Fos Km Met Pc Pef Tm	0.016
BM4367-R	4/1997	Urine and pulmonary tree		Em <sub>c</sub> Fos Km Met Pc Pef Rif Tm	4
BM4368	3/1997	Urine	Rifampin plus vancomycin	Pc Tc	0.016
BM4368-R <sub>1</sub>	3/1997	Pulmonary tree	Rifampin plus vancomycin	Pc Rif Tc	128
BM4368-R <sub>2</sub>	4/1997	Pulmonary tree		Pc Rif Tc	4

<sup>a</sup> Em<sub>c</sub>, constitutive resistance to macrolide, lincosamide, and streptogramin β-type antibiotics; Fos, resistance to fosfomycin; Fus, resistance to fusidic acid; Km, resistance to kanamycin; Met, resistance to methicillin; Pc, resistance to penicillin G; Pef, resistance to pefloxacin; Rif, resistance to rifampin; Tm, resistance to tobramycin.

tion of 100 mM, reaction buffer (United States Biochemicals), 2 μl of a template DNA sample containing 100 ng of DNA, and 1 U of *Taq* DNA polymerase (United States Biochemicals). The reactions were performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) for 35 cycles. The conditions were 4 min at 94°C for denaturation, 3 min at 54°C for preannealing, 45 s at 52°C for annealing, and 45 s at 72°C for polymerization for the 702-bp fragment. The PCR conditions for the 158-bp fragment differed by the lack of preannealing and by the use of an annealing temperature of 55°C. The amplification products were purified on Microspin S-400 HR columns (Pharmacia LKB Biotechnology, Uppsala, Sweden), cloned into pCRII vector (Original TA Cloning Kit; Invitrogen), and sequenced by the dideoxy chain termination method (20) with T7 DNA polymerase (T7 Sequencing kit; Pharmacia) and [ $\alpha$ -<sup>35</sup>S]dATP (Amersham Radiochemical Center, Amersham, England).

## RESULTS AND DISCUSSION

**Population distribution of *S. aureus*.** The MICs of rifampin for 4,644 clinical *S. aureus* strains isolated at Hopital Henri Mondor between 1993 and 1996 are presented in Fig. 1. On the basis of this multimodal distribution, the strains were categorized into categories of susceptible (MICs,  $\leq 0.5$  μg/ml), low-level resistant (MICs, 1 to 4 μg/ml), and high-level resistant (MICs,  $\geq 8$  μg/ml) according to the indicated breakpoints.

**Typing of clinical isolates.** The members of each of the five pairs and of the three isolates from the same patient had similar resistance phenotypes except for that for rifampin (Table 1). Pulsed-field gel electrophoresis of total DNA digested with *Sma*I is suitable for discrimination of *S. aureus* clones (25). The two members of each pair and the three strains from the same patient had indistinguishable DNA profiles (data not shown), indicating that the resistant mutants were selected in vivo under therapy with rifampin. The five pairs of clinical strains and the three isolates from the same patient displayed six *Sma*I patterns (CS range, 0.5 to 0.7) and were therefore not clonally related.

**Selection of rifampin-resistant mutants.** Rifampin-resistant mutants were obtained at frequencies of  $10^{-7}$  to  $10^{-8}$  by plating *S. aureus* RN4220 (MIC, 0.008 μg/ml) and clinical isolate MSSA BM4368 (MIC, 0.016 μg/ml) onto solid medium containing rifampin at various concentrations. The mutation frequencies to low- and high-level rifampin resistance were similar and were not affected by the concentration of the selecting agent, which was less than 2 μg/ml. This result suggests that resistance to high levels of rifampin does not arise by sequential independent events but, rather, arises in a single-step fashion. The relative ease with which rifampin-resistant *S. aureus* mutants were obtained confirms that this antibiotic must not be prescribed alone and that considerable

attention must be paid to interactions between rifampin and other antimicrobial agents. In fact, rifampin-resistant *S. aureus* BM4627 and BM4364-R mutants emerged in vivo under combination therapy. Ten mutants of *S. aureus* RN4220 obtained in vitro in two independent experiments were selected for further studies.

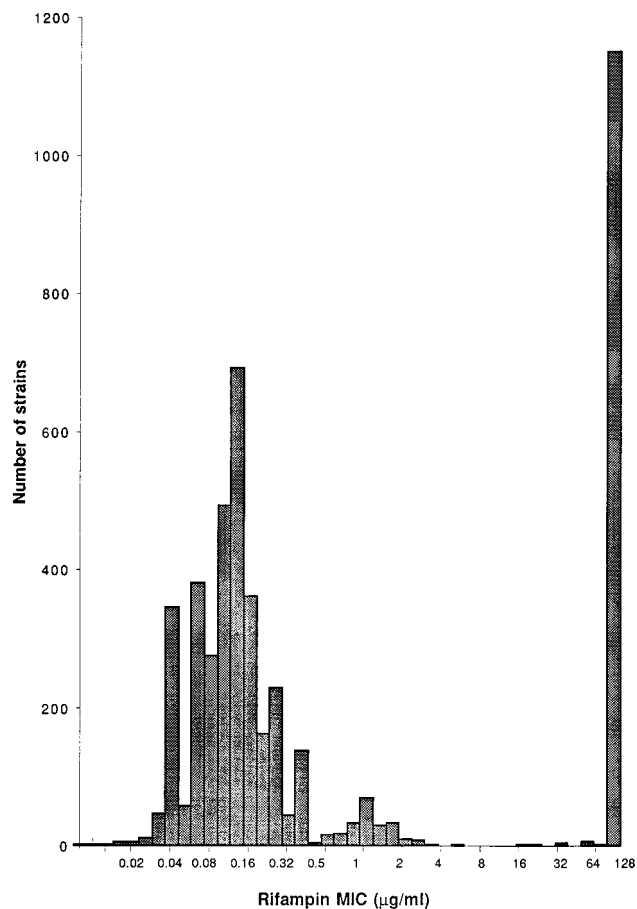


FIG. 1. Distribution of rifampin MICs for 4,644 *S. aureus* strains isolated at Henri Mondor Hospital between 1993 and 1996.

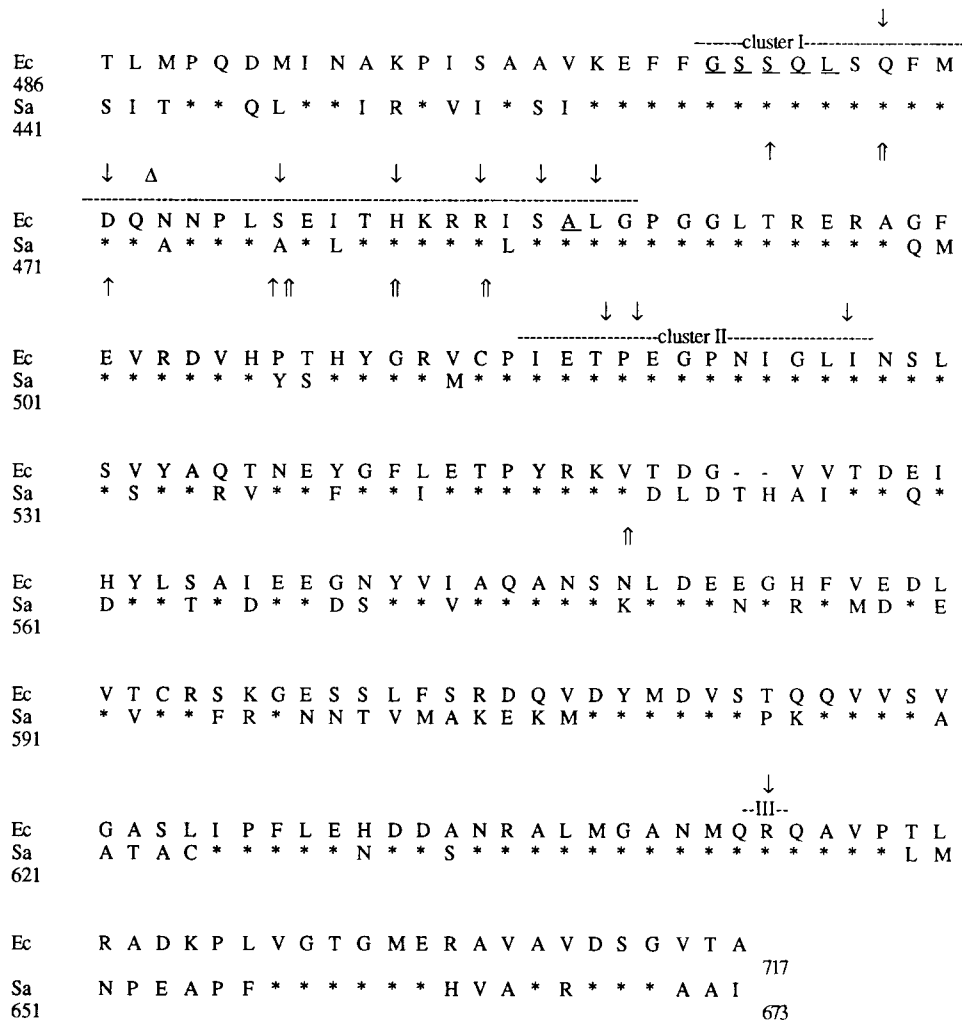


FIG. 2. Amino acid sequence comparison of the Rif regions of *E. coli* (Ec) and *S. aureus* (Sa). Clusters I, II, and III are indicated by dashed lines. Identical amino acids are indicated by asterisks, and gaps are indicated by dashes. Positions known to be involved in rifampin resistance in *E. coli* are indicated by downward-pointing arrows (mutations) or triangles (insertions) or are underlined (deletions). Mutations leading to low- and high-level rifampin resistance in *S. aureus* are indicated by single and double upward-pointing arrows, respectively.

**DNA sequence analysis and susceptibility to rifampin.** The sequences of the *rpoB* Rif gene region from nucleotides 441 to 673 (*S. aureus* coordinates) of seven high-level-resistant clinical isolates and of their susceptible counterparts, two in vitro low-level-resistant mutants (RN4220-R<sub>1</sub> and RN4220-R<sub>2</sub>), and eight high-level-resistant mutants were determined (Fig. 2). Relative to their susceptible counterparts, the 17 in vitro and in vivo mutants had a single base pair change in the Rif region of the *rpoB* gene that resulted in an amino acid substitution, although the occurrence of other mutations in nonsequenced regions of the *rpoB* gene cannot be excluded. Eight mutational changes were found at seven positions: six clustered from nucleotide positions 464 to 484 and one was at position 550 (Table 2). The mutations in the *E. coli* *rpoB* gene involved in rifampin resistance can be assigned to three clusters, clusters I, II, and III (13). Since no strain had mutations in clusters II and III, we do not know if these regions are involved in rifampin resistance in *S. aureus*, as has been described for high- and low-level (cluster II) or very low level (cluster III) resistance in *M. tuberculosis* (24) and *E. coli* (13) (Fig. 2). Replacement of aspartate 550 by a glycine was a new substitution found outside the clusters at a

position where *E. coli* and *M. tuberculosis* have a threonine and a valine, respectively.

Certain mutations in cluster I were frequently found: the His481/Tyr substitution occurred in two clinical isolates and in four high-level-resistant mutants obtained independently in vitro; the Asp471/Tyr substitution was present in two in vivo low-level-resistant mutants, BM4368-R<sub>2</sub> and BM4367-R, and in the in vitro low-level-resistant mutant RN4220-R<sub>2</sub>. The MICs of rifampin for mutants that were obtained in vivo and in vitro and that had identical mutations were similar and were within 1 dilution, suggesting that reduced affinity of the enzyme for the antibiotic following the occurrence of a mutation in the Rif region of the *rpoB* gene is the major mechanism of resistance in *S. aureus*.

Comparative analysis of the level of resistance to rifampin in *S. aureus* and of the mutation sites indicated that high-level resistance correlated with mutations at codons 468 and 481 and that low-level resistance was associated with a mutation at codon 471 (*S. aureus* coordinates). These codons correspond to those associated with respective similar levels of resistance in *E. coli* (13), *N. meningitidis* (5), *M. tuberculosis* (24), and *Mycobacterium*

TABLE 2. Resistance to rifampin of *S. aureus* strains and mutations in the *rpoB* gene

Strain	Rifampin concn ( $\mu\text{g/ml}$ ) used for selection	Rifampin MIC ( $\mu\text{g/ml}$ )	Mutation (amino acid substitution) <sup>a</sup>	Corresponding mutation in the <i>E. coli</i> $\beta$ subunit
RN4220-R <sub>5</sub>	2	4	TCT→CCT (Ser464→Pro)	$\Delta$ [Gly507-Leu511] <sup>b</sup>
RN4220-R <sub>6</sub>	2	4	TCT→CCT (Ser464→Pro)	$\Delta$ [Gly507-Leu511] <sup>b</sup>
RN4220-R <sub>10</sub>	32	256	CAA→CGA (Gln468→Arg)	Gln513→Pro/Leu
RN4220-R <sub>2</sub>	0.016	2	GAC→TAC (Asp471→Tyr)	Asp516→Val/Asn
BM4368-R <sub>2</sub>	In vivo	4	GAC→TAC (Asp471→Tyr)	Asp516→Val/Asn
BM4367-R	In vivo	4	GAC→TAC (Asp471→Tyr)	Asp516→Val/Asn
RN4220-R <sub>1</sub>	0.016	1	GCT→GTT (Ala477→Val)	Ser522→Phe
RN4220-R <sub>3</sub>	0.016	256	GCT→GAT (Ala477→Asp)	Ser522→Phe
BM4368-R <sub>1</sub>	In vivo	128	GCT→GAT (Ala477→Asp)	Ser522→Phe
RN4220-R <sub>4</sub>	0.032	256	CAT→TAT (His481→Tyr)	His526→Tyr
RN4220-R <sub>7</sub>	2	256	CAT→TAT (His481→Tyr)	His526→Tyr
RN4220-R <sub>8</sub>	2	256	CAT→TAT (His481→Tyr)	His526→Tyr
RN4220-R <sub>9</sub>	16	256	CAT→TAT (His481→Tyr)	His526→Tyr
BM4365-R	In vivo	256	CAT→TAT (His481→Tyr)	His526→Tyr
BM4364-R	In vivo	256	CAT→TAT (His481→Tyr)	His526→Tyr
BM4366-R	In vivo	128	CGT→CAT (Arg484→His)	Arg529→Cys/Ser
BM4627	In vivo	128	GAT→GGT (Asp550→Gly)	ND <sup>c</sup>

<sup>a</sup> The underscores indicate the base changes.

<sup>b</sup> Deletion.

<sup>c</sup> ND, not described.

*bacterium leprae* (12). However, replacement of alanine at position 477 by a valine led to low-level resistance (mutant RN4220-R<sub>1</sub>), and replacement of alanine at position 477 by an aspartate led to high-level resistance (mutants RN4220-R<sub>3</sub> and BM4368-R<sub>1</sub>). This is not surprising since alanine and valine are both hydrophobic amino acids, whereas aspartate is acidic. It thus appears that the level of rifampin resistance in *S. aureus* depends not only on the position of the mutation but also on the nature of the new amino acid.

Relative to the corresponding susceptible strain, BM4368, resistant clinical isolates BM4368-R<sub>1</sub>, selected under rifampin therapy (1,800 mg per day), and BM4368-R<sub>2</sub>, obtained 3 days after the end of the treatment, had single base pair changes that resulted in a different level of rifampin resistance. They shared the same mutation as in vitro low-level-resistant mutant RN4220-R<sub>2</sub> and high-level-resistant mutant RN4220-R<sub>3</sub>, respectively (Table 2). The low- and high-level-resistant mutants characterized in the present study are adequately categorized by using the breakpoints based on the population distribution of a large number of clinical isolates (Fig. 1).

Mutations at positions 468 and 481 led to resistance to very high concentrations of rifampin and might point to amino acid changes in the  $\beta$  subunit that prevent the binding of rifampin to RNA polymerase. Indeed, it has been shown in *E. coli* that residues 516 to 540 (positions 471 to 495 in Rif region cluster I; *S. aureus* coordinates) are part of the target of rifampin (28) and participate, along with residues 1065 and 1237 (*E. coli* coordinates), in the formation of the initiation site when the  $\beta$  subunit is assembled in the RNA polymerase complex (21).

We also sequenced the *rpoB* region, from codons 94 to 144, which corresponds to the region containing a Rif mutation (Val146/Phe) in *E. coli* (22). None of the 17 *S. aureus* mutants examined in the present study had substitutions in their N-terminal cluster, which is distant, by almost 400 amino acids, from the central Rif region. This mutation, not detected in subsequent studies, is probably rare but suggests that the amino acid residue at position 146 and the central Rif region might jointly form the rifampin binding site (21).

In conclusion, using gene amplification and sequencing, we have established that rifampin resistance in *S. aureus* is probably due to mutations in the Rif region of the *rpoB* gene and

that the resistance levels are dependent on both the location and the nature of the amino acid substitution. With one exception, the resistance mutations detected in *S. aureus* were identical to or were in the same codon as those in other eubacteria, confirming that the regions implicated in the interaction with rifampin are conserved among prokaryotes.

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