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Cross-Resistance of *Escherichia coli* RNA Polymerases Conferring Rifampin Resistance to Different Antibiotics

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In this study we further defined the rifampin-binding sites in *Escherichia coli* RNA polymerase (RNAP) and determined the relationship between rifampin-binding sites and the binding sites of other antibiotics, including two rifamycin derivatives, rifabutin and rifapentine, and streptolydigin and sorangicin A, which are unrelated to rifampin, using a purified in vitro system. We found that there is almost a complete correlation between resistance to rifampin (Rif^r) and reduced rifampin binding to 12 RNAPs purified from different *rpoB* Rif^r mutants and a complete cross-resistance among the different rifamycin derivatives. Most Rif^r RNAPs were sensitive to streptolydigin, although some exhibited weak resistance to this antibiotic. However, 5 out of the 12 Rif^r RNAPs were partially resistant to sorangicin A, and one was completely cross-resistant to sorangicin A, indicating that the binding site(s) for these two antibiotics overlaps. Both rifampin and sorangicin A inhibited the transition step between transcription initiation and elongation; however, longer abortive initiation products were produced in the presence of the latter, indicating that the binding site for sorangicin A is within the rifampin-binding site. Competition experiments of different antibiotics with ^{3}H-labeled rifampin for binding to wild-type RNAP further confirmed that the binding sites for rifampin, rifabutin, rifapentine, and sorangicin A are shared, whereas the binding sites for rifampin and streptolydigin are distinct. Because Rif^r mutations are highly conserved in eubacteria, our results indicate that this set of Rif^r mutant RNAPs can be used to screen for new antibiotics that will inhibit the growth of Rif^r pathogenic bacteria.

RNA polymerase (RNAP) is the sole enzyme responsible for transcribing RNA from DNA template in eubacteria (3). Because of its essential role in gene expression, RNAP has been a target for antibiotic studies since its discovery in the 1960s. Several antibiotics inhibiting the functions of RNAP have been discovered. Among them, rifampin, a derivative of rifamycin (35), is the most important in clinical use (22, 33). Rifampin is part of the standard therapy of tuberculosis (8) which, after AIDS, is the leading cause of death by an infectious agent worldwide (23, 32) and is also used in prophylaxis of meningitis and against staphylococcal infections (19, 20).

Rifampin binds to RNAP with high affinity ($K_{eq} = 10^{-9}$ M at 37°C) (39). The mode of action of rifampin has been studied in most detail using *Escherichia coli* RNAP as a model system, as the overall structure and function of RNAPs from different eubacteria have been conserved. Rifampin inhibits RNAP's function by blocking the transition from transcription initiation to transcription elongation (24). In the presence of rifampin, RNAP can only synthesize short RNA oligomers, and it was proposed that rifampin exerts a steric hindrance of RNAP translocation along the nascent RNA path. Indeed, cross-linking experiments have indicated that rifampin blocks the channel leading a nascent RNA out of the catalytic center of RNAP (26).

Mutations in *E. coli* RNAP conferring rifampin resistance (Rif^r) were reported shortly after the antibiotic was discovered (7, 30). Rif^r mutations have been located exclusively on the second largest subunit of RNAP, the β peptide, encoded by the *rpoB* gene. Except for one located around the 5′ end, most of the Rif^r mutations in *E. coli* are found in three clusters near the middle of the *rpoB* gene and affect a limited amino acid segment of the β subunit (15, 21, 28, 36). Genetic evidence indicates that amino acid residues in cluster I and cluster III interact, forming the rif region (37). These mutations define the rifampin-binding sites in RNAP genetically.

Recently, the crystal structure of *Thermus aquaticus* core RNAP complexed with rifampin has been determined in the presence of a high concentration of rifampin, because *T. aquaticus* RNAP is naturally Rif^r (4). Several conserved amino acid residues in the rif region have been identified that interact with the antibiotic, which adequately accounts for all known Rif^r mutants. Rifampin binds to the rif region of the β subunit, which lies deep within the DNA-RNA channel. Clearly, the critical location of the rif region in RNAP is responsible for the multiple effects of Rif^r mutations on different aspects of transcription (13, 14, 18, 42).

Rif^r mutants from *E. coli* RNAP potentially could be used as a model system to screen new generations of antibiotics which inhibit the function of Rif^r mutants. It has been reported that clinical Rif^r isolates of a variety of pathogenic bacteria, including *Mycobacterium tuberculosis* and *Staphylococcus aureus*, affect a subset of the conserved amino acid residues within the rif region in the β subunit of RNAP, further demonstrating that

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the rif region is well conserved among eubacteria (2, 31). A new generation of rifamycins with improved pharmaceutical properties has been developed (22, 29). Among them are rifabutin and rifapentine. There are reports that Rif^r mutant RNAPs are only partially cross-resistant to rifabutin (6, 40). In addition, a new macrolide polyether antibiotic, sorangicin A, which has a different chemical structure from the rifamycins, has been described to be an inhibitor of RNAP (11). Interestingly, it was reported that Rif^r mutant RNAP are partially cross-resistant to sorangicin A (27, 34).

We are interested in further defining the rifampin-binding sites in *E. coli* RNAP and in studying the mechanism(s) of cross-resistance of Rif^r mutant RNAP to different antibiotics. In particular, we would like to know which amino acid residue(s) in the rif region is responsible for the possible multiple drug interaction site(s) in RNAP. Using a set of purified *E. coli* Rif^r mutant RNAPs described previously (15), we first studied their ability to bind rifampin, because the effects of those *E. coli* Rif^r RNAP on the binding of rifampin have not been analyzed. We then determined the effects of two rifamycin derivatives, rifabutin and rifapentine, as well as two other antibiotics unrelated to rifampin, sorangicin A and streptolydigin, on the functions of these Rif^r mutant RNAP and on the rifampin binding of wild-type RNAP. The sites in RNAP that are likely involved in interaction with sorangicin A within the rif region are discussed.

MATERIALS AND METHODS

Materials. Nucleotides were from Boehringer Mannheim. ^{32}P- or ^{3}H-labeled nucleotides were from Amersham or ICN. Poly(dA · dT) was from Sigma. Activated carbon Darco (100 mesh) was from Aldrich, and dextran T 70 was from Pharmacia. Rifampin, rifapentine, and [^{3}H]rifampin (152 mCi/mmol) were from Lepetit, Italy. Rifabutin was a kind gift from Anthony R. Imondi, Adria Laboratories, and sorangicin A was obtained from Hans Reichenbach, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany. Streptolydigin was obtained from Upjohn Co. The stock solutions of these antibiotics were prepared in *N,N*-dimethyl formamide. The chemical structures of these antibiotics are shown in Fig. 1. Plasmids were isolated by QIAGEN column (QIAGEN Inc.), and DNA fragments were purified as described elsewhere (12). Phage T7A1 promoter was from a PvuII fragment of pRL418, which was a gift from Robert Landick (University of Wisconsin—Madison). The DNA fragments containing the *gal* and *pyrBI* promoters were previously described (12, 17). RNAPs were purified from *E. coli* K-12 MG1655 derivatives by using Mono Q high-resolution ion-exchange chromatography (9).

Bacterial strains and techniques. Mutants used in this study are in an *E. coli* K-12 MG1655 background, originally obtained from Carol Gross' lab. The *rpoB* mutations were introduced into strain MG1655 by phage P1-mediated transduction using a linked Tn*10* as described previously (25). The mutations are described in Table 1.

Cells were grown in L broth (25). To determine the MICs of rifamycin derivatives for wild-type and Rif^r mutant strains, overnight cultures were diluted into Luria-Bertani broth containing different concentrations of antibiotics, and cell growth was followed at both 30 and 37°C. Spontaneous mutants conferring sorangicin A resistance (Sor^r) were selected by plating 2×10^8 to 5×10^8 cells from overnight cultures on L broth plates containing 50 μg of sorangicin A/ml, followed by incubation at 30 or 37°C for 24 to 48 h. To ensure independent events, usually only one Sor^r mutant was picked from each culture. The mutation rate was about 2×10^{-8}. The purified Sor^r mutants were then scored for their growth on L broth plates containing 50 μg of rifampin/ml. To score the Sor^r phenotype of the Rif^r mutants, growth of the Rif^r mutant strains on L broth plates containing different amounts of sorangicin A ranging from 50 to 500 μg/ml was determined.

In vitro transcription assays. The in vitro transcription assays were performed essentially as described elsewhere (16). Reaction mixtures (100 μl) containing 40 mM Tris-HCl (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 5% glycerol, acetylated bovine serum albumin (100 μg/ml), poly(dA · dT) (2 μg), and RNAP at ~2 nM, with or without antibiotics, were preincubated for ≥10 min at 37°C. The reaction was started by the addition of ATP and UTP (final concentrations, 0.2 mM), including 1 μCi of [^{3}H]-labeled UTP, and stopped after 15 min at 37°C by the addition of 1 ml of ice-cold 5% trichloroacetic acid. Transcription was monitored by the incorporation of [^{3}H]UTP into trichloroacetic acid-precipitable counts.

To analyze the transcription products in the presence of different antibiotics, the transcription assays (in 20 μl) were performed essentially as described above, except that different DNA templates were used and the antibiotic was present during the preincubation period. ATP, GTP, and CTP were present at 0.2 mM and UTP was at 0.02 mM, including ~5 μCi of ^{32}P-labeled UTP. After 15 min, reactions were terminated by addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.1% bromophenol, and 0.1% xylene cyanol) and analyzed on 24 and 8% sequencing gels (National Diagnostics) for productive and nonproductive products, respectively, as described elsewhere (12). The transcripts were visualized by autoradiography.

Determination of rifampin-RNAP complex. The rifampin-RNAP complexes were monitored using the dextran-coated charcoal method (41), with some modifications. The dextran-coated charcoal was prepared as described previously (41) and stored at 4°C. For the binding assays, reaction mixtures (200 μl in a siliconized microcentrifuge tube) containing 10 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol, acetylated bovine serum albumin (100 μg/ml), a fixed concentration of [^{3}H]rifampin (~250 nM), and the indicated concentrations of RNAPs were incubated for 10 min at 37°C. After adding 0.5 ml of ice-cold dextran-coated charcoal, reaction mixtures were mixed well and centrifuged at 12,000 rpm for 2 min in an Eppendorf microcentrifuge. About 0.55 ml of supernatant was carefully transferred into a new microcentrifuge tube and subjected to centrifugation again as described above. A 0.5-ml aliquot of clear supernatant was placed into scintillation vials containing 10 ml of Aquasol liquid scintillation fluid and counted with a scintillation counter for 1 min. Preliminary experiments showed the binding of rifampin to RNAP was stoichiometric (~1:1), and the nonspecific binding of rifampin to bovine serum albumin (0.1 mg/ml) was minimal (<3% of the total rifampin remained in the charcoal supernatant). To determine the inhibitory effects of different antibiotics on the binding of rifampin to wild-type RNAP, the procedures as described above were used with the following exceptions: a fixed concentration of [^{3}H]rifampin (~250 nM) and differing concentrations of a second cold antibiotic were presented in the reaction mixture prior to the addition of a fixed concentration of wild-type RNAP (~75 nM).

RESULTS

Binding and sensitivity to rifampin of different Rif^r mutant RNAP. Previously, the rif region was identified by sequencing a large number of Rif^r mutants of *E. coli* having a variety of phenotypes. Thus, this rif region defines the rifampin-binding site in RNAP genetically. However, there has been no systematic study of the effects of Rif^r mutations affecting different positions in the rif region on the binding of the antibiotic to RNAP. We addressed this issue by determining the binding of ^{3}H-labeled rifampin, as a function of protein concentration, to 12 highly purified, previously well-characterized mutant Rif^r RNAPs covering mutations in the three clusters of the rif region (Fig. 2). In the binding assays, as described in Materials and Methods, the concentration of [^{3}H]rifampin (250 nM) was fixed, whereas the concentrations of RNAPs were varied from 10 to 75 nM. We followed the binding of [^{3}H]rifampin to RNAP (RNAP + [^{3}H]rifampin ↔ RNAP-[^{3}H]rifampin) by using the dextran-coated charcoal method (41). In this procedure, dextran-coated charcoal absorbs the free unbound [^{3}H]rifampin, since the antibiotic is a small molecule, whereas the larger-sized RNAP-[^{3}H]rifampin complex will remain in the supernatant. The wild-type RNAP bound rifampin, and the amount of bound rifampin increased in proportion to the amount of RNAP added, as reported elsewhere (41). Under the conditions used, the apparent K_d of the wild-type RNAP for rifampin is estimated to be about 130 nM, which is in good agreement with the K_i value (0.1 μM) reported previously (4).

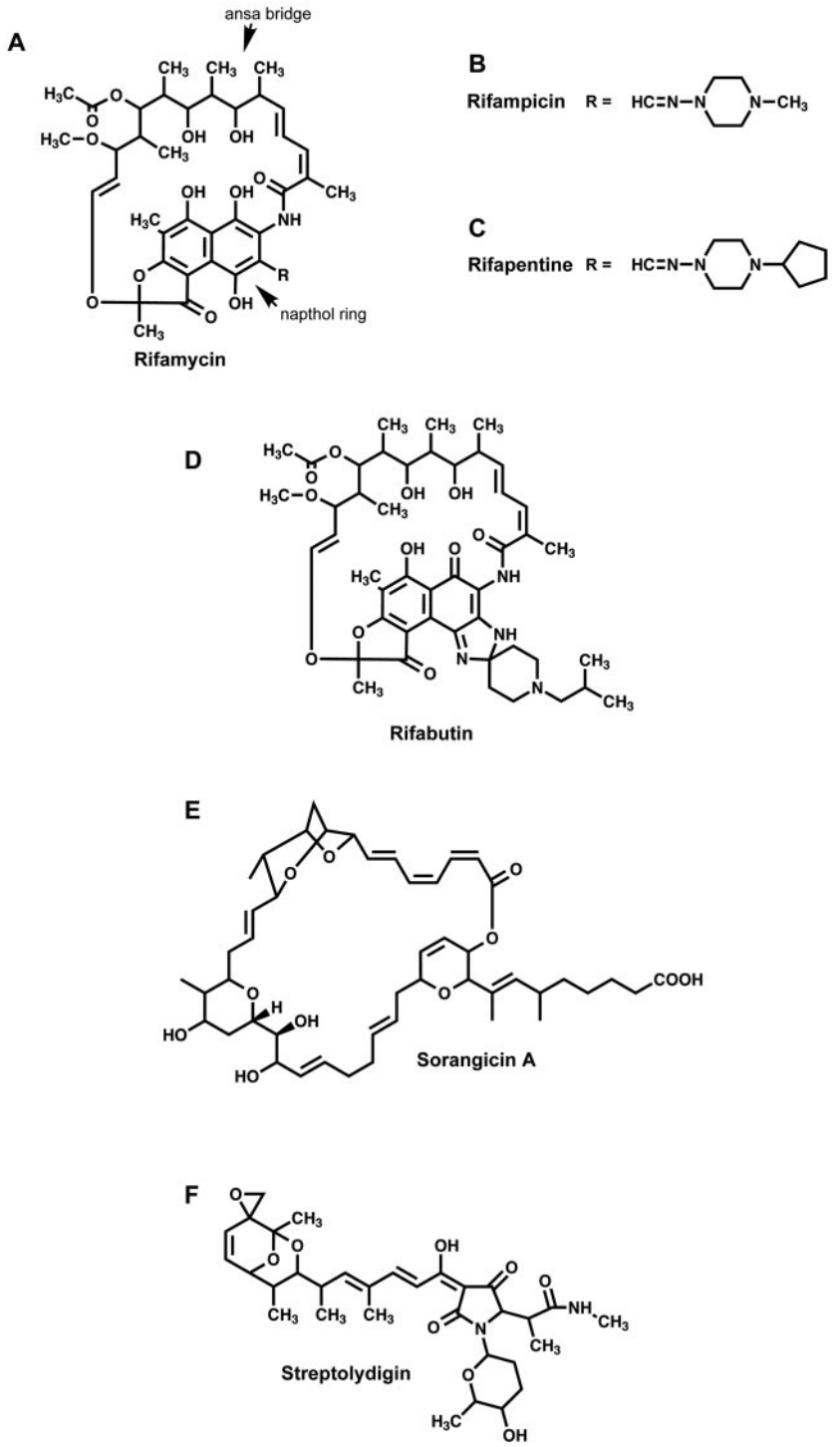

FIG. 1. Chemical structures of the different antibiotics used in the study. Rifampin (B), rifabutin (D), and rifapentine (C) are derivatives of rifamycin (A), which consists of ansa bridge and naphthol ring. Sorangicin A (E) and streptolydigin (F) are unrelated to rifamycins.

Interestingly, one Rif^r mutant RNAP, R687H, was able to bind rifampin as well as the wild-type RNAP in the assays. Six of the 12 Rif^r mutant RNAPs (D507-511, Q513L, Q513P, S522F, H526Y, and S531F, with approximate K_d values of 7, 20, 11, 8, 11, and 11 μM, respectively) barely bound [^{3}H]rifampin above the background, and there was essentially no increase in the binding as the amounts of the proteins increased. One mutant RNAP, P564L, with an approximate K_d of 5.0 μM, had minimal rifampin-binding ability, as its bind curve slope was only marginally above the background. The binding of rifampin to other four Rif^r mutant RNAP was reduced dramatically compared to that of the wild-type RNAP, and the amount of

TABLE 1. Resistance to different antibiotics of wild-type and different Rif^r RNAPs in vitro

rpoB allele	Amino acid residue affected	IC_{50} (μg/ml)[a]				
		Rifampin	Rifabutin	Rifapentine	Streptolydigin	Sorangicin A
Wild type		<0.5	<0.5	<0.5	2.5	<0.1
Cluster I[b]						
3445	Δ507-511	>100	>100	75.0	4.5	0.8
101	Q513L	>100	>100	>100	3.5	<0.1
8	Q513P	>100	>100	>100	5.0	1.2
113	D516N	8.0	6.0	5.0	3.5	0.5
3595	S522F	>100	>100	>100	3.5	1.2
2	H526Y	>100	>100	>100	1.5	>100
3401	R529C	5.0	5.0	5.0	3.0	<0.1
114	S531F	>100	>100	>100	5.0	<0.1
3449	Δ532	1.0	1.0	1.0	9.0	<0.1
Cluster II[b]						
3370	T563P	1.0	1.0	1.0	2.0	0.5
111	P564L	15.0	14.0	25.0	1.0	<0.1
Cluster III[b]						
3406	R687H	<0.5	<0.5	<0.5	3.0	<0.1

[a] IC_{50} is defined as the concentration of an antibiotic resulting in a 50% inhibition of transcription activity of an RNAP, determined using poly(dA · dT) as described in Materials and Methods.
[b] Cluster is a subregion within the rif region of the *rpoB* gene of *E. coli* (15).

rifampin bound increased slightly as the concentration of enzyme increased. The degree of rifampin binding of these four Rif^r RNAPs was in the following order: T563P = Δ532 (K_d ~1.5 μM) > R529C = D516N (K_d ~ 2.0 μM). It should be noted that except for wild-type RNAP and the mutant RNAP R687H, the binding activities of [^{3}H]rifampin to other Rif^r mutant RNAPs were measured under those conditions in which the concentration of [^{3}H]rifampin (250 nM) used were below their K_d values.

To determine whether the effects of the Rif^r RNAPs on the binding of rifampin correlate with their sensitivities to the antibiotic, we measured transcription activity of each RNAP as a function of the concentration of rifampin in an in vitro transcription assay. The concentrations of the antibiotic which inhibited RNA synthesis by 50% (IC_{50}) are presented in Table 1 for the 12 Rif^r mutant and the wild-type RNAPs. For the six Rif^r mutant RNAPs that did not bind rifampin, the IC_{50} values were the highest (IC_{50} > 100 μg/ml). For the mutant RNAP P564L, which only bound rifampin minimally, the IC_{50} was 15 μg/ml. For the four Rif^r mutant RNAPs that did retain some rifampin-binding activities, the IC_{50} values were lower (1.0 to 8.0 μg/ml) and in the order of T563P = Δ532 < R529C < D516N, which inversely correlates with their rifampin-binding capacities (i.e., the higher the IC_{50} the lower the affinity for rifampin). The only Rif^r RNAP, R687H, that bound rifampin as well as wild-type RNAP also had the same sensitivity to the antibiotic as the wild-type RNAP (IC_{50} < 0.5 μg/ml) in the assays.

These results from the in vitro transcription assays using the highly purified RNAPs generally agree with the results obtained previously by using partially purified mini preparations of these Rif^r RNAPs (15), with the exception of R687H. R687H is the Rif^r mutant that had the lowest resistance to rifampin in vivo, and in the previous in vitro transcription assays R687H exhibited only slight resistance to rifampin (15). We also rechecked the *rpoB3406* mutant cells, from which the larger preparation of the R687H RNAP was purified in this study, for their Rif^r phenotype and other growth phenotypes, and found that they could only grow on a broth plate containing ≤50 μg of rifampin/ml and were slow growers, temperature sensitive, and cold sensitive, as previously described (14). Furthermore, the purified R687H RNAP did exhibit some altered properties in transcription initiation and elongation compared to wild-type RNAP (unpublished data).

Cross-resistance of the Rif^r mutant RNAPs to different antibiotics. The sensitivities of different Rif^r RNAPs to two other rifamycins, rifabutin and rifapentine, and to streptolydigin and sorangicin A, two molecules which have different chemical structures (Fig. 1), were studied using the in vitro transcription assays as described above. The IC_{50} of each antibiotic for each of the 12 Rif^r mutant and wild-type RNAPs are shown in Table 1. There was complete cross-resistance between rifampin, rifabutin, and rifapentine. The extent of resistance for each RNAP, as measured by IC_{50}, was similar for the three rifamycin derivatives. The mutant RNAP R687H, which was completely sensitive to rifampin in vitro, was also as sensitive to rifabutin and rifapentine as the wild-type RNAP (Table 1). The sensitivity of the wild-type and the different Rif^r mutant strains to the three rifamycin derivatives were also compared in vivo, and the MICs for cell growth are presented in Table 2. Essentially, there was a complete correlation between the in vivo and in vitro results, with R687H being the only exception. The R687H mutant exhibited a low-level resistance to all three rifamycin derivatives tested in vivo but was sensitive to the antibiotics in vitro (compare Table 1 and 2).

The IC_{50} value of streptolydigin for wild-type RNAP (2.5 μg/ml) was higher than that of the rifamycins (<0.5 μg/ml) (Table 1). Although there were some small variations, most Rif^r mutant RNAPs had sensitivities to streptolydigin similar to that of wild-type RNAP. Only the mutant RNAP Δ532

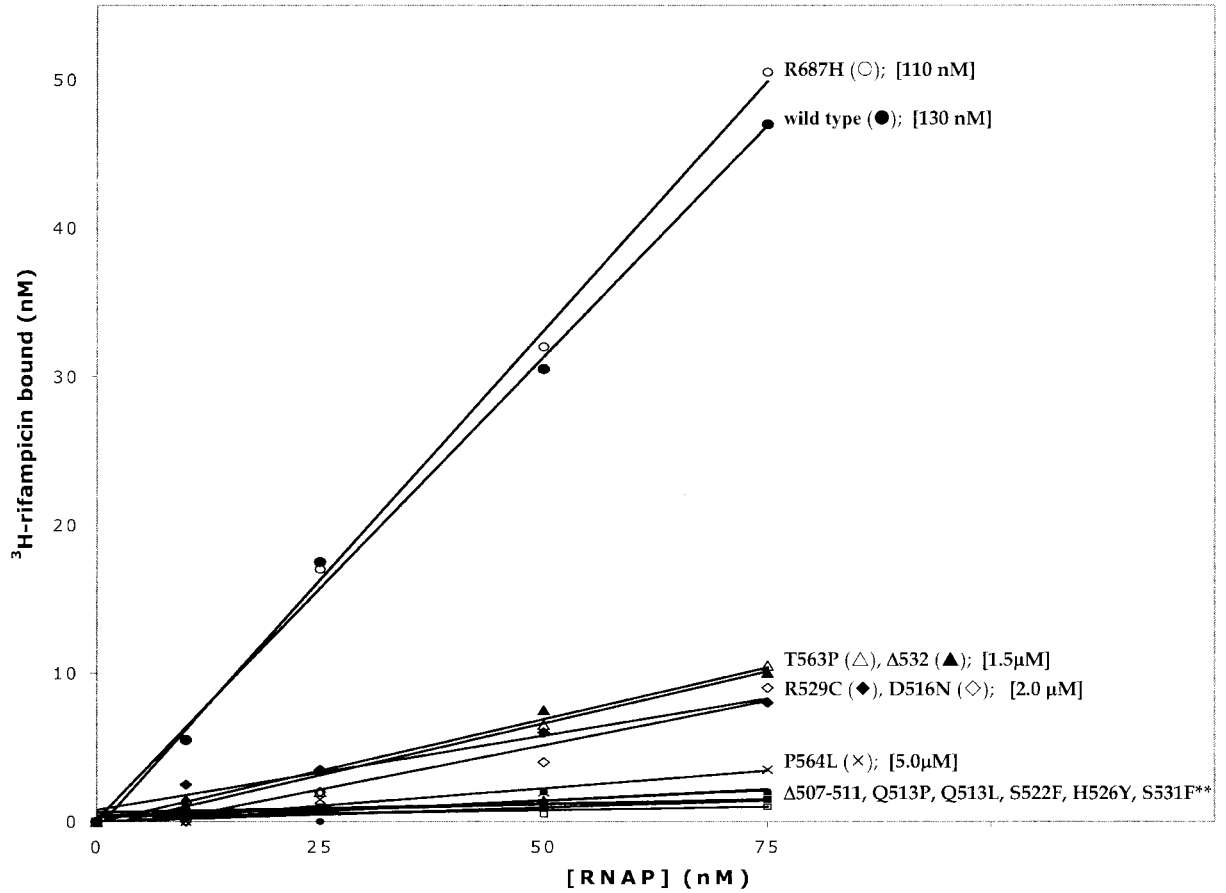

FIG. 2. Binding of [^{3}H]rifampin to different RNAPs as a function of the concentrations of the proteins. Formation and determination of the [^{3}H]rifampin-RNAP complexes were as described in Materials and Methods. In each reaction mixture, the total concentration of [^{3}H]rifampin present was ~250 nM and the concentration of each RNAP added was as indicated. The apparent K_d of each RNAP binding to rifampin is indicated, with the exceptions of those designated by **, which have K_d values ranging from 7 to 20 μM (see text for details). The results presented were from a typical set of assays. At least two sets of assays were performed for each enzyme, and similar results were obtained.

appeared to be significantly different from wild-type RNAP, and it was more resistant to streptolydigin (IC_{50} = 9 μg/ml). In this mutant, amino acid residue 532 of the β subunit of RNAP is deleted; this may determine an altered interaction with streptolydigin. Note that cross-resistance of Rif^r and Stl^r has been reported in some of the *rpoB* mutants of *S. aureus* (27).

Sorangicin A inhibited transcription by wild-type RNAP (IC_{50} < 0.1 μg/ml) (Table 1). Six of the 12 Rif^r mutant RNAPs were more resistant to the inhibitory effect of sorangicin A, and the other six RNAPs were as sensitive to the antibiotic as was the wild-type RNAP. There seems to be no correlation between the levels of resistance to rifampin and levels of resistance to sorangicin A. Q513L and S531F, which were resistant to high levels of rifampin (IC_{50} > 100 μg/ml), were as sensitive to sorangicin A as wild-type RNAP. Among the six Rif^r mutant RNAPs that exhibited cross-resistance to the two antibiotics, two RNAPs (T563P and D516N) which had low IC_{50} values to rifampin also exhibited low IC_{50} values to sorangicin A (≤0.5 μg/ml); three RNAPs (Δ507-511, Q513P, and S522F) which were resistant to high levels of rifampin (IC_{50} > 100 μg/ml) were only partially resistant to sorangicin A (IC_{50} ≤ 1.2 μg/ml); only one mutant RNAP, H526Y, was resistant to very high levels of both antibiotics (IC_{50} > 100 μg/ml), exhibiting a complete cross-resistance.

To determine whether these Rif^r *rpoB* mutations also confer resistance to sorangicin A in vivo, we checked the growth phenotypes on L broth plates containing different amounts of the antibiotic (Table 3). *E. coli* K-12 is rather impermeable to sorangicin A, just as in the case of rifampin. Thus, the wild-type *rpoB*$^+$ cells could grow on an L broth plate containing low levels of sorangicin A (≤20 μg/ml). Those *rpoB* mutants that contained Rif^r RNAPs sensitive to sorangicin A in vitro (Table 1) all behaved like the wild-type cells and could not grow on the plates containing 50 μg of sorangicin A/ml, whereas the six *rpoB* mutants that contained Rif^r RNAPs resistant to sorangicin A in vitro (Table 1) were all able to grow at this concentration, exhibiting Sor^r phenotypes. However, at higher concentrations of sorangicin A, some of these *rpoB* mutants failed to grow, and only the H526Y mutant grew at the highest concentration of sorangicin A tested (500 μg/ml). The extent of resistance to sorangicin A of the six *rpoB* mutants is in the following order: T563P < D516N = Δ507-511 < Q513P = S522F < H526Y. This order from the in vivo results appears to correlate well with the order from the in vitro results (compare

TABLE 2. Sensitivity of wild-type and Rif^r mutant strains to different rifamycin derivatives in vivo

rpoB allele	Amino acid residue affected	MIC (μg/ml)[a]					
		Rifampin		Rifabutin		Rifapentine	
		30°C	37°C	30°C	37°C	30°C	37°C
Wild type		4–8	8–16	4–8	1–4	4–8	16–32
Cluster I							
3445	Δ507-511	>128	>128	>128	>128	>128	>128
101	Q513L	>128	>128	>128	>128	>128	>128
8	Q513P	>128	>128	>128	>128	>128	>128
113	D516N	>128	>128	64–128	64–128	>128	>128
148	D516V	>128	>128	128	>128	>128	>128
3051	Ω517DQ	128	>128	32–64	128	>128	>128
3595	S522F	>128	>128	128	128	>128	>128
2	H526Y	>128	>128	>128	>128	>128	>128
3401	R529C	>128	>128	16–32	16–32	>128	>128
3402	R529S	>128	>128	64–128	32–64	>128	>128
114	S531F	>128	>128	>128	>128	>128	>128
3449	Δ532	>128	>128	64–128	64–128	>128	>128
3443	L533P	>128	>128	128	>128	>128	>128
Cluster II							
3370	T563P	64–128	>128	16–32	64	128	>128
111	P564L	>128	>128	128	>128	>128	>128
7	I572F	>128	>128	128	>128	>128	>128
Cluster III							
3406	R687H	64	128	8–16	16	8–16	128

[a] The MIC values were determined as described in Materials and Methods.

Tables 1 and 3). Five additional previously described Rif^r *rpoB* mutants, D516V, Ω517DQ, R529S, L533P, and I572F, which were not included in the IC_{50} experiments in vitro, were tested for in vivo cross-resistance to sorangicin A; three of them were partially cross-resistant to the antibiotic at relatively low levels (≤100 μg/ml) (Table 3). It is likely that the Rif^r RNAPs from these three *rpoB* mutants would also be resistant to relatively low levels of sorangicin A in vitro.

To determine whether mutants selected on sorangicin A are resistant to rifampin, we isolated 125 independent Sor^r mutants from MG1655, an *E. coli* K-12 strain. We screened the Sor^r mutants for their Rif^r phenotypes and found that all of the Sor^r mutants simultaneously acquired the Rif^r phenotype. Therefore, in contrast to Rif^r mutants, only some of which are cross-resistant to sorangicin A, all Sor^r mutants appear to be cross-resistant to rifampin.

Effects of different antibiotics on the binding of rifampin to wild-type RNAP. The cross-resistance of Rif^r mutant RNAPs to different antibiotics indicates that the binding sites for these antibiotics are overlapping. We therefore expected those antibiotics to interfere with the binding of rifampin to wild-type RNAP as competitive inhibitors. Thus, we compared the binding of [^{3}H]rifampin to RNAP in the presence of a particular nonradioactive antibiotic with that in the absence of the challenging antibiotic, and the effects of different antibiotics on the binding of rifampin to wild-type *E. coli* RNAP are shown in Fig. 3. When unlabeled rifampin was added to the reaction mixture, the amount of radioactive rifampin bound to RNAP was reduced in a concentration-dependent manner. When equal amounts of unlabeled rifampin and [^{3}H]rifampin were present, radioactivity bound was reduced about 50% as ex-

TABLE 3. Resistance to sorangicin of different Rif^r mutant strains in vivo[a]

rpoB allele	Amino acid residue affected	Resistance to sorangicin A at (μg/ml):			
		50	100	250	500
Wild type		−	−	−	−
Cluster I					
3445	Δ507-511	+	+/−	−	−
101	Q513L	−	−	−	−
8	Q513P	+	+	+/−	−
113	D516N	+	+/−	−	−
148	D516V	+	−	−	−
3051	Ω517DQ	+	+/−	−	−
3595	S522F	+	+	+/−	−
2	H526Y	+	+	+	+
3401	R529C	−	−	−	−
3402	R529S	−	−	−	−
114	S531F	−	−	−	−
3449	Δ532	−	−	−	−
3443	L533P	−	−	−	−
Cluster II					
3370	T563P	+	−	−	−
111	P564L	−	−	−	−
7	I572F	+	−	−	−
Cluster III					
3406	R687H	−	−	−	−

[a] Cell growth was monitored after incubation for 24 h at 37°C on L broth plates containing the specified amount of sorangicin A. +, colonies had the same size as those on L broth plate without sorangicin A; +/−, colonies had significantly reduced size (≤50%) compared to that on L broth plate without sorangicin A; −, no growth. All strains grew on Luria-Bertani medium in the absence of sorangicin A.

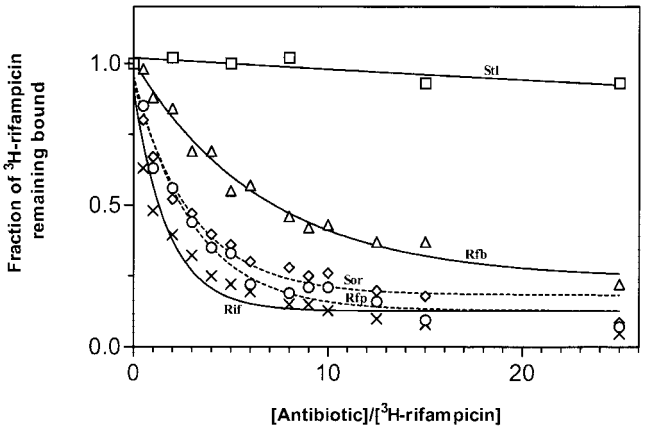

FIG. 3. Effects of different antibiotics on binding of [^{3}H]rifampin to wild-type RNAP. The experiments were performed essentially as described in the legend to Fig. 2, with some modifications as described in Materials and Methods. A fixed concentration of [^{3}H]rifampin (~250 nM) and differing concentrations of a second nonradioactive antibiotic (relative to the concentration of [^{3}H]rifampin) (antibiotic concentration/[^{3}H]rifampin concentration) were mixed prior to the addition of a fixed concentration of wild-type RNAP (~75 nM). In the absence of nonradioactive antibiotics, RNAP formed complexes with [^{3}H]rifampin; the value was designated as 1.0 (100%) binding. The remaining fraction of the [^{3}H]rifampin-RNAP complexes was plotted as a function of increasing ratio of antibiotic concentration/[^{3}H]rifampin concentration in the assay mixtures. ×, Rif rifampin; ○ Rfp, rifapentine; △ Rfb, rifabutin; ◇ Sor, sorangicin A; □ Stl, streptolydigin. Since the approximate K_d of wild-type RNAP binding to rifampin is 130 nM (Fig. 2), to account for the observed inhibition curves approximate K_d values for the binding of rifabutin, rifapentine, and sorangicin A were estimated to be 780, 260, and 325 nM, respectively. The results presented were from a typical set of assays. At least two sets of assays were performed for each antibiotic, and both assays were in close agreement.

pected. Streptolydigin had no effect on rifampin binding to RNAP, indicating that the binding sites for the two antibiotics are distinct. This is consistent with the reports that the mutations conferring streptolydigin resistance are located at different positions, although close to the Rif^r mutations, in the β subunit of RNAP (10, 36).

As expected, both rifabutin and rifapentine inhibited the binding of rifampin to RNAP. However, they competed with radioactive rifampin somewhat less effectively than rifampin itself, requiring approximately sixfold and twofold excesses, respectively, to inhibit rifampin binding by 50%. Interestingly, sorangicin A inhibited the binding of rifampin very effectively. A 50% inhibition of the binding of rifampin to wild-type RNAP was achieved with about a 2.5-fold excess of sorangicin A relative to the radioactive rifampin. Because the approximate K_d of rifampin binding to wild-type RNAP is 130 nM (Fig. 2), it is estimated that the approximate K_d values for the binding of rifabutin, rifapentine, and sorangicin A are 780, 260, and 325 nM, respectively. Thus, the inhibition of rifampin binding to RNAP by sorangicin A was almost as effective as that of rifapentine and more effective than that of rifabutin.

Mode of action of sorangicin A. Although it was reported that sorangicin A inhibits RNA synthesis (11), the step(s) at which transcription is blocked by sorangicin A has not been defined. We investigated whether the mode of inhibition by sorangicin A is the same as that of rifampin by analyzing transcription products from several DNA templates. In the absence of any antibiotic, RNAP made both nonproductive initiation products and productive full-length transcripts at several DNA templates used in the assays (Fig. 4). Operationally, rifampin inhibited the transition between transcription initiation and transcription elongation, since RNAP only synthesized abortive products (small-sized RNA oligomers) and produced no productive full-length transcripts from different promoters in the presence of rifampin. In the presence of sorangicin A, RNAP also made abortive products and made none of the productive full-length transcripts from these DNA templates. However, there were some noticeable subtle differences between sorangicin A and rifampin. For example, on a poly(dA · dT) template, RNAP was able to make a few additional longer aborted products (5-mer and 6-mer) in the presence of sorangicin A, which were absent in the presence of rifampin (mostly 3-mers were made with rifampin). This difference is not limited to the synthetic DNA template, because RNAP also made an extra longer RNA oligomer (AAUUU) at the *pyrBI* promoter in the presence of sorangicin A compared to that in the presence of rifampin. Nevertheless, both rifampin and sorangicin A prevented RNAP from entering the elongation mode. For comparison we also analyzed the transcription products in the presence of other antibiotics. The same small-sized RNA oligomers were made in the presence of the other two rifamycins as in the presence of rifampin. Interestingly, however, there was a subtle difference in the distribution of the set of aborted products in the presence of rifabutin (for example, more 4-mer and AAUU were made) compared to that in the presence of rifampin or rifapentine, on both the *pyrBI* and poly(dA · dT) templates. As expected, streptolydigin had no effect on nonproductive initiation and only inhibited elongation, resulting in reduced production of the full-length transcripts.

DISCUSSION

The analysis of a set of purified Rif^r RNAPs covering the rif region for their sensitivity and ability to bind rifampin establishes that there is a complete correlation between levels of resistance to rifampin and reduced capacity to bind the antibiotic (Tables 1 and 2 and Fig. 2), with the exception of the mutant RNAP R687H. This study confirms that the rifampin-binding site(s) lies within the sites identified by these *rpoB* mutations. The mutant RNAP R687H purified from the *rpoB3406* mutant was found to be as sensitive to rifampin and as able to bind to rifampin as the wild-type RNAP. Note that R687H is the only mutation mapping in the cluster III of the rif region, which is far away from the other two clusters. However, genetic evidence suggested that amino acid residue 687 interacts with amino acid residue 529 of the β subunit of RNAP, a site in cluster I of the rif region (37). Also, R687H is the only mutant with the ability to grow on L broth plates containing only very low concentrations of rifampin (15). It is possible that such a weak resistance cannot be detected in our in vitro assays. Alternatively, the R687H mutation might alter the permeability of cell walls.

Our results showed that the amino acid residues 507-511, 513, 522, 526, and 531 of the *E. coli* β subunit are important for

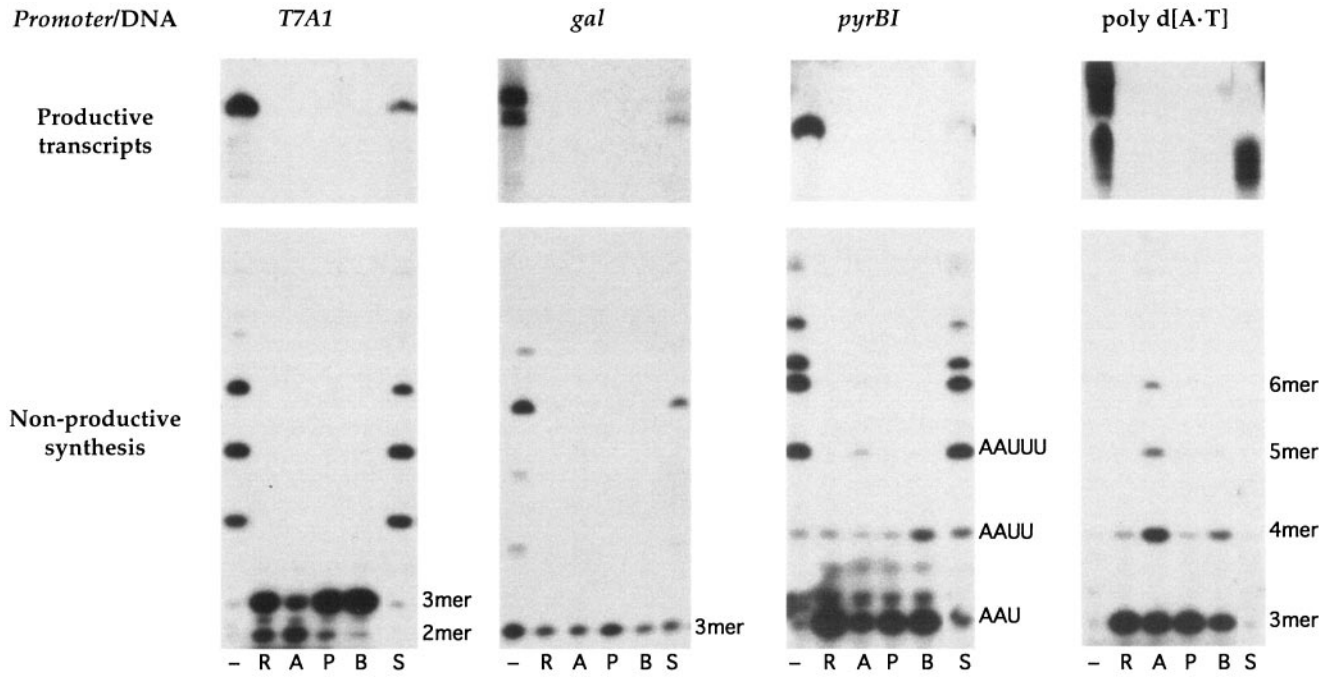

FIG. 4. Transcription products in the presence of different antibiotics. Transcription was performed either in the absence (-) or in the presence of a 50-μg/ml concentration of the indicated antibiotic as described in Materials and Methods. The nonproductive initiation products were analyzed on a 24% gel, and the productive full-length transcripts were analyzed on an 8% gel, followed by autoradiography. R, rifampin; A, sorangicin A; P, rifapentine; B, rifabutin; S, streptolydigin. The estimated abortive initiation products from each promoter in the presence of rifamycins and sorangicin A are indicated.

the binding of rifampin, since changes in these positions lead to a high level of resistance and no binding of rifampin (Fig. 5). Intriguingly, among these residues, only three of the equivalents in the *T. aquaticus* RNAP β subunit are in direct contact with the rifampin in the structure of the *T. aquaticus* RNAP-rifampin complex (4). On the other hand, changes in the amino acid residues 516, 529, 532, and 563 of the *E. coli* β subunit lead to a low level of resistance and fractional binding of rifampin. However, among these, two of the equivalents in the *T. aquaticus* RNAP β subunit are in direct contact with the rifampin in the structure of the *T. aquaticus* RNAP-rifampin complex. It is possible that the microenvironments of the rif region are different in the two bacterial RNAPs, as the *T. aquaticus* RNAP is Rif^r and the *E. coli* RNAP is Rif^s. A

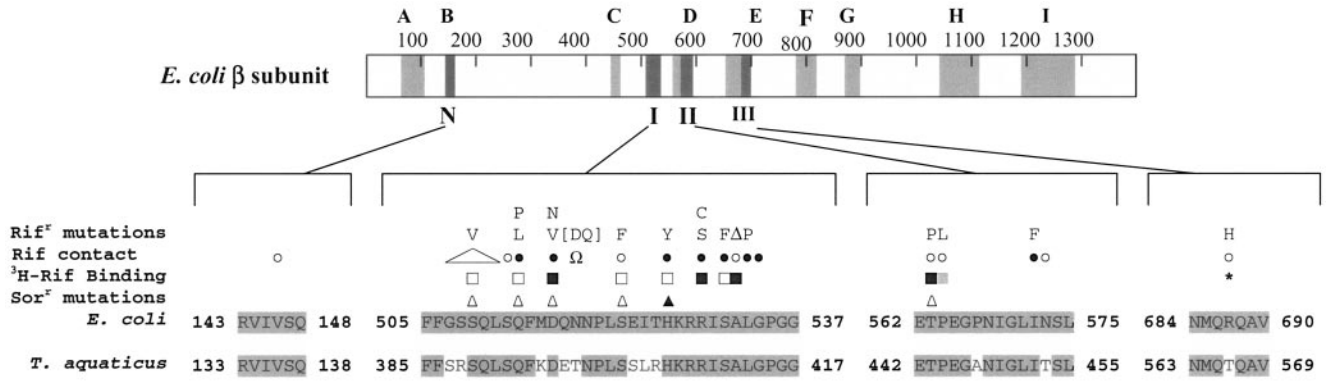

FIG. 5. rif region of the *E. coli* RNAP β subunit. A graphic representation of the *E. coli* β subunit is shown, with the lightly shaded areas indicating the evolutionally conserved regions A through I (1, 38) and the darkly shaded areas highlighting the four regions in which Rif^r mutations have been located: the N-terminal cluster (N) and clusters I, II, and III (15, 21, 28, 36). The sequence alignment for these four clusters from *E. coli* and *T. aquaticus* is located below the diagram, with identical amino acids shaded in grey. The nature of the Rif^r mutations is denoted as follows: Δ, deletion; Ω, insertion; circle, amino acid substitution. Only the substitutions for the Rif^r mutations used in this study are shown. ●, residue that has direct contact to bound rifampin in *T. aquaticus* RNAP (4); ○, residue that does not directly interact with bound rifampin due to distance. The strength of [^{3}H]rifampin binding is expressed as follows: open square, binding around baseline levels; lightly shaded square, binding slightly above baseline; filled square, significant binding. An asterisk indicates binding equivalent to that of the wild-type RNAP. The strength of resistance to sorangicin A of different Rif^r mutants is similarly indicated, as follows: Δ, low-level resistance; ▲, high-level resistance; no triangle, Sor^s.

structural study of the *E. coli* RNAP-rifampin complex is necessary for further understanding the rifampin-binding sites.

The studies of cross-resistance of different Rif^r RNAPs to several antibiotics and of the effects of different antibiotics on rifampin binding to RNAP reveal the relationship between the rifampin-binding sites and the other antibiotic-binding sites in RNAP. We found that there is essentially a complete cross-resistance between rifampin and two other rifamycin derivatives. This is in contrast to other reports (6, 40). Our results indicate that the binding sites for all three rifamycin derivatives are the same in RNAP. However, the efficiencies in competition for rifampin binding sites are reduced by about two- and sixfold relative to rifampin for rifapentine and rifabutin, respectively. This correlates well with the fact that rifabutin has a relatively larger modification on the naphthol ring of rifamycin than rifapentine (Fig. 1). The MICs of the three rifamycin derivatives to wild-type *E. coli* cells are essentially the same at 30°C, although it appears that some Rif^r mutants exhibit lower MICs of rifabutin than of the other two rifamycin derivatives in vivo.

Although the chemical structures of rifampin and sorangicin A are different (Fig. 1), the binding sites for these two antibiotics overlap since sorangicin A competes effectively for the binding of rifampin to RNAP and their modes of action are essentially the same. Indeed, recently the structure of the *T. aquaticus* RNAP-sorangicin complex has been determined, and it was found that rifampin and sorangicin bind RNAP in the same β subunit pocket (5). While all Sor^r mutants were resistant to rifampin, only some of the Rif^r RNAPs were resistant to sorangicin A, indicating that the binding sites for sorangicin A are within a subset of the rifampin-binding sites in RNAP (Fig. 5). This conclusion is consistent with the subtle differences in the synthesis of abortive initiation products in the presence of different antibiotics (Fig. 4). It is possible that sorangicin A-binding sites are further upstream of the active center compared to the rifampin-binding site, so that sorangicin A causes less steric hindrance than rifampin; thus, at some promoters, a slightly larger RNA oligomer(s) can be synthesized in the presence of sorangicin A than in the presence of rifampin. Interestingly, most of the Rif^r RNAPs that were resistant to sorangicin A were only resistant at low levels ($IC_{50} < 1.5$ μg/ml) in vitro, indicating that residues 513, 516, 522, 563, and probably 572 of the β subunit of RNAP are only peripherally involved in the binding of sorangicin A. Only one mutant enzyme, H526Y, was resistant to a high level of sorangicin A ($IC_{50} > 100$ μg/ml), indicating that amino acid residue 526 of the β subunit of RNAP is critical in the binding of the two antibiotics. The analysis of the three-dimensional structures of the two antibiotics complexed with *E. coli* RNAP should shed light on the potential groups or structures in the antibiotics that interact with the sites in the RNAP. Furthermore, because some of the Rif^r mutant RNAPs are only partially resistant to or sensitive to sorangicin A, this new antibiotic will be able to inhibit those Rif^r mutant RNAPs both in vivo and in vitro, a very desirable feature for some studies.

To develop a new generation of antibiotics that would inhibit Rif^r RNAPs is a challenging task. This study is an attempt to understand the cross-resistance of Rif^r RNAPs to different antibiotics. Our work indicates the usefulness of this set of Rif^r RNAPs to counter-screen in the future for such new potential antibiotics that have a mode of action different than that of rifampin.

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