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Rifampin-Resistant RNA Polymerase Mutants of *Chlamydia trachomatis* Remain Susceptible to the Ansamycin Rifalazil

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Stable, homotypic mutants of *Chlamydia trachomatis* for which MICs of rifampin and rifalazil are elevated were isolated by serial passage at sub-MIC concentrations of these compounds. An alternative approach, in which *Chlamydia* cells were incubated and subsequently passaged three times, all in the presence of the selective agent at concentrations above the MIC, appeared to be a more effective means of selecting for mutants. In every instance where an elevation in the MIC occurred, one or more mutations in the *rpoB* gene, encoding the rifampin binding site, were detected. With one exception, all *rpoB* mutants that contained a single mutation conferred lower levels of resistance than mutants containing multiple mutations. Some *rpoB* mutations conferred very high levels of resistance to rifampin, up to 512 μg/ml. In all cases, mutants remained susceptible to concentrations of rifalazil at or below 0.064 μg/ml. Thus, rifalazil, a compound that is extremely potent against *Chlamydia* wild-type cells (MIC of 0.00025 μg/ml), may also protect against the selection of mutants at physiologically achievable concentrations.

Rifalazil [3′-hydroxy-5′-(4-isobutyl-1-piperazinyl) benzoxazinorifamycin] is one of the newest generation of ansamycins (Fig. 1), compounds that inhibit bacterial RNA polymerases isolated from a wide variety of microorganisms. The ansamycins rifampin, rifabutin, and rifapentine have been utilized clinically predominantly as components of multiple-drug therapy, most commonly to treat tuberculosis (TB) patients (12). Rifalazil, otherwise known in the literature as KRM-1648 and, more recently, as ABI-1648, was originally developed by Kaneka Corporation, Osaka, Japan, through phase II clinical trials as an improved antimycobacterial agent to replace rifampin in the multidrug regimen for the treatment of TB (3, 12).

However, rifalazil also has extraordinary potency against both *Chlamydia pneumoniae* (6, 11) and *Chlamydia trachomatis* (11, 14). MICs have been reported in the range between 0.0025 and 0.00025 μg/ml, depending on some variation in methodology. All of the studies were in agreement that rifalazil was considerably more potent in cell culture than any compound that has advanced to human clinical trials. The MICs of rifalazil were reported to be 50 to 500 times lower than azithromycin, the standard of care for urogenital infections caused by *C. trachomatis* or respiratory infections caused by *C. pneumoniae*. Studies in an acute respiratory mouse model suggest potent activity in vivo (6). In addition, experiments in which susceptibility is tested by exposing *Chlamydia* to a compound, followed by three compound-free passages, suggest that rifalazil uniquely eradicates *Chlamydia* in cell culture (14).

Taken together with the possibility that *Chlamydia* may have the capability of persisting after standard treatment (2), these results suggest that rifalazil could be a very promising agent to treat chlamydial infections. However, one factor that could play a decisive role in its success or failure is the development of resistance. Rifampin has been generally limited to combination therapy in the treatment of TB and gram-positive infections due to the emergence of resistance caused by mutations in the *rpoB* gene, which are selected for during monotherapy. The RpoB protein is one of the large subunits of the core bacterial RNA polymerase and contains the rifampin binding site, recently described by X-ray diffraction studies (1). Evaluation of clinical isolates indicates that rifalazil shares with rifampin the loss of activity against some strains that are highly resistant to rifampin, including both *Mycobacterium tuberculosis* (7, 10, 18, 19) and *Staphylococcus aureus* (16, 17). Furthermore, the recent report of the selection of stable homotypic mutants of *C. trachomatis* that are resistant to high concentrations of rifampin following multiple passages of *C. trachomatis* serovar K in cell culture in the presence of the drug indicate that RNA polymerase mutants may arise in the clinical setting (4). Given the propensity of rifampin to select for mutants, clinicians have been reluctant to utilize rifampin in therapy to treat *C. trachomatis*, despite its activity in cell culture (13). Therefore, we initiated a study to determine the potential for resistance to develop when *Chlamydia* is exposed to rifalazil and rifampin in cell culture. Our goals were to compare the frequency of mutant selection of the two compounds and to determine the MICs of rifalazil and rifampin for resistant mutants.

MATERIALS AND METHODS

Organisms. The laboratory reference strain *C. trachomatis* serovar L2 (L2/434/BU) was used.

Antimicrobial susceptibility testing. MICs were tested by inoculating *Chlamydia* strains onto monolayers of the McCoy cell line on 96-well microtiter plates as described previously (15). Cells were maintained in antimicrobial-free growth

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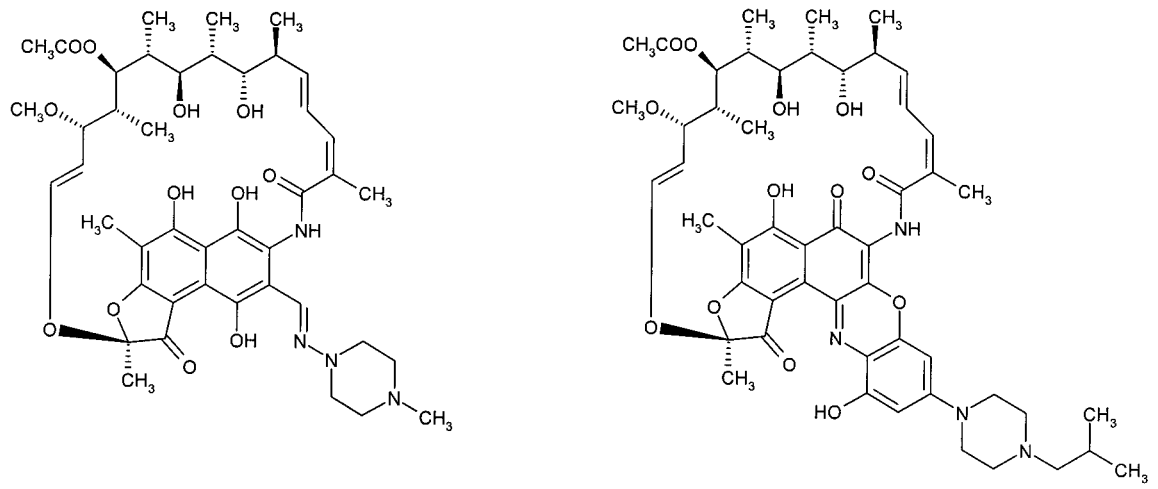


FIG. 1. Chemical structures of ansamycins. The structure of rifampin is shown on the left; the structure of rifalazil is shown on the right.

medium consisting of minimal essential medium with 10% fetal bovine serum and 220 mg of L-glutamine/liter added. The inoculum size of infectious chlamydial forms was 10,000 to 50,000 inclusion-forming units (IFU) per well. Within 30 min of addition of *Chlamydia* cells, the monolayer was centrifuged by using a Beckman model J-6 M centrifuge at 1,200 × *g* for 1 h at 37°C, a procedure which can increase the efficiency of infection for particular serovars of *C. trachomatis*. Following the removal of supernatant, the same growth medium containing 1 μg of cycloheximide/ml and the appropriate concentration of rifampin or rifalazil was applied in a volume of 100 μl. Cells were incubated at 37°C in 4% CO_2 for 48 h and fixed with methanol. Chlamydial inclusions were detected by fluorescence with a genus-specific monoclonal antibody CF-2 (Washington Research Foundation, Seattle). Rifampin (Sigma) and rifalazil (provided by ActivBiotics, Inc.) were prepared by solubilization in dimethyl sulfoxide to 10 mg/ml and then appropriate dilution in growth medium.

Strain purification. The wild-type *C. trachomatis* L2 and mutant derivatives were purified by making twofold dilutions in cell culture to the limit of detection of *Chlamydia* particles. The lowest dilution that resulted in the appearance of inclusions was assumed to originate from a single bacterium or a limited number of bacteria that were genetically identical.

Making wild-type preparations. Four independent wild-type preparations, 1 to 4, were made by purifying our wild-type stock by limiting dilution as described above and growing out 4 wild-type clones. This procedure assures that independent mutants are isolated from each preparation.

Selection for resistance. (i) Selection for resistance of *Chlamydia* by serial passages with methods 1 and 2. Four isolates of *C. trachomatis* serovar L2/432 cloned by limiting dilution were individually passed in 75-cm^2 flasks onto monolayers of McCoy cells without centrifugation. After 2 h at 37°C, with agitation every 20 min, the infected monolayer was overlaid with growth medium containing one-half the MIC of either rifampin or rifalazil. After 48 h of incubation, the monolayer was lysed, debris was removed by slow-speed centrifugation, half of the supernatant was reinoculated on a fresh McCoy monolayer in 75-cm^2 flasks, and the process was repeated at one-half the MIC. In the initial infection, a total of 3×10^7 mammalian cells in the monolayer was infected with a total of 3×10^8 IFU of *C. trachomatis* at a multiplicity of infection of 10. The number of viable IFU transferred at each subsequent passage was dependent on the number of surviving and/or resistant *Chlamydia* cells resulting from the inhibitory effect of the selective agent at each passage. Survival and/or resistance was monitored at each passage by testing for MICs in parallel cultures with monolayers in 48-well microtiter plates. To maintain a concentration of one-half the MIC, the concentration of the selective agent at the initiation of each passage was suitably elevated as resistant *Chlamydia* cells emerged by using the MIC determined on the 48-well monitoring plate. Passages were discontinued once maximum resistance was observed (i.e., MIC of rifampin of 512 μg/ml).

As resistance emerged, mutants were either cloned by limiting dilution (method 1) or left as a mixed population (method 2) and inoculated onto monolayers in 75-cm^2 flasks and overlaid with one-half the resistant MIC of appropriate drug. These passages were repeated until the highest attainable level of resistance was reached (i.e., resistance to 512 μg of rifampin/ml). Azithromycin was passed at one-half the MIC as a control for one cloned L2 isolate.

(ii) Method 3. To select more effectively for resistance with rifalazil as the selective agent, the vial passage method, similar to the MCC3 method described previously (15), was employed. Cloned L2 isolates were inoculated onto monolayers of McCoy cells in 12-mm^2 shell vials at an approximate multiplicity of infection of 25 (a total of 10^7 IFU of *C. trachomatis*). Serial twofold dilutions of either rifampin or rifalazil in growth medium were overlaid onto inoculated monolayers. The highest concentrations for rifampin and rifalazil were 0.064 μg/ml. MICs were monitored on corresponding wells of a 96-well plate to observe emerging resistance. Plates were stained and read at 48 h, and vials were passed onto new shell vial monolayers and overlaid with previous corresponding drug concentrations. After three passages under the influence of appropriate serial twofold drug concentrations, vials positive for *C. trachomatis* growth were cloned by limiting dilutions and MICs were determined.

DNA sequencing of the *rpoB* gene. Standard PCR methods and sequencing were used to determine the DNA sequence of the *rpoB* gene in wild-type and mutant strains. Using the GenBank sequence of *C. trachomatis* serovar D as a guide, both strands of the entire 3,756-bp *rpoB* gene plus 390 bp of the promoter of the parent L2 strain were sequenced from PCR products directly without interim cloning by using the appropriate primers shown in Table 1. The parent (wild type) L2 strain contained polymorphisms compared to *C. trachomatis* D at codons 520 (CTG-CTA, neutral), 572 (GAT-AAT, D changed to N), and 577 (CCC-CCT, neutral). The entire *rpoB* gene of one mutant isolated after 20 passages in the presence of rifalazil for preparation 2 (see Table 4) was also sequenced, and only one codon change, from isoleucine to methionine at codon 517 (I 517 → M), was revealed. For all other resistant mutants, both strands of the amplification were sequenced with the primers RpoBCTD5 and RpoBCTD3. This region of the *rpoB* gene encodes amino acids 442 to 644, a segment that is well known to encompass the vast majority of mutations conferring resistance to rifampin.

RESULTS

Mutant selection by serial passages of *Chlamydia* in the presence of rifampin. To select for mutants resistant to rifampin, *C. trachomatis* L2 bacterial preparations 1 to 4 were tested for resistance development by using methods 1 and 2 detailed in Materials and Methods. DNA sequencing of the *rpoB* gene directly from passages indicated the mutation responsible for an elevation in MIC. In all cases, mutants were also purified by limiting dilution prior to DNA sequencing, so that the predominant clone would be assessed after each observed elevation in the MIC.

Using the methods described above, mutants for which

TABLE 1. Characteristics of primers used for PCR amplification and sequencing

Primer	Sequence (5′–3′)	Position[a]	Fragment length (bp)
Rpo1	GTG CTA AGG CTG TTG CTA AAG	−390	909
Rpo2	AGC TTC TAA CCA ACT TCC TCG	+519	
Rpo3	TTT CTC AAG TCC ACC GTT CTC	+407	958
Rpo4	TTG AGA ACG GCT GAA GAA ATC	+1365	
RpoBCTD5	CTC CAG GAA AGA TTA TTT CTG C	+1298	655
RpoBCTD3	CCC TTC TAA TCC AGT TCC AAC	+1953	
Rpo5	ACA TGC AAC GGC AGG CTG TAC	+1886	996
Rpo6	AAA TCA ACT AGC GAC TCT CTT TC	+2882	
Rpo7	TAC ACC GTC GTT CGG CAG ATA	+2789	967
Rpo8	AGC ATC TAC TAC CAT AGG GCG	+3756	

[a] Position is given according to the initiation of the translation codon ATG.

MICs of rifampin were elevated were readily detected. Mutants were initially observed at passage 6, 6, 2, or 4 for preparations 1 to 4, respectively (Table 2), or at passage 2, 6, 6, or 6 from preparations 1 to 4, respectively (Table 3). The MIC of rifampin for the parent strain was 0.008 μg/ml. The MICs for the initial mutants were either 0.5 μg/ml (mutants with an I 517 → M change in RpoB) or 4 μg/ml (when the I 517 → M change was found in combination with a second mutation of V 466 → A).

We planned to carry out each serial experiment for 20 passages, but when rifampin was the selective agent, no experiment was carried out beyond 19 passages because the maximum level of resistance (512 μg/ml) had already been reached. This highest level of resistance was caused by the acquisition of a second mutation, either D 461 → N or H 471 → N, in combination with I 517 → M. In one case (Table 3), the maximum MIC was reached after just 6 passages. Clearly the selection of mutant strains occurs with relative facility with either method 1 or method 2.

The *rpoB* mutations appeared to be stable even in the absence of selective pressure. Both MICs and DNA sequence modifications were monitored directly from passages as well as after strain purification by limiting dilution. This purification procedure results in the propagation of mutants for a total of 10^{10} bacterial generations in drug-free conditions (10-ml preparations containing ~10^9 IFU of *C. trachomatis* per ml in the absence of rifampin). We observed good agreement before and after purification by limiting dilution, in both the MIC and DNA sequence determinations, which suggests that these mutants are stable.

The MICs of rifalazil against the mutant clones were found to be unexpectedly low in all cases (Tables 2 and 3). Single lesions led to a rise in MIC from 0.00025 μg/ml to just 0.002 μg/ml, whereas the MIC for strains with multiple mutations rose to the range of 0.008 to 0.064 μg/ml, depending on the genotype.

Selecting for resistant mutants with rifalazil as the selective agent. Because there was a marked difference in the MICs of rifampin and rifalazil for the mutants, the question as to whether rifalazil would select for mutants with equal frequency as rifampin became a more compelling issue. Indeed, when *Chlamydia* was exposed to rifalazil, mutant selection occurred

TABLE 2. Evolution of mutant selection with rifampin by method 1

Chlamydia preparation no.	No. of passages, mutation(s)	MIC (μg/ml) of:	
		Rifampin	Rifalazil
1	6 16		
	I517M	0.5	0.002
	H471N, I517M	512	0.064
2	6 16 19		
	I517M	0.5	0.002
	V466A, I517M	4	0.008
	H471N, I517M	512	0.064
3	2 16		
	I517M	0.5	0.002
	H471N, I517M	512	0.064
4	4 17 19		
	I517M	0.5	0.002
	V466A, I517M	4	0.008
	D461N, I517M	512	0.064

TABLE 3. Evolution of mutant selection with rifampin by method 2

Chlamydia preparation no.	No. of passages, mutation(s)	MIC (μg/ml) of:	
		Rifampin	Rifalazil
1		0.5 512	0.002 0.064
2		4 512	0.008 0.064
3		4 512	0.008 0.064
4		4 512	0.008 0.064

at lower frequency (Table 4), even with method 2, which allows for any combination of multiple mutations during the 20 passages of constant selection at one-half the MIC. In two instances (preparations 1 and 3), no increase in MIC was detected after 20 passages. In the other two experiments, mutants containing a single lesion were selected at passages 12 and 16 from inocula from preparations 2 and 4, respectively. After 20 passages, the MIC of rifalazil for the survivors was 0.002 μg/ml. However, the MIC of rifampin for these same survivors had increased to 0.5 μg/ml, leaving no doubt that a mutant strain had taken over the culture. DNA sequencing showed the presence of the I 517 → M mutation in each case.

Selecting for mutants by serial passages at sub-MIC concentrations of rifalazil did not result in a high frequency of mutant selection. Therefore, we attempted to isolate mutants by serial passages in the presence of higher concentrations of rifalazil. Using method 3 (see Materials and Methods), *Chlamydia* was exposed to three successive passages at a fixed, higher drug concentration, regardless of the detection of inclusion bodies in the previous passage. One mutant strain was selected following exposure and passage at 0.032 μg/ml, considerably above the MIC of rifalazil (Table 5). Method 3 was indeed a more efficient process for selecting rifalazil-resistant mutants, in that an elevated MIC was detected approximately 50% of the time after three passages. It should be noted that fewer *Chlamydia* cells were transferred in method 3. Thus, it appears that use of rifalazil concentrations higher than the MIC enhances the chances of obtaining rifalazil-resistant mutants.

When rifalazil was the selective agent, the acquisition of a single mutation tended to result in only low-level resistance to rifalazil, 0.002 μg/ml, with the exceptions of mutations at position 471 when method 3 was used. The H471 → N mutation resulted in an MIC of 0.016, whereas the H471 → L and H471 → Y mutations resulted in a MIC of 0.032 μg/ml (Table 5). Mutants with multiple mutations were observed with method 3 when rifalazil was the selective agent (H 471 → N with I 517 → M and H 471→ L with I 517 → M). Multiple mutations were also observed when rifampin was used to select (again H 471→ N with I 517 → M, V 466 → A with I 517 → M, and the triple

TABLE 4. Evolution of mutant selection with rifalazil by method 2

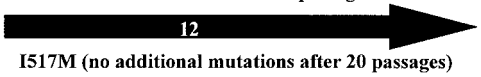
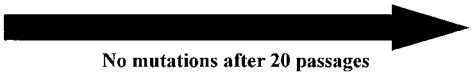
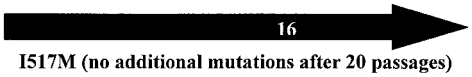
L2 preparation no.	No. of passages, mutation(s)	MIC (μg/ml) of:	
		Rifampin	Rifalazil
1		0.004	0.00025
2		0.5	0.002
3		0.004	0.00025
4		0.5	0.002

TABLE 5. Selection of mutants by method 3

L2 preparation no.	Selective agent	Selection concn (μg/ml)	Mutation at *Escherichia coli* (*C. trachomatis*) location designation:						MIC (μg/ml) of:	
			507 (R453)	516 (D461)	Not reported (V466)	522 (A467)	526 (H471)	572 (I517)	Rifampin	Rifalazil
1, 2, 3, 4	None								0.008	0.00025
1	Rifalazil	0.00025						I517M	0.5	0.002
		0.0005						I517M	0.5	0.002
		0.001						I517M	0.5	0.002
		0.002						I517M	0.5	0.002
		0.032					H471N	I517M	512	0.064
2	Rifalazil	0.00025					H471L	I517M	512	0.064
		0.0005	R453P						0.5	0.002
		0.001						I517M	0.5	0.002
		0.008					H471L		64	0.032
3	Rifalazil	0.00025						I517M	0.5	0.002
		0.001					H471N		16	0.016
		0.002						I517M	0.5	0.002
		0.004					H471L		64	0.032
4	Rifalazil	0.00025						I517M	8	0.008
		0.002					H471Y		64	0.032
2	Rifampin	0.064			V466A	A467T		I517M	64	0.032
2	Rifampin	0.064					H471N	I517M	512	0.064
2	Rifampin	0.05			V466A			I517M	4	0.008

mutant V 466 → A, A 467 → T, and I 517 → M) (Table 5). When multiple mutations were selected with method 3, the rifalazil MIC still did not rise above 0.064 μg/ml, whereas the MIC of rifampin was considerably higher, up to 512 μg/ml.

Correlation of mutant genotypes and phenotypes. Figure 2 shows the location of all *rpoB* mutations described in this study, aligned with lesions identified previously in *Chlamydia* (4) and with homologous positions of the *rpoB* genes of rifampin-resistant *M. tuberculosis* and *S. aureus* clinical isolates. As anticipated, there is a strong overlap in the location of mutations in *Chlamydia* and in these other resistant organisms, within the well-conserved region of *rpoB* encoding the rifampin binding site (1).

Table 6 shows the genotypic changes of all mutants and their phenotypic changes with regard to susceptibility to rifampin and rifalazil. In all cases, mutations conferred a large change in the MIC of rifampin. Even a single nucleotide change invariably led to an increase in the rifampin MIC of 64-fold or more. In contrast, the increase in MIC of rifalazil was approximately eightfold, except for the H 471 → L and H 471 → Y mutations, which led to a more substantial increase. When higher levels of resistance were obtained, multiple mutations were usually present. In the case of rifampin, multiple mutations led to MICs from 4 to 512 μg/ml, whereas the MIC of rifalazil did not increase above 0.064 μg/ml, regardless of the presence of single, double, or triple mutations in the *rpoB* gene.

DISCUSSION

Rifalazil has potential uses in treating acute and chronic bacterial infections caused by *Chlamydia* (urogenital infections, pelvic inflammatory disease, prevention of tubal infertility, trachoma, and possibly atherosclerosis). These possibilities make the best use of rifalazil's attributes (12), including high potency against *C. trachomatis* and *C. pneumoniae*, high intracellular concentrations, long half-life (possibly providing a prolonged protective advantage in the single-dose treatment of sexually transmitted diseases), and lack of P450 interaction (which contrasts with rifampin, a P450 inducer) (8).

Development of resistance is also an important consideration for any chemotherapeutic agent. In *S. aureus*, mutations in the *rpoB* gene can result in high levels of resistance to rifampin, probably from a single nucleotide change, and some of these mutant clinical isolates are also highly resistant to rifalazil (16, 17). Although rifampin is one of the most potent anti-*S. aureus* drugs against susceptible strains, the specter of resistant subpopulations has relegated rifampin to a combination drug for the treatment of serious gram-positive infections or for prophylaxis when the bioburden is minimal, so that the existence of a resistant subpopulations would not be a major risk. To assess the potential issue of resistance development in *Chlamydia*, in this work, we studied the nature and appearance of mutations in the *rpoB* gene following selection with rifampin and rifalazil.

Chlamydia strains present formidable obstacles in genetic studies because they are not amenable to genetic engineering (cloning and transformation, etc.), and furthermore, *Chlamydia* organisms are obligate intracellular pathogens. Even the isolation of a single bacterium, which is accomplished by streaking free-living bacteria on agar plates, is not straightforward for *Chlamydia*. To assemble a collection of independently isolated resistant mutants, selections for resistant bacteria were carried out with four wild-type preparations by using the limiting dilution procedure to make sure that each wild-type preparation originated from a single wild-type bacterium or a low number of genetically identical wild-type bacteria, as described in Materials and Methods. Thus, in each of the four wild-type preparations, resistant mutants were independently generated as a result of errors in DNA replication during the growth of each preparation. Mutants were considered to be stable because MICs and DNA sequence changes in *rpoB* remained constant whether measured from passages or after extensive

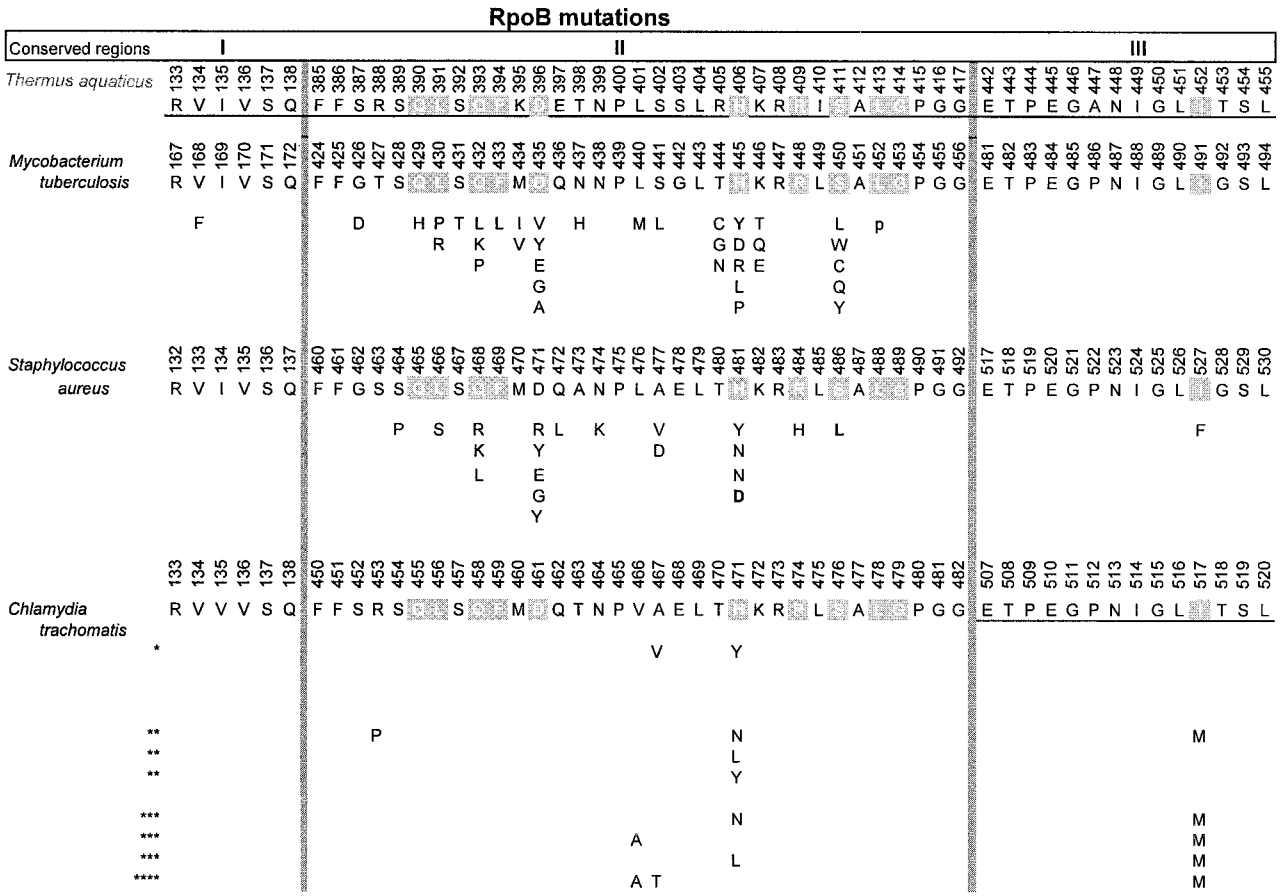


FIG. 2. Amino acid substitutions conferring rifampin resistance in *C. trachomatis*. Positions in RpoB resulting in resistance to rifampin of clinical isolates of *M. tuberculosis* (7, 10, 18, 19) and *S. aureus* (16, 17) as well as laboratory isolates of *S. aureus* (9) are shown within the conserved regions of RpoB known to be involved in resistance to rifampin. The *C. trachomatis* mutated RNA polymerases are as follows: *, single amino acid changes reported previously; **, single changes observed in this study; ***, double amino acid changes from this study; ****, triple amino acid changes from this study. The sequence of the rifampin binding site determined from the X-ray crystal structure with *Thermus aquaticus* RpoB is shown. The residues known to contact rifampin are shown with a grey background and are conserved in all sequences shown.

growth in drug-free medium during strain purification (data not shown).

When rifampin was the selective agent in passaging experiments, a sequence of mutational events was observed (Tables 2 and 3). The I 517 → M mutation occurred first, leading to a low level of resistance (MIC of rifampin of 0.5 μg/ml). Then a transiently observed V 466 → A mutation was found in combination with I 517 → M, resulting in an intermediate level of resistance (MIC of rifampin of 4 μg/ml). Subsequently, with the exposure to higher rifampin concentrations, mutants carrying the V 466 → A lesion were replaced by mutants carrying either the H 471 → N change or the D 461 → N change in combination with I 517 → M, conferring the high resistance (rifampin MIC of 512 μg/ml).

Using methods 1 to 3, we detected a total of eight different mutations within six codons in the *rpoB* gene (Fig. 2; Table 6). Mutations arose several independent times at codon 471 as well as at codon 517. In addition, a mutation altering codon 453 was observed once. Mutations in codons 461, 466, and 467 appeared only in combination with other mutations (Fig. 2). Mutations in positions 461, 466, and 467 appear to affect resistance because the MICs for combination mutants were higher than that of the isogenic strain carrying the wild-type allele at positions 461, 466, and 467. Mutations at positions 453, 461, 466, 467, 471, and 517 have been reported in other species at the corresponding codon positions (Fig. 2). In addition, Dreses-Werringloer et al. previously described the isolation of stable rifampin-resistant mutants of *C. trachomatis* with alterations in codons 467 and 471 (4).

Our investigation of *C. trachomatis* mutants in cell culture suggests a clear potential benefit of rifalazil over rifampin with regard to resistance development. Specifically, (i) the frequency of mutant selection was much reduced when rifalazil rather than rifampin was the selective agent, and (ii) mutants containing up to three lesions in *rpoB* and mutants that conferred total resistance to rifampin remained susceptible to rifalazil.

Rifalazil has greater potency against *Chlamydia* than rifampin, which is in contrast to the equivalent MICs that rifampin and rifalazil have against some gram-positive pathogens such as *S. aureus* (5). The results showing that rifalazil retains activity against *Chlamydia* strains that are resistant to rifampin was unanticipated, particularly in light of the cross-resistance of a high proportion of clinical isolates of both *S.*

TABLE 6. Summary of ansamycin-resistant mutants

Nucleotide change(s) in *rpoB*	Amino acid change	Selective agent	Method no.	MIC (μg/ml) of:		Mutant/wild type MIC ratio for:		Rifampin/rifalazil MIC ratio
				Rifampin	Rifalazil	Rifampin	Rifalazil	
None	None	None	None	0.008	0.00025			32
ATT → ATG	I 517 → M	Rifampin and rifalazil	1, 2, 3	0.5	0.002	64	8	256
CGT → CCT	R 453 → P	Rifalazil	3	0.5	0.002	64	8	256
CAC → CTC	H 471 → L	Rifalazil	3	64	0.032	8,192	128	2,048
CAC → TAC	H 471 → Y	Rifalazil	3	64	0.032	8,192	128	2,048
CAC → AAC	H 471 → N	Rifalazil	3	16	0.016	2,048	64	1,024
CAC → AAC ATT → ATG	H 471 → N I 517 → M	Rifampin and rifalazil	1, 2, 3	512	0.064	65,535	256	8,192
GAT → AAT ATT → ATG	D 461 → N I 517 → M	Rifampin	1	512	0.064	65,535	256	8,192
GTC → GCC ATT → ATG	V 466 → A I 517 → M	Rifampin	1, 2, 3	4	0.008	512	32	512
CAC → CTC ATT → ATG	H 471 → L I 517 → M	Rifalazil	3	512	0.064	65,535	256	8,192
GTC → GCC GCA → ACA ATT → ATG	V 466 → A A 467 → T I 517 → M	Rifampin	3	64	0.032	8,192	128	2,048

aureus (16, 17) and *M. tuberculosis* (7, 10, 18, 19) to both rifampin and rifalazil. It would be most interesting to isolate RNA polymerase from wild-type and mutant cells to determine whether rifalazil's special potency is related to increased affinity to its target.

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