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Detection of Rifampin- and Ciprofloxacin-Resistant *Mycobacterium tuberculosis* by Using Species-Specific Assays for Precursor rRNA

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rRNA precursor (pre-rRNA) molecules carry terminal stems which are removed during rRNA synthesis to form the mature rRNA subunits. Their abundance in bacterial cells can be markedly affected by antibiotics which directly or indirectly inhibit RNA synthesis. We evaluated the feasibility of rapidly detecting antibiotic-resistant *Mycobacterium tuberculosis* strains by measuring the effects of brief in vitro antibiotic exposure on mycobacterial pre-rRNA. By hybridizing extracted *M. tuberculosis* nucleic acid with radiolabeled nucleic acid probes specific for pre-16S rRNA stem sequences, we detected clear responses to rifampin and ciprofloxacin within 24 and 48 h, respectively, of exposure of cultured cells to these drugs. Detectable pre-rRNA was depleted in susceptible cells but remained abundant in resistant cells. In contrast, no measurable responses to isoniazid or ethambutol were observed. Probes for pre-rRNA were specific for the *M. tuberculosis* complex when tested against a panel of eight *Mycobacterium* species and 48 other bacteria. After 24 h of incubation with rifampin, resistant *M. tuberculosis* strains were detectable in a reverse transcriptase PCR assay for pre-rRNA with a calculated lower limit of sensitivity of approximately 10^2 cells. Susceptible cells were negative in this assay at over 500 times the calculated lower limit of sensitivity. This general approach may prove useful for rapidly testing the susceptibility of slowly growing *Mycobacterium* species to the rifamycin and fluoroquinolone drugs and, with possible modifications, to other drugs as well.

The prevalence of drug-resistant strains of *Mycobacterium tuberculosis* and related pathogens is increasing worldwide, resulting in high-level mortality, inappropriate treatment, and prolonged infectivity (5, 25, 26). Even with improvements upon classical culture approaches, antibiotic susceptibility testing of the slowly growing mycobacteria remains time-consuming and costly. Substantial effort is currently directed toward developing more-rapid antibiotic susceptibility tests for these pathogens.

Existing and proposed methods for detecting drug-resistant *M. tuberculosis* strains can be divided into phenotypic and genotypic categories. Phenotypic methods, which include culture and the BACTEC method (10, 23), measure the physiological response of mycobacteria to antibiotic challenge. Such methods can detect antibiotic resistance regardless of its genetic basis, but they typically require a relatively long wait for results due to the slow growth of the pathogen. The proposed firefly luciferase assay (12) may be the exception to this rule if reliable methods for introducing exogenous luciferase genes into pathogenic mycobacteria in a clinical laboratory setting can be devised. Genotypic methods include proposed PCR assays for the genetic determinants of antibiotic resistance (2, 6, 27, 31). These methods can be rapid and highly specific, and they are very compatible with future PCR-based *M. tuberculosis* detection assays (23, 25). However, the diverse genetics of antibiotic resistance pose a challenge to the development of reliable genetic tests.

New phenotypic approaches which may combine the advantages of both phenotypic and genotypic tests have been proposed. These approaches measure bacterial RNA levels as physiological indicators of mycobacterial response to in vitro antibiotic exposure. The rationale is that certain RNA molecules are abundant in actively growing cells but much less so in growth-inhibited cells. By using assays for species-specific RNA sequences, it may be possible to assess the physiological states of individual microbial species present in complex samples, ignoring other organisms which may also be present (1, 21). This raises the possibility of measuring antibiotic susceptibility simultaneously with species identification in mixed samples. Toward this end, two laboratories have proposed assays targeting mature mycobacterial rRNA by direct DNA probe hybridization (14) or in vitro RNA amplification (28). However, the exceptional stability of mature rRNA limits these approaches to the detection of cell death over extended periods. Much more rapid results may be obtainable by in vitro amplification of specific mRNA molecules, which are very unstable even in growing cells. This approach has been proposed for *Mycobacterium leprae* (20). However, the low abundance of bacterial mRNA is a potential limitation to this approach.

We hypothesized that by targeting bacterial rRNA precursors (pre-rRNAs) we could avoid some of the limitations of mRNA- and mature rRNA-targeted assays. Pre-rRNA molecules are intermediates in rRNA synthesis generated by RNase III cleavage of primary transcripts of bacterial *rrn* operons. Leader and tail sequences (stems) are removed from pre-rRNA during the secondary steps in rRNA processing, to yield the mature rRNA (17, 18). Pre-rRNAs are typically more abundant than even strongly expressed mRNA species (18), making them easier to detect. However, as biosynthetic intermediates, they are less persistent in growth-inhibited cells than are mature rRNAs (17), potentially making them more-sensitive indicators of bacterial physiological response to antibiotic exposure.

A preliminary analysis of this concept with *Escherichia coli* supported its feasibility for detecting resistance to rifampin, an

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important antituberculosis drug (3). Rifampin is a transcriptional inhibitor which could be expected to deplete pre-rRNA in susceptible cells by inhibiting de novo pre-rRNA synthesis while allowing maturation to proceed. DNA probe assays for pre-rRNA in cultured *E. coli* cells could distinguish rifampin-resistant strains from rifampin-susceptible strains harvested within minutes of exposure to the drug. Resistant cells were detectable by this method in the presence of a 100-fold excess of susceptible cells, showing that the method can detect resistant mutants even when they are present as a small percentage of a bacterial population.

The next step was to evaluate this approach with *M. tuberculosis*. Previous attempts at detecting *M. tuberculosis* pre-rRNA were unsuccessful, presumably because of the low ribosome copy number of *M. tuberculosis* relative to *E. coli* and the former organism's resistance to lysis (13). We report methods for releasing and detecting pre-rRNA from cultured *M. tuberculosis* cells, the effects of brief in vitro antibiotic exposure on pre-rRNA abundance in susceptible and resistant cells, the sensitivity of DNA probe and reverse transcriptase PCR (RT-PCR) assays for mycobacterial pre-rRNA, and the phylogenetic specificity of pre-rRNA-targeted probes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Mycobacterium* strains and other bacteria used in this analysis are listed in Table 1. *M. tuberculosis* TK33, a ciprofloxacin-resistant laboratory derivative of strain H37Ra (ATCC 25177), was provided to us by H. F. Chambers, University of California, San Francisco. *M. tuberculosis* AFB4690, a rifampin-resistant clinical isolate, was provided to us by Marie Coyle, Harborview Medical Center, Seattle, Wash. All other bacterial strains originated from the American Type Culture Collection. *Mycobacterium* cells were maintained on Lowenstein-Jensen slants (Remel Microbiology Products, Lenexa, Kans.) or Dubos plates with albumin enrichment (30). Broth cultures of *Mycobacterium* strains were grown in Dubos broth with albumin enrichment (Difco Laboratories, Detroit, Mich.) at 37°C with continuous shaking under a 5% CO_2 atmosphere. Other bacteria were cultured at MicroProbe Corporation, Bothell, Wash., under conditions recommended for each species by the American Type Culture Collection. The purity and identity of bacterial cultures harvested for DNA probe specificity tests were verified by colony morphology, pigment, growth characteristics, conventional biochemical tests, and microscopic morphology by Kinyoun acid-fast staining (16) and/or Gram staining.

Antibiotic challenge and lysate preparation. Broth cultures were diluted three- to fivefold in fresh broth in 250-ml plastic culture flasks (Fisher Scientific, Santa Clara, Calif.) to a final optical density at 600 nm of approximately 0.05. Pretreatment (0-h time point) samples were taken as described below, immediately prior to the addition of 7.5 μg of ciprofloxacin (Miles Laboratories, Kanaskee, Ill.) per ml, 2.5 μg of rifampin per ml, 2.5 μg of isoniazid (pasoniazid) per ml, or 5 μg of ethambutol (Sigma Chemical Co., St. Louis, Mo.) per ml. Additional samples were taken over the course of incubation at time points indicated below.

At each time point, culture optical density was measured where indicated, and samples were harvested and lysed as follows. Ten milliliters of broth culture was centrifuged, and cell pellets were resuspended in 1.7 ml of freshly prepared enzyme solution (10 mM Tris [pH 7.5], 1 mM EDTA, 10 mg of lysozyme [Sigma Chemical Co.] per ml, 10 μg of proteinase K [Boehringer Mannheim] per ml). Suspensions were incubated for 30 min at 37°C, centrifuged again, and resuspended in 0.6 ml of lysing solution (100 mM Tris [pH 7.5], 10 mM EDTA, 2% [wt/vol] *N*-lauryl sarcosine, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 0.1% Proclin 150 [Supelco, Inc., Bellefonte, Pa.]). These suspensions were incubated in a dry heating block at 85°C for 5 min, and then 0.9 ml of concentrated guanidine solution (100 mM Tris [pH 7.5], 10 mM EDTA, 8.75% [vol/vol] formamide, 5.25 M guanidine thiocyanate, 2.1% *N*-lauryl sarcosine) was added. The resulting lysates were stored at −20°C until use.

Nucleic acid extraction and blotting. Nucleic acid was extracted from 0.1 ml of each 1.5-ml lysate by phenol-chloroform extraction and applied to 0.22-μm-pore-size Magna NT membrane filters (MSI, Westboro, Mass.) by using a slot blot apparatus as described previously (3, 4, 19) except that nucleic acid was fixed to membrane filters using a Stratalinker 1800 UV cross-linker (Stratagene, La Jolla, Calif.) set at 120,000 μJ. Each sample was divided between two filters which were in turn cut in half before hybridization with probes, resulting in four replicate blotted samples per 0.1 ml of lysate. Thus, nucleic acid from approximately 2×10^6 CFU was present on each blot, as calculated from serial dilution and plate counts of test cultures which had been vortexed with nylon beads to disperse clumps (data not shown).

Deoxyribonucleotide probe labeling and hybridization. Synthetic oligonucleotide probe MTB030 (5′-ACC CAA ACA CTC CCT TTG GAA AAG GG-3′) was designed to recognize 26 bases of the pre-16S rRNA leader immediately adjacent to the 5′ terminus of the mature 16S rRNA (15). Probe UP041 (5′-CTG CTG CCT CCC GTA GGA GT-3′), which is complementary to a conserved region within the mature 16S rRNA (29), was used to detect combined precursor and mature 16S rRNA. Since less than 10% of the total bacterial rRNA is typically in the precursor form (17, 18), signal intensity with this probe primarily reflects mature rRNA abundance. Probe MTB030-C is complementary to MTB030 and was used to detect the chromosomal *rrn* operon DNA coding for pre-16S rRNA (rDNA). Probes were synthesized and end labeled with ^{32}P as described previously (19, 29). Hybridizations were carried out either at room temperature in a 3 M guanidine thiocyanate hybridization solution or at 42°C in formamide-SDS-Denhardt's solution (19) prepared with diethyl pyrocarbonate (DEPC)-treated water. Filters were washed at 52°C in a solution containing 9 mM Tris (pH 8.0), 90 mM NaCl, 0.6 mM EDTA, and 0.2% SDS and exposed to Kodak X-Omat autoradiography film for 1 to 10 days, depending on the specific activity of the labeled probe.

Ribonucleotide probe synthesis and hybridization. ^{32}P-labeled RNA probe MTBT71 was generated as follows. The PCR was used to amplify a 174-base section of the *M. tuberculosis* genome coding for the pre-16S rRNA immediately upstream of the 5′ terminus of the mature 16S rRNA. Primers were MTBPCR09

TABLE 1. Specificities of DNA probes for *M. tuberculosis* pre-16S rRNA

Source of nucleic acid[a]	Hybridization reaction result with[b]:		
	UP041	MTB030	MTBT71
Mycobacterium strains			
M. tuberculosis H37Ra	+	+	+
M. tuberculosis TK33 (H37Ra-Cp^R)	+	+	+
M. tuberculosis ATCC 35838 (H37Rv-Rp^R)	+	+	+
M. tuberculosis AFB4690 (Rp^r)	+	+	+
M. bovis BCG	+	+	+
M. gastri	+	−	W[c]
M. gordonae	+	−	−
M. intracellulare ATCC 13950	+	−	−
M. intracellulare ATCC 25169	+	−	−
M. nonchromogenicum	+	−	W
M. smegmatis	+	−	W
M. terrae	+	−	−
Other bacteria			
Actinobacillus actinomycetemcomitans	+	−	−
Bacteroides gracilis	+	−	−
Capnocytophaga spp. (2)	+	−	−
Corynebacterium matruchotii	+	−	−
Eikenella corrodens	+	−	−
Enterococcus spp. (2)	+	−	−
Fusobacterium spp. (2)	+	−	−
Haemophilus spp. (3)	+	−	−
Kingella oralis	+	−	−
Lactobacillus spp. (3)	+	−	−
Leptotrichia buccalis	+	−	−
Listeria monocytogenes	+	−	−
Mitsuakella multiacidus	+	−	−
Mobiluncus mulieris	+	−	−
Moraxella catarrhalis	+	−	−
Neisseria spp. (3)	+	−	−
Peptostreptococcus spp. (3)	+	−	−
Porphyromonas gingivalis	+	−	−
Prevotella spp. (2)	+	−	−
Selenomonas noxia	+	−	−
Staphylococcus spp. (2)	+	−	−
Streptococcus spp. (12)	+	−	−
Veillonella parvula	+	−	−
Wolinella succinogenes	+	−	−

[a] Strain designations are specified when more than one strain of a species is listed. The numbers of tested species of genera other than *Mycobacterium* are indicated in parentheses.

[b] A duplicate experiment yielded similar results. +, reaction observed; −, no reaction.

[c] W, barely detectable signal.

(5′-AAT TTA ATA CGA CTC ACT ATA GGG ACC CAA ACA CTC CCT TTG GAA AAG-3′) and MTBPCR07 (5′-ACT CTA GAC GGA AAC AAG CAA GCG TGT TG-3′). Primer MTBPCR09 has a 24-base T7 RNA polymerase promoter and spacer sequence followed by a 24-base sequence complementary to the pre-16S rRNA leader immediately adjacent to the 5′ terminus of the mature 16S rRNA (15). MTBPCR07 is complementary to the opposite DNA strand at bases 153 to 174 upstream of the 5′ terminus of the mature 16S rRNA coding region (15) (it has an 8-base *Xba*I site and spacer sequence at its 5′ end for unrelated experiments). Amplification of *M. tuberculosis* DNA with these primers yielded a 206-bp fragment containing the T7 promoter. The fragment was purified by phenol-chloroform extraction and ethanol precipitation prior to T7 RNA polymerase-driven transcription. A ^{32}P-labeled RNA probe was transcribed in vitro from the amplified fragment by using T7 Transcription and Ribonucleotide Pac-Kits (Epicentre Technologies, Madison, Wis.). Transcription was carried out in the presence of 20 μCi of [α-^{32}P]UTP (DuPont NEN, Boston, Mass.) in a 20-μl total reaction volume at 37°C for 4 h. To maximize incorporation of radiolabel, 12.5 U of T7 RNA polymerase was added both at the outset of the procedure and halfway through it. Upon completion of incubation, the transcript was separated from unincorporated nucleotides by adding 20 μl of 5 M ammonium acetate and 1 ml of 100% ethanol, storing the solution at −20°C for 1 h, centrifuging it at 13,000 × *g* for 15 min, and discarding the supernatant. After resuspension of pellets in 250 μl of DEPC-treated water, 250 μl of 4.5 M guanidine thiocyanate (Sigma Chemical Co.) was added to inhibit RNase activity. Probes were stored frozen until use. Hybridization was carried out in formamide-SDS-Denhardt's solution (19) containing approximately 9×10^5 cpm of RNA probe per ml at 42°C. Filters were washed as described above, except that washes were carried out at 70°C, and exposed to autoradiography film for 16 to 24 h.

DNase I pretreatment for slot blot hybridization. Where indicated, extracted nucleic acid was pretreated with DNase I as follows. Two microliters of a magnesium chloride-calcium chloride stock solution (50 mM each), followed by 1 μl (10 U) of RNase-free DNase I (Boehringer Mannheim), was added to 16 μl of nucleic acid dissolved in DEPC-treated water. This sample was incubated for 60 min at 37°C, another 1 μl of DNase I was added, and incubation was continued for 90 min. Untreated (control) aliquots received 4 μl of DEPC-treated water and were incubated at 37°C for 150 min concurrently with treated aliquots. Samples were diluted to 200 μl in Tris-EDTA (TE) buffer and applied to filters.

RT-PCR amplification. The three-step pre-rRNA amplification procedure consisted of enzymatic digestion of rDNA, reverse transcription of pre-rRNA, and PCR amplification of the transcripts. For the first step, each 5-μl sample was treated with RNase-free DNase I (Boehringer Mannheim) in the presence of 34 U of RNasin (Promega, Madison, Wis.) as described previously (9). The mixture was centrifuged briefly at the outset of the reaction to assure digestion of all DNA in the tube. Incubation of the reaction mixture, heat inactivation of DNase, and subsequent cooling were all carried out in a Perkin-Elmer GeneAmp 2400 thermocycler.

For the second step, each reaction mixture in a 200-μl thin-walled PCR tube received 1 μl (15 nmol) of oligonucleotide UP041, 4 μl of Superscript II RT 5× First Strand Buffer (Gibco BRL Products, Gaithersburg, Md.), and 2 μl of 0.1 M dithiothreitol (final volume, 18 μl). After incubation for 5 min at 70°C, 1.5 μl of 10 mM deoxynucleoside triphosphate (dNTP) stock (Perkin-Elmer GeneAmp PCR kit) and 0.5 μl (25 U) of Superscript II RT were added. This reaction mixture was incubated for 10 min at 70°C to generate 355-base cDNAs from the 5′ ends of mycobacterial rRNA molecules, as well as longer cDNAs from intact pre-rRNA molecules, when present.

In the final step, 2 μl of each cDNA synthesis reaction mixture was transferred to a fresh PCR tube containing 83.5 μl of DEPC-treated water, 10 μl of 10× PCR buffer, 0.5 μl (2.5 U) of *Taq* polymerase (Fisher Biotech), 2 μl of 10 mM dNTP stock, 1 μl (15 nmol) of primer UP041, and 1 μl (15 nmol) of primer MTB030-C, which recognizes cDNAs generated from intact pre-rRNA molecules. PCR amplification was carried out over 35 cycles of denaturation at 94°C, annealing at 50°C, and extension at 72°C. A 10-μl aliquot of each 100-μl reaction mixture was then analyzed by 2% agarose gel electrophoresis for the expected 381-bp product.

RESULTS

Release and DNA probe detection of *M. tuberculosis* pre-rRNA. We evaluated over 10 methods for lysing *M. tuberculosis* cells and releasing intact pre-rRNA, including treatment with organic solvents, bead beating, heat, and sonication, and found the procedure described above to be the most convenient and consistently effective. This procedure resulted in >99.99% loss of mycobacterial cell viability as measured by colony counts before and after treatment; however, rare CFU were detectable in the lysates.

Nucleic acid extracted from lysates of actively growing cells was applied to membrane filters by using a slot blot apparatus and hybridized with oligonucleotide probe MTB030, a 26-mer complementary to the pre-16S rRNA leader sequence immediately preceding the 5′ terminus of the mature 16S rRNA. This yielded a signal of much lower intensity than that resulting from hybridization with probe UP041, which recognizes a segment of the mature 16S rRNA 355 bases downstream of the 5′ terminus (Fig. 1A, 0-h time point). This is consistent with the relative abundances of pre-rRNA and mature rRNA typically observed in growing bacterial cells (18). A second pre-rRNA-targeted probe complementary to the first 23 bases of the pre-16S rRNA tail immediately following the mature 3′ terminus (15) gave an autoradiographic signal equivalent to that of MTB030 (data not shown). Probe MTB030-C, which is complementary to MTB030 and would therefore hybridize with the DNA sequences encoding pre-rRNA (rDNA) but not with the pre-rRNA itself, gave a significantly fainter signal than MTB030. Pretreatment of nucleic acid with DNase-free RNase reduced the MTB030 signal to the level of the MTB030-C signal, suggesting that the excess signal generated by MTB030 was due to hybridization with RNA (data not shown). These observations are consistent with the release from *M. tuberculosis* cells and DNA probe detection of multicopy pre-16S rRNA molecules. This conclusion is reinforced by the effects of antibiotics described below.

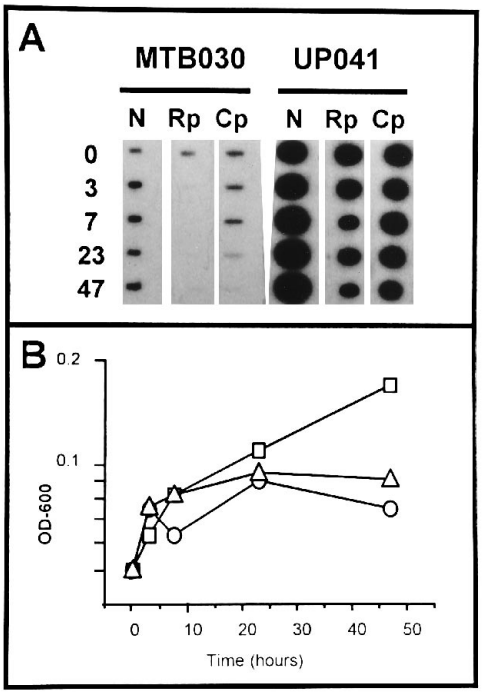

FIG. 1. Effects of rifampin and ciprofloxacin on pre-rRNA abundance in *M. tuberculosis* H37Ra. Cultures were exposed to antibiotics or left untreated as described in the text. (A) Slot blot hybridization assays for pre-rRNA (probe MTB030) and mature rRNA (probe UP041) extracted from approximately 2×10^6 CFU. Time in hours after addition of antibiotics is shown on the left. N, no treatment; Rp, rifampin; Cp, ciprofloxacin. (B) Optical densities at 600 nm (OD-600) of the same cultures at the same time points. Squares, no treatment; circles, rifampin; triangles, ciprofloxacin. Similar results were observed in three replicate experiments.

Response of pre-rRNA to antibiotic challenge. To determine the response of *M. tuberculosis* pre-rRNA to in vitro antibiotic challenge, parallel cultures of *M. tuberculosis* H37Ra were exposed to antibiotics or left untreated and samples taken at various time points over 47 h were probed for mature rRNA and pre-rRNA. Pre-rRNA detected by probe MTB030 remained almost constant in nucleic acid extracted from un-

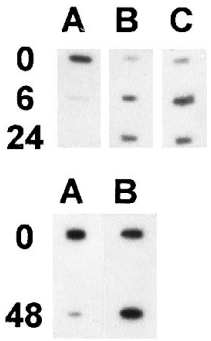

FIG. 2. (Top) Effects of rifampin on pre-rRNA detected by probe MTBT71 in rifampin-susceptible *M. tuberculosis* H37Ra (lane A), rifampin-resistant strain ATCC 35838 (lane B), and rifampin-resistant strain 4690 (lane C). (Bottom) Effects of ciprofloxacin on pre-rRNA detected by probe MTBT71 in ciprofloxacin-susceptible strain H37Ra (lane A) and ciprofloxacin-resistant strain TK33 (lane B). Time in hours after rifampin addition is shown on the left. Similar results were observed in three replicate experiments.

treated cells, but it decreased to nearly undetectable levels after 3 h of rifampin treatment and after 47 h of ciprofloxacin treatment (Fig. 1A). The divergence in pre-rRNA abundance between treated and untreated cells was much greater than the divergence over the same period in mature rRNA abundance measured with probe UP041 (Fig. 1A). It was also much greater than the divergence in culture optical density over the same period (Fig. 1B). In contrast, isoniazid and ethambutol did not measurably reduce cellular pre-rRNA or mature rRNA abundance over the same periods (data not shown).

We next compared cellular pre-rRNA abundance in rifampin- and ciprofloxacin-resistant *M. tuberculosis* cells with that in susceptible cells after brief exposure to these drugs. Cultures were incubated in the presence of each drug and assayed for pre-rRNA as described above, except that oligonucleotide probe MTB030 was replaced with the more sensitive riboprobe MTBT71. This probe recognizes a stretch of the 5′ pre-16S rRNA leader overlapping the MTB030 recognition site plus an additional 148 bases preceding it (whether *M. tuberculosis* pre-16S rRNA actually extends that far upstream is unknown). Rifampin-resistant *M. tuberculosis* strains H37Rv-Rp^R (ATCC 35838) and AFB4690 were clearly distinguishable from rifampin-susceptible strain H37Ra within 24 h of exposure to the drug (Fig. 2, top). Ciprofloxacin-resistant *M. tuberculosis* TK33, a derivative of H37Ra, was also clearly distinguishable from its parent strain after 48 h of culture in the presence of ciprofloxacin (Fig. 2, bottom).

Abundance of pre-rRNA in rifampin-treated cells. In most experiments, rifampin decreased pre-rRNA in susceptible cells to barely detectable levels which remained constant over time. Stable background signal levels may reflect hybridization of probes with residual pre-rRNA in susceptible cells or alternatively with rDNA. We conducted two experiments to help distinguish these possibilities. In the first experiment, parallel cultures of *M. tuberculosis* were treated with rifampin or left untreated as for Fig. 1 except that the cell concentration was increased fivefold. Replicate filters were hybridized with MTB030 and its complement, MTB030-C, which is specific for the coding strand of rDNA. After extended autoradiography, MTB030 signal (representing both pre-rRNA and rDNA) did not exceed MTB030-C signal (representing rDNA only) in nucleic acid extracted from rifampin-treated cells (Fig. 3). These data suggest that pre-rRNA abundance in nucleic acid extracted from rifampin-treated cells does not measurably exceed that of the DNA encoding it.

In the second experiment, rifampin-susceptible and -resistant *M. tuberculosis* cells were treated with rifampin as for Fig. 2 and extracted nucleic acid was split into two aliquots, one of which was treated with RNase-free DNase I prior to blotting. After hybridization with pre-rRNA-targeted probe MTBT71 and extended autoradiography, background signal was visible in nucleic acid from susceptible cells, but not when it had been digested with DNase I prior to hybridization (Fig. 4). This observation is consistent with the presence in rifampin-treated susceptible cells of detectable rDNA but very little pre-rRNA.

Detection of *M. tuberculosis* pre-rRNA by RT-PCR. We next evaluated a three-step in vitro RNA amplification method (RT-PCR) with the aim of boosting assay sensitivity without compromising resolution of resistant and susceptible cells. First, nucleic acid extracted from *M. tuberculosis* cells was digested with DNase I to degrade rDNA. Reverse transcription of the 5′ portion of the 16S rRNA and its adjoining leader sequence (when present) was then carried out with oligonucleotide UP041 as a primer. Finally, cDNAs were amplified by PCR using oligonucleotides UP041 and MTB030-C as primers. MTB030-C is specific for cDNA transcribed from the pre-16S rRNA leader; therefore, this primer set amplified only those cDNAs generated from intact pre-rRNA molecules, to yield a 381-bp product. When tested with serial dilutions of nucleic acid extracted from rifampin-resistant cells, the assay had a calculated lower limit of sensitivity of approximately 10^2 CFU.

Results of a typical experiment are shown in Fig. 5. Starting with nucleic acid extracted from approximately 5×10^4 rifampin-susceptible and -resistant CFU, 381-bp amplification products were obtained from untreated cells (0-h time points) and from rifampin-treated resistant cells but not from rifampin-treated susceptible cells. Omission of the RT step eliminated signal from otherwise positive samples, indicating that DNase I treatment was effective and RNA was being amplified. In nine RT-PCR assays conducted with three separate sets of cultures, faint signals were observed in two instances with nucleic acid extracted from susceptible cells after 6 h of rifampin exposure, but never after 24 h of exposure. Positive signals were always observed with nucleic acid from resistant or untreated cells.

Phylogenetic specificity of pre-rRNA-targeted probes. To test the species specificity of pre-rRNA-targeted probes, we attempted to hybridize them with nucleic acid extracted from eight *Mycobacterium* species as well as from 48 other gram-

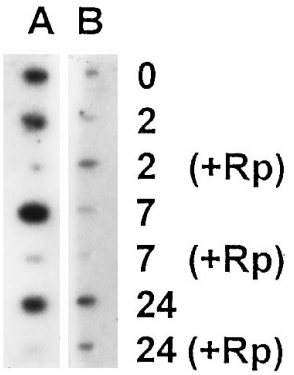

FIG. 3. Relative abundances of pre-16S rRNA detected by probe MTB030 (lane A) and rDNA detected by complementary probe MTB030-C (lane B) in nucleic acid extracted from *M. tuberculosis* H37Ra. Cultures were exposed to rifampin (+Rp) or left untreated as described in the text. Time in hours after addition of the antibiotic is shown on the right. Similar results were observed in duplicate experiments.

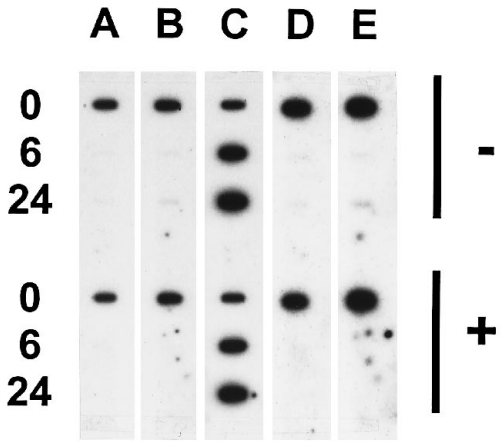

FIG. 4. Sensitivity to DNase I digestion of background signal generated by hybridization of pre-rRNA-targeted probe MTBT71 with nucleic acid extracted from *M. tuberculosis* cells treated with rifampin. Rifampin-susceptible strains H37Ra (lane A) and TK33 (lane B) and rifampin-resistant strain ATCC 35838 (lane C) were sampled at 0, 6, and 24 h after addition of rifampin as for Fig. 2. Extracted nucleic acid was treated with DNase I (+) or left untreated (−) prior to slot blot hybridization. Lanes D and E are overexposed replicates of lanes A and B, respectively, and are presented to more clearly display background signal.

positive and gram-negative bacterial species representing an oropharyngeal specificity panel (4). Universal prokaryotic mature rRNA-targeted probe UP041 hybridized strongly with all bacterial nucleic acid samples, indicating that cell lysis and RNA extraction were effective in all cases. However, the 26-base pre-rRNA-targeted probe MTB030 hybridized only with RNA from *M. tuberculosis* complex strains (*M. tuberculosis* and *M. bovis*). The much-longer (174-base) RNA probe MTBT71 hybridized only with RNA from *M. tuberculosis* complex strains, and the reactions with RNA from *Mycobacterium gastri*, *Mycobacterium smegmatis*, and *Mycobacterium nonchromogenicum* were very weak (Table 1). Neither probe hybridized with nucleic acid extracted from approximately 10^5 human T47D cells (data not shown).

DISCUSSION

The results of our experiments show that pre-rRNA responds very rapidly to certain antibiotics. The method appears particularly useful for detecting susceptibility to rifampin, a first-line antituberculosis drug which reduced pre-rRNA to undetectable levels within hours of exposure. This rapid response was presumably due to direct inhibition of RNA polymerase activity by rifampin, which halts synthesis of pre-rRNA while allowing its processing to proceed (17). Response to the fluoroquinolone drug ciprofloxacin was slower than that to rifampin, possibly reflecting indirect inhibition of transcription through inhibition of DNA unwinding activity (11). In both cases, pre-rRNA responses were much more rapid and visible than changes in turbidity of the pure cultures. Resistant strains did not exhibit pre-rRNA responses and were clearly distinguishable from susceptible strains. Although only a limited number of strains were tested in this study, it is reasonable to expect similar results from other phenotypically rifampin- or ciprofloxacin-resistant strains, regardless of the genetic basis for resistance.

The decline in cellular abundance of pre-rRNA in susceptible cells exposed to rifamycin and fluoroquinolone drugs was steeper and more complete than that of mature rRNA, as measured in hybridization assays using oligonucleotide probes for each (Fig. 1). These results are consistent with the effects of rifampin and ofloxacin on mature rRNA in the fast-growing saprophyte *M. smegmatis*, which doubles about once every 2.5 h. When the NASBA in vitro RNA amplification method was used to measure *M. smegmatis* mature rRNA, 3 to 7 days (approximately 29 to 67 *M. smegmatis* doubling times) was required to measure the effects of these drugs (28). In our experiments using RT-PCR to measure *M. tuberculosis* pre-rRNA, only 6 to 48 h (approximately 0.3 to 2.6 *M. tuberculosis* doubling times) was required to measure similar effects.

The pre-rRNA method described here has a significant limitation in that no measurable responses to isoniazid or ethambutol were observed. A previous evaluation of mature rRNA-targeted assays did not include these drugs (28), but in our experiments mature rRNA also remained stable in their presence. These drugs may inhibit pre-rRNA synthesis to an extent no greater than that to which they inhibit its processing, resulting in stabilization of pre-rRNA abundance. The same may be expected of other important antituberculosis drugs which do not act directly on transcription. To render the method useful for such drugs, it may be necessary to use additional manipulations to deplete bacterial pre-rRNA prior to starting the assay and then to measure inhibition by the drugs of pre-rRNA replenishment. We are evaluating several such approaches, the simplest of which takes advantage of normal fluctuations in pre-rRNA abundance during cell starvation and reinitiation of growth. The success of such approaches will depend upon the kinetics and regulation of rRNA biogenesis in the slowly growing mycobacteria and on the effects of individual antituberculosis drugs on these processes.

There are two particularly compelling theoretical advantages to the pre-rRNA approach. One is its potential for species specificity. If the nucleotide sequences of pre-rRNA stems are phylogenetically specific, stringent hybridization assays for such stems could in theory measure the physiological activity of individual microbial species present in complex samples, ignoring other species which may also be present. This could circumvent the need for separate microbial identification tests and antibiotic susceptibility tests on isolated cultures. Comparison of published mycobacterial pre-rRNA sequences (7, 8, 13, 15, 22, 24) revealed significant divergence (data not shown), indicating that such specificity may be possible. The empirical results in Table 1 are consistent with this observation; however, broader specificity analysis is still needed. Of equal importance is the observation that 5′ pre-16S rRNA-targeted probes recognized all *M. tuberculosis* complex strains used in this study

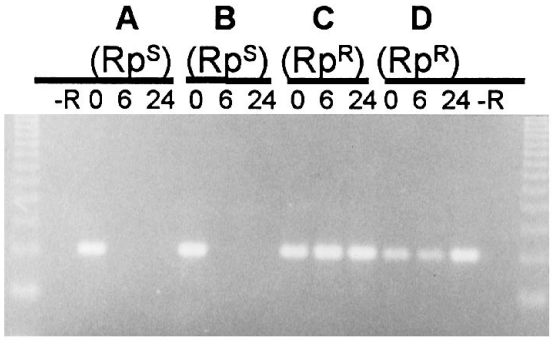

FIG. 5. RT-PCR assay for pre-rRNA in rifampin-susceptible strains H37Ra (A) and TK33 (B), and rifampin-resistant strains ATCC 35838 (C) and AFB4690 (D). Time in hours after addition of rifampin is shown (0, 6, and 24 h). Replicates of sample A at 0 h and sample D at 24 h which were amplified by PCR without prior reverse transcription are shown on each side (−R).

(Table 1). In contrast to the hypervariable internal transcribed spacer regions of the rDNA (8), pre-rRNA stems have secondary structure thought to be important to rRNA processing (18), and this may exert sufficient evolutionary pressure to conserve these sequences within a species. However, broader testing is needed in this regard as well.

The second important advantage to RNA-based susceptibility testing is that it can be carried out by using RNA amplification methods such as RT-PCR. This makes it compatible with in vitro DNA and RNA amplification tests for pathogen identification, which may come into common use in future clinical mycobacteriology laboratories (23, 25). A suite of pathogen identification and antibiotic susceptibility tests which use the same basic molecular technology and can be carried out within a few days of each other would greatly improve diagnostic turnaround time.

We have not yet determined whether pre-rRNA-targeted assays can detect small numbers of antibiotic-resistant cells within larger populations of susceptible cells of the same species (proportion method). For rifampin susceptibility testing, we observed previously that hybridization assays for *E. coli* pre-rRNA could detect resistant cells in the presence of a 100-fold excess of susceptible cells (3). In the present study of *M. tuberculosis*, we observed that pre-rRNA is very scarce in rifampin-susceptible *M. tuberculosis* cells, raising the hope that small subpopulations of resistant clones will be detectable among such cells.

We also have not yet determined whether pre-rRNA-targeted assays can quantitatively measure antibiotic MICs or whether they can be conducted on unpurified patient samples such as sputum. However, our preliminary analysis suggests that the pre-rRNA approach has attractive features in terms of rapid response, species specificity, sensitivity, and compatibility with future amplification-based diagnostic methods and that further development and evaluation are warranted. Since all cellular organisms synthesize and process pre-rRNA, this general approach may also prove useful for a broad range of other fastidious or noncultivatable organisms.

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