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Genetic Basis of Macrolide Resistance in *Mycobacterium avium* Isolated from Patients with Disseminated Disease

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Clarithromycin (CLM) and azithromycin (AZM) are important agents in the treatment of disseminated *Mycobacterium avium* complex disease; however, monotherapy with these macrolides often leads to clinically significant resistance. The underlying resistance mechanism was investigated by comparing 23S rRNA gene sequences in the domain V region of 10 CLM-susceptible *M. avium* strains and 8 CLM-resistant strains. Four of the CLM-resistant strains were derived from CLM-susceptible strains included in this study. The only differences in the domain V sequences associated with CLM resistance were at position 2274 of the complete *M. avium* 23S rRNA gene (GenBank accession no. X74494). All the CLM-susceptible strains had an A residue at this site, whereas seven of the eight CLM-resistant strains had either a C, G, or T. Four of these seven CLM-resistant strains emerged during monotherapy with CLM and two emerged during AZM monotherapy, showing that resistance selected by either macrolide was associated with mutation of the 23S rRNA gene. Thermodynamic analysis of secondary rRNA structure suggests that the observed mutations cause an alteration in free energy associated with rRNA folding, which may result in a localized conformation change in assembled ribosomes. Such a shift may be important in the resistance of ribosomes to the effects of macrolides. This study therefore establishes a link between mutations within the 23S rRNA gene and clinically significant macrolide resistance in *M. avium* and also identifies a possible molecular mechanism of resistance at the level of the ribosome.

Macrolides such as clarithromycin (CLM), azithromycin (AZM), and roxithromycin are important agents for the treatment of a variety of mycobacterial infections, including disseminated *Mycobacterium avium* complex (MAC) infection (11). Initial clinical trials of CLM and AZM demonstrated that these agents were effective in reducing or eliminating the mycobacteremia commonly associated with disseminated MAC disease and that this microbiological response was reflected in a positive clinical response (3, 5, 10, 30). However, when any one of these agents was used for monotherapy, macrolide resistance developed usually within 12 weeks of the start of therapy (5, 10). As a consequence, the recommendation was made that these agents be used to treat disseminated MAC disease only in combination with at least one additional agent, such as ethambutol (16). Nevertheless, at present it is unknown whether combination therapy actually prevents or delays the emergence of macrolide resistance.

Macrolide resistance in other bacteria has been studied, and the predominant mechanism of clinically significant resistance appears to be posttranscription methylation of an adenine residue within the peptidyl transferase loop of 23S rRNA (28). Other bacterial mechanisms of macrolide resistance that have been described include macrolide-inactivating enzymes (4), changes in membrane permeability (8), active drug efflux (4), mutated ribosomal proteins (4), and mutations within the 23S rRNA (6, 7). However, with the exception of 23S rRNA mutation, the clinical significance of these mechanisms is unclear.

Recently, Meier et al. (17) characterized point mutations in the 23S rRNA genes of macrolide-resistant *Mycobacterium intracellulare* strains. These strains were isolated from patients with pulmonary disease who were enrolled in a clinical trial to assess the treatment efficacy of CLM (27). The mutations in the 23S rRNA genes of *M. intracellulare* were homologous to mutations reported for *Escherichia coli* with experimentally selected macrolide resistance (6, 7).

In this study, we extended the observations of Meier et al. (17) by analyzing the association between macrolide resistance and mutations in the 23S rRNA of *M. avium* isolates obtained from AIDS patients with symptomatic disseminated MAC disease. Furthermore, we analyzed the effect of these mutations on potential RNA secondary structure and propose that alterations in free energy associated with base pairing in the peptidyl transferase region of ribosomes influence local conformation of the ribosomal macrolide binding site.

MATERIALS AND METHODS

Antimicrobial agents. CLM (Abbott Laboratories, Abbott Park, Ill.) and AZM (Pfizer Inc., Groton, Conn.) were both dissolved in methanol to give a solution with a maximum concentration of 5 mg/ml, which was then immediately diluted threefold with 0.1 M phosphate buffer (pH 6.8). Stock solutions were stored at −20°C for a maximum of 1 month.

Susceptibility (MIC) determination in vitro. Susceptibility to CLM and AZM was assessed by using a radiometric broth macrodilution assay and the T100 method of data analysis (11). This assay determines the MIC relative to a 99.9% inhibition of growth. Resistances to CLM and AZM were defined as MICs of ≥32 and >128 μg/ml, respectively, although the latter has not been clinically defined.

Mycobacteria. Table 1 lists the *M. avium* and *Mycobacterium tuberculosis* strains used in this study and the MICs of CLM and AZM for those strains. All *M. avium* strains were originally isolated from patients with AIDS. The list includes three pairs of *M. avium* isolates (504-511, 505-512, and 506- 513) obtained from three human immunodeficiency virus-infected pediatric patients enrolled in a clinical trial of CLM monotherapy for disseminated *M. avium* infection (10). For each pair, the strain with the lower number was isolated prior to the initiation of CLM therapy and the strain with the higher number was isolated after 3 months of therapy. In addition, two pairs of strains (TB0001.1-TB0001.2 and JJL004.1-JJL004.2), kindly supplied by L. E. Bermudez, Kuzell Institute, San Francisco, Calif., were isolated from patients prior to initiation of

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TABLE 1. Partial 23S gene sequences and macrolide susceptibilities of various *M. avium* and *M. tuberculosis* strains

Strain[a]	Partial 23S gene sequence[b]	MIC (μg/ml)	
		CLM	AZM
503	GCGCGGCAGGACGAAAAGACCCCGGGACCT	2	ND[c]
Co	.....	8	128
Fr	.....	2	32
Ma	.....	8	≥128
225076-C	.....	128	≥128
Wa	.....C.....	128	64
101	.....	<1	<8
101R	.....G.....	>128	≥128
504	.....	2	16
511	.....C.....	>128	>128
505	.....	4	32
512	.....C.....	>128	>128
506	.....	2	8
513	.....G.....	>128	>128
JJL004.1	.....	4	32
JJL004.2	.....T.....	>32	>256
TB0001.1	.....	4	32
TB0001.2	.....C.....	>32	>256
H37Ra	.....	16	32
H37Rv	.....	ND	ND

[a] All strains are *M. avium* except for H37Ra and H37Rv, which are *M. tuberculosis*.

[b] Region corresponds to positions 2260 to 2289 of the *M. avium* 23S rRNA gene (GenBank accession no. X74494) determined by van der Giessen et al. (25).

[c] ND, not determined.

AZM therapy and after either 4 (TB0001.2) or 10 (JJL004.2) months of therapy. Strain 101R was isolated from a beige mouse given daily oral CLM following experimental infection with the CLM-susceptible *M. avium* strain 101 (kindly supplied by L. E. Bermudez). Thus, strain 101R was a spontaneous mutant that had been selected in vivo. *M. avium* 225076-C was isolated from a baseline bone marrow specimen (prior to treatment) obtained from a patient enrolled in a CLM-plus-ethambutol treatment trial. The remaining *M. avium* strains, Co, Ma, Fr, and Wa, were isolated from blood specimens drawn from four independent patients. *M. tuberculosis* H37Ra (ATCC 25177) and H37Rv (ATCC 27294) were obtained from American Type Culture Collection (Rockville, Md.). All mycobacteria were maintained on either Middlebrook 7H11 agar (Difco, Detroit, Mich.) or Middlebrook 7H9 broth supplemented with 10% OADC (BBL, Becton Dickinson, Cockeysville, Md.). Mycobacterium species were determined by AccuProbe culture identification assay (Gen-Probe, Inc., San Diego, Calif.).

DNA isolation and PCR. Mycobacteria were suspended in 400 μl of TE buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA) and mixed with 400 μl of phenol-chloroform-isoamyl alcohol (pH 8.0) (Sigma Chemical Co., St. Louis, Mo.) and 500 μl of 0.1-mm-diameter glass beads (Sigma). This mixture was vigorously shaken with a Mini-Beadbeater (Biospec Products, Bartlesville, Okla.) for 3 min at room temperature. The organic and aqueous phases were separated by centrifugation at ≥7,200 × *g* (≥10,000 rpm) at room temperature for 15 min, the aqueous phase was removed to a new tube, and the DNA was precipitated with 1/10 volume of sodium acetate (pH 5.2) and 2 volumes of ethanol. After sedimentation by centrifugation (≥7,200 × *g* at 4°C for 30 min), the nucleic acid pellet was dissolved in 50 μl of TE buffer. The DNA was stored at −20°C until needed.

The mycobacterial DNA was amplified by a PCR designed to span domain V of the 23S rRNA gene using primers 23.1 (5′ AATGGCGTAACGACTTCT CAACTGT 3′) and 23.2 (5′ GCACTAGAGGTTCGTCCGTCCC 3′). These primers were designed by using OLIGO software (National Biosciences, Inc., Plymouth, Minn.) with reference to the 23S rRNA gene sequence of *Mycobacterium kansasii* (GenBank assession no. Z17212). The basic 100-μl amplification reaction mixture consisted of 50 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Eastman Kodak, Rochester, N.Y.), and 5 μl of DNA preparation. Each reaction mixture was overlaid with 50 μl of mineral oil. The cycling conditions were 94°C for 1.5 min, 60°C for 2 min, and 72°C for 3 min, and each reaction mixture was subjected to 35 cycles in a model 480 Thermal Cycler (Perkin-Elmer, Norwalk, Conn.). Amplification products were analyzed by electrophoresis in a 3% agarose gel. The amplification products of this PCR were of the expected size, i.e., 721 bp.

Sequencing. The domain V PCR products were sequenced in both directions by using primers 23.2c(see above), 23.3 (5′ CCGACTTTCGTCCCTGCTTGA 3′), 23.4 (5′ ACTCGCCGAAATTGCACTACGA 3′), and 23.5 (5′ TCAGGT GGCGAGTGTAAGTGC 3′). These primers were designed with reference to preliminary *M. avium* 23S rRNA sequence data obtained with primers 23.1 and 23.2. Sequencing was performed on an Automatic Sequencer (ABI, San Francisco, Calif.) according to the manufacturer's recommended protocols.

rRNA secondary structure analysis. The domain V sequences were used to study the effect of base changes on the predicted secondary rRNA structures. RNA folding was investigated with the computer program MulFold (coded for Macintosh computers by Don Gilbert at Indiana University, Bloomington) using free energy minimization algorithms (12, 13, 31). rRNA structures were drawn by using the program LoopDloop, also written by Don Gilbert.

PFGE. Mycobacterial DNA plugs for pulsed-field gel electrophoresis (PFGE) were prepared by a modification of the procedure described by Maslow et al. (15). Briefly, after lysozyme lysis, up to six plugs were incubated for 4 h at 60°C in 1 ml of PT buffer (10 mM Tris-Cl [pH 7.6], 1 M NaCl, 10 mM EDTA, 1% sodium lauroyl sarcosine) containing 15 U of PreTAQ protease (Gibco BRL, Grand Island, N.Y.). The plugs were then washed three times for 30 min each with 5 to 10 volumes of TE buffer (pH 7.6) at room temperature. The processed plugs were stored in 0.5 M EDTA (Sigma). Restriction digestion of the mycobacterial DNA was done as described elsewhere (15), using 30 U of *Ase*I per plug in 250 μl of NEBuffer 3 (New England Biolabs, Beverly, Mass.). The restricted DNA was analyzed by field inversion PFGE in a 0.8% SeaKem GTG agarose (Intermountain, Bountiful, Utah) gel run in 0.5× Tris-borate-EDTA buffer (Intermountain) with a PC 750 pulse controller (Hoefer Scientific Instruments, San Francisco, Calif.). The gels were run at 4°C for 20 h at 75 V (13-cm electrode spacing) with pulse times set to initial values of 2.4 s forward and 0.8 s reverse and a ramp factor of 0.6. The gels were stained with ethidium bromide and photographed with Polaroid type 55 film. The DNA size markers were lambda concatemers embedded in low-melting-point agarose (New England Biolabs).

Southern blot. Whole-genome mycobacterial DNA plugs were restricted for 16 h with combinations of *Xba*I (New England Biolabs), *Bsp*106I, *Not*I, and *Spe*I (Stratagene, La Jolla, Calif.). The plugs were washed twice in TE buffer (pH 7.6), and the agarose was digested by using β-Agarase I (New England Biolabs). The DNA was ethanol precipitated and resuspended in 20 μl of TE buffer (pH 8.3). DNA fragments were separated by PFGE in a 1% SeaKem GTG agarose gel run at room temperature for 3 h using pulse times of 0.6 s forward and 0.2 s reverse and a ramp factor of 0.6. The DNA was transferred to a MagnaGraph nylon membrane (MSI, Westborough, Mass.) under neutral conditions according to procedures described by Sambrook et al. (20). The probe used to analyze the rRNA operon was based on a 319-bp fragment of the 16S rRNA gene of *M. avium* generated by PCR (18, 29) and was labeled with fluorescein isothiocyanate by using the Illuminator Prime-It kit (Stratagene). The blots were hybridized overnight at 42°C with the rRNA probe in Hybrisol I hybridization solution (Oncor, Gaithersburg, Md.). Bound probe was visualized with the Illuminator nonradioactive detection system (Stratagene) and Kodak XRP-1 film.

RESULTS

rRNA operon number. The presence of multiple copies of the rRNA operon in mycobacterial genomes was investigated by restriction enzyme digestion of genomic DNA followed by Southern blot analysis using a probe specific for the 16S rRNA gene. Restriction mapping of a cosmid containing the rRNA operon of *M. avium* 512 confirmed that the rRNA genes were contiguous and arranged in the order 16S, 23S, and 5S (19), thus justifying the use of a 16S rRNA gene probe. This restriction map also showed that *Not*I, *Spe*I, *Xba*I, and *Bsp*106I would be candidate restriction enzymes for analyzing variability in DNA upstream of the rRNA operon.

Analysis of genomic DNA extracted from *M. avium* 505 following digestion with either a combination of *Not*I and *Spe*I or *Xba*I and *Bsp*106I or *Xba*I alone revealed only single-band profiles after hybridization with the 16S rRNA probe (Fig. 1). Identical analysis of *M. avium* 512 and 225076-C also produced single-band profiles (data not shown). In contrast, digestion of the *Mycobacterium smegmatis* mc^2155 genome with a combination of either *Not*I and *Xba*I or *Xba*I and *Bsp*106I generated patterns consisting of two bands (Fig. 1). These results provide evidence that *M. avium* possesses only a single rRNA operon

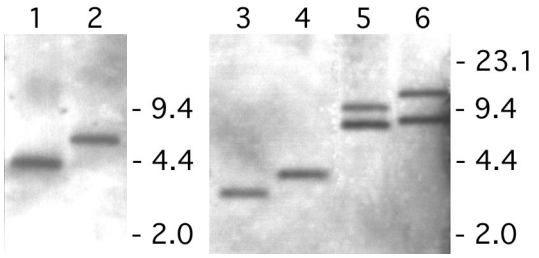

FIG. 1. Southern blots of DNA extracted from *M. avium* 505 restricted with *Not*I and *Spe*I (lane 1), *Not*I (lane 2), *Xba*I and *Bsp*106I (lane 3), and *Xba*I (lane 4) and DNA extracted from *M. smegmatis* mc^{2}155 restricted with *Xba*I and *Bsp*106I (lane 5) and *Xba*I and *Not*I (lane 6). DNA sizes (in kilobases) are indicated.

per genome and that *M. smegmatis* (a rapidly growing species) possesses two copies of the operon.

Strain identity analysis. Of the 18 *M. avium* strains, 5 were isolated from patients who had received either CLM (strains 511, 512, and 513) or AZM (strains TB0001.2 and JJL004.2) monotherapy for disseminated MAC disease and had experienced recrudescence of clinical symptoms and a rebound mycobacteremia. For each of the CLM- and AZM-resistant strains, a pretreatment CLM- and AZM-susceptible isolate was also obtained (strains 504, 505, 506, TB0001.1, and JJL004.1, respectively).

Figure 2 shows the large-fragment restriction fragment length polymorphism (RFLP) patterns of the *M. avium* isolate pairs 504 and 511 (lanes 2 and 3), 505 and 512 (lanes 4 and 5), and 506 and 513 (lanes 6 and 7). Of these pairs, strains 504 and 511 show identical patterns, and strains 506 and 513 differ by only a single band shift (between approximately 340 and 388 kb). According to the criteria proposed by Maslow et al. (15), strain 504 can be considered to be congenic with strain 511 and strain 506 can be considered congenic with strain 513. Identity was also established by RFLP for the strain pair TB0001.1-TB0001.2 (data not shown). In contrast, strains 505 and 512 were shown to have widely dissimilar RFLP patterns (Fig. 2), as did strains JJL004.1 and JJL004.2 (data not shown), indicating that the pre- and posttreatment isolates from each patient were unrelated. Strain 101R was derived from strain 101 and on this basis alone was considered to be congenic. Thus, of the paired, posttreatment resistant strains, four (511, 513, TB0001.2, and 101R) were derived from the corresponding pretreatment strains (504, 506, TB0001.1, and 101, respectively).

Although the RFLP pattern of strain 512 does not match that of strain 505, the pattern of strain 512 does match that of strain 506 (Fig. 2). Review of the laboratory records confirmed that strains 505 and 512 were isolated from blood specimens drawn from the same patient and not from the patient from whom strains 506 and 513 were isolated. There was an epidemiological link between the two patients in that both were enrolled in the same clinical trial and, on occasion, were present at the same clinic. This suggests that horizontal transmission of *M. avium* may have occurred. Interestingly, strain 512 does not have the RFLP band shift of strain 513 (Fig. 2), and also the two resistant strains have different mutations (Table 1). The significance of this is unclear.

In addition to being tested for strain identity, strain 505 was examined for the presence of a subpopulation within a CLM-resistant phenotype by spreading 10^7 CFU on five 7H11 agar plates containing 32 μg of CLM per ml. We failed to detect any CLM-resistant *M. avium* organisms within a population of strain 505. This screening, however, was limited by the spontaneous emergence of a CLM-resistant phenotype, which occurs at a frequency of approximately 10^{-8} (9). This suggests that the emergence of a CLM-resistant strain is not due to the selection of an extant CLM-resistant subpopulation at the initiation of therapy.

Correlation of CLM and AZM susceptibility phenotype and sequence analysis. A sequence analysis of the domain V region of the 23S rRNA genes of 18 strains of *M. avium* and the H37Ra and H37Rv strains of *M. tuberculosis* was performed. The region is believed to include the site of mutations associated with macrolide resistance on the basis of studies of *E. coli* (6, 7) and *M. intracellulare* (17).

Comparison of the *M. avium* domain V sequences indicated that the only difference that could be associated with the CLM-resistant phenotype is at position 2274 of the *M. avium* 23S rRNA gene; Table 1 shows the sequence alignments of the strains in the region of this variation. Strains Wa and Fr have identical sequence variation from the other *M. avium* strains further downstream in the domain V region (data not shown); however, these differences cannot be associated with the susceptibility phenotype, since Wa is CLM resistant and Fr is CLM susceptible. All nine of the CLM-susceptible *M. avium* strains show an A at position 2274; however, in all but one (strain 225076-C) of the eight CLM-resistant *M. avium* strains, this position is either a C, G, or T. Thus, base substitution at site 2274 of a susceptible *M. avium* strain appears to be associated with the expression of a resistant phenotype. Furthermore, emergence of macrolide resistance in vivo was associated with a position 2274 mutation whether the selective (therapeutic) agent was CLM (101R, 511, 512, and 513) or AZM (TB0001.2 and JJL004.2). The wild-type genotype of resistant strain 225076-C suggests that there is an alternative mechanism of macrolide resistance in *M. avium*.

Although the MIC breakpoint for clinically significant resistance to AZM has not been defined, the susceptibility results

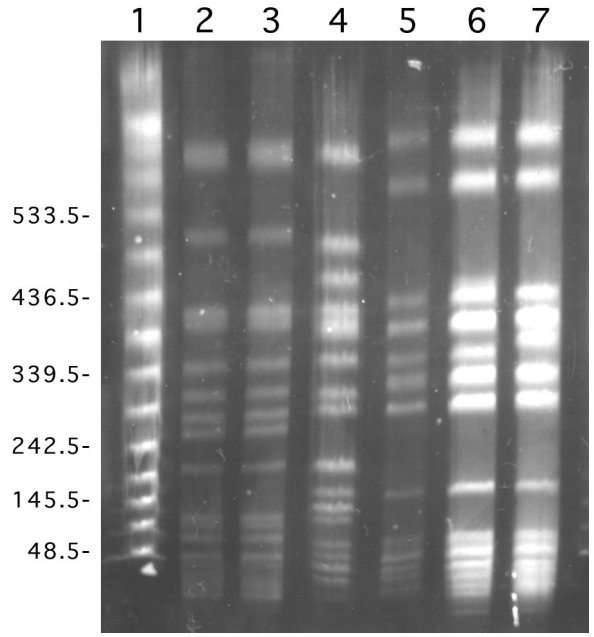

FIG. 2. PFGE profiles of three pairs of *M. avium* strains (504 and 511, 505 and 512, and 506 and 513) restricted with *Ase*I. Lanes: 1, lambda concatemers; 2, 504; 3, 511; 4, 505; 5, 512; 6, 506; 7, 513. DNA sizes (in kilobases) are indicated on the left.

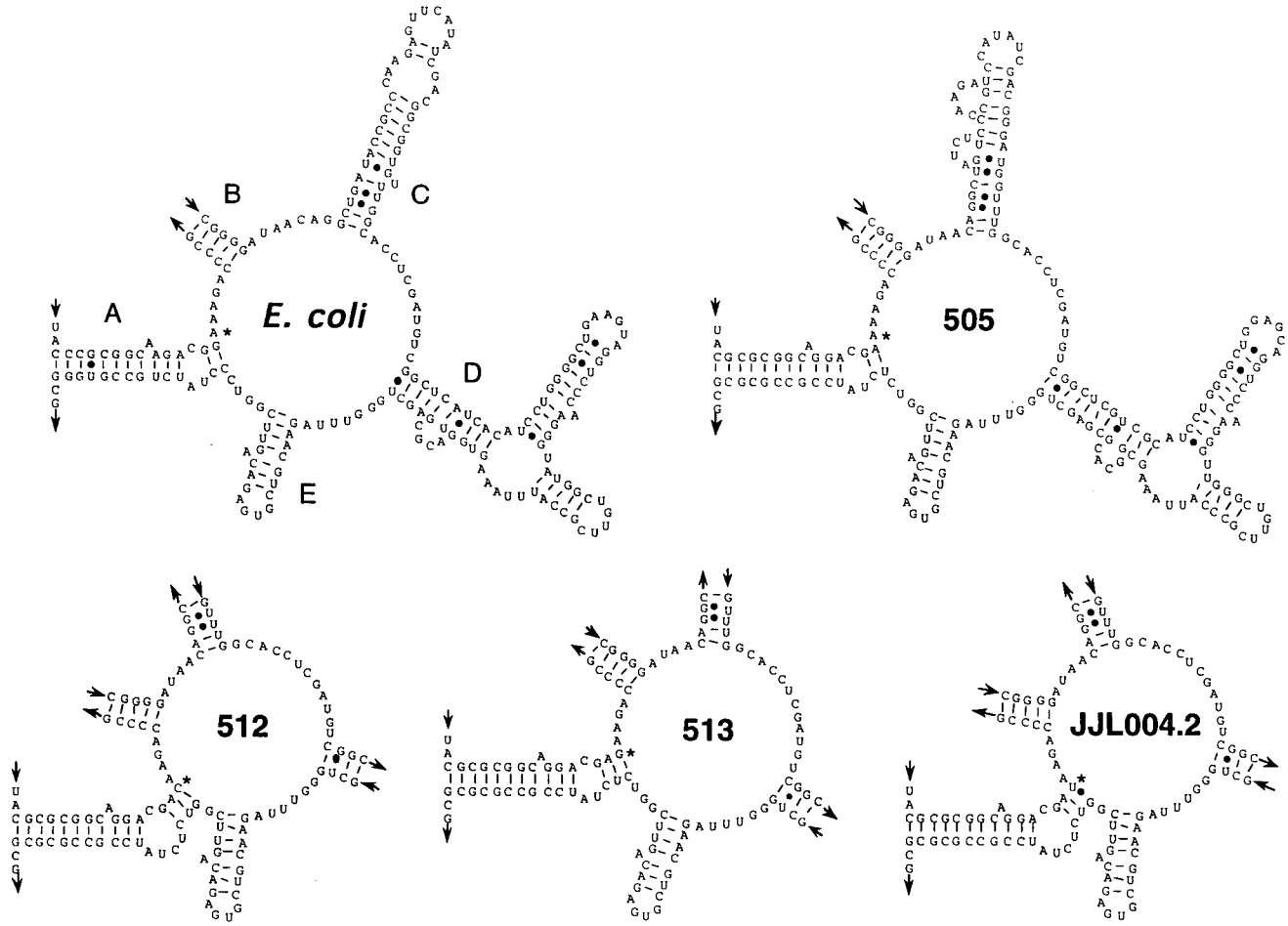

FIG. 3. Predicted secondary structures of part of the 23S rRNA domain V region of *E. coli* (from GenBank accession no. J01695) and *M. avium* 505, 512, 513, and JJL004.2; structures were determined by free energy minimization (12, 13, 31). The regions of double-stranded RNA associated with domain V of *E. coli* have been labeled (A to E). The corresponding labels for the predicted *M. avium* rRNA structures are not shown in order to avoid cluttering the diagrams but follow the same clockwise sequence as for the structure of *E. coli* rRNA. To reduce the size of the figures, the majority of region B is not shown, and regions C and D are reduced in the structures of strains 512, 513, and JJL004.2. The 5′-to-3′ orientation of the rRNA is shown (arrows). The site (residue 2274) of the point mutations observed in the macrolide-resistant *M. avium* strains and the equivalent position in *E. coli* (residue 2058) are indicated (asterisks). Strain 505 is CLM susceptible, whereas strains 512, 513, and JJL004.2 are CLM resistant.

for isolates TB0001.2 and JJL004.2 (Table 1) suggest that a threshold MIC of ≥256 μg/ml correlates with therapeutic failure. The MICs of AZM for seven of the eight isolates resistant to CLM were ≥256 μg/ml. Thus, once a strain gains a mutation at position 2274 of the 23S rRNA gene, a phenotype of resistance to both CLM and AZM is expressed. The exception to this trend is strain Wa, the significance of which is unclear.

The *M. tuberculosis* strains analyzed, H37Ra and H37Rv, both have the wild-type A residue at the position equivalent to 2274. Thus, CLM or AZM should be active against *M. tuberculosis* at the level of the ribosome.

23S rRNA secondary structure analysis. In addition to the obvious chemical changes, base substitution in the 23S rRNA may also cause a significant change in the rRNA structure in assembled ribosomes. To explore this issue, local changes in the free energy of the peptidyl transferase region were analyzed by determining RNA secondary structure on the basis of free energy minimization algorithms (12, 13, 31). The determined structures of the peptidyl transferase regions for *M. avium* strains covering all four possible residues at position 2274 and for the homologous region of *E. coli* are shown in Fig. 3.

The proposed structures of *M. avium* 505 and *E. coli* are very similar, which is as expected, since rRNA sequences are highly conserved throughout the prokaryotes. If the A residue at position 2274 of the *M. avium* 23S rRNA is substituted with either a C, G, or U residue, there is a change in the free energy of the peptidyl transferase loop, shown by a significant shift in the structure of loop A (Fig. 3). In this study, no information concerning the base pairings of rRNA within ribosomes of *M. avium* was obtained; however, to prevent a structure from forming because of entropy, rRNA folding must be constrained (e.g., by ribosomal proteins) during ribosome assembly. Furthermore, a shift in the balance between the free energy of the rRNA and the constraining mechanism may cause a local conformational change in the assembled ribosomes. Thus, these results suggest that a mutation at position 2274 of the *M. avium* 23S rRNA not only causes a base change, but is also likely to cause a conformational change within the ribosome.

DISCUSSION

On the basis of studies of other bacteria, high-level macrolide resistance requires that at least 50% of ribosomal targets be of the resistant phenotype (22). Therefore, resistance based

on a single mutational event within an rRNA gene is limited to microorganisms with only one or two rRNA operons per genome. Our observation that *M. avium* possesses only a single rRNA operon per genome is consistent with previous reports on other slowly growing mycobacteria (2, 21, 24). Furthermore, rapidly growing mycobacteria, such as *M. smegmatis*, appear to possess two operons per genome (2, 23). Therefore, for both slowly and rapidly growing mycobacteria, rRNA mutation is a feasible and likely mechanism of resistance to drugs that target ribosomes.

We found that seven of eight CLM- and AZM-resistant *M. avium* isolates have a base substitution within domain V of the 23S rRNA gene, resulting in a residue change from A to C, G, or T at position 2274. All 10 CLM-susceptible *M. avium* isolates have the wild-type A residue in this position. While this base substitution originated by random mutation, the extremely restricted nature of the sequence differences between the CLM-susceptible and CLM-resistant *M. avium* isolates suggests that these specific mutations persist as a consequence of the selective pressure of CLM or AZM therapy. Furthermore, the base substitutions do not reflect strain-related polymorphisms, since four of the resistant isolates were shown to be derived from susceptible isolates on the basis of a separate analysis of strain lineage (large-fragment RFLP analysis). Therefore, the discrete sequence differences in the V domain of the 23S rRNA genes are most likely associated with the development of macrolide resistance. The results of our study of the emergence of macrolide resistance in *M. avium* strains isolated from AIDS patients with disseminated MAC disease are consistent with previous findings for *M. intracellulare* strains isolated from patients with pulmonary disease (17). Furthermore, homologous mutations have been reported for *E. coli* strains transformed with a multicopy plasmid containing a complete *E. coli* rRNA operon and experimentally selected for macrolide resistance (6, 7).

The resistant phenotypes of the *M. avium* strains show a significant trend, in that the resistance acquired during monotherapy with CLM (511, 512, and 513) or AZM (TB0001.2 and JJL004.2) cross-reacts with the other macrolide. This trend is also apparent from data from other studies (9, 17). Moreover, the mechanism of clinically significant acquired resistance is the same (i.e., 23S rRNA mutation) with either macrolide. The sequence evidence is compelling support for the hypothesis that resistance to CLM and AZM can occur by mutation during monotherapy. Furthermore, we speculate that this may be the major mechanism of clinically significant resistance (at least when patients receive monotherapy) rather than the emergence of a preexisting (extant) resistant subpopulation (9). Interestingly, the domain V genotypes of the *M. tuberculosis* strains, H37Ra and H37Rv, suggest that the ribosomes of these strains should be susceptible to macrolides; however, the macrolides perform poorly in animal models of tuberculosis (14). This, along with the results for *M. avium* 225076-C, suggests that there are other mechanisms of intrinsic and acquired or adaptive macrolide resistance in mycobacteria.

Other mutations within 23S rRNA genes of *E. coli* have been associated with resistance to macrolides. Vannuffel et al. (26) found that a mutation (C→T) at position 2611 of *E. coli* 23S rRNA genes conferred resistance to erythromycin. This mutation site is believed to be opposite positions 2057 and 2058 (*E. coli* numbering) of the folded rRNA. We have not found any evidence of macrolide resistance-associated mutations at an equivalent position within 23S rRNA genes of any of the resistant *M. avium* strains. In addition, preliminary screening for rRNA methylases using a degenerate primer PCR assay (1) did not reveal any evidence of *erm*-like genes in either *M. avium* 225076-C or *M. tuberculosis* H37Ra and H37Rv (unpublished data).

The *E. coli* ribosomal binding site for macrolides has been shown to involve the unpaired residues A-2058 and A-2059 of the 23S rRNA (6). Residue A-2058 of *E. coli* is homologous to residue A-2274 of *M. avium*. Mutations in residue A-2058 reduced macrolide binding affinity of the ribosomes (6), resulting in resistance at the level of the ribosome (6) and the bacterium (6, 7). Although the reduced macrolide binding affinity of mutant ribosomes may be due to the actual base change within the binding site, there is evidence for a localized conformation change in the peptidyl transferase loop of the 23S rRNA. Our analysis of the *M. avium* 23S rRNA shows that mutations in the peptidyl transferase loop change the thermodynamics of the region, which could affect both the rRNA-rRNA and rRNA-protein interactions within the assembled ribosome. Douthwaite and Aagaard (6) found that if base substitutions were present at positions 2032 and 2058 of the *E. coli* 23S rRNA, then position 2063 became susceptible to chemical modification within assembled ribosomes. This suggests that the conformation of the peptidyl transferase region has changed significantly. Thus, we conclude that in *M. avium*, ribosomal resistance to macrolides involves both a base mutation and a conformational change in the ribosome at the macrolide binding site.

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