# Molecular Mechanisms of Clarithromycin Resistance in *Mycobacterium avium*: Observation of Multiple 23S rDNA Mutations in a Clonal Population

**Albrecht Meier, Leonid Heifets, Richard J. Wallace, Jr., Yansheng Zhang, Barbara A. Brown, Peter Sander, and Erik C. Böttger**

*Institute for Medical Microbiology, Medical School, Hannover, Germany; Department of Microbiology, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado; Department of Microbiology and Center for Pulmonary Infectious Disease Control, University of Texas Health Center, Tyler, Texas*

**The peptidyltransferase region of the 23S rRNA gene (the probable target site for the macrolides) was investigated in blood isolates of *Mycobacterium avium* recovered from 38 patients before and after the development of clarithromycin resistance. Point mutations were identified in 100% of the 74 resistant relapse blood isolates but in none of 69 susceptible pretreatment isolates. Multiple mutations were identified in isolates from 23 (61%) of 38 patients. Of the 63 identified mutations, 95% involved adenine at bp 2058. Single-colony clones from cultures that were mixtures of more than one mutation revealed a single mutation within each clone. Pulsed field gel electrophoresis of genomic DNA restriction fragments revealed that 13 (81%) of 16 multiple mutations identified in the same patient were derived from a single infecting strain. In vitro investigation revealed the same point mutations observed in vivo. This study defines the probable mechanism of clarithromycin resistance in *M. avium* and provides in vivo evidence that mutational resistance is random and selection-directed.**

The genetic mechanisms of antibiotic resistance have been studied intensely over the past 20 years, with most commonly encountered types of resistance having been well-defined. Resistance usually relates to a transferable gene or resistance determinant on a plasmid or transposon that encodes for an enzyme (such as $\beta$-lactamase or aminoglycoside-modifying enzymes) that inactivates the antibiotic. Other resistance determinants result in accelerated export of the drug from the cell (such as the *tetB* gene) [1] or modify the target or binding site of the antibiotic (such as the erythromycin ribosomal methylase [*erm*] genes that modify the 23S rRNA erythromycin-binding site) [2]. Some resistance relates to nontransferable genes, such as the *mecA* gene of methicillin-resistant *Staphylococcus aureus* [3]. Resistance due to mutational changes that involve chromosomal genes among bacterial species is relatively rare. Exceptions to this are acquired resistance to rifampin (RNA polymerase gene; *rpoβ*) [4] and the quinolones (gyrase gene; *gyrA*) [5].

It was demonstrated in vitro some 40 years ago that mutational resistance is not adaptive but selection-directed; that is, bacteria resistant to a drug were present before addition of the drug used to select the mutants [6]. However, in vivo evidence demonstrating in humans the selection of undirected resistance mutations while being treated for an infectious pathogen is still missing.

Studies of resistance of mycobacterial species to antimicrobial agents is essentially in its infancy, having been done at the genetic level only within the past 5 years. Most work has centered on *Mycobacterium tuberculosis* [7–9]. The emergence of *Mycobacterium avium* complex as a serious pathogen in patients with AIDS [10, 11] and in an increasing number of patients with chronic lung disease [12, 13] has focused interest on this pathogen, including how antimicrobials work and what mechanisms of resistance develop to thwart drug therapy [12]. The newer macrolides clarithromycin and azithromycin have emerged as the pillars of therapy for *M. avium* complex [14–19], but acquired resistance follows relatively quickly when monotherapy is used [18, 19].

Previous studies from our laboratories with a small number of isolates of *Mycobacterium intracellulare* suggested acquired resistance related to mutational changes in the central loop of domain V of the 23S rRNA [20]. Nash and Inderlied [21] studied 8 macrolide-resistant strains of *M. avium* from patients with disseminated disease and identified a mutation in 7 strains at the same position (homologous to *Escherichia coli* position A-2058) as identified with *M. intracellulare* [21]. We have expanded this study to include a large number of isolates of *M. avium* from patients with AIDS, as well as spontaneous

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Informed consent was obtained from the patients in the clarithromycin treatment trial from whence the study isolates were obtained in accordance with guidelines of the US Department of Health and Human Services and the individual institution where the treatment was undertaken.

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Reprints or correspondence: Dr. Richard J. Wallace, Jr., Dept. of Microbiology, University of Texas Health Center, Box 2003, Tyler, TX 75710.

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laboratory-resistant mutants. In addition, the clinical isolates were analyzed for their restriction fragment length polymorphism (RFLP) pattern using pulsed-field gel electrophoresis (PFGE).

## Methods

*Clinical isolates of M. avium for genetic evaluation.* Blood isolates of *M. avium* complex were obtained from patients with AIDS before clarithromycin therapy and again after clinical and microbiologic failure. When possible (or within 1 month of onset of therapy), 2 pretreatment and 2 relapse samples were obtained from each patient for culture. Details of culture methods and MICs have been published [18, 22]. Quantitative blood cultures were done by plating lysed blood samples on 7H11 agar [23], with the peak pretreatment colony count assessed for each patient. Sweeps from organisms plated on Middlebrook 7H10 agar were frozen at −70°C, then submitted for genetic evaluation. Organisms were identified as *M. avium* using commercial RNA/DNA probes (Accu-Probe; GenProbe, San Diego).

*Selection of clarithromycin-resistant in vitro mutants.* Two reference strains of *M. avium,* ATCC 35712 and ATCC 35718, and a reference strain of *M. intracellulare,* ATCC 35761, were obtained from American Type Culture Collection (Rockville, MD). The organisms were grown on Middlebrook 7H10 agar plates containing 32 μg/mL clarithromycin. Single colonies were picked, and isolates with MICs ≥32 μg/mL were submitted for genetic evaluation.

*Clarithromycin susceptibility testing.* Clinical isolates of *M. avium* were tested for susceptibility to clarithromycin using the BACTEC radiometric system (Becton Dickinson Diagnostic Instrument Systems, Towson, MD) as described [22]. The MIC range was 0.125–1024 μg/mL. Results of these MIC studies have been published [22]. The in vitro mutants obtained from the 3 ATCC reference strains were evaluated by broth microdilution using cation-supplemented Mueller-Hinton broth with 5% oleic acid, albumin, and dextrose [24]. Selected isolates, especially those in whom a mutation could not be identified, were regrown with single-colony isolates being retested using the BACTEC system.

*23S rDNA polymerase chain reaction (PCR) and gene sequencing.* The peptidyltransferase region of the 23S rDNA was subjected to PCR and sequenced [20]. Nucleic acids were extracted by mechanical lysis. A small loopful of bacterial mass was resuspended in 1.0 mL of TRIS-EDTA buffer (TE) in a 1.5-mL microcentrifuge tube and incubated for 10 min at 80°C to inactivate the mycobacteria. The sample was centrifuged in a microcentrifuge for 10 min at maximum speed, the supernatant was discarded, and 100 μL of TE and a loopful of acid-washed glass beads with a diameter of 100 μm (Sigma, Munich) were added. The sample was placed for 2 min at maximum speed in a tissue disintegrator (Mickle Laboratory, Gomshall, UK) to disrupt the cells. The sample was subsequently centrifuged at maximum speed in a microcentrifuge for 2 min, and a 5-μL aliquot of the supernatant was used for PCR.

PCR was done with a 50-μL reaction mixture containing 50 m*M* KCl, 10 m*M* TRIS-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 0.01% (wt/vol) gelatin, 200 m*M* each dNTP, 1.25 U of Taq polymerase (Perkin-Elmer Cetus, Uberlingen, Germany), 30 pmol of primer 18 (5′-AGTCGGGACCTAAGGCGAG-3′, corresponding to *E. coli* 23S rRNA positions 1342–1360), and 10 pmol of biotinylated primer 21 (5′-TTCCCGCTTAGATGCTTTCAG-3′, corresponding to *E. coli* positions 2765–2745). Next, 5 μL of crude nucleic acids was added to the PCR mixture while the reaction was maintained at 70°C. The thermal profile involved 39 cycles with a 1-min denaturation step at 94°C, a 2-min annealing step at 62°C, and a 2-min extension step at 72°C. Successful amplification was confirmed by agarose gel electrophoresis.

The primers that were used exhibit broad reactivity, amplifying 23S rRNA gene fragments from a variety of gram-positive and gram-negative bacteria; therefore, a combined psoralen UV treatment was used to eliminate contaminating DNA within PCR reagents [25]. Multiple reactions without adding exogenous nucleic acids were run to control for contamination as well as for the success of the decontamination procedure.

The PCR product was sequenced directly. In brief, the biotinylated single-stranded DNA template was prepared by use of Dynabeads-280-streptavidin (Dynal, Hamburg, Germany) and a Dynal MPC-E magnetic separator essentially as described by the manufacturer. A 20-μL bead solution (10 μg/μL) was used in each PCR. The beads were resuspended in 20 μL of $H_2O$. Sequencing was done with 2–5 μL of the bead single-stranded DNA solution, 2 pmol of sequencing primer 19 (5′-GTAGCGAAATTC-CTTGTCGG-3′, corresponding to *E. coli* 23S rRNA positions 1930–1949) per reaction, 0.5–1.0 μCi of [α-$^{35}$P]dCTP at 3000 Ci/mmol (Amersham Buchler, Braunschweig, Germany), and Sequenase version 2.0 (USR, Bad Homburg, Germany) by standard procedures (USB and Dynal manuals). After electrophoresis, gels were fixed in 10% (vol/vol) acetic acid with 12% (vol/vol) methanol, dried, and exposed to radiographic film for 6–12 h.

The clinical isolates of *M. avium* were tested blindly without knowledge of the clarithromycin susceptibility results or whether the isolates were pretreatment or relapse. Clarithromycin-resistant in vitro mutants were streaked on 7H10 agar with and without 50 μg/mL clarithromycin. Sequence analysis was done using bacterial mass grown on the control plate as well as the drug-containing plate. For isolates with a mixed mutation (i.e., change in >1 bp noted in the same blood culture isolate), 4 single-colony clones were prepared and the sequencing was repeated on the individual clones.

*Comparison of genomic DNA large restriction fragment patterns by PFGE.* Subcultures of *M. avium* were grown in broth, heated to 80°C for 30 min to kill the organisms, lysed with lysozyme (2 mg/mL), SDS (1%), and proteinase K (1 mg/mL), and incorporated into agarose plugs as described [20, 26]. Genomic DNA was digested with *Dra*I and *Xba*I (Boehringer Mannheim, Indianapolis) according to the manufacturer's instructions. PFGE of the large restriction fragments was done using a contour-clamped homogeneous electric field mapper (Bio-Rad, Richmond, CA) at 14°C for 20 h at 6 V/cm. Pulse time was ramped from 3 to 12 s after *Xba*I digestion and from 5 to 15 s for 14 h and then from 60 to 70 s for 6 h after *Dra*I digestion.

PFGE was done on all available blood isolates from patients with a relapse culture that showed evidence of a mixed mutation (i.e., >1 mutation in the same blood isolate) or multiple mutations (i.e., different mutations in different blood culture isolates), as

well as the single-colony clones selected for repeat sequencing. In addition, for any culture whose RFLP pattern suggested the presence of >1 organism (too many bands or bands of different intensity), 10 single colonies were chosen from an isolation plate and the PFGE was repeated on subcultures made from the single colonies.

## Results

*Clinical isolates for genetic analysis.* Thirty-eight patients were identified with disseminated *M. avium* disease who received clarithromycin monotherapy with subsequent relapse of their bacteremia. A total of 69 pretreatment isolates and 74 relapse isolates were evaluated for clarithromycin susceptibility and 23S rRNA gene sequences. All isolates were *M. avium* as determined by DNA probe.

*Selection of clarithromycin-resistant in vitro mutants.* Forty-two single-colony mutants from the 3 ATCC reference strains with clarithromycin MICs ≥32 μg/mL were submitted for 23S rDNA sequencing.

*Clarithromycin-susceptibility testing.* The 69 pretreatment blood isolates of *M. avium* were all susceptible to clarithromycin, with broth-determined MICs of 0.125–2.0 μg/mL. Of the 74 relapse blood isolates, the clarithromycin MICs ranged from 256 to 1024 μg/mL [22].

Of the 42 in vitro mutants selected from the ATCC reference strains, 18 had clarithromycin MICs of 32–64 μg/mL and 24 had MICs ≥128 μg/mL.

*23S rDNA PCR and gene sequencing.* Of the 69 pretreatment blood isolates, none had a mutation identified in the peptidyltransferase region. Of the 74 relapse blood isolates that had clarithromycin MICs ≥128 μg/mL, 5 isolates from 3 patients had a wild type sequence. Repeat sequencing done after growth on plain 7H10 agar plates revealed a wild type sequence in 4 of 5 strains, while sequencing of isolates grown on clarithromycin containing 7H10 agar revealed mutational sequences in all 5, demonstrating that these cultures were mixtures of both wild types and mutations. Of interest, blood cultures from these 3 patients were never negative while the patients were receiving therapy. The blood cultures that were sequenced were obtained between day 75 and day 174 of therapy and were the first cultures in these patients to show a major increase in clarithromycin MIC. In the remaining 69 samples, mutations were identified on initial sequencing. Thus, 100% (all 74) of the relapse blood isolates from the 38 patients had identified mutations in the peptidyltransferase region.

The mutations were not all uniform. Fifteen patients had the same single mutation identified in all blood isolates tested (27 total). An additional 17 patients had 18 cultures with 2 different mutations at the A-2058 position in the same blood culture. Single-colony isolations with repeat sequencing revealed in each case the presence of 2 clones that each had a different but single nucleotide mutation. Of the remaining 6 patients, 5 had different A-2058 mutations identified in different blood cultures taken on different days, and 1 patient had an A-2058 and an A-2059 mutation in different cultures on different days. Thus, of the 38 patients, only 15 (39%) had a single nucleotide mutation identified in their relapse strain of *M. avium*. In contrast, 21 patients (56%) had 2 nucleotide mutations involving bp A-2058 or A-2059 (or both), and 2 patients (5%) had 3 mutations that involved both nucleotides A-2058 and A-2059.

The recognition of mixed or multiple mutations was related to the number of blood culture isolates analyzed. The presence of >1 mutation was identified in 40% (2/5) of patients with a single blood isolate analyzed, 60% (18/30) of those with 2 blood isolates analyzed, and 100% (3/3) of those with 3 blood isolates evaluated. There was no relationship between the identified level of bacteremia of pretreatment blood cultures and the likelihood of having multiple or mixed mutations.

Thus, 63 mutations involving the 23S rDNA peptidyltransferase region were identified in the isolates of *M. avium* recovered from the 38 patients with clarithromycin therapy failure (table 1). Sixty of these mutations (95%) involved A-2058, with the most common substitutions being adenine to cytosine (33/63, 52%) and adenine to guanine (25/63, 40%). Thus, 92% of all mutations involved either a guanine or cytosine substitution for A-2058. Only 3 mutations (5% of the total) involved A-2059, with all 3 containing an adenine-to-guanine substitution. No strain exhibited a mutational change in both A-2059 and A-2058.

Of the 42 spontaneous in vitro mutants, 21 (50%) had a point mutation in the peptidyltransferase region. These most commonly involved a guanine or cytosine substitution for A-2058 (12/21, 57%) or A-2059 (7/21, 33%). The remaining 21 isolates (50%) had a wild type sequence. There was a direct relationship between the degree of clarithromycin resistance as measured by MICs and the likelihood of finding a mutation. Of 21 strains with mutations in the peptidyltransferase region, 19 (91%) had MICs ≥128 μg/mL. In contrast, of the 21 mutants with wild type peptidyltransferase regions, 16 (76%) had MICs of 32–64 μg/mL using the broth microdilution susceptibility method. The presence of low-level resistance in these strains was confirmed using BACTEC, and efforts to show a mixed susceptible/resistant population were unsuccessful.

*PFGE.* Fifty-seven blood culture isolates from 16 patients with multiple or mixed mutations were studied by PFGE. In addition, 32 single-colony (sequenced) clones from 8 patient isolates with mixed mutations and 30 single (unsequenced) colony clones from 3 isolates that appeared to have a mixed RFLP pattern were also studied (table 1). By PFGE, each patient's isolates were unique. The 2 mutations in 12 (86%) of 14 patients and the 3 mutations in 1 of 2 patients (13/16, 81% overall) were due to multiple mutations in the same *M. avium* strain (clone), with the pretreatment and subsequent peptidyltransferase mutants having the same PFGE pattern (table 1, figures 1 and 2). Lanes 1–8 in figure 1 show the PFGE patterns of a patient with mixed mutations in a single strain, while the

**Table 1.** Results of pulsed-field gel electrophoresis (PFGE) genomic DNA large restriction fragment analysis of cultures containing >1 identified mutation in 23S r-DNA peptidyltransferase region.

| Group | No. of patients evaluated | No. of blood cultures evaluated* | No. of single-colony clones sequenced | No. of strains determined by PFGE to be present per patient | | |
|---|---|---|---|---|---|---|
| | | | | 1 | 2 | >2 |
| Same blood culture, 2 different mutations at bp 2058 | 9 | 30 | 24 | 9 | 0 | 0 |
| Same blood culture, 2 different mutations at bp 2058, second blood culture with mutation at bp 2059 | 2 | 7 | 8 | 1 | 0 | 1 |
| Different blood cultures with different single mutations | 5 | 20 | 0 | 3 | 2 | 0 |
| Total | 16 | 57 | 32 | 13 | 2 | 1 |

* Includes pretherapy and relapse isolates.

PFGE pattern of a patient with 2 infecting strains is shown in lanes 9–12. Results of the sequencing gel and PFGE of a patient with a mixed mutation in a single strain is shown in figure 2. One of the 16 patients (6%) had evidence of 5 different strains (clones) in the *M. avium* isolates recovered from the blood.

## Discussion

These studies clearly define that clarithromycin resistance following clarithromycin therapy in patients with disseminated *M. avium* infection relates to mutations involving 1 of 2 bp in the peptidyltransferase region of the 23S rDNA. This finding supports a preliminary study from our laboratory done with *M. avium* and *M. intracellulare* that showed mutations involving A-2058 or A-2059 in 7 of 10 strains [20] and a recent study by Nash and Inderlied [21] that showed mutations at A-2058 in 7 of 8 strains of *M. avium* that had developed clarithromycin resistance.

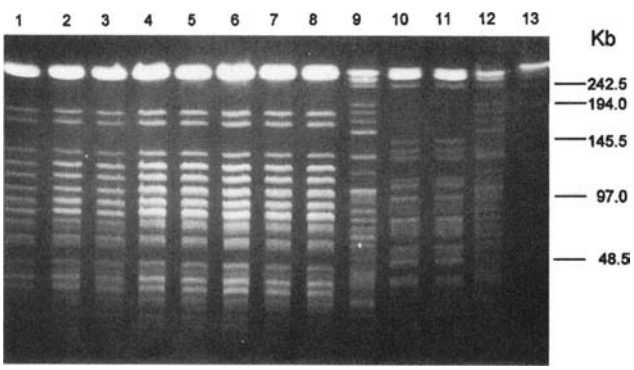


**Figure 1.** *Xba*I large restriction fragment (LRF) patterns of genomic DNA from *M. avium* cultures from 2 patients with mixed mutations. Lanes 1–8: isolates from patient 602. Lane 1, pretreatment isolate; lane 3, culture from day 8 of therapy; lane 2, relapse culture from day 84 of therapy, which contained A→C mutation at bp 2058; lane 4, relapse culture from day 168 which contained A→G/C mixed mutation at bp 2058; lanes 5, 6: single-colony clones from previous culture with 2058 A→G mutation; lanes 7, 8, single-colony clones from previous culture with 2058 A→C mutation. Lanes 9–12: isolates from patient 421. Lane 10, pretreatment isolate; lane 12, last positive culture before culture conversion (although not easily seen, 2 LRF patterns are present in lane 12); lane 9, relapse isolate from treatment day 114 which contains A→G mutation at bp 2058; lane 11, relapse isolate from treatment day 141 with A→C mutation at bp 2058; lane 13, linear bp standards.

A-2058 is thought to be involved in macrolide binding for several reasons. The same position in rRNA is the site of methylation by the *erm* [2] genes, the most common mechanism of macrolide resistance among gram-positive bacteria, which acts by blocking ribosomal binding. In addition, erythromycin binding affinity to the 23S ribosome has been shown to be reduced with mutations involving *E. coli* A-2058 [27], presumably related to conformational changes in the peptidyltransferase loop [21, 27]. Since all macrolides are thought to have a common ribosomal binding site, it would explain the same mutation developing after therapy with either azithromycin or clarithromycin [20, 21] and the cross-resistance between clarithromycin and azithromycin previously observed among isolates of *M. avium* complex identified as being resistant to either agent [22].

No mutation involving A-2058 or A-2059 was detected in 4 (22%) of 18 clarithromycin-resistant isolates combined from two previous studies [20, 21] and initially in 5 (7%) of 74 isolates in the current study. Subsequent testing with the latter after regrowth in clarithromycin-containing medium suggested these were false-negative tests because of a mixed resistant/susceptible population.

Surprisingly, similar mutations were identified in only 50% of in vitro–selected clarithromycin-resistant mutants of *M. avium–M. intracellulare*. A feature of the mutants with wild type peptidyltransferase regions was their relatively low clarithromycin MICs, usually in the range of 32–64 μg/mL. This contrasts with the higher MICs seen in the peptidyltransferase

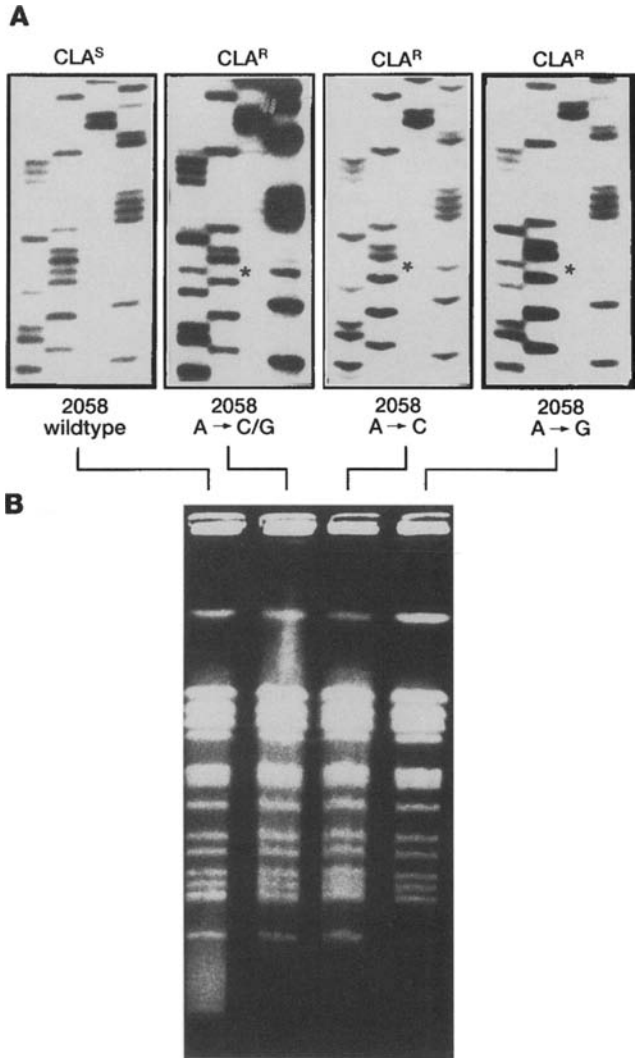


**Figure 2.** Sequencing gels of peptidyltransferase region (**A**) and pulsed-field gel electrophoresis (**B**) of isolates obtained from patient with mixed mutation: 2058 wild type, clarithromycin-susceptible pretreatment isolate ($CLA^S$); 2058 A→C/G, drug-resistant ($CLA^R$) relapse culture with mixed mutations; 2058 A→C, 2058 A→G, single-colony clones from culture with mixed mutation. Columns in gels were loaded in order G-A-T-C. ★ = position of adenine 2058 on sequencing gels.

mutants, which were almost always >128 μg/mL, and among the clinical isolates, which averaged 1024 μg/mL. This suggests that some other mutation is responsible for these low-level clarithromycin-resistant mutants, perhaps involving cellular or cell wall transport. Of interest, similar low-level mutants with wild type peptidyltransferase regions were not encountered among clinical isolates. Given the very high macrophage and tissue concentrations achieved with both azithromycin and clarithromycin [28–31], these concentrations may actually be achieved in tissues in vivo and hence the isolates are not clinically resistant.

One previous study of *M. avium–M. intracellulare* identified mutations at A-2059 in 3 of 7 isolates with macrolide resistance [20]. The current study confirmed the importance of this position, as point mutations at A-2059 were identified in 5% of the 60 in vivo mutants and 33% of 21 in vivo mutants. The effects of base pair changes at A-2059 on ribosomal structure or macrolide binding are not as well defined as with A-2058. Resistance to erythromycin in *Mycoplasma pneumoniae* [32] and of lincosamides in chloroplasts [33] has also been reported following point mutations at locations homologous to A-2059. Thus, this position appears to play an important role in macrolide binding in addition to the previously recognized A-2058.

Clearly the most astonishing finding of the study was the presence of multiple mutations in *M. avium* isolates from the same patient. This was identified in 61% of patients with clarithromycin-resistant *M. avium* but was directly proportional to the number of blood cultures studied and may well have been present in most if not all patients. By RFLP analysis, the majority of such patients (13/16 studied) had multiple mutations in the same strain as determined by PFGE. The presence of multiple resistance mutations in a single strain of organisms (i.e., clonal population) is indicative of the random nature of these mutational events occurring in vivo.

One concern might be that the presence of >1 mutation in the same culture reflects an error in the PCR and sequencing technique. This is highly unlikely for a number of reasons, including that the isolates underwent PCR and sequencing with the investigator blinded to the susceptibility results. Mixed mutations were seen only among resistant isolates. The sequencing was done directly from the PCR product without cloning, a technique that minimizes amplification errors. Finally, single-colony clones from the cultures with a mixed mutation gave single mutations that reflected the mixed mutation seen in the parent strain.

The reason for these multiple mutations at first glance appears unclear, as the emergence of a single drug-resistant mutant would likely replace most of the population at any given site. However, *M. avium* infection in patients with AIDS is usually widely disseminated, with foci involving the liver, spleen, multiple sites within the bone marrow, gastrointestinal tract, lymph nodes, and other organs. Although the frequency of spontaneous resistant mutants to clarithromycin was not determined in this study, several previous studies have determined it to be generally $10^{-8}$ to $10^{-9}$ [22].

The total body burden of *M. avium* in patients with positive blood cultures is variable, as up to 30% of patients with *M. avium* bacteremia will have no detectable foci histopathologically at postmortem examination, while ~50% will have three or more sites of involvement [34]. The number of visible sites of involvement increased with duration of survival after the first positive blood culture [34]. Since most of these organs are acid-fast smear–positive, it is likely that each of these organisms potentially harbors some spontaneous clarithro-

mycin mutants. The large numbers of organisms at each site may provide good conditions for the development of multiple clones containing different mutations. The same mutational change, such as adenine to guanine at bp 2058, could actually occur at multiple sites but be undetectable by current genetic methodologies.

Ribosomal mutations are a rare cause of antibiotic resistance among bacterial species, in part related to the finding that most gram-positive and gram-negative bacteria contain multiple copies of the 23S rRNA gene in the chromosome. Hence, a mutation involving only one of these genes would have a lesser impact (probably not a clinical one) than mutations involving other genes that are present as single copies. Previous studies in *M. avium* complex have shown that they contain only a single chromosomal copy of the 23S rRNA gene [21, 35] and hence would be highly susceptible to mutations involving the gene. As has been noted previously [22] and was confirmed in the current study, spontaneous clarithromycin-resistant mutants are readily recovered from a single culture in the laboratory.

Since the introduction of antibiotics in clinical medicine, it is clear that drug treatment may lead to selection of resistant mutants. Definitive evidence of spontaneous, undirected bacterial mutation was furnished by an in vitro statistical approach called fluctuation analysis, designed by Luria and Delbrück [6] in 1943. Lederberg and Lederberg [36] subsequently used replica plating to demonstrate the same effect more directly. Three unique circumstances have allowed us to provide evidence in vivo for the stochastic nature of acquired mutational resistance: the existence of a drug resistance that is exclusively mutational, the functional equivalence of different resistance-conferring mutations, and the excessive bacterial load present in patients with disseminated *M. avium* infection that occurs in patients with AIDS.

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