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Identification of Mutations in 23S rRNA Gene of Clarithromycin-Resistant *Mycobacterium intracellulare*

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Clarithromycin is a potent macrolide that has been used for treating infections with nontuberculous mycobacteria. Pairs of susceptible and resistant *Mycobacterium intracellulare* strains were obtained from patients with chronic pulmonary *M. intracellulare* infections undergoing monotherapy with clarithromycin. Nucleotide sequence comparisons of the peptidyltransferase region in 23S rRNAs from parental and resistant strains revealed that in three of six resistant strains, for which the MIC was >32 μg/ml, a single base was mutated (*Escherichia coli* equivalent, A-2058→G, C, or U). As the modification of adenine 2058 by dimethylation is a frequent cause of macrolide resistance in a variety of different bacteria, we suggest that mutation of A-2058 confers acquired resistance to clarithromycin in *M. intracellulare*.

Slowly growing mycobacteria, including *Mycobacterium kansasii*, *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. szulgai*, *M. nonchromogenicum*, *M. intracellulare*, and *M. avium* complex (MAC), have been implicated in various types of human diseases, including skin and soft tissue infections and pulmonary disease. Notably, a common complication in patients with late-stage AIDS is disseminated infection with MAC. In relation to the CD4 cell count, the prevalences of disseminated MAC infection in these patients were reported to be 21% at 1 year and 43% at 2 years following the diagnosis of AIDS (17).

Recently, new macrolides, including clarithromycin, roxithromycin, and azithromycin, were synthesized. The in vitro evaluations of these new macrolides as potential therapeutic agents for mycobacterial infections have demonstrated that clarithromycin in particular has activity against isolates of nontuberculous mycobacteria (1, 2, 9). A double-blind clinical trial showed that within 6 weeks, monotherapy with clarithromycin produced a dramatic decrease in the number of viable bacteria in the blood of patients with AIDS and disseminated MAC infection (3). However, it was simultaneously noted that monotherapy with clarithromycin can lead to drug resistance (21).

Macrolides are bacteriostatic antibiotics which inhibit the peptidyltransferase region of the 50S ribosomal subunit (for a review, see reference 6). A few years after the introduction of erythromycin for therapy, resistance of *Staphylococcus aureus* to this drug emerged. For these resistent strains, simultaneous resistance to other macrolides, to lincomycin, and to streptogramin B antibiotics was noted. This is the so-called MLS phenotype (29). Although the exact mechanism of translational inhibition by these drugs is still undetermined, a peptidyltransferase center in a small region of 23S rRNA has been identified by mapping resistance to several transferase inhibitors. In particular, the *Escherichia coli* equivalent A-2058 in 23S rRNA is modified by a methylase to yield resistance to erythromycin and to MLS antibiotics (22). This position lies in a generally conserved loop of domain V, which has been implicated in peptidyl transfer (18). The MLS cross-resistance phenotype due to modification of the drug target is widely distributed and has been detected in a number of gram-positive and gram-negative bacteria. Clinically acquired resistance of the MLS phenotype is due to the plasmid-mediated production of methylases by a group of genes known collectively as the *erm* genes (for a review, see reference 13).

We showed previously that within the eubacteria, slowly growing mycobacteria exhibit a unique mechanism of acquired resistance. Namely, a mutated small-subunit rRNA was found to mediate resistance to streptomycin (5). This finding prompted us to investigate the primary sequence of the large-subunit rRNA in *M. intracellulare* strains with clinically acquired clarithromycin resistance.

Patients with abnormal chest radiographs and sputa positive for *M. intracellulare* were enrolled a clinical treatment trial in which clarithromycin was given at 250 to 1,000 mg orally twice a day for 4 months alone and then with multiple other drugs. Informed consent was obtained from all patients. Sputa were processed with *N*-acetylcysteine–2% NaOH and plated on Middlebrook 7H10 and Lowenstein-Jensen agar before, during, and after therapy. Details of this trial are currently unpublished (28a). The isolates were identified as MAC by use of gene probes (Gen-Probe, Inc., San Diego, Calif.) and subsequently identified as *M. intracellulare* by 16S rRNA gene sequence determinations (11). Broth microdilution MICs were determined with either Middlebrook 7H9 broth or Mueller-Hinton broth supplemented with 5% oleic acid, albumin, and dextrose (OAD) as previously described (1, 2). The pH of the supplemented 7H9 broth was corrected to 7.4. Plates were inoculated to a concentration of 10^4 CFU/ml and read after 14 days of incubation at 35°C.

Pulsed-field gel electrophoresis with infrequently cutting restriction endonucleases (*Xba*I and *Asn*I) and genomic DNA was performed on pretreatment and subsequent clarithromycin-resistant isolates by published techniques (14). DNA extraction, PCR amplification, and nucleic acid sequencing were done as described previously (11). The primers chosen for amplification were based on published oligonucleotides (12, 27) after comparison with the 23S rRNA sequences of *M. leprae*, *Streptomyces griseus*, *Micrococcus luteus*, and a *Frankia* sp., which were obtained from the EMBL data base. These

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TABLE 1. Nucleotide sequences of the oligonucleotide primers used[a]

Oligonucleotide primer	5′→3′ Sequence	Direction	Position[b]
18	AGTCGGGACCTAAGGCGAG	Forward	1342–1360
19	GTAGCGAAATTCCTTGTCGG	Forward	1930–1949
20	TCGCTCAACGGATAAAAGGTA	Forward	2419–2439
21	TTCCCGCTTAGATGCTTTCAG	Reverse	2765–2745
22	AGTTTAACTGGGGCGGT	Forward	2241–2257

[a] Oligonucleotide primers 18 and 21 were used for amplification.
[b] Corresponding *E. coli* 23S rRNA position, in nucleotides.

primers 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 (15). The decontamination procedure was controlled by running blank samples. The oligonucleotide primers used are shown in Table 1.

Table 2 lists the features of the isolates investigated. Six posttherapy isolates were found to be resistant to clarithromycin (MIC, >32 μg/ml). The large restriction fragment patterns obtained by pulsed-field gel electrophoresis of *Xba*I digests of paired clarithromycin-susceptible (pretherapy) and clarithromycin-resistant (posttherapy) *M. intracellulare* clinical isolates are shown in Fig. 1. The large restriction fragment patterns of both susceptible and resistant clinical isolates were identical. These results were verified by comparison with large restriction fragment patterns generated by digestion with *Asn*I and *Ase*I (data not shown). The pairs of clarithromycin-susceptible and -resistant isolates were analyzed by gene amplification techniques to determine the primary structure of the 23S rRNA gene. The primers used directed the synthesis of an approximately 1.5-kb gene fragment. Subsequently, more than 400 nucleotide positions, including the peptidyltransferase region, were determined for each isolate. For three (577-3, 682-7, and 968-2) of the six independent clarithromycin-resistant isolates but for none of the susceptible parental strains, a single point mutation was found in domain V of 23S rRNA. The position mutated is homologous to *E. coli* position 2058. An adenine at this position was replaced by guanine (isolate 968-2), cytosine (isolate 577-3), or thymidine (isolate 682-7) (Fig. 2). Figure 3

TABLE 2. Isolates investigated

Patient	Date of culture (day/mo/yr)	Isolate tested	Clarithromycin MIC (μg/ml)
1	20/03/92	968	1
	23/07/92	968-2	>64
2	02/02/91	682-4	4
	22/04/92	682-7	>64
3	13/04/92	577-2	1
	22/09/92	577-3	>64
4	14/07/92	1070-2	2
	11/12/92	1070-4	64
5	02/05/91	531-2	2
	16/12/91	531-6	>32
6	02/02/93	1088	2
	06/10/93	1088-4	>64

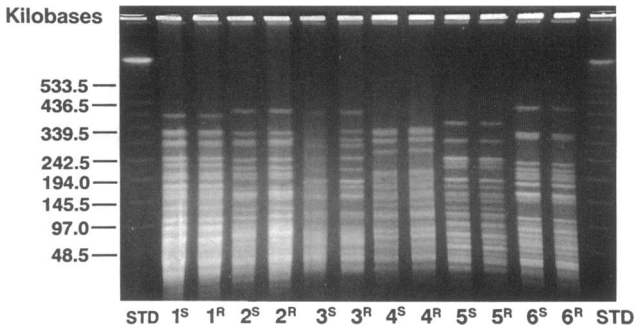

FIG. 1. *Xba*I LRF patterns of six pairs of clarithromycin-susceptible (S) (pretherapy) and clarithromycin-resistant (R) (posttherapy) *M. intracellulare* clinical isolates. Lanes: 1^S, 531-2; 1^R, 531-6; 2^S, 682-4; 2^R, 682-7; 3^S, 968; 3^R, 968-2; 4^S, 577-2; 4^R, 577-3; 5^S, 1070-2; 5^R, 1070-4; 6^S, 1088; 6^R, 1088-4; STD, lambda phage 48.5-kb concatamers.

depicts the alterations within a secondary structure model of the peptidyltransferase region. For three isolates (531-6, 1070-4, and 1088-4), no mutations in the peptidyltransferase region were found; specifically, positions 2505 and 2611, which have been implicated in macrolide resistance (7, 8, 24), were found to be wild type.

We suggest that mutation of A-2058 confers clarithromycin resistance in a subgroup of resistant *M. intracellulare* isolates. The data do not prove absolutely that the change of A-2058 to G, C, or U is responsible for the observed resistance. Nevertheless, the central loop of domain V is known to contain the peptidyltransferase region of the 50S ribosomal subunit, and many mutations conferring resistance to peptidyltransferase inhibitors are due to alterations of this region. Specifically, macrolide resistance mutations or modifications have been found at a position homologous to A-2058 of *E. coli* (Fig. 2). This adenine is frequently dimethylated by plasmid-encoded methylases, and the modification is sufficient to confer MLS resistance (13). An A→G substitution was found to mediate resistance of the MLS type in *S. ambofaciens* (20), yeast mitochondria (23), and chloroplasts of *Chlamydomonas reinhardtii* (8). In the vicinity of this adenine, other resistance mutations have been located (e.g., 7, 8, 24). In the secondary structure, positions 2057 and 2611 are base paired and, together with A-2058, appear to define the RNA binding site of erythromycin (16). On the basis of phylogenetic evidence, it has been suggested that only organisms with a composition

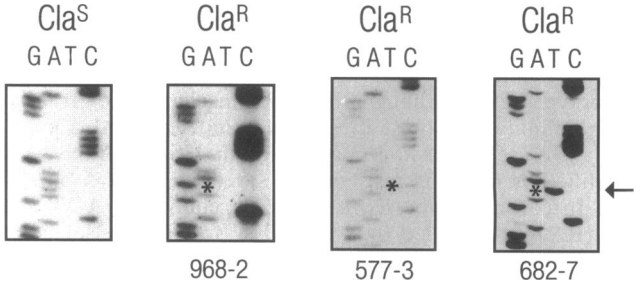

FIG. 2. Nucleotide sequence of a short region of the 23S rRNA gene demonstrating the mutations at homologous *E. coli* position 2058 (A→G, C, or T) that are associated with clarithromycin resistance. The wild-type (Cla^S) sequence is shown along with those of resistant (Cla^R) isolates. The mutated base is indicated by an asterisk, and the location of the mutation is marked by an arrow.

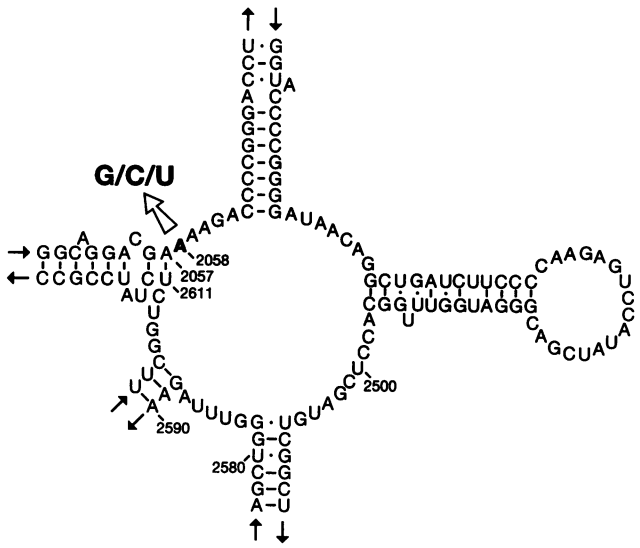

FIG. 3. Proposed secondary structure of a portion of *M. intracellulare* 23S rRNA (central loop of domain V) according to the model of Noller (18). The base substitutions observed in clarithromycin-resistant *M. intracellulare* isolates are indicated. Numbering corresponds to that for *E. coli*.

equivalent to G-2057, A-2058, and C-2611 are erythromycin susceptible (28). More recently, disruption of base pairing between positions 2057 and 2611 was shown to confer erythromycin resistance (4, 7, 8). Clarithromycin-susceptible and -resistant *M. intracellulare* strains have an adenine at homologous *E. coli* position 2057 and a uracil at homologous *E. coli* position 2611 but differ at residue 2058. On the basis of our results, we suggest that susceptibility to macrolides such as erythromycin and clarithromycin requires an adenine at position 2058 and Watson-Crick base pairing between residues 2057 and 2611, i.e., G · C or U · A.

Mutations conferring resistance to some antibiotics of the MLS group have been found in the genes coding for ribosomal components: proteins or rRNA. Erythromycin resistance mutations have been located in the genes coding for proteins L4 and L22 in *E. coli* (19, 25, 26, 30). These ribosomal proteins represent a possible resistance mechanism in the three (531-6, 1070-4, and 1088-4) of six clarithromycin-resistant *M. intracellulare* isolates with a wild-type domain V of 23S rRNA. Resistance mutations located in rRNA genes could be observed when a single set of rRNA genes was present, as in yeast mitochondria and plant chloroplasts (7, 8, 23, 24). When several sets of rRNA genes were present, as in most bacterial pathogens, such mutations could not be isolated as a result of acquired resistance, but resistance was found to be mediated by enzymes which act in *trans*, i.e., by methylation of nucleotide positions (22). In contrast, slowly growing mycobacteria, such as *M. intracellulare* and MAC, show a single rRNA operon, allowing normally recessive mutations to exert a dominant phenotype. It is of interest that direct selection in the laboratory for macrolide resistance in *S. ambofaciens*, which resulted in a mutation at A-2058 (20), also appears to have occurred in a clinical setting, in which it was responsible for the selection of clarithromycin-resistant mycobacteria.

In a mouse model, the frequency of clarithromycin-resistant mutants was estimated to be between 10^{-8} and 10^{-9} (10). Given the mechanism of resistance demonstrated here, which reflects single-step mutational resistance, monotherapy of extensive MAC lung disease or other mycobacterial infections with large numbers of organisms should not be advocated, as it may result in the frequent occurrence of resistant strains. Likewise, it may be questioned whether attempts to use clarithromycin as monoprophylaxis against disseminated MAC infection in patients with AIDS should be encouraged in light of the molecular mechanisms of resistance described.

We recently had the opportunity to investigate two *M. avium* patient isolates with acquired resistance to azithromycin (these isolates were kindly provided by Lowell Young); both showed a single point mutation in 23S rRNA, A-2058→T and A-2059→C, respectively (data not shown). As expected, the clarithromycin-resistant *M. intracellulare* strains investigated in this study showed cross-resistance to azithromycin (data not shown), indicating that the two macrolides seem to select for the same type of genetic resistance. The results of our study provide evidence that for a subgroup of macrolide-resistant *M. intracellulare* and MAC strains, resistance is due to an altered 23S RNA gene, the mutations being localized to the central loop of domain V, the peptidyltransferase region. The mutations in rRNA genes described previously (5) and in this paper demonstrate that mutations in rRNA genes play a crucial role in the development of mycobacterial resistance to antibiotics which act by inhibiting protein synthesis. Identification of the molecular mechanisms underlying drug resistance offers the possibility of the rapid recognition of resistance genes directly in clinical samples. This rapid recognition is especially important for organisms which grow slowly.

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ADDENDUM IN PROOF

We have recently characterized two more clarithromycin-resistant *M. avium-M. intracellulare* strains, both of which showed an A→C transition at position 2059, indicating that an adenine at position 2059 is somehow involved in susceptibility to macrolides. In total, of 10 clarithromycin-resistant *M. avium-M. intracellulare* isolates investigated, 7 showed a mutation of 23S rRNA at position 2058 or 2059.

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