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Transition Mutations in the 23S rRNA of Erythromycin-Resistant Isolates of *Mycoplasma pneumoniae*

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Erythromycin is the drug of choice for treatment of *Mycoplasma pneumoniae* infections due to its susceptibility to low levels of this antibiotic. After exposure of susceptible strains to erythromycin in vitro and in vivo, mutants resistant to erythromycin and other macrolides were isolated. Their phenotypes have been characterized, but the genetic basis for resistance has never been determined. We isolated two resistant mutants (M129-ER1 and M129-ER2) by growing *M. pneumoniae* M129 on agar containing different amounts of erythromycin. In broth dilution tests both strains displayed resistance to high levels of several macrolide-lincosamide-streptogramin B (MLS) antibiotics. In binding studies, ribosomes isolated from the resistant strains exhibited significantly lower affinity for [^{14}C]erythromycin than did ribosomes from the M129 parent strain. Sequencing of DNA amplified from the region of the 23S rRNA gene encoding domain V revealed an A-to-G transition in the central loop at position 2063 of M129-ER1 and a similar A-to-G transition at position 2064 in M129-ER2. Transitions at homologous locations in the 23S rRNA from other organisms have been shown to result in resistance to MLS antibiotics. Thus, MLS-like resistance can occur in *M. pneumoniae* as the result of point mutations in the 23S rRNA gene which reduce the affinity of these antibiotics for the ribosome. Since they involve only single-base changes, development of resistance to erythromycin in vivo by these mechanisms could be a relatively frequent event.

Mycoplasma pneumoniae is a common cause of respiratory tract infections particularly in school-aged children and young adults. All levels of the respiratory tract are involved, and while the most characteristic manifestations are acute bronchitis and pneumonia (8, 11), serious complications can occur (5). The sensitivity of *M. pneumoniae* to erythromycin and many other macrolide antibiotics makes them the drugs of choice for chemotherapy (3, 33). Treatment with erythromycin results in relatively rapid alleviation of symptoms; however, viable *M. pneumoniae* can frequently be isolated from infected individuals for a prolonged period of time following therapy (6, 41, 45). Isolation of resistant strains from patients following treatment is not uncommon (26, 28, 43–45), and erythromycin-resistant mutants are readily derived by selection in vitro (27, 43, 44). Phenotypic studies have demonstrated that most of these strains simultaneously developed resistance to macrolide, lincosamide, and group B streptogramin (MLS) antibiotics (48). It was suspected that development of resistance to erythromycin contributed to the prolonged colonization of the respiratory tract following chemotherapy.

As part of our efforts to understand what role, if any, development of antibiotic resistance may play in the course of *M. pneumoniae* infection, we are attempting to determine the molecular mechanisms by which resistance to erythromycin can arise. While the genetic basis for MLS resistance has been extensively studied for other bacteria, except for a rudimentary study of *Ureaplasma urealyticum* (30) it has not been explored in mycoplasmas. We show that MLS-type resistance in two isolates derived in vitro is correlated with A-to-G transitions at two conserved sites in the central loop in domain V of the 23S rRNA which are known to result in similar patterns of resistance in other organisms. The possible clinical implications of these mechanisms are discussed.

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MATERIALS AND METHODS

Media and growth conditions. *M. pneumoniae* M129-B16 (ATCC 33530) was grown in glass culture bottles or 96-well microtiter plates in modified Hayflick's medium (19) without penicillin. One-percent Bacto-Agar (Difco, Detroit, Mich.) was added for growth on solid medium. Antibiotics were obtained from Sigma (St. Louis, Mo.) and added from concentrated solutions prepared in ethanol or deionized water. For applications other than determination of MIC, resistant isolates were grown in medium containing 1 μg of erythromycin per ml. All isolates were stored in Hayflick's medium without antibiotics at −70°C.

Determination of MICs of antibiotics. Resistance to various antibiotics was determined by the broth dilution method in 96-well microtiter plates (37). Each well contained an initial inoculum of 10^4 CFU in 0.2 ml of Hayflick's medium without penicillin. MIC was defined as the lowest concentration of antibiotic at which (i) medium color did not change during the 3-week incubation at 37°C and (ii) microscopic examination of the bottom of the well failed to find evidence of mycoplasmal growth. No further growth or change in the results occurred in any of the tests after the 14th day of incubation.

Ribosome isolation and erythromycin binding assays. Ribosomes were isolated by methods similar to those described for other bacteria (16, 30). For each ribosome preparation, cells harvested from 20 bottles were suspended in TMMKA-100 (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 100 mM KCl, 30 mM NH_4Cl, 6 mM β-mercaptoethanol) and ruptured by sonication. Ribosomes were then isolated by differential centrifugations at 4°C. The concentration of 70S ribosomes was determined by measuring the optical density at 260 nm with a DMS 80 spectrophotometer (Varian, Walnut Creek, Calif.). Ribosomes were stored in small aliquots at −70°C. They were used in binding assays immediately after thawing and never refrozen.

Binding assays were performed by incubating various concentrations of 70S ribosomes with 100 pmol of [*N-methyl*-^{14}C]erythromycin (54.0 mCi/mmol) (NEN DuPont, Wilmington, Del.) in 200 μl of TMMKA-100 at 22°C for 30 min. This solution was then filtered through 0.22-μm-pore-size GS nitrocellulose filters (Millipore, Bedford, Mass.) and rinsed three times with 5.0 ml of TMMKA-100. Bound ^{14}C was measured by scintillation counts of these filters in a Wallac 1410 liquid scintillation counter (Wallac Oy, Turku, Finland).

PCR amplification, cloning, and sequencing of domain V from the 23S rRNA gene. A forward primer extending from position 1758 to 1775 (GCAGTGAA GAAGAACGAGGGG) in the known sequence of the *M. pneumoniae* 23S rRNA (22) and a reverse primer from position 2684 to 2664 (GTCCTCGCT TCGGTCCTCTCG) of the same sequence were used to amplify the peptidyl transferase region of the 23S rRNA from M129, M129-ER1, and M129-ER2.

TABLE 1. MICs for the *M. pneumoniae* M129 and two erythromycin-resistant mutants

Antibiotic	MIC (μg/ml)[a]		
	M129	M129-ER1	M129-ER2
Erythromycin	0.004–0.01	>1,000	125–250
Oleandomycin	0.01–0.06	>1,000	125–500
Spiramycin	0.16–0.63	20.0	>160
Midecamycin	0.08	5.0	>100
Tylosin	0.003–0.01	0.01–0.2	6.3
Clindamycin	1.6–3.1	>1,000	50
Lincomycin	3.1–12.6	>1,000	500
Tetracycline	0.08–0.63	0.16–0.31	0.31
Mikamycin A	0.16–1.25	0.31	0.31
Kanamycin	20.0	20.0	10.0
Chloramphenicol	7.8	3.9	0.5

[a] In two to six separate tests.

PCR was performed by using buffers and *Taq* DNA polymerase from Cetus (Emoryville, Calif.) with *M. pneumoniae* genomic DNA as template. Thirty-five cycles of polymerization reactions (1 min at 95°C, 1 min at 45°C, and 1.5 min at 72°C) resulted in a single fragment of the expected size (928 bp), which was purified by electrophoresis in 1% NuSieve agarose (FMC, Rockland, Maine) followed by extraction with B-agarase (New England Biolabs, Beverly, Mass.). Fragments from M129 and M129-ER1 were ligated to the *Srf*I site in pCR-Script SK(+) (Stratagene, La Jolla, Calif.), and the fragment from M129-ER2 was ligated to the PCR cloning site of pGEM-T (Promega, Madison, Wis.). Recombinant plasmids were introduced into *Escherichia coli* XL-1 Blue (7) by chemical transformation (36). Sequences of the cloned fragments were determined by using Sequenase 2.0 (U.S. Biochemical, Cleveland, Ohio) and internal primers designed from the published sequence.

RESULTS

Selection for mutants resistant to erythromycin was conducted by inoculating 10^6 CFU of *M. pneumoniae* M129-B16 on each of a series of petri dishes containing increasing concentrations of erythromycin (0.05, 0.5, and 5.0 μg/ml). Two erythromycin-resistant colonies which grew on the 0.05-μg/ml plates were used to inoculate Hayflick's broth containing 0.05 μg of erythromycin per ml for growth of sufficient quantities for further studies and production of frozen stocks. The resistance phenotypes of these two mutants (M129-ER1 and M129-ER2) were characterized by broth dilution assays to determine MICs of several macrolide, lincosamide, group A streptogramin, tetracycline, chloramphenicol, and aminoglycoside antibiotics (Table 1). MICs for the erythromycin-sensitive M129 strain were consistent with previous reports for this and other strains of *M. pneumoniae* (1, 3, 4, 9, 20, 26–28, 43, 44). Both mutants demonstrated significantly increased resistance to macrolide and lincosamide antibiotics, while sensitivities to tetracycline, group A streptogramin (mikamycin A), chloramphenicol, or aminoglycosides were unaffected. M129-ER2 may be more sensitive to chloramphenicol and kanamycin than the other strains; however, this difference was not dramatic. While both mutant strains exhibited MLS-like patterns of resistance, there were differences between them. M129-ER1 showed greater resistance to the 14-member macrolides (erythromycin and oleandomycin) and lincosamides (clindamycin and lincomycin), while M129-ER2 was resistant to greater concentrations of the 16-member macrolides (midecamycin, spiramycin, and tylosin).

MLS antibiotics inhibit growth of susceptible cells by interfering with peptidyl transferase activity of the 50S ribosomal subunit (14, 16). They are thought to achieve this by binding to the ribosome near the central loop in domain V of the 23S rRNA. Known examples of MLS resistance result from modifications of the rRNA or ribosomal proteins which reduce the affinities of MLS antibiotics for the ribosome (12, 13, 21, 31, 46, 47). To test for reduced affinity of erythromycin for the ribosomes from the resistant mutants, we measured binding of [^{14}C]erythromycin to ribosomes isolated from M129, M129-ER1, and M129-ER2 in vitro. The results of these binding assays are shown in Fig. 1. Affinities of the ribosomes from both resistant mutants were significantly reduced relative to the sensitive parent strain. Thus, we designed experiments to test for sequence changes in the 23S rRNA of the 50S ribosomal subunit which are known to result in MLS resistance in other organisms.

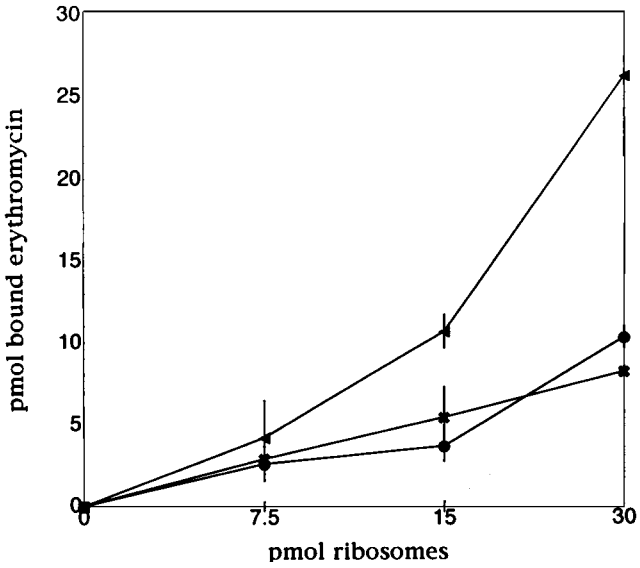

FIG. 1. Binding of [^{14}C]erythromycin to 70S ribosomes isolated from *M. pneumoniae* M-129 (triangles), M129-ER1 (circles), and M129-ER2 (x). Each point is the average of two to six independent tests. Vertical lines, standard errors.

To investigate the possibility that point mutations in the central loop of domain V in the 23S rRNA could be responsible for resistance (13, 39), we used the known sequence of the *M. pneumoniae* 23S rRNA to design primers for amplification and sequencing of the region from nucleotide 1758 to 2684. The M129 sequence matched the GenBank entry for *M. pneumoniae* (22); however, M129-ER1 had an A-to-G transition at nucleotide 2063, and M129-ER2 had an A-to-G transition at nucleotide 2064. Both transitions are within a highly conserved region of the central loop of domain V (Fig. 2) (17, 29) which is believed to be part of the binding site for MLS antibiotics (12, 13, 25). Thus, these mutations should account for the sudden onset of high-level resistance in M129-ER1 and M129-ER2.

DISCUSSION

Several lines of evidence suggest that the A-to-G transitions in M129-ER1 and M129-ER2 are responsible for the MLS resistance phenotypes, although they do not constitute absolute proof. RNA protection studies indicate considerable overlap in binding sites for MLS antibiotics in the central loop of domain V of the 23S rRNA, and there are numerous reports of MLS resistance being associated with point mutations at specific sites within this loop (reviewed in reference 47). A-2063 of the *M. pneumoniae* 23S rRNA is homologous to A-2058 in *E. coli* (17). A-to-C, -G, or -U mutations at this site have been

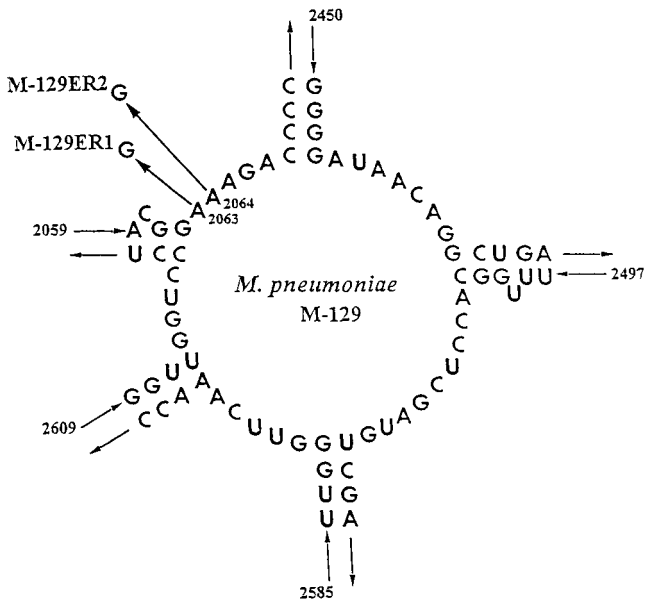

FIG. 2. Proposed structure of the central loop in domain V of the 23S rRNA from *M. pneumoniae* M129. The sequence was determined in this study and is identical to that reported by Ludwig et al. (22). Numbers indicate the position in the molecule. A-to-G transitions at sites 2063 in M129-ER1 and 2064 in M129-ER2 are indicated.

implicated in MLS resistance in *E. coli* (38, 39, 46), *Mycobacterium intracellulare* and *Mycobacterium avium* (24), *Streptomyces ambofaciens* (32), yeast mitochondria (42), and chloroplasts from *Chlamydomonas reinhardtii* (18) and *Nicotiana plumbaginifolia* (10) and have been shown to interfere with binding of erythromycin to the ribosome (13). This along with the reduced affinity of labeled erythromycin for ribosomes isolated from M129-ER1 provides convincing evidence that the transition at position 2063 is responsible for the observed resistance phenotype.

The effects of a transition at locations homologous to A-2064 in *M. pneumoniae* (A-2059 in *E. coli*) are not as well defined. Erythromycin and some lincosamides protect this position from chemical modification (12, 25), although in *E. coli* lincosamides protected A-2058 more strongly. The lesser resistance of M129-ER2 to lincosamides is consistent with these observations. A-to-G transitions at this site have been correlated with resistance to clarithromycin and azirithromycin in *Mycobacterium intracellulare* and *Mycobacterium avium* (24) and with resistance to lincosamides in chloroplasts (10). The greater resistance of M129-ER2 to 16-member macrolides suggests that A-2064 is particularly important for binding of these antibiotics.

Since there is only a single rRNA operon in the *M. pneumoniae* genome (15), the mutations in M129-ER1 and M129-ER2 are dominant, and emergence of resistance by this mechanism should occur more readily in response to antibiotic pressure than in bacteria with multiple rRNA operons. The presence of a single rRNA operon in *Mycobacterium intracellulare* and *Mycobacterium avium* may account for mutations at homologous positions in their 23S rRNA being associated with resistance to clarithromycin and azirithromycin (24). Other factors which would facilitate the appearance of these mutant forms are the presumed high rate of transition mutations in *M. pneumoniae* (23) and the inability of erythromycin to quickly eliminate apparently sensitive strains from the respiratory tract (6, 40, 41, 45). So, selection for resistance due to transitions at A-2063 and A-2064 may be quite effective in vivo following chemotherapy and may account for previously described resistant isolates from clinical samples with phenotypes similar to those of M129-ER1 and M129-ER2 (26–28, 43, 44).

Dimethylation at A-2063 homologous locations by methylases encoded by a family of phylogenetically related *erm* genes also results in MLS resistance (21, 47). Some of these genes are carried by plasmids or transposons which have the potential for intergeneric transfer (2). Whether *M. pneumoniae* could acquire resistance by this mechanism from other respiratory tract bacteria is unknown. However, acquisition of *tetM* by *Mycoplasma hominis* and *U. urealyticum* as a result of incorporation of the streptococcal transposon Tn*916* (34, 35) supports this possibility. The presence of a plasmid-encoded *erm* gene in *M. pneumoniae* was suggested by the report of a clinically isolated strain that demonstrated erythromycin-inducible MLS resistance which was lost upon exposure of cultures to acridine orange (43). Since the existence of plasmids in *M. pneumoniae* has never been demonstrated, this observation is potentially of great interest. Future isolation and molecular analysis of patient-derived MLS-resistant strains should help determine to what extent different mechanisms contribute to the development of resistance in vivo.

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