ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2004, p. 460–465 Vol. 48, No. 2
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.2.460–465.2004
Copyright © 2004, American Society for Microbiology. All Rights Reserved.

In Vitro Selection and Characterization of Resistance to Macrolides and Related Antibiotics in *Mycoplasma pneumoniae*

S. Pereyre, C. Guyot, H. Renaudin, A. Charron, C. Bébéar, and C. M. Bébéar*

Laboratoire de Bactériologie, Université Victor Segalen Bordeaux 2, 33076 Bordeaux Cedex, France

Received 17 June 2003/Returned for modification 25 August 2003/Accepted 13 October 2003

Macrolide-resistant mutants of *Mycoplasma pneumoniae* were selected in vitro from the susceptible reference strain M129, by 23 to 50 serial passages in subinhibitory concentrations of macrolides and related antibiotics, erythromycin A, azithromycin, josamycin, clindamycin, quinupristin, quinupristin-dalfopristin, pristinamycin, and telithromycin. Mutants for which the MICs are increased could be selected with all antibiotics except the streptogramin B quinupristin. Portions of genes encoding 23S rRNA (domains II and V) and ribosomal proteins L4 and L22 of mutants were amplified by PCR, and their nucleotide sequences were compared to those of the susceptible strain M129. No mutation could be detected in domain II of 23S rRNA. Two point mutations in domain V of 23S rRNA, C2611A and A2062G, were selected in the presence of erythromycin A, azithromycin, josamycin, quinupristin-dalfopristin, and telithromycin. Mutants selected in the presence of clindamycin and telithromycin harbored a single amino acid change (H70R or H70L, respectively) in ribosomal protein L4, whereas insertions of one, two, or three adjacent glycines at position 60 (*M. pneumoniae* numbering) were selected in the presence of both streptogramin combinations. Telithromycin was the sole antibiotic that selected for substitutions (P112R and A114T) and deletions ($_{111}$IPRA$_{114}$) in ribosomal protein L22. Three sequential mutational events in 23S rRNA and in both ribosomal proteins were required to categorize the strain as resistant to the ketolide. Azithromycin and erythromycin A were the only selector antibiotics that remained active (MICs, 0.06 and 1 μg/ml, respectively) on their mutants selected after 50 passages.

Mycoplasma pneumoniae is a common etiological agent of community-acquired respiratory tract infections in children and young adults. Macrolide and related antibiotics are the drugs of choice for the treatment of these infections, and strains with acquired resistance to macrolides have been rarely described. However, resistant strains of *M. pneumoniae* have been obtained in vitro, by selection in the presence of erythromycin A (20, 22, 27), and few macrolide-resistant *M. pneumoniae* isolates have been reported from patients treated with these antibiotics (8, 22, 27).

Macrolide, lincosamide, streptogramin, and ketolide antibiotics (MLSKs) inhibit protein synthesis by binding to domain V of 23S rRNA, and domain II is in the vicinity of this binding site (1, 12, 17, 26). Three main mechanisms of resistance have been reported: drug inactivation, active efflux, and modification of the target sites by methylation or mutation (29, 34). Mutations in domains II and V of 23S rRNA and in ribosomal proteins L4 and L22 were involved in resistance to MLSKs, first in bacteria with a small number of *rrn* operons and more recently in bacteria like *Streptococcus pneumoniae* and *Staphylococcus aureus* with four and six *rrn* operons, respectively (25, 29, 32). In mycoplasmas, gram-positive related bacteria which possess one or two copies of 23S rRNA, only resistance by point mutations in the peptidyltransferase loop of domain V of 23S rRNA has been described (13, 20, 22, 23). The genome of *M. pneumoniae* is entirely sequenced (18) and possesses only one copy of the rRNA gene operon. In this species, an A2058G transition (*Escherichia coli* numbering) was found in a clinical clindamycin-resistant strain isolated from a 9-year-old girl (22), whereas mutations at positions 2058 and 2059 were described in erythromycin-selected resistant mutants (20, 22).

As in *M. pneumoniae* little is known on mechanisms of resistance to macrolides and related antibiotics, the aim of this study was to select for mutants resistant to MLSKs, by serial passages of the reference strain M129 in subinhibitory concentrations of different macrolides (erythromycin A, azithromycin, and josamycin), of a lincosamide (clindamycin), a streptogramin B (quinupristin), two streptogramin combinations (quinupristin-dalfopristin and pristinamycin), and a ketolide (telithromycin). Mechanisms of resistance of these mutants were investigated by sequencing portions of genes encoding domain II and V of 23S rRNA and ribosomal proteins L4 and L22.

MATERIALS AND METHODS

Bacterial strains. The reference strain *M. pneumoniae* M129 (ATCC 29342) was used to select for macrolide-resistant mutants. Mycoplasmas were grown at 37°C in Hayflick modified agar or broth medium supplemented with glucose (33).

Antibiotics. The following antimicrobial agents were provided by the indicated manufacturers: erythromycin A, josamycin, quinupristin, quinupristin-dalfopristin, pristinamycin, and telithromycin (Aventis, Romainville, France); azithromycin (Pfizer, Orsay, France); and clindamycin (The Upjohn Co., Guyancourt, France).

Selection of macrolide-resistant mutants of *M. pneumoniae* M129. Selection of macrolide-resistant mutants was performed by serial transfers of *M. pneumoniae* M129 in Hayflick modified broth medium containing subinhibitory concentrations of erythromycin A, azithromycin, josamycin, clindamycin, quinupristin, quinupristin-dalfopristin, pristinamycin, or telithromycin. For the first passage, the reference strain *M. pneumoniae* M129 was inoculated in Hayflick modified medium with increasing twofold dilutions of each antibiotic. The MIC was determined as the lowest concentration of antimicrobial agent that prevented a color change in the medium at the time when the drug-free growth control first showed a color change (after about 5 days of incubation at 37°C). The culture containing the highest antibiotic concentration with visible growth was used to

* Corresponding author. Mailing address: Laboratoire de Bactériologie, Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France. Phone: (33) 5.57.57.16.25. Fax: (33) 5.56.93.29.40. E-mail: cecile.bebear@u-bordeaux2.fr.

TABLE 1. Oligonucleotides used in this study

Primer target and designation	Primer position (nucleotide no.)	Primer sequence	Product size (bp)
23S rRNA domain II			
MP23S-17b	560[a]	5′-CGTGCGTTTTGAAGTATGAG-3′	331
MP23S-24	886[a]	5′-TGGCGCCATCATACATTCAG-3′	
23S rRNA domain V			
MH23S-11	1,911[a]	5′-TAACTATAACGGTCCTAAGG-3′	352
MP23S-22	2,261[a]	5′-ACACTTAGATGCTTTCAGCG-3′	
MH23S-9	2,421[a]	5′-GCTCAACGGATAAAAGCTAC-3′	342
MP23S-23	2,762[a]	5′-ACACTTAGATGCTTTCAGCG-3′	
Ribosomal protein L4			
MPL4-1	46[b]	5′-GAACCAGTGAAACTAAGCCC-3′	420
MPL4-2	465[b]	5′-TTTGTCCAAGAGCTTGGCAC-3′	
Ribosomal protein L22			
MPL22-1	99[b]	5′-CCGTGTGAGAATCTCACCCC-3′	404
MPL22-2	502[b]	5′-CTGCTTTTTGACGTGCCATC-3′	

[a] *E. coli* numbering.
[b] *M. pneumoniae* numbering.

inoculate another antibiotic dilution panel for the following passage. When the MIC of the selector antibiotic increased fourfold, the culture was plated on Hayflick modified agar without antibiotic and five clones were subcultured, except for the 47th pristinamycin selection passage for which 10 clones were studied. MICs of each macrolide and related antibiotics were then determined for these clones by an agar dilution method, as previously described (2).

Fifty passages were performed for each selector antibiotic except for josamycin, for which high-level-resistant mutants were obtained after 23 passages. At least five consecutive subcultures in antibiotic-free medium were realized for each drug selection experiment. At the 50th and the 55th passages (23rd and 28th passages for josamycin), MICs of all drugs were determined again to check whether the phenotype was stable without selection pressure.

PCR amplifications and DNA sequencing. The macrolide-resistant mutants were screened for the erythromycin resistance genes *erm*(A), *erm*(B), *erm*(C), *mef*(A), and *msr*(A) by PCR amplification with primers previously described (28). Moreover, random amplified polymorphic DNA analysis was performed according to the method of Cousin-Allery et al. (7) on the reference strain *M. pneumoniae* M129 before antibiotic exposure and on its macrolide-resistant clones issued from the last passage of each antibiotic selection.

Specific oligonucleotidic primers for regions of interest in genes encoding 23S rRNA domain II and domain V and L4 and L22 ribosomal proteins of *M. pneumoniae* M129 were designed from the complete genome sequence (18) and are shown in Table 1. PCRs were performed as previously described for *M. pneumoniae* (20) with some modifications. Briefly, PCRs were carried out with a Perkin-Elmer Cetus thermal cycler with a 1 μM concentration of each primer, *Taq* DNA polymerase (Eurobio), and 2.5 μl of a broth culture as the template. Amplification was achieved with an initial denaturation step of 10 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at the appropriate hybridizing temperature, and 1 min at 72°C; and a final 10-min extension step at 72°C. Hybridizing temperature was 60°C for amplification of both ribosomal protein gene fragments and 57°C for a 2,178-bp fragment of 23S rRNA encompassing regions of interest in domain II and domain V (primers MP23S-17b and MP23S-23 [Table 1]). After purification with the Wizard PCR preps DNA purification system (Promega), both strands of the PCR products were sequenced with an ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit and an ABI PRISM 377 sequencer (Applied Biosystems), according to the manufacturer's instructions. With primers designated in Table 1, three DNA fragments of 23S rRNA and one fragment of ribosomal proteins L4 and L22 were sequenced. For each passage with a significantly increased MIC of antibiotic, two of the five clones subcultured were characterized by amplification and sequencing. Two clones subcultured from passages 45 to 50, depending on the selector antibiotic, were systematically characterized, except for josamycin. Ten clones issued from the 47th passage in pristinamycin were studied.

RESULTS

Selection of MLSK-resistant mutants *of M. pneumoniae* M129. Mutants resistant to macrolides and related antibiotics were isolated by serial passages of the parental strain *M. pneumoniae* M129 in subinhibitory concentrations of erythromycin A, azithromycin, josamycin, clindamycin, quinupristin, quinupristin-dalfopristin, pristinamycin, and telithromycin. When the passage MIC increased fourfold, two out of the five clones subcultured were studied. For each passage of interest, only MICs of one clone are shown in Table 2 since no significant difference was observed between the susceptibilities of the two clones. Moreover, for each antibiotic selection experiment, resistance phenotype remained stable in all mutants after five consecutive subcultures in antibiotic-free medium.

Mutants with significantly increased MICs could be selected with all the selector antibiotics except with quinupristin. With this streptogramin B antibiotic, MICs did not increase even after 50 passages. According to the definitions of the Comité de l'Antibiogramme de la Société Française de Microbiologie (6), passaged strains categorized as resistant to all MLSKs except to azithromycin could be obtained. Indeed, after 50 passages in subinhibitory concentrations of azithromycin, MICs for selected clones remained below the breakpoint (MIC, 0.06 μg/ml). Moreover, whatever the selecting MLSK drug, all *M. pneumoniae* selected strains remained categorized as susceptible or intermediate-susceptible to azithromycin (MICs $\leq$ 1 μg/ml). High-level resistance to erythromycin A and to telithromycin (MICs $\geq$ 64 μg/ml) was obtained only with telithromycin as the selector drug. Resistance to clindamycin developed in selection experiments with streptogramins and clindamycin. Moreover, MICs of josamycin, a 16-membered macrolide, and of streptogramin combinations, pristinamycin and quinupristin-dalfopristin, were significantly increased only when selections were performed with these three antibiotics.

TABLE 2. MICs for and ribosomal mutations observed in *M. pneumoniae* M129 mutants selected with macrolides and related antibiotics[a]

Antibiotic and *M. pneumoniae* strain[b]	MIC (μg/ml)								Nucleotidic and amino acid changes		
	ERY	AZM	JOS	CLI	QUI	PRI	Q-D	TEL	23S rRNA[c]	L4[d]	L22[d]
None											
M129	0.03	0.015	0.06	2	4	0.12	0.12	0.007	—[e]	—	—
ERY											
E3	1	0.06	0.12	2	8	0.25	0.25	0.12	C2611A	—	—
AZM											
A2	1	0.06	0.06	2	8	0.25	0.25	0.12	C2611A	—	—
CLI											
C30	0.03	0.015	0.25	16	4	0.25	0.12	0.015	—	H70R	—
TEL											
T2	1	0.06	0.06	2	8	0.25	0.25	0.12	C2611A	—	—
T20	2	0.25	0.06	2	8	0.25	0.25	0.25	C2611A	—	A114T
T32	64	0.5	0.25	2	8	0.25	0.12	4	C2611A	H70L	A114T
T37	64	1	0.25	2	8	0.25	0.12	16	C2611A	H70L	A114T P112R
T48	64	1	0.5	2	8	0.25	0.25	128	C2611A	H70L	$_{111}$IPRA$_{114}$ deletion
JOS											
J20	0.03	0.015	0.25	2	4	0.12	0.12	0.015	—	—	—
J23	0.25	0.06	512	2	16	4	4	0.06	A2062G	—	—
PRI											
P20	0.25	0.06	0.25	8	8	0.25	0.12	0.06	—	$_{60}$G insertion	—
P47	0.25	0.06	2	16	8	2	1	0.06	—	$_{60}$GG or $_{60}$GGG insertion[f]	—
Q-D											
S20	0.03	0.015	0.25	8	8	0.25	0.25	0.007	—	$_{60}$G insertion	—
S35	0.12	0.015	256	8	8	8	2	0.06	A2062G	$_{60}$G insertion	—

[a] Abbreviations: ERY, erythromycin A; AZM, azithromycin; JOS, josamycin; CLI, clindamycin; QUI, quinupristin; PRI, pristinamycin; Q-D, quinupristin-dalfopristin; TEL, telithromycin.

[b] *M. pneumoniae* M129 is the parental strain. Mutants are designated by the initial of the selector antibiotic followed by the passage number. Fifty passages were performed for each selector antibiotic except for josamycin (23 passages). Only passages with a significant MIC increase (at least fourfold) are presented in Table 2. For these passages, two of the five clones subcultured were studied. Only MICs for one clone are shown since no significant difference (no more than one dilution) was observed between the susceptibilities of both clones. Their nucleotide sequences were always identical.

[c] *E. coli* numbering.

[d] *M. pneumoniae* numbering.

[e] —, no mutation.

[f] Ten clones issued from the 47th passage in pristinamycin were studied. Six of them harbored a two-glycine insertion and four of them harbored a three-glycine insertion at position 60 of the L4 protein, but their MICs remained identical.

The macrolide-resistant mutants were screened for the erythromycin resistance genes *erm*(A), *erm*(B), *erm*(C), *mef*(A), and *msr*(A). None of the isolates had a PCR product specific to any of these resistance determinants (data not shown). Moreover, to ensure that the *M. pneumoniae* strain was the same before and after antibiotic exposure, random amplified polymorphic DNA analysis was performed with the reference strain *M. pneumoniae* M129 and with mutants issued from the last passage of each antibiotic selection. As expected, mutant profiles were identical to the parental strain profile (data not shown).

23S rRNA mutations in passaged mutants. Two of the five clones subcultured were analyzed by amplification and sequencing of the domains II and V. Nucleotidic sequences were always identical for both mutants (Table 2).

The mutants selected at the second passage in the presence of azithromycin (mutant A2) and telithromycin (T2), and at the third passage in the presence of erythromycin A (E3) harbored a C2611A mutation in domain V of 23S rRNA. For these mutants, this transversion resulted in the same phenotype of resistance with 4- to 33-fold-increased MICs of 14- and 15-membered macrolides and of telithromycin. However, the MIC differences did not constitute defined resistance, and there were no changes in MICs of the other MLS antibiotics. Note that clones issued from the last passages with erythromycin A or azithromycin were also sequenced and did not harbor any additional mutations in portions of the 23S rRNA, L4, and L22 genes investigated.

With josamycin as the selector antibiotic, a 4-fold-increased MIC of this 16-membered macrolide was observed at the 20th passage (J20), but no alteration was observed in the 23S rRNA, L4, and L22 characterized sequences (Table 2). At the 23rd passage (J23), a A2062G substitution was selected. The MIC of josamycin dramatically increased (MIC, 512 μg/ml), and MICs of pristinamycin and quinupristin-dalfopristin were also significantly higher (33-fold increase). Other antimicrobials were less affected, with a 4- to 8-fold-increased MIC, whereas the MIC of clindamycin remained unchanged. Consequently, after

23 passages, the selected clone J23 was categorized as resistant to the 16-membered macrolide and to both streptogramin combinations. This mutation was also present in mutants selected at the 35th passage in the presence of quinupristin-dalfopristin. When this mutation appeared, MICs of the same antibiotics increased similarly, 1,024 times for josamycin and 8 to 32 times for both streptogramin combinations.

Mutations in ribosomal protein L4. Mutants selected with clindamycin, telithromycin, and streptogramins harbored mutations in ribosomal protein L4 (Table 2). Clindamycin-selected mutants (C30) had a point transition (A209G) that led to a single amino acid change from histidine to arginine at position 70 (H70R) (*M. pneumoniae* numbering), whereas telithromycin selected a point transversion at the same position (A209T) that replaced the histidine with a leucine (H70L) for mutants T32, T37, and T48. These mutants, which exhibited an amino acid change at the same position, did not have an identical resistance MIC profile. The H70R but not the H70L substitution led to a significantly increased MIC of clindamycin (MIC for mutant C30, 16 μg/ml), whereas the H70L substitution led to a significantly increased MIC of erythromycin A (MIC for mutant T32, 64 μg/ml) and of telithromycin (MIC, 4 μg/ml), but not of clindamycin (MIC, 2 μg/ml). However, both substitutions led to a fourfold increase of the josamycin MIC.

At the 20th passage, mutants selected with both streptogramin combinations, pristinamycin (P20) and quinupristin-dalfopristin (S20), harbored a three-nucleotide insertion (GGT) leading to an insertion of a glycine (Table 2). This insertion is located in a region of the protein where three glycines are already present at positions 60 to 62 (*M. pneumoniae* numbering). Consequently, it was not possible to know where this insertion really took place between positions 60 and 62, and we arbitrarily located it at position 60. In both cases, this insertion moderately affected the activity of josamycin and clindamycin (fourfold MIC increase). However, resistance phenotypes of mutants P20 and S20, which harbored the same glycine insertion, were not identical. For mutant P20, MICs of erythromycin A, azithromycin, and telithromycin were four- to eightfold increased, whereas for mutant S20, MICs of these antimicrobials were unchanged. Interestingly, mutants selected at the 47th passage in the presence of pristinamycin (P47) harbored one or two additional GGT insertions at the same position that led to an insertion of a total of two or three glycines. Among the 10 subcultured clones obtained from this passage, 6 of them harbored a two-glycine insertion and 4 of them harbored a three-glycine insertion. This additional insertion of one or two glycines increased MICs of josamycin and streptogramins eightfold. However, it should be noted that insertion of a total of two or three glycines at this position led to an identical resistance phenotype (Table 2).

Mutations in ribosomal protein L22. Mutations in ribosomal protein L22 were selected only in the presence of telithromycin (Table 2). Three successive changes were obtained. At the 20th passage (T20), mutants differed from the parental strain by one base (G340A), leading to a single-amino-acid change from alanine to threonine at position 114 (*M. pneumoniae* numbering). This substitution increased by two- to fourfold the MICs of erythromycin A, azithromycin, and telithromycin. At the 37th passage (T37), another substitution (C335G) led to an additional P112R. This mutation increased by four times the MIC of telithromycin. At least, at the 48th passage (T48), a 12-bp deletion led to the suppression of four amino acids between threonine 110 and lysine 115. Consequently, both amino acids substituted in the preceding passages were deleted. This last change led to a complete loss of activity of telithromycin (MIC, 128 μg/ml), the activity of other MLS being unchanged (Table 2).

Combined mutations in different ribosomal targets. In this study, 14-, 15-, and 16-membered macrolides selected only one mutation in 23S rRNA. Clindamycin, a lincosamide, and pristinamycin, a streptogramin mixture, selected one mutation in ribosomal protein L4, whereas the other streptogramin association, quinupristin-dalfopristin, selected a modification in both structures, 23S rRNA and L4. Interestingly, the ketolide telithromycin successively selected five changes. 23S rRNA was first affected, and then ribosomal protein L22, ribosomal protein L4, and again protein L22, with amino acid substitutions replaced by a four amino acid deletion, were affected. At each stage, an increase of the ketolide MIC could be noted. Three sequential mutational events were required to categorize the strain as resistant to telithromycin (mutant T32 [Table 2]), and two additional modifications led to a high-level resistance to the ketolide (T48).

DISCUSSION

In this study, selection of macrolide-resistant mutants of *M. pneumoniae* M129 was associated with a variety of mutations in 23S rRNA and in ribosomal proteins L4 and L22. In *M. pneumoniae*, resistance by mutation is the most expected mechanism of resistance, and so far it is the only one described in this species (20, 22), since this mycoplasma harbors only one copy of the rRNA gene operon (18). Thus, heterozygous strains or gene dosage effect, previously described for *Mycoplasma hominis* (13, 23) or other bacteria (19, 25) with at least two copies of rRNA, could not exist in *M. pneumoniae*.

MLSKs are not equally active in selecting resistance in *M. pneumoniae.* After serial passages in subinhibitory concentrations, erythromycin A, azithromycin, clindamycin, or josamycin selected only one mutation, but these mutations did not appear at the same stage of the selection. Erythromycin A and azithromycin selected a 23S rRNA substitution very early, as soon as the second or the third passage, but these antimicrobials remained active. In contrast, josamycin and clindamycin selected mutations later, at the 23rd or the 30th passage, leading to the complete loss of activity. In telithromycin, three mutations and 32 passages were required to categorize *M. pneumoniae* as resistant. However, this is the only MLSK that selected five mutations in 23S rRNA or in ribosomal proteins. This is in contrast with a previous selection study reported for *S. pneumoniae* (5, 9), where telithromycin selected macrolide-resistant mutants less often than the other drugs. Moreover, all *S. pneumoniae* mutants for which the MIC of telithromycin was ≥ 1 μg/ml were cross-resistant to other macrolides. In our work, the resistant mutant of *M. pneumoniae* that was highly resistant to telithromycin (T48; MIC, 128 μg/ml) remained susceptible or intermediate-susceptible to azithromycin, josamycin, clindamycin, and streptogramin combinations. Interestingly, among the eight selecting drugs, erythromycin A and azithromycin are the sole antibiotics which are still active against their selected

isolates after 50 serial passages. Moreover, a concentration of azithromycin of 1 μg/ml inhibited all *M. pneumoniae* resistant mutants selected in this study.

Although mutations in 23S rRNA at position 2058 and 2059 are the only mutations described so far in *M. pneumoniae* (20, 22) and are the most commonly associated with macrolide resistance in other species of bacteria (29, 32), no mutation was found at these positions in this study. Moreover, no mutation was observed in domain II of 23S rRNA even in mutants selected with telithromycin. Nevertheless, two substitutions, C2611A and A2062G (*E. coli* numbering) were selected in 23S rRNA. Note that the use of only one parental strain for this selection could introduce a bias and may explain why different mutations were seen here. However, Lucier et al. selected *M. pneumoniae* resistant mutants harboring A2058G or A2059G mutations also by growing an M129 *M. pneumoniae* strain in the presence of erythromycin (20). To our knowledge, mutations C2611A and A2062G had never been reported in *M. pneumoniae*, but substitutions at these positions had been described in *M. hominis*, a genital human mycoplasma (13, 23). The C2611A transversion was previously described in *S. pneumoniae* alone (30) or associated with a substitution in ribosomal protein L22, at the same position as the 114 substitution found in the telithromycin-selected *M. pneumoniae* mutant (T20). In both studies, those two mutations led to the same resistance profile, with 14- and 15-membered macrolides and ketolides affected. This C2611A substitution disrupts the base pair G2057-C2611, leading to an opening of the stem preceding the single-stranded portion of the peptidyltransferase region. As described for *S. pneumoniae* (5), it gave only small advantage to the bacteria as it led to a low-level resistance.

The substitution at position 2062 has been described in a josamycin-selected strain of *M. hominis* (13) and in a clinical strain of *S. pneumoniae* (11). As in our study, 16-membered macrolides and streptogramins were significantly affected. This is consistent with the protection seen at position A2062 with streptogramins in rRNA footprinting studies (24). Moreover, the recently published crystal structure of complexes between the large ribosomal subunit from *Haloarcula marismortui* and three 16-membered macrolides, tylosin, spiramycin, and carbomycin A, showed a covalent bond between the ethylaldehyde at C-6 of the macrolide and the N-6 of A2062 (16). As josamycin possesses the same ethylaldehyde grouping at C-6, we could speculate that a mutation at position 2062 of 23S rRNA may therefore highly perturb the binding of this macrolide to the ribosome and account for the high-level resistance harbored by the mutants. Lastly, the crystal structure of 14-membered macrolides binding on the 50S ribosomal subunit showed that the 6-hydroxy group of erythromycin A is within hydrogen bonding distance of N-6 of A2062 in *Deinococcus radiodurans* (26). This is consistent with the eightfold increase of the erythromycin MIC found for our *M. pneumoniae* A2062G mutant (J23). Interestingly, an A2062C transversion was described in a mutant of *Halobacterium halobium* resistant to chloramphenicol (21) but our mutants did not show any increased MIC of this antibiotic (data not shown).

Mutations obtained in ribosomal protein L4 are all located in a highly conserved disordered loop of the protein, which is the main rRNA-binding site of the ribosomal protein (35). Consequently, since crystallographic studies showed no direct interaction between ribosomal protein and macrolides (16, 26), resistance presumably results from an indirect conformational change in the tunnel region that forms a major portion of the macrolide binding site (15). Clindamycin and telithromycin selected substitutions at position 70 (*M. pneumoniae* numbering). This position has previously been described to be mutated in *S. pneumoniae* (30). Interestingly, in our study, the H70R and the H70L substitutions did not lead to the same phenotype of resistance, but the reason of this difference remained unknown. Streptogramins, pristinamycin and quinupristin-dalfopristin, selected insertions of one, two, or three adjacent glycines in a region of the L4 protein where three glycines are already present. These three-, six-, or nine-nucleotide inserts (GGT, GGTGGT, or GGTGGTGGT) may result from a slippage of the DNA polymerase, as the preceding nucleotides (GGTGGTGGT) are identical to those of the insert. However, the reason for the different resistance phenotypes observed between mutants P20 and S20 despite the same L4 protein insertion remains unknown. For these mutants, sequencing the entire 23S rRNA, L4, and L22 genes could be useful to detect any additional mutation.

Only mutants selected by telithromycin harbored mutations in ribosomal protein L22. This preferential selection of L22 mutations by this ketolide was also described for *S. pneumoniae* (5). Interestingly, the same proline 91 (P112 in *M. pneumoniae*) and alanine 93 (A114 in *M. pneumoniae*) were substituted, indicating a preferential site of mutation for developing telithromycin resistance. These amino acids are located in the protruding β hairpin of the protein, between β strands 2 and 3, in a highly conserved region (31). Nevertheless, these substitutions only slightly increased MICs of MLSKs. Interestingly, the *M. pneumoniae* mutants selected after 48 passages in telithromycin in this study (T48) harbored a deletion of 4 amino acids between the end of the β strand 2 and the highly conserved glycine 116 (31). This deletion only affects the activity of telithromycin (eightfold MIC increase). The β hairpin is involved in interactions with all domains of 23S rRNA (1). Recently, the crystal structure of *Thermus thermophilus* L22 mutant showed that the L22 β hairpin tip is in close proximity with the nucleotides of helix 35 in domain II (10). Arginine 109 and alanine 110 of L22 protein, corresponding to the deleted arginine 113 and alanine 114 in *M. pneumoniae* mutants T48, are close to nucleotides G748 and A750 in domain II of 23S rRNA (*E. coli* numbering). Moreover, the crystal structure of telithromycin bound to the *D. radiodurans* large ribosomal subunit showed that the antibiotic is located in close proximity to nucleotides of the helix 35 (4). Consequently, deletion of the four amino acids in *M. pneumoniae* mutants may disrupt the contact of the L22 β hairpin with domain II of 23S rRNA, thereby altering the binding site of the ketolide leading to the observed increased resistance to telithromycin. Another hypothesis is that the deletion could decrease the length of the β hairpin, which is constant in all species. As the β hairpin is forming a large part of the surface of the polypeptide exit channel (1), the deletion could modify the width of the tunnel. If this deletion leads to a widening of the tunnel entrance, as described for *E. coli* mutants (14), a resistance could develop by a lack of obstruction of the tunnel by the antibiotic. However, this hypothesis does not explain

why telithromycin is the only antimicrobial affected by this deletion in L22 ribosomal protein.

At least, it should be noted that no mutation was found in the sequenced portions of 23S rRNA, L4, and L22 genes at the 20th passage for the josamycin-selected mutant, despite a four-fold-increased MIC. Other experiments need to be performed to detect additional mechanisms, like mutations elsewhere in the 23S rRNA or in ribosomal proteins, or eventually a drug efflux system that may contribute to the resistant phenotype of this mutant.

In conclusion, this study showed that a variety of mutations can be selected in vitro in *M. pneumoniae* with different MLSKs. To date, this is the first description of mutations in ribosomal proteins L4 and L22 associated with macrolide resistance in mycoplasmas. These laboratory-derived mutants could be predictive for mutations observed in clinical strains, as has been noted for resistance to fluoroquinolones (3).

ACKNOWLEDGMENTS

This study was supported in part by a grant from Aventis and Pfizer.

REFERENCES

1. **Ban, N., P. Nissen, J. Hansen, P. B. Moore, and T. A. Steitz.** 2000. The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. Science **289:**905–920.
2. **Bébéar, C., and J. Robertson.** 1996. Determination of minimal inhibitory concentration, p. 189–199. *In* J. G. Tully and S. Razin (ed.), Molecular and diagnostic procedures in mycoplasmology, vol. II. Academic Press, San Diego, Calif.
3. **Bébéar, C. M., and C. Bébéar.** 2002. Antimycoplasmal agents, p. 545–566. *In* S. Razin and R. Herrmann (ed.), Molecular biology and pathogenicity of mycoplasmas. Kluwer Academic/Plenum Publishers, London, United Kingdom.
4. **Berisio, R., J. Harms, F. Schluenzen, R. Zarivach, H. A. S. Hansen, P. Fucini, and A. Yonath.** 2003. Structural insight into the antibiotic action of telithromycin against resistant mutants. J. Bacteriol. **185:**4276–4279.
5. **Canu, A., B. Malbruny, M. Coquemont, T. A. Davies, P. C. Appelbaum, and R. Leclercq.** 2002. Diversity of ribosomal mutations conferring resistance to macrolides, clindamycin, streptogramin, and telithromycin in *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **46:**125–131.
6. **Carret, G., J.-D. Cavallo, H. Chardon, C. Chidiac, P. Choutet, P. Courvalin, H. Dabernat, H. Drugeon, L. Dubreuil, F. Goldstein, V. Jarlier, R. Leclercq, M.-H. Nicolas-Chanoine, A. Philippon, C. Quentin-Noury, B. Rouveix, J. Sirot, and C.-J. Soussy.** 2003. Comité de l'Antibiogramme de la Société Française de microbiologie report 2003. Int. J. Antimicrob. Agents. **21:**364–391.
7. **Cousin-Allery, A., A. Charron, B. de Barbeyrac, G. Fremy, J. S. Jensen, H. Renaudin, and C. Bébéar.** 2000. Molecular typing of *Mycoplasma pneumoniae* strains by PCR-based methods and pulsed-field gel electrophoresis. Application to French and Danish isolates. Epidemiol. Infect. **124:**103–111.
8. **Critchley, I. A., M. E. Jones, P. D. Heinze, D. Hubbard, H. D. Engler, A. T. Evangelista, C. Thornsberry, J. A. Karlowsky, and D. F. Sahm.** 2002. In vitro activity of levofloxacin against contemporary clinical isolates of *Legionella pneumophila*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* from North America and Europe. Clin. Microbiol. Infect. **8:**214–221.
9. **Davies, T. A., B. E. Dewasse, and P. C. Appelbaum.** 2000. In vitro development of resistance to telithromycin (HMR 3647), four macrolides, clindamycin, and pristinamycin in *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **44:**414–417.
10. **Davydova, N., V. Streltsov, M. Wilce, A. Liljas, and M. Garber.** 2002. L22 ribosomal protein and effect of its mutation on ribosome resistance to erythromycin. J. Mol. Biol. **322:**635–644.
11. **Depardieu, F., and P. Courvalin.** 2001. Mutation in 23S rRNA responsible for resistance to 16-membered macrolides and streptogramins in *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **45:**319–323.
12. **Douthwaite, S., L. H. Hansen, and P. Mauvais.** 2000. Macrolide-ketolide inhibition of MLS-resistant ribosomes is improved by alternative drug interaction with domain II of 23S rRNA. Mol. Microbiol. **36:**183–192.
13. **Furneri, P. M., G. Rappazzo, M. P. Musumarra, P. Dipietro, L. S. Catania, and L. S. Roccasalva.** 2001. Two new point mutations at A2062 associated with resistance to 16-membered macrolide antibiotics in mutant strains of *Mycoplasma hominis.* Antimicrob. Agents Chemother. **45:**2958–2960.
14. **Gabashvili, I. S., S. T. Gregory, M. Valle, R. Grassucci, M. Worbs, M. C. Wahl, A. E. Dahlberg, and J. Frank.** 2001. The polypeptide tunnel system in the ribosome and its gating in erythromycin resistance mutants of L4 and L22. Mol. Cell **8:**181–188.
15. **Gregory, S. T., and A. E. Dahlberg.** 1999. Erythromycin resistance mutations in ribosomal proteins L22 and L4 perturb the higher order structure of 23S ribosomal RNA. J. Mol. Biol. **289:**827–834.
16. **Hansen, J. L., J. A. Ippolito, N. Ban, P. Nissen, P. B. Moore, and T. A. Steitz.** 2002. The structures of four macrolide antibiotics bound to the large ribosomal subunit. Mol. Cell **10:**117–128.
17. **Hansen, L. H., P. Mauvais, and S. Douthwaite.** 1999. The macrolide-ketolide antibiotic binding site is formed by structures in domains II and V of 23S ribosomal RNA. Mol. Microbiol. **31:**623–631.
18. **Himmelreich, R., H. Hilbert, H. Plagens, E. Pirkl, B. C. Li, and R. Herrmann.** 1996. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae.* Nucleic Acids Res. **24:**4420–4449.
19. **Leclercq, R., and P. Courvalin.** 2002. Resistance to macrolides and related antibiotics in *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **46:**2727–2734.
20. **Lucier, T. S., K. Heitzman, S. K. Liu, and P. C. Hu.** 1995. Transition mutations in the 23S rRNA of erythromycin-resistant isolates of *Mycoplasma pneumoniae.* Antimicrob. Agents Chemother. **39:**2770–2773.
21. **Mankin, A. S., and R. A. Garrett.** 1991. Chloramphenicol resistance mutations in the single 23S rRNA gene of the archaeon *Halobacterium halobium.* J. Bacteriol. **173:**3559–3563.
22. **Okazaki, N., M. Narita, S. Yamada, K. Izumikawa, M. Umetsu, T. Kenri, Y. Sasaki, Y. Arakawa, and T. Sasaki.** 2001. Characteristics of macrolide-resistant *Mycoplasma pneumoniae* strains isolated from patients and induced with erythromycin in vitro. Microbiol. Immunol. **45:**617–620.
23. **Pereyre, S., P. Gonzalez, B. de Barbeyrac, A. Darnige, H. Renaudin, A. Charron, S. Raherison, C. Bébéar, and C. M. Bébéar.** 2002. Mutations in 23S rRNA account for intrinsic resistance to macrolides in *Mycoplasma hominis* and *Mycoplasma fermentans* and for acquired resistance to macrolides in *M. hominis.* Antimicrob. Agents Chemother. **46:**3142–3150.
24. **Porse, B. T., and R. A. Garrett.** 1999. Sites of interaction of streptogramin A and B antibiotics in the peptidyl transferase loop of 23S rRNA and the synergism of their inhibitory mechanisms. J. Mol. Biol. **286:**375–387.
25. **Prunier, A. L., B. Malbruny, D. Tande, B. Picard, and R. Leclercq.** 2002. Clinical isolates of *Staphylococcus aureus* with ribosomal mutations conferring resistance to macrolides. Antimicrob. Agents Chemother. **46:**3054–3056.
26. **Schlunzen, F., R. Zarivach, J. Harms, A. Bashan, A. Tocilj, R. Albrecht, A. Yonath, and F. Franceschi.** 2001. Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. Nature **413:**814–821.
27. **Stopler, T., and D. Branski.** 1986. Resistance of *Mycoplasma pneumoniae* to macrolides, lincomycin and streptogramin B. J. Antimicrob. Chemother. **18:**359–364.
28. **Sutcliffe, J., A. Tait-Kamradt, and L. Wondrack.** 1996. *Streptococcus pneumoniae* and *Streptococcus pyogenes* resistant to macrolides but sensitive to clindamycin: a common resistance pattern mediated by an efflux system. Antimicrob. Agents Chemother. **40:**1817–1824.
29. **Sutcliffe, J. A., and R. Leclercq.** 2002. Mechanisms of resistance to macrolides, lincosamides, and ketolides, p. 281–317. *In* W. Schönfeld and H. A. Kirst (ed.), Macrolide antibiotics. Birkhauser Verlag, Boston, Mass.
30. **Tait-Kamradt, A., T. Davies, M. Cronan, M. R. Jacobs, P. C. Appelbaum, and J. Sutcliffe.** 2000. Mutations in 23S rRNA and ribosomal protein L4 account for resistance in pneumococcal strains selected in vitro by macrolide passage. Antimicrob. Agents Chemother. **44:**2118–2125.
31. **Unge, J., A. Aberg, S. Al-Kharadaghi, A. Nikulin, S. Nikonov, N. Davydova, N. Nevskaya, M. Garber, and A. Liljas.** 1998. The crystal structure of ribosomal protein L22 from *Thermus thermophilus*: insights into the mechanism of erythromycin resistance. Structure **6:**1577–1586.
32. **Vester, B., and S. Douthwaite.** 2001. Macrolide resistance conferred by base substitutions in 23S rRNA. Antimicrob. Agents Chemother. **45:**1–12.
33. **Waites, K. B., C. M. Bébéar, J. A. Roberston, D. F. Talkington, and G. E. Kenny.** 2001. Cumitech 34, Laboratory diagnosis of mycoplasmal infections. Coordinating ed., F. S. Nolte. American Society for Microbiology, Washington, D.C.
34. **Weisblum, B.** 2000. Resistance to macrolide-lincosamide-streptogramin antibiotics, p. 694–710. *In* V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (ed.), Gram-positive pathogens. American Society for Microbiology, Washington, D.C.
35. **Worbs, M., R. Huber, and M. C. Wahl.** 2000. Crystal structure of ribosomal protein L4 shows RNA-binding sites for ribosome incorporation and feedback control of the S10 operon. EMBO J. **19:**807–818.