

Two New Mechanisms of Macrolide Resistance in Clinical Strains of *Streptococcus pneumoniae* from Eastern Europe and North America

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Resistance to macrolides in pneumococci is generally mediated by methylation of 23S rRNA via *erm(B)* methylase which can confer a macrolide (M)-, lincosamide (L)-, and streptogramin B (S_B)-resistant (MLS_B) phenotype or by drug efflux via *mef(A)* which confers resistance to 14- and 15-membered macrolides only. We studied 20 strains with unusual ML or MS_B phenotypes which did not harbor *erm(B)* or *mef(A)*. The strains had been isolated from patients in Eastern Europe and North America from 1992 to 1998. These isolates were found to contain mutations in genes for either 23S rRNA or ribosomal proteins. Three strains from the United States with an ML phenotype, each representing a different clone, were characterized as having an A2059G (*Escherichia coli* numbering) change in three of the four 23S rRNA alleles. Susceptibility to macrolides and lincosamides decreased as the number of alleles in isogenic strains containing A2059G increased. Sixteen MS_B strains from Eastern Europe were found to contain a 3-amino-acid substitution (${}_{69}GTG_{71}$ to TPS) in a highly conserved region of the ribosomal protein L4 (${}_{63}KPWRQKGTGRAR_{74}$). These strains formed several distinct clonal types. The single MS_B strain from Canada contained a 6-amino-acid L4 insertion (${}_{69}GTGREKGTGRAR$), which impacted growth rate and also conferred a 500-fold increase in MIC on the ketolide telithromycin. These macrolide resistance mechanisms from clinical isolates are similar to those recently described for laboratory-derived mutants.

The most prevalent mechanisms of macrolide resistance in *Streptococcus pneumoniae* are mediated by *mef(A)*, a gene encoding an efflux pump specific for 14- and 15-membered macrolides, and *erm(B)*, a 23S rRNA methylase that dimethylates A2058, resulting in macrolide-lincosamide-streptogramin B resistance (MLS_B) (10, 15, 16, 29, 38, 40, 51). The *Mef(A)* pump is found in other species of streptococci (12, 32, 41) and other bacterial genera (18, 28; H. Fraimow and C. Knob, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. A-125, 1997). *Erm(B)* or another homologous methylase is found in almost every bacterial species, including macrolide-producing strains (10, 15, 29, 38, 40, 51). Both mechanisms are common, but geographically, North American pneumococcal isolates have a greater tendency to harbor *mef(A)* while European isolates more frequently contain *erm(B)* (5, 9, 13, 14, 33, 35).

During studies to determine the frequency as well as the nature of macrolide resistance in pneumococcal clinical isolates, we identified strains with unusual MS_B or ML phenotypes. We have recently shown that mutations obtained in vitro in a highly conserved region of ribosomal protein L4 or in one of four different nucleotides in domain V of 23S rRNA are responsible for macrolide resistance in *S. pneumoniae* isolates (43; W. Fu, M. Anderson, S. Williams, A. Tait-Kamradt, J. Sutcliffe, and J. Retsema, Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones, abstr. 7.10, 2000). This study documents our investigation of 20 clinical isolates with an MS_B or ML phenotype. These isolates had

been recovered from patients in Eastern Europe and North America from 1992 to 1998.

MATERIALS AND METHODS

Bacterial strains. The clinical isolates of *S. pneumoniae* were collected as part of surveillance studies in Eastern Europe, Canada, and the United States (Table 1). Strain Bu41 was an isolate from the ear of a 1-year-old infant in Bulgaria, collected by A. Marton and provided by R. Hakenbeck. A series of strains (Sof289, Bul23, Bul29, Bul34, Bul40, Bul91, and Bul115) from separate individuals in Bulgaria collected in 1992 to 1995 was kindly provided by L. Setchanova. All but one of these strains were from patients under the age of four. These serotype 19F strains were previously characterized as a single clone called K_1 , and Sof289 is representative of this clone (33). Strains 395, 398, 404, 407, 381, 386, 387, and 442 are from Eastern Europe. Strain BM4418 is from a blood culture of a 64-year-old man with Crohn's disease who was hospitalized in Toronto, Ontario, Canada, for an episode of community-acquired bacteremic pneumococcal pneumonia. The strain was collected by the Toronto Invasive Bacterial Diseases Network (20) and provided by A. McGeer. 02J1200 is a 1996 blood isolate from a 5-year-old male in New Jersey, E40 is a 1996 serotype 23F isolate from Tennessee obtained from Linda McDougal (Centers for Disease Control and Prevention), and 117-891 was isolated in 1998 from the sputum of a 78-year-old male in Washington state.

S. pneumoniae R6 and CP1000 were used as recipients in transformation experiments, and strain A9, a spontaneous streptomycin-resistant derivative of R6, served as a control in transformation experiments (1). Strains used in this study are listed with their mutations in Tables 3 and 5.

Susceptibility testing. Phenotypes were determined by disk diffusion using 2- μ g-clindamycin, 15- μ g-erythromycin, and 25- μ g-streptogramin B disks as described previously (42). MICs were determined in microtiter trays using Mueller-Hinton broth supplemented with 2.5% lysed horse blood according to the recommendations of the NCCLS (23). The *S. pneumoniae* strain recommended by the NCCLS, ATCC 49619, was included in the MIC evaluations. All compounds were purchased from Sigma or made by published methods at Pfizer, Inc.

DNA amplification and sequencing. Primers were purchased from Sigma/Genosys Biotechnologies (The Woodlands, Tex.). Primers for internal fragments of *erm(A)*, *erm(B)*, *erm(C)*, *erm(TR)*, *msr(A)*, *mef(A)*, *mph(A)*, *mph(B)*, *mph(C)*, *ere(A)*, and *ere(B)* have been described previously (39, 43). Primers for amplifying 23S rRNA, 3' ends of individual 23S rRNA genes, and L4 and L22 ribosomal protein genes from *S. pneumoniae* have been described previously, as have methods for purification of PCR products and DNA sequencing (43).

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TABLE 1. Source and phenotype of clinical isolates

Strain	Country	Year	Source	Phenotype
Bu41	Bulgaria	Unknown	Middle ear exudate	MS _B
Sof289	Bulgaria	1994	Nasopharynx	MS _B
Bul23	Bulgaria	1992	Nose	MS _B
Bul29	Bulgaria	1992	Nose	MS _B
Bul34	Bulgaria	1994	Conjunctiva	MS _B
Bul40	Bulgaria	1993	Middle ear exudate	MS _B
Bul91	Bulgaria	1995	Middle ear exudate	MS _B
Bul115	Bulgaria	1995	Sputum	MS _B
395	Bulgaria	1994	Sputum	MS _B
398	Bulgaria	1994	Nose	MS _B
404	Bulgaria	1994	Eye	MS _B
407	Bulgaria	1994	Middle ear exudate	MS _B
381	Slovakia	1994	Middle ear exudate	MS _B
386	Slovakia	1994	Nasopharynx	MS _B
387	Slovakia	1994	Nasopharynx	MS _B
442	Poland	1994	Nasopharynx	MS _B
BM4418	Canada	1994–1996	Blood	MS _B
E40	Tennessee	1993	Blood	ML
02J1200	New Jersey	1996	Blood	ML
117-891	Washington	1998	Sputum	ML

PFGE. Genomic DNA from each isolate was restricted with *Sma*I and analyzed by pulsed-field gel electrophoresis (PFGE) as previously described (43).

Transformation. Synthetic competence-stimulating peptide I and the modified method of Havarstein et al. (7) were used to induce *S. pneumoniae* to move into a transformation-competent state. MS_B transformants were selected on Todd-Hewitt agar containing 5% sheep blood and 0.5 µg of erythromycin per ml. When genomic DNA from BM4418 was transformed into CP1000, 2 µg of pristinamycin per ml was used as the selecting agent. ML transformants were selected with 0.05 or 0.1 µg of erythromycin per ml. Plates were incubated overnight at 37°C in 5% CO₂. Cell suspensions were monitored for competence by determining the transformation frequency of streptomycin resistance from *S. pneumoniae* A9, which ranged from 0.1 to 0.5%.

In vitro translation and cellular viability. Methods for isolation of *S. pneumoniae* ribosomes and in vitro translation have been described previously (42). To determine cellular viability, aliquots from strains growing in Todd-Hewitt broth containing 0.5% yeast extract were removed initially and hourly thereafter, diluted, and plated onto Trypticase soy agar containing 5% sheep blood. Following incubation at 37°C for 22 to 24 h, colonies were counted.

RESULTS

Of the 20 strains studied, 17 were found to have an MS_B phenotype and 3 displayed an ML phenotype (Table 1; Fig. 1). MICs of representative macrolide-lincosamide-streptogramin B agents are shown in Table 2. Genomic DNA from these isolates was subjected to PCR analysis using primers specific for macrolide esterases [*ere*(A) and *ere*(B)], phosphotrans-

ferases [*mph*(A), *mph*(B), and *mph*(C)], an ATP binding cassette transporter [*msr*(A)], a proton motive force transporter [*mef*(A)], and rRNA methylases [*erm*(A), *erm*(TR) (belongs to class A) (29), *erm*(B), and *erm*(C)]. None of the isolates gave a PCR product specific to any of these known resistance determinants described for enteric bacteria, staphylococci, or streptococci.

Analysis of strains with an ML phenotype. Three strains, 02J1200, E40, and 117-891, had an ML phenotype (Fig. 1A). As these strains did not contain any of the known resistance mechanisms, we reasoned that mutations in the 23S rRNA alleles and/or in ribosomal proteins L4 and L22 might account for resistance. This was based on literature reports where mutations in L4, L22, or 23S rRNA genes were responsible for macrolide resistance in *Escherichia coli* and *Bacillus* spp. as well as our recent experience in characterizing resistance mechanisms in *S. pneumoniae* strains passaged with macrolides (2, 4, 25, 31, 34, 43, 46, 50, 52; Fu et al., Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolid Oxazolidinones). Using primers designed from the genomic database available for *S. pneumoniae* (43), we found that the sequences of the structural genes for L4 and L22 were identical to those of the wild-type strain, whereas the sequences for 23S rRNA

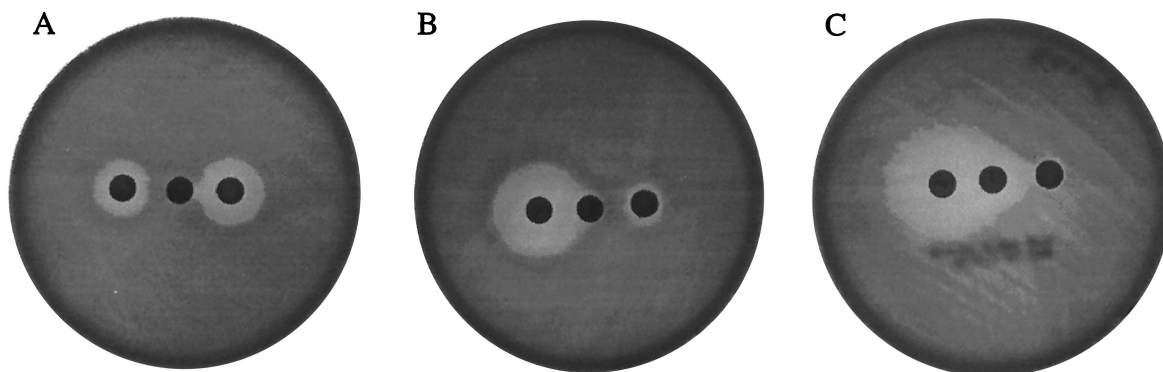


FIG. 1. Phenotypes of pneumococcal clinical isolates. (A) 02J1200, ML pattern; (B) Bu41, MS_B pattern; (C) BM4418, MS_B pattern. Disks: left, 2 µg of clindamycin; center, 15 µg of erythromycin; right, 25 µg of streptogramin B.

TABLE 2. MICs of macrolide-lincosamide-streptogramin B antibiotics against MS_B and ML strains

Strain	MIC ($\mu\text{g/ml}$) of compound ^a :									
	ERY	CLR	AZM	JOS	TEL	LIN	CLX	STA	STB	PEN
E40	100	25	>100	>100	0.01	12.5	0.78	100	6.25	6.25
02J1200	50	12.5	>100	>100	0.01	12.5	0.78	100	3.12	0.01
117-891	50	12.5	>100	>100	0.01	12.5	0.78	50	3.12	0.03
Bu41	100	12.5	>100	100	0.03	0.78	0.05	100	12.5	6.25
Sof289 ^b	>100	25	>100	100	0.1	0.78	0.1	100	12.5	3.12
395	>100	25	>100	100	0.1	1.56	0.1	100	12.5	1.56
398	100	25	>100	100	0.03	0.78	0.05	100	12.5	3.12
404	>100	25	>100	>100	0.2	1.56	0.1	>100	25	6.25
407	>100	25	>100	100	0.05	1.56	0.1	100	25	6.25
381	>100	25	>100	100	0.05	0.39	0.05	100	25	6.25
386	>100	50	>100	100	0.1	0.39	0.1	100	25	3.12
387	>100	50	>100	100	0.1	0.39	0.1	100	25	1.56
442	>100	25	>100	100	0.1	0.78	0.1	100	12.5	6.25
BM4418	6.25	12.5	12.5	6.25	3.12	0.39	0.05	50	25	0.01
ATCC 49619	0.03	0.03	0.1	0.2	0.006	0.78	0.05	25	3.12	0.39

^a Fourteen-membered macrolides: erythromycin (ERY) and clarithromycin (CLR); 15-membered macrolide: azithromycin (AZM); 16-membered macrolide: josamycin (JOS); ketolide: telithromycin (TEL); lincosamides: lincomycin (LIN) and clindamycin (CLX); streptogramins: streptogramin A (STA) and streptogramin B (STB). PEN, penicillin G.

^b The MICs for Sof289 are representative of those for six other identical isolates (Bul23, Bul29, Bul34, Bul40, Bul91, and Bul115).

revealed that all three strains had a mixture of adenine and guanine at position 2059 of domain V. The nature of the mutation was confirmed to be heterozygous, since the three isolates had an A2059G mutation in three of the four alleles when each allele was amplified separately and sequenced.

The ML strains were resistant to 14-, 15-, and 16-membered macrolides, with MICs ranging from 12.5 to >100 $\mu\text{g/ml}$ (Table 2). As expected from their phenotype, the strains were also 16-fold-more resistant to the lincosamides clindamycin and lincomycin than was ATCC 49619. The isolates remained susceptible to streptogramin B and to the ketolide telithromycin. Only E40 was resistant to penicillin G. Genomic DNA from each isolate was restricted with *Sma*I and analyzed by PFGE (Fig. 2A). Each strain had a unique pattern, indicating that the

strains did not share a recent common origin (45).

To confirm that the A2059G changes were sufficient to confer ML, the PCR product containing 23S rRNA from strain 117-891 was transformed into R6 and transformants were selected with low levels of erythromycin. The sequences of 23S rRNA, L4, and L22 genes of two transformants (ML1 and ML2) that were phenotypically ML were determined. Both had A2059G mutations in two of the four alleles of 23S rRNA, while the genes for the ribosomal proteins were wild type. When strain ML1 was phenotyped, colonies were noted in the zones of inhibition on erythromycin and clindamycin disks. Twelve "zone clones" were selected (Z1 to Z12) and streaked on Trypticase soy agar containing 5% sheep blood devoid of antibiotic. As shown by disk diffusion analysis, the majority of the zone clones had smaller zones of inhibition by erythromycin and clindamycin than did their parent, ML1, more analogous to the zone sizes seen with 117-891. When the zone clones were sequenced, all were found to have wild-type sequences for L4 and L22 ribosomal genes and 3 of the 12 had three of the four alleles of 23S rRNA as A2059G mutations. The remaining nine clones had two of the four alleles of 23S rRNA as A2059G. To see if there were any cells that had converted all four alleles to 2059G, samples from the wells containing inhibitory concentrations of spiramycin were streaked onto nonselective agar. The susceptibility of individual colonies growing from these wells to macrolide-lincosamide-streptogramin B antibiotics was determined by disk diffusion, and clones that had no zones of inhibition by erythromycin were identified (Z10-Spir, Table 3). Sequencing of the PCR products from 23S rRNA-specific primers revealed that all four alleles were present as A2059G with no changes in L4 or L22 sequences in Z10-Spir (Table 3).

The isogenic strains were checked for susceptibilities to macrolide-lincosamide-streptogramin B antibiotics (Table 3). MICs of azithromycin increased >100-fold with two or more A2059G changes. The MICs of erythromycin and clarithromycin increased about 100-fold with two A2059G changes and a further four- to eightfold with three or four A2059G changes. MICs of josamycin, tylosin, and spiramycin increased 10- to 20-fold with two A2059G changes and a further 30- to 100-fold with three or four A2059G changes. MICs of lincomycin but

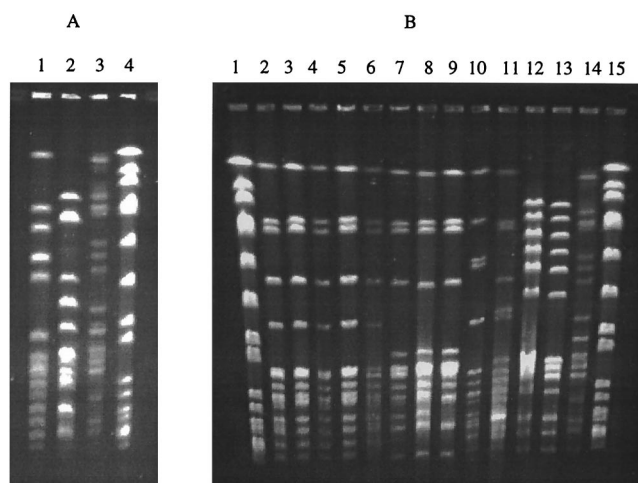


FIG. 2. PFGE of *Sma*I digests of genomic DNA from ML and MS_B strains. (A) Lane 1, strain 02J1200; lane 2, strain E40; lane 3, strain 117-891; lane 4, *S. aureus* *Sma*I markers. (B) Lanes 1 and 15, *S. aureus* *Sma*I markers; lane 2, Sof289 (Bulgaria); lane 3, Bu41 (Bulgaria); lane 4, 404 (Bulgaria); lane 5, 407 (Bulgaria); lane 6, 398 (Bulgaria); lane 7, 381 (Slovakia); lane 8, 386 (Slovakia); lane 9, 387 (Slovakia); lane 10, 395 (Bulgaria); lane 11, 442 (Poland); lane 12, BM4418 (susceptible revertant of BM4418); lane 13, BM4418 (Canada); lane 14, 117-891 (United States; ML isolate).

TABLE 3. The impact of mutational frequency at position 2059 in 23S rRNA on MICs^a

Strain	A2059:G2059 ^b	MIC (μg/ml) of compound:										Disk diffusion zone diam (mm) for compound:			
		ERY	CLR	AZM	JOS	TYL	SPI	TEL	LIN	CLX	STA	STB	CLX	ERY	STB
R6	4:0	0.02	0.05	0.10	0.10	0.39	0.20	0.006	0.39	0.05	25.00	3.12	30.0	30.5	22.1
ML1	2:2	3.12	1.56	400	1.56	6.25	3.12	0.006	6.25	0.10	50.00	3.12	22.1	20.6	19.3
Z10	1:3	12.5	6.25	>400	200	200	400	0.006	12.5	0.39	50.00	3.12	18.2	14.0	19.4
Z10-Spir	0:4	25	6.25	>400	200	400	800	0.006	12.5	0.78	50.00	3.12	13.8	0	18.4

^a Fourteen-membered macrolides: erythromycin (ERY) and clarithromycin (CLR); 15-membered macrolide: azithromycin (AZM); 16-membered macrolides: josamycin (JOS), tylosin (TYL), and spiramycin (SPI); ketolide: telithromycin (TEL); lincosamides: lincomycin (LIN) and clindamycin (CLX); streptogramins: streptogramin A (STA) and streptogramin B (STB).

^b The ratio of the number of 23S rRNA genes harboring adenine at position 2059 to the number of genes harboring guanine there.

not clindamycin increased about 10-fold with two A2059G changes; MICs of lincomycin remained at similar values with three or four A2059G changes, while those of clindamycin increased a further four- to eightfold. MICs of telithromycin, streptogramin A, and streptogramin B were not affected by any of the A2059G changes. Disk diffusion analysis of these strains also confirmed that the effect of the mutation was to render the cells increasingly resistant to macrolides and lincosamides (Table 3).

Since mutations in the ribosome could alter the growth rate, viability was monitored for R6, ML1 (transformant with two A2059G changes), Z10 (zone clone with three A2059G changes), and Z10-Spir (four A2059G changes) in Todd-Hewitt broth–0.5% yeast extract at 35°C. The generation time increased as a function of the number of A2059G mutations. In two separate analyses, R6 had a generation time of 24 min while the generation times of ML1, Z10, and Z10-Spir were 29, 32, and 38 min, respectively.

Purification of ribosomes from this isogenic set and testing for translation inhibition by macrolide-lincosamide-streptogramin B antibiotics were done to ascertain ribosomal sensitivity. The 50% inhibitory concentrations (IC₅₀) for macrolides increased 20- to over 50-fold as the number of alleles containing the A2059G mutation increased, while there was no change in the IC₅₀ for telithromycin and a fourfold change for streptogramin B (Table 4).

Analysis of strains with an MS_B phenotype. Strains that had an MS_B phenotype displayed a large zone of inhibition around clindamycin and a reduced or no zone of inhibition for erythromycin or streptogramin B (Fig. 1B and C). These 17 strains did not harbor any known resistance determinants by PCR

analysis, and sequencing of genes for 23S rRNA and L22 ribosomal protein did not reveal any mutation. By contrast, two types of mutations within the L4 sequence were found. The 16 Eastern European isolates contained a 3-amino-acid substitution (₆₉GTG₇₁ to TPS) resulting from mutations in a highly conserved region (₆₃KPWRQKGTGRAR₇₄) (Fig. 3). These strains were highly resistant to erythromycin, azithromycin, and josamycin (MIC, ≥100 μg/ml) and more moderately resistant to clarithromycin (MIC, 12.5 to 25 μg/ml) (Table 2). The strains remained susceptible to lincosamides and telithromycin, but all were resistant to streptogramin B and penicillin. We showed that the nucleotide changes (seven changes, two of which are silent) resulting in the ₆₉GTG₇₁ to TPS (nucleotide region 205 to 240) substitution are sufficient to confer MS_B by analysis of R6 transformants transformed with the L4-specific PCR product from Bu41 (Table 5). Erythromycin-resistant transformants (i.e., R6 MS_B in Table 5) contained the same seven changes in the nucleotide 205 to 240 region, and MICs for them were similar to MICs for the parent strain. Other changes outside of the nucleotide 205 to 240 region that were found in some of the wild-type strains and some of the transformants did not appear to impact the expression of MS_B as measured by MICs of the transformants (data not shown).

The second type of L4 mutation was seen in the Canadian strain BM4418 and consisted of an 18-bp insertion resulting in the addition of six amino acids (₆₉GTGREKGTGRAR) between ₇₁G and ₇₂R (Fig. 3). The MICs for this strain were different, most notably in that this strain was moderately resistant to all macrolides and more highly resistant to telithromycin (Table 2). Confirmation that this change in L4 sequence could confer an MS_B phenotype was obtained from the study of transformants derived using genomic DNA to transform CP1000 (i.e., BM4440 in Table 5). BM4440 contained the same change as that in BM4418. Further, a susceptible revertant of BM4418, called BM4419, was obtained by growth of BM4418 for 80 generations in the absence of antibiotic. Sequence analysis of the genes for 23S rRNA and L4 and L22 ribosomal proteins in BM4419 revealed no mutations; the L4 DNA sequence revealed a clean deletion of the 18-bp insertion in BM4418 (Fig. 3).

The MICs of the macrolide-lincosamide-streptogramin B antibiotics against the isogenic pairs of MS_B strains provided the best indicator of the impact of the L4 mutation (Table 5). A differential of ca. 300- to 500-fold for each of the macrolides, including the ketolide telithromycin, was seen in strains derived from BM4418 harboring the 6-amino-acid insert. Against the transformant R6 MS_B containing the ₆₉GTG₇₁ to TPS mutation derived from Bu41, the increase in MICs of 14- and

TABLE 4. In vitro poly(A)-directed translation using ribosomes from isogenic strains

Strain	IC ₅₀ (μg/ml) of compound ^a :					
	ERY	AZM	JOS	TEL	CLX	STB
R6	0.02	0.02	0.2	0.02	0.03	0.15
ML1	0.08	0.08	>10	0.04	0.09	0.3
Z10	0.9	1.2	>10	0.02	0.3	0.3
Z10-Spir	0.5	0.8	>10	0.04	0.6	0.6
CP1000	0.07	0.01	0.003	0.005	0.03	0.1
BM4440	10	7	8	0.4	0.05	>10
R6	0.01	0.01	0.2	0.08	0.01	0.3
R6 MS _B	>10	>10	>10	0.15	0.03	>10

^a Fourteen-membered macrolide: erythromycin (ERY); 15-membered macrolide: azithromycin (AZM); 16-membered macrolide: josamycin (JOS); ketolide: telithromycin (TEL); lincosamide: clindamycin (CLX); streptogramin: streptogramin B (STB).

Type 1: GTG to TPS

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      63 K P W R Q K G T G R A R Q G S I R S
R6    187 AAA CCA TGG CGT CAA AAA GGA ACT GGA CGT GCT CGT CAA GGT TCT ATC CGC TCA 240
R6 MSB 187 AAA CCA TGG CGT CAA AAA ACA CCT AGC CGT GCG CGT CAA GGT TCT ATC CGC TCT 240
      63 K P W R Q K T P S R A R Q G S I R S
    
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Type 2: 18bp insert

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      K P W R Q K G T G R
BM4418 187 AAA CCA TGG CGT CAA AAA GGA ACT GGA --- --- --- --- --- --- CGT 216
BM4419 187 AAA CCA TGG CGT CAA AAA GGA ACT GGA CGT GAA AAA GGA ACT GGA CGT 234
      R Q K G T G
    
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FIG. 3. Nucleotide and amino acid changes (boldface) in strains with MS_B phenotypes.

16-membered macrolides was >2,000-fold while the differential observed for azithromycin (15-membered macrolide) was ca. 60-fold. The MIC of telithromycin against R6 MS_B (0.02 µg/ml) was approximately eightfold higher than that against R6 (0.006 µg/ml), but still in the susceptible range. The large increase in resistance to telithromycin of BM4440 and BM4418 led to a MIC in the putative intermediate range of 2 to 3 µg/ml (M. M. Traczewski, S. D. Brown, and A. L. Barry, Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones, abstr. 2.08, 2000; G. A. Pankuch, M. R. Jacobs, and P. C. Appelbaum, Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones, abstr. 2.09, 2000). Comparison of IC₅₀ of inhibition by different macrolide-lincosamide-streptogramin B antibiotics using the purified ribosomes from isogenic pairs R6 and R6 MS_B and CP1000 and BM4440 indicated that resistance also occurred at the translational level (Table 4). However, the IC₅₀ differential observed for telithromycin in CP1000 versus BM4440 was considerably less than that observed by MIC evaluation (Table 5).

When total DNA from the MS_B isolates was digested with *Sma*I and examined by PFGE, four profiles were found (Fig. 2B). The first one (clone 1) consisted of Bulgarian strains Sof289, Bu41, 398, 404, and 407. The three isolates 381, 386, and 387 from Slovakia are clearly related to the first clone, differing by two bands and therefore assigned to clone 1 (45). The Bulgarian isolate 395 had a different restriction pattern by PFGE and was assigned to clone 2 (Fig. 2B, lane 10). Strains 442 from Poland (Fig. 2B, lane 11) and BM4418 from Canada also appeared to be separate clones.

Changes in ribosomal proteins have resulted in a tempera-

ture-sensitive phenotype in *E. coli* L4 mutants (3, 6). To determine if the L4 changes observed in *S. pneumoniae* strains resulted in growth defects, the generation times of R6 and its isogenic derivatives were measured in Todd-Hewitt-0.5% yeast extract broth at 35°C. R6 transformed with the L4 PCR product of BM4418 had a generation time of 38 min versus a generation time of 24 min for R6. However, R6 MS_B (₆₉GTG₇₁ to TPS change) had the same generation time as did its isogenic parent, R6.

DISCUSSION

Target modification of the adenine at position 2058 (*E. coli* numbering) in 23S rRNA by an Erm methylase is a common resistance mechanism seen in a variety of pathogenic bacteria (10, 29, 38, 40, 51). In *S. pneumoniae*, *erm*(B) is the predominant methylase and is generally part of Tn1545 or Tn3872 (19, 47). Alteration of A2058 to another residue has been described previously (8, 11, 17, 21, 22, 24, 26, 30, 36, 37, 43, 44, 48, 49; Fu et al., Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones). Modification of adenine at position 2059 (A2059G transition) has been found in macrolide-resistant clinical strains of *Mycobacterium* spp. (21, 49), *Helicobacter pylori* (8, 24, 37, 48), and *Propionibacterium* spp. (30). In laboratory-derived mutants that contained A2059G, lincomycin resistance was conferred on *Nicotiana plumbaginifolia* chloroplast (50), streptogramin A resistance was conferred on *Halobacterium halobium* (27), macrolide resistance was conferred on *Mycoplasma pneumoniae* (17), and macrolide resistance and increased lincosamide resistance were con-

TABLE 5. MICs of macrolide-lincosamide-streptogramin B antibiotics against MS_B strains and transformants

Strain	MIC (µg/ml) of compound ^a :								Mutation
	ERY	CLR	AZM	JOS	TEL	CLX	STB	PEN	
BM4419	0.05	0.03	0.10	0.20	0.006	0.05	1.56	0.03	None
BM4418	6.25	12.5	12.5	6.25	3.12	0.05	25	0.03	₆₃ KPWROKGTGREKGTGRAR ₈₀
CP1000	0.03	0.01	0.05	0.05	0.003	0.03	1.56	0.01	None
BM4440	3.12	3.12	3.12	3.12	1.56	0.03	25	0.01	₆₃ KPWROKGTGREKGTGRAR ₈₀
R6	0.02	0.05	0.10	0.10	0.003	0.02	1.56	0.006	None
R6 MS _B	50	>100	6.25	50	0.024	0.02	12.5	0.01	₆₃ KPWROKTPSRAR ₇₄
Bu41	100	>100	12.5	100	0.05	0.05	12.5	3.12	₆₃ KPWROKTPSRAR ₇₄

^a Fourteen-membered macrolides: erythromycin (ERY) and clarithromycin (CLR); 15-membered macrolide: azithromycin (AZM); 16-membered macrolide: josamycin (JOS); ketolide: telithromycin (TEL); lincosamide: clindamycin (CLX); streptogramin: streptogramin B (STB). PEN, penicillin G.

ferred on *S. pneumoniae* (43; Fu et al., Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones). We explored the dose effect of this mutation by isolating isogenic strains carrying two, three, or four alleles of A2059G. As the number of alleles increased, so did the MICs of 14-, 15-, and 16-membered macrolides. The effect on clindamycin was not as pronounced in the isogenic strains, but for the three clinical strains that carry three 2059G alleles and one A2059 allele the MICs were $\sim 1 \mu\text{g/ml}$, consistent with a resistant phenotype ($\geq 1 \mu\text{g/ml}$) according to NCCLS standards (Table 2).

The MS_B phenotype was seen in laboratory studies when susceptible isolates of *S. pneumoniae* were passaged with macrolides (43). It was due to a C2611A or C2611G change in three or four alleles of 23S rRNA, respectively, or to L4 mutations in which there was either a change at amino acid 71 from glycine to cysteine or a 2-amino-acid insert (₆₃KPWRQ SQKGTGRAR) in a highly conserved region of L4. Interestingly, two types of mutations in L4 were observed in the clinical isolates, neither of which was seen in the laboratory-derived mutants. The majority of the clinical strains had mutations that resulted in the amino acid substitutions of TPS for ₆₉GTG₇₁. One isolate, BM4418, had a 6-amino-acid insert (₆₉GTGREKGTGRAR) in the highly conserved region of L4 and differed from the other MS_B isolates by its increased resistance to telithromycin. However, the latter mutant paid a price, as was noted by a 60% increase in generation time at 35°C. Thus, the L4 mutation ₆₉GTG₇₁ to TPS could be more easily maintained since it apparently does not alter growth.

As more macrolide-resistant isolates are characterized, it will be interesting to see how many contain 23S rRNA and/or ribosomal protein mutations in addition to or as the sole mechanism responsible for MLS_B. Recently, there have been several reports of clinical strains of *S. pneumoniae* that do not contain either *erm*(B) or *mef*(A) (9; E. Di Modugno, A. Felici, M. Guerrini, H. Mottl, A. Zamperlin, and D. Sabatini, Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones, abstr. 7.06, 2000; K. Waites, C. Johnson, B. Gray, K. Edwards, M. Crain, and W. Benjamin, Jr., Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones, abstr. 7.08, 2000; E. Di Modugno, A. Felici, M. Guerrini, H. Mottl, P. Piccoli, and D. Sabatini, Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones, abstr. 7.14, 2000). A surveillance study reported the number of *erm*(B)- and *mef*(A)-negative isolates to be as high as 2.2% of the total macrolide-resistant isolates (Di Modugno et al., Abstr. Fifth Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones, abstr. 7.06).

What are the clinical implications of these findings? Since the majority of clinical laboratories do not test streptogramin B, an ML phenotype will likely be interpreted as a strain carrying an Erm methylase, whereas a strain that is MS_B may be interpreted as harboring *mef*(A). If the MS_B phenotype results from a mutation in L4, the isolate is likely to be susceptible to clindamycin, whereas if it is the result of a C2611A/G change in 23S rRNA, as was seen previously for passaged isolates (43), there may be some uncertainty, as MICs of 0.2 to 0.4 μg of clindamycin per ml indicate intermediate resistance (23). The MICs of clindamycin for an ML strain also indicated intermediate to low-level resistance (0.39 to 0.78 $\mu\text{g/ml}$), and it remains to be determined if these isolates are susceptible to clindamycin therapy. Further, in MS_B strain BM4418, we have seen a hint of one mechanism that may confer ketolide resistance. Use of disk diffusion analysis to infer the likely mechanism responsible for macrolide resistance needs to be modified in light of these new resistance mecha-

nisms; however, using erythromycin, clindamycin, telithromycin, and streptogramin B disks will be helpful.

In summary, this work characterized two resistance mechanisms not previously recognized in clinical isolates of *S. pneumoniae* from Eastern Europe and North America. These isolates had changes in 23S rRNA genes (A→G at position 2059) or changes in a highly conserved region of ribosomal protein L4 (₆₉GTG₇₁ to TPS mutation or a 6-amino-acid L4 insertion, ₆₉GTGREKGTGRAR). Introduction of new macrolide-lincosamide-streptogramin B agents will necessitate ongoing vigilance in testing the susceptibility of isolates to new and existing macrolide-lincosamide-streptogramin B agents and the determination of resistance mechanisms in strains with decreased susceptibility.

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