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Mutation in 23S rRNA Responsible for Resistance to 16-Membered Macrolides and Streptogramins in *Streptococcus pneumoniae*

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Streptococcus pneumoniae **clinical isolate BM4455 was resistant to 16-membered macrolides and to streptogramins. This unusual resistance phenotype was due to an $A_{2062}C$ (*Escherichia coli* numbering) mutation in domain V of the four copies of 23S rRNA.**

Macrolide and lincosamide antibiotics exhibit high activity against streptococci and are among the drugs that can be used for the treatment of infections due to *Streptococcus pneumoniae* (10). The macrolides are largely prescribed for empiric therapy of community-acquired respiratory tract infections and may be useful in case of intolerance or resistance to β-lactams. In France, oral streptogramins are the second line treatment in the macrolide, lincosamide, streptogramin (MLS) group of antibiotics, in which they replace erythromycin and related macrolides in case of resistance of *S. pneumoniae* to macrolides (11). The streptogramin antibiotics produced by *Streptomyces pristinaespiralis* contain two active components, A and B (II and I in pristinamycin, M and S in virginiamycin, and the semisynthetic derivatives dalfopristin and quinupristin in Synercid), that inhibit peptide elongation synergistically; individually they are bacteriostatic, whereas together they can be bacteriocidal. The presence of the A component strongly enhances ribosomal binding of the B component (19). The prevalence of macrolide-resistant strains of *S. pneumoniae* has increased during the last 10 years (17, 22). There is, thus, a need for alternative drugs. The ketolides, such as telithromycin, constitute a new semisynthetic 14-membered macrolide class of antimicrobial agents (8). Telithromycin is a 3-keto derivative of clarithromycin. The ketolides have the same antibacterial spectrum as macrolides but also display good activity against erythromycin-resistant isolates of gram-positive cocci (8).

The macrolide, lincosamide, and streptogramin B antibiotics (MLS_B) are three chemically distinct but functionally related drug classes. They act by binding to the 50S subunit of bacterial ribosomes and inhibit protein synthesis by blocking elongation of the nascent peptide chain (2, 10).

Resistance to macrolides in *S. pneumoniae* is due to two mechanisms: target site modification or active efflux. Target modification is secondary to acquisition of an *erm* gene which encodes an enzyme that methylates a specific adenine residue (A2058) in 23S rRNA (Fig. 1) (10). This alteration induces a conformational change in the 50S ribosomal subunit that blocks binding of the MLS_B to the ribosome (10). Expression of resistance can be inducible or constitutive. Streptococci, as opposed to staphylococci, are cross-resistant to 14-, 15-, and 16-membered MLS_B whether resistance is inducible or constitutive. Streptogramins A are not affected, and synergy between the two components of streptogramins against MLS_B-resistant strains is maintained. Thus, in streptococci, constitutive resistance cannot be distinguished from inducible resistance on the sole basis of elevated MICs of erythromycin and lincomycin (21). The high prevalence of the MLS_B-inducible phenotype in *S. pneumoniae* explains why weak inducers like the ketolides are active against most of the isolates of this species.

The second resistance mechanism, macrolide-specific efflux from the cells, is effected by a membrane protein encoded by the *mef* gene (25). This leads to the M phenotype, which is resistance to 14- and 15-membered macrolides and susceptibility to 16-membered macrolides, ketolides, lincosamides, and streptogramins. The *mef* gene is the most common macrolide resistance determinant among *S. pneumoniae* isolates in the United States, whereas *erm* is more prevalent in Europe (17, 22).

Recently, two other mechanisms associated with unusual resistance phenotypes to MLS antibiotics have been identified in clinical isolates of *S. pneumoniae*. Macrolide-streptogramin resistance is due to a 3-amino-acid substitution, whereas further resistance to ketolides is due to a 6-amino-acid insertion in a highly conserved region of ribosomal protein L4 ($_{63}$KPWRQKGTGRAR$_{74}$ [*S. pneumoniae* numbering]) (A. Tait-Kamradt, T. Davies, L. Brennan, F. Depardieu, P. Courvalin, J. Duignan, J. Petitpas, L. Wondrack, M. Jacobs, P. Appelbaum, and J. Sutcliffe, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. LB-8, 1999). The macrolide-lincosamide resistance phenotype observed in clinical isolates from the United States or in laboratory mutants is due to an $A_{2059}G$ (*Escherichia coli* numbering) change in two, three, or four copies of 23S rRNA (26). A gene dosage effect on the level of macrolide and lincosamide resistance was observed in isogenic strains depending upon the number of mutated *rrl* alleles.

S. pneumoniae BM4455, of capsular serovar 18F, was isolated in 1988 in France from a blood culture (6) and had a new resistance phenotype (Table 1). There was a dissociation between resistance to 16-membered macrolides and susceptibility to 14- and 15-membered macrolides associated with resistance to the two components of streptogramins. The MICs of certain

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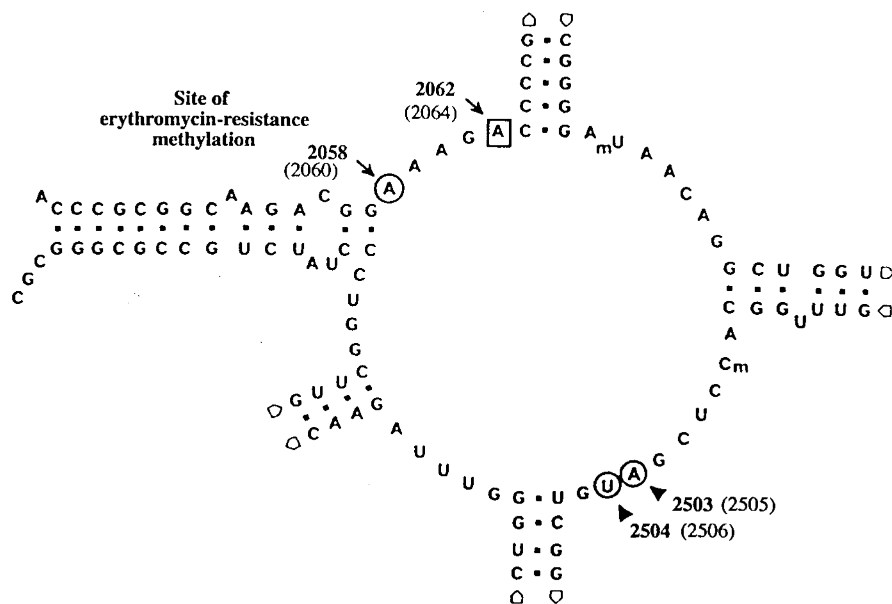

FIG. 1. Secondary structure of the peptidyl transferase loop in domain V of 23S rRNA. The mutated position in *S. pneumoniae* BM4455 is indicated by a square. The positions of the other binding sites of streptogramins (m^2A2503-U2504) are indicated by a circle. Nucleotide sequence and numbering are those of *E. coli* 23S rRNA, and the corresponding *S. pneumoniae* numbering is given in parentheses.

MLS antibiotics for *S. pneumoniae* BM4455 and susceptible *S. pneumoniae* BM4203 and CP1000 (Table 1) were determined by agar dilution in Mueller-Hinton medium supplemented with 5% horse blood with an inoculum of 10^4 CFU per spot (Table 2) (3). Strain BM4455 was resistant to high levels of 16-membered macrolides and to intermediate levels of streptogramins A and B, but synergy between the two components was retained. The strain remained susceptible to the 14- and 15-membered macrolides, the ketolides, and the lincosamides.

S. pneumoniae BM4455 was tested for the presence of known MLS resistance determinants. Attempts to amplify genomic DNA using primers specific for *ermA*, *ermB*, and *ermC* genes encoding an rRNA methylase; *ereA* and *ereB* specifying a macrolide esterase; *mphA* and *mphB* macrolide phosphotransferases; *msrA*, an ATP binding cassette-type transporter; and *mefA*, a proton-motive force macrolide transporter (24), were unsuccessful (data not shown).

The possibility that strain BM4455 carried mutations in ribosomal proteins L4, L16, or L22 or in 23S rRNA was explored. Ribosomal proteins L4 and L22 are involved in the binding of spiramycin to the 50S subunit of the ribosome (1, 7, 31). Proteins L16 and L22 interact with the streptogramins (4, 5). L4 and L22 bind primarily to domain I of 23S rRNA, but erythromycin resistance mutations in these proteins perturb the conformation of residues in domains II, III, and V and thereby affect the action of antibiotics known to interact with nucleotide residues in the peptidyl transferase center of domain V (7). Mutations conferring resistance to macrolides were first identified in proteins L4 and L22 of *E. coli* (18, 31) and subsequently in 23S rRNA (30). There is compelling evidence that the peptidyltransferase loop in domain V of 23S rRNA may contain at least part of the site at which the MLS antibiotics physically bind to the ribosome (14, 19, 30). Mutants selected for resistance to individual MLS antibiotics show changes in A2058 (*E. coli* numbering) and neighboring nucleotides, suggesting their involvement in the binding of these antibiotics (30).

The primers used to amplify the entire structural genes *rplD*, *rplP*, and *rplV* for ribosomal proteins L4, L16, and L22, respectively, and of part of *rrl* for domains II and V of 23S rRNA were designed complementary to conserved regions (Table 3). Amplifications were performed with total DNA of strain

TABLE 1. Strains of *S. pneumoniae* studied

Strain	Resistance phenotype[a]	Reference or source
BM4203	Susceptible	27
CP1000	Sm^r	15
BM4455	M_{16}^r S_A^r S_B^r S^r	6
BM4456	Sm^r M_{16}^r S_A^r S_B^r S^r	Transformation of BM4455 total DNA into CP1000
BM4457	Sm^r M_{16}^r S_A^r S_B^r S^r	Transformation of BM4455 *rrl* gene into CP1000

[a] Abbreviations: M_{16}, 16-membered macrolides; S_A, pristinamycin II; S_B, pristinamycin I; S, pristinamycin I and II; Sm, streptomycin.

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TABLE 2. MICs of antibiotics against *S. pneumoniae* strains[a]

Strain	MIC (μg/ml) of[b]:											
	Macrolides[c]						Lincosamides		Streptogramins			
	ERY (14)	AZI (15)	SPI (16)	JOS (16)	TYL (16)	TEL (14)	LIN	CLI	S_B	S_A	S	QUI + DAL
BM4203	<0.25	0.5	0.5	0.5	0.5	<0.0075	0.5	0.125	2	16	0.125	0.25
CP1000	<0.25	<0.125	<0.125	0.125	<0.25	<0.0075	0.5	0.03	1	<2	<0.0625	0.25
BM4455	<0.25	0.5	512	64	64	<0.0075	0.5	<0.015	32	16	2	2
BM4456	<0.25	0.5	512	32	32	<0.0075	0.5	<0.015	64	32	4	2
BM4457	<0.25	0.5	512	32	32	<0.0075	0.5	<0.015	64	32	4	2

[a] The MICs were determined by the agar dilution method after 24 h at 37°C in an atmosphere enriched with 5% CO_2.
[b] Abbreviations: AZI, azithromycin; ERY, erythromycin; CLI, clindamycin; JOS, josamycin; LIN, lincomycin; QUI + DAL, 30% quinupristin–70% dalfopristin; S_A, pristinamycin II; S_B, pristinamycin I; S, pristinamycin I and II; SPI, spiramycin; TEL, telithromycin; TYL, tylosin.
[c] Numbers in parentheses are numbers of ring members.

BM4455 as a template using *Pfu* polymerase (Stratagene) in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.) for 30 cycles. The conditions were 1 min at 95°C for denaturation, 1 min at 50°C (*rplD*, *rplP*, *rplV*, and *rrl* for domain II) or at 54°C (four alleles of *rrl* corresponding to domain V) for annealing, and 2 min at 72°C for elongation. The amplification products were purified, cloned into pCR-Blunt vector (Invitrogen), and sequenced by the dideoxy chain termination method using T7 DNA polymerase (T7 Sequencing kit; Pharmacia) and [α-^{35}S]-dATP (Amersham Radiochemical Centre). The sequences obtained were compared with those of *S. pneumoniae* obtained from The Institute for Genomic Research (TIGR)'s Website (http://www.tigr.org.). No mutations were observed in the *rplP* and *rplV* genes, whereas the sequence of *rplD* for L4 differed by a point mutation in codon 20, leading to a Ser-to-Asn substitution (Table 3).

Four copies of the 23S rRNA *rrl* gene are present in *S. pneumoniae*, and a strategy has been developed to amplify these copies individually for domain V (26). It consists in using primers complementary to unique sequences downstream from each *rrl* gene and a primer (23S rRNA-V$^+$) common to the four alleles and complementary to a region upstream from the peptidyl transferase region in domain V (Table 3). The four *rrl* genes were amplified separately, the region encoding domain V was sequenced, and each copy was found to contain an $A_{2062}C$ (*E. coli* numbering) substitution. No mutations were observed in the *rrl* portion corresponding to domain II.

To determine if the mutations in the *rplD* gene for ribosomal protein L4 and in the portion of *rrl* corresponding to domain V of 23S rRNA were necessary and sufficient to confer the 16-membered macrolide–streptogramin (M_{16}-S) resistance phenotype to the host, total DNA from strain BM4455 or the PCR products corresponding to the *rplD* gene and domain V of the *rrl* gene were introduced into *S. pneumoniae* CP1000 by transformation (Table 1). Chromosomal or amplified DNA was added to competent cells of CP1000, and the mixture was incubated at 37°C until the optical density at 550 nm reached 0.2. The bacteria were then plated onto horse blood agar and incubated at 37°C for 2 h without antibiotic. Transformants were selected with spiramycin at 10 μg/ml and 60 μg/ml or pristinamycin at 1 μg/ml by the overlay procedure and incubated 24 to 48 h at 37°C in an atmosphere enriched with 5%

TABLE 3. Oligodeoxynucleotides used and mutations in *S. pneumoniae* strains

Gene (ribosomal protein) template for amplification	Primer		Position[b]	Product size (bp)	Reference or source	Mutation (amino acid substitution) in strain:			
	Designation	Sequence (5′ to 3′)[a]				CP1000	BM4455	BM4456	BM4457
rplD (L4)	L4 NH2	+ GTGCACGAGTGTCAACT	1337–1353	855	This study	None	AGC→AAC (Ser_{20}-Asn)	None	None
	L4 COOH	− GTTGTACAAGTTGTTCC	499–515						
rplP (L16)	L16 NH2	+ AGTATGGATCTACCGTG	2177–2193	551	This study	None	None	None	None
	L16 COOH	− CGCGAGTTCTTCTTGAG	2713–2729						
rplV (L22)	L22 NH2	+ TGCACCAACTCGTACTT	1155–1171	479	This study	None	None	None	None
	L22 COOH	− TCACGGATGATGCCGAC	1617–1633						
rrl (23S rRNA)									
Domain II	23S rRNA-II$^+$	+ CTCCCTAGTGACCGATA	465–481	865	This study	None	None	None	None
	23S rRNA-II$^-$	− TACGGTGGACAGGATTC	1312–1328						
Domain V	23S rRNA-V$^+$	+ TCAGCCGCAGTGAATAG	1749–1765		This study				
	DS18	− GCCAGCTGAGCTACACCGCC		2,002	26	None	$A_{2062}C$	$A_{2062}C$	$A_{2062}C$
	DS23	− TACACACTCACATATCTCTG		2,004	26	None	$A_{2062}C$	$A_{2062}C$	$A_{2062}C$
	DS30	− TTTTACCACTAAACTACACC		1,296	26	None	$A_{2062}C$	$A_{2062}C$	$A_{2062}C$
	DS91	− TACCAACTGAGCTATGGCGG		1,217	26	None	$A_{2062}C$	$A_{2062}C$	$A_{2062}C$

[a] +, sense primer; −, antisense primer.
[b] For the *rplD* gene, positions were determined from the sp19 contig as found on TIGR's Website (http://www.tigr.org); for the *rplP* and *rplV* genes, positions are according to the sequence of *S. pneumoniae* R6 (accession number AF126059); for domains II and V of the *rrl* gene, positions are according to the *S. pneumoniae* numbering obtained from the sp23 contig in TIGR's Website and aligned on the *E. coli rrl* gene sequence.

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CO_2. Transformants were obtained only with chromosomal DNA or with the 1,013-bp *rrl* PCR fragment of domain V containing the $A_{2062}C$ mutation and on spiramycin (10 μg/ml). Their phenotype was indistinguishable from that of the donor strain BM4455 (Table 1). Experiments performed with the 855-bp *rplD* PCR product containing the G-to-A substitution at position 59 (Table 3) did not yield any transformant. The four *rrl* alleles corresponding to domain V and the *rplD* gene of transformants BM4456 and BM4457 obtained with total DNA or the *rrl* PCR product of domain V (Table 1), respectively, were amplified and sequenced as described above. The two transformants were found to harbor the same $A_{2062}C$ mutation in the four *rrl* copies as in the donor but not the substitution in the *rplD* gene for ribosomal protein L4 (Table 3). The MICs of various MLS antibiotics against the transformants were determined and found to be similar to those against the wild strain (Table 2). Taken together these data indicate that the mutation in the four *rrl* genes corresponding to domain V is solely responsible for resistance to 16-membered macrolides and to streptogramins. Gene conversion could be responsible for the presence of the $A_{2062}C$ mutation in the four *rrl* copies (9).

To the best of our knowledge, a single example of mutation at position 2062 of 23S rRNA has been reported. A chloramphenicol-resistant mutant was isolated in *Halobacterium halobium*, which possesses a single copy of 23S rRNA, that contained an A-to-C substitution at position 2062 (*E. coli* numbering) in domain V (13). The target site for macrolides lies within 23S rRNA at the peptidyltransferase center of the 50S subunit (Fig. 1). The peptidyltransferase activity is associated with the central loop of domain V, where macrolides make several contacts with the rRNA (14). Recently, the binding sites of streptogramins B in *E. coli* were localized by UV-induced modifications at positions A2503-U2504 and G2061-A2062 in the peptidyltransferase loop (Fig. 1) (19, 20). Mutation at position 2062 of 23S rRNA may therefore prevent binding of pristinamycin I and account for resistance in strain BM4455 (Fig. 1).

Mutations at A2058 presumably perturb the site were the MLS drugs interact with the ribosome and thereby affect their binding in a manner similar to methylation at this position (23). In *Helicobacter pylori*, clarithromycin resistance is due to mutations at position 2058 or 2059 in the 23S rRNA (28). Mutations at position 2058 confer an MLS_B phenotype with high-level clarithromycin resistance, an A-to-G substitution being the most common in clinical isolates (29). Mutations at position 2059 confer a lower level of resistance to clarithromycin and no resistance to the streptogramins (16). *Mycoplasma pneumoniae* displays phenotypes similar to those of *H. pylori* following $A_{2058}G$ and $A_{2059}G$ mutations, and it has been shown that the G2059 mutant is more resistant to 16-membered macrolides such as tylosin and spiramycin (12). These results could reflect subtle differences in the mode of interaction of 14- and 16-membered macrolides in the 2058 region of 23S rRNA (14). Erythromycin-resistant mutants that remain susceptible to spiramycin also support the notion that the ribosome binding sites for the two groups of macrolides are not identical (1). In addition, the drugs with 16 atoms are much larger than those with 14 atoms, and the number of sugar residues also differs. Together these observations could explain the dissociated resistance between 16- or 14- and 15-membered macrolides.

In conclusion, we have shown that an $A_{2062}C$ mutation in 23S rRNA confers M_{16}-S resistance in *S. pneumoniae*. With the emergence of new resistance mechanisms, it seems advisable to test in vitro the activity of one member each of the 14-, 15-, and 16-membered macrolide, ketolide, lincosamide, and streptogramin class of drugs.

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