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Clinical Isolates of *Staphylococcus aureus* with Ribosomal Mutations Conferring Resistance to Macrolides

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Six strains of *Staphylococcus aureus* isolated from cystic fibrosis patients after treatment with azithromycin were cross-resistant to azithromycin and erythromycin. None of the isolates contained *erm* or *msr*(A) genes, but they all carried either A2058G/U or A2059G mutations within the *rrl* genes, with a majority of the rRNA copies bearing the mutation. One strain displayed an additional mutation in the *rplV* gene, encoding the L22 ribosomal protein.

Emergence of resistance to macrolides in staphylococci shortly after the therapeutic use of erythromycin has been reported (3). In most cases, macrolide resistance in clinical isolates of staphylococci has been linked to target site alteration due to methylation of adenosine 2058 (A2058) of 23S rRNA within the large ribosomal subunit (8). These ribosomal methylases are encoded by *erm* genes. In some cases, ABC transporters encoded by plasmid-borne *msr*(A) genes cause active efflux of 14-member-ring (erythromycin, clarithromycin, roxithromycin, and dirithromycin) or 15-member-ring (azithromycin) macrolides (14). Rare staphylococcal strains have been reported to produce a macrolide phosphotransferase which inactivates some of these antimicrobials (12, 20). Overall, in several survey studies, drug efflux and ribosomal methylation have been found to be responsible for macrolide resistance in all of the strains studied (10, 15).

The use of macrolides for the treatment of staphylococcal infections is generally limited to uncomplicated soft tissue infections. Recently, it has been inferred from anti-inflammatory and antiadhesion effects of macrolides observed in vitro that these antimicrobials may have a favorable action at low concentrations for treatment of *Pseudomonas aeruginosa* infections (7). These indirect effects might be helpful in the case of cystic fibrosis, where the major cause of morbidity and mortality remains respiratory disease, with *P. aeruginosa* as the most frequently organism isolated, followed by *Staphylococcus aureus* (5). We report on six strains of erythromycin-resistant *S. aureus*, isolated from patients suffering from cystic fibrosis, with unusual mutations of the ribosomal target of macrolides.

Bacterial strains and antimicrobial susceptibility testing. Among 12 *S. aureus* strains resistant to erythromycin and isolated from cystic fibrosis patients, 6 did not contain *erm* or *msr*(A) genes as determined by PCR (1). These strains, *S. aureus* UCN13, UCN14, UCN15, UCN16, UCN17, and UCN18, were isolated at the hospitals of Brest and Caen, France, from the sputa of five patients (*S. aureus* UCN13 and UCN14 were isolated from the same patient) suffering from cystic fibrosis who were treated with azithromycin (10 mg/kg/day) for a minimum of 3 months and a maximum of 1 year. The strains were considered to be genetically unrelated since the patterns of *Sma*I-restricted DNA differed by more than three fragments after pulsed-field gel electrophoresis analysis (data not shown) (18). All isolates were resistant to erythromycin as determined by the disk diffusion technique. Macrolide-susceptible *S. aureus* ATCC 29213 was included as a control. MICs of antibiotics were determined by the agar dilution method with Mueller-Hinton medium. Dalfopristin (RP54476), erythromycin, quinupristin (RP57669), quinupristin-dalfopristin, pristinamycin, and spiramycin were from Aventis Pharma (Romainville, France), and lincomycin was from Pharmacia-Upjohn (Kalamazoo, Mich.).

PCR and DNA sequence analysis. As mentioned above, no rRNA methylase genes [*erm*(A), *erm*(B), and *erm*(C)] or efflux gene [*msr*(A)] could be detected by PCR with specific primers (1). Since mutations in genes coding for L4 or L22 ribosomal proteins or in domains II and V of 23S rRNA have been reported to be responsible for macrolide resistance in a variety of bacterial species, we hypothesized that similar mutations might account for resistance in the strains studied (2, 16, 19). Portions of *rrl* genes for domains II and V of 23S rRNA and the genes for ribosomal proteins L4 and L22 were amplified by PCR from total genomic DNA with the oligonucleotides shown in Table 1. The amplification primers were designed after analysis of the sequence of *S. aureus* COL obtained from The Institute for Genomic Research website (http://www.tigr.org). Mutations were screened for by PCR-SSCP, as described previously (2). After heat denaturation, the single-stranded PCR products were separated by nondenaturing polyacrylamide gel electrophoresis. Fragments with mobilities different from those of susceptible controls were sequenced.

We also determined the copy numbers of the *rrl* genes carrying 23S rRNA mutations. A strategy was developed to amplify the different copies of *rrl* domain V individually. Sequence analysis of *S. aureus* COL showed that the strain carried six copies of the *rrl* gene. We used primers complementary to unique sequences downstream from each *rrl* gene (V LA to V LF in Table 1) and a primer common to the six alleles and complementary to a region upstream from the peptidyl transferase region in domain V (V U1). Internal primers (V U2 and V L2) were then used to amplify a 144-bp fragment encompassing the domain V region, which was subsequently sequenced.

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TABLE 1. Oligodeoxynucleotides used for the amplification of fragments of the 23S rRNA gene and of ribosomal protein genes

Gene	Primer designation	Primer sequence (5′ to 3′)[a]	Position	Product size (bp)
rrl (23S rRNA)				
Domain II	II U1	+CGGAAGGGGAGTGAAATAGAAC	486[b]	478
	II L1	−CCTTATCACCCATGTTCTGAC	963[b]	
	II U2	+GCCTCAAGTGATGATTATTGG	837[b]	508
	II L2	−ACTAACCCAGAGCGGACGAGC	1344[b]	
Domain V	V U1	+GAAAGGCGTAATGATTTGGG	1968[b]	437
	V L1	−GGAACCACCGGATCACTAAG	2404[b]	
	V U3	+GTATAAGGGAGCTTGACTG	2331[b]	439
	V L3	−GGGTTTCACACTTAGATG	2769[b]	
	V LA	−TGTGAAAAAGACTGGATGACAG	2161[c]	2,251
	V LB	−CGTTGACATATTGTCATTCAG	1145[c]	1,235
	V LC	−CATACTTAGACAATCGAAAGTG	1319[c]	1,409
	V LD	−CTAGGCCGGCAATATGTAAG	1138[c]	1,228
	V LE	−CAGGTGCATTGAGAGAATTTG	1438[c]	1,528
	V LF	−TCCACAGGTAGGACTCGAAC	1087[c]	1,177
	V U2	+CTGTCTCAACGAGAGACTC	1990[b]	144
	V L2	−CTTAGACTCCTACCTATCC	2133[b]	
rplD (L4)	RPL 4 a	+AATAATAAGAAGTGAAAGGAGG	−31[d]	414
	RPL 4 a′	−GCGTCAACTACAGTTAAGCC	383[d]	
	RPL 4 b	+CTCAGCATTATCTTTCAAAGC	335[d]	411
	RPL 4 b′	−GCCATTTTTACTTGTGTTTTG	745[d]	
rplV (L22)	RPL 22 a	+CAAAGGACACGTTGCAGACGACAAGAAA	−68[d]	456
	RPL 22 b	−ATTTTTTGACCCACAGTATTCCCTCCTT	388[d]	

[a] +, sense primer; −, antisense primer.
[b] *E. coli* numbering.
[c] Base relative to adenine 2058 on each copy.
[d] Base relative to ATG.

Susceptibility to antimicrobials. The six isolates were highly resistant to erythromycin and azithromycin (MIC of >128 μg/ml) (Table 2). Five strains were also resistant to the 16-member-ring macrolide spiramycin, whereas the spiramycin MIC for strain UCN16 was lower (8 μg/ml). MICs of quinupristin, a streptogramin B, and clindamycin were more widely distributed. MICs of dalfopristin, a streptogramin A, were similar to those for susceptible strains (9). All strains except strain UCN15 were susceptible to quinupristin-dalfopristin and pristinamycin.

Identification of ribosomal mutations. Sequencing showed that SSCP mobilities of fragments different from those of the controls amplified from *S. aureus* ATCC 29213 were associated with point mutations or a deletion. Four strains, *S. aureus* UCN13, UCN15, UCN16, and UCN17, carried an A2058G (*E. coli* numbering) transition; *S. aureus* UCN14 contained an A2058T transversion; and *S. aureus* UCN18 had an A2059G transition. No mutation was detected in domain II of 23S rRNA or in the *rplD* gene (encoding the L4 protein). In strain UCN15, a deletion of nine nucleotides which would result in the deletion of three amino acids at position 101 of the deduced amino acid sequence of protein L22 was associated with the A2058G mutation. Mutations at positions A2058 and A2059 were associated with macrolide-lincosamide-streptogramin B and macrolide-lincosamide phenotypes, respectively, similar to those already reported for other organisms (19). Relative to other rRNA mutations, A2058G gives the highest level of resistance to 14-member-ring macrolides and confers macrolide-lincosamide-streptogramin B resistance, defined as high resistance to all of the antimicrobials in this group. However, MICs of clindamycin and quinupristin were lower than expected for strains UCN14, UCN15, UCN16, and UCN18. This might be due to the fact that these staphylococci grew slowly on agar, as has been reported for most *S. aureus* strains isolated from cystic fibrosis patients, which frequently yield small-colony variants (6). The A-to-U or A-to-G substitutions

TABLE 2. MICs of macrolides, lincosamides, and streptogramins

S. aureus strain	Ribosomal mutation(s) (gene)	MIC (μg/ml)[a] of:							
		ERY	AZI	SPI	CLI	QUI	DAL	QUI-DAL	PRI
ATCC 29213	None	0.25	0.5	4	0.06	4	2	1	0.12
UCN13	A2058G (*rrl*)	>128	>128	>128	16	64	2	1	0.5
UCN14	A2058T (*rrl*)	>128	>128	128	1	32	0.5	0.25	0.12
UCN15	A2058G (*rrl*), deletion (*rplV*)	>128	>128	128	2	32	4	4	1
UCN16	A2058G (*rrl*)	>128	>128	8	0.5	8	0.25	0.25	0.12
UCN17	A2058G (*rrl*)	>128	>128	128	16	16	4	0.5	0.25
UCN18	A2059G (*rrl*)	>128	>128	128	1	2	2	0.25	0.12

[a] Abbreviations: AZI, azithromycin; CLI, clindamycin; DAL, dalfopristin; ERY, erythromycin; PRI, pristinamycin; QUI, quinupristin; QUI-DAL, quinupristin-dalfopristin; SPI, spiramycin.

TABLE 3. Mutations of the *rrl* genes

S. aureus strain	Mutation in *rrl*	Nucleotide in *rrl* copy[a]						No. of wild-type copies/no. of mutated copies
		A (533061)	B (576663)	C (581841)	D (1977965)	E (2113031)	F (2229521)	
UCN13	A2058G	G	—[b]	A	G	G	G	1 (A)/4 (G)
UCN14	A2058T	A	—	T	T	T	T	1 (A)/4 (T)
UCN15	A2058G	A	—	G	G	G	G	1 (A)/4 (G)
UCN16	A2058G	A	—	G	G	G	G	1 (A)/4 (G)
UCN17	A2058G	G	G	G	G	A	A	2 (A)/4 (G)
UCN18	A2059G	G	—	A	G	A	G	2 (A)/3 (G)

[a] Numbers in parentheses indicate the position of A2058 in *S. aureus* COL.
[b] —, copy B could not be amplified.

gave a similar level of resistance. The A2059 mutation gave the macrolide-lincosamide phenotype, with moderate resistance to clindamycin and no resistance to streptogramins B, as previously reported for *Helicobacter pylori* and *Streptococcus pneumoniae* (17, 19).

Resistance by mutation in 23S rRNA has generally been reported for bacteria with few copies of *rrn* operons, such as *H. pylori, Mycoplasma pneumoniae*, *Mycobacterium intracellulare*, and *Mycobacterium avium.* However, mutations in 23S rRNA have been reported for *S. pneumoniae*, which contains four *rrn* operons (2, 4, 16). In PCR experiments carried out to determine the copy numbers of mutated *rrl* genes, one copy, named B, could not be amplified from five of our strains (Table 3). In silico analysis of strain N315 DNA, available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov), revealed that it also contained only five *rrl* copies and that the copy at a position similar to that of the B copy was missing. In every erythromycin-resistant strain, three or four copies were mutated, confirming that mutation in a majority of *rrl* operons was associated with significant resistance, as reported for *S. pneumoniae* (Table 3).

Resistance to streptogramins was observed in strain UCN15, which combined a *rplV* mutation with an A2058G mutation. Recently, resistance to quinupristin-dalfopristin in staphylococcal strains selected under treatment with this antibiotic has been explained by similar mutations in the conserved 3′ end of the *rplV* gene (11)).

Isolation of staphylococci with resistance to macrolides conferred by ribosomal mutations is unusual. It may be related to the specific context of cystic fibrosis. Half of 12 erythromycin-resistant strains of *S. aureus* isolated from cystic fibrosis patients in our institutions were mutants. Indeed, patients suffering from this genetic disorder receive multiple courses of antibiotics. In addition, administration of macrolides at low doses aimed at preventing *Pseudomonas* infections might favor the emergence of mutants, although no definitive relationship could be proven in this study. Finally, colonization of cystic fibrosis patients by hypermutable strains of *P. aeruginosa* has been reported (13). A similar possibility for the staphylococcal isolates is currently under investigation.

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