ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2005, p. 3586–3589 Vol. 49, No. 8
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.8.3586–3589.2005

Prevalence of Resistance Mechanisms against Macrolides and Lincosamides in Methicillin-Resistant Coagulase-Negative Staphylococci in the Czech Republic and Occurrence of an Undefined Mechanism of Resistance to Lincosamides

Gabriela Novotna,[1] Václava Adamkova,[2] Jiri Janata,[1]* Oto Melter,[3] and Jaroslav Spizek[1]

Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic[1]*; Third Faculty of Medicine, Charles University, Prague, Czech Republic*[2]*; and National Institute of Public Health, Prague, Czech Republic*[3]

Received 1 October 2004/Returned for modification 30 November 2004/Accepted 14 May 2005

High occurrence of the non-macrolide-lincosamide-streptogramin B resistance genes *msrA* (53%) and *linA*/*linA′* (30%) was found among 98 methicillin-resistant coagulase-negative staphylococci additionally resistant to macrolides and/or lincosamides. The gene *msrA* predominated in *Staphylococcus haemolyticus* (43 of 62 isolates). In *Staphylococcus epidermidis*, it was present in 7 of 27 isolates. A novel mechanism of resistance to lincosamides appears to be present in 10 genetically related isolates of *S. haemolyticus* in the absence of *ermA*, *ermC*, *msrA*, and *linA*/*linA′*.

Three basic mechanisms of resistance to macrolides, lincosamides, and streptogramin B (MLS_B) have been described for staphylococci. The first one is a cross-resistance to all three structurally different groups of antibiotics having similar effects on bacterial protein synthesis. The responsible genes, *ermA* and *ermC*, protecting the ribosome from the drug binding by 23S rRNA methylation, can be expressed constitutively or inducibly (25, 27). Due to the distinct predominance of the *erm* resistance mechanism in staphylococci (1, 13, 17, 21, 23, 26), resistances to macrolides, lincosamides, and streptogramin B form one MLS_B resistance group. In the second mechanism of antibiotic modification, cells harboring the *linA* gene (12) inactivate both lincomycin and clindamycin but resist high levels of lincomycin alone (L resistance). The occurrence of this resistance type remains quite low in staphylococci (11, 14). The third mechanism, a partial cross-resistance to 14- and 15-membered macrolides and streptogramin B (MS resistance), is the active efflux of antibiotics, which is conferred by the gene *msrA* (20). Although the early isolates of staphylococci with MS resistance (4, 9) and also with lincosamide-inactivating resistance (3, 8) originated from Eastern Europe, no detailed study on the distribution of these resistance mechanisms has so far been performed in the area. The goal of our study was to characterize the distribution of genes coding for resistance to macrolides and lincosamides in clinical isolates of methicillin-resistant coagulase-negative staphylococci (MRCoNS) resistant to at least one of the respective antibiotics.

A series of 919 isolates of coagulase-negative staphylococci (CoNS) were isolated during two periods in 1996 (March to June and September to November) in seven large (general) hospitals located throughout the Czech Republic. Single-patient isolates were mainly from superficial colonization or infection, respiratory tract specimens, pus, and blood. The isolates were placed in a mannitol salt agar medium and sent to the National Institute of Public Health in Prague. They were tested by the Api Staph (Biomerieux, Marcy l'Etoile, France), clumping factor, and coagulase production tests. Finally, 98 isolates, in particular, *Staphylococcus haemolyticus* ($n = 62$), *Staphylococcus epidermidis* ($n = 27$), *Staphylococcus hominis* ($n = 5$), *Staphylococcus capitis* ($n = 3$), and *Staphylococcus warneri* ($n = 1$), were selected by the disk diffusion method (19) for their cross-resistance to oxacillin and to at least one of the three antibiotics erythromycin, lincomycin, and clindamycin. The numbers of resistant strains obtained from each hospital are listed in Table 1. The most numerous species, *S. haemolyticus* and *S. epidermidis*, are listed in the table separately. Susceptibility to quinupristin-dalfopristin was additionally tested in all selected isolates. The phenotypic characterization was complemented by a modified triple disk induction test as described previously (10, 27). In the test, lincomycin and clindamycin disks were placed at the sides of an erythromycin disk 15 mm apart.

DNA for genetic analyses was extracted by using the simple salting-out procedure of Miller et al. (18). To ensure a complete lysis, the cells were incubated overnight in 5 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% glycine. For determining the resistance genotype, digoxigenin-labeled hybridization probes were synthesized by PCR using primers specific for *ermC* (5′-AGGTG TAATTTCGTAACTGC-3′ and 5′-GCAAACCCGTATTCC ACG-3′), *ermA* (5′-AAGCGGTAAACCCCTCTG-3′ and 5′-ATACTTTTGTAGTCCTTCTTT-3′), *msrA* (5′-GCAAATG GTGTAGGTAAG-3′ and 5′-ATCATGTGATGTAAACAA AAT-3′), and *linA*/*linA′* (5′-GTAGATGTATTAACTGGA A-3′ and 5′-GAAAAAGAAGTTGAGCTTC-3′). In the

* Corresponding author. Mailing address: Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, Prague 14220, Czechoslovakia. Phone: 0420241062370. E-mail: janata@biomed.cas.cz.

TABLE 1. Distribution of resistance genes *erm* (*ermA* or *ermC*), *msrA*, and *linA*/*linA′* among various MRCoNS isolated in seven Czech hospitals and correlation of resistance genotypes with phenotypes

Resistance genotype	No. of isolates with genotype														Total no. of isolates
	In hospital[a] (*S. haemolyticus*/*S. epidermidis*):							With phenotype[b]:							
	BB	KR	KV	OL	OV	PL	UL	ELC	ELiCi	ELCi	E	EL	L	LC	
erm		5 (2/2)	1 (0/1)	3 (1/2)	1 (0/1)	2 (2/0)	9 (2/6)	9	8	3			1		21
erm + *msrA*	4 (3/0)	1 (0/1)	2 (1/1)	4 (4/0)			1 (1/0)	4	7		1				12
erm + *linA*		1		2 (0/2)			2 (1/0)	2		3					5
erm + *msrA* + *linA*		2 (2/0)		1 (1/0)			1 (1/0)		1	2		1			4
msrA	2 (2/0)	3 (3/0)	2 (2/0)	7 (5/2)	3 (2/1)		4 (3/1)	1			18	2			21
msrA + *linA*	3 (3/0)	2 (1/0)	1 (1/0)	5 (4/1)	2 (2/0)	1 (1/0)	1 (1/0)	3			4	8			15
linA				1 (0/1)	1 (0/1)	1 (1/0)	2 (0/2)						5		5
No gene		4 (4/0)		3 (2/0)	1 (0/1)	2 (1/0)	5 (3/1)	1					1	13	15
Total	9 (8/0)	18 (12/3)	6 (4/2)	26 (17/8)	8 (4/4)	6 (5/0)	25 (12/10)	20	16	8	23	11	7	13	98

[a] BB, Brno hospital; KR and KV, Prague hospitals; OL, Olomouc hospital; OV, Ostrava hospital; PL, Plzen hospital; and UL, Usti nad Labem hospital.
[b] E, erythromycin; L, lincomycin; C, clindamycin; i, inducible.

labeling reaction, deoxynucleoside triphosphate was replaced by PCR DIG labeling mix (ROCHE, Mannheim, Germany) including DIG-11-dUTP. Accuracy of the probes was verified by DNA sequencing. Southern blots with DNA digested by EcoRI were hybridized under stringent conditions (hybridization at 68°C overnight in Standard hybridization buffer, post-hybridization washes twice with 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at room temperature and twice with 0.5× SSC at 68°C for 15 min) according to Roche Molecular Biochemicals (DIG Application Manual for Filter Hybridization, 2000). The DIG Luminescent Detection kit for nucleic acids (Roche, Mannheim, Germany) was used for detection. The DNA of clinical isolates, from which specific probes were synthesized, was positively detected in each blot as a control. Pulsed-field gel electrophoresis (PFGE) analysis of SmaI-digested DNA was performed using the CHEF 2015 Pulsaphor electrophoresis system (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described previously (2).

Out of 98 MRCoNS additionally resistant to at least one of the three antibiotics erythromycin, lincomycin, or clindamycin, the full cross-resistance occurred in only 20 strains. Even in the presence of an inducer (erythromycin), more than half of the tested isolates (54 of 98) were susceptible to at least one of the applied MLS antibiotics. All 98 isolates were susceptible to quinupristin-dalfopristin (combination of streptogramins A and B). The triple-disk induction test distinguished three basic (Fig. 1a to c) and two combined (Fig. 1d to e) resistance patterns between 78 erythromycin-resistant isolates. Surprisingly, the most common was the E phenotype (23 strains), which together with the EL phenotype (11 strains) indicated the presence of MS resistance.

Of 20 erythromycin-susceptible isolates, 7 were resistant to lincomycin only (Fig. 2a) and 12 isolates were resistant to both lincomycin and clindamycin (Fig. 2b). One isolate (not shown) exhibited intermediate resistance to only clindamycin. The phenotypic patterns indicated an unusual distribution of resistance determinants in the collection and occurrence of a new, so far undefined, resistance to lincosamides.

Genetic analysis confirmed a high occurrence of the non-MLS-type resistance genes *msrA* (53%) and *linA* (30%) as well as a frequent combination of two (33%) or three (4%) resistance genes (Table 1). The combination of genes *msrA* and *linA* partially mimicked the MLS resistance phenotype, conferring resistance to macrolides and lincomycin (and presumably to streptogramin B; not tested) but not to clindamycin. In consequence, the genetic analysis revealed a relatively high number of 41 strains (42%) susceptible to clindamycin. With regard to other studies on CoNS, the predominance of MS resistance and a high occurrence of L resistance were remarkable. Even though studies published on the distribution of MLS_B resistance genes in staphylococci are not fully compat-

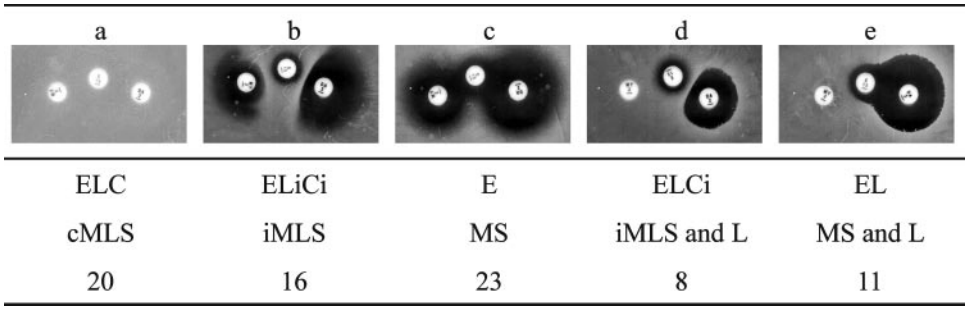

FIG. 1. Phenotypes identified by a triple-disk induction test in erythromycin-resistant strains. Disk order, from left: lincomycin, erythromycin, clindamycin. Upper row: phenotype description (E, erythromycin resistant; L, lincomycin resistant; C, clindamycin resistant; Li and Ci, resistant after induction by erythromycin). Middle row: resistance mechanism corresponding to the pattern. Bottom row: frequency of the phenotype. cMLS, constitutive MLS resistance; iMLS, inducible MLS resistance.

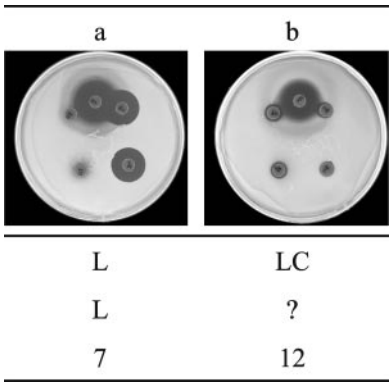

FIG. 2. Phenotypes identified by a triple-disk induction test in erythromycin-sensitive strains. Disk order from the left: top, lincomycin, erythromycin, clindamycin; bottom, lincomycin, clindamycin. Upper row: phenotype description (L, lincomycin resistant; C, clindamycin resistant). Middle row: resistance mechanism corresponding to the pattern. Bottom row: frequency of the phenotype.

ible with our collection, we unified the available data corresponding to this study in Table 2.

While *S. epidermidis* was the most frequent in the original set of CoNS, *S. haemolyticus* distinctly (63%) prevailed in the selective subset of 98 resistant isolates. The higher rate of resistance to erythromycin, methicillin, and other drugs for *S. haemolyticus* than for other CoNS was described previously (5, 7).

For *S. haemolyticus*, the *msrA* gene was present in 43 of 62 isolates (69%), followed by the *linA*/*linA′* (29%) and *erm* (27%) genes. Increased occurrence of MS resistance was reported for *S. haemolyticus* formerly (6, 14), whereas the *erm* mechanism was predominant in *S. epidermidis* (59%; 16 of 27 strains), followed by *msrA* (26%) and *linA* (11%) (Table 1). Even the lower frequency of *msrA* in *S. epidermidis* was still high compared to the data of Martineau et al. (16) and Fiebelkorn et al. (6), who reported only 8 of 142 and 11 of 68 strains, respectively.

The 43 isolates of *S. haemolyticus* harboring *msrA* constitute a relatively heterogeneous group in terms of the distribution of different resistance genotypes in each individual hospital as well as between different hospitals (Table 1). Accordingly, a variety of *msrA* hybridization patterns were observed. Among eight distinctive patterns, the most frequent was the 5-kb (±0.1) probe-hybridizing fragment detected in 16 strains, followed by a 7.2-kb (±0.1) fragment in 6 strains and two fragments of 4.6 kb (±0.1) and 7.7 kb (±0.1), both detected in five strains. Interestingly, these *msrA* hybridization pattern groups did not correspond to groups based on resistance genotypes. PFGE analysis confirmed the genetic heterogeneity among *msrA*-carrying *S. haemolyticus* isolates. Two isolates of each resistance genotype from each hospital were analyzed. Based on a comparison of SmaI restriction patterns, 25 different genotypes were discerned among 32 isolates. Out of these, 11 genotypes could be clustered into 3 groups. In the first group, four genotypes comprising six isolates differed from each other by six bands. In the second group, five isolates, each representing one genotype, differed from each other by five or six bands, and in the third group, two isolates differed by four bands. According to the criteria of Tenover et al. (24), isolates within these groups are possibly related.

The majority of the 15 isolates without any detected resistance gene displayed an unusual lincomycin-clindamycin resistance phenotype (13 isolates), indicating the presence of new resistance determinant(s) (Table 1). Ten of them formed a phenotypically homogeneous group of *S. haemolyticus*. Although isolated in five different hospitals, the strains are closely related because their PFGE patterns differed from each other by no more than three bands (24). We expect cross-resistance to streptogramin A in these strains, since similar types of resistance have already been found in *Enterococcus faecalis* and *Streptococcus agalactiae* (15, 22). A representative strain of lincosamide-resistant *S. haemolyticus* was deposited in the Czech Collection of Microorganisms (CCM 7296).

To sum up, we confirmed a high incidence of non-MLS-type resistance determinants among MRCoNS in the Czech Republic, which contrasts with the data from other regions. Experiments aiming at elucidating the mechanism of the unknown resistance to lincosamides are now under way.

TABLE 2. Comparison of relevant studies on distribution of resistance genes *erm*, *msrA*, and *linA*/*linA′* among clinical isolates of CoNS

Resistance phenotype[a]	Species (n^b)	% of isolates with:			Applied method	Reference
		erm genes	*msrA*	*linA*		
Met^s Met^r Ery^r	*S. epidermidis* (142)	94	6	ND	PCR	16[c]
Ery^r	*S. epidermidis* (92), *S. haemolyticus* (22), *S. hominis* (22), other CoNS (36)	63	41	ND	Dot blot hybridization	4[c]
Met^s, resistant to at least one of the following: ERY, LIN, PRI, Q/D, Q and D	CoNS (41)	73	20	2	PCR	14[c]
Met^r, resistant to at least one of the: ERY, LIN, PRI, Q/D, Q and D	CoNS (109)	82	15	5	PCR	14[c]
Met^r, resistant to at least one of the following: ERY, LIN, CLI	*S. epidermidis* (27), *S. haemolyticus* (62), *S. hominis* (5), other CoNS (4)	43	53	24	Southern blot hybridization	This paper

[a] MET, methicillin; ERY, erythromycin; LIN, lincomycin; CLI, clindamycin; PRI, pristinamycin; Q/D, quinupristin/dalfopristin; Q, quinupristin; D, dalfopristin.
[b] *n*, no. of isolates.
[c] From the published data, only isolates with comparable characteristics are presented.

This study was supported by the Czech Science Foundation (grant no. 204/04/0801) and the Grant Agency of the Academy of Sciences (grant no. IAA600200519).

We are grateful to Juri Schindler for providing access to a large collection of staphylococcal strains and to Petr Petras for consultations. We thank Marketa Mareckova and Jan Kopecky for carefully reading the manuscript.

REFERENCES

1. **Almer, L. S., V. D. Shortridge, A. M. Nilius, J. M. Beyer, N. B. Soni, M. H. Bui, G. G. Stone, and R. K. Flamm.** 2002. Antimicrobial susceptibility and molecular characterization of community-acquired methicillin-resistant *Staphylococcus aureus*. Diagn. Microbiol. Infect. Dis. **43:**225–232.
2. **Chung, M., H. de Lencastre, P. Matthews, A. Tomasz, I. Adamsson, M. Aires de Sousa, T. Camou, C. Cocuzza, A. Corso, I. Couto, A. Dominguez, M. Gniadkowski, R. Goering, A. Gomes, K. Kikuchi, A. Marchese, R. Mato, O. Melter, D. Oliveira, R. Palacio, R. Sa-Leao, I. Santos Sanches, J. H. Song, P. T. Tassios, and P. Villari.** 2000. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. Microb. Drug Resist. **6:**189–198.
3. **Devriese, L. A.** 1980. Two new types of resistance to lincomycin in pathogenic staphylococci from animals. Ann. Microbiol. (Paris) **131B:**261–266.
4. **Eady, E. A., J. I. Ross, J. L. Tipper, C. E. Walters, J. H. Cove, and W. C. Noble.** 1993. Distribution of genes encoding erythromycin ribosomal methylases and an erythromycin efflux pump in epidemiologically distinct groups of staphylococci. J. Antimicrob. Chemother. **31:**211–217.
5. **Ferreira, R. B., A. P. Nunes, V. M. Kokis, N. Krepsky, S. Fonseca Lde, C. Bastos Mdo, M. Giambiagi-deMarval, and K. R. Santos.** 2002. Simultaneous detection of the mecA and ileS-2 genes in coagulase-negative staphylococci isolated from Brazilian hospitals by multiplex PCR. Diagn. Microbiol. Infect. Dis. **42:**205–212.
6. **Fiebelkorn, K. R., S. A. Crawford, M. L. McElmeel, and J. H. Jorgensen.** 2003. Practical disk diffusion method for detection of inducible clindamycin resistance in *Staphylococcus aureus* and coagulase-negative staphylococci. J. Clin. Microbiol. **41:**4740–4744.
7. **Froggatt, J. W., J. L. Johnston, D. W. Galetto, and G. L. Archer.** 1989. Antimicrobial resistance in nosocomial isolates of *Staphylococcus haemolyticus*. Antimicrob Agents Chemother. **33:**460–466.
8. **Hajek, V.** 1976. *Staphylococcus intermedius*, a new species isolated from animals. Int. J. Syst. Bacteriol. **26:**401–408.
9. **Janosi, L., and E. Ban.** 1982. Localisation of the gene(s) determining inducible type macrolide and streptogramin B resistance on the penicillinase plasmid of isolates of an epidemic *Staphylococcus aureus* strain, p. 918–919. *In* P. Periti and G. G. Grassi (ed.), Current chemotherapy and immunotherapy: proceedings of the 12th International Congress of Chemotherapy, vol. 2. American Society for Microbiology, Washington, D.C.
10. **Jenssen, W. D., S. Thakker-Varia, D. T. Dubin, and M. P. Weinstein.** 1987. Prevalence of macrolides-lincosamides-streptogramin B resistance and *erm* gene classes among clinical strains of staphylococci and streptococci. Antimicrob. Agents Chemother. **31:**883–888.
11. **Leclercq, R., A. Brisson-Noel, J. Duval, and P. Courvalin.** 1987. Phenotypic expression and genetic heterogeneity of lincosamide inactivation in *Staphylococcus* spp. Antimicrob. Agents Chemother. **31:**1887–1891.
12. **Leclercq, R., C. Carlier, J. Duval, and P. Courvalin.** 1985. Plasmid-mediated resistance to lincomycin by inactivation in *Staphylococcus haemolyticus*. Antimicrob. Agents Chemother. **28:**421–424.
13. **Lim, J. A., A. R. Kwon, S. K. Kim, Y. Chong, K. Lee, and E. C. Choi.** 2002. Prevalence of resistance to macrolide, lincosamide and streptogramin antibiotics in Gram-positive cocci isolated in a Korean hospital. J. Antimicrob. Chemother. **49:**489–495.
14. **Lina, G., A. Quaglia, M. E. Reverdy, R. Leclercq, F. Vandenesch, and J. Etienne.** 1999. Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. Antimicrob. Agents Chemother. **43:**1062–1066.
15. **Malbruny, B., A. M. Werno, T. P. Anderson, D. R. Murdoch, and R. Leclercq.** 2004. A new phenotype of resistance to lincosamide and streptogramin A-type antibiotics in *Streptococcus agalactiae* in New Zealand. J. Antimicrob. Chemother. **54:**1040–1044.
16. **Martineau, F., F. J. Picard, N. Lansac, C. Menard, P. H. Roy, M. Ouellette, and M. G. Bergeron.** 2000. Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **44:**231–238.
17. **Melter, O., M. Aires de Sousa, P. Urbaskova, V. Jakubu, H. Zemlickova, and H. de Lencastre.** 2003. Update on the major clonal types of methicillin-resistant *Staphylococcus aureus* in the Czech Republic. J. Clin. Microbiol. **41:**4998–5005.
18. **Miller, S. A., D. D. Dykes, and H. F. Polesky.** 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. **16:**1215.
19. **National Committee for Clinical Laboratory Standards.** 1995. Performance standards for antimicrobial disk susceptibility tests. National Committee for Clinical Laboratory Standards, Villanova Pa.
20. **Ross, J. I., E. A. Eady, J. H. Cove, W. J. Cunliffe, S. Baumberg, and J. C. Wootton.** 1990. Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport super-gene family. Mol. Microbiol. **4:**1207–1214.
21. **Schmitz, F. J., R. Sadurski, A. Kray, M. Boos, R. Geisel, K. Kohrer, J. Verhoef, and A. C. Fluit.** 2000. Prevalence of macrolide-resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European university hospitals. J. Antimicrob. Chemother. **45:**891–894.
22. **Singh, K. V., G. M. Weinstock, and B. E. Murray.** 2002. An *Enterococcus faecalis* ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. Antimicrob. Agents Chemother. **46:**1845–1850.
23. **Spiliopoulou, I., E. Petinaki, P. Papandreou, and G. Dimitracopoulos.** 2004. erm(C) is the predominant genetic determinant for the expression of resistance to macrolides among methicillin-resistant *Staphylococcus aureus* clinical isolates in Greece. J. Antimicrob. Chemother. **53:**814–817.
24. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
25. **Thakker-Varia, S., A. C. Ranzini, and D. T. Dubin.** 1985. Ribosomal RNA methylation in *Staphylococcus aureus* and *Escherichia coli*: effect of the "MLS" (erythromycin resistance) methylase. Plasmid **14:**152–161.
26. **Tunckanat, F., and S. Arikan.** 2000. Phenotypes of staphylococcal resistance to macrolides, lincosamides and streptogramin B (MLS) in a Turkish university hospital. Zentbl. Bakteriol. **289:**827–833.
27. **Weisblum, B., and V. Demohn.** 1969. Erythromycin-inducible resistance in *Staphylococcus aureus:* survey of antibiotic classes involved. J. Bacteriol. **98:**447–452.