ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1990, p. 985–988
0066-4804/90/060985-04$02.00/0

Vol. 34, No. 6

High-Level Chromosomal Gentamicin Resistance in *Streptococcus agalactiae* (Group B)

ANNIE BUU-HOÏ,[1] CHANTAL LE BOUGUENEC,[2]† AND THEA HORAUD[2]*

Centre Hospitalo-Universitaire, Broussais-Hôtel Dieu, Paris,[1] and Laboratoire des Staphylocoques et des Streptocoques, Institut Pasteur, 75724 Paris Cedex 15,[2] France

Received 8 November 1989/Accepted 21 March 1990

This is the first report of high-level gentamicin resistance in a group B streptococcus. Strain B128 of serotype II was isolated from an infected leg wound in 1987. B128 was resistant to high levels of gentamicin as well as of all other available aminoglycosides and was also resistant to tetracyclines. No bactericidal synergism was found between ampicillin or vancomycin and any of these aminoglycosides. Gentamicin, kanamycin, streptomycin, and tetracycline resistance determinants transferred by conjugation into a plasmid-free group B streptococcus recipient at a frequency of 10^{-8} to 10^{-9} transconjugants per donor cell. No transconjugants were detected when streptococci of groups A, C, and G, *Streptococcus sanguis*, or *Enterococcus faecalis* was used as a recipient. No plasmids were detected in B128 or in any of the four transconjugants tested. By DNA-DNA hybridization, homology was detected between gene *aac6/aph2*, of *E. faecalis* origin, and a 2.4-kilobase *Hin*dIII chromosomal fragment of B128; homology to the genes *aph3* and *aadE*, of *E. faecalis* origin, was found with *Hin*dIII chromosomal fragments of the same size (3.0 kilobases). Strains like B128, which potentially can be responsible for severe neonatal infections, are of great clinical concern, since there are to date no antibiotic combinations exhibiting bactericidal synergism against them.

During the last two decades, group B streptococci (GBS) have become the most frequent cause of life-threatening infections in newborns; the diseases this organisms cause, particularly septicemia and meningitis, are associated with a high mortality rate (1). Severe GBS infections also occur in adults in two distinct populations: young healthy females in which GBS are a complication of pregnancy, abortion, or the postpartum period (28) and elderly and immunocompromised patients with serious underlying diseases (25). Although uniformly susceptible to beta-lactam antibiotics, GBS require higher concentrations of these antibiotics for growth inhibition and are killed at much lower rates than are streptococci of group A, C, or G (5). As for other streptococcal infections, penicillin G is the antibiotic of choice for the current treatment of GBS infections. However, the use of penicillin G alone is not indicated when the GBS strain is penicillin G tolerant (18) or when it causes septicemia and meningitis in neonates. In the latter cases, it is now known that the number of bacteria per milliliter (10^6 to 10^8) found in vivo is higher than the number of bacteria per milliliter (10^5) used in vitro to evaluate a standard MIC of penicillin G. If one increases the inoculum in an in vitro test from 10^5 to 10^8 bacteria per ml, the MIC of penicillin G increases from 0.03 to 0.1 μg/ml to 2 to 4 μg/ml (1). Therefore, penicillin G alone cannot be the recommended therapy for severe GBS neonatal infections. Antibiotic combinations of penicillin G or ampicillin with an aminoglycoside usually demonstrate in vitro synergism against GBS, as they do against a variety of other streptococci and enterococci (1, 5). High-level aminoglycoside resistance in enterococci and streptococci has been shown to confer resistance to bactericidal synergism between aminoglycosides and antibiotics, such as penicillins or vancomycin, that act on the cell wall (33). The combination of ampicillin plus gentamicin is the current treatment in France for serious GBS infections in newborns.

For the last 10 years, all streptococcal and enterococcal clinical isolates received by our laboratory have been routinely tested for high-level resistance to gentamicin, kanamycin, and streptomycin (10): more than 10% of GBS are highly resistant to streptomycin and kanamycin-neomycin, and about 50% of *Enterococcus faecalis* strains are resistant to different aminoglycosides, including 5 to 8% of *E. faecalis* isolates which are highly resistant to gentamicin. High-level resistance to gentamicin in GBS has not yet been reported.

GBS strain B128, highly resistant to gentamicin as well as to all other clinically available aminoglycosides, was recently isolated at Broussais Hospital. The purpose of the present study was to localize and identify the aminoglycoside resistance genes of this strain. B128 was investigated with respect to the conjugative transfer of its antibiotic resistance markers and to the presence of plasmids. DNA-DNA hybridization experiments were performed with probes bearing aminoglycoside resistance genes originating from different plasmids harbored by *E. faecalis* strains.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. In addition, strain B96(pIP501) (11) was used as a control. Serogroup and serotype were determined by the methods of Fuller (9) and Lancefield (19), respectively. The plasmid-free recipient strains used in the mating experiments were BM137 (16), BM132 (14), BM138 (16), BM140 (16), BM90 (27), and JH2-2 (17), which represent group A, B, C, and G streptococci, *Streptococcus sanguis* (strain Challis), and *E. faecalis*, respectively. All the recipient strains were resistant to both rifampin and fusidic acid.

Media. Media and growth conditions have been described previously (11).

Determination of antibiotic susceptibility. The disk diffusion method was performed on Mueller-Hinton agar (Diagnostics Pasteur, Marne-la-Coquette, France) supplemented

* Corresponding author.
† Present address: Unité des Entérobactéries, Institut Pasteur, Paris, France.

TABLE 1. Bacterial strains and DNA-DNA hybridization results

Strain (origin or reference)	Antibiotic resistance markers[a]	Size (kb) of *Hin*dIII fragments hybridizing with aminoglycoside resistance gene[b]:		
		aac6/aph2	*aph3*	*aadE*
B128 (Leg wound)	Ak^r Gm^r Km^r Nm^r Nt^r Sm^r Tm^r Tc^r Mn^r	2.4	3.0	3.0
BM132 (14)	Fus^r Rif^r	—[c]	—	—
BM5236 (B128 × BM132)	Ak^r Km^r Nm^r Sm^r Fus^r Rif^r	—	3.0	3.0
BM5237 (B128 × BM132)	Ak^r Gm^r Km^r Nt^r Tm^r Tc^r Mn^r Fus^r Rif^r	2.4	—	—
BM5238 (B128 × BM132)	Ak^r Gm^r Km^r Nm^r Nt^r Sm^r Tm^r Fus^r Rif^r	2.4	3.0	3.0
BM5239 (B128 × BM132)	Ak^r Gm^r Km^r Nm^r Nt^r Sm^r Tm^r Fus^r Rif^r	2.4	3.0	3.0

[a] Ak^r, High-level amikacin resistance; Gm^r, high-level gentamicin resistance; Km^r, high-level kanamycin resistance; Nm^r, high-level neomycin resistance; Nt^r, high-level netilmicin resistance; Sm^r, high-level streptomycin resistance; Tm^r, high-level tobramycin resistance; Tc^r Mn^r, tetracycline-minocycline resistance; Fus^r and Rif^r, resistance to fusidic acid and rifampin, respectively (Fus^r and Rif^r are chromosomal markers obtained by mutation and carried by BM132).

[b] *aac6/aph2*, Gene encoding gentamicin-kanamycin resistance; *aph3*, gene encoding kanamycin-neomycin resistance; *aadE*, gene encoding streptomycin resistance.

[c] —, No homology detected.

with 5% horse blood by using different disks (Diagnostics Pasteur). Resistance to aminoglycosides was tested with overloaded disks of streptomycin, gentamicin, and kanamycin containing 500, 250, and 1,000 μg per disk, respectively. The MICs of different aminoglycosides were determined by the agar dilution method as described by Chabbert (4) on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% horse serum and serial dilutions of antibiotics.

Mating experiments. Matings were performed on membrane filters (HAEP, 0.45-μm pore diameter, 47mm; Millipore Corp., Bedford, Mass.) as described earlier (3). For counterselection of the donor, we used fusidic acid and rifampin, and for selection of transconjugants, we used gentamicin, kanamycin, streptomycin, and tetracycline (final concentrations, 25, 100, 1,000, 2,000, 1,000, and 4 μg/ml, respectively).

DNA isolation. DNA was extracted from B128, from its corresponding transconjugants, and from B96 by ultracentrifugation in dye-buoyant density gradients as described earlier (21). Plasmid DNA from *Escherichia coli* strains was isolated as described by Birnboim and Doly (2).

DNA analysis. Chromosomal DNA was digested by the restriction endonuclease *Hin*dIII (Amersham International, Little Chalfont, England). Electrophoresis of digested chromosomal DNA was carried out on horizontal submerged agarose gels (0.7%; Sigma Chemical Co., St. Louis, Mo.). The molecular sizes of linear DNA fragments were calculated with λDNA doubly digested by *Hin*dIII and *Eco*RI (Amersham International) and a 1-kilobase (kb) DNA ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as molecular size markers.

Probes for DNA-DNA hybridization. The following plasmids were used as probes in hybridization experiments: (i) pSF815A (7) (*aac6/aph2* probe), which carries the gene specifying the bifunctional enzyme 6′-aminoglycoside acetyltransferase/2″-aminoglycoside phosphotransferase; (ii) pAT93 (30) (*aph3* probe), which carries the gene specifying the 3′,5″-aminoglycoside phosphotransferase type III; and (iii) an MP13mp18 Ω 467-base-pair *Hpa*II fragment from pJH1 (26) (*aadE* probe), which carries the gene specifying the 6-streptomycin adenylyltransferase.

DNA blotting and hybridization. DNA was transferred from agarose gels to nitrocellulose filters by the bidirectional method described by Smith and Summers (29). The probes were labeled with [α-^{32}P]dCTP by using the Multiprime DNA labeling system (Amersham International). Hybridization was carried out under stringent conditions (65°C) as reported previously (21). The radioactivity of the probe (specific activity, about 10^9 cpm/μg) corresponded to approximately 10^7 cpm per filter.

RESULTS AND DISCUSSION

Wild-type strain description. B128 was isolated in 1987 at Broussais Hospital from pus recovered from an adult patient with an infected leg wound. B128 was initially identified as a beta-hemolytic group B *Streptococcus* of serotype II; after several subcultures in broth this strain became nonhemolytic. B128 was resistant to high levels of gentamicin and of all other clinically available aminoglycosides and was also resistant to tetracycline-minocycline (Table 1).

The MICs of gentamicin, netilmicin, tobramycin, kanamycin, lividomycin A, neomycin, ribostamycin, amikacin, and streptomycin were >16,000, 1,000, >2,000, >32,000, >2,000, >2,000, >2,000, 2,000, and >128,000 μg/ml, respectively.

Mating experiments and plasmid isolation. B128 was mated with BM137, BM132, BM138, BM140, BM90, and JH2-2. No transfer was observed with any of these recipients, except BM132. Antibiotic resistance determinants transferred into BM132 at a frequency of 3×10^{-9}, 1×10^{-8}, 7×10^{-9}, or 2×10^{-8} transconjugants per donor cell when selection was done with gentamicin, kanamycin, streptomycin, or tetracycline, respectively. Two hundred ten transconjugants were analyzed by replica plating. Eighty of these clones carried only the Tc^r-Mn^r marker, 101 clones carried the same antibiotic resistance markers as B128, and 27 clones which were susceptible to tetracycline-minocycline had an aminoglycoside resistance pattern identical to that of B128. The two remaining clones had aminoglycoside resistance profiles different from that of B128: BM5236 was resistant to kanamycin-neomycin and streptomycin, and BM5237 was resistant to gentamicin-kanamycin (as well as to tetracycline-minocycline) (Table 1).

All attempts to detect plasmids either in B128 or in the four transconjugant clones tested, BM5236, BM5237, BM5238, and BM5239 (Table 1), were unsuccessful, unlike the current isolation of pIP501 from B96. Chromosomal location and low frequency of transfer of antibiotic resistance determinants are often associated in streptococci (3, 12, 15).

Gene identification. DNA-DNA hybridization experiments were carried out between *Hin*dIII-digested chromosomal DNAs of the strains listed in Table 1 and the probes described in Materials and Methods. B128, BM5238, and

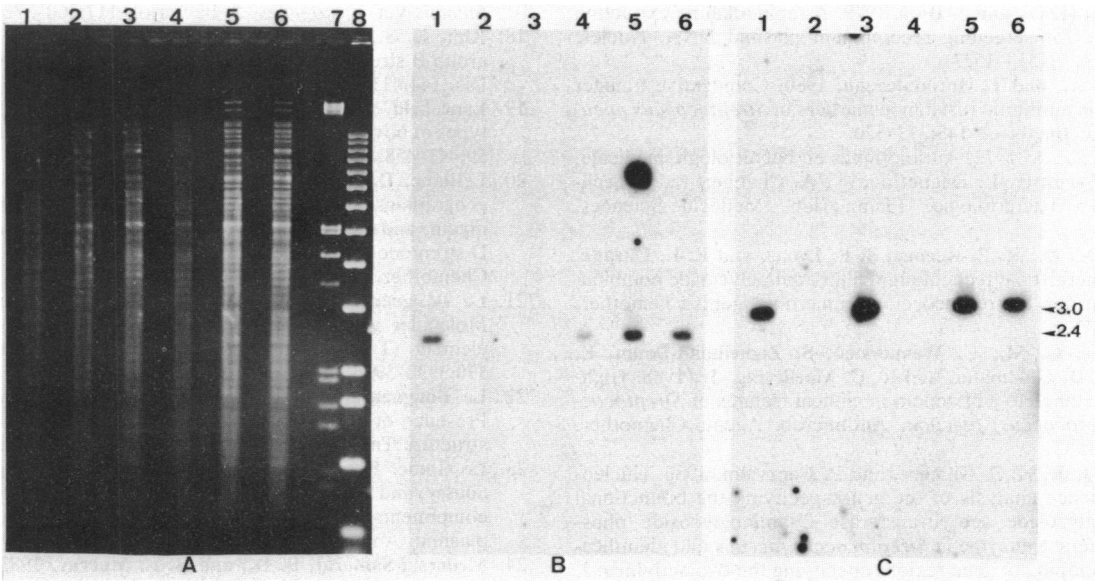

FIG. 1. Hybridization of chromosomal DNA with aminoglycoside resistance genes. (A) Electrophoretic pattern of *Hin*dIII-digested DNAs. Lanes: 1, B128; 2, BM132;3, BM5236; 4, BM5237; 5, BM5238; 6, BM5239; 7, λ fragments obtained by double digestion with *Eco*RI and *Hin*dIII; 8, 1-kb ladder. λ fragments and the 1-kb ladder were used as molecular size markers. (B and C) Same DNAs as in panel A but transferred to a nitrocellulose filter and probed with α-^{32}P-labeled pSF815A (encoding resistance to gentamicin-kanamycin) (B) and pAT93 (encoding resistance to kanamycin-neomycin) (C). Molecular sizes (in kilobases) of the hybridizing fragments are indicated on the right.

BM5239 carried the three genes, *aac6/aph2*, *aph3*, and *aadE*, whereas BM5236 carried only *aph3* and *aadE* and BM5237 carried only *aac6/aph2* (Table 1 and Fig. 1). Gene *aac6/aph2* was located in each strain on a *Hin*dIII fragment of 2.4 kb, while the other two genes were located on *Hin*dIII fragments of the same size (3.0 kb). That the sizes of the fragments hybridizing with both *aph3* and *aadE* were the same in B128 and in the corresponding transconjugants would suggest that these two genes are linked. The genetic analysis of B128 transconjugants favors this hypothesis, since none of the 129 clones resistant to kanamycin-neomycin and streptomycin carried either of these determinants without the other. The analysis of transconjugants resistant to kanamycin-neomycin and streptomycin and obtained previously from other streptococci (groups A, B, and G, *Streptococcus bovis*, and viridans group streptococci) (15), as well as the study of the locations of *aph3* and *aadE* on plasmids harbored by *E. faecalis* strains (20), further supports the notion that *aph3* and *aadE* are linked genes.

High-level gentamicin resistance is reported here for the first time to be chromosome borne in a streptococcal strain. The genetic analysis of the transconjugants obtained from B128 suggests that B128 carried three distinct chromosomal conjugative elements encoding resistance to tetracycline-minocycline, gentamicin-kanamycin, and kanamycin-neomycin and streptomycin. The presence of at least two chromosomal elements carried by one strain has been reported recently in streptococci of groups A, B, C, and G (22). The nature of the elements carried by B128 is under study in our laboratory.

Since the initial report of resistance to high levels of gentamicin and related aminoglycosides carried by plasmids in *E. faecalis* (13), plasmid-borne resistance to these antibiotic has been reported in other *E. faecalis* strains (24, 32) as well as, more recently, in *Enterococcus faecium* (6). Most of these strains, like B128, are also resistant to high levels of streptomycin and, consequently, the high-level aminoglycoside resistance, either plasmid or chromosome borne, confers resistance to bactericidal synergism between all available aminoglycosides and antibiotics which inhibit cell wall synthesis, such as beta-lactams and vancomycin. The high-level gentamicin resistance in B128 may be mediated by the bifunctional enzyme 6′-aminoglycoside acetyltransferase/2″-aminoglycoside phosphotransferase, which has previously been detected in gentamicin-resistant strains of *E. faecalis* (7), *E. faecium* (6), *Staphylococcus aureus* (23, 31), *Staphylococcus epidermidis* (31), and *Staphylococcus haemolyticus* (8).

GBS are important human pathogens, responsible for severe early-onset and nosocomial late-onset newborn diseases. In diseases which require bactericidal therapy, such as neonatal infections and enterococcal subacute endocarditis, the treatment of choice is the combination of gentamicin with an active agent against the cell wall. As expected, combinations of ampicillin or vancomycin with gentamicin, netilmicin, tobramycin, amikacin, kanamycin, or streptomycin failed to show bactericidal synergism against B128 (data not shown). Therefore, the emergence of GBS like B128 is an event of great clinical concern, since there are to date no antibiotic combinations exhibiting bactericidal synergism against such strains.

ACKNOWLEDGMENTS

We thank Karen Pepper for criticism of the manuscript, Gilda de Cespédès for technical assistance, and Odette Rouelland for secretarial assistance. We are grateful to J. Ferretti for pSF815A and to P. Courvalin for pAT93 and for the M13mp18 Ω 467-base-pair *Hpa*II fragment.

This work was supported by grant 873008 (Molecular Biology of Streptococci and Enterococci: Mobile Genetic Elements and Virulence Factors) from the Institut National de la Santé et de la Recherche Médicale to T.H.

LITERATURE CITED

1. **Backer, C. J.** 1977. From the National Institutes of Health: summary of the workshop on perinatal infections due to group B *Streptococcus*. J. Infect. Dis. **136**:137–152.

2. **Birnboim, H. L., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Buu-Hoï, A., and T. Horodniceanu.** 1980. Conjugative transfer of multiple antibiotic resistance markers in *Streptococcus pneumoniae*. J. Bacteriol. **143**:313–320.
4. **Chabbert, Y. A.** 1972. Antibiotiques en bactériologie médicale, p. 152–154. *In* G. L. Daguet and Y. A. Chabbert (ed.), Techniques en bactériologie. Flammarion, Médecine-Sciences, Paris.
5. **Cooper, M. D., R. E. Keeney, S. F. Lyons, and E. L. Cheatle.** 1979. Synergistic effects of ampicillin-aminoglycoside combinations on group B streptococci. Antimicrob. Agents Chemother. **15**:484–486.
6. **Eliopoulos, G. M., C. Wennersten, S. Zighelboim-Daum, E. Reiszner, D. Goldmann, and R. C. Moellering, Jr.** 1988. High-level resistance to gentamicin in clinical isolates of *Streptococcus* (*Enterococcus*) *faecium*. Antimicrob. Agents Chemother. **32**:1528–1532.
7. **Ferretti, J. J., K. S. Gilmore, and P. Courvalin.** 1986. Nucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase 2″-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J. Bacteriol. **167**:631–638.
8. **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.
9. **Fuller, A. T.** 1938. The formamide method for the extraction of polysaccharides from hemolytic streptococci. Br. J. Exp. Pathol. **19**:130–139.
10. **Horaud, T., and F. Delbos.** 1987. *Streptococcaceae* and antibiotics (a review). Lett. Infect. **2**:595–604.
11. **Horodniceanu, T., D. H. Bouanchaud, G. Bieth, and Y. A. Chabbert.** 1976. R plasmids in *Streptococcus agalactiae* (group B). Antimicrob. Agents Chemother. **10**:795–801.
12. **Horodniceanu, T., L. Bougueleret, and G. Bieth.** 1981. Conjugative transfer of multiple-antibiotic resistance markers in beta-hemolytic group A, B, F, and G streptococci in the absence of extrachromosomal deoxyribonucleic acid. Plasmid **5**:127–137.
13. **Horodniceanu, T., L. Bougueleret, N. El Solh, G. Bieth, and F. Delbos.** 1979. High-level, plasmid-borne resistance to gentamicin in *Streptococcus faecalis* subsp. *zymogenes*. Antimicrob. Agents Chemother. **16**:686–689.
14. **Horodniceanu, T., L. Bougueleret, N. El Solh, D. H. Bouanchaud, and Y. A. Chabbert.** 1979. Conjugative R plasmids in *Streptococcus agalactiae* (group B). Plasmid **2**:197–206.
15. **Horodniceanu, T., A. Buu-Hoï, F. Delbos, and G. Bieth.** 1982. High-level aminoglycoside resistance in group A, B, G, D (*Streptococcus bovis*), and viridans streptococci. Antimicrob. Agents Chemother. **21**:176–179.
16. **Horodniceanu, T., A. Buu-Hoï, C. Le Bouguénec, and G. Bieth.** 1982. Narrow host range of some streptococcal R plasmids. Plasmid **8**:199–206.
17. **Jacob, A. E., and S. J. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J. Bacteriol. **117**:360–372.
18. **Kim, K. S., and B. F. Anthony.** 1981. Penicillin tolerance in group B streptococci isolated from infected neonates. J. Infect. Dis. **144**:411–419.
19. **Lancefield, R. C.** 1934. Serological differentiation of specific types of bovine haemolytic streptococci (group B). J. Exp. Med. **59**:441–458.
20. **LeBlanc, D. J., J. M. Inamine, and L. N. Lee.** 1986. Broad geographical distribution of homologous erythromycin, kanamycin, and streptomycin resistance determinants among group D streptococci of human and animal origin. Antimicrob. Agents Chemother. **29**:549–555.
21. **Le Bouguénec, C., G. de Cespédès, and T. Horaud.** 1988. Molecular analysis of a composite chromosomal conjugative element (Tn*3701*) of *Streptococcus pyogenes*. J. Bacteriol. **170**:3930–3936.
22. **Le Bouguénec, C., G. de Cespédès, and T. Horaud.** 1990. Presence of chromosomal elements resembling the composite structure Tn*3701* in streptococci. J. Bacteriol. **172**:727–734.
23. **Le Goffic, F., A. Martel, N. Moreau, M. L. Capmau, C. J. Soussy, and J. Duval.** 1977. 2″-*O*-Phosphorylation of gentamicin components by a *Staphylococcus aureus* strain carrying a plasmid. Antimicrob. Agents Chemother. **12**:26–30.
24. **Mederski-Samoraj, B. D., and B. E. Murray.** 1983. High-level resistance to gentamicin in clinical isolates of enterococci. J. Infect. Dis. **147**:751–757.
25. **Opal, S. M., A. C. Cross, M. Palmer, and R. Almazan.** 1988. Group B streptococcal sepsis in adults and infants. Contrasts and comparisons. Arch. Intern. Med. **148**:641–645.
26. **Ounissi, H., and P. Courvalin.** 1987. Nucleotide sequences of streptococcal genes (appendix B), p. 275. *In* J. J. Ferretti and R. Curtiss III. (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
27. **Pepper, K., T. Horaud, C. Le Bouguénec, and G. de Cespédès.** 1987. Location of antibiotic resistance markers in clinical isolates of *Enterococcus faecalis* with similar antibiotypes. Antimicrob. Agents Chemother. **31**:1394–1402.
28. **Sexton, D. J., S. G. Rockson, R. E. Hempling, and C. W. Cathey.** 1985. Pregnancy-associated group B streptococcal endocarditis: a report of two fatal cases. Obstet. Gynecol. **66**(Suppl.):44S–47S.
29. **Smith, G. E., and M. D. Summers.** 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem. **109**:123–139.
30. **Trieu-Cuot, P., and P. Courvalin.** 1983. Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3′*5*″-aminoglycoside phosphotransferase type III. Gene **23**:331–341.
31. **Ubukata, K., N. Yamashita, A. Gotoh, and M. Konno.** 1984. Purification and characterization of aminoglycoside-modifying enzymes from *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **25**:754–759.
32. **Zervos, M. J., T. S. Mikesell, and D. R. Schaberg.** 1986. Heterogeneity of plasmids determining high-level resistance to gentamicin in clinical isolates of *Streptococcus faecalis*. Antimicrob. Agents Chemother. **30**:78–81.
33. **Zimmerman, R. A., R. C. Moellering, Jr., and A. N. Weinberg.** 1971. Enterococcal resistance to antibiotic synergism, p. 517–521. Antimicrob. Agents Chemother. 1970.