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Outer Membrane Permeability in *Pseudomonas cepacia*: Diminished Porin Content in a β-Lactam-Resistant Mutant and in Resistant Cystic Fibrosis Isolates

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Since β-lactam resistance is a feature of *Pseudomonas cepacia* isolates causing pulmonary infections in cystic fibrosis (CF), this study was undertaken to determine whether alterations in β-lactam permeability mediate drug resistance in this species. A β-lactam-susceptible non-CF isolate (strain 75-26), a resistant mutant derived from 75-26 by selection for cross-resistance to ciprofloxacin and ceftazidime, and two resistant CF isolates of *P. cepacia* were used. Permeability constants were calculated from the rate of nitrocefin hydrolysis in intact bacterial cells. Qualitative changes in outer membrane proteins were determined electrophoretically. The permeability constants of the mutant and the resistant CF isolates were lower than the value for the reference strain, 75-26. Whereas the lipopolysaccharide side chains were present in the test and reference strains, the resistant mutant and the CF isolates contained reduced amounts of the 36-kilodalton (kDa) outer membrane protein and failed to express the 27-kDa outer membrane protein. These observations suggest that the 27-kDa outer membrane protein may be a major porin or a major protein component of the porin complex in *P. cepacia* and that decreased expression of the 36-kDa outer membrane and loss of the 27-kDa porin are associated with high-level β-lactam resistance in some CF isolates of *P. cepacia*.

Pseudomonas cepacia has emerged as a significant cause of pulmonary infections among children with cystic fibrosis (CF) (14, 23). Most CF sputum isolates of *P. cepacia* are β-lactam resistant (3, 9, 15); furthermore, significantly more CF isolates than non-CF isolates of *P. cepacia* are resistant to ceftazidime, the most active agent against this species (4). The mechanism(s) that mediates high-level β-lactam resistance in CF isolates of *P. cepacia* is not known. Most CF and non-CF isolates of *P. cepacia* contain a chromosomal β-lactamase which focuses on isoelectric gels in the pH 7.9 to 8.1 range (4). A resistant mutant, developed from a β-lactam-susceptible parental strain of *P. cepacia* by selection on ceftazidime-containing agar, was highly resistant to piperacillin and ceftazidime and produced 40 times more chromosomal β-lactamase than did the drug-susceptible parent (2). Among 13 CF isolates of *P. cepacia*, moderate increases in base-line chromosomal β-lactamase content far below the levels observed in the derepressed mutant were associated with low-level piperacillin resistance only. Nevertheless, 12 of these strains were resistant to ceftazidime (8). These observations suggest that although derepressed production of chromosomal β-lactamase in *P. cepacia* can mediate high-level β-lactam resistance, this mechanism does not appear frequently in resistant CF isolates of this organism.

In a resistant mutant of *Pseudomonas aeruginosa*, loss of the outer membrane porin (F protein) decreased passive diffusion of β-lactam antibiotics into the bacterial cell and resulted in drug resistance (17). Since either of two *P. cepacia* outer membrane proteins may function as a porin (19), this study was undertaken to determine whether decreased expression of either protein mediated high-level β-lactam resistance in *P. cepacia*.

MATERIALS AND METHODS

Test strains. Four test strains of *P. cepacia* were used. Strain 75-26 was a β-lactam-susceptible, non-CF isolate (8). Serial dilutions of this strain were inoculated onto Mueller-Hinton agar containing 16 μg of ciprofloxacin per ml and incubated at 35°C for 24 h. The initial inoculum was determined from viable counts plated on drug-free agar. Resistant colonies that emerged on antibiotic-containing medium were counted and subcultured onto antibiotic-free medium, and then each isolate was replated onto medium containing ciprofloxacin (8 μg/ml) and ceftazidime (16 μg/ml) in order to screen the ciprofloxacin-resistant strains for cross-resistance. Selected cross-resistant isolates were subcultured onto Mueller-Hinton agar and stored at −70°C in 5% skim milk medium. One resistant mutant, designated 75-26Mc, was selected for this study. Strains 16901 and 619i were β-lactam-resistant *P. cepacia* CF isolates; the latter isolate was included in a previous study (8). *Escherichia coli* ATCC 25218, which contains TEM-1 and chromosomal β-lactamases, was used as a control for isoelectric focusing. Cell-free lysates of *P. cepacia* 75-26z, a derepressed chromosomal β-lactamase-producing strain (2), were used to determine the Michaelis-Menten kinetics of nitrocefin hydrolysis. *P. aeruginosa* ATCC 27853 was used as a control in the drug susceptibility assays.

β-Lactamase preparations. Cell-free lysates of the *P. cepacia* test strains, *P. cepacia* 75-26z, and *E. coli* ATCC 25218 were prepared without induction as previously described (8).

Preparation of cell envelopes. Cells from overnight cultures of the test strains were harvested from Mueller-Hinton agar in 0.05 M Tris-EDTA (pH 7.8), washed twice with buffer, suspended in 8 ml of 0.05 M Tris-EDTA containing 1 mg each of RNase and DNase (Sigma Chemical Co., St. Louis, Mo.), crushed in a French press at 6,000 to 7,000 lb/in^2, and cleared of cellular debris by centrifugation. The cleared lysates were sterilized by passage through a 0.45-μm-pore-size filter. The cell envelopes were separated from the cleared lysate by ultracentrifugation at 150,000 × g for 1 h,

suspended in 0.05 M phosphate buffer (pH 7.0), and stored at −70°C (22).

Outer membrane preparations. Outer membranes were derived from cell envelopes by selective solubilization in 2% *n*-lauroyl sarcosine–0.01 M phosphate buffer (pH 7.0) and ultracentrifugation (5). The final pellets were suspended in 0.1 M phosphate buffer and stored at −70°C.

Antibiotic susceptibility. The MICs of ceftazidime and piperacillin against the test strains were determined by agar dilution (25). Overnight broth cultures of the *P. cepacia* test strains and *P. aeruginosa* ATCC 27853 were adjusted with fresh broth, using a 0.5 McFarland standard. To achieve final bacterial concentrations of 10^4 CFU per spot, Mueller-Hinton agar plates containing doubling dilutions of either drug were inoculated with a Steers replicator. The plates were incubated at 35°C overnight. The MIC was defined as the lowest concentration of drug that totally inhibited visible bacterial growth.

Protein assay. The β-lactamase preparations, cell envelopes, and outer membrane preparations were assayed for protein by the method of Bradford (6). Bovine serum albumin was used as the standard.

β-Lactamase activity. β-Lactamase preparations of the test strains of *P. cepacia* were assayed for enzyme activity by nitrocefin hydrolysis (8, 18). The specific β-lactamase activity for each test strain was defined as nanomoles of nitrocefin hydrolyzed per minute per milligram of bacterial protein. The hydrolysis rates of five initial concentrations of nitrocefin were determined by using cell-free lysates from *P. cepacia* 75-26z, a strain derepressed for chromosomal β-lactamase production (4). The Michaelis-Menten constant (K_m) and the V_{max} were determined from a Lineweaver-Burk plot.

Isoelectric focusing. Cell-free preparations of the *P. cepacia* test strains and *E. coli* ATCC 25218 were isoelectrically focused on commercial polyacrylamide gels (Integrated Separation Systems, Newton, Mass.), stained for β-lactamase activity with nitrocefin, and photographed (4). The gels were restained with Coomassie blue, and the migration of pI markers included on each gel was used to determine the isoelectric focusing points of each β-lactamase band.

Outer membrane permeability. The rate of nitrocefin hydrolysis in intact cells was determined by the method of Nicas and Hancock in order to correct for extracellular β-lactamase (17). Cells from overnight cultures of the test strains were harvested by centrifugation and washed three times with 0.05 M phosphate buffer (pH 7.0). The final pellets were resuspended in buffer. Equal portions of the final suspensions were cleared of cells by centrifugation, and the cell-free supernatant was retained. Equal volumes of the supernatant and the respective cell suspension were placed in 1-ml matched optical cuvettes. Nitrocefin was then added to both cuvettes. The nitrocefin concentration was adjusted for each strain. The lowest concentrations that produced a reliable curve were selected to avoid saturating the periplasmic enzyme. The supernatant mixture was placed in the reference path and the cell suspension was placed in the sample path of a double-beam DMS 70 spectrophotometer (Varian, Palo Alto, Calif.). The A_{517} was measured continuously by recorder, and the hydrolysis rate of nitrocefin was calculated from the steady-state portion of the tracing.

Electrophoretic separation of outer membrane proteins. The protein components of the outer membrane preparations from the *P. cepacia* test strains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a 5% stacking gel overlying a 12.5% resolving gel (5, 12).

TABLE 1. Characterization of test strains of *P. cepacia*

Strain	β-Lactamase sp act (U)[a]	MIC (μg/ml)		Source
		Piperacillin	Ceftazidime	
75-26	33	2	2	Non-CF[b]
75-26Mc	37	>128	64	See text
619i	24	64	128	CF[c]
16091	58	32	32	CF

[a] Defined as nanomoles of nitrocefin hydrolyzed per minute per milligram of bacterial protein (without induction).
[b] See reference 4.
[c] See reference 8.

Molecular size standards (Bio-Rad Laboratories, Richmond, Calif.) were included on each gel. Each sample was thawed at room temperature and then placed over boiling water for 40 min (19). Equal portions of each preparation were mixed in a 1:1 ratio with Laemmli buffer, and 0.3 μg of protein from each mixture was loaded onto the gel. The gels were stained with silver to demonstrate protein bands (16).

Characterization of cell envelope LPS. Portions containing 40 μg of protein from cell envelope preparations of each test strain were incubated at room temperature for 2 h with 40 μg of trypsin (Sigma) (13). The lipopolysaccharide (LPS) components were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a 5% stacking gel and a 15% resolving gel containing 4 M urea. The gels were developed by using the silver stain of Tsai and Frasch (24).

RESULTS

Characteristics of test strains. Ciprofloxacin was used as the initial selecting agent, since cross-resistance between oxyquinolones and β-lactams has been associated with changes in the expression of bacterial outer membrane proteins, suggesting an alteration in outer membrane permeability (20). Inoculation of 7.5×10^7 CFU of strain 75-26 per ml onto ciprofloxacin-containing agar produced 7.5×10^2 resistant colonies (frequency, 10^{-5}). All 370 ciprofloxacin-resistant colonies that were replated onto plain agar subsequently grew on ciprofloxacin-ceftazidime agar. Since this last observation suggests that most, if not all, of the ciprofloxacin-resistant mutants were cross-resistant to ceftazidime, the frequency of appearance of cross-resistant, ciprofloxacin-selected mutants of strain 75-26 was comparable to the rate of β-lactamase derepression in the same strain (2).

The specific β-lactamase activity and the MICs of piperacillin and ceftazidime against 75-26, 75-26Mc, and the two CF isolates of *P. cepacia* are shown in Table 1. Levels of β-lactamase production by all of the strains were comparable and well below 1,000 U, the specific enzyme activity associated with derepressed enzyme production (2). All of the strains produced a major β-lactamase band that focused in the pH range of 7.9 to 8.1, similar to previous observations and comparable to the β-lactamase focusing pattern of the derepressed strain, 75-26z (2, 4, 8). The MICs of piperacillin and ceftazidime against the CF isolates (619i and 16901) and the resistant mutant (75-26Mc) were comparable and higher than the MICs of these two compounds against strain 75-26. The parent and the mutant strains were equally susceptible to the hydrophobic, non-β-lactam agents chloramphenicol (MIC, 16 μg/ml) and novobiocin (MIC, 16 μg/ml). These observations suggested that derepressed β-lactamase production was not an explanation for the degrees of β-lactam resistance in the resistant mutant and the CF isolates and

TABLE 2. Outer membrane permeability of *P. cepacia* test strains

Strain	S_O range (μM)	S_e/S_O range	$C \pm$ SE[a]
75-26	48–775	0.051–0.077	0.019 ± 0.003
75-26Mc	48–775	0.002–0.009	0.005 ± 0.001
619i	24–387	0.004–0.026	0.003 ± 0.001
16091	97–1,550	0.012–0.064	0.006 ± 0.001

[a] In milliliters per minute per milligram of bacterial protein as determined from linear regression analysis of V_{int} versus S_O.

that all of the test strains produced the same chromosomal enzyme.

Outer membrane permeability. Limitation of β-lactam diffusion by the bacterial outer membrane into the periplasmic space is defined quantitatively by the permeability constant, C, described by Zimmermann and Rousselet and calculated from Fick's law of diffusion (26). At steady state, the nitrocefin hydrolysis rate measured in intact cells (V_{int}) is equal to the diffusion rate of nitrocefin (V_{diff}) such that:

$$V_{int} = V_{diff} = C(S_O - S_e) \qquad (1)$$

in which S_e is the periplasmic concentration of nitrocefin and S_O is the initial concentration of nitrocefin added to the mixture (26). Since the test strains contained low concentrations of chromosomal β-lactamase (Table 1), the addition of extremely high concentrations of nitrocefin (S_O) to the reaction mixtures was required in order to accurately measure V_{int} (17). Under these conditions, the calculation of C from equation 1 is inaccurate; however, V_{int} is directly proportional to S_O (1, 17). If S_O is significantly greater than S_e, then equation 1 can be rewritten as:

$$C = V_{int}/S_O \qquad (2)$$

Therefore, V_{int} was measured at five nitrocefin concentrations (S_O) for each test strain of *P. cepacia*. By linear regression analysis (least-square method), the line of best fit between V_{int} and S_O and the correlation coefficient were determined for each strain. From equation (2), the permeability constant (C) for each strain was defined as the slope of each line. The periplasmic concentration of nitrocefin (S_e) was calculated at each concentration of S_O for every organism, using the Michaelis-Menten equation (26).

The permeability constants (C) of the four *P. cepacia* test strains are shown in Table 2. The relationship between V_{int} and S_O was linear for all strains over the range of added nitrocefin concentrations studied (range of r^2, 0.86 to 0.97). For the β-lactam-susceptible strain 75-26, the value for C determined by this method was similar to the permeability constant reported for *P. cepacia* PC715J (19). The permeability constants and ratios of S_e to S_O for the mutant and the resistant CF isolates were much lower than those for strain 75-26, consistent with the inverse relationship between MIC and outer membrane permeability observed with other bacterial species (1, 21, 26).

Outer membrane proteins. Parr et al. identified an 81-kilodalton (kDa) porin complex in the outer membrane of *P. cepacia* (19). After being heated, the complex was resolved electrophoretically into two protein components with apparent molecular sizes of 36 and 27 kDa. Separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer membrane protein components from each test strain is shown in Fig. 1. The level of the 27-kDa porin was markedly lower in strain 75-26Mc than in strain 75-26, and it was absent in the two resistant CF isolates. The 36-kDa outer membrane protein was present in all of the test strains.

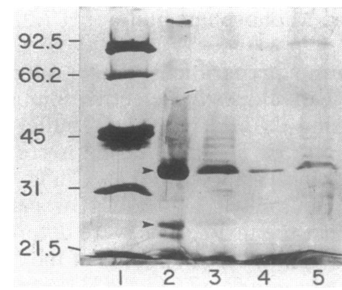

FIG. 1. Electrophoretic separation of the outer membrane proteins of the test strains of *P. cepacia*. Lanes: 1, molecular size markers (in kilodaltons); 2, strain 75-26; 3, strain 75-26Mc; 4, strain 619i; 5, strain 16901. Arrows indicate the 36-kDa (top) and 27-kDa (bottom) outer membrane proteins.

Characteristics of LPS. The LPS banding patterns in trypsinized cell envelopes derived from each test strain are shown in Fig. 2. All of the strains maintained the ladder pattern that has been associated with repeating sugar side chains in smooth strains of *P. aeruginosa* (7).

DISCUSSION

Reduced β-lactam diffusion across the bacterial outer membrane is a recognized mechanism of β-lactam resistance in *P. aeruginosa*. A mutant of *P. aeruginosa* deficient in porin (F protein) was less permeable to nitrocefin than the porin-bearing parental strain (17). In our study, partial or total loss of the 27-kDa outer membrane protein was associated with decreased β-lactam permeability and susceptibility in *P. cepacia*. This observation suggests that (i) the 27-kDa outer membrane protein is either a major porin or a major protein component of the porin complex in *P. cepacia*, (ii) decreased bacterial expression of this protein reduces β-lactam diffusion across the outer membrane, and (iii) decreased expression of the 27-kDa porin results in high-level β-lactam resistance in *P. cepacia*.

Loss of LPS side chains independent of porin protein deficiency has also been associated with β-lactam resistance in *P. aeruginosa*. Godfrey et al. developed four β-lactam-resistant permeability mutants of *P. aeruginosa* PAO503 (10). Although the permeability coefficients of the mutant strains were significantly lower than that of the parent strain when penicillin G was used as the probe, the porin protein

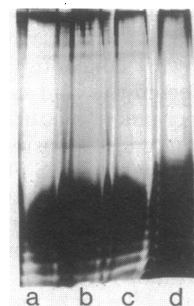

FIG. 2. Electrophoretic separation of LPS components in cell envelopes from the test strains of *P. cepacia*. Lanes: a, strain 75-26; b, strain 75-26Mc; c, strain 619i; d, strain 16901.

was present in the outer membrane of all of the mutants. Analysis of the sugar moieties that made up the outer membrane suggested an association between loss of side chain sugars and reduced β-lactam permeability. A subsequent study from the same laboratory demonstrated that the semiquantitative bindings of monoclonal antibodies directed against the porin protein or core LPS were similar in PAO503 and its impermeable mutant PCC118; however, binding of antibody directed against the LPS side chains was markedly reduced in the mutant (11). Using silver-stained, sodium dodecyl sulfate-polyacrylamide gel electrophoresis to demonstrate the ladder pattern associated with the presence of LPS side chains in *P. aeruginosa*, Bryan et al. demonstrated impermeable-type aminoglycoside resistance in transductants of PAO503 lacking LPS side chains (7). In our study, the LPS ladder patterns were similar in all of the test strains of *P. cepacia*.

In conclusion, decreased β-lactam permeability appears to be a mechanism of high-level resistance to this class of agents among CF isolates of *P. cepacia*. Two outer membrane proteins have been identified as possible porins in this species; decreased expression of the 36-kDa outer membrane protein and nonexpression of the 27-kDa outer membrane protein are associated with decreased permeability. From these observations, I speculate that the 27-kDa porin may be a major porin or a major protein constituent of the porin complex in *P. cepacia*. Loss of LPS side chains, a mechanism of reduced β-lactam permeability in *P. aeruginosa*, was not demonstrated in any of the strains studied.

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LITERATURE CITED

1. **Angus, B. L., A. M. Carey, D. A. Caron, A. M. B. Kropinski, and R. E. W. Hancock.** 1982. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. Antimicrob. Agents Chemother. **21**:299–309.
2. **Aronoff, S. C.** 1988. Derepressed beta-lactamase production as a mediator of high level beta-lactam resistance in *Pseudomonas cepacia*. Pediatr. Pulmonol. **4**:72–77.
3. **Aronoff, S. C., and J. D. Klinger.** 1985. Comparison of HR-810 and four antipseudomonal beta-lactam agents against *Pseudomonas* isolates from children with cystic fibrosis. J. Antimicrob. Chemother. **15**:545–549.
4. **Aronoff, S. C., and P. H. Labrozzi.** 1986. Differences in drug susceptibility between isolates of *Pseudomonas cepacia* recovered from patients with cystic fibrosis and other sources and its relationship to beta-lactamase focusing pattern. Pediatr. Pulmonol. **2**:368–372.
5. **Aronoff, S. C., and R. C. Stern.** 1988. Serum IgG antibody against the outer membrane antigens of *Pseudomonas cepacia* and *Pseudomonas aeruginosa* in cystic fibrosis. J. Infect. Dis. **157**:934–940.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Bryan, L. E., K. O'Hara, and S. Wong.** 1984. Lipopolysaccharide changes in impermeable-type aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26**:250–255.
8. **Chiesa, C., P. H. Labrozzi, and S. C. Aronoff.** 1986. Decreased baseline beta-lactamase production associated with increased piperacillin susceptibility of *Pseudomonas cepacia* isolated from children with cystic fibrosis. Pediatr. Res. **20**:1174–1177.
9. **Conrad, D. A., R. K. Scribner, A. H. Weber, and M. I. Marks.** 1985. In vitro activity of BMY-28142 against pediatric pathogens, including isolates from cystic fibrosis sputum. Antimicrob. Agents Chemother. **28**:58–63.
10. **Godfrey, A. J., L. Hatlelid, and L. E. Bryan.** 1984. Correlation between lipopolysaccharide structure and permeability resistance in β-lactam-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26**:181–186.
11. **Godfrey, A. J., M. S. Shahrabadi, and L. E. Bryan.** 1986. Distribution of porin and lipopolysaccharide antigens on a *Pseudomonas aeruginosa* permeability mutant. Antimicrob. Agents Chemother. **30**:802–805.
12. **Hancock, R. E. W., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140**:902–910.
13. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
14. **Isles, A., I. Maclusky, M. Corey, R. Gold, C. Prober, P. Fleming, and H. Levison.** 1984. *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. J. Pediatr. **104**:206–210.
15. **Klinger, J. D., and S. C. Aronoff.** 1985. *In vitro* activity of ciprofloxacin and other antibacterial agents against *Pseudomonas aeruginosa* and *Pseudomonas cepacia* from cystic fibrosis patients. J. Antimicrob. Chemother. **15**:679–684.
16. **Meril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert.** 1980. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science **211**:1437–1438.
17. **Nicas, T. I., and R. E. W. Hancock.** 1983. *Pseudomonas aeruginosa* outer membrane permeability: isolation of a porin protein F-deficient mutant. J. Bacteriol. **153**:281–285.
18. **O'Callahan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283–288.
19. **Parr, T. R., Jr., R. A. Moore, L. V. Moore, and R. E. W. Hancock.** 1987. Role of porins in intrinsic antibiotic resistance of *Pseudomonas cepacia*. Antimicrob. Agents Chemother. **31**:121–123.
20. **Sanders, C. C., W. E. Sanders, Jr., R. V. Goering, and V. Werner.** 1984. Selection of multiple antibiotic resistance by quinolones, β-lactams, and aminoglycosides with special reference to cross-resistance between unrelated drug classes. Antimicrob. Agents Chemother. **26**:797–801.
21. **Sawai, T., K. Matsuba, and S. Yamagishi.** 1977. A method for measuring the outer membrane permeability of beta-lactam antibiotics in gram negative bacteria. J. Antibiot. **30**:1134–1136.
22. **Schnaitman, C. A.** 1970. Examination of the protein composition of the cell envelope of *Escherichia coli* by polyacrylamide gel electrophoresis. J. Bacteriol. **104**:882–889.
23. **Tablan, O. C., T. L. Chorba, D. V. Schidlow, J. W. White, K. A. Hardy, P. H. Gilligan, W. M. Morgan, L. A. Carson, W. J. Martone, J. M. Jason, and W. R. Jarvis.** 1985. Pseudomonas cepacia colonization in patients with cystic fibrosis: risk factors and clinical outcome. J. Pediatr. **107**:382–387.
24. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
25. **Washington, J. A., II, and V. Sutter.** 1980. Dilution susceptibility test: agar and macro-broth procedures, p. 453–458. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of Clinical Microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
26. **Zimmermann, W., and A. Rosselet.** 1977. Function of the outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. Antimicrob. Agents Chemother. **12**:368–372.