Enterobacter species are becoming increasingly important nosocomial pathogens (29). In the most recent National Nosocomial Infections Study data published, Enterobacter is the third-most-common pathogen recovered from the respiratory tract (16). Data from isolates recovered from intensive care units revealed that this organism was also the fourth-most-common pathogen recovered from surgical wounds, the fifth-most-common pathogen recovered from the urinary tract, and the fifth-most-common pathogen recovered from blood (16). Risk factors for nosocomial Enterobacter infection include the prior use of antimicrobial agents, a prolonged hospital stay, a serious underlying illness, immunosuppression, and the presence of a foreign device (29).

Resistance to expanded-spectrum cephalosporins commonly develops in Enterobacter aerogenes during therapy due to selection of mutants producing high levels of the chromosomal Bush group 1 β-lactamase. Recently, resistant strains producing plasmid-mediated extended-spectrum β-lactamas (ESBLs) have been isolated as well. A study was designed to investigate ESBL production among 31 clinical isolates of Enterobacter aerogenes from Richmond, Va., with decreased susceptibility to expanded-spectrum cephalosporins and a positive double-disc potentiation test. Antibiotic susceptibility was determined by standard disk diffusion and agar dilution procedures. β-Lactamas were investigated by an isoelectric focusing overlay technique which simultaneously determined isoelectric points (pIs) and substrate or inhibitor profiles. Decreased susceptibility to cefotaxime, ceftazidime, and aztreonam (MIC range, 1 to 64 μg/ml) was detected and associated with resistance to gentamicin and trimethoprim-sulfamethoxazole. All strains produced an inducible Bush group 1 β-lactamase (pI 8.3). Twenty-nine of the 31 isolates also produced an enzyme similar to SHV-4 (pI 7.8), while 1 isolate each produced an enzyme similar to SHV-3 (pI 6.9) and to SHV-5 (pI 8.2). The three different SHV-derived ESBLs were transferred by transconjugation to Escherichia coli C600N and amplified by PCR. Plasmid profiles of the clinical isolates showed a variety of different large plasmids. Because of the linkage of resistance to aminoglycosides and trimethoprim-sulfamethoxazole with ESBL production, it is possible that the usage of these drugs was responsible for selecting plasmid-mediated resistance to extended-spectrum cephalosporins in Enterobacter aerogenes. Furthermore, it is important that strains such as these be recognized, because they can be responsible for institutional spread of resistance genes.
designed to investigate and describe the possible mechanisms responsible for the resistance to the expanded-spectrum cephalosporins that was observed in *E. aerogenes* strains isolated from this hospital.

**MATERIALS AND METHODS**

**Bacterial strains.** Among all *E. aerogenes* strains (n = 184) recovered from clinical specimens during an 18-month period (September 1993 to March 1995), 31 (16.8%) strains showing a resistance phenotype different from that observed with the derepressed mutants normally encountered at the Hunter Holmes McGuire Medical Center were selected for this study. The strains selected were shown to possess an intermediate to ceftriaxone but resistant to cefazolin and cefotaxime by both the double-disk test and automated susceptibility system (bioMérieux Vitek, St. Louis, Mo.). Derepressed mutants previously isolated from this center were usually resistant to both ceftriaxone and cefazolin.

**Susceptibility testing.** Antibiotic susceptibilities were determined by standard disk diffusion (21) and agar dilution (22) procedures. Disks were obtained from Becton Dickinson Microbiology Systems (Cockeysville, Md.). Disk diffusion susceptibilities to the following antibiotics were determined: ampicillin, amoxicillin-clavulanic acid, aztreonam, cefazolin, cefoxitin, cefotaxime, ceftriaxone, ceftazidime, cepacime, imipenem, gentamicin, trimethoprim-sulfamethoxazole, and ciprofloxacin. Standard powders of antimicrobial agents for MIC determinations were kindly provided by the following companies: Merck (Rahway, N.J.) (cefalexin and imipenem), Hoechst-Roussel Pharmaceuticals Inc. (Somerville, N.J.) (ceftizidine), Glaxo Group Research Ltd. (Greenford, England) (ceftizidine), Bristol-Myers Squibb (Princeton, N.J.) (ceftazidime and cepacime), and Schering-Plough (Lanbury, N.J.) (gentamicin). The following quality control strains were run simultaneously with the test organisms: *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *E. coli* ATCC 35218. Throughout this study, results were interpreted with National Committee for Clinical Laboratory Standards criteria for disk diffusion (21) and broth dilution (22).

**Double-disk potentiation test.** This test, described by Jarlier et al. (15) with cefotaxime, ceftriaxone, cefazolin, and aztreonam disks, was performed on the strains to screen for possible ESBL production. This test is a modification of the disk diffusion susceptibility test in that cefotaxime, ceftriaxone, ceftazidime, and aztreonam disks are placed 30 mm from disks containing amoxicillin-clavulanic acid. A potentiation of the zones of cefotaxime, ceftriaxone, ceftazidime, or aztreonam by clavulanic acid represented a positive test and was indicative of possible ESBL production.

**β-Lactamase preparation, IEF, and assays.** Overnight cultures in 5 ml of Mueller-Hinton broth were diluted with 95 ml of fresh broth and incubated with shaking for 90 min at 37°C. One-fourth of the cefotaxin MIC was added for induction, while sterile medium was used in the noninduced cultures and incubated for an additional 2 h. The induction process was stopped by the addition of 1 mM 8-hydroxyquinoline solution to each culture. Cells were harvested by centrifugation at 4°C, washed with 1 M potassium-phosphate buffer (pH 7.0), suspended, and sonicated. After sonication, crude extracts were obtained by centrifugation at 6,000 rpm for 1 h. The β-lactamases in the sonicate extracts were assayed for isoelectric points (pI) and substrate and inhibitor profiles in polyacrylamide gels with overlays of 0.75 g of cefotaxime per ml, 1,000 µg of clavulanic acid, and 1,000 µg of clavulanic acid prior to overlay with nitrocefin agar (1, 19, 28). Cephalexin inhibition tests were determined by UV spectrophotometric assay (25). Inducibility of the Bush group 1 β-lactamase was inferred from the induction of isoelectric focusing (IEF) patterns for uninduced and induced β-lactamase extracts. As controls, crude β-lactamase preparations from the following organisms possessing different SHV enzymes were evaluated simultaneously with those obtained from the *Enterobacter* strains: SHV-1 [from *E. coli* J53 (R1010)], SHV-2 (from *Klebsiella aerourea* 2180), SHV-3 [from *E. coli* J53-2p(UD18)], SHV-4 [from *E. coli* J53-2p(UD21)], and SHV-5 [from *E. coli* Cia Nap(PP2F)].

**Isolation of plasmids.** The organisms were inoculated into 5 ml of Luria Bertani (LB) broth (Difco, Detroit, Mich.) and incubated for 20 h at 37°C with shaking. Cells from 1.5 ml of overnight culture were harvested by centrifugation in an Eppendorf centrifuge for 5 min. After the supernatant was decanted, the pellet was resuspended in 500 µl of sterile deionized water. The cells were lysed by heating to 95°C for 10 min, and cellular debris was removed by centrifugation for 5 min at 13,000 rpm. The supernatant was used as the source of template for amplification. Oligonucleotide primers specific for SHV genes were selected from a consensus alignment sequence generated by the MacVector 4.5 ( Kodak/IJI) software package from the published nucleotide sequences of *SHV-1* (20), SHV-2 (13), SHV-5 (2), and SHV-7 (4). The sequences of the PCR primers used were A 5′-CACCTAAGGATGATGTGGTGT-3′ and B 5′-TTAGGTTGCG AGTGCCTG-3′, which amplified a 781-base pair fragment. Primer specificity controls included the TEM-1, MIN-1, and SHV-7 β-lactamase genes. PCR amplifications were carried out on a DNA Thermal Cycler 480 instrument (Perkin-Elmer Cetus, Norwalk, Conn.) with the Gene/Ampl DNA amplification kit containing AmpliTaq polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, N.J.). The composition of the reaction mixture was as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 10 mM dithiothreitol, 0.2 mM each dNTP, and 1.2 U of AmpliTaq in a total volume of 49 µl. A total of 1 µl of sample lysate was added to the reaction mixture, which was centrifuged briefly before 50 µl of mineral oil was layered onto the surface. The PCR program consisted of an initial denaturation step at 96°C for 30 s followed by 24 cycles of DNA denaturation at 96°C for 30 s, primer annealing at 50°C for 15 s, and primer extension at 72°C for 2 min. After the last cycle, the products were stored at 4°C. The PCR products were analyzed by electrophoresis with 1.4% agarose gels in TAE buffer. The gels were stained with ethidium bromide, and the PCR products were visualized with UV light.

**RESULTS**

**Bacterial strains.** Twenty-four of the 31 strains from Hunter Holmes McGuire Medical Center originated from patients in two spinal cord injury wards (SCW1 and SCW2), while 3 strains were isolated from patients in the medical intensive care unit, 3 isolates were recovered from patients attending the surgical outpatient clinic, and 1 isolate was recovered from a patient in a general surgery ward. Disk diffusion susceptibility tests showed all the strains to be resistant to ampicillin, amoxicillin-clavulanate, cefazolin, cefoxitin, ceftazidime, and trimethoprim-sulfamethoxazole and all the strains to be susceptible to ciprofloxacin. MICs of cefotaxime, ceftriaxone, ceftazidime, aztreonam, cefepime, imipenem, and gentamicin are summarized in Table 1. All strains were susceptible to cefepime and imipenem but showed decreased susceptibility to cefotaxime, ceftazidime, and aztreonam. MICs of gentamicin ranged from 8 to >128 µg/ml for 25 of 37 (67%) isolates. All the strains selected for this study showed a positive double-disk test when cefotaxime and ceftriaxone disks were used.

**Characteristics of β-lactamases.** All the *Enterobacter* isolates possessed a Bush group 1 inducible β-lactamase with an alkaline pI of 8.3 which was sensitive to inhibition by clavulanic acid but not clavulanic acid (Table 1). Additional Bush group 2b enzymes with pI similar to SHV-β-lactamases were also present in all the strains (Table 1). Three different group 2b enzymes were detected in the species of *Enterobacter* (Table 1); the majority of isolates (29 of 31) produced an enzyme with a pI of 7.8, which aligned with SHV-4. One isolate produced an enzyme with a pI of 6.8, which aligned with SHV-3, and one isolate produced an enzyme with a pI of 8.2, which aligned with SHV-5.

**Plasmid profiles.** A variety of different plasmids with sizes ranging from 10 to approximately 60 kb were visualized with electrophoresis (Table 2). Furthermore, eight different plasmid patterns were observed, with the number of plasmids ranging from 0 to 5 per organism (Table 2). No plasmids were visualized in three strains, including the strain which produced an enzyme resembling SHV-5 (Table 2). Three different susceptibility profiles were identified (Table 2). The majority of organisms isolated were resistant to cefotaxime, aztreonam, trimethoprim-sulfamethoxazole, and gentamicin. This antibiotic was associated with the production of β-lactamases resembling SHV-4 and SHV-5 and isolated from SCW1 and SCW2, the medical intensive care unit, the general surgical ward, and the outpatient clinic (Table 2). Eight of thirty strains...
showing three different plasmid profiles (a, b, and f) and producing an enzyme resembling SHV-4 isolated from SCW1 as well as from the surgical outpatient clinic were susceptible to gentamicin, while the E. aerogenes strain producing an enzyme resembling SHV-3 appeared susceptible to ceftazidime and aztreonam (Table 2). Seven different plasmid profiles (b to h) were observed among E. aerogenes strains isolated from SCW1, while only four patterns (c, d, g, and h) were observed among those strains recovered from SCW2 (Table 2). Plasmid profile b, consisting of five plasmids ranging from 50 to 10 kb (observed in six isolates), and plasmid profile f, consisting of three plasmids ranging from 60 to 10 kb (observed in one isolate), were unique to SCW1 (Table 2). Two of the three strains isolated from the medical intensive care unit possessed four plasmids ranging from 60 to 10 kb (plasmid profile c), while no plasmids from the other strain were visualized (plasmid profile h) (Table 2). These organisms produced an enzyme resembling SHV-4 and were resistant to ceftazidime, aztreonam, trimethoprim-sulfamethoxazole, and gentamicin. The E. aerogenes strains originating from the surgical outpatient clinic had

### Table 1. MICs and characteristics of β-lactamases produced by E. aerogenes

<table>
<thead>
<tr>
<th>No. of strains</th>
<th>β-Lactamase group (enzyme type)*</th>
<th>Enzyme characteristic</th>
<th>MIC (µg/ml) of β-Lactamase**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pl</td>
<td>CTX</td>
</tr>
<tr>
<td>1 1 2be (SHV-3)</td>
<td>8.3</td>
<td>6.9</td>
<td>No</td>
</tr>
<tr>
<td>29 1 2be (SHV-4)</td>
<td>8.3</td>
<td>7.8</td>
<td>No</td>
</tr>
<tr>
<td>1 1 2be (SHV-5)</td>
<td>8.3</td>
<td>8.0</td>
<td>No</td>
</tr>
<tr>
<td>4 1’ (wild type)</td>
<td>8.3–8.8</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5 1’ (derepressed)</td>
<td>8.3–8.9</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; FEP, cefepime; IMI, imipenem; GM, gentamicin.

**Based on Bush-Jacoby-Medeiros classification (5). The β-lactamase listed in parentheses is the one most similar to the group 2be enzyme produced by the Enterobacter strains.

### Table 2. Plasmid profiles of E. aerogenes

<table>
<thead>
<tr>
<th>Plasmid profile</th>
<th>No. of plasmids</th>
<th>Approximate size (kb)</th>
<th>Ward (no. of isolates)*</th>
<th>Antibiogram results**</th>
<th>Most likely ESBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>5</td>
<td>50, 35, 20, 15, 12</td>
<td>OPC (1)</td>
<td>R R R GM</td>
<td>SHV-4</td>
</tr>
<tr>
<td>b</td>
<td>5</td>
<td>50, 45, 35, 20, 10</td>
<td>SCW1 (6)</td>
<td>R R R</td>
<td>SHV-4</td>
</tr>
<tr>
<td>c</td>
<td>4</td>
<td>60, 45, 20, 10</td>
<td>SCW1 (5)</td>
<td>R R R R</td>
<td>SHV-4</td>
</tr>
<tr>
<td>d</td>
<td>4</td>
<td>45, 35, 20, 10</td>
<td>SCW1 (2)</td>
<td>R R R R</td>
<td>SHV-4</td>
</tr>
<tr>
<td>e</td>
<td>3</td>
<td>60, 50, 14</td>
<td>SCW1 (2)</td>
<td>R R R R</td>
<td>SHV-4</td>
</tr>
<tr>
<td>f</td>
<td>3</td>
<td>60, 50, 10</td>
<td>SCW1 (1)</td>
<td>R R R</td>
<td>SHV-4</td>
</tr>
<tr>
<td>g</td>
<td>2</td>
<td>50, 10</td>
<td>SCW1 (1)</td>
<td>R R R R</td>
<td>SHV-3</td>
</tr>
<tr>
<td>h</td>
<td>0</td>
<td></td>
<td>SCW1 (1)</td>
<td>R R R R</td>
<td>SHV-5</td>
</tr>
</tbody>
</table>

*OPC, outpatient clinic; SCW1, spinal cord injury ward 1; SCW2, spinal cord injury ward 2; MICU, medical intensive care unit; SGW, surgical general ward.

**CAZ, ceftazidime; ATM, aztreonam; SXT, trimethoprim-sulfamethoxazole; GM, gentamicin.

R, resistant.
two different antibiograms and plasmid profiles. Two of these isolates, possessing plasmid profile a, were resistant to cef-
tazidime, aztreonam, trimethoprim-sulfamethoxazole, and gentamicin, while the remaining isolate, with plasmid profile b, appeared to be susceptible to gentamicin (Table 2). All these organisms produced an enzyme resembling SHV-4.

**Conjugation experiments.** The following strains were se-
lected for conjugation with *E. coli* C600N: *E. aerogenes* 187, pro-
ducing an enzyme with a pI of 6.8, resembling SHV-3; *E. aerogenes* 200 and *E. aerogenes* 220, producing enzymes with 
plIs of 7.8, resembling SHV-4; and *E. aerogenes* 184, producing an enzyme with a pI of 8.2, resembling SHV-5. All the strains also possessed an inducible Bush group 1 β-lactamase with a pI of 8.3. A plasmid of approximately 50 kb was transferred from *E. aerogenes* 187, *E. aerogenes* 200, and *E. aerogenes* 220 to *E.
coli* C600N (Table 3). No plasmids were visualized in *E. aerogenes* 184 or its transconjugant, *E. coli* JP04/tr (Table 3). IEF performed on the *Enterobacter* strains and their respective transconjugants showed the β-lactamases resembling SHV-3, SHV-4, and SHV-5 present in both donors and recipients. The transfer of plasmids encoding SHV β-lactamase genes into *E.
coli* C600N was accompanied by resistance to gentamicin and trimethoprim-sulfamethoxazole and decreased susceptibility to cefotaxime, cefazidime, and aztreonam (Table 3).

**DNA amplification.** The strains used in the conjugation ex-
periments were selected for amplification with PCR (as fol-
ows): *E. aerogenes* 187 (pl 6.8), *E. aerogenes* 200 (pl 7.8), *E. 
aerogenes* 220 (pl 7.8), *E. aerogenes* 184 (pl 8.2) as well as their respective transconjugants, *E. coli* JP01/tr (pl 6.8), *E.
coli* JP02/tr (pl 7.8), *E. coli* JP03/tr (pl 7.8), and *E. coli* JP04/tr (pl 8.2). Strains producing SHV-3, SHV-4, SHV-5, and SHV-7 were used as positive controls, while *E. coli* C600N was used as a negative control. A 781-bp fragment specific for SHV β-la-
tamases was amplified in *E. aerogenes* 187, *E. aerogenes* 200, *E. aerogenes* 220, *E. aerogenes* 184, and their respective transcon-
jugants as well as in the positive controls (Table 3). No ampli-
fication was observed with *E. coli* C600N (Table 3). Therefore, the ESBLs produced by these strains are indeed derivatives of an SHV β-lactamase.

**DISCUSSION**

The prevalence of resistance among *Enterobacter* strains to expanded-spectrum β-lactam antibiotics varies between diverse geographic locations (29). To our knowledge, this is the first study describing a large number of strains of *E. aerogenes* producing different SHV-derived extended-β-lacta-
mases. The different β-lactamases resembling SHV-3, SHV-4, and SHV-5 as well as resistance to gentamicin and tri-
methoprim-sulfamethoxazole were transferred into *E. coli* C600N. This was accompanied by a plasmid of approximately 50 kb in some of the strains (Table 3). Although it may seem surprising that an organism with an inducible cephalosporinase would acquire an extended-spectrum β-lactamase, resistance to other agents such as the aminoglycosides and trimethoprim-sulfamethoxazole, which is encoded on the same plasmid as the extended-spectrum β-lactamase, may often be the major factor behind the acquisition of these plasmids by *Enterobacter* (29, 30).

It is important to detect *Enterobacter* strains producing ex-
tended-spectrum β-lactamases in a clinical laboratory and to
differentiate them from derepressed mutants. Plasmids encod-
ing extended-spectrum β-lactamases may also encode resis-
tance to other classes of antibiotics, such as the aminoglyco-
sides and trimethoprim-sulfamethoxazole, limiting the options of physicians treating infections caused by organisms producing these enzymes (30). Therefore, factors leading to the selec-
tion and spread of strains producing ESBLs need to be iden-
tified and, where possible, eliminated (29, 30). In this study, strains of *E. aerogenes* producing enzymes resembling SHV-4 and SHV-5 were resistant to cefazidime, aztreonam, gentamicin, and trimethoprim-sulfamethoxazole, but not neces-
sarily to cefotaxime (Table 1). Thus, for a clinical laboratory to effectively detect species of *Enterobacter* producing extend-
ed-spectrum β-lactamases, a combination of cefotaxime with cefazidime and aztreonam should be included in the test panel for routine susceptibility testing. Strains of *Enterobacter* which are resistant to cefazidime and aztreonam but which appear susceptible to cefotaxime should be screened for possible extended-spectrum β-lactamase production by a method such as the double-disk potentiation test. This will ensure that the majority of extended-spectrum β-lactamase-producing *Entero-
bacter* strains will be detected. The detection of these strains is of vital importance, because they can be responsible for the spread of resistance genes in a hospital setting (29). One iso-
late in this study, *E. aerogenes* 187, producing an enzyme re-
sembling SHV-3, showed decreased susceptibility but not re-
sistance to cefazidime, cefotaxime, and aztreonam. The detection of these strains not showing frank resistance to the expanded-spectrum cephalosporins or aztreonam remains a challenge for the clinical laboratory.

*Enterobacter* spp. are important nosocomial pathogens (29). Because of the popularity of the expanded-spectrum cephalo-
spirins, the prevalence of *Enterobacter* spp. will probably con-
tinue to increase. Thus, the challenge to clinicians and micro-
biologists to recognize susceptibility patterns indicative of the presence of specific β-lactamases, such as the extended-spec-

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### TABLE 3. Characteristics of *Enterobacter* strains and their respective transconjugants

<table>
<thead>
<tr>
<th>Strain¹</th>
<th>pl(s) of β-lactamases</th>
<th>Approximate size (kb) of plasmid(s)</th>
<th>Antibiotic inhibition zone diam (mm)²</th>
<th>Most likely β-lactamase</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli C600N</td>
<td>8.3, 6.8</td>
<td>50, 10</td>
<td>28</td>
<td>CAZ, ATM, GM, SXT</td>
</tr>
<tr>
<td><em>E. aerogenes</em> 187</td>
<td>6.8</td>
<td>50</td>
<td>20</td>
<td>AmpC, SHV-3</td>
</tr>
<tr>
<td><em>E. coli</em> JP01/tr</td>
<td>8.3, 7.8</td>
<td>60, 50, 14</td>
<td>22</td>
<td>SHV-3</td>
</tr>
<tr>
<td><em>E. aerogenes</em> 200</td>
<td>7.8</td>
<td>50, 10</td>
<td>14</td>
<td>AmpC, SHV-4</td>
</tr>
<tr>
<td><em>E. aerogenes</em> 220</td>
<td>8.3, 7.8</td>
<td>60, 50, 10</td>
<td>17</td>
<td>SHV-4</td>
</tr>
<tr>
<td><em>E. aerogenes</em> JP03/tr</td>
<td>7.8</td>
<td>50</td>
<td>15</td>
<td>AmpC, SHV-5</td>
</tr>
<tr>
<td><em>E. aerogenes</em> 184</td>
<td>8.3, 8.2</td>
<td>50</td>
<td>15</td>
<td>SHV-5</td>
</tr>
<tr>
<td><em>E. coli</em> JP04/tr</td>
<td>8.2</td>
<td></td>
<td>17</td>
<td>SHV-5</td>
</tr>
</tbody>
</table>

¹ *E. aerogenes* 187, 200, 220, and 184 were donors; *E. coli* C600N served as the recipient; and *E. coli* JP01, -2, -3, and -4 were the respective transconjugants.

² CAZ, cefazidime; ATM, aztreonam; GM, gentamicin; SXT, trimethoprim-sulfamethoxazole.
trum β-lactamases, will become even more important as this genus acquires additional antimicrobial resistance mechanisms, as shown in this study.

REFERENCES