Antibiotic Susceptibility of Beta-Lactamase-Producing Strains of Branhamella (Neisseria) catarrhalis

GARY V. DOERN,† KATHY G. SIEBERS, LESLEY M. HALLICK, and STEPHEN A. MORSE

Department of Clinical Pathology and Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon 97201

All 11 clinically significant isolates of Branhamella catarrhalis examined in this study were found to produce β-lactamase. The enzyme was apparently not plasmid associated since extrachromosomal deoxyribonucleic acid was not detected in any of the strains. The β-lactamase activity of all strains was significantly depressed by the β-lactamase inhibitors clavulanic acid and CP 45899. Based on comparisons of relative susceptibility to various β-lactam antibiotics, it was inferred that the β-lactamase of B. catarrhalis was significantly more active against penicillin congeners than against cephalosporin congeners. Most strains were not inhibited by readily achievable serum concentrations of the penicillinase-sensitive penicillins, penicillin G, ampicillin, and amoxicillin. Meticillin was equally ineffective. With rare exceptions, most strains of B. catarrhalis were inhibited by achievable serum concentrations of seven cephalosporins (cephalothin, cephalaparin, cephaloridine, cephalexin, cephemadone, cefaclor, and cefuroxin) and one cephamycin (cefotaxin). All strains were uniformly resistant to clindamycin but were inhibited by achievable serum concentrations of erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. Comparison of geometric mean minimum inhibitory concentrations of all antimicrobial agents tested suggested that B. catarrhalis was most susceptible to cefoxitin, erythromycin, and tetracycline.

MATERIALS AND METHODS

Organisms. The 11 clinically significant isolates of B. catarrhalis and other organisms employed in this study are described in Table 1.

β-Lactamase determinations. β-Lactamase production was determined by the iodometric penicillin-agar overlay method of Baldwin et al. (1) with organisms grown on GC agar (Difco) with or without 1 μg of penicillin G per ml for 16 h at 37°C in 10% CO₂ and by the rapid aciometric tube method of Escamilla (6) with organisms grown on IsoVitaleX-enriched (BBL Microbiology Systems) chocolate agar for 16 h at 35°C in 10% CO₂. A 10-μg/ml solution of penicillin G (GIBCO Laboratories, Grand Island, N.Y.) was used as a substrate in the iodometric penicillin-agar overlay method. A 120-μg/ml solution of penicillin G (Pfizer Inc.) was used as a substrate in the rapid aciometric tube method.

Isolation of plasmid DNA. Cultures of B. catarrhalis and N. gonorrhoeae were grown to a density of 10⁶ colony-forming units per ml in glucose-containing liquid medium as previously described (11). To prevent the loss of plasmids, penicillin G (1 μg/ml) was added to all medium used to grow β-lactamase-producing strains.

Two independent isolation procedures were used to purify supercoiled plasmid deoxyribonucleic acid (DNA). The first procedure was essentially as described by Baron et al. (2). In the second technique, a modification of the Hirt lysis procedure was employed (7). The cells were concentrated 50- to 100-fold by
centrifugation and resuspension in 0.05 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8) containing 25% sucrose. They were then digested with 1 mg of lysozyme (Calbiochem) per ml for 5 min on ice, adjusted to a final concentration of 0.05 M ethylenediaminetetraacetate (EDTA), and incubated for an additional 15 min on ice. The spheroplasts were lysed in 1% (wt/vol) sodium dodecyl sulfate, mixed gently to minimize shearing, and incubated for 20 min at 50°C. The viscous solution was then adjusted to a final concentration of 1 M NaCl and allowed to sit overnight in ice. The resulting viscous lysate was centrifuged at 48,000 x g for 90 min in a Beckman SW 50.1 rotor at 4°C. The supernatant was adjusted to a final concentration of 1.5% Sarkosyl and 1 mg of pronase (Calbiochem, grade B, preincubated for 30 min at 37°C) per ml and incubated for 4 h at 50°C.

The supercoiled DNA was banded by density equilibration centrifugation in cesium chloride-ethidium bromide by adding to the supernatants from either lysis procedure 3.9 g of CsCl and 100 μl of ethidium bromide (10 mg/ml) per 4.0 ml of DNA solution. They were centrifuged 147,000 x g in a Beckman SW 50.1 rotor for 72 h at 17°C. The lower supercoiled band in the B. catarrhalis strains were collected, extracted several times with buffer-saturated butanol, and dialyzed overnight against 0.1 M Tris-hydrochloride, pH 8.1, containing 0.001 M EDTA.

Gel electrophoresis. Vertical 1% agarose (Biorad) gels with 40 mM Tris-hydrochloride-5 mM sodium acetate-0.5 mM EDTA (pH 8.0) electrophoresis buffer were run at 3 V/cm for 9 to 16 h. The gels were stained in electrophoresis buffer containing ethidium bromide (0.5 μg/ml) for 1 h and photographed with Polaroid type 105 film with a mineralight short-wave lamp (Ultra Violet Products, Inc., San Gabriel, Calif.) and a Kodak 23 A orange filter.

Agar dilution MICs. Agar dilution minimum inhibitory concentrations (MICs) were determined essentially by the method described by Washington and Barry (15). B. catarrhalis was propagated on chocolate agar enriched with 1% (vol/vol) IsoVitaleX for 16 h at 35°C in 10% CO. Several colonies were then removed and suspended in 5.0 ml of Mueller-Hinton broth. After incubation at 35°C for 6 to 8 h, the cell suspension was diluted in Mueller-Hinton broth to yield an optical density equivalent to a 0.5 McFarland standard. With a Steers replicator device, 0.001 ml of this suspension was transferred to the surface of Mueller-Hinton agar plates containing 5% chcolatized sheep blood and serial twofold dilutions (0.02 to 80 μg/ml) of the appropriate antibiotics. In this manner, approximately 104 viable log-phase colony-forming units were present in each inoculum spot. The inoculated plates were incubated at 35°C in 10% CO and examined for growth after 20 h. MICs were defined as the lowest concentration of a given antibiotic that inhibited visible surface growth.

The antibiotics tested and the solvents in which they were initially dissolved were as follows: penicillin G (Pfizer), methicillin, cephalothin, cefaclor, cephaloridine, cephalaxin, cephamandole (Lilly), cefoxitin (Merck, Sharp and Dome), cefuroxime (Glaxo Research Ltd.), clindamycin (Upjohn), tetracycline (Lederle), and trimethoprim (Burroughs-Wellcome) in glass-distilled water; ampicillin (Bristol) and amoxycillin (Parke-Davis) in 0.1 M phosphate buffer, pH 8.0; erythromycin (Abbott) and chloramphenicol (Parke-Davis) in 70% ethanol; and sulfamethoxazole (Burroughs-Wellcome) in 0.1 N lactic acid.
Inhibition of \( \beta \)-lactamase by clavulanic acid and CP 45899. Inhibition of \( \beta \)-lactamase activity by clavulanic acid (Beecham Pharmaceuticals) and CP 45899 (Pfizer, Inc.) was determined by comparing broth dilution MICs of penicillin G, ampicillin, methicillin, cefalothin, and cefoxitin against three strains of \( B. \) catarrahuis (318, A1262, A1246) in the presence and absence of each \( \beta \)-lactamase inhibitor. Bacteria were propagated on chocolate agar enriched with 1% (vol/vol) IsoVitaleX for 16 h at 35°C in an atmosphere containing 10% \( \text{CO}_2 \). Inocula were prepared by suspending several colonies in Mueller-Hinton broth containing 5% (vol/vol) Fildes enrichment (MH-F), incubating for 6 to 8 h at 35°C, and then diluting in MH-F to an optical density equivalent to a 1.0 McFarland standard. This suspension was further diluted 1:200 in MH-F to yield a final inoculum of approximately 10\(^3\) viable log-phase bacteria per ml. Portions (1 ml) were transferred to 10-ml volumes of MH-F containing serial twofold dilutions (0.02 to 80 \( \mu \text{g} / \text{ml} \)) of appropriate antibiotics either alone or in the presence of a 10-\( \mu \text{g} / \text{ml} \) final concentration of clavulanic acid or CP 45899. Tubes were incubated at 35°C for 16 h and examined visually for the presence of turbidity. MICs were defined as the lowest concentration of antibiotic in which turbidity was not observed. Incubation of tubes for 24 or 48 h did not alter results.

RESULTS

\( \beta \)-Lactamase production. All 11 clinically significant isolates of \( B. \) catarrahuis produced \( \beta \)-lactamase when tested by the iodometric penicillin-agar overlay method. The clear zones surrounding the colonies were narrow, suggesting that the \( \beta \)-lactamase was essentially cell-associated. No increase in zone diameter was observed after the growth of these isolates on GC agar (Difco) containing 1 \( \mu \text{g} / \text{ml} \) of penicillin G per ml. ATCC 8176 did not produce detectable \( \beta \)-lactamase activity by this procedure. When tested by the rapid acidometric tube assay, these isolates required incubation times of 60 to 75 min for development of the color change indicative of \( \beta \)-lactamase activity. \( \beta \)-Lactamase activity was not evident with shorter periods of incubation. ATCC 8176 remained negative after incubation for up to 180 min.

Extrachromosomal DNA. Agarose gel electrophoresis of the supercoiled DNA from two strains of \( N. \) gonorrhoeae revealed the presence of plasmids. \( N. \) gonorrhoeae strain CS-7 contained the cryptic 2.6 \( \times \) 10\(^2\)-dalton plasmid. The \( \beta \)-lactamase-producing \( N. \) gonorrhoeae strain 76-073389 contained 2.6 \( \times \) 10\(^2\)-dalton, 4.4 \( \times \) 10\(^3\)-dalton, and 24.5 \( \times \) 10\(^3\)-dalton plasmids. No plasmids were detected in any of the strains of \( B. \) catarrahuis examined. Electron microscopy revealed the presence of covalently closed circles with preparations from \( N. \) gonorrhoeae strains CS-7 and 76-073389. No circles were observed with preparations from \( B. \) catarrahuis (G. Wiesehahn, personal communication).

Antibiotic susceptibility. The MICs of 17 antibiotics for the 11 \( \beta \)-lactamase-producing strains of \( B. \) catarrahuis and ATCC 8176 are presented in Table 2. The geometric mean MICs of each antibiotic for the clinical isolates are also shown. A reasonably narrow range of MICs (\( \leq \) 16-fold difference) was observed with the cephalosporins, cephalexin, erythromycin, clindamycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. A wider variation was observed with penicillin G, ampicillin, amoxicillin, and methicillin.

The geometric mean MICs of all 17 antibiotics for the clinical isolates were uniformly higher than the MICs of the corresponding antibiotic for ATCC strain 8176. This variation was most significant with penicillin G, ampicillin, amoxicillin, methicillin, and cefoxitin. The only exceptions were one strain which was susceptible to penicillin and three strains which were susceptible to methicillin. Conversely, nearly all strains were susceptible to each of the other antibiotics tested, the sole exception being a single strain which was not susceptible to cephalixin. ATCC 8176 was uniformly susceptible to all antibiotics except clindamycin. Based on analysis of individual and geometric mean MICs, the 11 clinical isolates of \( B. \) catarrahuis examined in this study were most susceptible to cefoxitin, erythromycin, and tetracycline.

Inhibition of \( \beta \)-lactamase by clavulanic acid and CP 45899. Inhibition of \( \beta \)-lactamase activity was examined by comparing broth dilution MICs of penicillin G, ampicillin, methicillin, cefalothin, and cefoxitin for three strains of \( B. \) catarrahuis (strains 318, A-1262, and A-1246) in the presence and absence of clavulanic acid and CP 45899 (Table 3). The broth dilution MICs of these five antibiotics determined in the absence of \( \beta \)-lactamase inhibitors were equivalent to the MICs of these antibiotics when determined by agar dilution as described above. Penicillin MICs for all three strains were significantly diminished (94 to 98%) by both clavulanic acid and CP 45899. Similarly, both inhibitors depressed the MIC of ampicillin for strains 318 and A-1262. Strain A-1246, a strain with a relatively low ampicillin MIC (0.32 \( \mu \text{g} / \text{ml} \)) in the absence of inhibitors, was not affected by clavulanic acid or CP 45899. The methicillin, cephalothin, and cefoxitin MICs for all three strains...
### Table 2. MICs of 17 antibiotics for isolates of B. catarrhalis

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of strains susceptible at concn (µg/ml):</th>
<th>% Susceptible</th>
<th>Geometric mean MIC</th>
<th>MIC (µg/ml) for ATCC 8176</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤0.16 0.32 0.64 1.25 2.5 5.0 10 20 40 80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin</td>
<td>1 1 6 2 1 1 9 10 1</td>
<td>10.1 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>3 4 2 2 0 1.5 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>1 1 5 1 1 2 0 10.8 0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>2 1 6 1 1 27 12.3 0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cephalothin</td>
<td>1 4 6 100 3.8 0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>1 2 8 100 4.2 0.32</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cephalexin</td>
<td>1 1 3 1 91 0.64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefaclor</td>
<td>5 5 1 100 2.7 0.32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cephamandole</td>
<td>1 2 8 100 4.2 0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>10 1 100 0.5 0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>1 4 2 100 1.2 0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>3 3 4 100 0.4 0.16</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Clindamycin</td>
<td>5 7 0 32.7 20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>9 2 100 1.5 0.32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2 8 1 100 1.5 0.64</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TMP-SMX*</td>
<td>3 3 4 1 100 3.8 0.64</td>
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</tr>
</tbody>
</table>

*Based on susceptibility correlates previously described (3). An MIC of ≤0.2 µg/ml was used as the susceptibility breakpoint for ampicillin and is representative of that applied to staphylococci and other organisms usually susceptible to penicillin. An MIC of ≥10 µg/ml was used as the susceptibility breakpoint for all of the cephalosporin and cefamycin class antibiotics.

**Ampicillin**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Inhibitor</th>
<th>MIC</th>
<th>% Reduction in MIC</th>
<th>MIC</th>
<th>% Reduction in MIC</th>
<th>MIC</th>
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<tbody>
<tr>
<td>Penicillin</td>
<td>None</td>
<td>20 2</td>
<td>98</td>
<td>10 2</td>
<td>98</td>
<td>10 2</td>
<td>97</td>
</tr>
<tr>
<td>CP 45899</td>
<td>Clavulanic acid</td>
<td>0.32 0.16</td>
<td>98</td>
<td>0.32 0.16</td>
<td>98</td>
<td>0.32 0.16</td>
<td>97</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>None</td>
<td>5 5</td>
<td>1.0</td>
<td>5 5</td>
<td>1.0</td>
<td>5 5</td>
<td>1.0</td>
</tr>
<tr>
<td>CP 45899</td>
<td>Clavulanic acid</td>
<td>0.64 0.32</td>
<td>97</td>
<td>0.32 0.16</td>
<td>97</td>
<td>0.32 0.16</td>
<td>50</td>
</tr>
<tr>
<td>Methicillin</td>
<td>None</td>
<td>20 5</td>
<td>0.32</td>
<td>5 5</td>
<td>0.32</td>
<td>5 5</td>
<td>0.32</td>
</tr>
<tr>
<td>CP 45899</td>
<td>Clavulanic acid</td>
<td>10 5</td>
<td>0.5</td>
<td>5 5</td>
<td>0.5</td>
<td>5 5</td>
<td>0.5</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>None</td>
<td>25 5</td>
<td>2.5</td>
<td>5 5</td>
<td>2.5</td>
<td>5 5</td>
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</tr>
<tr>
<td>CP 45899</td>
<td>Clavulanic acid</td>
<td>5 5</td>
<td>0.32</td>
<td>5 5</td>
<td>0.32</td>
<td>5 5</td>
<td>0.32</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>None</td>
<td>0.32 0.32</td>
<td>0.32</td>
<td>0.32 0.32</td>
<td>0.32</td>
<td>0.32 0.32</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*Clavulanic acid or CP 45899 was added at a final concentration of 10 µg/ml.

**A** A 50% reduction in MIC represents a single twofold (one-tube) change which is within the experimental variation of this test and thus cannot be considered significant.

were not significantly depressed by either inhibitor. It should be emphasized that a 50% reduction in MIC represents a single twofold (one-tube) change, which is well within the experimental variation of this test and thus cannot be considered significant.

**DISCUSSION**

Several investigators have reported that clinically significant isolates of *B. catarrhalis* produce beta-lactamase (8, 10, 12-14). In the present study, 11 of 11 strains of *B. catarrhalis*...
implicated as the etiological agents of significant human disease were found to produce \( \beta \)-lactama-
se. \( \beta \)-Lactamase was not detected after short incubation times by a rapid acidometric assay. This assay is useful in detecting constitutive, extracellular \( \beta \)-lactamases such as those produced by Haemophilus influenzae and Neisseria gonorrhoeae. Only after prolonged periods of incubation (60 to 75 min) in the presence of penicillin was the B. catarrhalis \( \beta \)-lactamase detected. Conversely, the iodometric penicillin-
agar overlay method clearly detected \( \beta \)-lacta-
mase production by all clinical isolates of B. catarrhalis. This assay includes preincubation of test organisms in the presence of penicillin for 10 min. No difference was observed when organ-
isms were grown in the presence of penicillin. These results are consistent with the notion that the \( \beta \)-lactamase of B. catarrhalis is strongly cell-associated such that minimal amounts of enzyme are released into the surrounding envi-
ronment when organisms are suspended in fluid media.

Since extrachromosomal DNA was not found in any of the strains of B. catarrhalis, it was con-
cluded that the \( \beta \)-lactamase of B. catarrhalis was not plasmid mediated, but rather of chromo-
osomal origin. This is consistent with the ob-
servations of Hoi-Dang Van et al. (8) who con-
cluded that the \( \beta \)-lactamase of the single B. catarrhalis strain that they examined was also not plasmid mediated. Furthermore, attempts to transfer the \( \beta \)-lactamase phenotype from B. ca-
tarrhalis to other bacteria by means known to transfer plasmid DNA have been unsuccessful (14).

The substrate specificity of the \( \beta \)-lactamase of the 11 strains of B. catarrhalis examined in this study could be inferred from the differential susceptibility of these organisms to various beta-
lactam antibiotics. Three penicillin-sensitive penicillins were significantly less active (i.e., demonstrated higher MICs) than seven cepha-
losporins and one cephamycin. These results are consistent with the observations of Hoi-Dang Van et al. (8) and Percival et al. (14). In contra-
distinction to the \( \beta \)-lactamase of N. gonorrhoeae (4, 8), our data suggest that the \( \beta \)-lactamase of B. catarrhalis possesses minimal or no cepha-
losporinase activity. If this is so, then \( \beta \)-lacta-
mase inhibitors such as clavulanic acid and CP 45899 would depress the penicillin-sensitive penicillin MICs, but would not affect the MICs of the penicillinase-resistant penicillins, cepha-
losporins, or cephamycin. Indeed, both clavu-
lanic acid and CP 45899 markedly lowered pen-
icillin and ampicillin MICs to B. catarrhalis while not significantly altering the MICs of methicillin, cephalothin, or cefoxitin. Similarly, Ninane et al. (12) found that clavulanic acid rendered three \( \beta \)-lactamase-producing clinical isolates of B. catarrhalis very susceptible to amoxicillin. When three patients with presumed B. catarrhalis pneumonia who had failed to respond to either ampicillin or amoxicillin were treated with BRL 25000, a drug containing both amoxicillin and clavulanic acid, clinical cures were rapidly achieved.

Several investigators (8, 14) have observed multiple bands of \( \beta \)-lactamase activity when ex-
tracts of B. catarrhalis were analyzed by isoe-
lectic focusing in polyacrylamide or agarose gels. It is not known whether these bands rep-
resent \( \beta \)-lactamases with different substrate pro-
files or merely isoenzymes with different isoelec-
tric points. Results of the present study suggest that the former might be true. Not only were wide variations observed in the levels of resistance to penicillinase-sensitive penicillins, but the effect of \( \beta \)-lactamase inhibitors on these antibi-
otics was strain dependent. For example, strain A 1246 was significantly less resistant to ampi-
cillin (MIC = 0.32 \( \mu \)g/ml) than penicillin (MIC = 10 \( \mu \)g/ml). The penicillin MIC was reduced 97% by clavulanic acid and 94% by CP 45899. The ampicillin MIC was not affected by either \( \beta \)-lactamase inhibitor. The possibility arises that among the B. catarrhalis \( \beta \)-lactamases, there exists a heterogenous population of enzymes that vary with regard to their activity against the penicillin-sensitive penicillins. Such hetero-
genecity would be manifest in strain-to-strain variation in susceptibilities to these antibiotics.

Since B. catarrhalis is apparently not suscept-
tible to the penicillinase-sensitive penicillins, due in large part to the elaboration of \( \beta \)-lacta-
mase, it was of interest to know what alternative antibiotics might be used in treating human infections caused by this organism. Based on the results of MIC determinations of 17 antibiotics against 11 clinically significant isolates of B. catarrhalis, cefoxitin, erythromycin, and tetra-
cycline were found to be most effective. The geometric mean MICs of these agents were 0.5, 0.4, and 0.4 \( \mu \)g/ml, respectively.

Finally, it should be emphasized that the B. ca-
tarrhalis ATCC 8176 is significantly different from the strains of B. catarrhalis described in this study which were associated with human disease. As previously reported (G. V. Doern and S. A. Morse, submitted for publication), ATCC 8176 possessed several growth character-
istics (growth on nutrient agar at 22°C and ab-
sence of growth on Thayer-Martin medium) dif-
ferent from those of numerous clinically signifi-
cant strains. Similarly, in the present study, ATCC 8176 was uniformly more susceptible to all 17 antibiotics tested when compared with 11
clinical isolates of *B. catarrhalis*. Furthermore, the ATCC strain did not possess \(\beta\)-lactamase activity, whereas the clinical isolates did. The possibility exists that the ATCC strain is representative of nonpathogenic, commensal strains of *B. catarrhalis* and that isolates from clinical material represent a distinct subvariety of this bacterium which in addition to enhanced virulence possesses altered growth and antibiotic susceptibility characteristics. This hypothesis awaits further investigation.

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LITERATURE CITED