Characterization of Ampicillin-Resistant Haemophilus parainfluenzae

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Carriage of ampicillin-resistant (Amp') Haemophilus parainfluenzae has become frequent among children in our community, although carriage of Amp' Haemophilus influenzae remains uncommon. In this study we characterized the mechanism of ampicillin resistance in 27 representative isolates of H. parainfluenzae. As determined by isoelectric focusing, each isolate had a TEM-1 β-lactamase; substrate profiles assessed for enzymes from 10 strains were also consistent with TEM-1 enzyme. Agarose gel electrophoresis revealed a plasmid of 23 to 34 megadaltons in each isolate and a small plasmid (≤4 megadaltons) in 14 isolates. Transfer of ampicillin resistance to H. influenzae Rd was achieved during membrane mating with 14 of 15 donors. The transconjugants exhibited high-level ampicillin resistance (≥50 μg/ml), which was stable despite serial passage of isolates on antibiotic-free media. The transconjugants tested retained fertility. Cryptic plasmids were discovered in 7 of 25 antibiotic-susceptible H. parainfluenzae isolates. Our data suggest that H. parainfluenzae may play an important role in the exchange of Amp' genes among throat bacteria.

Ampicillin resistance in Haemophilus parainfluenzae, which was first described in 1976 (6), usually results from the production of a β-lactamase. A report from a children's hospital in 1978 (33) described equal frequencies of resistance among concurrent isolates of H. parainfluenzae and Haemophilus influenzae. In subsequent surveys, ampicillin resistance has been reported more frequently in H. parainfluenzae. In a national survey of hospital isolates in New Zealand in 1978 (5), β-lactamase activity (β-Lac+) was almost five times more frequent in H. parainfluenzae than in H. influenzae (15.3 versus 3.4%). In 1980 a report from England (18) showed that ampicillin resistance was three times more frequent in H. parainfluenzae than in H. influenzae (21 versus 7.7%). In a recent survey of ambulatory children (26), we found an even greater difference; 86% of the H. parainfluenzae isolates were β-Lac+, compared with 12% of the H. influenzae isolates (P < 0.001). Moreover, 72% of the children carried ampicillin-resistant (Amp') H. parainfluenzae strains, whereas only 3% harbored Amp' H. influenzae strains.

The high frequency of ampicillin resistance in H. parainfluenzae in our community stimulated our interest in defining the basis of resistance in this species. In this report we demonstrate that ampicillin resistance in H. parainfluenzae is mediated by TEM-1 β-lactamases that are specified by plasmids comparable in size to the plasmids in β-Lac+ H. influenzae. Moreover, plasmids were readily transferred from H. parainfluenzae to H. influenzae, producing transconjugants with stable ampicillin resistance and continued fertility.

MATERIALS AND METHODS

Bacterial isolates. The strains included in this study were obtained during a previous survey (26) and were preserved in skim milk suspensions at −70°C. Strains were identified as H. parainfluenzae on the basis of colony and Gram stain morphology, dependence for growth on NAD, and ability to synthesize porphyrins from levulinic acid (13). β-Lactamase activity was detected with a chromogenic cephalosporin substrate (Nitrocefin; Glaxo-Allenburys Research, Greenford, England) (20).

A total of 27 strains were chosen for detailed study; these strains represented the range of colony types, biotypes, and ampicillin resistance levels (as determined by methods described previously) which we encountered (26). H. influenzae strains Rd (Rif' Str' Ery') (23) and DC57 (Str') were used as recipients in mating experiments.

Screening for antibiotic resistance. Tests for resistance to six antibiotics were performed by using a screening procedure (4) in which spot inocula containing approximately 104 colony-forming units of stationary phase bacteria were applied to the surface of brain heart infusion (BHI) agar containing hemin, NAD, thiamine (1 μg/ml), and a critical concentration of antibiotic. The antibiotics used included tetracycline hydrochloride (1 μg/ml), gentamicin sulfate (2 μg/ml), erythromycin lactobionate (16 μg/ml), chlorampheni-
col (2 \( \mu\)g/ml), kanamycin sulfate (16 \( \mu\)g/ml), and rifampin (5 \( \mu\)g/ml). The minimal inhibitory concentration (MIC) of an antibiotic was measured by broth dilution if a strain grew at the screening concentration.

Characterization of \( \beta \)-lactamases. \( \beta \)-Lactamases were first characterized by isoelectric focusing of crude cell lysates, as described by Simpson et al. (28). Bacteria were grown overnight on chocolate agar containing 10 \( \mu\)g of NAD per ml and 2 \( \mu\)g of ampicillin per ml, and the resulting colonies were harvested and suspended in 1.0 ml of 0.01 M phosphate buffer (pH 7.0). The cells were disrupted by ultrasonic treatment for 30 s in an ice water bath. The cell debris was pelleted by centrifugation at 12,000 \( \times \) g for 5 min, and the supernatant (crude enzyme extract) was stored at −20°C. The crude \( \beta \)-lactamase extracts were focused on plates supporting thin layers of polyacrylamide gel containing carrier ampholytes (pH 3.5 to 10; LKB Instruments, Ltd., Bromma, Sweden) (15); the voltage was gradually increased over a period of 2.5 h to 1,000 V and then maintained at this level for an additional 3 h. Enzyme bands were detected by overlaying the gel with Nitrocefin. The positions of bands from clinical isolates were compared with those of defined enzymes, including purified TEM-2 from Escherichia coli (Public Health Laboratory Service, Centre for Applied Microbiology, Porton Down, England) and lysates of \( E.\) coli strains containing TEM-1 (strain 1193E), TEM-2 (strain 1725E), and SHV-1 (strain 2008E), which were kindly provided by Cynthia O’Callaghan, Glaxo Group Research (Greenford) Ltd., Middlesex, England (28). Serial photographs of the enzyme bands were taken with a Polaroid model MP4 Land camera to aid interpretation. The pI's of the major band locations were measured at the stain-free gel margin with a surface electrode.

\( \beta \)-Lactamases were also characterized on the basis of substrate profile and susceptibility to inhibition by cloxacillin (31). Crude extracts were prepared as described above from \( E.\) coli 1193E containing TEM-1 \( \beta \)-lactamase, from two \( \beta \)-Lac\(^+\) \( H.\) influenzae isolates, and from 10 \( \beta \)-Lac\(^+\) \( H.\) parainfluenzae strains. The microiodometric method of Novick (19), as modified by Sykes and Nordström (32), was used to compare the rates of hydrolysis of cloxacillin, carbenicillin, and ampicillin relative to the rate of hydrolysis of benzylpenicillin for each enzyme extract. Susceptibilities of enzymes to inhibition by cloxacillin (10\( ^{-4} \) M) were assessed by the method of Jack and Richmond (10), using Nitrocefin as the substrate (20).

Agarose gel electrophoresis. Isolates were examined for plasmids by using the agarose gel electrophoresis screening technique of Meyers et al. (17). Bacteria were grown overnight on chocolate agar containing NAD and 5 \( \mu\)g of ampicillin per ml and harvested with a spreader. Included in each gel were lysates of strains containing plasmids of known sizes, which were used to calculate the relationship between plasmid mass and distance travelled; the lysates used were from \( H.\) influenzae HK7(RSF007) (30 Mdal) (24), \( E.\) coli J53(S-a) (23 Mdal) (8), \( E.\) coli J53(RP4) (34 Mdal) (9), and \( H.\) parainfluenzae HR-885 (4.1 Mdal) (22). The relationship between plasmid mass and distance travelled was used to estimate the masses of unknown plasmids.

Mating procedure. The ability of \( H.\) parainfluenzae to transfer ampicillin resistance to \( H.\) influenzae strain Rd was assessed by using a membrane mating tech-

nique (23, 29). Donor strains were grown overnight in BHI broth containing NAD and 5 \( \mu\)g of ampicillin per ml. Because of the tendency of most isolates to clump, quantitation of donor cells was difficult: for these experiments, the climax population after overnight incubation was assumed to be 10\(^3\) colony-forming units per ml. The recipient strain (\( H.\) influenzae Rd) was also grown overnight in supplemented BHI broth. Then 10 ml of each broth culture was passed through a membrane filter (pore size, 0.45 \( \mu\)m; type 7102; Falcon Plastics, Oxnard, Calif.), which was placed on a chocolate agar plate and overlaid with 1 mg of DNase (type 1 bovine pancreas; Sigma Chemical Co., St. Louis, Mo.) in 1 ml of buffer. After overnight incubation at 37°C, the bacteria on the filter were suspended in BHI broth, and samples were distributed to chocolate agar plates containing 100 \( \mu\)g of streptomycin per ml, 20 \( \mu\)g of erythromycin per ml, and 15 \( \mu\)g of ampicillin per ml. Colonies growing on this medium were subcultured at least once to fresh plates to verify resistance, identified to species, and tested for ampicillin resistance by the broth tube dilution MIC method. The number of transconjugant colonies on the original plates was counted, and the approximate transfer frequency was estimated. Each strain was mated at least twice.

Testing of transconjugants. The stability of ampicillin resistance in transconjugants was tested by serial passage on antibiotic-free NAD-supplemented chocolate agar. Measurement of the MIC was repeated after 10 subcultures at 2- to 3-day intervals. The isolated colonies on the final subculture plates were flooded with a Nitrocefin solution to test for segregation of \( \beta \)-lactamase activity.

The fertilities of two transconjugants were examined. Resistance was first transferred to \( H.\) influenzae DC57 (Str\(^+\)), and the transcient was subsequently mated with strain Rd. Transfer frequencies were calculated for each mating.

Screening for hemagglutinin. The ability of isolates to agglutinate sheep erythrocytes was determined by using a modification of the method of Kahn and Gromkova (11). Bacteria which had been grown in BHI broth overnight were mixed with a 1% suspension of washed sheep erythrocytes in 0.15 M saline (100 \( \mu\)l of each preparation was added to a round-bottomed microtiter plate). After the mixtures had incubated for 1 h at room temperature, the erythrocyte settling patterns were examined. Suspensions of \( H.\) influenzae served as negative controls. The effects on hemagglutination of 5% D-mannose and 5% D-fructose were also determined.

RESULTS

Resistance to non-\( \beta \)-lactam antibiotics. Included in the tests were 50 Amp\(^+\) and 51 Amp\(^+\) \( H.\) parainfluenzae isolates. One Amp\(^+\) isolate was found to be resistant to tetracycline (MIC, 8 \( \mu\)g/ml), and another Amp\(^+\) strain was resistant to gentamicin (MIC, 4 \( \mu\)g/ml). No antibiotic resistance was detected among the Amp\(^+\) isolates.

Characterization of \( \beta \)-lactamases. The \( \beta \)-lactama-

ses of 27 representative Amp\(^+\) \( H.\) parainfluenzae isolates and 10 Amp\(^+\) \( H.\) influenzae isolates were compared by isoelectric focusing;
each of these enzymes had a pH of 5.4 and aligned precisely with the major band of the TEM-1 control enzymes (Fig. 1). Satellite bands developed with most test enzymes and generally aligned with the satellite bands of the TEM-1 prototype.

The substrate profiles of the 10 *H. parainfluenzae* enzymes were identical, matching those of the *H. influenzae* and *E. coli* TEM-1 enzymes (Table 1). The enzymes from all three species were similarly inhibited by cloxacillin and readily hydrolyzed Nitrocefin.

**Demonstration of plasmids in β-Lac⁺*H. parainfluenzae***. Using the gel screening method, we detected large plasmids in lysates of 24 of 27 strains (Fig. 2). In three strains, plasmid DNA was detected only after ampicillin resistance was transferred to strain Rd, bringing the ultimate plasmid detection rate to 100%. The following four types of large plasmids were detected: 23 megadaltons (Mdal) (eight isolates), 28 Mdal (seven isolates), 30 Mdal (eight isolates), and 34 Mdal (four isolates). In addition, small plasmids (≤4 Mdal) were detected in 14 of the 27 isolates.

We also screened for plasmids 25 *H. parainfluenzae* isolates which were susceptible to all seven antibiotics tested. Four (16%) contained plasmids of 23 Mdal (two isolates) or 28 Mdal (two isolates), and another three harbored small plasmids (≤4 Mdal). The plasmid-containing isolates had no predominant biotype or distinctive characteristics, such as encapsulation, hemolysis, or hemagglutinin.

**Transfer of plasmids to*H. influenzae***. Transfer of ampicillin resistance from *H. parainfluenzae* to *H. influenzae* Rd was achieved with 14 of 15 donors. Each of six strain Rd transconjugants examined by agarose gel electrophoresis had acquired a plasmid of the same size as the plasmid of the donor. Small plasmids did not cotransfer from either of two donors which had both large and small plasmids. The frequency of plasmid transfer was estimated in duplicate for each strain, with eight isolates transferring within the range of 10⁻⁴ to 10⁻⁶ per donor cell and six transferring within the range of 10⁻⁷ to 10⁻⁹ per donor cell. The strain which did not transfer resistance was tested three times.

**Characterization of transconjugants**. The MICs of ampicillin for the transconjugants ranged from 50 to 800 μg/ml, equaling or exceeding the ampicillin MICs for the donor strains. After 10 subcultures on antibiotic-free media, the MICs were unaltered in the nine isolates tested, and no β-lactamase-negative segregants were evident.

Both transconjugants of *H. influenzae* DC57 tested were able to transfer ampicillin resistance to strain Rd; one transferred resistance at a frequency of 10⁻⁴ per donor cell, and the other transferred resistance at a frequency of 10⁻⁸ per donor cell. The *H. parainfluenzae* donors and

![FIG. 1. Crude enzyme extracts were isoelectrically focused, and the β-lactamase bands were located by using a chromogenic cephalosporin substrate. The major enzyme bands of Amp¢ *H. parainfluenzae* strains Hp-255 and Hp-210 and Amp¢ *H. influenzae* strains Hi-123 and Hi32 align with the major band of TEM-1 enzyme at pH 5.4, well separated from the major band of the TEM-2 and SHV-1 enzymes. Satellite enzyme bands of strains Hp-255, Hp-210, and Hi32 were evident after a longer incubation and aligned with the satellite bands of the TEM-1 and strain Hi-123 prototypes. TEM-1d, Dilute TEM-1.](http://aac.asm.org/)

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of strains tested</th>
<th>Rates of hydrolysis of the following antibiotic substrates:</th>
<th>Cloxacillin inhibition of Nitrocefin hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (TEM-1)</td>
<td>1</td>
<td>100</td>
<td>116</td>
</tr>
<tr>
<td><em>H. influenzae</em> b</td>
<td>2</td>
<td>100</td>
<td>125</td>
</tr>
<tr>
<td><em>H. parainfluenzae</em></td>
<td>10</td>
<td>100</td>
<td>114 ± 4.3a</td>
</tr>
</tbody>
</table>

* Rates of hydrolysis relative to penicillin, expressed as percentages.
| Mean ± standard deviation. |
strain Rd transconjugants had identical ampicillin MICs (200 μg/ml).

When the donor strain which was Amp' Tet' was mated with strain Rd, the transconjugant was also Amp' Tet' (tetracycline MIC, 25 μg/ml). Transconjugants with dual resistance were obtained with equal frequency whether ampicillin or tetracycline selection was used. When the donor strain which was Amp' Gen' was mated with strain Rd, the transconjugant was only Amp' (gentamicin MIC, 2 μg/ml).

**Hemagglutinin activity.** Of 30 Amp' isolates, 27 (90%) agglutinated sheep erythrocytes, whereas 24 of 31 Amp' strains were similarly positive (no significant difference). This activity was not associated with particular biotypes or the presence of cryptic plasmids; the strain Rd transconjugants from 11 donor strains with hemagglutinin activity were uniformly negative for this property. Hemagglutination was not blocked by D-mannose or D-fructose. Hemagglutination occurred with all strains that grew as clumps in broth (n = 31) and with some non-clumped strains (20 of 30 strains) (χ² = 9.68; P < 0.01).

**DISCUSSION**

Ampicillin resistance in *H. parainfluenzae* is usually due to the production of a β-lactamase which inactivates ampicillin (5, 6, 16, 18, 26, 33). In the isolates which we studied, the *H. parainfluenzae* enzymes had the same isoelectric focusing characteristics as the TEM-1 enzymes isolated from Amp' *E. coli* and Amp' *H. influenzae* (Fig. 1). Furthermore, the *H. parainfluenzae* enzymes had substrate profiles and susceptibilities to cloxacillin inhibition typical of TEM enzymes (Table 1) (31). The gene coding for the TEM-1 enzyme is located on a transposon (Tn4) (25).

In each β-Lac⁺ *H. parainfluenzae* isolate studied, we found evidence of a large plasmid (≥22 Mdal). In most isolates (89%), plasmids were demonstrated in the strain by gel electrophoresis. In contrast, few (30%) plasmids were identified when β-Lac⁺ *H. influenzae* isolates were screened. Similar results for resistant *H. influenzae* strains have been reported by others (23, 29, 30). Stuy (30) suggested that plasmids are not revealed in resistant plasmid-free strains because they are integrated into the chromosome, whereas Roberts and Smith (23) indicated that plasmids may be broken in lysing and migrate with the chromosome band during electrophoresis.

The following four sizes of large plasmids were detected in β-Lac⁺ *H. parainfluenzae* strains: 23, 28, 30, and 34 Mdal. Plasmids of 22 to 23 Mdal were frequent in our analysis of *H. parainfluenzae* strains but have not been reported previously in *H. influenzae*. However, among 10 recent local β-Lac⁺ *H. influenzae* isolates we found two 23-Mdal R-plasmids. Other large plasmids (28 to 46 Mdal) have been reported in Amp' *H. influenzae* isolates (7, 24, 25).

The large plasmids and β-lactamase production were transferable in vitro from *H. parainfluenzae* donors to *H. influenzae* strain Rd, with frequencies similar to the frequencies in matings between *H. influenzae* donors and *H. influenzae* strain Rd as the recipient (29). The characteristics of the *H. influenzae* transconjugants are noteworthy; resistance to ampicillin was high (within the range usually reported for clinical isolates) and stable upon serial subculturing. In some cases the transconjugant had a significantly higher level of ampicillin resistance than the donor. The fertility of both transconjugants tested was maintained; each was able to transfer its plasmid and ampicillin resistance to another *H. influenzae* strain.

*H. parainfluenzae* strains resistant to tetracycline (1, 27), chloramphenicol (1, 27), co-trimoxazole (1, 5), or combinations of antibiotics have been described previously. However, resistance to non-β-lactam antibiotics was uncommon among our isolates. Two Amp' strains were resistant to tetracycline or gentamicin, but only the tetracycline resistance was transferable.

In view of the high frequency of Amp' *H. parainfluenzae* strains in the children whom we have studied (26), we wondered whether these strains possess an additional factor that promotes colonization. Kahn and Gromkova (11)
recently described a mannose-resistant hemagglutinin in 11 of 18 nonpiliated \textit{H. parainfluenzae} isolates, which might enhance colonization of mucosal surfaces. We confirmed the presence of a hemagglutinin in \textit{H. parainfluenzae}, detecting activity in 51 of 61 isolates (84\%). This hemagglutinin did not appear to confer a selective advantage upon Amp\(^{\beta}\) isolates since this property was equally frequent in Amp\(^{\beta}\) strains and was not plasmid associated.

As information accumulates, \textit{H. parainfluenzae} seems increasingly suited as a vector for transfer of antibiotic resistance genes from the enteric pool to \textit{H. influenzae}. A missing link has been postulated (3) because the core plasmids of \textit{H. influenzae} do not resemble those of enteric bacteria but have guanine-plus-cytosine contents similar to the content of the \textit{H. influenzae} chromosome. However, cryptic plasmids which might accept transposons from enteric plasmids have been detected rarely in \textit{H. influenzae} (3, 12). We identified cryptic plasmids in 7 of 25 \textit{H. parainfluenzae} isolates; 4 of these plasmids had 23 or 28 Mda mass. Other workers (14) have reported small cryptic plasmids (≤4 Mda). Recently, Palmer (21) detected \textit{Haemophilus} strains (largely \textit{H. parainfluenzae}) in 26\% of the fecal samples tested, often in large numbers, suggesting that \textit{H. parainfluenzae} may also reside in the gastrointestinal tract. Proximities with enteric bacteria in the gut and with \textit{H. influenzae} in the throat would set the stage for \textit{H. parainfluenzae} to act as a gene transfer vector. Further studies will be needed to assess the molecular relationships between plasmids in \textit{Haemophilus} species and the ability of \textit{H. parainfluenzae} to receive R-plasmids or transposons from enteric bacteria.

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\textbf{LITERATURE CITED}


