**bla**<sub>ROB-1</sub> Presence on pB1000 in *Haemophilus influenzae* Is Widespread, and Variable Cefaclor Resistance Is Associated with Altered Penicillin-Binding Proteins<sup>7</sup>

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Approximately 95% of β-lactamase-positive *Haemophilus influenzae* isolates have bla<sub>TEM-1</sub>-like, with the remainder having bla<sub>ROB-1</sub> (5). bla<sub>TEM-1</sub> is widespread in *Enterobacteriaceae* and probably transposed onto native *Haemophilus* plasmids, where it is usually on large (40 kb) chromosomally integrated conjugative elements (ICEs) or occasionally on small (4 kb) non-conjugative plasmids (11, 16). bla<sub>ROB-1</sub> has been reported on small (4 to 5 kb) plasmids in various human and animal isolates belonging to the family *Pasteurellaceae* (16). Recently, bla<sub>ROB-1</sub> was described on a small plasmid (pB1000) in clonally identical *Haemophilus parasuis* isolates in diseased pigs in Spain (12). The same plasmid and derivatives (pB1000<sup>'</sup> and pB1002) were subsequently found in animal isolates of *Pasteurella multocida* and in genetically unrelated bla<sub>ROB-1</sub>-Positive human *Haemophilus influenzae* isolates, also from Spain (13, 14). The plasmid was shown to be mobilizable into *H. influenzae* from *Escherichia coli* with broad-host-range IncP conjugation machinery, but mobilization within *Pasteurellaceae* or *Haemophilus* was not investigated (13, 14). The presence of bla<sub>ROB-1</sub> is usually associated with cefaclor resistance; however, in some studies, only 70% of bla<sub>ROB-1</sub>-positive *H. influenzae* isolates were resistant to cefaclor by CLSI criteria (5, 8).

The aim of this study was to determine if bla<sub>ROB-1</sub> is found on pB1000-like plasmids in *H. influenzae* isolates outside Spain, to investigate the plasmid’s mobility within *H. influenzae*, and to determine the molecular basis for variable cefaclor resistance.

Eleven clinical isolates of bla<sub>ROB-1</sub>-positive *H. influenzae* from North America (n = 9) and Australia (n = 2), collected between 1999 and 2003 (5, 18), were characterized by PCR for bla<sub>ROB-1</sub>/TEM-1<sup>1</sup> and ICEs and by pulsed-field gel electrophoresis (PFGE) for strain relatedness, as previously described (4, 11, 18). Extracted plasmids were characterized with PstI and Sau3 digests, and various transformants were produced using electroporation as previously described (19). Susceptibility testing was performed using CLSI methodology (1–3) and Etest, and sequencing of bla<sub>ROB-1</sub>, *ftsI*, and the pB1000 plasmid performed as previously described (7, 12, 19). Results are summarized in Table 1.

Of the 11 bla<sub>ROB-1</sub>-positive isolates, 5 were also bla<sub>TEM-1</sub>-positive, which is surprising, as isolates with both β-lactamases are uncommon (5, 8). The presence of ICEs in the bla<sub>TEM-1</sub>-positive isolates is consistent with the results of previous studies, but the presence of an ICE in one bla<sub>TEM-1</sub>-negative isolate was unexpected, as these “cryptic plasmids” are very rare (11). There were major discrepancies for cefaclor susceptibility, as all isolates tested susceptible by disk diffusion and three tested intermediate or resistant by Etest, but all tested intermediate or resistant by reference broth microdilution. However, the range of cefaclor MICs seen is consistent with that seen by Karlowsky et al. (8). To further investigate these discrepancies, the 11 isolates were similarly tested for cefotaxime susceptibility, and 9 previously characterized bla<sub>TEM-1</sub>-positive isolates were similarly tested for cefaclor susceptibility. In both cases, the disk diffusion and Etest results correlated with the broth microdilution results, indicating that the initial discrepancies are specific to bla<sub>ROB-1</sub> and cefaclor. The most likely explanation is “the inoculum effect,” observed in broth methods but not diffusion methods and, in this instance, seen with ROB-1 and cefaclor because cefaclor is a good substrate for the enzyme but not with ROB-1 and cefotaxime or TEM-1 and cefaclor because of the weak activity of the enzymes against the respective substrates (6). Similar discrepancies between Etest and broth microdilution results have been reported for TEM-1 and ampicillin, which the enzyme readily hydrolyzes (17).

Previous suggestions for variation in cefaclor MICs in ROB-1-producing isolates include bla<sub>ROB-1</sub> mutations and associated changes in β-lactamase activity or expression or coexisting alterations in penicillin-binding protein 3 (PBP3) (5, 8). The bla<sub>ROB-1</sub> sequences for the 11 isolates in this study were identical to the published sequence (GenBank accession no. AF022114), ruling out changes in expression associated with
promoter variation or changes in activity being responsible for the variation in cefaclor MICs. \textit{H. influenzae} Rd was transformed with plasmids from representative isolates, and the identical cefaclor MICs in the transformants, irrespective of the MIC of the organism from which the plasmid originated, support the conclusion that the variability in cefaclor MICs in the wild isolates is independent of \textit{bla}_{ROB-1} and the plasmid carrying it. However, the finding of PBP3 substitutions in isolates with the highest cefaclor MICs may be significant. The substitutions R517H, V547I, D350N, and N569S have all been associated with \textit{bla}\textsubscript{BLNAR} isolates, and V489G and M503R are adjacent to positions 490 and 501/502 where BLNAR-associated substitutions are known to occur (16, 19). We propose that, in our isolates, these substitutions augment the cefaclor resistance associated with \textit{ROB-1} to produce higher MICs. Altered PBP3 substitutions in \textit{ICE}\textsubscript{H. influenzae} Rd were transconjugants with \textit{pB1000} and \textit{bla}\textsubscript{TEM-1} alone) with streptomycin and cefaclor. Both donors produced numerous transconjugants on cefaclor plates (even at concentrations as high as 32 \(\mu\)g/ml), but unfortunately, of 100 transconjugants tested, all were \textit{bla}\textsubscript{TEM-1} positive but \textit{bla}\textsubscript{ROB-1} negative by PCR. When tested by broth microdilution, these transconjugants had cefaclor MICs of 1 \(\mu\)g/ml as expected with \textit{TEM-1}, but at the postconjugation inoculum required to detect cells with both conjugatively transferred \textit{bla}_{TEM-1} and the mobilized \textit{bla}_{ROB-1} produced breakthrough growth.

An alternative strategy was devised using \textit{pHS-Tet} (GenBank accession no. AY862435) as a \textit{pB1000} surrogate, as \textit{pHS-Tet} is almost identical, but with \textit{tet} (B) instead of \textit{bla}_{ROB-1} and identical mobilization genes (10, 12). Using \textit{H. influenzae} Rd \textit{Str}\textsuperscript{r} \textit{ICE}_{HinRd} (\textit{pHS-Tet}) constructed during this study as donor and an \textit{H. influenzae} Rd (\textit{Nat}\textsuperscript{r}) strain as recipient, transconjugants with only ICE of \textit{H. influenzae} isolate \textit{F48} (\textit{ICE}_{HinF48}) were produced at a frequency of 10\textsuperscript{-3} per recipient, and those with both \textit{ICE}_{HinRd} and the mobilized \textit{pHS-Tet} were produced at a frequency of 10\textsuperscript{-8} per recipient. The genotypes of transconjugants were confirmed using PCR for ICEs, \textit{bla}_{TEM-1}, and \textit{pHS-Tet} (10); spontaneous nalidixic acid-resistant mutant donors were excluded by demonstrating streptomycin susceptibility in the transconjugants, and transfer of \textit{pHS-Tet} by transformation was excluded by use of DNase I during conjugation as previously described (9).

Although we have not demonstrated mobilization of \textit{pB1000} between isolates of \textit{H. influenzae} using conjugative plasmids native to \textit{H. influenzae}, it seems reasonable to conclude that, given their similarity, \textit{pB1000} would be mobilized comparably to \textit{pHS-Tet}. A recent study demonstrated a fitness cost in \textit{H. influenzae} with \textit{pB1000} over isolates without and suggested that this might explain the relatively low frequency of \textit{bla}_{ROB-1}

### TABLE 1. Results for characterization of clinical strains and transconformants

<table>
<thead>
<tr>
<th>Strain</th>
<th>\textit{bla} type(s)</th>
<th>Presence of ICE</th>
<th>Cefaclor MIC (mg/liter) \textsuperscript{a}</th>
<th>Substitutions in PBP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3</td>
<td>ROB-1</td>
<td>−</td>
<td>4 (S)</td>
<td>None</td>
</tr>
<tr>
<td>F50</td>
<td>ROB-1</td>
<td>−</td>
<td>2 (S)</td>
<td>None</td>
</tr>
<tr>
<td>F48</td>
<td>ROB-1, TEM-1</td>
<td>+</td>
<td>6 (S)</td>
<td>None</td>
</tr>
<tr>
<td>F52</td>
<td>ROB-1, TEM-1</td>
<td>+</td>
<td>6 (S)</td>
<td>None</td>
</tr>
<tr>
<td>H23</td>
<td>ROB-1</td>
<td>−</td>
<td>2 (S)</td>
<td>None</td>
</tr>
<tr>
<td>H62</td>
<td>ROB-1</td>
<td>−</td>
<td>6 (S)</td>
<td>None</td>
</tr>
<tr>
<td>F45</td>
<td>ROB-1</td>
<td>+</td>
<td>24 (I)</td>
<td>D350N, V547I, N569S</td>
</tr>
<tr>
<td>F49</td>
<td>ROB-1, TEM-1</td>
<td>+</td>
<td>16 (I)</td>
<td>D350N, V547I, N569S</td>
</tr>
<tr>
<td>F54</td>
<td>ROB-1, TEM-1</td>
<td>+</td>
<td>32 (R)</td>
<td>V489G, M503R, R517H, V547I, N569S</td>
</tr>
<tr>
<td>F41</td>
<td>ROB-1</td>
<td>−</td>
<td>4 (S)</td>
<td>None</td>
</tr>
<tr>
<td>F47</td>
<td>ROB-1, TEM-1</td>
<td>+</td>
<td>4 (S)</td>
<td>None</td>
</tr>
<tr>
<td>Rd</td>
<td>None</td>
<td>−</td>
<td>0.5 (S)</td>
<td>None</td>
</tr>
<tr>
<td>Rd (\Omega)</td>
<td>ROB-1</td>
<td>−</td>
<td>4–8 (S)</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

\(\text{a}\) CLSI breakpoints for cefaclor: susceptible (S), \(\leq 8\) mg/liter; intermediate (I), 16 mg/liter; resistant (R), \(\geq 32\) mg/liter.

\(\Omega\), strain Rd transformants with \textit{pB1000} and \textit{bla}_{ROB-1} from donors F3, F50, F45, F49, F54, and F47.
compared to that of blaTEM-1 in H. influenzae isolates (14). Our work suggests that the relatively higher frequency with which ICEs bearing blaTEM-1 can be conjugatively transferred compared to that at which pB1000 is likely to be mobilized might also be a significant factor. This hypothesis is supported by the observation that, of the approximately 95% of β-lactamase-positive H. influenzae isolates that are blaTEM-1 positive, only 5% have blaTEM-1 on small nonconjugative plasmids rather than the more common ICEs (11). Therefore, it could be proposed that the nature of the replicon, i.e., ICE with blaTEM-1 (90%) or with small nonconjugative plasmids (5% each for blaTEM-1 and blaROB-1), might be a major determining factor in the frequency of these genes in H. influenzae.

**Nucleotide sequence accession numbers.** The nucleotide sequences for pB1000 in strains F50 and F45 and for ftsI genes in strains F45, F49, and F54 have been assigned GenBank accession numbers HM236408 to HM236412, respectively.

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**REFERENCES**


