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bla$_{ROB-1}$ Presence on pB1000 in *Haemophilus influenzae* Is Widespread, and Variable Cefaclor Resistance Is Associated with Altered Penicillin-Binding Proteins▽

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Plasmid pB1000 is a small replicon recently identified as bearing *bla*$_{ROB-1}$ in animal and human *Pasteurellaceae* in Spain. We identified pB1000 in 11 *bla*$_{ROB-1}$-positive Australian and North American *Haemophilus influenzae* isolates, suggesting a wider role for pB1000 in disseminating *bla*$_{ROB-1}$. Native *H. influenzae* conjugative elements can mobilize plasmids similar to pB1000 at a low frequency of 10^{-8}, and this might account for the infrequency of *bla*$_{ROB-1}$ compared to the rate of occurrence of *bla*$_{TEM-1}$. Altered penicillin-binding protein 3 was associated with an increased cefaclor MIC in 3 isolates.

Approximately 95% of β-lactamase-positive *Haemophilus influenzae* isolates have *bla*$_{TEM-1}$, with the remainder having *bla*$_{ROB-1}$ (5). *bla*$_{TEM-1}$ 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) nonconjugative plasmids (11, 16). *bla*$_{ROB-1}$ has been reported on small (4 to 5 kb) plasmids in various human and animal isolates belonging to the family *Pasteurellaceae* (16). Recently, *bla*$_{ROB-1}$ 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′ and pB1002) were subsequently found in animal isolates of *Pasteurella multocida* and in genetically unrelated *bla*$_{ROB-1}$-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*$_{ROB-1}$ is usually associated with cefaclor resistance; however, in some studies, only 70% of *bla*$_{ROB-1}$-positive *H. influenzae* isolates were resistant to cefaclor by CLSI criteria (5, 8).

The aim of this study was to determine if *bla*$_{ROB-1}$ 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*$_{ROB-1}$-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*$_{ROB-1/TEM-1}$ 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*$_{ROB-1}$, *ftsI*, and the pB1000 plasmid performed as previously described (7, 12, 19). Results are summarized in Table 1.

Of the 11 *bla*$_{ROB-1}$-positive isolates, 5 were also *bla*$_{TEM-1}$ positive, which is surprising, as isolates with both β-lactamases are uncommon (5, 8). The presence of ICEs in the *bla*$_{TEM-1}$-positive isolates is consistent with the results of previous studies, but the presence of an ICE in one *bla*$_{TEM-1}$-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*$_{TEM-1}$-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*$_{ROB-1}$ 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*$_{ROB-1}$ mutations and associated changes in β-lactamase activity or expression or coexisting alterations in penicillin-binding protein 3 (PBP3) (5, 8). The *bla*$_{ROB-1}$ 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

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TABLE 1. Results for characterization of clinical strains and transformants

Strain	*bla* type(s)	Presence of ICE	Cefaclor MIC (mg/liter)[a] in:		Substitutions in PBP3
			Etest	Microbroth	
F3	ROB-1	−	4 (S)	16 (I)	None
F50	ROB-1	−	2 (S)	16 (I)	None
F48	ROB-1, TEM-1	+	6 (S)	16 (I)	None
F52	ROB-1, TEM-1	+	6 (S)	16 (I)	None
H23	ROB-1	−	2 (S)	16 (I)	None
H62	ROB-1	−	6 (S)	32 (R)	None
F45	ROB-1	+	24 (I)	64 (R)	D350N, V547I, N569S
F49	ROB-1, TEM-1	+	16 (I)	64 (R)	D350N, V547I, N569S
F54	ROB-1, TEM-1	+	32 (R)	64 (R)	V489G, M503R, R517H, V547I, N569S
F41	ROB-1	−	4 (S)	32 (R)	None
F47	ROB-1, TEM-1	+	4 (S)	32 (R)	None
Rd	None	−	0.5 (S)	1 (S)	None
Rd Ω[b]	ROB-1	−	4–8 (S)	32 (R)	Not tested

[a] CLSI breakpoints for cefaclor: susceptible (S), ≤8 mg/liter; intermediate (I), 16 mg/liter; resistant (R), ≥32 mg/liter.
[b] Ω, strain Rd transformants with pB1000 and $bla_{\text{ROB-1}}$ from donors F3, F50, F45, F49, F54, and F47.

promoter variation or changes in activity being responsible for the variation in cefaclor MICs. *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 $bla_{\text{ROB-1}}$ 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 β-lactamase-negative, ampicillin-resistant (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 ROB-1 to produce higher MICs. Altered PBP3 in combination with TEM-1 is well recognized in *H. influenzae*, but altered PBP3 has not previously been described in association with ROB-1 (16).

All 11 $bla_{\text{ROB-1}}$-positive isolates in this study produced a 4.6-kb plasmid with identical restriction patterns consistent with its identity as pB1000 (12, 13), which was confirmed when plasmid sequences from F45 and F50 differed from the sequence of pB1000 (GenBank accession no. DQ840517) at only 6 positions, none of which were in open reading frames. The PFGE results indicate that, although three pairs of isolates (F52/F48, F50/F3, and H62/H23) were closely related, the remainder were genetically diverse, as were the four pB1000-positive *H. influenzae* isolates reported from Spain (14). These observations are consistent with a wider role for pB1000 in the dissemination of $bla_{\text{ROB-1}}$.

Conjugation experiments were conducted using a 5:1 recipient-to-donor ratio on chocolate agar as previously described (15), with transconjugants selected on chocolate agar with a combination of streptomycin (500 μg/ml) or nalidixic acid (6 μg/ml) and cefaclor (8, 16, or 32 μg/ml), ampicillin (4 μg/ml), or tetracycline (2 μg/ml) as appropriate. Plasmid pB1000 from donor F3, F50, F45, F49, or F54 could not be mobilized into *H. influenzae* Rd (Str^r). This was expected for F3 and F50, as pB1000 itself is nonconjugative and the experiment was conducted to exclude mobilization by undetected donor conjugative elements. Isolate F45 was included because an ICE was present, and the failure of this element to mobilize pB1000 was not further investigated. However, the nature of this ICE is unknown, and the absence of a selectable marker made it impossible to check for conjugative transfer of the ICE irrespective of the mobilization of pB1000. To overcome this, F49 and F54 were used with the intention to select for transconjugants with mobilized pB1000 (but not those with ICE and $bla_{\text{TEM-1}}$ alone) with streptomycin and cefaclor. Both donors produced numerous transconjugants on cefaclor plates (even at concentrations as high as 32 μg/ml), but unfortunately, of 100 transconjugants tested, all were $bla_{\text{TEM-1}}$ positive but $bla_{\text{ROB-1}}$ negative by PCR. When tested by broth microdilution, these transconjugants had cefaclor MICs of 1 μg/ml, as expected with TEM-1, but at the postconjugation inoculum required to detect cells with both conjugatively transferred $bla_{\text{TEM-1}}$ and the mobilized $bla_{\text{ROB-1}}$, TEM-1 produced breakthrough growth.

An alternative strategy was devised using pHS-Tet (GenBank accession no. AY862435) as a pB1000 surrogate, as pHS-Tet is almost identical, but with *tet*(B) instead of $bla_{\text{ROB-1}}$ and identical mobilization genes (10, 12). Using *H. influenzae* Rd (Str^r $\text{ICE}_{Hin\text{F49}}$/pHS-Tet) constructed during this study as donor and an *H. influenzae* Rd (Nal^r) strain as recipient, transconjugants with only ICE of *H. influenzae* isolate F49 ($\text{ICE}_{Hin\text{F49}}$) were produced at a frequency of 10^{-3} per recipient, and those with both $\text{ICE}_{Hin\text{F49}}$ and the mobilized pHS-Tet were produced at a frequency of 10^{-8} per recipient. The genotypes of transconjugants were confirmed using PCR for ICEs, $bla_{\text{TEM-1}}$, and pHS-Tet (10); spontaneous nalidixic acid-resistant mutant donors were excluded by demonstrating streptomycin susceptibility in the transconjugants, and transfer of pHS-Tet by transformation was excluded by use of DNase I during conjugation as previously described (9).

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

compared to that of bla_{TEM-1} in *H. influenzae* isolates (14). Our work suggests that the relatively higher frequency with which ICEs bearing bla_{TEM-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 bla_{TEM-1} positive, only 5% have bla_{TEM-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 bla_{TEM-1} (90%) or with small nonconjugative plasmids (5% each for $bla_{TEM-1}$and bla_{ROB-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

1. **Clinical and Laboratory Standards Institute.** 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 8th ed. Approved standard M07-A8. CLSI, Wayne, PA.
2. **Clinical and Laboratory Standards Institute.** 2009. Performance standards for antimicrobial disk susceptibility tests, 10th ed. Approved standard M02-A10. CLSI, Wayne, PA.
3. **Clinical and Laboratory Standards Institute.** 2010. Performance standards for antimicrobial susceptibility testing; 20th informational supplement. M100-S20. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Dabernat, H., C. Delmas, M. Seguy, R. Pelissier, G. Faucon, S. Bennamani, and C. Pasquier.** 2002. Diversity of β-lactam resistance-conferring amino acid substitutions in penicillin-binding protein 3 of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **46:**2208–2218.
5. **Farrell, D. J., I. Morrissey, S. Bakker, S. Buckridge, and D. Felmingham.** 2005. Global distribution of TEM-1 and ROB-1β-lactamases in *Haemophilus influenzae*. J. Antimicrob. Chemother. **56:**773–776.
6. **Galan, J.-C., M.-I. Morosini, M.-R. Baquero, M. Reig, and F. Baquero.** 2003. *Haemophilus influenzae* bla_{ROB-1} mutations in hypermutagenic ΔampC *Escherichia coli* conferring resistance to cefotaxime and β-lactamase inhibitors and increased susceptibility to cefaclor. Antimicrob. Agents Chemother. **47:**2551–2557.
7. **Juteau, J. M., and R. C. Levesque.** 1990. Sequence analysis and evolutionary perspectives of ROB-1 β-lactamase. Antimicrob. Agents Chemother. **34:** 1354–1359.
8. **Karlowsky, J. A., G. Verma, G. G. Zhanel, and D. J. Hoban.** 2000. Presence of ROB-1 β-lactamase correlates with cefaclor resistance among recent isolates of *Haemophilus influenzae*. J. Antimicrob. Chemother. **45:**871–875.
9. **Kuipers, E. J., D. A. Israel, J. G. Kusters, and M. J. Blaser.** 1998. Evidence for a conjugation-like mechanism of DNA transfer in Helicobacter pylori. J. Bacteriol. **180:**2901–2905.
10. **Lancashire, J. F., T. D. Terry, P. J. Blackall, and M. P. Jennings.** 2005. Plasmid-encoded Tet B tetracycline resistance in *Haemophilus parasuis*. Antimicrob. Agents Chemother. **49:**1927–1931.
11. **Leaves, N. I., I. Dimopoulou, I. Hayes, S. Kerridge, T. Falla, O. Secka, R. A. Adegbola, M. P. Slack, T. E. Peto, and D. W. Crook.** 2000. Epidemiological studies of large resistance plasmids in Haemophilus. J. Antimicrob. Chemother. **45:**599–604.
12. **San Millan, A., J. A. Escudero, A. Catalan, S. Nieto, F. Farelo, M. Gibert, M. A. Moreno, L. Dominguez, and B. Gonzalez-Zorn.** 2007. β-Lactam resistance in *Haemophilus parasuis* is mediated by plasmid pB1000 bearing bla_{ROB-1}. Antimicrob. Agents Chemother. **51:**2260–2264.
13. **San Millan, A., J. A. Escudero, B. Gutierrez, L. Hidalgo, N. Garcia, M. Llagostera, L. Dominguez, and B. Gonzalez-Zorn.** 2009. Multiresistance in Pasteurella multocida is mediated by coexistence of small plasmids. Antimicrob. Agents Chemother. **53:**3399–3404.
14. **San Millan, A., S. Garcia-Cobos, J. A. Escudero, L. Hidalgo, B. Gutierrez, L. Carrilero, J. Campos, and B. Gonzalez-Zorn.** 2010. Haemophilus influenzae clinical isolates with plasmid pB1000 bearing blaROB-1: fitness cost and interspecies dissemination. Antimicrob. Agents Chemother. **54:**1506–1511.
15. **Stuy, J. H.** 1985. Transfer of genetic information within a colony of Haemophilus influenzae. J. Bacteriol. **162:**1–4.
16. **Tristram, S., M. R. Jacobs, and P. C. Appelbaum.** 2007. Antimicrobial resistance in *Haemophilus influenzae*. Clin. Microbiol. Rev. **20:**368–389.
17. **Tristram, S. G.** 2008. A comparison of Etest, M.I.C.Evaluator strips and CLSI broth microdilution for determining {beta}-lactam antimicrobial susceptibility in Haemophilus influenzae. J. Antimicrob. Chemother. **62:**1464–1466.
18. **Tristram, S. G., and S. Nichols.** 2006. A multiplex PCR for β-lactamase genes of Haemophilus influenzae and description of a new blaTEM promoter variant. J. Antimicrob. Chemother. **58:**183–185.
19. **Ubukata, K., Y. Shibasaki, K. Yamamoto, N. Chiba, K. Hasegawa, Y. Takeuchi, K. Sunakawa, M. Inoue, and M. Konno.** 2001. Association of amino acid substitutions in penicillin-binding protein 3 with β-lactam resistance in β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob. Agents Chemother. **45:**1693–1699.