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Genetic and Molecular Characterization of β-Lactamase-Negative Ampicillin-Resistant *Haemophilus influenzae* with Unusually High Resistance to Ampicillin

Frank S. Kaczmarek,[1] Thomas D. Gootz,[1]* Fadia Dib-Hajj,[1] Wenchi Shang,[1] Shawn Hallowell,[2] and Melissa Cronan[2]

Department of Immunology and Infectious Disease[1] and Department of Molecular Sciences,[2] Pfizer Global Research and Development, Groton, Connecticut 06340

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Previous studies with beta-lactamase-negative, ampicillin-resistant (BLNAR) *Haemophilus influenzae* from Japan, France, and North America indicate that mutations in *ftsI* encoding PBP3 confer ampicillin MICs of 1 to 4 μg/ml. Several BLNAR strains with ampicillin MICs of 4 to 16 μg/ml recently isolated from North America were studied. Pulsed-field gel electrophoresis identified 12 unique BLNAR strains; sequencing of their *ftsI* transpeptidase domains identified 1 group I and 11 group II mutants, as designated previously (K. Ubukata, Y. Shibasaki, K. Yamamoto, N. Chiba, K. Hasegawa, Y. Takeuchi, K. Sunakawa, M. Inoue, and M. Konno, Antimicrob. Agents Chemother. 45:1693-1699, 2001). Geometric mean ampicillin MICs for several clinical isolates were 8 to 10.56 μg/ml. Replacement of the *ftsI* gene in *H. influenzae* Rd with the intact *ftsI* from several clinical isolates resulted in integrants with typical BLNAR geometric mean ampicillin MICs of 1.7 to 2.2 μg/ml. Cloning and purification of His-tagged PBP3 from three clinical BLNAR strains showed significantly reduced Bocillin binding compared to that of PBP3 from strain Rd. Based on these data, changes in PBP3 alone could not account for the high ampicillin MICs observed for these BLNAR isolates. In an effort to determine the presence of additional mechanism(s) of ampicillin resistance, sequencing of the transpeptidase regions of *pbp1a*, *-1b*, and *-2* was performed. While numerous changes were observed compared to the sequences from Rd, no consistent pattern correlating with high-level ampicillin resistance was apparent. Additional analysis of the resistant BLNAR strains revealed frame shift insertions in *acrR* for all four high-level, ampicillin-resistant isolates. *acrR* was intact for all eight low-level ampicillin-resistant and four ampicillin-susceptible strains tested. A knockout of *acrB* made in one clinical isolate (initial mean ampicillin MIC of 10.3 μg/ml) lowered the ampicillin MIC to 3.67 μg/ml, typical for BLNAR strains. These studies illustrate that BLNAR strains with high ampicillin MICs exist that have combined resistance mechanisms in PBP3 and in the AcrAB efflux pump.

Despite the extensive utilization of antimicrobial therapies and the availability of the *Haemophilus influenzae* type b vaccine, *H. influenzae* remains a major pathogen in bronchopulmonary as well as ear, nose, and throat infections. Following the broad use of the *H. influenzae* type b vaccine in developed countries, noninvasive, noncapsulated *H. influenzae* are the principle source of such respiratory tract infections (26, 31). Macrolides and beta-lactams have been the most commonly prescribed antimicrobials used to treat infections with *H. influenzae*; however, it has proven difficult to improve the modest potency of macrolides against this organism due to expression of the efflux pump AcrAB found in clinical strains (25, 32). AcrAB plays an important role in efflux of erythromycin, azithromycin, and clarithromycin as well as new ketolides, such as ketek, in clinical isolates of *H. influenzae*.

The most common mechanism of resistance to aminopenicillins in *H. influenzae* from many countries involves the expression of TEM-1 and ROB-1 beta-lactamases (8, 11, 29). Beta-lactamase inhibitor–beta-lactam combination products are effective against *bla*$^+$ *H. influenzae*; however, mutations in PBP3A and -3B leading to reduced affinity to beta-lactams can occur, conferring elevated MICs to beta-lactams and inhibitor combinations (6, 7, 11, 34, 35). Beta-lactamase-negative, ampicillin-resistant (BLNAR) *H. influenzae* first observed in the 1980s (16, 20) occur at very low frequency in North America and Europe (3, 8, 11, 13, 29, 34) but can constitute 25 to 30% of ampicillin-resistant isolates in Japan, although rates can vary between children and adults (11, 23, 33, 34, 35). A recent study comparing resistance mechanisms in *H. influenzae* isolated in either Japan or the United States found that the frequency of *bla*$^-$, ampicillin-susceptible isolates recovered was 55.1 and 46%, respectively, in each country (11). In *bla*$^+$, ampicillin-resistant strains 3 and 26% of isolates from Japan and the United States, respectively, had TEM-1, while 0 and 10%, respectively, expressed ROB-1 beta-lactamase. In that study, 13.2 and 0% of *H. influenzae* were BLNAR from Japan and the United States, respectively (11). BLNAR strains in that study were defined as having ampicillin MICs of 1 to 16 μg/ml, with the mode MIC being 2 μg/ml.

H. influenzae has five penicillin-binding proteins (1A, 1B, 2, 3, and 4), and these have been studied as determinants of beta-lactam resistance. PBP3 is encoded by the *ftsI* gene, and mutations in the transpeptidase domain of *ftsI* have correlated with resistance (6, 34). This is consistent with studies examin-

* Corresponding author. Mailing address: Department of Antibiotics, Immunology and Cancer, MS 8118W-211, Pfizer Global Research and Development, Eastern Point Rd., Groton, CT 06340. Phone: (860) 715-6627. Fax: (860) 715-8162. E-mail: thomas_d_gootz@groton.pfizer.com.

ing the crystal structure of PBP2x from *Streptococcus pneumoniae* R6 and mutants, indicating that amino acid substitutions clustering around the active site of the transpeptidase domain lead to resistance (37). Several studies have described the mechanism of ampicillin resistance in *bla*$^-$ strains of *H. influenzae* (6, 7, 11, 27, 34, 35). Clairoux et al. (6) studied BLNAR *H. influenzae* isolated in Canada and found that low-level resistance to ampicillin correlated with decreased affinity of ^{125}I-labeled penicillin for PBP3A and -3B in clinical isolates compared to that of *H. influenzae* Rd. Ampicillin MICs for these BLNAR strains clustered into three groups: group 1 was 0.5 to 1.0 μg/ml, group II was 2 to 4 μg/ml, and group III had higher MICs of ≥8 μg/ml. Transformants of strain Rd made from whole-cell DNA from the BLNAR strains from groups I and II had ampicillin MICs equal to those of the donors and possessed PBP3A and -3B with decreased affinity for beta-lactams (6). Interestingly, transformants made from DNA from group III BLNAR had moderate ampicillin MICs in the 1- to 4-μg/ml range, although PBP3A and -3B had reduced affinity to ampicillin comparable to that of group I and II BLNAR. The mechanism responsible for the higher level of ampicillin resistance in the group III BLNAR was not determined.

More recent studies by Ubukata et al. (34, 35) characterized mutations in the transpeptidase region of *ftsI* that encodes PBP3 of BLNAR strains from Japan. The changes in sequence compared to the sequence of *ftsI* of strain Rd were classified into three groups. In group I, His is substituted for Arg-517 (R517→H); group II strains had Lys substituted for Asn-526 (N526→K). In group III strains, three residues (Met-377, Ser-385, and Leu-389) were replaced by Ile, Thr, and Phe, respectively, in addition to replacement of Asn-526 with Lys. Transformation of *H. influenzae* Rd with the *ftsI* gene of the clinical strains produced transformants with ampicillin and cephalosporin MICs equal to those for the original BLNAR strains (1 to 4 μg/ml). Some strains also had a 7-bp deletion in *dacB*, which encodes PBP4, although this change did not appear to correlate with elevated ampicillin MICs (35).

Similar results were observed in a more recent study of BLNAR strains from France (7). Examination of the transpeptidase region of *ftsI* from bp 960 to 1618 identified 18 different mutation patterns that were divided into group I and groups IIa to IId that were similar to substitutions observed in the Japanese BLNAR strains. No group III BLNAR strains were identified in this study. Typically, the ampicillin MICs in these BLNAR strains ranged from 1 to 4 μg/ml. The PBP proteins from these strains were not examined for affinity for beta-lactams.

We obtained a collection of BLNAR *H. influenzae* collected from North America from 1996 to 2001 (3) that possessed higher level resistance to ampicillin in the 8 to 16 μg/ml range, being more resistant than other BLNAR previously characterized. Extensive sequence analysis of all PBP genes and construction of transformants of strain Rd with *ftsI* and the *acrR* regulatory gene of the AcrAB efflux pump from these clinical isolates revealed that mutations in both loci led to the higher level of ampicillin resistance observed. These studies show that lowered affinities of beta-lactams to PBP3 as well as increased drug efflux mediated by overexpression of AcrAB can confer such higher level resistance to ampicillin in BLNAR *H. influenzae*. Global spread of such resistant strains could compromise therapy, even with new high-dose amoxicillin regimens.

TABLE 1. Ampicillin MICs for all nonclonal BLNAR *H. influenzae*, control strains, and transformants

Strain	Assigned group[a]	*n*[b]	Geometric mean MIC (μg/ml)	MIC range[e]
Rd	S	11	0.16	0.063–0.5
1100	S	6	0.11	0.063–0.125
1311	II	9	8.00[c]	2–16
Rd/1311	II(tx)	7	2.21	1–4
1100/1311	II(tx)	5	1.15	1–2
Rd/N→K	II(tx)	6	2.24	1–4
1312	I	20	10.29[c]	4–16
Rd/1312	I(tx)	5	1.74	1–4
1100/1312	I(tx)	5	1.32	1–2
Rd/R→H	I(tx)	7	1.10	0.125–2
ATCC49247[d]	II	10	2.83	1–4
Rd/49247	II(tx)	5	2.64	1–4
1176	II, β-lac$^+$	5	>128	ND
Rd/1176	II(tx)	3	2.00	2
1316	II	5	9.19[c]	8–16
1318	II	5	10.56[c]	8–16
1360	II	4	2.00	1–4
1365	II	4	3.36	2–8
1368	II	4	2.38	1–4
1369	II	4	2.83	2–4
1370	II	4	0.84	0.5–2
1371	II	4	3.36	2–4
1373	II	4	2.38	0.5–4

[a] S, susceptible; I or II indicates the defining amino acid substitution in the transpeptidase domain of PBP3. Group I is characterized by R517→H substitution; group II is characterized by N526→K substitution. (tx), transformant containing full-length *ftsI*. β-lac, beta-lactamase.
[b] Designates the number of times the MIC was evaluated on different days.
[c] BLNAR with higher level resistance to ampicillin. Magnitude of ampicillin MIC significantly different from MICs for other BLNAR strains tested ($P \leq 0.01$).
[d] ATCC 49247 is a control strain; the NCCLS QC range for ampicillin is 2 to 8 μg/ml (22).
[e] ND, not determined.

MATERIALS AND METHODS

Bacterial strains and media. The BLNAR clinical isolates used in this study (Table 1) were obtained from Steve Brown of the Clinical Microbiology Institute (Wilsonville, Oreg.). Twenty-six strains were selected based on having an ampicillin MIC of ≥2 μg/ml. These strains were isolated in North America between 1996 and 2001. The beta-lactamase-positive, ampicillin-resistant (BLPAR) strain was obtained from Dan Sahm of Focus Technologies (Herndon, Va.), and the ampicillin and amoxicillin-clavulanate (2:1) MICs for this strain were >128 and 1 μg/ml, respectively. The isolates were maintained on chocolate agar plates (Remel, Lenexa, Kans.). Broth cultures were grown at 37°C in BXV medium brain heart infusion broth (BHI; Becton Dickinson, Sparks, Md.) supplemented with 10 μg of β-NAD$^+$ (Sigma Chemicals, St. Louis, Mo.) per ml and 10 μg of hemin (Fluka BioChemika, Buchs, Switzerland) per ml. Two ampicillin-susceptible *H. influenzae* strains, Rd and a clinical isolate in our Pfizer collection, *H. influenzae* 1100, were used as recipients in transformation experiments. *H. influenzae* ATCC 49247 was included in our studies as a BLNAR control.

Antimicrobial susceptibility testing. MICs for clinical and isogenic strains were determined by the National Committee for Clinical Laboratory Standards (NCCLS) broth dilution method (22) in Haemophilus Test Medium (HTM; BBL Microbiology Systems, Cockeysville, Md.) with *H. influenzae* ATCC 49247 as control. A final inoculum of 3×10^5 CFU/ml was used, and susceptibility test plates were incubated for 20 h in an ambient atmosphere. Ampicillin, erythromycin, penicillin G, and amoxicillin-clavulanate were purchased from Sigma Chemical. Azithromycin and sulopenem were synthesized at Pfizer (Groton, Conn.). Given the variability observed with ampicillin MIC results for BLNAR *H. influenzae* (3), susceptibility tests were run multiple times in this study, from

3 to 20 separate days, and the geometric means were calculated for each organism.

Amplification of *ftsI* genes. The primers used to PCR amplify the transpeptidase region of *pbp1a*, *-1b*, *-2*, and *-3* are listed in Table 2. PCR products from two independent reactions were analyzed for each gene evaluated from all strains tested.

DNA sequencing and analysis of sequence data. Terminator kits were obtained from Applied Biosystems (Foster City, Calif.). Primers were synthesized by Operon/Qiagen (Valencia, Calif.). Cycle sequencing was performed according to the Applied Biosystems BigDye Terminator *Taq*FS v3.0 cycle sequencing protocol with the following specific modifications: quarter reaction in a 10-μl volume with a final 1× concentration of dilution buffer (200 mM Tris-HCl [pH 9.0], 5 mM $MgCl_2$), 10 pmol of primer, and 5% dimethyl sulfoxide. A modified thermal cycle program was performed on the DNA Engine Tetrad (M. J. Research, Watertown, Mass.) as follows: 95°C for 1 min (1 cycle); 98°C for 45 s, 50°C for 10 s, 60°C for 4 min (1 cycle); 98°C for 15 s, 50°C for 10 s, 60°C for 4 min (29 cycles); 4°C for 5 min (1 cycle); 10°C until needed. Unincorporated terminators were removed as per the Applied Biosystems isopropanol precipitation protocol. Samples were analyzed on the ABI PRISM 3700 DNA analyzer (Applied Biosystems) with Data Collection 2.0, POP6 polymer. Chromatograms were analyzed by using the Sequencher program, enhanced for Sequence Collector v4.1.4b5 (Gene Codes Corp., Ann Arbor, Mich.).

Cloning of *ftsI* and expression and purification of PBP3. The gene encoding soluble PBP3 (Δ1-Q66, in which the transmembrane domain was deleted) was amplified by PCR with the primer set described in Table 2. The upstream primer contained an NdeI site, and the downstream primer contained a BamHI site to facilitate the cloning process. The 1,653-bp product was purified and inserted into pGEM-T Easy plasmid (Promega, Madison, Wis.). The resulting plasmid was digested with NdeI and BamHI, and the NdeI-BamHI fragment was cloned into pET15b (Novagen, Madison, Wis.), resulting in the introduction of a hexa-His tag at the N terminus of the expressed protein. Recombinant PBP3 from Rd, three clinical strains, and the two single-point-mutant constructs were expressed in *Escherichia coli* BL21(DE3) and were purified according to standard Ni^{2+} column procedures. Briefly, the culture was grown in 1 liter of Terrific broth (Sigma) at 37°C until the A_{600} reached 1.0. Isopropyl-[exists]-D-thiogalactopyranoside was then added to a final concentration of 0.5 mM, and the cells were shifted to 15°C and grown overnight. The cells were harvested by centrifugation, decanted, and resuspended in 20 ml of binding buffer (50 mM HEPES [pH 7.5], 500 mM NaCl, 5 mM imidazole, and 5% glycerol). The cells were lysed by adding 250 U of Benzonase per ml and 50 mg of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate per ml of cell suspension followed by incubation at room temperature for 45 min. Cells were then sonicated and centrifuged at 19,700 × *g* for 60 min at 4°C. The supernatant from the cell lysate was applied to a DE52 column and allowed to drip into an Ni-nitrilotriacetic acid column. Once all supernatant had passed through the DE52 column, 20 ml of binding buffer was applied to the column. The Ni-nitrilotriacetic acid column was washed with 200 ml of buffer containing 50 mM HEPES (pH 7.5) with 500 mM NaCl, 30 mM imidazole, and 5% glycerol. His-tagged protein was eluted with 17 ml of binding buffer containing 250 mM imidazole. After dialysis against 10 mM HEPES (pH 7.5), 500 mM NaCl, and 15% glycerol, PBP3 was concentrated and stored at −20°C. The proteins were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Determination of PBP3 binding affinity. Membrane preparations from *H. influenzae* cells labeled with radiolabeled beta-lactams sometimes produce two labeled species of PBP3, designated PBP3A and -3B (35). To avoid this problem, purified recombinant PBP3 proteins were used in binding studies. Binding assays were performed as previously described (36). Purified recombinant PBP3 protein was incubated with different concentrations of ampicillin in HEPES buffer (10 mM HEPES [pH 7.5], 500 mM NaCl) at 37°C for 10 min. Bocillin FL penicillin (Molecular Probes, Eugene, Oreg.) was added to a final concentration of 6.5 μM, and the reaction mixture was kept at 37°C for 30 min. The reaction was stopped by adding excess cold penicillin V (200 μg per reaction mixture). Samples were denatured by adding NuPage sample buffer and boiling for 3 min. Samples were loaded onto 4 to 12% NuPage Bis-Tris gels. Fluorescence-labeled PBP proteins were visualized by using a Storm 860 PhosphorImager (Molecular Dynamics, Amersham Pharmacia, Piscataway, N.J.). For determination of the binding affinity of different PBP3 constructs, various amounts of Bocillin FL were added to 170 ng of each recombinant protein and were incubated at 37°C for 30 min. Results were recorded as relative fluorescent units of bound label.

Assay for measuring transpeptidase activity of recombinant PBP3 proteins. In order to assess the effects of mutations within the transpeptidase region of *ftsI* on the functional activity of PBP3, the transpeptidase activity of each recombinant PBP3 was determined and compared with that of PBP3 from *H. influenzae* Rd. Transpeptidase activities of the PBP3 from Rd, 1312, 1311, 1369, R517→H, and N526→K were assayed by measuring their ability to hydrolyze a thiol-ester analog of cell wall stem peptide (21, 36) with the structure C_6H_5-CONH-CH-CH_3-COS-CH-CH_3-COOH (synthesized at Pfizer). The thiol group release that was coupled to 2,2′-dithiodipyridine was measured by monitoring the increase in optical density at 325 nm. Reaction mixtures (200 μl each) contained 50 mM sodium phosphate (pH 7.0), 3 mM thiol-ester substrate, 0.8 mM 4,4′-dithiodipyridine, and 2.4 μg of PBP3. The increase in optical density was monitored at 37°C for 1 h by using a SpectraMax 250 (Molecular Devices, Sunnyvale, Calif.).

Construction of group I and group II mutations of *ftsI*. Group I (CGT→CAT, R517→H) or group II (AAT→AAG, N526→K) single-amino-acid substitutions in the *ftsI* gene were introduced by overlapping PCR utilizing genomic DNA from *H. influenzae* Rd with the primers described in Table 2.

Transformation. The PCR-generated *pbp* and *acrR* gene products used in this study were transformed by electroporation into the recipient strain *H. influenzae* Rd (10, 30). The strains used to make the *acrB* knockouts were transformed by using chemically defined M-IV competence-inducing medium (2, 30).

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed according to the procedure described by Saito et al. (31).

Analysis of OMP. Outer membrane proteins (OMPs) were isolated according to the rapid outer membrane protein procedure, and the migration and intensity of the putative OMP P2 from each clinical strain was compared to that of the preparation made from *H. influenzae* Rd (1, 5).

***acrAB* gene disruptants and *acrR* gene mutations.** The *acrA* and *acrB* gene disruptants were obtained by PCR from *H. influenzae* strains Rd 0894::kan and Rd 0895::kan (32) with the primer set described in Table 2. Transformants were selected on chocolate agar supplemented with 20 μg of kanamycin/ml. The strains designated Rd/1311*acrR* and Rd/1316*acrR* were obtained by PCR from *H. influenzae* clinical strains 1311 and 1316 by using the primer set listed in Table 2. Resulting transformants were selected on s-BHI agar supplemented with 3 μg of erythromycin/ml.

***acrR* sequence analysis.** The repressor gene, *acrR*, from select *H. influenzae* isolates was sequenced (nucleotide [nt] 1 to 564) by colony PCR with the primer set listed in Table 2. PCR products from two independent reactions were analyzed for each gene evaluated.

PCR conditions. PCR amplification was carried out with *Haemophilus* cell lysates. Template DNA was obtained by placing several small colonies of each respective strain in 100 μl of TE (10 mM Tris, 1 mM EDTA [pH 8.0]) and boiling the sample for 10 min. After being cooled on ice, 1 to 2 μl of each lysate was used in the PCR. PCRs were carried out for 30 cycles using high-fidelity *Taq* polymerase (Invitrogen, Carlsbad, Calif.) with the following cycle parameters: an initial hold at 95°C for 1 min, followed by 94°C for 1 min, 52°C for 1 min, 72°C for 3 min, and a final 72°C for 10 min. Elongase enzyme mix (Invitrogen) was used for *acrAB* gene disruptants in PCR with the following cycles: an initial hold at 94°C for 30 s followed by 94°C for 30 s, 54°C for 30 s, 68°C for 10 min, and a final 72°C for 10 min. All PCR products were purified with a PCR purification kit (Qiagen, Inc.) and were subjected to automated DNA sequencing.

Morphological analysis of *H. influenzae* BLNAR strains by scanning electron microscopy. The BLNAR strains were streaked onto chocolate agar plates and were incubated overnight at 37°C. A 0.02-μm-pore-size filter (Whitman no. A30113NB) was then placed onto several colonies, and the filter was then immediately removed for processing. The filters were fixed in 0.1 M sodium cacodylate buffer containing 4% glutaraldehyde for 60 min. The filters were washed with 0.2 M sodium cacodylate buffer. The filters were then postfixed with 0.1 M sodium cacodylate buffer containing 2% osmium tetroxide (OsO_4) for 60 min followed by a wash with distilled water. The filters were next dehydrated in a series of graded ethanols (50, 70, 95, and 100%) twice for 15 min each. The filters were then chemically dried by using hexamethyldisilane for 5 min followed by removal of the fluid and air drying. In the final step the filters were sputter coated with gold-palladium (40 mA for 63 s).

Assay of [^{14}C]erythromycin accumulation. The intracellular accumulation of radiolabeled [^{14}C]erythromycin (40 to 60 mCi per mmol; New England Nuclear, Boston, Mass.) was determined by the methods of Sánchez et al. (32) and Li et al. (14). Briefly, a standardized cell suspension was incubated with labeled drug for 5 min, after which the cultures were split and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma) was added to a final concentration of 0.2 mM, and accumulation of radiolabel was followed for 25 min.

RESULTS

Analysis of BLNAR strains. Twenty-six *H. influenzae* BLNAR isolates were obtained from the Clinical Microbiology Insti-

TABLE 2. Source of DNA primers used in this study

Primer set designation and use	Sense primer	Reverse primer	Source or reference
To amplify conserved transpeptidase region of each *pbp*[a]			
pbp1a	GACAGGCAGAAGCAATCCTGTG	CATCAAACCCGACATAAGTTGTG	This study
pbp1b	CTTTACAAGCAGATTTACGCCGGTCAGCGACCTTTAGGTGTG	GAGAGAGGGAGATGAGTTTCG	This study
		GAGCGGGTGGATTGGGATTATCC	This study
pbp2	GGACTAAAAGGTGCTGTAGTTGTGC	GCGGTAAACCAAGCGTGATCATG	This study
pbp3	GTTGCACTATCTCCGATGAG	CAGCTGCTTCAGCATCTTGC	35
To amplify entire coding region of selected *pbp* genes			
pbp1a	CTCTGCTTACGATGAATGCCG	CAAGACATCGGCATGATTGCCAG	This study
pbp1b	GCCTTGCTTTTTGCGGTACGCAAG	GTGTCATCGTTTTGCACCTGTATG	This study
pbp2	GTATCGTGCGTGGTCGCTAAC	CACCAGCATACCATAAGCAG	This study
pbp3	GACGATTTGGATAACCCATA	CTGGATAATTCTGTCTCAGA	35
To amplify upstream (225-bp) region of *ftsI*			
pbp3	GTTGCTTTAATGTGGAATTACTTGCAAGTTGAGC	CATCCATCTCACATTCAGTGGC	This study
To introduce single-point mutations in wild-type *ftsI*			
Arg→His	CAGGTACGGCACATAAGATTGAAAATGGAC	GTCCATTTTCAATCTTATGTGCCGTACCTG	This study
Asn→Lys	GGACATTATGTAAAGAAGTATGTGGCATTTACTGC	GCAGTAAATGCCACATACTTCTTTACATAATGTCC	This study
pbp3	GACGATTTGGATAACCCATA	CTGGATAATTCTGTCTCAGA	35
To clone *ftsI* for expression	GCGGCGGCCCATATGTCTATTAATGCCGATACG	GCGCGGATCCCACTTTTTGATTCTTGTGTTCG	This study
For *acrAB* gene disruptants			
acrA	CATCTTGTGGGTTTACGGCTTACC	CTGGATATGCGGTACTCACTG	This study
acrB	CATTGTGGGTGCAATAGAGCCTGCA	CCGCACTTATGCAGGTTATGCTC	This study
For *acrR* sequence analysis	CCTACAGAATAAGTATTGTTACG	CTCGGCCTTATCATTACGCC	This study
For *acrR* transformations	GGTACAGATTAGTAATGACGCAG	CTGGATATGCGGTACTCACTG	This study

[a] Primer sets used to amplify the transpeptidase region of selected *pbp* genes: *pbp1a*, bp 1142 to 2270; *pbp1b*, bp 1114 to 2269 or 1048 to 2264; *pbp2*, bp 825 to 1750; *pbp3*, bp 796 to 1741.

TABLE 3. Distribution of deduced amino acid substitutions in the transpeptidase region of PBP3 in BLNAR isolates of *H. influenzae* compared to the sequence of strain Rd

Strain	AA273 (Ser→Ala)	AA350 (Asp→Asn)	AA357 (Ser→Asn)	AA377 (Met→Iso)	AA436 (Val→Ala)	AA443 (Thr→Ala)	AA449 (Iso→Val)	AA490 (Gly→Glu)	AA502 (Ala→Val)	AA511 (Val→Ala)	AA517 (Arg→His)	AA526 (Asn→Lys)
A51907[a]												
1311												*
1312	*		*								*	
1316												*
1318			*			*						*
1360										*		*
1365					*							*
1368												*
1369		*		*			*					*
1370		*		*					*			*
1371	*											*
1373								*				*
49247[b]	*											*
1176[c]							*					*

[a] ATCC control strain Rd.
[b] Strain 49247 designates the ATCC BLNAR control.
[c] Clinical isolate 1176 is a beta-lactamase-positive amoxicillin–clavulanate-resistant strain.

tute. The strains were grouped according to their level of ampicillin resistance. Those isolates exhibiting ampicillin MICs of 1 to 4 μg/ml were considered low-level BLNAR strains, while a second group with ampicillin MICs of 8 to 16 μg/ml were designated higher level resistant BLNAR. PFGE analysis revealed that there were 12 nonclonal BLNAR isolates plus the ATCC 49247 BLNAR control strain (data not shown). Given the variability that can be encountered with MIC results for BLNAR strains (3), MICs for each nonclonal isolate in this study were determined from 3 to 20 different test days; the results have been reported as geometric mean MICs. Among the 12 unique strains, 4 were categorized as higher level ampicillin-resistant BLNAR (strains 1311, 1312, 1316, and 1318), while 8 fell into the low-level resistance category (Table 1). The geometric mean MICs for the higher level ampicillin-resistant isolates were statistically different (Student's *t* test) from the eight low-level BLNAR clinical isolates ($P < 0.01$). Penicillin MICs for the four clinical strains ranged from 8 to 32 μg/ml, while the penem antimicrobial, sulopenem, inhibited all strains with MICs of 0.5 to 2.0 μg/ml (data not shown).

One isolate (strain 1176) was beta-lactamase-positive and was subsequently shown to have mutations in *ftsI*. Ampicillin and amoxicillin-clavulanate (2:1) MICs for this strain were >128 and 1 μg/ml, respectively.

***ftsI* mutation patterns.** The nt sequence of *ftsI* encoding the transpeptidase region (nt 796 to 1741) was determined for the 12 nonclonal BLNAR, the *bla*$^+$ strain 1176, and two susceptible strains of *H. influenzae* (strains Rd and 1100). Table 3 summarizes the deduced amino acid changes observed for all of the nonclonal strains examined. Two distinct groups emerged as originally designated by Ubukata et al. (35). Group II strains characterized by a substitution of N526→K was the most common group encountered (11 out of 12 clinical strains). The R517→H change indicative of group I was observed in only one strain (1312). Of the BLNAR isolates with higher ampicillin MICs (geometric mean MICs of 8 to 10.56 μg/ml), three were from group II and one was from group I (Table 1). ATCC *H. influenzae* 49247 demonstrated a geometric mean MIC of 2.83 μg/ml and fell in group II. Group III isolates, which comprise three separate amino acid substitutions (Met377 to Ile, Ser385 to Thr, and Leu389 to Phe) as identified by Ubukata et al. (35) were not observed among our strains. Based on scanning electron microscopy, all of the BLNAR strains examined displayed the characteristic filamentous phenotype (6) of *ftsI* mutants (data not shown).

Construction of isogenic sets of resistant transformants in susceptible *H. influenzae* strains. PCR-amplified *ftsI* genes from the BLNAR strains 1311, 1312, 1176, ATCC 49247, and *bla*$^+$ 1176 were used to transform the ampicillin-susceptible *H. influenzae* Rd. The *ftsI* gene from strains 1311 and 1312 was also used to transform another ampicillin-susceptible strain, *H. influenzae* 1100, in order to assess ampicillin resistance in a different background. In addition, strain Rd was also transformed with the *ftsI* alleles containing a single-amino-acid substitution that defines either group I or group II. Table 1 contains the ampicillin MICs for the transformants and the corresponding parent strains. In all examples the transformants had ampicillin MICs significantly lower than those of their corresponding parent strain (compare MICs for transformant Rd/1312 with those for clinical strain 1312). This suggested that expression of low-affinity PBP3 alone did not account for the higher level of ampicillin resistance observed in the original BLNAR clinical isolates.

Sequencing of *pbp1a*, *-1b*, and *-2*. In order to look for additional mechanisms of ampicillin resistance, the transpeptidase regions of *pbp1a*, *pbp1b*, and *pbp2* were also sequenced from the four higher level ampicillin-resistant BLNAR strains. Although no common amino acid substitutions were identified among the strains (data not shown), full-length coding sequences for each *pbp* gene were PCR amplified and transformed into the susceptible strain Rd and into two strains containing the single-amino-acid substitution in *ftsI* that characterized group I and group II BLNAR strains. Cefaclor (Sigma) and ampicillin were used for selecting transformants. No ampicillin-resistant transformants were recovered at selecting concentrations of 1 to 4 μg/ml. In addition, a 225-bp region upstream of the start codon for *ftsI* was also sequenced in all nonclonal strains. No changes between the sequence in this

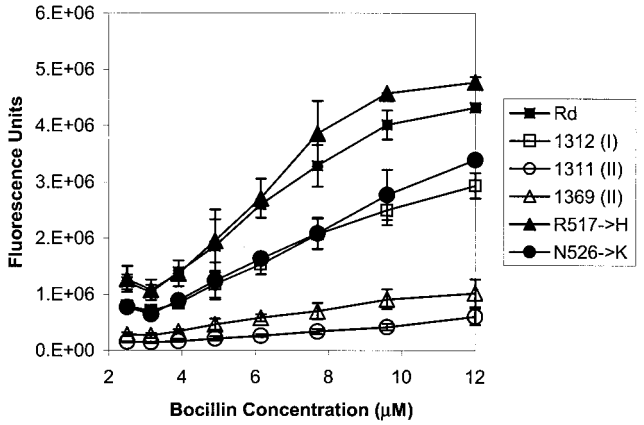

FIG. 1. Affinity of recombinant PBP3 proteins for fluorescent-labeled Bocillin. One hundred-seventy nanograms of each PBP3 was incubated for 30 min at 37°C with 2 to 12 μM Bocillin. Error bars indicate ±1 standard deviation. PBP3 proteins tested were expressed from *ftsI* of *H. influenzae* Rd (control); BLNAR strains 1312 (group I), 1311, and 1369 (group II); and single-substitution alleles of group I (Arg517 to His) and group II (Asn526 to Lys) PBP3.

region and the sequence in the homologous region of *H. influenzae* Rd were identified.

Binding of beta-lactams to recombinant PBP3 of BLNAR strains. Figure 1 shows results of fluorescent Bocillin binding to recombinant PBP3 cloned from ampicillin-susceptible (strain Rd), low-level (strain 1369), and higher level (strains 1311 and 1312) BLNAR isolates and the alleles with the single-amino-acid substitutions R517→H (group I) and N526→K (group II). At Bocillin concentrations of ≥7.5 μM the single allele substitution of R517→H bound slightly more Bocillin than PBP3 from the control strain Rd. All of the other recombinant PBP3s demonstrated reduced binding of Bocillin. However, all of the PBP3-recombinant proteins bound within a fivefold amount of labeled Bocillin.

When the recombinant PBP3 proteins were evaluated for transpeptidase activity by using a synthetic peptide substrate (Fig. 2), as with the Bocillin affinity assay, the group I recombinant R517→H had higher transpeptidase activity than the recombinant protein from control strain Rd. The other PBP3 proteins evaluated had lower transpeptidase activity than PBP3 from Rd.

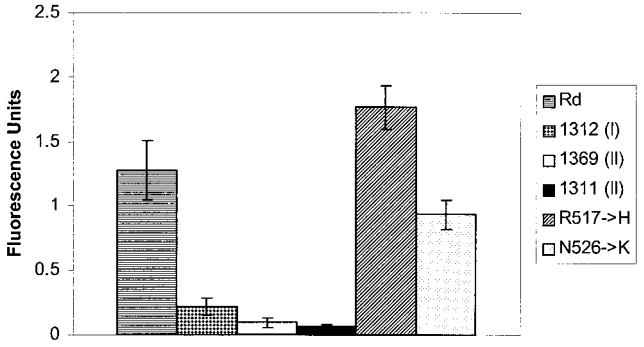

FIG. 2. Transpeptidase activity of each recombinant PBP3 expressed in relative fluorescent units. Reaction mixtures contain 2.4 μg of each PBP3. Error bars represent means ± 1 standard deviation.

TABLE 4. Competitive binding data for ampicillin and PBP recombinant proteins[a]

Strain	Assigned group[b]	Geometric mean MIC	IC_{50} (μg/ml) ± SD
Rd	S	0.16	0.18 ± 0.09
1369	II	2.83	1.77 ± 0.40
1312	I	10.29	0.96 ± 0.10
Rd/1312	I(tx)	1.74	
1311	II	8.00	2.92 ± 1.93
Rd/1311	II(tx)	2.21	

[a] Purified PBP3 proteins were incubated with unlabelled ampicillin, followed by Bocillin FL and penicillin.
[b] S, ampicillin-susceptible *H. influenzae* Rd; I or II, class I or II PBP3; (tx), transformant of Rd containing full-length *ftsI* from clinical strains.

The recombinant PBP3s were also evaluated in a competitive binding assay with various amounts of unlabeled ampicillin and a fixed amount of fluorescent Bocillin. The data listed in Table 4 indicate that the 50% inhibitory concentration (IC_{50}) values for ampicillin binding correlate well with the geometric mean ampicillin MIC for *H. influenzae* Rd and the low-level BLNAR clinical isolate 1369. In contrast, the geometric mean ampicillin MICs for the two high-level BLNAR clinical isolates were 3- to 11-fold higher than the ampicillin IC_{50}. These data suggest that some additional factor must account for the level of ampicillin resistance in these strains.

OMP pattern. The OMPs extracted were compared between clinical and control strains, particularly with regard to the relative amount of the 40-kDa putative P2 porin (data not shown). While size variations of the putative P2 porin were evident and have been observed previously with nontypeable *H. influenzae* (4, 9, 12), there was no obvious change in the intensity of this protein between the ampicillin-susceptible control and the ampicillin-resistant clinical strains and their transformants that could account for differences in the ampicillin resistance levels observed.

Sequence analysis of *acrR* of the AcrAB efflux pump. In order to determine whether increased efflux of ampicillin plays a role in conferring higher ampicillin MICs in selected BLNAR strains, the coding sequence of *acrR* from nt 1 to 564 was determined and compared to that of *H. influenzae* Rd. The data listed in Table 5 indicate that none of the susceptible strains nor the BLNAR *H. influenzae* strains with ampicillin MICs of ≤4 μg/ml had changes in the *acrR* regulatory gene. In contrast, all four of the higher level, ampicillin-resistant BLNAR strains contained a single nt insertion of either a T after nt 40 (strains 1311, 1316, and 1318) or an A inserted after nt 150 (strain 1312) in *acrR*. In both cases these changes predicted early termination of the *acrR* reading frame.

In order to assess the role played by an intact AcrAB efflux pump in determining the magnitude of the ampicillin MIC, a knockout in *acrB* was made in the clinical strain 1312. The data listed in Table 6 indicate that the Δ*acrB* mutant of 1312 had a geometric mean ampicillin MIC of 3.67 μg/ml, while strain 1312 that had a mean MIC of 10.29 μg/ml. The geometric mean ampicillin MIC for the transformant of strain Rd containing the full-length *ftsI* from 1312 was 1.74 μg/ml. Ampicillin as well as erythromycin had reduced MICs for *H. influenzae* Rd

TABLE 5. Analysis of nonclonal BLNAR strains regarding the presence of an intact *acrR* coding sequence

Strain	Mean ampicillin MIC (μg/ml)	*acrR* gene frameshift
Rd	0.16	N
1100	0.11	N
ATCC49766	0.21	N
ATCC10211	0.21	N
ATCC49247	2.83	N
1360	2.00	N
1365	3.36	N
1368	2.38	N
1369	2.83	N
1370	0.84	N
1371	3.36	N
1373	2.38	N
1311	8.00	Y[a]
1312	10.29	Y[b]
1316	9.19	Y[a]
1318	10.56	Y[a]

[a] T inserted after nt 40 in the coding sequence.
[b] A inserted after nt 150 in coding sequence.

Δ*acrB*, underscoring the importance of an intact AcrAB efflux pump in limiting the activity of both antibiotics in this species.

In order to make a direct assessment on MICs of ampicillin for the *acrR* mutation observed in clinical isolates 1311 and 1316, *H. influenzae* Rd was transformed with each *acrR* mutation found in these strains. Transformants were selected on plates containing 3 μg of erythromycin/ml, and the presence of the *acrR* mutation was confirmed by sequencing the gene. Transformants of strain Rd containing an intact *acrR* from 1311 or 1316 showed statistically significant ($P < 0.01$) increases in geometric mean MICs for both ampicillin and erythromycin compared to that for Rd (Table 6). This is consistent with the observation that high-level resistance to ampicillin and erythromycin in these clinical isolates is in part mediated by disruption of *acrR*, likely producing an increase in AcrAB-mediated efflux.

Accumulation studies with radiolabeled erythromycin. In order to confirm the phenotype of the Δ*acrB* mutants, the accumulation of [^{14}C]erythromycin was measured in wild-type and knockout mutants of *H. influenzae* Rd and clinical isolate *H. influenzae* 1312. A standardized cell suspension was incubated for 5 min with labeled drug, after which 0.2 mM CCCP was added to half of the culture in order to dissipate proton motive force. Accumulation of labeled erythromycin was followed for 25 min. In the absence of CCCP, both *acrB* knockouts accumulated about twice the amount of erythromycin as the corresponding wild-type strain (Fig. 3). After addition of CCCP, erythromycin accumulation was increased further in all four strains. These results are consistent with those observed by Sanchez et al. (32) with similar disruption mutants of *acrA* or *acrB* in *H. influenzae*.

TABLE 6. Comparison of ampicillin and erythromycin MICs for isogenic strains of *H. influenzae*

Strain tested	Ampicillin		Erythromycin	
	n[a]	Geometric mean MIC (μg/ml)	*n*[a]	Geometric mean MIC (μg/ml)
Rd	11	0.16	11	2.26
Rd Δ*acrB*	6	0.09	9	0.16
Rd/1311*acrR*	7	0.55[b]	7	4.42[b]
Rd/1316*acrR*	7	0.50	7	4.42
1312	20	10.29	12	7.55
Rd/1312	5	1.74	2	4.00
1312 Δ*acrB*	8	3.67	4	0.16
ATCC 49247	10	2.83	4	4.00
Rd/49247	5	2.64	2	4.00

[a] Designates the number of times the MIC was determined on different days.
[b] Statistically different ($P < 0.01$) from MICs for strain Rd.

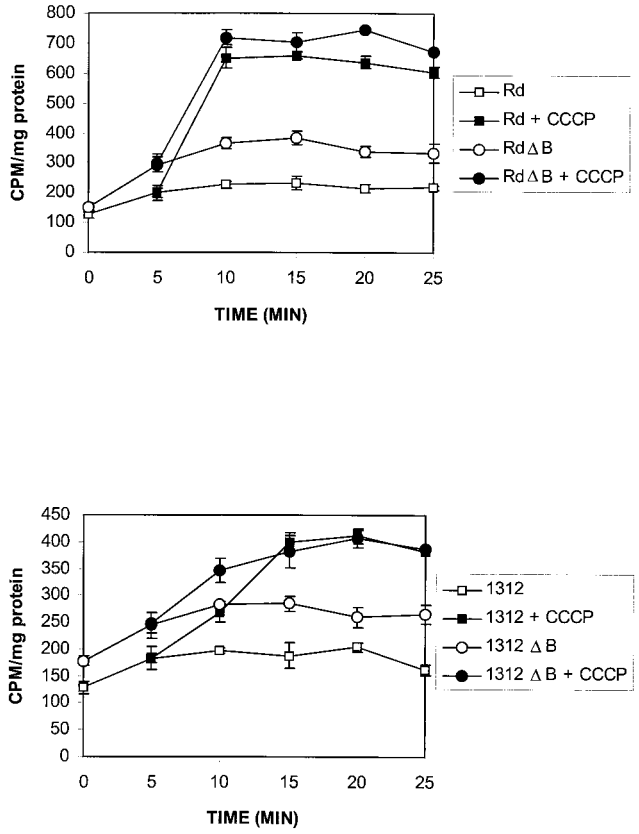

FIG. 3. Accumulation of [^{14}C]erythromycin in strains of *H. influenzae*. Cells were grown and treated as described by Sánchez et al. (32). The experiment was started after 5 min of preincubation of the cells by the addition of radiolabeled erythromycin. After 5 min, CCCP was added to a final concentration of 0.2 mM. Open symbols represent untreated cultures; closed symbols represent CCCP-treated cultures. Each datum point represents the average of two experiments. CPM, counts per minute.

DISCUSSION

BLNAR strains of *H. influenzae* are rare globally but represent another mechanism by which clinical resistance to beta-lactams can occur. Two issues complicate the importance that BLNAR strains play when choosing therapy for infections caused by *H. influenzae*. First, it is unclear whether BLNAR *H. influenzae* isolates are sufficiently pathogenic to cause respiratory tract infections. All of the strains characterized in this study were collected as part of a North American surveillance study and presumably were cultured from the respiratory tract

of patients with respiratory infection (3). A previous study found that a nontypeable strain of *H. influenzae* with altered PBP proteins was recovered from the spinal fluid of a patient with meningitis, further suggesting the pathogenic potential of these organisms (19). These observations suggest that BLNAR strains can be involved with infections in humans under circumstances requiring antimicrobial intervention.

A second issue complicating the detection of BLNAR strains involves the variability observed in the magnitude of ampicillin MICs obtained for these isolates. The NCCLS definition of BLNAR *H. influenzae* consists of those strains with no detectable beta-lactamase and ampicillin MICs of ≥4 μg/ml (22). Strains for which the ampicillin MIC is 2 μg/ml are defined as intermediate, or more appropriately, indeterminant, because such strains can be susceptible, intermediate, or resistant if retested on another day or by another method. Barry et al. (3) examined the effect of methodological changes on the reproducibility of ampicillin MICs for isolates taken from a surveillance study, and the strains in this report were a subset of those isolates. Given the inherent variability of ampicillin MICs in BLNAR strains, we used the recommended HTM broth method and developed geometric mean ampicillin MICs for our isolates from tests done on several different days. We also used PFGE to eliminate replicate isolates (data not shown), focusing on nonclonal strains with consistently high ampicillin MICs (8.0 to 10.56 μg/ml [Table 1]), significantly above those of typical BLNAR strains (1 to 4 μg/ml) documented to have mutations in *ftsI* (7, 34, 35). Four such strains were studied in detail along with the BLNAR reference strain ATCC 49247.

Sequencing of the transpeptidase region of *ftsI* (nt 796 to 1741) of these strains, compared with the transpeptidase region of *ftsI* of *H. influenzae* Rd, revealed several patterns of amino acid substitution similar to those observed in previous studies. All strains belonged to group II (Asn526→Lys), with the exception of strain 1312, which possessed the Arg517→His substitution characteristic of group I BLNAR (35). Numerous other mutations were detected in this conserved region compared to the Rd sequence, but none presented an obvious correlation with the higher level of ampicillin resistance observed (Table 3).

Full-length *ftsI* genes from two of the four BLNAR strains (1311 and 1312) with the highest ampicillin MICs were used to transform ampicillin-susceptible *H. influenzae* Rd and 1100, selecting for transformants with ampicillin or cefaclor. In agreement with the *ftsI* sequence data, all transformants demonstrated reduced geometric mean ampicillin MICs (1.15 to 2.10 μg/ml) typical for BLNAR strains, suggesting that a second locus contributed to the ampicillin resistance observed in the original clinical strains. In addition, strain 1176 expressed beta-lactamase and had an ampicillin MIC of >128 μg/ml. The transformant selected with the *ftsI* gene from this isolate (Rd/1176 in Table 1) demonstrated a geometric mean ampicillin MIC of 2.0 μg/ml and had substitutions in *ftsI* consistent with group II BLNAR. The amoxicillin-clavulanate MIC for this strain was 2 μg/ml, indicating the existence of a dual mechanism of resistance to beta-lactams that is masked by the beta-lactamase expression. Results for this strain confirm those from other studies (34, 35), indicating that both low-affinity PBP3 and beta-lactamase production can occur in the same isolate of *H. influenzae*, raising the MICs to amoxicillin as well as to amoxicillin-clavulanate.

In order to confirm that changes in the PBP3 proteins in our clinical isolates conferred a reduced binding of beta-lactams, full-length *ftsI* from *H. influenzae* Rd, 1311 and 1369 (group II), and 1312 (group I) as well as the single-substitution alleles Asn526→Lys and Arg517→His, defining groups II and I, respectively, were cloned, expressed, and evaluated for binding of fluorescent Bocillin. Interestingly, the recombinant protein representing the single group II allele demonstrated similar or slightly enhanced Bocillin binding compared to that of recombinant PBP3 from Rd. All of the other recombinant PBP3 proteins demonstrated reduced binding of Bocillin in these tests (Fig. 1). These data are consistent with the idea that mutations in PBP3 lead to reduced drug binding and resistance. It is also apparent that multiple changes in PBP3 lead to greater reduction in binding. Recombinant PBP3 proteins from both group II BLNAR mutants appeared to confer lower binding of Bocillin compared to that of either the group I protein or either single-allele-substituted proteins. These data are consistent with other studies describing substitutions in PBP2x from *S. pneumoniae*, where substituted PBP2x from resistant strains had reduced acylation efficiency for penicillins and cephalosporins compared to that of PBP2x from susceptible strain R6 (37). Interestingly, with the exception of the single group II allele PBP3, the other recombinant PBP3 proteins had reduced transpeptidase activity compared to that of PBP3 from Rd as well (Fig. 2). Similar observations have been made in studies with resistant PBP2x from *S. pneumoniae* using synthetic peptide substrates or beta-lactams in transpeptidase assays (37). The discordant results observed in the present study with the group I Arg517→His single allele are difficult to interpret; however, it is likely that native PBP3 containing numerous substitutions possess complicated secondary structural relationships. There are likely multiple mutations and subsequent compensatory mutations that lead to the final PBP3 with low beta-lactam affinity and transpeptidase activity necessary for cell wall synthesis. In the case of *S. pneumoniae*, low-affinity *pbp* mosaic genes are acquired via transformation of DNA from other species of streptococci in the environment (21). This may allow for acquisition of large regions of *pbp* genes that already contain the optimum combination of low affinity and catalytically functional motifs. It is uncertain whether acquisition of such mosaic *pbp* gene regions occurs in *H. influenzae*, although it is clear that this species is transformable in vitro. A better understanding of these structural considerations will await the first crystal structures of wild-type and mutant PBP3 proteins from *H. influenzae*.

In competitive binding studies with unlabeled ampicillin and fluorescent Bocillin, the IC_{50} values for ampicillin binding correlated well with the geometric mean ampicillin MIC for *H. influenzae* Rd and the low-level BLNAR clinical isolate 1369 (Table 4). This was not the case for the three higher level BLNAR clinical strains, indicating that some additional determinant of resistance was present.

In an attempt to find an additional mechanism to *ftsI* mutations contributing to ampicillin resistance in the clinical BLNAR isolates, the sequence of the regulatory region *acrR* of the AcrAB efflux pump was examined. AcrAB-mediated efflux of several antibiotics has been shown to be an important re-

sistance mechanism in some *Enterobacteriaceae* (15, 17, 18, 24, 28). Of all the BLNAR strains tested and described in Table 5, only those strains expressing elevated levels of resistance to ampicillin were found to have insertions in this regulatory region. Therefore, loss of a functional AcrR repressor appeared to correlate with higher level ampicillin MICs. In order to explore this observation further, an *acrB* knockout was made in the clinical isolate *H. influenzae* 1312 (Table 6). Consistent with the role of efflux contributing to ampicillin resistance, the ampicillin MIC in 1312 Δ*acrB* was markedly reduced compared to that of the clinical 1312 strain. Ampicillin MICs for the Rd transformant Rd/1312 containing the full-length *ftsI* only were consistent with those for typical BLNAR strains. The effect of the *acrB* knockout was even more profound in terms of lowering the erythromycin MIC, a compound long recognized as being affected by AcrAB-mediated efflux in *H. influenzae*.

Direct demonstration of efflux on resistance to ampicillin and erythromycin was obtained by putting each mutant allele of *acrR* from clinical isolates 1311 and 1316 in *H. influenzae* Rd. In both instances, the *acrR* mutant transformant had significantly higher ($P < 0.01$) geometric mean ampicillin MICs than Rd, demonstrating the effect of disrupting the regulatory gene (Table 6).

Whole-cell accumulation of [^{14}C]erythromycin was substantially greater in our Δ*acrB* mutants of Rd or clinical isolate 1312 than in the corresponding parent organism, confirming the efflux-defective phenotype of the disruptants. The proton conductor, CCCP, increased erythromycin accumulation in both the disruptants and parent organisms. An identical observation was made in a previous study with *acrAB* disruptants in *H. influenzae* (32), and it was hypothesized that CCCP treatment promoted spontaneous influx of erythromycin due to its decoupling effect of the cellular pH gradient. In addition, a recent paper by Peric et al. (25) emphasizes the importance of efflux in conferring elevated resistance to macrolides in clinical isolates of *H. influenzae*. That study found increased accumulation of labeled azithromycin or clarithromycin after treatment with CCCP in strains for which both drugs MICs were 0.25 to >4 μg/ml. In hypersusceptible strains with drug MICs of <0.25 μg/ml, treatment with CCCP had no effect on drug accumulation, suggesting the absence of a functional efflux pump. Interestingly, in a paper by Sanchez et al. (32) describing the construction of *acrAB* disruption mutants in this species, it was stated that an intact AcrAB pump did not appear to hinder the potency of beta-lactams in this species. Our data conclude that regulation of AcrAB efflux can indeed decrease the potency of ampicillin against *H. influenzae* and, in combination with reduced PBP3 binding resulting from mutations in *ftsI*, can produce elevated ampicillin MIC levels in the range of 8 to 16 μg/ml.

Surveillance studies in the United States suggest that BLNAR strains with ampicillin MICs of 16 μg/ml are rare. However, in Japan such isolates have been identified in recent studies (11). Classification of such BLNAR strains has been confusing with respect to the magnitude of ampicillin resistance and the presence of *ftsI* mutations. Hasegawa et al. (11) have classified such strains as either low-level BLNAR (ampicillin MICs of 0.5 to 2.0 μg/ml) or BLNAR (ampicillin MICs of 1 to 16 μg/ml). With this designation, low-level BLNAR strains constitute *ftsI* groups I and II as defined by Ubukata et al. (35), while BLNAR strains are all from *ftsI* group III. None of the higher level BLNAR isolates from the present study had all of the changes from *ftsI* group III, yet the geometric mean ampicillin MICs for these strains were 8 to 10.56 μg/ml. Given the broad range of ampicillin MICs in the BLNAR isolates from Japan, our results suggest that in the future, such *H. influenzae* isolates should be examined for dual mutations in both *ftsI* and *acrR*.

Should such *H. influenzae* strains with dual *ftsI*/*acrR* mutations become more widespread, it is important to consider how this might affect the therapeutic effects of aminopenicillin regimens. Augmentin XR is the newest high-dose form of amoxicillin made available in the marketplace; this formulation contains 2 g of a sustained release formulation of amoxicillin that, when given orally twice a day, provides adequate blood levels for over 40% of the dosing interval to treat pathogens with amoxicillin MICs of ≤4 μg/ml (B.-P. Richard and B. Wynne, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 952, 2001). While this product is mainly directed against penicillin-resistant *S. pneumoniae*, it is very possible that BLNAR strains of *H. influenzae* with amoxicillin-ampicillin MICs of 8 to 16 μg/ml would be inadequately covered by even this new, high-dose regimen. Interestingly, given the important role of AcrAB in conferring elevated MIC levels in *H. influenzae* to macrolides, ketolides, and ampicillin, extensive use of beta-lactams and macrolide derivatives may further select for strains overexpressing AcrAB. Because BLNAR strains have been isolated with the highest frequency in Japan, it might be predicted that such dual-target mutants may increase in that country most rapidly. Such strains may already have been isolated in Canada, but the role of AcrAB overexpression was not appreciated (6).

In summary, our study indicates that BLNAR strains of *H. influenzae* with mutations in the AcrAB repressor gene *acrR* can occur clinically and that such dual-target mutants can have elevated ampicillin MICs in the 8- to 16-μg/ml range. Increased isolation of such multilocus mutants may complicate therapy of respiratory tract infections with ampicillin or amoxicillin, as well as beta-lactam–beta-lactamase inhibitor combinations.

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