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# Genetic Approach To Study the Relationship between Penicillin-Binding Protein 3 Mutations and *Haemophilus influenzae* β-Lactam Resistance by Using Site-Directed Mutagenesis and Gene Recombinants

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**To clarify the relationship between mutations commonly found for penicillin-binding protein 3 (PBP 3) of β-lactamase-nonproducing ampicillin-resistant (BLNAR) *Haemophilus influenzae* isolates and β-lactam resistance, single and multiple amino acid mutations at positions 377, 385, 389, 517, and 526 were introduced into PBP 3 of a β-lactam-susceptible Rd strain by site-directed mutagenesis. Twelve isogenic recombinant strains were challenged with nine β-lactam antibiotics. Replacement of the asparagine at position 526 with lysine (N526K) increased the resistance to imipenem eightfold and increased the resistance to various cephalosporins two- to eightfold. Substitution of threonine for serine at position 385 (S385T) and/or substitution of phenylalanine for leucine at position 389 (L389F), in addition to the N526K mutation, led to two- to fourfold additional increases in cephalosporin resistance. An isoleucine-to-methionine substitution at position 377 did not change the antibiotic sensitivity of any of the recombinant strains also carrying other PBP 3 mutations tested. Thirty-six clinical isolates carrying a PBP 3 gene (*ftsI*) with the S385T, L389F, R517H, and/or N526K mutation were chosen from among 279 clinical isolates collected in Japan, and the isolates were grouped into six classes on the basis of the patterns of the four mutations in PBP 3. Rd recombinants were made with each of the *ftsI* genes. The levels of resistance to β-lactams varied between recombinants of different classes but were comparable for those of the same class. The levels of resistance to cephalosporins of these recombinants were similar to those of the parent clinical isolates, while those to ampicillin and carbapenems were lower. These results indicate that resistance to β-lactams, at least to cephalosporins, depends in large part on the PBP 3 mutations R517H, N526K, S385T, and L389F.**

*Haemophilus influenzae* has two β-lactam resistance mechanisms. One involves enzymatic hydrolysis of β-lactams by the TEM-1 or the ROB-1 β-lactamase (7). The other mechanism involves decreased β-lactam affinities for penicillin-binding protein 3 (PBP 3) (11). The β-lactam resistance phenotype mediated by the second mechanism is named β-lactamase-nonproducing ampicillin resistance (BLNAR). Since 1992 the frequency of BLNAR strains has increased exponentially among Japanese isolates from community-acquired infections (9, 12). Recently, strains with both resistance mechanisms were found among clinical isolates, and such *H. influenzae* strains are termed β-lactamase-producing ampicillin-clavulanic acid resistant (2, 5, 10).

From the genetic analysis of the *ftsI* gene, which encodes PBP 3 in BLNAR strains, the amino acid mutations surrounding the conserved KTG (Lys512-Thr-Gly) and SSN (Ser379–Ser-Asn) motif would be relevant to β-lactam resistance (1, 2, 8, 11). Amino acid substitutions, such as N526K and R517H, near the KTG motif are commonly found in isolates with cefotaxime intermediate resistance (MICs, 0.063 to 0.25 μg/ml). Additional mutations (M377I, S385T, and/or L389F), which are near the SSN motif, are associated with higher levels of cefotaxime resistance (MICs, 1 to 2 μg/ml).

To clarify the relationship between PBP 3 mutations and β-lactam resistance, we used oligonucleotide-directed mutagenesis to construct *ftsI* sequences with defined mutations at positions M377I, S385T, L389F, R517H, and N526K and tested the β-lactam susceptibilities of isogenic mutants constructed from the *H. influenzae* Rd susceptible strain. We also constructed 36 *ftsI* recombinant strains by transformation of the Rd strain with *ftsI* genes from different clinical isolates carrying PBP 3 with the S385T, L389F, R517H, and N526K mutations and compared their β-lactam susceptibilities with those of the corresponding clinical isolates.

## MATERIALS AND METHODS

**Bacterial strains.** Two hundred seventy-nine clinical isolates of *H. influenzae* were collected between 1998 and 2000 as part of the Community-Acquired Bacterial Infections Working Group in Japan (K. Ubukata, N. Chiba, K. Hasegawa, Y. Shibasaki, M. Konno, and the Study Group for Communty-Acquired Infections. Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-682, 2001) and a working group for postmarketing surveillance of cefditoren-pivoxil (4). From the results of sequencing of the *ftsI* gene sequences, 151 of 279 isolates had *ftsI* gene mutations, including S385T, L389F, R517H, and/or N526K. Thirty-six isolates that contained common amino acid substitutions in PBP 3 were chosen from among those 151 isolates for further investigation. The Rd

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TABLE 1. Primers used for PCR amplification of the *ftsI* gene and site-directed mutagenesis

| Use | Forward primer[a] | Reverse primer[a] |
|---|---|---|
| PCR amplification of complete *ftsI* gene | 5′-CTCGTTATCCGTTACAGCAG-3′ | 5′-GCCAAACCGTGTGATGAAAC-3′ |
| Introduction of R517H change | 5′-AAAACGGGTACGGCACATAAGATTGAA-3′ | 5′-TTCAATCTTATGTGTGCCGTACCCGTTTT-3′ |
| Introduction of N526K change | 5′-GGACATTATGTAAAGAAGTATGTGGCA-3′ | 5′-TGCCACATACTTCTTTACATAATGTCC-3′ |
| Introduction of M377I change | 5′-GAGATTTTAATTAACTCAAGTAACCGTGGT-3′ | 5′-ACCACGGTTACTTGAGTTAATTAAAATCTC-3′ |
| Introduction of S385T change | 5′-AACCGTGGTGTAACTCGTCTTGCATTA-3′ | 5′-TAATGCAAGACGAGTTACACCACGGTT-3′ |
| Introduction of S385T + L389F changes | 5′-GGTGTAACTCGTCTTGCATTTCGTATGCCA-3′ | 5′-TGGCATACGAAATGCAAGACGAGTTACACC-3′ |

[a] Oligonucleotide sequences were based on the *H. influenzae* Rd genomic sequence (GenBank accession number NC000907).

strain was obtained from the American Type Culture Collection and was used as the recipient strain for all transformations.

**Media, antibiotics, and antimicrobial susceptibility testing.** Chocolate II agar (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) and brain heart infusion (BHI) broth (Becton Dickinson Company, Sparks, Md.) supplemented with 2% defibrinated, heat-treated horse blood plus 15 μg/ml β-NAD was routinely used as the substrate for bacterial growth. MICs were determined by the agar dilution method (6). The medium for the MIC tests was Mueller-Hinton agar (Becton Dickinson Company). The following β-lactam antibiotics were obtained from the indicated commercial sources: penicillin, ampicillin, cefotaxime, and cefaclor, Sigma-Aldrich Chemie GmbH (Steinheim, Germany); cefditoren, Meiji Seika Kaisha, Ltd. (Tokyo, Japan); cefdinir, Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan); cefozopran, Takeda Chemical Industries, Ltd. (Osaka, Japan); imipenem, Banyu Pharmaceuticals (Tokyo, Japan); and meropenem, Sumitomo Pharmaceuticals Co., Ltd. (Tokyo, Japan). Cefcapene and cefpodoxime were prepared from the commercial prodrugs (Shionogi & Co., Ltd., Osaka, Japan; Sankyo Co., Ltd., Tokyo, Japan) at the Medicinal Chemistry Research Laboratories of Meiji Seika Kaisha (Yokohama, Japan).

**PCR and nucleotide sequencing.** The full-length *ftsI* genes were amplified by PCR with the sets of primers listed in Table 1 and Ex *Taq* polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan). PCR amplification was performed with a PCR System 9700 apparatus (Applied Biosystems, Foster City, CA), as follows: 2 min of denaturalization at 94°C and 30 cycles of denaturalization at 94°C for 30s, annealing at 53°C for 30s, and extension at 72°C for 2 min. The PCR products were sequenced by using an ABI PRISM 3700 DNA (Applied Biosystems).

**Cloning of the *ftsI* gene and oligonucleotide-directed mutagenesis.** The DNA fragment amplified from the parent Rd *ftsI* gene by PCR was cloned into the pTrcHis-TOPO vector (Invitrogen Co., Carlsbad, CA). Site-directed mutagenesis of the cloned *ftsI* fragment was carried out with the QuikChange site-directed mutagenesis kit (Strategene, La Jolla, CA). The primers used for amplification of the *ftsI* gene and site-directed mutagenesis are listed in Table 1.

**Homologous recombination.** The *ftsI* genes amplified from 36 clinical isolates and the recombinant *ftsI* genes obtained by site-directed mutagenesis were transformed into the parent Rd strain by electroporation, as described previously (11).

Recombinants were selected on BHI-chocolate agar containing 15 μg/ml NAD, 2% defibrinated, heat-treated horse blood, and β-lactam antibiotics (0.1 or 0.5 μg/ml cefotaxime, 0.25 μg/ml cefdinir, or 0.25 μg/ml cefozopran). Mutations in the recombinant strains were confirmed by DNA sequencing. No unexpected mutations were found in the recombinant strains. Transformed *ftsI* genes from clinical isolates and those obtained by site-directed mutagenesis were stable in the recombinant on BHI-chocolate agar without antibiotics.

**Nucleotide sequence accession numbers.** The complete DNA sequences of the *ftsI* genes from the 36 clinical isolates were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB186930 to AB186965.

## RESULTS

**Site-directed mutagenesis of the *ftsI* gene and resistance to β-lactam antibiotics.** To clarify the relationship between the naturally occurring mutations R517H, N526K, M377I, S385T, and L389F and β-lactam resistance, we used site-directed mutagenesis to introduce the aforementioned mutations into the *ftsI* gene of the parent Rd strain. We successfully obtained the fol-

TABLE 2. MICs of several β-lactams for strain Rd recombinants carrying defined mutations in PBP 3 and for strain Rd for comparison

| Strain | Amino acid mutations by site-directed mutagenesis | | | | | MIC (μg/ml)[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 377M[a] | 385S | 389L | 517R | 526N | CTX | CEC | CDR | CPD | CDN | CPN | AMP | IPM | MEM |
| Rd | | | | | | 0.008 | 1 | 0.25 | 0.031 | 0.008 | 0.008 | 0.125 | 0.125 | 0.031 |
| Recombinant | | | | | | | | | | | | | | |
| 1 | | | | | K | 0.016 | 8 | 0.5 | 0.125 | 0.016 | 0.031 | 0.25 | 1 | 0.031 |
| 2 | | T | | | K | 0.031 | 16 | 1 | 0.25 | 0.016 | 0.063 | 0.5 | 1 | 0.031 |
| 3 | I | T | | | K | 0.031 | 8 | 1 | 0.125 | 0.016 | 0.063 | 0.5 | 1 | 0.031 |
| 4 | | T | F | | K | 0.063 | 32 | 2 | 0.25 | 0.031 | 0.125 | 0.5 | 2 | 0.031 |
| 5 | I | T | F | | K | 0.063 | 16 | 2 | 0.25 | 0.031 | 0.125 | 0.5 | 1 | 0.031 |
| 6 | | T | | H | | 0.016 | 16 | 1 | 0.125 | 0.008 | 0.031 | 0.25 | 0.125 | 0.031 |
| 7 | I | T | | H | | 0.016 | 8 | 0.5 | 0.125 | 0.008 | 0.031 | 0.25 | 0.125 | 0.031 |
| 8 | | T | F | H | | 0.031 | 16 | 2 | 0.25 | 0.016 | 0.063 | 0.5 | 0.125 | 0.031 |
| 9 | I | T | F | H | | 0.031 | 8 | 1 | 0.25 | 0.016 | 0.063 | 0.5 | 0.125 | 0.031 |
| 10 | | T | F | H | K | 0.031 | 16 | 2 | 0.25 | 0.016 | 0.063 | 0.5 | 0.5 | 0.031 |
| 11 | I | T | F | H | K | 0.031 | 8 | 2 | 0.25 | 0.016 | 0.063 | 0.5 | 0.125 | 0.031 |
| 12 | I | T | F | | | 0.031 | 2 | 1 | 0.25 | 0.016 | 0.063 | 0.25 | 0.125 | 0.031 |

[a] The amino acid on PBP 3 in strain Rd.
[b] CTX, cefotaxime; CEC, cefaclor; CDR, cefdinir; CPD, cefpodoxime; CDN, cefditoren; AMP, ampicillin; IPM, imipenem; MEM, meropenem.

TABLE 3. Amino acid mutations PBP 3 from the 36 clinical isolates

| Class | Total no. of isolates | No. of strains with the following amino acid mutation[a]: | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | S7P | A22T | Q28K | P31S | V34L | C56G | A60T | N75S | N122H | L124I | A125V | A131S | E135Q | E141K | S152A | L165S | S166N |
| I | 12 | 3 | | | 6 | 4 | 3 | 1 | 4 | 1 | 3 | 1 | 4 | 4 | 2 | 4 | 4 | 4 |
| II | 4 | | | | 3 | | | | | 1 | | 1 | 1 | 1 | 2 | 2 | 2 | 2 |
| III | 11 | | 1 | | 5 | | | | | | | | | | 5 | 1 | 1 | |
| IV | 5 | 1 | | 1 | 2 | 1 | | | 1 | | 1 | | 1 | 1 | 1 | 1 | 2 | 1 |
| V | 3 | | | | 1 | | | | | 2 | | 2 | 2 | 2 | | 2 | 3 | 2 |
| VI | 1 | | | | 1 | | | | | | | 1 | | | | | | |

[a] Mutations are in reference to the PBP3 sequence of the Rd strain. The amino acid substitutions used for classification are set in boldface type.

lowing recombinants: N526K, N526K + S385T, N526K +S385T + M377I, N526K + S385T + M377I + L389F, R517H + S385T, R517H + S385T + M377I, and R517H + S385T + M377I + L389F but not the recombinants R517H and R517H + M377I. We tested the susceptibilities of the various recombinant strains to several β-lactam antibiotics, and the results are given in Table 2.

The cephalosporin MICs for recombinants with the N526K mutation increased by two- to eightfold compared to those for the parent Rd strain. Introduction of the S385T mutation alone or in combination with L389F into an N526K background resulted in additional MIC increases for cephalosporins. However, the addition of the M377I mutation did not further increase β-lactam resistance. The presence of either the S385T or the L389F mutation into an R517H background also resulted in elevated MICs of all cephalosporins except cefditoren. On the other hand, the imipenem MIC depended entirely on the N526K mutation, while no change in the meropenem MIC was observed for any of the recombinants. For recombinants with both N526K and N526K + S385T, the ampicillin MICs were two- to fourfold greater than that of the parent Rd strain.

**Amino acid mutations in PBP 3 for clinical isolates.** Thirty-six *H. influenzae* clinical isolates carrying PBP 3 with the R517H, N526K, S385T, and/or L389F mutation were selected for further investigation. The PBP 3 sequences of those isolates were grouped into six classes (classes I to VI) on the basis of the patterns of the four mutations shown to be associated with β-lactam resistance in the site-directed mutagenesis study. Table 3 lists all the mutations found in the *ftsI* gene products (PBP 3) for the six classes. No mutations were found near the conserved motif, STVK, at positions 327 to 330 in any of the isolates. Five mutations, A239E, D350N, S357N, V547I, and N569S, were commonly found in members of all classes; indeed, all of the isolates with PBP 3 of classes III, V, and VI had these mutations. In isolates with the class III PBP 3, with the S385T and N526K mutations, the amino acid sequences between positions 174 and 585 positions were identical.

**Resistance to β-lactam antibiotics of clinical isolates and of recombinants carrying the *ftsI* genes from clinical isolates.** Thirty-six Rd recombinant strains were made, and each of these recombinants carried the *ftsI* gene from 1 of the 36 clinical isolates. The β-lactam susceptibilities of the 36 clinical isolates, grouped according to their PBP 3 class, and of the Rd

TABLE 4. β-Lactam susceptibilities of clinical isolates and Rd recombinants carrying the corresponding *ftsI* genes

| Class[a] | No. of strains | Strain[b] | Geometric mean MIC (μg/ml [range]) | | | |
|---|---|---|---|---|---|---|
| | | | Cefotaxime | Cefaclor | Cefdinir | Cefpodoxime |
| | | Rd | 0.008 | 1 | 0.25 | 0.031 |
| I | 12 | R | 0.071 (0.063–0.12) | 38 (16–64) | 1.8 (0.5–4) | 0.22 (0.12–0.25) |
| | | C | 0.059 (0.031–0.12) | 16 (1–128) | 1.3 (0.5–4) | 0.2 (0.12–0.5) |
| II | 4 | R | 0.42 (0.25–0.5) | 64 (64) | 4.8 (4–8) | 0.84 (0.5–1) |
| | | C | 0.35 (0.12–0.5) | 32 (32) | 6.7 (4–8) | 0.84 (0.5–2) |
| III | 11 | R | 0.50 (0.5) | 68 (64–128) | 4.0 (4) | 1.1 (1–2) |
| | | C | 0.57 (0.5–1) | 110 (8–256) | 8.5 (4–32) | 1.8 (0.25–4) |
| IV | 5 | R | 0.041 (0.031–0.12) | 18 (16–32) | 1.3 (0.5–4) | 0.14 (0.12–0.25) |
| | | C | 0.031 (0.031) | 4.0 (1–16) | 0.87 (0.5–1) | 0.12 (0.12) |
| V | 3 | R | 0.16 (0.12–0.25) | 40 (32–64) | 4.0 (4) | 0.31 (0.25–0.5) |
| | | C | 0.12 (0.063–0.5) | 6.3 (2–64) | 4.0 (4) | 0.40 (0.25–1) |
| VI | 1 | R | 0.25 | 64 | 4 | 0.5 |
| | | C | 0.5 | 64 | 4 | 0.5 |

[a] Amino acid mutations used as the basis of a classification, as follows: I, N526K; II, N526K + S385T; III, N526K + S385T + L389F; IV, R517H;V, R517H + S385T; VI, R517H + S385T + L389F.
[b] R, recombinants with the *ftsI* genes from clinical isolates; C, clinical isolates.

TABLE 3—*Continued*

| No. of strains with the following amino acid mutation[a]: | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N169D | R173K | I180N | L219I/M | T228I | A239E | S242A | A271V | S273A | E274D | G280A | D350N | S357N | M377I | **S385T** | **L389F** | A437S | T443A/S | I449V |
| | 3 | | 0/4 | 1 | 9 | | | | 1 | | 9 | 7 | | | | | | 1 |
| 1 | 3 | 1 | 0/3 | 1 | 4 | 1 | 1 | | | 1 | 4 | 4 | 1 | **4** | | 1 | 0/1 | |
| | 2 | | | | 11 | | | | | | 11 | 11 | 11 | **11** | **11** | | | |
| | 1 | | 2/0 | | 5 | | 1 | 1 | | | 3 | 1 | | | | 2 | | |
| | | | 0/2 | | 3 | | | | | | 3 | 3 | 2 | **3** | | | 1/0 | |
| | | | 0/1 | | 1 | | | | | | 1 | 1 | 1 | **1** | **1** | | | |

recombinants carrying the corresponding *ftsI* gene are reported in Table 4. Although there were few common mutations for recombinants of a given class, excluding the class-determining mutation(s), the β-lactam susceptibilities, as measured by the individual MICs, were usually similar (within fourfold) for isolates of each class (Table 4).

Resistance to cephalosporins was similar between clinical isolates and the corresponding recombinants, with the notable exception of resistance to cefaclor for the class IV and V isolates. For class I and IV clinical isolates and the corresponding recombinants with N526K or R517H, the level of resistance to cephalosporins increased 4- to 16-fold compared with that of the susceptible Rd strain. Addition of the S385T substitution (classes II, III, V, and VI) led to further increases in resistance.

Resistance to ampicillin also increased, and the level of resistance depended on the type of mutation for both the clinical isolates and the recombinant strains except those of class III. Four of the 11 clinical isolates of class III showed high-level resistance to ampicillin (MIC, 8 to 16 μg/ml). However, the resistance to ampicillin of the corresponding recombinants was clearly lower than that of the clinical isolates (MICs, 0.5 to 1 μg/ml).

Similar discrepancies in resistance to imipenem and meropenem between the class II and III clinical isolates and the corresponding recombinants were found.

## DISCUSSION

Studies of the β-lactam resistance caused by *H. influenzae* PBP 3 mutations have been reported by several investigators. Ubukata et al. (11) showed that R517H and N526K mutations downstream from the KTG motif and the M377I, S385T, and L389F mutations near the SSN motif of PBP 3 were commonly found in Japanese BLNAR isolates. Dabernat et al. (1) classified 108 French clinical isolates into two classes based on the R517H and N526K mutations. We sequenced the *ftsI* genes of 279 Japanese clinical isolates; and the R517H, N526K, M377I, S385T, and L389F mutations were most commonly found in isolates with increased β-lactam resistance (unpublished data).

To clarify the roles of the R517H, N526K, M377I, S385T, and L389F PBP 3 mutations in β-lactam resistance, we constructed, using site-directed mutagenesis, Rd strains containing the aforementioned mutations in the *ftsI* gene. The site-directed mutagenesis studies indicated that addition of S385T

TABLE 4—*Continued*

| Geometric mean MIC (μg/ml [range]) | | | | |
|---|---|---|---|---|
| Cefditoren | Cefcapene | Ampicillin | Imipenem | Meropenem |
| 0.008 | 0.008 | 0.12 | 0.12 | 0.031 |
| 0.029 (0.016–0.063)<br>0.037 (0.016–0.12) | 0.11 (0.063–0.25)<br>0.089 (0.063–0.12) | 0.56 (0.5–1)<br>0.69 (0.5–4) | 2.5 (0.5–8)<br>2.2 (0.25–8) | 0.071 (0.031–0.12)<br>0.12 (0.063–0.25) |
| 0.089 (0.063–0.12)<br>0.18 (0.063–0.25) | 0.71 (0.25–1)<br>0.84 (0.5–1) | 1.0 (1)<br>2.0 (1–4) | 0.18 (0.063–0.25)<br>3.4 (2–8) | 0.063 (0.063)<br>0.18 (0.12–0.25) |
| 0.12 (0.12)<br>0.21 (0.12–0.25) | 1.0 (1)<br>1.8 (1–4) | 0.78 (0.5–1)<br>3.8 (0.5–16) | 0.12 (0.12–0.25)<br>4.8 (1–32) | 0.04 (0.031–0.063)<br>0.23 (0.031–1) |
| 0.018 (0.016–0.031)<br>0.021 (0.016–0.031) | 0.041 (0.031–0.12)<br>0.041 (0.031–0.063) | 0.50 (0.5)<br>0.38 (0.063–0.5) | 0.38 (0.12–0.5)<br>0.29 (0.063–0.5) | 0.031 (0.031)<br>0.036 (0.016–0.063) |
| 0.032 (0.016–0.12)<br>0.040 (0.016–0.12) | 0.31 (0.25–0.5)<br>0.20 (0.12–0.5) | 0.5 (0.5)<br>1.3 (1–2) | 0.12 (0.12)<br>0.20 (0.12–0.5) | 0.031 (0.031)<br>0.049 (0.031–0.12) |
| 0.063<br>0.063 | 0.5<br>1 | 0.5<br>1 | 0.12<br>0.12 | 0.031<br>0.031 |

TABLE 3—*Continued*

| No. of strains with the following amino acid mutation[a]: | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G490E | A502V/T | V511A | **R517H** | I519L | **N526K** | A530S | T532S | V547I | D551N/Y/V | G555E | Y557H | V562L | N569S | A586S | S594T | A595T | I601L | E603D/G |
| 1 | 3/6 | | | | **12** | 1 | 1 | 9 | 0/1/1 | | | | 7 | 9 | 8 | 8 | | 8/0 |
| | 2/0 | 2 | | 2 | **4** | | 2 | 4 | | | | | 2 | | 1 | 1 | 1 | 1/1 |
| | | | | | **11** | | | 11 | | | | 11 | 11 | 3 | 3 | 3 | | 3/0 |
| | 2/0 | | **5** | | | | | 3 | 1/0/0 | 1 | 1 | | 3 | 2 | 3 | 3 | 2 | 3/1 |
| | | | **3** | | | | 2 | 3 | | 1 | 1 | | 3 | 3 | 3 | 3 | | 3/0 |
| | | 1 | **1** | | | | | 1 | | | | | 1 | 1 | 1 | 1 | | 1/0 |

and S385T + L389F mutations to N526K or R517H further increased the levels of cephalosporin resistance. The reason that a mutant with only the R517H mutation could not be selected is unclear. However, we think that this mutation actually influences resistance to cephalosporins, since it was one of the mutations commonly found in BLNAR strains, and the resistance of the recombinants with the *ftsI* genes from BLNAR strains (Table 4, class IV) to cephalosporins was increased compared with that of the Rd strain. On the other hand, M377I did not influence the degree of antibiotic resistance, but it just might be incidental to the S385T mutation. Thus, four amino acid mutations, R517H, N526K, S385T, and L389F, would play important roles in β-lactam resistance.

We used those four amino acid mutations to classify the clinical isolates on the basis of their PBP 3 sequences, a criterion which appeared to be reasonable, since overall β-lactam resistance was similar for isolates of the same class.

When the MICs for the recombinants with a mutation(s) introduced by site-directed mutagenesis were compared with those for recombinants possessing *ftsI* genes from clinical isolates, the β-lactam resistance of the site-directed recombinants in any class were lower. These results indicate that although the four mutations, R517H, N526K, S385T, and L389F, are important for β-lactam resistance, the other mutations of PBP 3 found in BLNAR clinical isolates could also contribute to resistance, at least when they are combined with the former four mutations.

If the levels of antibiotic resistance of the recombinants with the *ftsI* gene of the BLNAR strains and the parent Rd strain are compared, it is apparent that S385T + R517H or S385T + N526K contributes to the increased resistance to all cephalosporins; however, the absolute degree of resistance depends on the individual cephalosporin. For example, while the MICs of cefotaxime, cefditoren, and cefcapen for the Rd strain were the same (0.008 μg/ml) for the class I recombinants, those of cefotaxime and cefcapene were overall higher than that of cefditoren. This difference was even higher for recombinants with both the S385T and the N526K mutations. Since these three compounds have an aminothiazole side chain at the C-7 position of the cephem skeleton but each has a unique C-3 side chain, we propose that the C-3 methylthiazole side chain of cefditoren could be responsible for a different interaction with the binding pocket of mutated PBP 3.

Although the PBP 3 mutations studied herein could account for much of the resistance to cephalosporins observed in clinical isolates, these mutations were not fully responsible for penicillin or carbapenem resistance. In fact, within a given class, the resistance to imipenem was greater for the clinical isolates than for the recombinants, even though both contained the same *ftsI* gene. A reduction in the affinities of other PBPs in clinical isolates for ampicillin and the two carbapenems could be an explanation for the difference in imipenem susceptibilities. However, an analysis of the full-length DNA sequences coding for all PBPs in the 36 isolates used in this study (unpublished data) did not reveal any of the usual mutations commonly found in PBPs except those of PBP 3 in ampicillin- and carbapenem-resistant strains. Kaczmarek et al. (3) recently showed that the point mutation of *acrR* caused the repressor of the AcrAB pump to lose function, resulting in unusually high resistance to ampicillin when it was combined with a PBP 3 alteration. We have not sequenced the *acrR* gene of Japanese BLNAR isolates. Further investigation is needed to identify what factors affect ampicillin and carbapenem resistance in BLNAR strains.

The main conclusion of this study is that increased levels of resistance to β-lactams, in particular, the cephalosporins, are determined by certain PBP 3 mutations. Such mutations are commonly found in BLNAR strains. The relative degree of resistance depends on both the specific mutation and the type of antibiotic.

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