

## Complete Sequences of Six Penicillin-Binding Protein Genes from 40 *Streptococcus pneumoniae* Clinical Isolates Collected in Japan

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Received 11 September 2003/Returned for modification 20 November 2003/Accepted 21 February 2004

All six penicillin-binding protein (PBP) genes, namely, *pbp1a*, *pbp1b*, *pbp2a*, *pbp2b*, *pbp2x*, and *pbp3*, of 40 *Streptococcus pneumoniae* clinical isolates, including penicillin-resistant *S. pneumoniae* isolates collected in Japan, were completely sequenced. The MICs of penicillin for these strains varied between 0.015 and 8 µg/ml. In PBP 2X, the Thr550Ala mutation close to the KSG motif was observed in only 1 of 40 strains, whereas the Met339Phe mutation in the STMK motif was observed in six strains. These six strains were highly resistant (MICs  $\geq 2$  µg/ml) to cefotaxime. The MICs of cefotaxime for 27 strains bearing the Thr338Ala mutation tended to increase, but the His394Leu mutation next to the SSN motif did not exist in these strains. In PBP 2B, the Thr451Ala/Phe/Ser and Glu481Gly mutations close to the SSN motif were observed in 24 strains, which showed penicillin resistance and intermediate resistance, and the Thr624Gly mutation close to the KTG motif was observed in 2 strains for which the imipenem MIC (0.5 µg/ml) was the highest imipenem MIC detected. In PBP 1A, the Thr371Ser/Ala mutation in the STMK motif was observed in all 13 strains for which the penicillin MICs were  $\geq 1$  µg/ml. In PBP 2A, the Thr411Ala mutation in the STIK motif was observed in one strain for which with the cefotaxime MIC (8 µg/ml) was the highest cefotaxime MIC detected. On the other hand, in PBPs 1B and 3, no mutations associated with resistance were observed. The results obtained here support the concept that alterations in PBPs 2B, 2X, and 1A are mainly involved in *S. pneumoniae* resistance to  $\beta$ -lactam antibiotics. Our findings also suggest that the Thr411Ala mutation in PBP 2A may be associated with  $\beta$ -lactam resistance.

*Streptococcus pneumoniae* is a crucial pathogen that causes community-acquired infections such as pneumonia, otitis media, and meningitis. Since an *S. pneumoniae* strain showing intermediate resistance to penicillin was first isolated in 1967 in Australia (13), the emergence of penicillin-resistant *S. pneumoniae* (PRSP) strains has been reported throughout the world.

One of the common mechanisms of resistance to  $\beta$ -lactam antibiotics is the alterations of penicillin-binding proteins (PBPs). PBPs are the enzymes that catalyze polymerization and cross-linking of peptidoglycan precursors in the bacterial cell wall biosynthesis step (20). The penicillin-binding module contains three conserved motifs that form the active cavity. They are the Ser-X-X-Lys (SXXK), the Ser-X-Asn (SXN), and the Lys-Thr/Ser-Gly (KT/SG) motifs (11). Alterations of the PBPs reduce the affinities of  $\beta$ -lactam antibiotics, resulting in resistance. In *S. pneumoniae*, it has been documented that among the six PBPs, PBPs 1A, 2B, and 2X are the most often associated with penicillin resistance (4, 6, 11).

Although several studies have described the mutations of PBPs and some amino acids residues that must be responsible for resistance to  $\beta$ -lactam antibiotics, only one or two PBP genes or their partial sequences were investigated and compared to the sequences of the genes of penicillin-sensitive *S.*

*pneumoniae* strains in those studies (2, 3, 10, 16, 21, 22, 23, 24, 28). The *pbp1a*, *pbp2b*, and *pbp2x* genes of 2 and 18 clinical isolates collected mainly in the United States (5, 17), 8 clinical isolates collected in France (9), and 15 clinical isolates collected in Canada (21) were sequenced. In the present study, we completely sequenced the six PBP genes of 40 clinical isolates of *S. pneumoniae*, including PRSP isolates, collected in Japan and describe the mutations in these genes.

### MATERIALS AND METHODS

**Bacterial strains.** Forty clinical isolates of *S. pneumoniae* were selected from among more than 6,600 isolates collected between 1998 and 2000 by the Community-Acquired Bacterial Infections Working Group in Japan (27). The isolates were selected on the basis of of penicillin, cefotaxime, and imipenem MICs (see Results). To find out the common features of the penicillin-susceptible *S. pneumoniae*, penicillin-intermediate *S. pneumoniae*, and PRSP strains spread throughout Japan, we chose a variety of strains in terms of the diseases that they caused and the districts where they were isolated. Strain R6, obtained from American Type Culture Collection, was used as a reference strain.

**Identification of *S. pneumoniae*.** To identify *S. pneumoniae*, the *lytA* gene encoding pneumococcal autolysin was detected by PCR (18). The primers used to detect the *lytA* gene are shown in Table 1. They were designed on the basis of the nucleotide sequence reported by Kawamura et al. (14). Furthermore, the partial sequence of the *sodA* gene, which encodes superoxide dismutase, was determined to confirm the identification (14). The primers were designed as shown in Table 1, and the DNA sequences were compared with those of other streptococci obtained from the GenBank database.

**Antimicrobial susceptibility testing.** MICs were determined by the microdilution method recommended by the National Committee for Clinical Laboratory Standards (19). Antibiotics were obtained from Eiken Chemical Co., Ltd. (Tokyo, Japan).

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TABLE 1. Oligonucleotide primers for PCR amplification

Gene	Sense primer sequence	Antisense primer sequence
<i>pbp1a</i>	5'-CCAGCAACAGGTGAGAGTC-3'	5'-GTAACACAAGCCAAGACAC-3'
<i>pbp1a<sup>a</sup></i>	5'-GAACTTCAAGACAAGGCAGT-3'	5'-GTAACACAAGCCAAGACAC-3'
<i>pbp1b</i>	5'-TTCATACCCTTATTGTAACACG-3'	5'-TAGCTCATCAAAAATGTGCATG-3'
<i>pbp2a</i>	5'-CCGCTGATCTTGATTGAATAG-3'	5'-ATGCGTTTTTCATCCCCTCTG-3'
<i>pbp2b</i>	5'-CCGTCTTAATCCCGATACC-3'	5'-ATTTTTGGGTGACTTGTGAG-3'
<i>pbp2x</i>	5'-GGAATTGGTGTCCCCTAAGC-3'	5'-CATCTGCTGGCCTGTAATTTG-3'
<i>pbp3</i>	5'-ATACGCAGTTGAGCTGAACC-3'	5'-GCACGCTAACAAAAGGGTTAA-3'
<i>lytA</i>	5'-CTGTTTCAATCGTCAAGCCG-3'	5'-TAAGAACAGATTTGCCTCAAGT-3'
<i>sodA</i>	5'-ACAATGCATTTGCACCATGAC-3'	5'-GGTTTGCTGTTGAAGTCACTT-3'

<sup>a</sup> Used for strains SP00081, SP00087, and SP00090.

**PCR and nucleotide sequencing.** The six sets of primers used for PCR were designed on the basis of the DNA sequences of strain R6 (DDBJ/EMBL/GenBank accession number NC003098). The oligonucleotide sequences used for DNA amplification are shown in Table 1. The *pbp1a* gene was not successfully amplified from strains SP00081, SP00087, and SP00090 with the set of primers described above. Therefore, an additional set of primers was designed to amplify the *pbp1a* gene; the primers were based on the DNA sequence obtained by direct sequencing with a GenomicTip G20 kit (Qiagen K. K., Tokyo, Japan). The oligonucleotide sequences used to amplify the DNA of these three strains are shown in Table 1. PCR amplification was performed with a PCR System 9700 (Applied Biosystems, Foster City, Calif.). The PCR products were sequenced with an ABI PRISM 3700 DNA analyzer (Applied Biosystems). Both strands were amplified by PCR, and a given position was sequenced at least three times with different primers.

**Nucleotide sequence accession numbers.** The complete sequences of the six PBP genes from 40 clinical isolates appear in the DDBJ/EMBL/GenBank nucleotide sequence database. The serial numbers for the 40 clinical isolates are SP00051 to SP00092, with SP00066 and SP00071 missing. The corresponding accession numbers for the sequences of the PBP genes from the 40 clinical isolates in the DDBJ/EMBL/GenBank database are as follows: AB119753 to AB119792 for *pbp1a*, AB119793 to AB119832 for *pbp1b*, AB119833 to AB119872 for *pbp2a*, AB119873 to AB119912 for *pbp2b*, AB119913 to AB119952 for *pbp2x*, and AB119953 to AB119992 for *pbp3*.

RESULTS AND DISCUSSION

**Amino acid substitutions in PBPs and β-lactam antibiotic susceptibilities.** The deduced amino acid substitutions in PBPs 2X, 2B, 1A, and 2A are shown in Table 2, which summarizes the mutations for groups of strains with identical or similar mutations. The activities of the β-lactam antibiotics against the *S. pneumoniae* isolates used in this study are shown in Table 3, which provides the range of MICs for each group in Table 2. The activities of carbapenems and faropenem were more potent than those of the penicillins and cephalosporins against PRSP strains. In particular, panipenem showed the most potent activity, and its geometric mean MIC was 0.091 μg/ml. Among the cephalosporins, cefditoren, the active form of cefditoren pivoxil, showed the most potent activity against PRSP strains.

**Substitutions in PBP 2X.** The deduced amino acid substitutions in PBP 2X are shown in Table 2. Mutations at positions close to each of three conserved motifs, SXXK, SXN, and

TABLE 2. Deduced amino acid substitutions in PBPs

Strain	Amino acid substitution <sup>a</sup>																		
	PBP 2X					PBP 2B				PBP 1A				PBP 2A					
	338T	339M	394H	550T	431T	Insertion	432Q	451T	481E	624A	371T	432P	574T	575S	576Q	577F	411T	461S	586V
R6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SP00051, SP00053, SP00054	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/.	I/.
SP00052	.	.	.	<b>A</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I
SP00058, SP00059, SP00060, SP00061	<b>A</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I/.
SP00069, SP00074	<b>A</b>	.	.	.	.	.	.	<b>A</b>	<b>G</b>	.	.	.	.	.	.	.	.	A	.
SP00055, SP00067, SP00073	.	.	<b>L</b>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I/.
SP00072	.	.	<b>L</b>	.	.	.	.	<b>A</b>	<b>G</b>	.	.	.	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	.
SP00057, SP00070	.	.	<b>L</b>	.	.	P/-	<b>J/A</b>	<b>S/P</b>	<b>G</b>	.	.	.	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	I/.
SP00081	.	.	<b>L</b>	.	<b>K</b>	.	<b>L</b>	<b>A</b>	<b>G</b>	.	<b>S</b>	<b>T</b>	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	I
SP00056, SP00063, SP00064, SP00068	<b>A</b>	.	.	.	.	.	.	.	.	.	<b>S/.</b>	<b>T/.</b>	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	I/.
SP00085, SP00088	<b>A</b>	.	.	.	.	.	.	<b>A</b>	<b>G</b>	.	<b>S/.</b>	<b>T</b>	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	I
SP00075, SP00076, SP00078, SP00079, SP00082, SP00083, SP00086, SP00087, SP00089	<b>A</b>	.	.	.	<b>K</b>	.	<b>L</b>	<b>A</b>	<b>G</b>	.	<b>S/A</b>	<b>T</b>	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	I/.
SP00062	<b>P</b>	.	.	.	.	WYT	.	<b>S</b>	<b>G</b>	.	.	.	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	I
SP00065	<b>A</b>	<b>F</b>	.	.	.	.	.	.	.	.	<b>S</b>	.	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	I
SP00077	<b>A</b>	<b>F</b>	.	.	.	.	.	<b>A</b>	<b>G</b>	.	<b>A</b>	<b>T</b>	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	.
SP00080, SP00091	<b>A</b>	<b>F</b>	.	.	<b>K</b>	.	<b>L</b>	<b>A</b>	<b>G</b>	.	<b>S</b>	<b>T</b>	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	I
SP00090	<b>A</b>	<b>F</b>	.	.	.	WYT	.	<b>S</b>	<b>G</b>	.	<b>S</b>	<b>T</b>	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	I
SP00092	<b>A</b>	.	.	.	<b>K</b>	.	<b>L</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	<b>T</b>	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	.	A	.
SP00084	<b>A</b>	<b>F</b>	.	.	<b>K</b>	.	<b>L</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>S</b>	<b>T</b>	<b>N</b>	<b>T</b>	<b>G</b>	<b>Y</b>	<b>A</b>	<b>A</b>	.

<sup>a</sup> Identity with the amino acid from strain R6 is indicated by a dot. The strains were typed by the mutations indicated in boldface, which are mainly close to the conserved motifs: 337STMK, 395SSN, and 547KSG for PBP 2X; 391SVVK, 448SSN, and 619KTG for PBP 2B; 370STMK, 428SRN, and 557KTG for PBP 1A; and 410STIK, 465SLN, and 590KTG for PBP 2A.

TABLE 3. Antibacterial activities of  $\beta$ -lactam antibiotics

Strain	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>										
	PEN	AMX	CTX	CRO	CPD	CDR	CDN	IPM	MEM	PAM	FRM
R6	0.016	0.016	0.016	0.031	0.031	0.008	0.063	0.008	0.016	0.004	0.002
SP00051, SP00053, SP00054	0.016-0.063	0.016-0.031	0.031-0.125	0.031-0.125	0.063-0.125	0.125-0.25	0.016-0.063	0.008	0.016	0.004	0.008-0.016
SP00052	0.008	0.008	0.25	0.125	0.5	0.5	0.063	0.016	0.016	0.002	0.008
SP00058, SP00059, SP00060, SP00061	0.031-0.063	0.031-0.063	0.25-0.5	0.25-0.5	0.5-1	0.25-0.5	0.125-0.25	0.008-0.016	0.016-0.031	0.002-0.008	0.008-0.016
SP00069, SP00074	0.125-0.25	0.125	0.5	0.25-0.5	1-2	0.5-1	0.25	0.016	0.063	0.008	0.016-0.063
SP00055, SP00067, SP00073	0.031-0.125	0.031-0.063	0.125-0.25	0.125-0.25	0.25	0.25-0.5	0.063-0.125	0.008	0.016	0.002-0.004	0.008-0.016
SP00072	0.031	0.25	0.25	0.25	0.5	0.5	0.25	0.031	0.063	0.008	0.031
SP00057, SP00070	0.25	0.25	0.125-0.25	0.25-0.5	0.25-0.5	0.5	0.125	0.031-0.063	0.063-0.125	0.008	0.063
SP00081	0.5	0.25	0.25	0.25	0.25	0.5	0.25	0.125	0.25	0.031	0.125
SP00056, SP00063, SP00064, SP00068	0.063-0.5	0.031-0.25	0.5-1	0.5-1	1-2	2-8	0.25-1	0.008-0.063	0.016-0.125	0.002-0.016	0.008-0.063
SP00085, SP00088	0.5-0.1	0.25	1-2	1-2	2	2-8	0.5-1	0.063	0.125	0.016-0.031	0.125
SP00075, SP00076, SP00078, SP00079, SP00082, SP00083, SP00086, SP00087, SP00089	0.5-2	0.5-1	0.5-1	0.5-2	1-4	1-8	0.25-1	0.125-0.25	0.25-0.5	0.031-0.125	0.125-0.5
SP00062	0.25	0.125	0.063	0.063	0.125	0.125	0.031	0.063	0.063	0.008	0.063
SP00065	0.063	0.063	2	2	16	2	0.5	0.016	0.031	0.004	0.016
SP00077	1	0.5	2	2	16	8	1	0.125	0.25	0.031	0.125
SP00080, SP00091	2-4	1-2	4	2-4	4-16	16	1-2	0.25-0.5	0.5	0.063-0.25	0.25-0.5
SP00090	4	1	4	4	16	32	4	0.25	0.5	0.125	0.25
SP00092	2	8	4	2	4	16	1	0.5	1	0.25	1
SP00084	4	2	8	4	16	16	2	0.5	2	0.125	1

<sup>a</sup> Abbreviations: PEN, penicillin; AMX, amoxicillin; CTX, cefotaxime; CRO, ceftriaxone; CPD, cefpodoxime; CDR, ceftidizime; CDN, ceftidizole; IPM, imipenem; MEM, meropenem; PAM, panipenem; and FRM, faropenem.

KT/SG, or those which seemed to be related to susceptibility are indicated in Table 2. The Met339Phe mutation in the STMK motif was observed in 6 of the 40 strains, and all of them were highly resistant to cefotaxime (MICs  $\geq 2$   $\mu\text{g/ml}$ ). On the other hand, the Thr338Ala mutation was observed in 28 strains, but not all of them were necessarily highly resistant to cefotaxime. Asahi et al. (2) reported that both the Met339Phe and the Thr338Ala mutations in PBP 2X are important substitutions leading to high levels of resistance to cefotaxime. However, our findings do not completely support their results in the case of strains bearing the Thr338Ala mutation. Interestingly, the His394Leu mutation just before the SSN motif did not exist in the strains bearing the Thr338Ala mutation. Although there might be an association between Thr338Ala and His394Leu mutations, the influences of these substitutions on  $\beta$ -lactam resistance are not clear. The Thr550Ala mutation just after the KSG motif was observed in only 1 of the 40 strains, and no strain bore the Gln552Glu mutation. It has been clearly shown by site-directed mutagenesis that these two mutations in PBP 2X are responsible for  $\beta$ -lactam resistance (5, 9). However, these mutations are not found in clinical isolates too frequently (9).

**Substitutions in PBP 2B.** The deduced amino acids substitutions in PBP 2B are shown in Table 2. The insertion of Trp-Tyr-Thr between residues 431 and 432, described by Yamane et al. (28), was observed in two strains. The insertion of Phe between residues 431 and 432 was observed in one strain. The short mosaic sequence Ala-Phe-Ser-Arg/Val-Pro-Asn from residues 432 to 437 described by Dowson et al. (7) was observed as Ala-Phe-Ser-Gly-Ala-Met in one strain in this study.

The Thr446Ala mutation adjacent to the SSN motif was not observed in this study. This mutation was confirmed by site-directed mutagenesis to be associated with  $\beta$ -lactam resistance (10). However, no clinical isolates bearing this mutation were found in Japan or France (9), similar to the case of the Thr550Ala and the Gln552Glu mutations in PBP 2X. The Thr451Ala/Phe/Ser and the Glu481Gly mutations, close to the SSN motif, were observed in 18 of the 19 strains for which penicillin MICs were  $\geq 0.5$   $\mu\text{g/ml}$ . These findings are consistent with the observation that these mutations are common in resistant strains isolated in both South Africa and France (14, 23). The Thr624Gly mutation, close to the KTG motif, was observed in two strains; and the imipenem MICs for these two strains were the highest (0.5  $\mu\text{g/ml}$ ) among those for the strains tested. This mutation was reported in a  $\beta$ -lactam-resistant recombinant of *S. pneumoniae* obtained by transformation with chromosomal DNA prepared from a *Streptococcus mitis* isolate (12). Although strains bearing the Thr624Gly mutation are rare among clinical isolates, this mutation seems to be important for the acquisition of high-level resistance to carbapenems.

**Substitutions in PBP 1A.** The deduced amino acids substitutions in PBP 1A are shown in Table 2. The Thr371Ser/Ala mutation in the STMK motif was observed in 18 of the 19 strains for which the penicillin MICs were  $\geq 0.5$   $\mu\text{g/ml}$ . Four consecutive substitutions, Thr574Asn, Ser575Thr, Gln576Gly, and Phe577Tyr, were found in the 19 strains for which the penicillin MICs were  $\geq 0.5$   $\mu\text{g/ml}$  and in 3 of 6 strains for which they were 0.12 to 0.25  $\mu\text{g/ml}$ . These two types of mutations

were confirmed to be essential for penicillin resistance by using reverse amino acid substitution by site-directed mutagenesis (24, 25). Interestingly, one isolate had a 16-amino-acid deletion, from residues 670 to 685, which did not appear to be necessary for resistance to  $\beta$ -lactams. In strains SP00081, SP00087, and SP00090, the DNA from the *pbp1a* genes was not amplified when the set of primers designed from the sequence data for strain R6 were used. We determined the DNA sequences of downstream regions by direct sequencing and detected a rearrangement of the mosaic elements, which would be the partial *pena* gene of *S. mitis*. The PBP encoded by the *pena* gene has been known as a low-affinity PBP (1). Therefore, these three strains probably acquired the mosaic element on the *pbp1a* gene by interspecies gene transfer and, as a result, showed high-level resistance to  $\beta$ -lactams.

An insertion sequence (IS) of about 1.4 kb was detected in three strains (strains SP00055, SP00065, and SP00073) downstream (from 10 to 20 bases) from the *pbp1a* gene. The DNA sequences of these IS elements corresponded to IS1167, which encodes the transposase of *S. pneumoniae* (30). Compared with the DNA sequence of IS1167 (DDBJ/EMBL/GenBank accession number AE07425), the percent identities for SP00055 and SP00073 (98.0% for both) were higher than that for SP00065 (88.5%). Furthermore, the DNA sequences at the 3' region of the *pbp1a* gene were also identical between strains SP00055 and SP00073, as were those of the IS elements. This observation suggests that the IS element contributes to horizontal gene transfer and the genetic rearrangement of *pbp* genes.

**Substitutions in PBP 2A.** The deduced amino acid substitutions in PBP 2A are shown in Table 2. The Thr411Ala mutation in the STIK motif was observed in only one strain, which showed a high level of resistance to cefotaxime (MIC = 8  $\mu\text{g/ml}$ ). This mutation was also seen in  $\beta$ -lactam-resistant recombinants of *S. pneumoniae* obtained through transformation of chromosomal DNA from *S. mitis* strain B6, resulting in low-affinity variants (12). Although Zhao et al. (29) and Du Plessis et al. (8) reported that the substitutions in PBP 2A would not be associated with the acquisition of  $\beta$ -lactam resistance, our findings suggest that the Thr411Ala mutation in PBP 2A would be important for additional resistance.

As was the case for *pbp1a*, the IS element was observed downstream from the *pbp2a* gene in three strains (strains SP00065, SP00069, and SP00084). However, the DNA sequences of the IS element and its upstream region were slightly different among these strains. The significance of these insertions near the *pbp2a* gene is not clear at present.

**Substitutions in PBP 1B and PBP 3.** The deduced amino acids substitutions in PBP 1B and PBP 3 were also investigated. No mutations at positions close to their conserved motifs that would be associated with  $\beta$ -lactam resistance were noted in this study. However, it has not been clarified whether PBP 1B plays an important role in the development of resistance (8, 12). As for PBP 3, Krauss and Hakenbeck (15) detected the Thr242Ile mutation in laboratory mutants resistant to cefotaxime. We believe that the Thr242Ile mutation is not necessarily common in clinical isolates, since this mutation was not observed in this study.

**Alteration ratio of the TP domain and complete PBPs.** We investigated the relationship between the ratio of mutations in

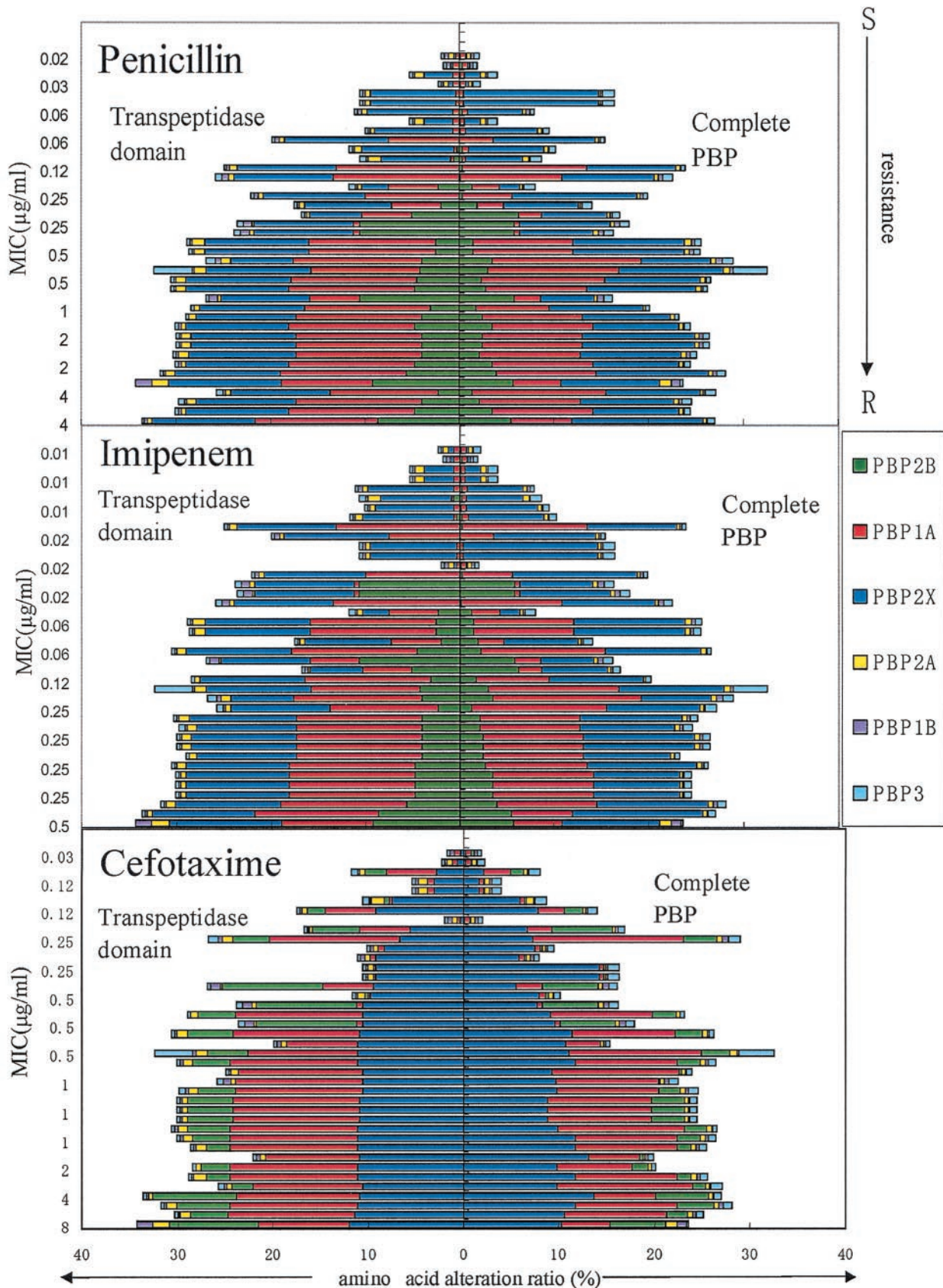


FIG. 1. Relationship between the amino acid alteration ratios for six PBPs and susceptibility to penicillin, imipenem, and cefotaxime for 40 clinical isolates of *S. pneumoniae*. The alteration ratios for the TP domain and the complete PBP were calculated on the basis of the sequence of reference strain R6.

the PBPs and susceptibility to penicillin, cefotaxime, and imipenem. The alteration ratio within the transpeptidase (TP) domain and complete PBPs was calculated on the basis of the sequence of reference strain R6, and the results are shown in Fig. 1. The alteration ratios increased in the following order: PBP 2B (alteration ratio for TP domain, 0 to 10.6%; alteration ratio for complete PBP, 0 to 6.3%), PBP 2X (alteration ratio for TP domain, 0.28 to 11.9%; alteration ratio for complete PBP, 0.13 to 14.3%), and PBP 1A (alteration ratio for TP domain, 0.31 to 17.3%; alteration ratio for complete PBP, 0.42 to 15.7%). Furthermore, the alteration ratios for the TP domain tended to be higher than those for the complete PBP. On the other hand, the alteration ratios for the other three PBPs, namely, PBPs 2A, 1B, and 3, were low, regardless of whether the strain was susceptible or resistant to  $\beta$ -lactams (for PBP 1B, the alteration ratio for the TP domain was 0 to 1.2% and that for the complete PBP was 0 to 1.0%; for PBP 2A, the alteration ratio for the TP domain was 0 to 1.8% and that for the complete PBP was 0 to 1.2%; and for PBP 3, the alteration ratio for the TP domain was 0 to 4.0% and that for the complete PBP was 0.24 to 3.6%). Interestingly, the alteration ratios for PBPs 1A, 2B, and 2X, which are responsible for  $\beta$ -lactam resistance, were higher than those for PBPs 1B, 2A, and 3. These findings suggest that antibiotic pressure is capable of promoting genetic rearrangement in *S. pneumoniae*. The alteration ratios for PBPs have been reported by a few investigators (9, 21), but these pieces of information were restricted to the TP domains of PBPs. Clinical practitioners in Japan prefer to use cephalosporins instead of penicillins for the treatment of community-acquired infections, and the prevalence of cephalosporin-resistant *S. pneumoniae* strains in Japan has been increasing year by year (26). The sensitivity of *S. pneumoniae* to cephalosporins is related to the specific amino acid changes and also to the alteration ratio for PBP 2X.

**Concluding remarks.** In conclusion, the results obtained from the complete sequencing of the *pbp* genes support the concept that mutations in PBPs 1A, 2B, and 2X play an important role in the development of resistance to  $\beta$ -lactam antibiotics by *S. pneumoniae*. We also detected a mutation which would be important for additional resistance to cefotaxime in the STIK motif of PBP 2A but did not detect such a mutation in PBP 1B or PBP 3. The MICs of penicillins and carbapenems tended to be strongly influenced by mutations in PBP 2B, but those of cephalosporins tended to be strongly influenced by mutations in PBP 2X. Mutations in PBP 1A were associated with additional increases in the MICs for strains bearing mutations in PBP 2X or PBP 2B. Panipenem, a parenteral carbapenem, and cefditren, an active form of an oral cephalosporin, showed the most potent activities against *S. pneumoniae* strains, including PRSP.

#### ACKNOWLEDGMENT

We thank Kimiko Ubukata, Kitasato University, for kindly providing clinical isolates of *S. pneumoniae*.

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