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NOTES

Diversity of Substitutions within or Adjacent to Conserved Amino Acid Motifs of Penicillin-Binding Protein 2X in Cephalosporin-Resistant *Streptococcus pneumoniae* Isolates

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The sequence of an approximately 1.1-kb DNA fragment of the *pbp2x* gene, which encodes the transpeptidase domain, was determined for 35 clinical isolates of *Streptococcus pneumoniae* for which the cefotaxime (CTX) MICs varied. Strains with substitutions within a conserved amino acid motif changing STMK to SAFK and a Leu-to-Val change just before the KSG motif were highly resistant to CTX (MIC, ≧2 μg/ml). Strains with substitutions adjacent to SSN or KSG motifs had low-level resistance. The amino acid substitutions were plotted on the three-dimensional crystallographic structure of the transpeptidase domain of PBP2X. Transformants containing *pbp2x* from strains with high-level CTX resistance increased the CTX MIC from 0.016 μg/ml to 0.5 to 1.0 μg/ml.

Clinical isolates of penicillin (PC)-resistant *Streptococcus pneumoniae* (PRSP) for which cefotaxime (CTX) MICs are ≧2 μg/ml have been reported in recent years (3, 5, 8, 24, 30). In Japan, PRSP isolates for which CTX MICs ranged from 2 to 8 μg/ml have been recently isolated.

The high-molecular-weight PC-binding proteins (PBPs) 1A, 2X, and 2B, usually detected in *S. pneumoniae*, are involved in transpeptidase activity and contain conserved amino acid motifs of SXXK, including the active-site serine residue as a target of β-lactams, SXN, and KT(S)G. The decreased affinity of PBP 1A, 2B, and 2X for β-lactams has been shown to play an important role in the development of their resistance (2, 12, 17, 20, 29). In particular, alterations in PBP 2B mediate low-level resistance to PCs (25), while those in PBP 2X mediate low-level resistance to cephalosporins (7, 10, 13). Additional alterations in PBP 1A increased PC MICs to ≧1 μg/ml and CTX MICs to ≧0.5 μg/ml (20, 22, 29). The evidence that PBP 2A and PBP 1B with low affinity also affect β-lactam resistance was presented by Hakenbeck et al. (11).

Genetic analyses of *pbp1a* (1, 18), *pbp2x* (15, 16), and *pbp2b* (6) in PC-susceptible *S. pneumoniae* (PSSP) and PRSP have already been conducted. As for PBP 1A, of the many amino acid substitutions in the transpeptidase domain, substitution of Ala or Ser for Thr-371 in the conserved STMK motif has been most important for the development of PC resistance (1, 26). As for PBP 2B, substitutions of Ala or Ser for Thr just after the SSN motif, and of Gly for conserved Glu, were important in developing PC resistance (6, 25, 31). On the other hand, substitution of Ala for Thr just after the KSG motif in PBP 2X involved low-level resistance of cephalosporins (10, 23). Recently, structural evidence linking resistance to multiple β-lactams to amino acid substitutions for Thr-338 and/or Ser-571 within a buried cavity near the Ser-337 of a catalytic site in PBP 2X has been presented by Mouz et al. (19).

We determined the nucleotide sequence of a 1.1-kb region encoding transpeptidase activity, from bp 1018 to 2080, in the *pbp2x* gene sequence of *S. pneumoniae* strains ($n = 35$) isolated in Japan between 1993 and 1997. Amplification of DNA fragments and the sequencing reaction were carried out as described previously (1) with the following PCR primers: 5′-T_{958}ATGAAAAGGATCGTCTGGG$_{977}$ and 5′-A_{2105}GAG AGTCTTTCATAGCTGAAGC$_{2083}$. The correlation between amino acid substitutions in PBP 2X and the development of cephalosporin resistance was then examined.

The amino acid sequence of PBP 2X in the strains for which the CTX MICs were ≧0.125 μg/ml exhibited a variety of amino acid substitutions different from those of CTX-susceptible strains. On the basis of the substitution patterns within or adjacent to the three conserved amino acid motifs of STMK, SSN, and KSG, resistant strains were classified into five groups (Table 1). Table 1 also shows (i) the serotypes of the strains, (ii) the susceptibilities of the strains to PC, CTX, cefpodoxime, cefditoren, cefdinir, and cefaclor, and (iii) mutations in the *pbp1a* and *pbp2b* genes. Figure 1 shows the predicted amino acid sequence from residues 271 to 610 of a representative strain from each group. The nucleotide sequences of five PSSP strains were determined for comparison. They were identical to those of the strain R6.

Four strains were classified into group I. In this group, Thr-550 just after the KSG motif was replaced with Ala. From the X-ray crystallographic structure of a complex of a homologous DD-peptidase and CTX, Kuzin et al. (14) showed that the loss of a hydrogen bond between the Thr and CTX by the change to Ala can account for the higher CTX MICs. Other than this Thr, only one or two amino acid substitutions were confirmed. The CTX MIC for these strains was 0.125 μg/ml, which was four to eight times higher than that for PSSP.

Eight strains were classified into group II. Ala-393 and His-394 just before the SSN motif were replaced with Val and Leu,

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TABLE 1. Classification based on PBP 2X sequence differences and properties of *S. pneumoniae* strains

Group	Strain	Serotype	MIC (μg/ml)[a]						Amino acid motif[b]			PCR result[c]	
			PC	CTX	CPDX	CDTR	CFDN	CCL	STMK	AHSSNV	LKSGT	*pbp1a*	*pbp2b*
None	1/E9	15	0.031	0.031	0.125	0.016	0.125	1	----	------	-----	−	−
	2/HSB1	15	0.031	0.031	0.125	0.031	0.125	2	----	V-----	-----	−	−
	3/YO42	3	0.031	0.063	0.125	0.031	0.125	1	----	------	-----	−	−
	4/HSC21	6	0.031	0.063	0.125	0.031	0.25	1	----	------	-----	−	−
	5/H48	19	0.031	0.063	0.125	0.031	0.25	1	----	------	-----	−	−
I	6/HSB5	3	0.008	0.125	0.5	0.031	0.25	2	----	------	----A	−	−
	7/T37	6	0.008	0.125	0.25	0.031	0.25	2	----	------	----A	−	−
	8/TJ24	9	0.016	0.125	0.25	0.031	0.25	1	----	------	----A	−	−
	9/E13	9	0.016	0.25	0.5	0.031	0.25	2	----	------	----A	−	−
II	14/TO22	19	0.031	0.063	0.25	0.063	0.125	0.5	----	-L----	V----	−	−
	17/SU1	19	0.063	0.125	0.25	0.016	0.25	1	----	-L----	V----	−	−
	21/H3	10	0.031	0.125	0.25	0.063	0.125	1	----	-L----	-----	−	−
	16/MA37	23	0.063	0.125	0.25	0.125	0.25	1	----	VL----	-----	−	−
	22/H23	14	0.031	0.063	0.25	0.063	0.25	1	----	VL----	-----	−	−
III	11/KM99	3	0.031	0.125	0.5	0.063	0.25	0.5	-A--	------	-----	−	−
	15/HSC9	3	0.031	0.125	0.5	0.125	0.25	1	-A--	------	-----	−	−
	18/TJ41	3	0.031	0.125	0.5	0.063	0.25	2	-A--	------	-----	−	−
	23/H69	3	0.031	0.25	0.25	0.063	0.25	1	-A--	------	-----	−	−
	25/S19	3	0.031	0.125	0.5	0.125	0.25	1	-A--	------	----	−	−
IV	34/H31	6	1	0.5	1	0.5	4	32	-A--	------	V----	+	+
	36/Z21	6	2	0.5	2	0.5	8	64	-A--	------	V----	+	+
	37/H28	19	1	0.5	2	0.5	2	64	-A--	------	V----	+	+
	38/KM90	19	2	0.5	2	0.5	4	64	-A--	------	V----	+	+
	39/Z13	19	2	0.5	1	0.5	4	64	-A--	------	V----	+	+
	40/H29	23	1	0.5	2	0.5	4	64	-A--	------	V----	+	+
	41/B99	23	1	0.5	2	0.5	4	64	-A--	------	V----	+	+
	42/H0	14	4	1	4	1	8	64	-A--	------	V----	+	+
	27/S46	23	0.5	0.5	1	0.25	2	32	-A--	------	V----	−	+
	1A22/HA5	6	0.25	0.5	1	0.25	2	16	-A--	------	V----	−	+
V	20/SHA3	19	2	4	16	4	32	64	-A--	V-----	V----	+	+
	29/KK133	23	2	8	16	4	32	64	-AF-	------	V----	+	+
	30/NG44	23	2	2	16	2	16	64	-AF-	------	V----	+	+
	31/KU5	23	1	2	8	2	8	64	-AF-	------	V----	+	+
	32/KU81	19	4	4	16	2	16	64	-AF-	------	V----	+	+
	33/AK5	14	0.125	4	16	2	2	8	-AF-	------	V----	+	−

[a] Susceptibilities to PC, CTX, cefpodoxime (CPDX), cefditoren (CDTR), cefdinir (CFDN), and cefaclor (CCL) were determined by previously described methods (1).

[b] Only amino acid residues differing from PBP 2X conserved motif sequences of the PSSP strain R6 are shown. Conserved amino acid motifs are underlined.

[c] Altered PBP genes were detected by PCR as described previously (29). +, altered; −, not altered.

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                                 280        290        300        310        320        330        340        350        360        370        380
PSSP        R6     SFMETQMDAF QEKVKGKYMT ATLVSAKTGE ILATTQRPTF DADTKEGITE DFVWRDILYQ SNYEPGSTMK VMMLAAAIDN NTFPGGEVFN SSELKIADAT IRDWDVNEGL
PSSP        1/E9   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Group I     7/T37  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Group II    22/H23 ---------- ---------- ---------- ---------- -------L-K ---------- ---------- --T------- --Y------- -D-------- ---------V-
Group III   25/S19 ---------- ---------- ---------- ---------- N----D---K ---------- -------A-- --T--S---- -------Y-- ---------- -------D--
Group IV    34/H31 ----------L---------- ---------- ---------- N--------- ---------- -------A-- --T--SS--- ----S--Y-- ---F------ T------D--
Group V     31/KU5 ----------L---------- ---------- ---------- N--------- ---------- -------AF- -----SS--- ----S--Y-- ---F------ T---------
                390        400        410        420        430        440        450        460        470        480        490        500
R6      TGGRMMTFSQ GFAHSSNVGM TLLEQKMGDA TWLDYLNRFK FGVPTRFGLT DEYAGQLPAD NIVNIAQSSF GQGISVTQTQ MIRAFTAIAN DGVMLEPKFI SAIYDPNDQT ARKSQKEIVG
1/E9    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
7/T37   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
22/H23  ----------VL---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
25/S19  -T-G------ ----------S ---------- ---------- ---T------ ------M-A- ---------- -L-------- ---------- --L------S V---------
34/H31  -T-G----L- ----------S ---------- ------K--- ---------- ---------- ---S------ ---------- -L-------- ---------- -----T-N-S V---------
31/KU5  -T-G------ ---------T S---------- ------K--- ---------- ---------- ---S------ ---------- -L-------- ---------- -----T-N-S V---------
                510        520        530        540        550        560        570        580        590        600        610
R6      NPVSKDAASL TRTNMVLVGT DPVYGTMYNH STGKPTVTVP GQNVALKSGT AQIADEKNGG YLVGLTDYIF SAVSMSPAEN PDFILYVTVQ QPEHYSGIQL GEFANPILER
1/E9    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------
7/T37   ---------- ---------- ---------- ---------- ----------A ---------- ---------- ------
22/H23  ---------- ---H------ ---------- ---------- ---------- ---------- ------N--- ---------- ---------- ---------- ----------
25/S19  -----A---S --EH--M--- ---------- -----N-N-- ---------- ---------- --T-E-N--- -V---H---- ---------- -------V-- ----------
34/H31  -----E---T --NH-I---- --L------- Y----II--- -----V---- ---------- ----S-N--- ---T-N---- ---------- ---------- ----T-----
31/KU5  -----E---T --NH-I---- --L------- Y----II--- -----V---- ---------- ----S-N--- ---T-N---- ---------- ---------- ----T-----
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FIG. 1. Deduced amino acid sequences of part of PBP 2X from representative strains from each group. The sequence of PSSP R6 is shown on the top line. Numbering is based on published data on the R6 strain (15). Only amino acids differing from the R6 sequence are shown. Boxes represent conserved amino acid motifs.

TABLE 2. Susceptibilities to β-lactam antibiotics of *S. pneumoniae* transformants obtained with *pbp2x* DNA

Strain[a]	MIC (μg/ml)[b]					
	PC	CTX	CPDX	CDTR	CFDN	CCL
Recipient, KK97	0.031	0.016	0.031	0.016	0.063	0.5
Transformant						
$KK97^{2X34/H31}$	0.063	0.5	2.0	0.5	4.0	2.0
$KK97^{2X40/H29}$	0.063	0.5	2.0	0.5	4.0	2.0
$KK97^{2X29/KK133}$	0.063	1.0	2.0	0.5	8.0	2.0
$KK97^{2X32/KU81}$	0.063	1.0	4.0	0.5	8.0	2.0

[a] Nonencapsulated strain KK97 was transformed with PCR-amplified *pbp2x* from group IV and V strains as the donor DNA. Transformants were selected on plates containing 0.2 or 0.5 μg of CTX per ml. Example of transformant nomenclature, $KK97^{2X34/H31}$ corresponds to KK97 strain containing the *pbp2x* gene from strain 34/H31.

[b] For abbreviations of β-lactams, see Table 1, footnote *a*.

respectively, or His-394 and Leu-546 just before the SSN and KSG motifs were replaced with Leu and Val, respectively. The CTX MICs for these strains were also about 0.125 μg/ml.

Five strains that were classified into group III showed a substitution of Ala for Thr-338 in the STMK motif. The CTX MICs for these strains ranged from 0.125 to 0.25 μg/ml. The homology of amino acid sequences between these strains and the R6 strain was 90.7%.

PRSP strains classified into group IV were isolated predominantly in Japan. These strains had altered *pbp1a*, *pbp2x*, and *pbp2b* genes and two amino acid substitutions, Ala for Thr-338 in STMK and Val for Leu-546 adjacent to KSG, in the *pbp2x* gene product.

CTX MICs for strains that were classified into group V ranged from 2 to 8 μg/ml, which is four to eight times higher than those for group IV. The general amino acid substitutions in group V strains were virtually the same as those in the group IV strains, but Thr-Met in STMK was replaced with Ala-Phe. The amino acid sequence of these strains was highly homologous to that of the high-level CTX resistant strain CS111 isolated in the United States in 1991 (homology ranging from 99.7 to 100%) (5). The serotypes of the strains with high-level resistance were 14, 19, and 23.

Many strains in groups IV and V showed simultaneous alterations in PBP 1A, 2X, and 2B, while strains of other groups showed resistance mediated by an alteration in PBP 2X only. To clarify the effect of substitutions in the conserved amino acid motifs of PBP 2X on CTX MICs, the amplified *pbp2x* genes of strains in groups IV and V were used to transform a PSSP strain, KK97 (Table 2). Transformation of *pbp2x* genes was monitored by procedures previously described (27, 28). Transformants were selected on blood agar containing CTX and were confirmed by sequencing to contain the *pbp2x* gene of donor DNA. CTX MICs for transformants containing *pbp2x* DNA of the 34/H31 and 40/H29 strains (group IV) increased from 0.016 to 0.5 μg/ml. In contrast, CTX MICs for transformants from the 29/KK133 and 32/KU81 strains (group V) increased to 1.0 μg/ml. Although the *pbp2x* gene from group V strains could not transform a susceptible recipient strain to

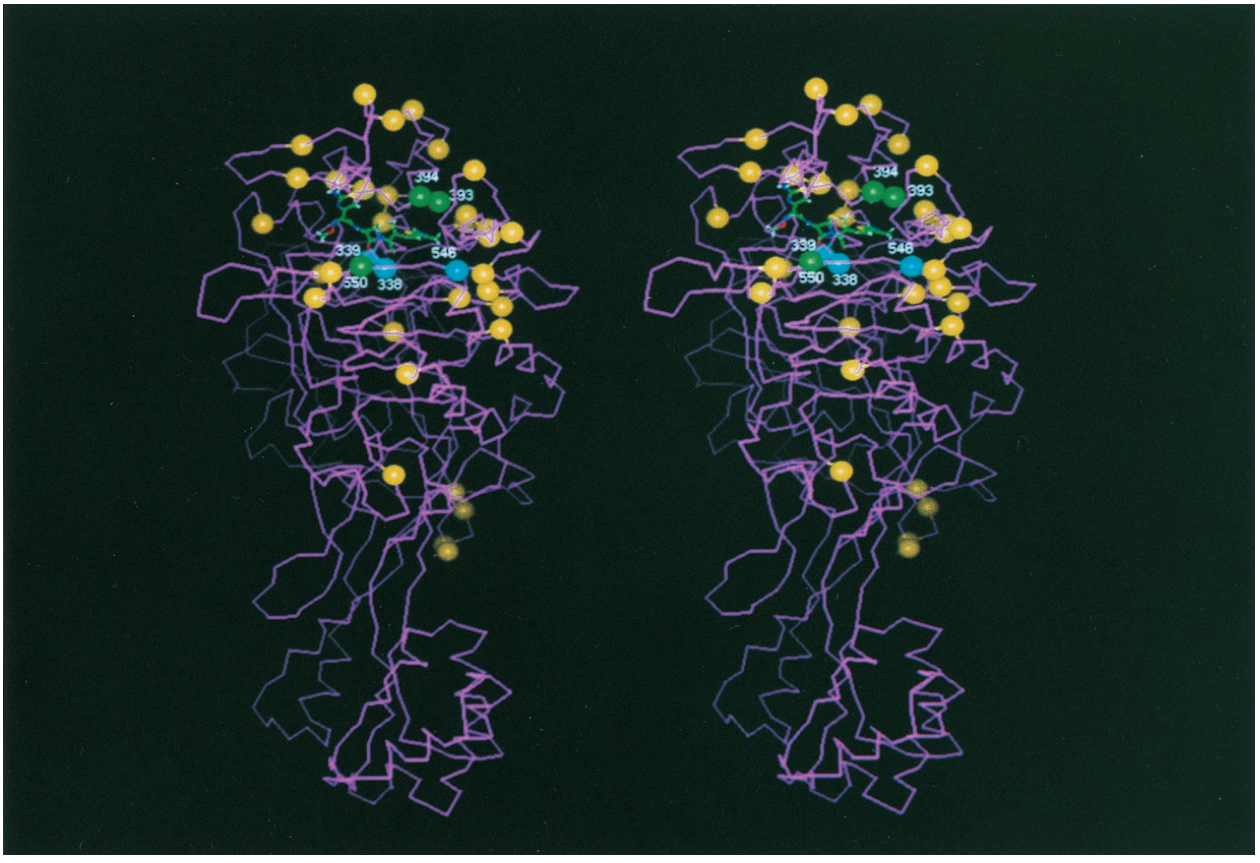

FIG. 2. Stereoview of the transpeptidase domain of PBP 2X of the high-level CTX-resistant PRSP strain 31/KU5. The structure was constructed by using the crystallographic coordinates of PBP 2X of Pares et al. (PDB entry code 1 PMD) (21). Cefditoren of oral cephalosporin was shown in the long groove as a β-lactam model. Amino acids differing from the R6 sequence are indicated by colored circles (blue, substitutions within or adjacent to conserved motifs of STMK and KTG; green, substitutions adjacent to SSN and KSG in strains classified into groups I and II; yellow, general substitutions).

donor-level CTX resistance by itself, it increased the resistance to a slightly higher level than that of the others.

Charlier et al. (4) crystallized PBP 2X, and Pares et al. (21) determined its three-dimensional structure by X-ray crystallography. According to their observations, PBP 2X consisted of three domains. Its central domain was the transpeptidase domain, with a long groove surrounded with conserved motifs of STMK, SSN, and KSG. The active-site serine to which β-lactam binds was located in the STMK motif at the center of this groove. Figure 2 shows a stereoview of the PBP 2X transpeptidase domain of the PRSP strain 31/KU5, in which amino acids differing from the R6 sequence are marked with yellow, green, and blue circles.

Distinct amino acid changes, as well as those near the conserved motif, are probably important but are more difficult to understand. An extended structural analysis, while far beyond the scope of this paper, would be worthwhile.

Zhao et al. (32) reported that the kinetics of the transpeptidase activity of PBP 2X differed significantly between penicillin-resistant and -susceptible strains of *S. pneumoniae*. The resistant strain used in their study was classified into group IV in the present study. Furthermore, Garcia-Bustos and Tomasz (9) used whole cells and documented that the products of pentaglycine bridge reactions differed between susceptible and resistant strains. It was speculated that these changes were attributable to modification of the three-dimensional structure of the active domain of high-molecular-mass PBPs in the resistant strains.

We have not yet isolated *S. pneumoniae* with high-level CTX resistance with substitutions such as Pro for Ser-571 in PBP 2X. Nevertheless, since many oral cephalosporins are currently in use in Japan, we fear that the strains with high-level cephalosporin resistance with the PBP 2X alterations so far described will predominate among strains isolated in the future.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study will appear in the DDBJ/EMBL/Gen Bank nucleotide sequence databases under the following accession numbers: AB011198 to AB011210 and AB015846 to AB015852.

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