

Penicillin-Binding Protein 1A, 2B, and 2X Alterations in Canadian Isolates of Penicillin-Resistant *Streptococcus pneumoniae*

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Received 30 October 2001/Returned for modification 16 March 2002/Accepted 10 July 2002

Alterations within the penicillin-binding domain of penicillin-binding protein (PBP) genes *pbp1a*, *pbp2b*, and *pbp2x* were determined for 15 Canadian isolates of *Streptococcus pneumoniae*. All penicillin-nonsusceptible *S. pneumoniae* isolates showed a variety of PBP 2X substitutions and contained a Thr445-Ala change after the PBP 2B SSN motif. Only isolates for which penicillin MICs were ≥ 0.5 $\mu\text{g/ml}$ had PBP 1A alterations near the STMK and SRN motifs. Sequence analysis revealed identical PBP 1A, PBP 2B, and PBP 2X substitution patterns among all isolates for which penicillin MICs were ≥ 1 $\mu\text{g/ml}$.

Streptococcus pneumoniae remains one of the most important bacterial pathogens associated with community-acquired pneumonia, meningitis, sinusitis, and acute otitis media (3). Penicillin has traditionally been used for the treatment of most pneumococcal infections. Recently, however, the efficacy of β -lactam antibiotics has been compromised by rapid increases in the prevalence of penicillin-resistant *S. pneumoniae* (3). In Canada, data from a 1997–1998 national surveillance study have demonstrated that *S. pneumoniae* strains with reduced susceptibility to penicillin constitute 21.2% of respiratory tract isolates (23).

The major antibacterial action of penicillin is derived from its ability to bind to and inactivate penicillin-binding proteins (PBPs). PBPs are membrane-associated serine peptidases that catalyze the polymerization and transpeptidation of glycan strands during the final steps of peptidoglycan biosynthesis (22). In all PBPs, the catalytic centers of transpeptidase activity are defined by three conserved amino acid motifs: SXXK (including the active-site serine residue), SXN and KXG (9). Six PBPs have been identified in *S. pneumoniae*, including five high-molecular-weight PBPs (1A, 1B, 2A, 2B, and 2X) and the low-molecular-weight PBP 3 (11). Pneumococcal resistance to penicillin is attributed to altered PBPs 1A, 2B, and 2X with reduced affinities for β -lactam antibiotics (4, 13, 15). The mosaic genes that encode these low-affinity variants are the product of interspecies recombinational events involving horizontal transfer of PBP genes from closely related species (6, 15, 17).

Genetic analyses of *pbp1a*, *pbp2b*, and *pbp2x* have previously been conducted in penicillin-susceptible and -resistant *S. pneumoniae* isolates from Czechoslovakia (6), Japan (1, 2), Papua New Guinea (6), South Africa (6, 10, 17, 19–21), Spain (6, 10, 17), and the United States (1, 6). To our knowledge, similar studies have not been conducted with penicillin-resistant isolates from across Canada. The purpose of this study was to determine the nucleotide sequence of a 1-kb region encoding

the penicillin-binding domain (PBD) of *pbp1a*, *pbp2b*, and *pbp2x* from Canadian isolates of *S. pneumoniae*.

Fifteen clinical isolates of *S. pneumoniae* were selected from more than 2,500 isolates collected between 1997 and 1999 as part of an ongoing Canadian national surveillance study (23). Selection of isolates was based upon the penicillin MIC and included five penicillin-susceptible (MIC, ≤ 0.06 $\mu\text{g/ml}$), five penicillin-intermediate (MIC, 0.12 to 1 $\mu\text{g/ml}$), and five penicillin-resistant (MIC, ≥ 2 $\mu\text{g/ml}$) isolates. The clinical and demographic parameters of the *S. pneumoniae* isolates examined in this study are listed in Table 1.

Chromosomal DNA was extracted and 1.1-, 1.3-, and 1.1-kb fragments encoding the PBDs of *pbp1a*, *pbp2b*, and *pbp2x*, respectively, were amplified by PCR. The primers used for amplification of these genes were as follows: *pbp1a*, 5'-T₁₈₂₇GGATGGATGTTTACACAAATG₁₈₄₉ and 5'-G₃₀₂₃TCGTA CTATTATTTGTGCTTGG₃₀₀₁; *pbp2b*, 5'-G₉₉₅GCTATTCTC TAAATGACCGT₁₀₁₅ and 5'-A₂₃₁₁GCTTAGCAATAGGTG TTGG₂₂₉₂; and *pbp2x*, 5'-T₉₅₈ATGAAAA(G/A)GA(T/C)CGT (C/G)T(G/A)GG₉₇₇ and 5'-A₂₁₀₅GAGAGTCTTTCATAGCT GAAGC₂₀₈₃. PCR was performed with a Perkin-Elmer GeneAmp PCR System 9700 under the following conditions: 94°C for 5 min; 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. The nucleotide sequences of the PBD of *pbp1a*, *pbp2b*, and *pbp2x* were determined by sequencing with a series of oligonucleotides that were primed at intervals of ± 275 nucleotides along each gene. Sequencing reactions were performed with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and analyzed with an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, Calif.). The nucleotide and deduced amino acid sequences of PBP 1A, PBP 2B, and PBP 2X were aligned and compared with the published sequence of penicillin-susceptible *S. pneumoniae* R6 (8, 14, 16).

Four of five penicillin-susceptible isolates (3203, 8099, 11184, and 14016) had PBP 2X nucleotide and amino acid sequences highly homologous ($\geq 99.7\%$) to R6 (Table 2). In contrast, isolate 12244 carried 57 (5.7%) nucleotide alterations, 9 of which were nonsynonymous substitutions, and possessed a Thr338-Ala alteration within the STMK motif. Al-

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TABLE 1. Penicillin susceptibility and demographics of *S. pneumoniae* isolates recovered in Canada

Isolate	Penicillin MIC ($\mu\text{g/ml}$)	Date isolated (mo/day/yr)	Geographic origin ^a	Source
6190	4	3/6/98	RUH	Sputum
8111	4	3/5/98	Van HSC	Sputum
742	2	1/17/98	VGH	Sputum
2848	2	10/1/97	HSC	Sputum
6363	2	5/14/98	UA	Trachea
3455	1	12/12/97	HSC	Endotracheal tube
14126	0.5	6/9/99	LHSC	Eye
11413	0.25	1/4/99	LHSC	Trachea
3996	0.12	8/28/97	CLS	Sputum
12276	0.12	1/3/99	MR	Sputum
3203	0.06	11/5/97	QEH	Sputum
11184	0.06	11/5/98	USL	Sputum
12244	0.06	2/11/99	CLS	Endotracheal tube
14016	0.06	1/7/99	SJH	Eye
8099	≤ 0.03	3/3/98	Van HSC	Lung aspirate

^a RUH, Royal University Hospital (Saskatoon, Saskatchewan); Van HSC, Vancouver Health Sciences Centre (Vancouver, British Columbia); VGH, Victoria General Hospital (Victoria, British Columbia); HSC, Health Sciences Centre (Winnipeg, Manitoba); UA, University of Alberta Hospitals (Edmonton, Alberta); LHSC, London Health Sciences Centre (London, Ontario); CLS, Calgary Lab Services (Calgary, Alberta); MR, Maisonneuve-Rosemont (Montreal, Quebec); QEH, Queen Elizabeth Hospital (Charlottetown, Prince Edward Island); USL, University de Sante de l'Estrie (Sherbrooke, Quebec); SJH, St. Joseph's Hospital (Hamilton, Ontario).

though alterations in PBP 2X may mediate low-level resistance to cephalosporins with almost no effect on penicillin susceptibility (1, 10), this isolate remained susceptible to cefotaxime and ceftriaxone (MICs, $\leq 0.06 \mu\text{g/ml}$) as well as related β -lactams (data not shown). *S. pneumoniae* isolates for which penicillin MICs were $\geq 0.5 \mu\text{g/ml}$ had identical *pbp2x* genes and revealed extensive sequence divergence from R6, differing by 177 (17.7%) nucleotide substitutions and 38 (11.4%) amino acid alterations. These isolates had altered *pbp1a*, *pbp2b*, and *pbp2x* genes and two key amino acid substitutions (Thr338-Ala

TABLE 2. Distribution of nucleotide and amino acid substitutions in the PBD of PBP 2X from clinical isolates of *S. pneumoniae*

Isolate	Penicillin MIC ($\mu\text{g/ml}$)	No. (%) of ^a :		Amino acid motif ^b		
		Nucleotides altered	Amino acids altered	STMK	AHSSNV	LKSGT
6190	4	177 (17.7)	38 (11.4)	-A--	-----	V----
8111	4	177 (17.7)	38 (11.4)	-A--	-----	V----
6363	2	177 (17.7)	38 (11.4)	-A--	-----	V----
742	2	177 (17.7)	38 (11.4)	-A--	-----	V----
2848	2	177 (17.7)	38 (11.4)	-A--	-----	V----
3455	1	177 (17.7)	38 (11.4)	-A--	-----	V----
14126	0.5	177 (17.7)	38 (11.4)	-A--	-----	V----
11413	0.25	67 (6.7)	12 (3.6)	----	-L----	----
3996	0.12	57 (5.7)	9 (2.7)	-A--	-----	----
12276	0.12	57 (5.7)	9 (2.7)	-A--	-----	----
3203	0.06	1 (0.1)	1 (0.1)	----	-----	----
11184	0.06	1 (0.1)	1 (0.1)	----	-----	----
12244	0.06	57 (5.7)	9 (2.7)	-A--	-----	----
14016	0.06	3 (0.3)	0	----	-----	----
8099	≤ 0.03	3 (0.3)	1 (0.3)	----	-----	----

^a Published sequence of penicillin-susceptible *S. pneumoniae* R6 was used for comparison.

^b Only amino acid residues different from the PBP 2X conserved motif sequences of *S. pneumoniae* R6 are shown. Conserved amino acid motifs are underlined.

TABLE 3. Distribution of nucleotide and amino acid substitutions in the PBD of PBP 2B from clinical isolates of *S. pneumoniae*

Isolate	Penicillin MIC ($\mu\text{g/ml}$)	No. (%) of ^a :		Amino acid motif ^b		
		Nucleotides altered	Amino acids altered	SVVK	SSNT	KTGTA
6190	4	73 (6.9)	13 (3.7)	----	---A	-----
8111	4	73 (6.9)	13 (3.7)	----	---A	-----
6363	2	74 (7.0)	13 (3.7)	----	---A	-----
742	2	73 (6.9)	13 (3.7)	----	---A	-----
2848	2	73 (6.9)	13 (3.7)	----	---A	-----
3455	1	73 (6.9)	13 (3.7)	----	---A	-----
14126	0.5	78 (7.4)	14 (4.0)	----	---A	-----
11413	0.25	54 (5.1)	13 (3.7)	----	---A	-----
3996	0.12	37 (3.5)	7 (2.0)	----	---A	-----
12276	0.12	37 (3.5)	7 (2.0)	----	---A	-----
3203	0.06	3 (0.3)	0	----	-----	-----
11184	0.06	0	0	----	-----	-----
12244	0.06	2 (0.2)	0	----	-----	-----
14016	0.06	3 (0.3)	0	----	-----	-----
8099	≤ 0.03	1 (0.1)	0	----	-----	-----

^a Published sequence of penicillin-susceptible *S. pneumoniae* R6 was used for comparison.

^b Only amino acid residues different from the PBP 2B conserved motif sequences of *S. pneumoniae* R6 are shown. Conserved amino acid motifs are underlined.

and Leu546-Val) within the *pbp2x* gene product. The majority of amino acid changes within the PBP 2X transpeptidase domain of these isolates were found to lie between the STMK and SSN motifs and/or within the locality of the C-terminal KSG motif. Recently, structural evidence linking penicillin resistance to the absence of a hydroxyl group following substitution of Ala for Thr338 has been presented (18). Kinetic parameters of PBP 2X variants have suggested that the substitution of Thr338 near the active-site serine residue significantly reduces the acylation efficiency of this resistance determinant by modifying the reactivity of Ser337 toward both the antibiotic and substrate analogues (18).

Within the PBD of *pbp2b*, penicillin-intermediate and -resistant isolates carried a variety of nonsynonymous substitutions (Table 3). The majority of these nucleotide and associated amino acid alterations occurred within a ± 250 -bp area between Asn404 and Thr488 and were located within the vicinity of the SVVK tetrad (which houses the active-site serine) and the SSN motif. Four prominent substitutions were common to all isolates for which the MIC was at least $0.12 \mu\text{g/ml}$. These included the replacement of Glu332 by Gly, Thr445 by Ala, Glu475 by Gly, and Thr488 by Ser or Ala. The importance of the exchange of Ala for Thr445, which has similarly been identified in all resistant isolates analyzed to date (10), was previously noted by Dowson and coworkers (7) and occurs adjacent to the conserved SSN motif. Because the Asn residue of this motif has been proposed to form a hydrogen bond with the carbonyl group of the penicillin R1 side chain, the substitution of Ala for Thr445 presumably disrupts this hydrogen bond and leads to a reduction in the affinity of the protein (12).

Analysis of *pbp2b* revealed highly similar patterns of nucleotide and amino acid sequence variation among all resistant isolates (MICs, $\geq 2 \mu\text{g/ml}$), as well as penicillin-intermediate isolate 3455 (MIC, $1 \mu\text{g/ml}$). These isolates showed simultaneous alterations in *pbp1a*, *pbp2b*, and *pbp2x* and substitution of Thr445-Ala immediately following the SSN motif. A similar

TABLE 4. Distribution of nucleotide and amino acid substitutions in the PBD of PBP 1A from clinical isolates of *S. pneumoniae*

Isolate	Penicillin MIC ($\mu\text{g/ml}$)	No. (%) of ^a :		Amino acid motif ^b		
		Nucleotides altered	Amino acids altered	<u>STMK</u>	<u>SRNVP</u>	<u>KTG</u>
6190	4	170 (18.3)	36 (11.6)	-A--	----T	---
8111	4	170 (18.3)	36 (11.6)	-A--	----T	---
6363	2	170 (18.3)	36 (11.6)	-A--	----T	---
742	2	170 (18.3)	36 (11.6)	-A--	----T	---
2848	2	170 (18.3)	36 (11.6)	-A--	----T	---
3455	1	170 (18.3)	36 (11.6)	-A--	----T	---
14126	0.5	203 (21.8)	43 (13.9)	-S--	----T	---
11413	0.25	4 (0.4)	1 (0.1)	----	----	---
3996	0.12	6 (0.6)	2 (0.6)	----	----	---
12276	0.12	5 (0.5)	2 (0.6)	----	----	---
3203	0.06	6 (0.6)	1 (0.1)	----	----	---
11184	0.06	5 (0.5)	2 (0.6)	----	----	---
12244	0.06	6 (0.6)	3 (1.0)	----	----	---
14016	0.06	6 (0.6)	1 (0.1)	----	----	---
8099	≤ 0.03	4 (0.4)	2 (0.6)	----	----	---

^a Published sequence of penicillin-susceptible *S. pneumoniae* R6 was used for comparison.

^b Only amino acid residues different from the PBP 1A conserved motif sequences of *S. pneumoniae* R6 are shown. Conserved amino acid motifs are underlined.

substitution pattern within or adjacent to the three conserved amino acid motifs was likewise seen when the penicillin MIC was 0.5 $\mu\text{g/ml}$. By comparison, penicillin MICs were 0.12 to 0.25 $\mu\text{g/ml}$ when the same Thr445-Ala substitution was detected, but only two of three PBP genes were altered.

All penicillin-susceptible isolates and three of five penicillin-intermediate (MICs, 0.12 to 0.25 $\mu\text{g/ml}$) isolates (12276, 3996, and 11413) showed a low degree of PBP 1A sequence variation (<1%) when compared to R6. The PBD of PBP 1A from these isolates revealed four to six nucleotide alterations and up to three amino acid substitutions (Table 4). In isolates with MICs between 0.03 and 0.25 $\mu\text{g/ml}$, nucleotide and amino acid alterations were essentially confined to an area surrounding the KTG motif. This included substitution of Asp533 by Glu or replacement of Ser540 with Thr. Widespread alterations within the PBD of PBP 1A were observed among five penicillin-resistant isolates (MICs, ≥ 2 $\mu\text{g/ml}$) and penicillin-intermediate isolate 3455 (MIC, 1 $\mu\text{g/ml}$), where nucleotide and amino acid sequences differed from those of R6 by 18.3 and 11.6%, respectively. Only isolates for which MICs were ≥ 0.5 $\mu\text{g/ml}$ had amino acid alterations within the locality of the STMK and SRN motifs. Two key changes within these regions included the substitution of Ser or Ala for Thr371 adjacent to the active-site serine residue and that of Thr for Pro432 just after the SRN motif.

Examination of the PBD of PBP 1A from resistant isolates has revealed that substitution of Thr371 by Ser or Ala is predominant and is furthermore associated with the high level of resistance in strains having simultaneous alterations in PBP 2X and PBP 2B. It has been hypothesized, therefore, that substitution of Thr371 adjacent to the active-site serine may change the three-dimensional structure of the transpeptidase domain and alter enzymatic activity for peptidoglycan synthesis (2). Consequently, for isolates with altered *pbp2x* and *pbp2b* genes and in which Thr371 was substituted by Ala in the PBP 1A STMK motif, penicillin MICs were ≥ 1 $\mu\text{g/ml}$. For isolate

14126, which likewise had altered *pbp2x* and *pbp2b* genes, but which carried a Thr371-to-Ser substitution in PBP 1A, the penicillin MIC was 0.5 $\mu\text{g/ml}$. For three isolates (11413, 3996, and 12276) with alterations in two PBP genes, but not in the PBP 1A STMK or SRN motifs, penicillin MICs were 0.12 to 0.25 $\mu\text{g/ml}$. No substitutions within or adjacent to the conserved amino acid motifs of PBP 1A were observed in penicillin-susceptible isolates.

Sequence analysis revealed identical PBP 1A, 2B, and 2X substitution patterns among all isolates for which penicillin MICs were ≥ 1 $\mu\text{g/ml}$. PBP gene sequences from these isolates showed 99 to 100% identity with those previously described in isolates from Japan, South Africa, and Spain (2, 17, 20). Evaluation of pulsed-field gel electrophoresis patterns showed that four of five penicillin-resistant isolates were genetically related and may be variants of the international Spanish/USA serotype 23F clone (data not shown).

In summary, penicillin MICs for clinical isolates of *S. pneumoniae* appear to be influenced by alterations in various combinations of the *pbp1a*, *pbp2b*, and *pbp2x* genes. Characterization of the PBP sequence profiles of these isolates, however, has suggested that not all steps of resistance increase are mediated solely by alterations in the genes encoding PBPs 1A, 2B, and 2X. In Canadian *S. pneumoniae* isolates, for example, identical mosaic PBP arrangements were found to occur at widely different MICs (1 to 4 $\mu\text{g/ml}$). This suggests that a limited number of analogous amino acid changes are associated with resistance in clinical isolates and that, at least theoretically, additional PBP and non-PBP genes may also be involved in the development of resistance. PBP 1B variants with reduced affinity have been previously described in interspecies transformations to penicillin resistance (5), and a low-affinity PBP 2A has been noted in several penicillin-resistant clinical isolates of *S. pneumoniae* (15). Further analysis will therefore determine whether amino acid substitutions in PBP 1B and PBP 2A contribute to the development of penicillin resistance in Canadian clinical isolates of *S. pneumoniae*.

We thank the participating centers, investigators, and laboratory site staff for their continued support. The technical assistance of L. Palatnick, B. Weshnoweski, and T. Belyou is appreciated.

Financial support from Abbott Laboratories, Ltd., Aventis Pharma, Bayer, Inc., Bristol-Myers Squibb Pharmaceutical Group, GlaxoSmithKline, Janssen-Ortho, Inc., Merck Frosst Canada & Co., and Pharmacia Upjohn is gratefully acknowledged.

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