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Alterations in Penicillin-Binding Protein 2B from Penicillin-Resistant Wild-Type Strains of *Streptococcus pneumoniae*

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The 1.5-kb transpeptidase-encoding region (TER) of penicillin-binding protein (PBP) 2B was amplified and sequenced from 18 penicillin-resistant isolates of *Streptococcus pneumoniae*, with each isolate representing a different DNA fingerprint profile of the TER. PBP 2B TERs from penicillin-resistant isolates revealed extensive sequence divergence from the penicillin-susceptible R6 strain, differing by up to 170 nucleotide substitutions and resulting in up to 38 alterations in the amino acid sequence of the protein. All penicillin-resistant isolates showed sequence divergence within a ±300-bp area at the center of the PBP 2B TER. Although a number of amino acid substitutions were found within this central area of PBP 2B, only two substitutions were common to all resistant isolates, namely, Thr-252 replacement by Ala and Glu-282 replacement by Gly. These two substitutions appear to be essentially associated with a decreased affinity of PBP 2B for penicillin. A second block of divergent nucleotide sequence was prominent amongst isolates with high levels of resistance. This was a ±100-bp area of the TER around nucleotide 1300 and included the substitution of Gly for Asp-431, which was the only amino acid substitution within this area that was common to all isolates. These data may assist in the definition of the structural changes in the penicillin-binding site of PBP 2B associated with penicillin resistance in *S. pneumoniae*.

β-Lactam antibiotics (penicillins and cephalosporins) inhibit the growth of pneumococci by the inactivation of penicillin-binding proteins (PBPs). PBPs are active-site serine peptidases which catalyze polymerization as well as cross-linking of peptidoglycan precursors in the assembly of bacterial cell walls (10, 19). The sensitivity of PBPs to β-lactams is related to the structural similarity between the β-lactam ring of the antibiotic and the carboxyl-terminal D-alanyl–D-alanine residues in peptidoglycan precursors (4). Pneumococcal resistance to β-lactams is due to the production of altered high-molecular-weight PBPs 1A, 2A, 2B, and 2X, which have a decreased affinity for the antibiotic (6, 8, 12, 20).

Nucleotide sequence analysis of the PBP 2B transpeptidase-encoding region (TER) in penicillin-resistant strains has shown more extensive alterations in these regions than in those of susceptible strains (1). Restriction endonuclease analysis of the PBP 2B TER has shown alteration of this region to occur in pneumococcal isolates that require penicillin MICs of ≥0.06 μg/ml but not at MICs lower than this (18). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorographic analysis of PBPs following the transformation of susceptible strains to progressively higher levels of penicillin resistance has revealed that PBP 2B is not observed in this assay, since the level of resistance in transformants reaches 0.05 μg of penicillin per ml (20), while the same PBP assay revealed that in clinical isolates that require MICs of ≥0.25 μg/ml, PBP 2B detection was diminished or completely absent from fluorograms (8, 13). These studies suggest that loss of penicillin binding to PBP 2B is associated with high-level penicillin resistance.

Using restriction endonuclease analysis, we have previously analyzed the PBP 2B TER among 58 penicillin-resistant South African pneumococci of serogroups 6 and 19 and have found the region to be diverse, with 18 different gene profiles (18). In the present study, we have sequenced the PBP 2B TER from 18 resistant isolates (each representing a different gene fingerprint profile) to identify nucleotide and amino acid alterations within this region of PBP 2B.

TABLE 1. Properties of pneumococcal isolates[a]

Isolate	Source	Serotype	Penicillin MIC (μg/ml)	Origin and date of isolation (mo/yr)
48478	CSF	19	4	BH, 11/89
13363	B/C	6	0.5	HH, 8/88
65654	CSF	6	0.25	RXH, 11/88
43	SPT	6	0.25	BH, 8/88
17230	B/C	6	0.25	RXH, 6/88
52075	P/ASP	19	0.5	BH, 5/89
52328	T/ASP	6	4	BH, 5/89
M11	N/S	6	4	HH, 9/89
56762	Pus	19	4	JH, 7/87
E957	E/S	19	0.25	HH, 7/87
27222	B/C	19	0.25	BH, 7/88
23884	SPT	6	0.25	CWH, 11/89
39030	B/C	19	0.25	CH, 5/91
21241	T/ASP	19	0.125	BH, 10/90
53135	B/C	6	0.125	LH, 11/89
8859	B/C	19	1	JH, 10/88
56739	SPT	19	1	RXH, 3/90
22012	B/C	6	0.06	CH, 8/89

[a] Each isolate represents a different fingerprint profile of the TER for PBP 2B, as found in a previous study of 58 penicillin-resistant isolates (18). R6, a penicillin-susceptible unencapsulated laboratory strain, was derived from Rockefeller University strain R36A. Abbreviations: B/C, blood culture; T/ASP, tracheal aspirate; SPT, sputum; CSF, cerebrospinal fluid; E/S, ear swab; P/ASP, pleural aspirate; N/S, nasal swab; CH, Coronation Hospital; BH, Baragwanath Hospital; CWH, Clairwood Hospital; RXH, Red Cross Hospital; HH, Hillbrow Hospital; LH, Livingstone Hospital; JH, Johannesburg Hospital.

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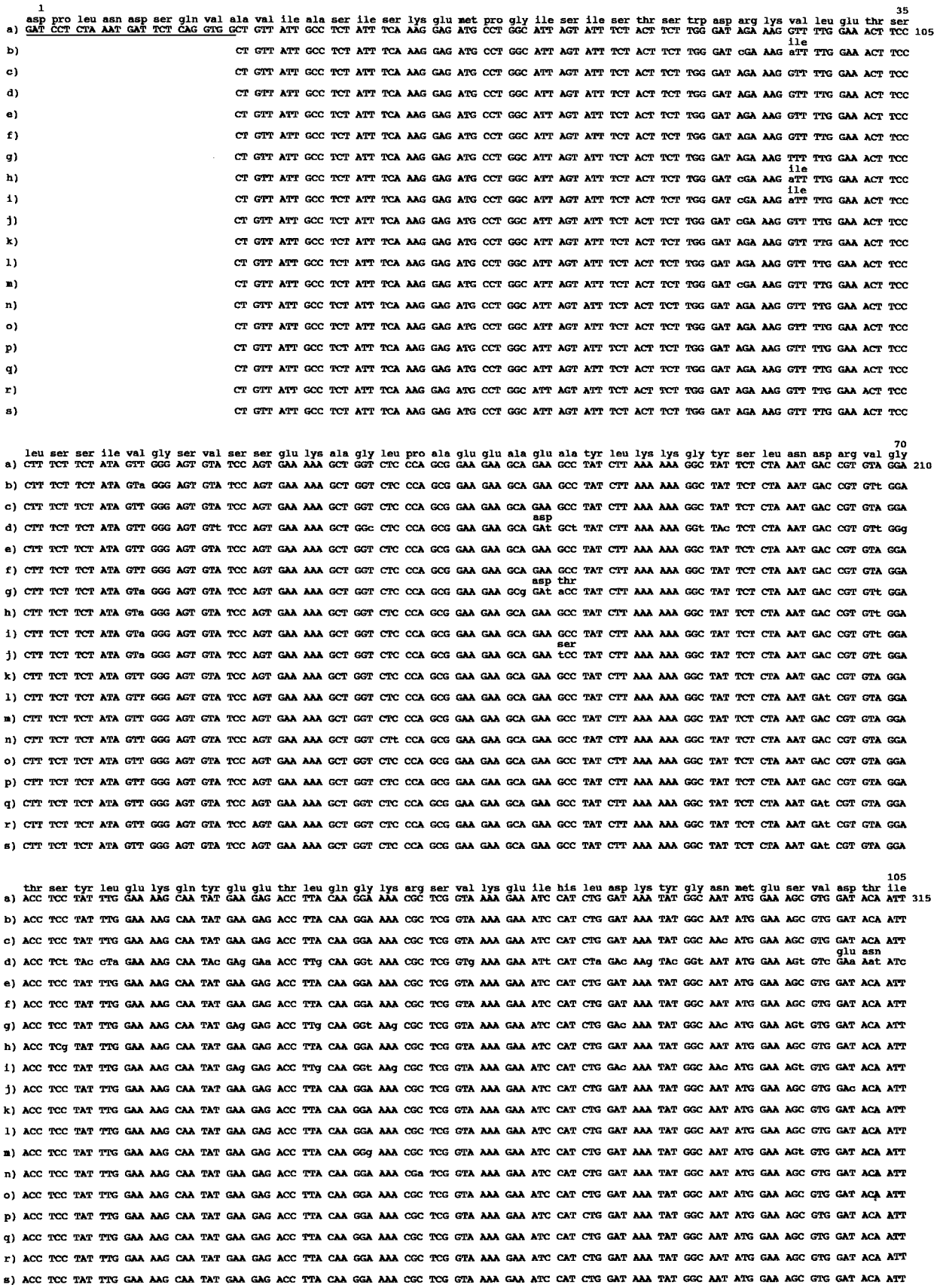

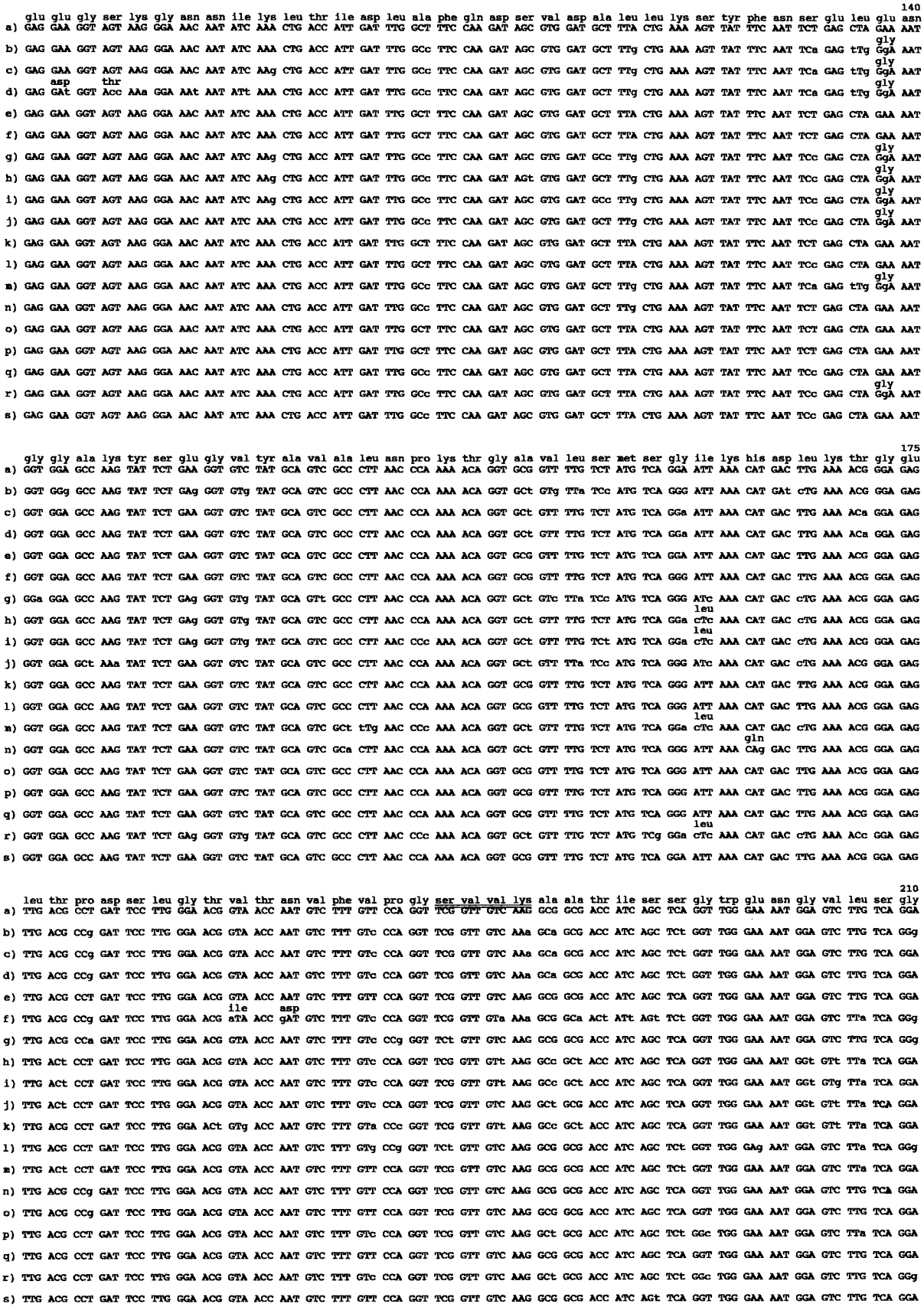

FIG. 1—*Continued.*

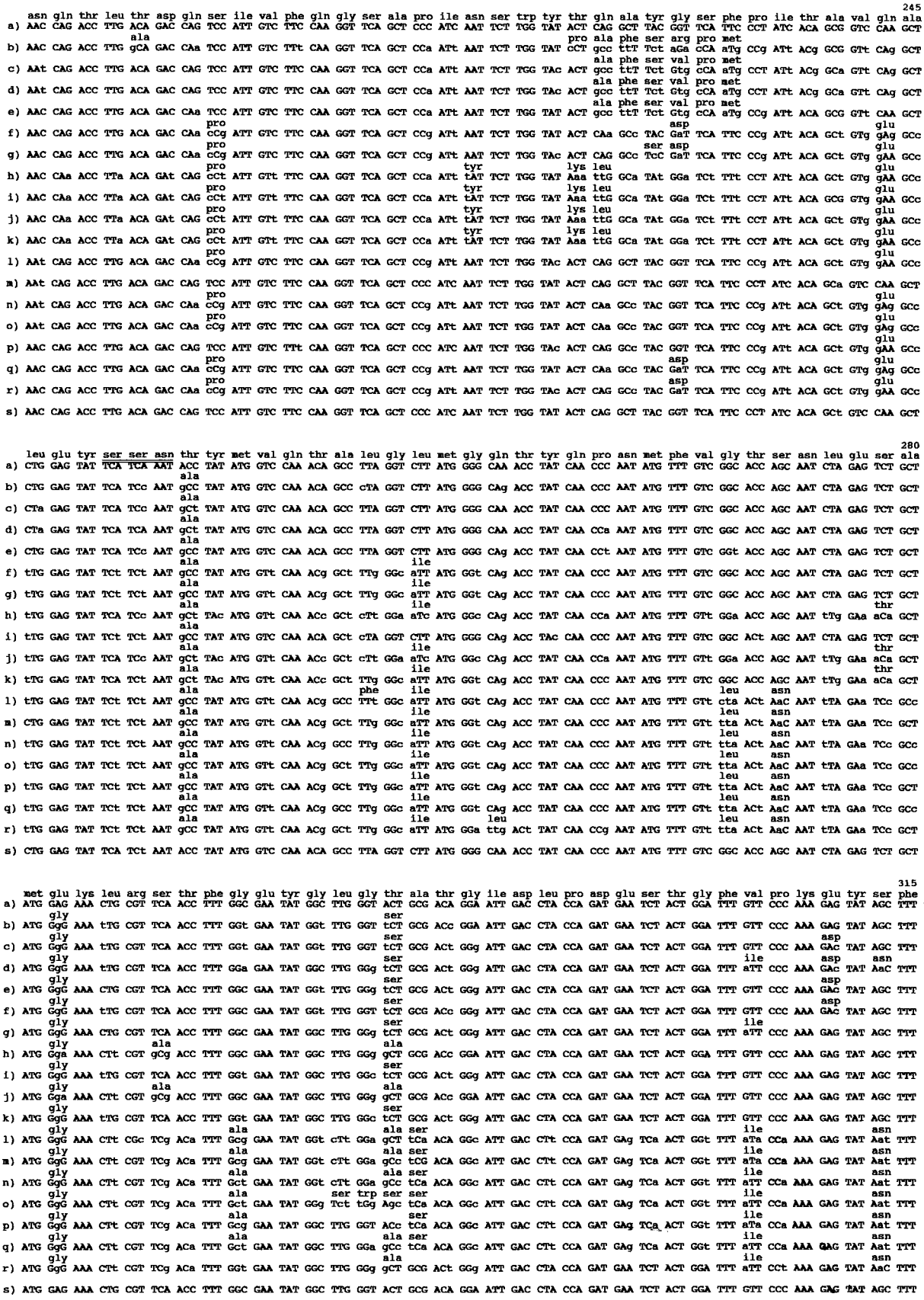

FIG. 1—*Continued.*

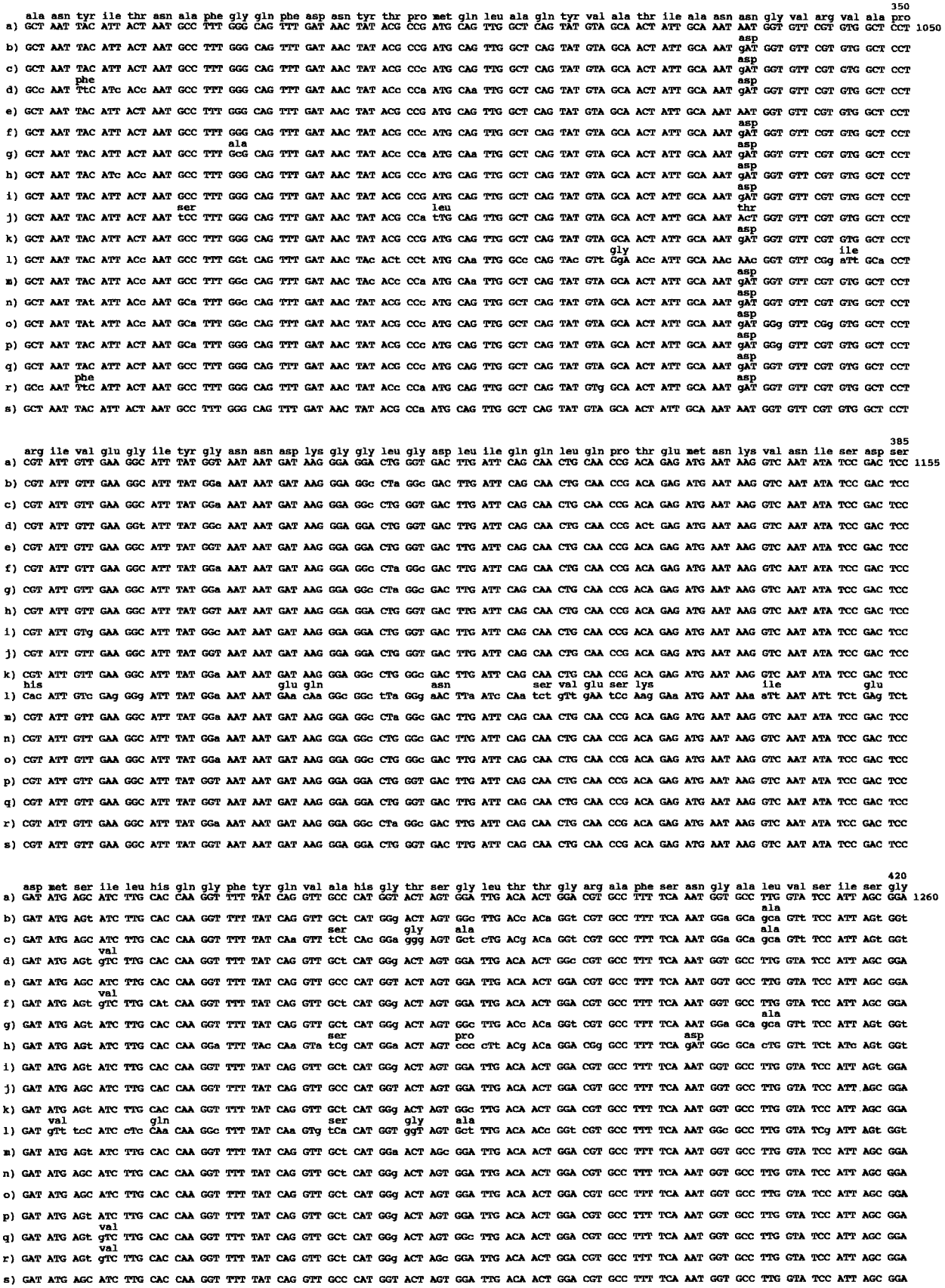

FIG. 1—*Continued.*

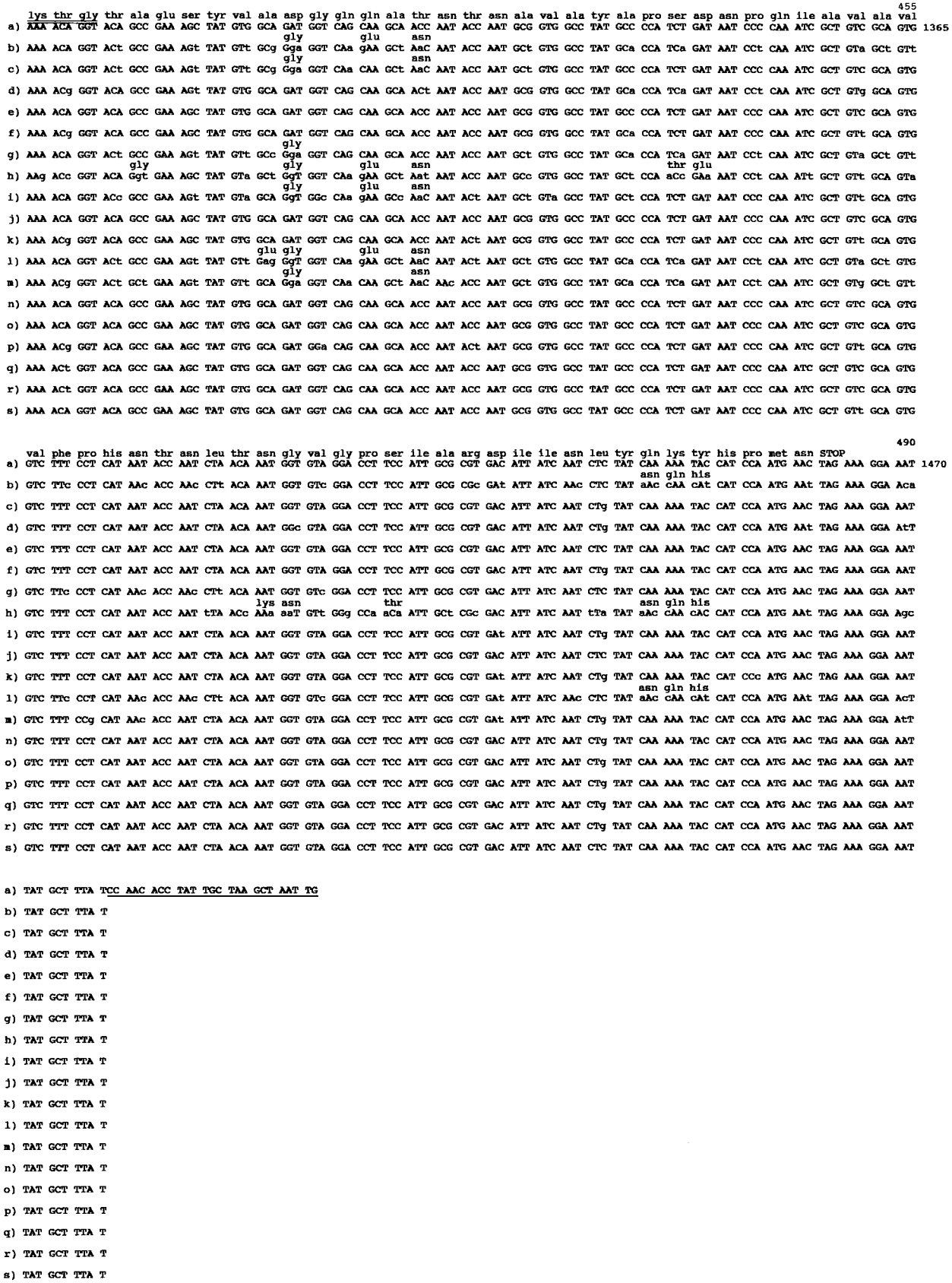

FIG. 1—*Continued.*

MATERIALS AND METHODS

Bacterial strains. Clinical isolates of *Streptococcus pneumoniae* were obtained from the South African Institute for Medical Research, a reference center for pneumococci in South Africa. The isolates were serotyped by the Quellung reaction (15), and penicillin MICs were determined by the broth microdilution method in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with 3% lysed horse blood (16). The properties of these isolates are listed in Table 1.

Isolation of PBP 2B DNA. Pneumococci were cultivated overnight (18 h) at 37°C in 5% CO_2 on brain heart infusion agar (Difco Laboratories), and the chromosomal DNAs were isolated from the bacterial cells by previously discussed methods (18). The 1.5-kb PBP 2B TER was amplified from the chromosomal DNA by PCR, incorporating primers Pn2B up and Pn2B down as described by Dowson and coworkers (2). The 100-μl PCR mixture contained 1 μg of chromosomal DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 1 μM (each) primer, 200 μM deoxynucleoside triphosphates (Boehringer GmbH, Mannheim, Germany), and 2 U of *Taq* DNA polymerase (Promega Corp., Madison, Wis.), with thermal cycling (30 times) at 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min.

Asymmetric PCR as described by McCabe (14) was used to obtain a single-stranded DNA template of the PBP 2B TER. This technique incorporates an unequal concentration of the two primers, in a ratio of 1:200, to generate an excess of one of the two strands. PCR conditions were as described above, incorporating 40 ng of purified PBP 2B DNA, 200 nM excess primer, and 1 nM limiting primer. Following PCR, the reaction mixture was made up to 400 μl with deionized H_2O, loaded in a Millipore MC 30,000 nominal molecular weight limit filter unit (Millipore Corp., Bedford, Mass.), and centrifuged at 6,400 rpm, resulting in the recovery of the amplified DNA on the filter of the unit. DNA from the filter was resuspended in 10 mM Tris–1 mM EDTA (pH 7.5) buffer.

DNA sequencing. Single-stranded DNA generated by PCR was sequenced by the Sanger dideoxynucleotide method of DNA sequencing (17), incorporating the Sequenase enzyme (United States Biochemical, Cleveland, Ohio), a genetic variant of bacteriophage T7 DNA polymerase. The protocol for sequencing was performed as described by the manufacturer of the Sequenase kit (United States Biochemical). The nucleotide sequence of the PBP 2B TER on both strands was determined by sequencing with a series of oligonucleotides that were primed at intervals of ±300 nucleotides along each strand. For each strain, a minimum of two independent PCR products were sequenced in order to eliminate any errors introduced by PCR. The approximate frequency of PCR error was <0.06%.

Unless otherwise stated, all chemicals used in the study described here were supplied by the Sigma Chemical Co., St. Louis, Mo.

Nucleotide sequence accession numbers. The sequence data for strain R6 appears in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under the accession number X16022, while data for isolates listed in lines b to s of Fig. 1 appear under accession numbers U20076, U20068, U20070, U20081, U20072, U20077, U20073, U20074, U20080, U20075, U20067, U20083, U20079, U20084, U20082, U20069, U20078, and U20071, respectively.

RESULTS AND DISCUSSION

We have previously used DNA fingerprinting to analyze the PBP 2B TER from 68 clinical isolates of South African serogroup 6 and 19 pneumococcal strains having a wide range of penicillin MICs (<0.015 to 8 μg/ml) (18). This region revealed a uniform profile among pneumococci inhibited at low MICs, with variation from this profile and extensive genetic diversity occurring only in isolates for which MICs were >0.06 μg/ml. These results suggest that PBP 2B alteration is associated with clinically relevant penicillin resistance.

In the present study, an isolate representing each of the 18 different PBP 2B DNA fingerprint profiles found amongst the 58 resistant isolates (18) was chosen for nucleotide sequencing analysis of the PBP 2B TER. This analysis therefore depicts the nucleotide diversity of the PBP 2B TER for a large group of penicillin-resistant isolates having a wide geographic spread across South Africa. The nucleotide sequence of this region from penicillin-susceptible strain R6, which was determined and found to agree with the published sequence of Dowson and coworkers (3), was used as the basis for comparison with resistant isolates. Isolate 22012 (inhibited by a penicillin concentration of 0.06 μg/ml) had seven nucleotide substitutions in the PBP 2B TER, which did not alter the corresponding amino acid sequence of the protein. This observation is of some relevance, as the changes in the PBP 2B DNA fingerprint associated with this low MIC (18) are not associated with any amino acid changes in the protein, and reveals that amino acid changes in the transpeptidase domain of PBP 2B are only associated with isolates requiring penicillin MICs of >0.06 μg/ml, which is the usual definition of clinically relevant intermediate penicillin resistance. The remaining 17 penicillin-resistant isolates (Table 1) had PBP 2B TERs which revealed extensive sequence divergence from strain R6, differing by up to 170 nucleotide substitutions and resulting in up to 38 alterations in the amino acid sequence of the protein (Fig. 1). As has been previously reported (1), PBP 2B TERs from penicillin-resistant isolates have a mosaic pattern, consisting of blocks containing the nucleotide substitutions alternating with blocks identical to sequences of susceptible strain R6 (Fig. 2). A large percentage of the nucleotide substitutions (≥50%) did not alter the amino acid sequence of the PBP.

The PBP 2B TER from isolate 43 with an intermediate level of resistance (MIC of 0.25 μg/ml) has nucleotide alterations occurring only within a ±300-bp area close to the center of this region (Fig. 1 and 2). As depicted in Fig. 2, all the resistant isolates are identical to each other with respect to nucleotide sequence diversity within this central area of the TER. This area lies within the locality of two of the three conserved amino acid motifs common to all penicilloyl serine transferases (5), namely, the Ser-X-X-Lys tetrad (residues 192 to 195 in Fig. 1) housing the active-site serine residue and the Ser-X-Asn triad (residues 249 to 251). According to Dowson and coworkers (2), this area very likely forms part of the penicillin binding site that interacts with the phenyl group of the R1 side chain of penicillin. It would therefore appear that changes within this central area of the PBP 2B TER may strongly influence penicillin resistance at least up to an intermediate level, with MICs of ±0.25 μg/ml. This area houses the majority of all nucleotide and amino acid substitutions occurring in the PBP 2B TER of resistant isolates. The amino acid substitutions occurring within this area between Asn-211 and Phe-315 can be grouped into five profiles based on a number of coinciding changes. These amino acid profiles are represented by the isolates whose sequences are shown in Fig. 1 as follows: profile 1 in line b, profile 2 in lines c to e, profile 3 in lines f and g, profile 4 in lines h to k, and profile 5 in lines l to r. These data therefore suggest that at least five mutational pathways exist for PBP 2B to remodel itself, in this region of the PBP, in order to inhibit the binding of penicillin. The most prominent amino acid alterations in the protein occur in profiles 1 and 2, with the substitution of seven consecutive residues (Thr-232 to Phe-238) and six consecutive residues (Gln-233 to Phe-238), respectively. Although striking at first glance, these six or seven consecutive substitutions do not seem critical to resistance development, since they do not occur in profiles 3 to 5. The replacements of Thr-252 by Ala and Glu-282 by Gly are the

FIG. 1. Nucleotide sequences and translations of the PBP 2B TER from pneumococcal isolates listed in Table 1. Line a is the sequence of this region from the penicillin-susceptible strain R6. The positions of PCR primers are underlined, while the conserved amino acid sequence motifs are doubly underlined. Lines b through s represent the following isolates: 48478 (b), 13363 (c), 65654 (d), 43 (e), 17230 (f), 52075 (g), 52328 (h), M11 (i), 56762 (j), E957 (k), 27222 (l), 23884 (m), 39030 (n), 21241 (o), 53135 (p), 8859 (q), 56739 (r), and 22012 (s). For lines b to s, nucleotide alterations from the sequence of strain R6 are indicated in lowercase letters, while amino acids are only shown where an alteration occurs. The nucleotide and amino acid sequences are numbered according to the published sequence of Dowson and coworkers (2).

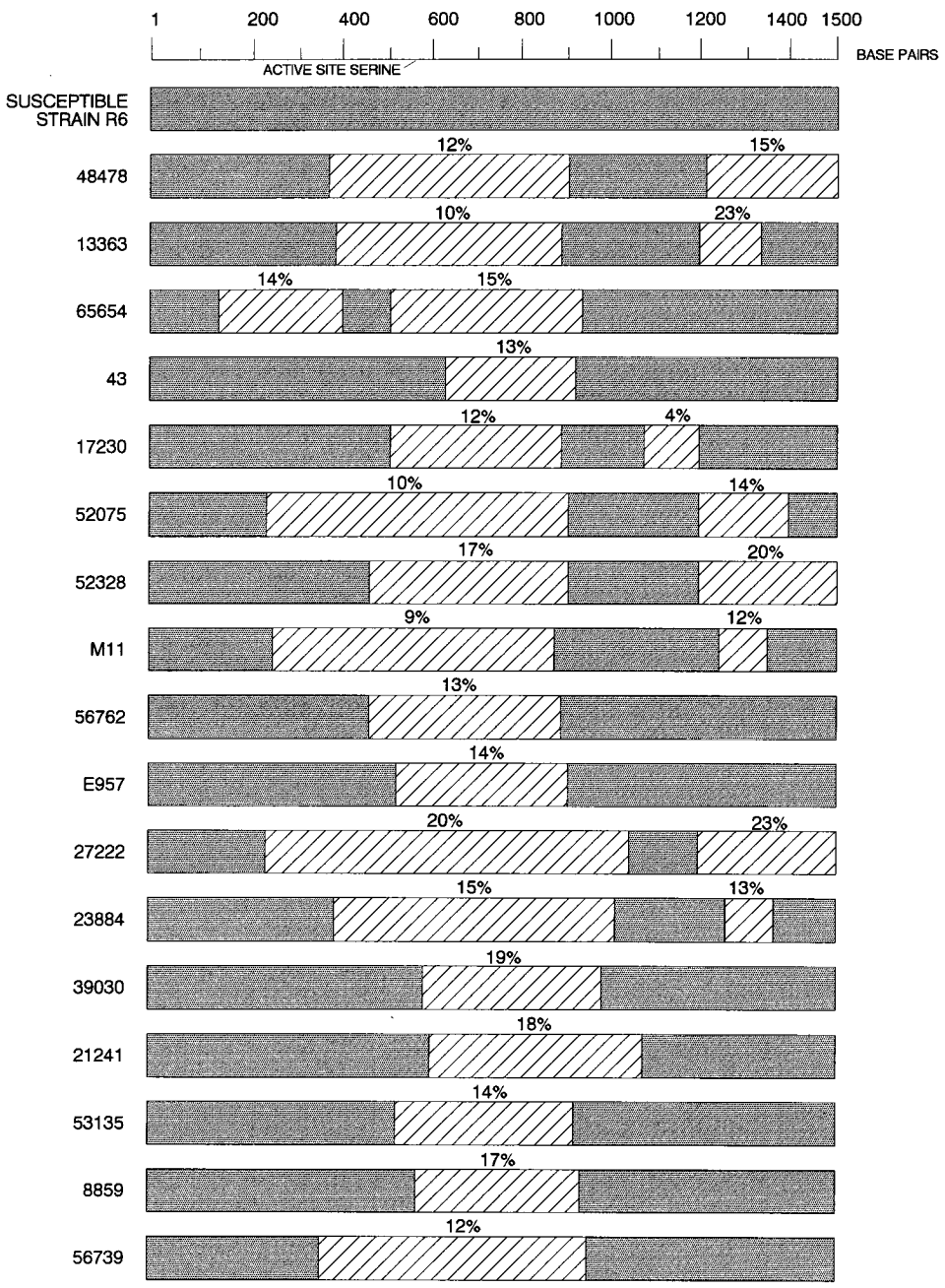

FIG. 2. Sequence diversity of the PBP 2B TERs from penicillin-resistant pneumococci (Table 1) compared with the susceptible strain R6 (isolate 22012 is not included since it has only seven nucleotide substitutions across the 1.5-kb region, which does not alter the amino acid sequence of the protein). The hatched blocks indicate the regions of the genes which have sequence divergence from the corresponding regions of the gene of strain R6 (the percent sequence diversity is indicated above each block). The nucleotide sequence is numbered according to the published sequence of Dowson and coworkers (2).

only two alterations common to all five amino acid substitution profiles and appear to be essentially associated with a decreased affinity of PBP 2B for penicillin. The importance of the substitution of Ala for Thr-252 has previously been noted by Dowson and coworkers (2) and occurs adjacent to the conserved Ser-X-Asn motif (residues 249 to 251). The Asn residue of this motif in class A β-lactamases has been proposed to form a hydrogen bond with the carbonyl group of the R1 side chain of penicillin (9), and the substitution of Ala for Thr-252 presumably disrupts this hydrogen bond. The significance of the second common substitution of Gly for Glu-282 has not previously been noted. Analysis of PBP 2B at amino acid residue 344 revealed another prominent substitution which occurred amongst most isolates, namely, Asn replacement by Asp or Thr.

A second block of the PBP 2B TER was prominent in the mosaic structure (Fig. 2) and appears to be relevant for resistance. This divergent block of the nucleotide sequence occurs in isolates 48478, 13363, 52075, 52328, M11, 27222, and 23884. This is a ±100-bp area around nucleotide 1300 and within the locality of the conserved amino acid motif Lys-X-Gly (residues 421 to 423 in Fig. 1). This area has previously been found to be

important for β-lactam interaction with PBPs. Amino acid mutations of this area coding for PBP 2X were identified in five independent cefotaxime-resistant laboratory mutants (11). Independently obtained piperacillin-resistant laboratory mutants were each found to have a single point mutation in this area coding for PBP 2B, namely, Gly-423 to Ala or Gly-466 to Asp (7). None of our penicillin-resistant clinical isolates have these particular amino acid substitutions in PBP 2B, but within the same area they do have a number of different amino acid mutations, including an analogous substitution of Gly for Asp-431. This area of the TER may be relevant to high-level resistance development, since the isolates having mutations within this area also have high levels of penicillin resistance (MICs of up to 4 μg/ml).

Alterations of PBP 2B very likely play a vital role in the development of penicillin resistance; however, alteration in PBP 2B alone would presumably not dictate the final level of penicillin resistance in pneumococci. The final resistance would be dependent on the collective action of multiple altered PBPs, i.e., the resistance phenotype that is expressed would depend on the genotype of all participating PBPs. The current data show that in the transpeptidase domain of PBP 2B a limited number of analogous amino acid substitutions can be associated with penicillin resistance in a wide variety of wild-type clinical isolates.

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