

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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$, 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 ($\mu\text{g/ml}$)	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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 a) glu gly ser lys gly asn asn ile lys leu thr ile asp leu ala phe gln asp ser val asp ala leu leu lys ser tyr phe asn ser glu leu leu 420
 GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG GCT TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCT GAG CTA GAA AAT
 b) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG GCT TTC CAA GAT AGC GTG GAT GCT TTg CTG AAA AGT TAT TTC AAT TCa GAG tTg gly Gga AAT
 c) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAg CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTg CTG AAA AGT TAT TTC AAT TCa GAG tTg gly Gga AAT
 d) GAG GAT GGT Acc Aaa GGA AAT AAT ATt AAA CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTg CTG AAA AGT TAT TTC AAT TCa GAG tTg gly Gga AAT
 e) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG GCT TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCT GAG CTA GAA AAT
 f) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG GCT TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCT GAG CTA GAA AAT
 g) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAg CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTg CTG AAA AGT TAT TTC AAT TCa GAG CTA gly Gga AAT
 h) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAg CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGT GTG GAT GCT TTg CTG AAA AGT TAT TTC AAT TCa GAG CTA gly Gga AAT
 i) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAg CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTg CTG AAA AGT TAT TTC AAT TCa GAG CTA gly Gga AAT
 j) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTg CTG AAA AGT TAT TTC AAT TCa GAG CTA gly Gga AAT
 k) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG GCT TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCT GAG CTA GAA AAT
 l) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG GCT TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCa GAG CTA GAA AAT
 m) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTg CTG AAA AGT TAT TTC AAT TCa GAG tTg gly Gga AAT
 n) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTg CTG AAA AGT TAT TTC AAT TCT GAG CTA GAA AAT
 o) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG GCT TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCT GAG CTA GAA AAT
 p) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG GCT TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCT GAG CTA GAA AAT
 q) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCa GAG CTA GAA AAT
 r) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCa GAG CTA gly Gga AAT
 s) GAG GAA GGT AGT AAG GGA AAC AAT ATC AAA CTG ACC ATT GAT TTG Gcc TTC CAA GAT AGC GTG GAT GCT TTA CTG AAA AGT TAT TTC AAT TCa GAG CTA GAA AAT

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 a) gly gly ala lys tyr ser glu gly val tyr ala val ala leu asn pro lys thr gly ala val leu ser met ser gly ile lys his asp leu lys thr gly glu 525
 GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCT GTg TPa Tcc ATg TCA GGG ATT AAA CAT GAT cTg AAA ACg GGA GAG
 b) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCT GTg TPa Tcc ATg TCA GGG ATT AAA CAT GAT cTg AAA ACg GGA GAG
 c) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCT GTT TTg TCT ATg TCA Gga ATT AAA CAT GAC TTg AAA ACa GGA GAG
 d) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCT GTT TTg TCT ATg TCA Gga ATT AAA CAT GAC TTg AAA ACa GGA GAG
 e) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCG GTT TTg TCT ATg TCA Gga ATT AAA CAT GAC TTg AAA ACg GGA GAG
 f) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCG GTT TTg TCT ATg TCA GGG ATT AAA CAT GAC TTg AAA ACg GGA GAG
 g) GGA GGA GCC AAG TAT TCT GAg GGT GTg TAT GCA GTt GCC CTT AAC CCA AAA ACA GGT GCT GTt TPa Tcc ATg TCA GGG ATc AAA CAT GAC cTg AAA ACg GGA GAG
 h) GGT GGA GCC AAG TAT TCT GAg GGT GTg TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCT GTt TTg TCT ATg TCA Gga cTc AAA CAT GAC TTg AAA ACg GGA GAG
 i) GGT GGA GCC AAG TAT TCT GAg GGT GTg TAT GCA GTC GCC CTT AAC Ccc AAA ACA GGT GCT GTT TTg TCT ATg TCA Gga cTc AAA CAT GAC cTg AAA ACg GGA GAG
 j) GGT GGA Gct Aaa TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCT GTT TPa Tcc ATg TCA GGG ATc AAA CAT GAC cTg AAA ACg GGA GAG
 k) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCG GTT TTg TCT ATg TCA GGG ATT AAA CAT GAC TTg AAA ACg GGA GAG
 l) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCG GTT TTg TCT ATg TCA GGG ATT AAA CAT GAC TTg AAA ACg GGA GAG
 m) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC Gct tTg AAC Ccc AAA ACA GGT GCT GTT TTg TCT ATg TCA Gga cTc AAA CAT GAC cTg AAA ACg GGA GAG
 n) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC Gca CTT AAC CCA AAA ACA GGT GCT GTT TTg TCT ATg TCA GGG ATT AAA CAg GAC TTg AAA ACg GGA GAG
 o) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCG GTT TTg TCT ATg TCA GGG ATT AAA CAT GAC TTg AAA ACg GGA GAG
 p) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCG GTT TTg TCT ATg TCA GGG ATT AAA CAT GAC TTg AAA ACg GGA GAG
 q) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCG GTT TTg TCT ATg TCA GGG ATT AAA CAT GAC TTg AAA ACg GGA GAG
 r) GGT GGA GCC AAG TAT TCT GAg GGT GTg TAT GCA GTC GCC CTT AAC Ccc AAA ACA GGT GCT GTT TTg TCT ATg Tcg Gga cTc AAA CAT GAC cTg AAA ACc GGA GAG
 s) GGT GGA GCC AAG TAT TCT GAA GGT GTC TAT GCA GTC GCC CTT AAC CCA AAA ACA GGT GCG GTT TTg TCT ATg TCA GGA ATT AAA CAT GAC TTg AAA ACg GGA GAG

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 a) leu thr pro asp ser leu gly thr val thr asn val phe val pro gly ser val val lys ala ala thr ile ser ser gly trp glu asn gly val leu ser gly 630
 TTG ACG CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GTC Aaa Gca GCG ACC ATC AGC Tct GGT TGG GAA AAT GGA GTC TTG TCA GGG
 b) TTG ACG Ccg GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GTC Aaa Gca GCG ACC ATC AGC Tct GGT TGG GAA AAT GGA GTC TTG TCA GGA
 c) TTG ACG Ccg GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GTC Aaa Gca GCG ACC ATC AGC Tct GGT TGG GAA AAT GGA GTC TTG TCA GGA
 d) TTG ACG Ccg GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GTC Aaa Gca GCG ACC ATC AGC Tct GGT TGG GAA AAT GGA GTC TTG TCA GGA
 e) TTG ACG CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTT CCA GGT TCG GTT GTC AAG GCG GCG ACC ATC AGC TCA GGT TGG GAA AAT GGA GTC TTG TCA GGA
 f) TTG ACG Ccg GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GPa Aaa GCG Gca Act ATt Agt Tct GGT TGG GAA AAT GGA GTC TPa TCA GGG
 g) TTG ACG Cca GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc Ccg GGT Tct GTT GTC AAG GCG GCG ACC ATC AGC TCA GGT TGG GAA AAT GGA GTC TTG TCA GGG
 h) TTG Act CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GTt AAg Gcc Gct Acc ATC AGC TCA GGT TGG GAA AAT GGT GTt TPa TCA GGA
 i) TTG Act CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GTt AAg Gcc Gct Acc ATC AGC TCA GGT TGG GAA AAT GGT GTt TPa TCA GGA
 j) TTG Act CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GTC AAG Gct GCG ACC ATC AGC TCA GGT TGG GAA AAT GGT GTt TPa TCA GGA
 k) TTG ACG CCT GAT TCC TTG GGA Act GTg AAT GTC TTT GTa Ccc GGT TCG GTT GTt AAg Gcc Gct Acc ATC AGC TCA GGT TGG GAA AAT GGT GTt TPa TCA GGA
 l) TTG ACG CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTg Ccg GGT Tct GTT GTC AAG GCG GCG ACC ATC AGC Tct GGT TGG GAg AAT GGA GTC TPa TCA GGG
 m) TTG Act CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTT CCA GGT TCG GTT GTC AAG GCG GCG ACC ATC AGC Tct GGT TGG GAA AAT GGA GTC TPa TCA GGA
 n) TTG ACG Ccg GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTT CCA GGT TCG GTT GTC AAG GCG GCG ACC ATC AGC TCA GGT TGG GAA AAT GGA GTC TTG TCA GGA
 o) TTG ACG Ccg GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTT CCA GGT TCG GTT GTC AAG GCG GCG ACC ATC AGC TCA GGT TGG GAA AAT GGA GTC TTG TCA GGA
 p) TTG ACG CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GTC AAG Gct GCG ACC ATC AGC Tct Ggc TGG GAA AAT GGA GTC TPa TCA GGA
 q) TTG ACG CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTT CCA GGT TCG GTT GTC AAG GCG GCG ACC ATC AGC TCA GGT TGG GAA AAT GGA GTC TTG TCA GGA
 r) TTG ACG CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTc CCA GGT TCG GTT GTC AAG Gct GCG ACC ATC AGC Tct Ggc TGG GAA AAT GGA GTC TTG TCA GGG
 s) TTG ACG CCT GAT TCC TTG GGA ACG GTA ACC AAT GTC TTT GTT CCA GGT TCG GTT GTC AAG GCG GCG ACC ATC Agt TCA GGT TGG GAA AAT GGA GTC TTG TCA GGA

FIG. 1—Continued.

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 a) asn gln thr leu thr asp gln ser ile val phe gln gly ser ala pro ile asn ser trp tyr thr gln ala tyr gly ser phe pro ile thr ala val gln ala
 AAC CAG ACC TTG ACA GAC CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAG GCT 735
 b) AAC CAG ACC TTG ACA GAC Caa TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAG GCT
 c) AAT CAG ACC TTG ACA GAC CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAC ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAG GCT
 d) AAT CAG ACC TTG ACA GAC CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAC ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAG GCT
 e) AAC CAG ACC TTG ACA GAC Caa TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 f) AAC CAG ACC TTG ACA GAC Caa TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 g) AAC CAG ACC TTG ACA GAC Caa TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 h) AAC Caa ACC TTa ACA GAT CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 i) AAC Caa ACC TTa ACA GAT CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 j) AAC Caa ACC TTa ACA GAT CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 k) AAC Caa ACC TTa ACA GAT CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 l) AAT CAG ACC TTG ACA GAC Caa TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAC ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 m) AAT CAG ACC TTG ACA GAC CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 n) AAT CAG ACC TTG ACA GAC Caa TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 o) AAT CAG ACC TTG ACA GAC Caa TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 p) AAC CAG ACC TTG ACA GAC CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 q) AAC CAG ACC TTG ACA GAC Caa TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 r) AAC CAG ACC TTG ACA GAC Caa TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAC ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT
 s) AAC CAG ACC TTG ACA GAC CAG TCC ATT GTC TTC CAA GGT TCA GCT CCA ATT AAT TCT TGG TAT ACT CAG GCT TAC GGT TCA TTC CCT ATC ACA GCG GTT CAA GCT

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 a) leu glu tyr ser ser asn thr tyr met val gln thr ala leu gly leu met gly gln thr tyr pro asn met phe val gly thr ser asn leu glu ser ala
 CTG GAG TAT TCA TCA AAT ACC TAT ATG GTC CAA ACA GCC TTA GGT CTT ATG GGG CAG ACC TAT CAA CCC AAT ATG TTT GTC GGC ACC AGC AAT CTA GAG TCT GCT 840
 b) CTG GAG TAT TCA TCC AAT gct ala TAT ATG GTC CAA ACA GCC TTA GGT CTT ATG GGG CAG ACC TAT CAA CCC AAT ATG TTT GTC GGC ACC AGC AAT CTA GAG TCT GCT
 c) CTA GAG TAT TCA TCC AAT gct ala TAT ATG GTC CAA ACA GCC TTA GGT CTT ATG GGG CAG ACC TAT CAA CCC AAT ATG TTT GTC GGC ACC AGC AAT CTA GAG TCT GCT
 d) CTA GAG TAT TCA TCA AAT gct ala TAT ATG GTC CAA ACA GCC TTA GGT CTT ATG GGG CAG ACC TAT CAA CCA AAT ATG TTT GTC GGC ACC AGC AAT CTA GAG TCT GCT
 e) CTG GAG TAT TCA TCC AAT gct ala TAT ATG GTC CAA ACA GCC TTA GGT CTT ATG GGG CAG ACC TAT CAA CCT AAT ATG TTT GTC GGT ACC AGC AAT CTA GAG TCT GCT
 f) TTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACg GCT TTg Ggc aTT ATG GGT CAG ACC TAT CAA CCC AAT ATG TTT GTC GGC ACC AGC AAT CTA GAG TCT GCT
 g) TTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACg GCT TTg Ggc aTT ATG GGT CAG ACC TAT CAA CCC AAT ATG TTT GTC GGC ACC AGC AAT CTA GAG TCT GCT
 h) TTG GAG TAT TCT TCT AAT gct ala TAc ATG GTC CAA ACC GCT cTT Gga aTc ATG GGC CAG ACC TAT CAA CCC AAT ATG TTT GTT GTC GGC ACC AGC AAT CTA GAG TCT GCT
 i) TTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACA GCT cTA GGT CTT ATG GGG CAG ACC TAc CAA CCC AAT ATG TTT GTC GGC Act AGC AAT CTA GAG TCT GCT
 j) TTG GAG TAT TCA TCC AAT gct ala TAc ATG GTC CAA ACC GCT cTT Gga aTc ATG GGC CAG ACC TAT CAA CCA AAT ATG TTT GTT GTC GGC ACC AGC AAT TTy Gaa aCa GCT
 k) TTG GAG TAT TCA TCT AAT gct ala TAc ATG GTC CAA ACC GCT TTg Ggc aTT ATG GGT CAG ACC TAT CAA CCC AAT ATG TTT GTC GGC ACC AGC AAT TTy Gaa aCa GCT
 l) TTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACg GCC TTt Ggc aTT ATG GGT CAG ACC TAT CAA CCC AAT ATG TTT GTT cta Act Aac AAT TTA Gaa Tcc Gcc
 m) CTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACg GCT TTg Ggc aTT ATG GGT CAG ACC TAT CAA CCC AAT ATG TTT GTT tta Act Aac AAT TTA Gaa Tcc GCT
 n) TTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACg GCC TTg Ggc aTT ATG GGT CAG ACC TAT CAA CCC AAT ATG TTT GTT tta Act Aac AAT TTA Gaa Tcc Gcc
 o) TTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACg GCT TTg Ggc aTT ATG GGT CAG ACC TAT CAA CCC AAT ATG TTT GTT tta Act Aac AAT TTA Gaa Tcc Gcc
 p) TTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACg GCT TTg Ggc aTT ATG GGT CAG ACC TAT CAA CCC AAT ATG TTT GTT tta Act Aac AAT TTA Gaa Tcc GCT
 q) TTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACg GCC TTg Ggc aTT ATG GGT CAG ACC TAT CAA CCC AAT ATG TTT GTT tta Act Aac AAT TTA Gaa Tcc Gcc
 r) TTG GAG TAT TCT TCT AAT gct ala TAT ATG GTC CAA ACg GCT TTg Ggc aTT ATG GGA leu ttg ACT TAT CAA CCG AAT ATG TTT GTT tta Act Aac AAT TTA Gaa Tcc GCT
 s) CTG GAG TAT TCA TCT AAT ACC TAT ATG GTC CAA ACA GCC TTA GGT CTT ATG GGG CAA ACC TAT CAA CCC AAT ATG TTT GTC GGC ACC AGC AAT CTA GAG TCT GCT

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 a) met glu lys leu arg ser thr phe gly glu tyr gly leu gly thr ala thr gly ile asp leu pro asp glu ser thr gly phe val pro lys glu tyr ser phe
 ATG GAG AAA CTG CGT TCA ACC TTT GGT GAA TAT GGT TTG GGT tCT GCG Acc GGA ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT 945
 b) ATG GgG AAA tTG CGT TCA ACC TTT GGT GAA TAT GGT TTG GGT tCT GCG Acc GGA ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 c) ATG GgG AAA tTG CGT TCA ACC TTT GGT GAA TAT GGT TTG GGT tCT GCG Act GgG ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 d) ATG GgG AAA tTG CGT TCA ACC TTT Gga GAA TAT GGC TTG GgG tCT GCG Act GgG ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 e) ATG GgG AAA CTG CGT TCA ACC TTT GGC GAA TAT GGT TTG GgG tCT GCG Act GgG ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 f) ATG GgG AAA tTG CGT TCA ACC TTT GGC GAA TAT GGT TTG GGT tCT GCG Acc GgG ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 g) ATG GgG AAA CTG CGT TCA ACC TTT GGT GAA TAT GGC TTG GgG tCT GCG Act GgG ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 h) ATG Gga AAA CTt CGT gCg ACC TTT GGC GAA TAT GGC TTG GgG gCT GCG Acc GGA ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 i) ATG GgG AAA tTG CGT TCA ACC TTT GGT GAA TAT GGC TTG GgG tCT GCG Act GgG ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 j) ATG Gga AAA CTt CGT gCg ACC TTT GGC GAA TAT GGC TTG GgG gCT GCG Acc GGA ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 k) ATG GgG AAA tTG CGT TCA ACC TTT GGT GAA TAT GGC TTG GgG tCT GCG Act GgG ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT AGC TTT
 l) ATG GgG AAA CTt CgC TcG AcA TTT GcG GAA TAT GGT cTT Gga gCT tCa ACA GGC ATT GAC CTt CCA GAT GAg TCa ACT GGT TTT aTa CCA AAA GAG TAT Aat TTT
 m) ATG GgG AAA CTt CGT TcG AcA TTT GcG GAA TAT GGT cTT Gga gCT tCa ACA GGC ATT GAC CTt CCA GAT GAg TCa ACT GGT TTT aTa CCA AAA GAG TAT Aat TTT
 n) ATG GgG AAA CTt CGT TcG AcA TTT GcT GAA TAT GGT cTT Gga gCT tCa ACA GGC ATT GAC CTt CCA GAT GAg TCa ACT GGT TTT aTT CCA AAA GAG TAT Aat TTT
 o) ATG GgG AAA CTt CGT TcG AcA TTT GcT GcT GcT Tct tGg Agc tCa ACA GGC ATT GAC CTt CCA GAT GAg TCa ACT GGT TTT aTT CCA AAA GAG TAT Aat TTT
 p) ATG GgG AAA CTt CGT TcG AcA TTT GcG GAA TAT GGC TTG GgG aCT tCa ACA GGC ATT GAC CTt CCA GAT GAg TCa ACT GGT TTT aTT CCA AAA GAG TAT Aat TTT
 q) ATG GgG AAA CTt CGT TcG AcA TTT GcT GAA TAT GGC TTG Gga aCT tCa ACA GGC ATT GAC CTt CCA GAT GAg TCa ACT GGT TTT aTT CCA AAA GAG TAT Aat TTT
 r) ATG GgG AAA CTt CGT TcG AcA TTT GGT GAA TAT GGC TTG GgG gCT GCG Act GgG ATT GAC CTA CCA GAT GAA TCT ACT GGA TTT GTT CCC AAA GAG TAT Aat TTT

FIG. 1—Continued.

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ala asn tyr ile thr asn ala phe gly gin phe asp asn tyr thr pro met gin leu ala gin tyr val ala thr ile ala asn asn gly val arg val ala pro
a) GCT AAT TAC ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT ACG CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT 1050
b) GCT AAT TAC ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT ACG CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
c) GCT AAT TAC ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT ACG CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
d) GcC AAT TAc ATc Acc AAT GCC TTT GGG CAG TTT GAT AAC TAT Acc CcA ATG CAa TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
e) GCT AAT TAC ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT ACG CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
f) GCT AAT TAC ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT ACG CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
g) GCT AAT TAC ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT Acc CcA ATG CAa TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
h) GCT AAT TAC ATc Acc AAT GCC TTT GGG CAG TTT GAT AAC TAT ACG CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
i) GCT AAT TAC ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT ACG CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
j) GCT AAT TAC ATT ACT AAT tCC TTT GGG CAG TTT GAT AAC TAT ACG CcA tTG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
k) GCT AAT TAC ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT ACG CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
l) GCT AAT TAC ATT Acc AAT GCC TTT GGT CAG TTT GAT AAC TAc Act Cct ATG CAa TTG GcC CAG TAc GTt GgA Acc ATT GCA AAc AAc GGT GTT CgG ATt GcA CcT
m) GCT AAT TAC ATT Acc AAT GCC TTT GGC CAG TTT GAT AAC TAc Acc CcA ATG CAa TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
n) GCT AAT TAc ATT Acc AAT GcA TTT GGC CAG TTT GAT AAC TAT Acc CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
o) GCT AAT TAc ATT Acc AAT GcA TTT GGC CAG TTT GAT AAC TAT Acc CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
p) GCT AAT TAc ATT ACT AAT GcA TTT GGG CAG TTT GAT AAC TAT Acc CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
q) GCT AAT TAc ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT Acc CCG ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
r) GcC AAT TAc ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT Acc CcA ATG CAG TTG GCT CAG TAT GTg GCA ACT ATT GCA AAT AAT asp GGT GTT CGT GTG GCT CCT
s) GCT AAT TAC ATT ACT AAT GCC TTT GGG CAG TTT GAT AAC TAT ACG CcA ATG CAG TTG GCT CAG TAT GTA GCA ACT ATT GCA AAT AAT GGT GTT CGT GTG GCT CCT

arg ile val glu gly ile tyr gly asn asn asp lys gly gly leu gly asp leu ile gin gin leu gin pro thr glu met asn lys val asn ile ser asp ser
a) CGT ATT GTT GAA GGC ATT TAT GGT AAT AAT GAT AAG GGA GGA CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC 1155
b) CGT ATT GTT GAA GGC ATT TAT GGA AAT AAT GAT AAG GGA GGC CTa GGC GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
c) CGT ATT GTT GAA GGC ATT TAT GGA AAT AAT GAT AAG GGA GGC CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
d) CGT ATT GTT GAA GGT ATT TAT GGC AAT AAT GAT AAG GGA GGA CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACT GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
e) CGT ATT GTT GAA GGC ATT TAT GGT AAT AAT GAT AAG GGA GGA CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
f) CGT ATT GTT GAA GGC ATT TAT GGA AAT AAT GAT AAG GGA GGC CTa GGC GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
g) CGT ATT GTT GAA GGC ATT TAT GGA AAT AAT GAT AAG GGA GGC CTa GGC GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
h) CGT ATT GTT GAA GGC ATT TAT GGT AAT AAT GAT AAG GGA GGA CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
i) CGT ATT GTg GAA GGC ATT TAT GGC AAT AAT GAT AAG GGA GGA CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
j) CGT ATT GTT GAA GGC ATT TAT GGT AAT AAT GAT AAG GGA GGA CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
k) CGT ATT GTT GAA GGC ATT TAT GGA AAT AAT GAT AAG GGA GGC CTG GGC GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
l) CAc ATT GTc GAg GgG ATT TAT GGA AAT AAT GAa CAa GGC GGC tTa GgG aAc TTa ATc CAa tct gTt gAA tCc AAg GAa ATG AAT Aaa sTt AAT ATt TcT GAg TcT
m) CGT ATT GTT GAA GGC ATT TAT GGA AAT AAT GAT AAG GGA GGC CTa GGC GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
n) CGT ATT GTT GAA GGC ATT TAT GGA AAT AAT GAT AAG GGA GGC CTG GGC GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
o) CGT ATT GTT GAA GGC ATT TAT GGA AAT AAT GAT AAG GGA GGC CTa GGC GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
p) CGT ATT GTT GAA GGC ATT TAT GGT AAT AAT GAT AAG GGA GGA CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
q) CGT ATT GTT GAA GGC ATT TAT GGT AAT AAT GAT AAG GGA GGA CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
r) CGT ATT GTT GAA GGC ATT TAT GGA AAT AAT GAT AAG GGA GGC CTa GGC GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC
s) CGT ATT GTT GAA GGC ATT TAT GGT AAT AAT GAT AAG GGA GGA CTG GGT GAC TTG ATT CAG CAA CTG CAA CCG ACA GAG ATG AAT AAG GTC AAT ATA TCC GAC TCC

asp met ser ile leu his gin gly phe tyr gin val ala his gly thr ser gly leu thr thr gly arg ala phe ser asn gly ala leu val ser ile ser gly
a) GAT ATG AGC ATC TTG CAC CAA GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA 1260
b) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
c) GAT ATG AGC ATC TTG CAC CAA GGT TTT TAT CAa GTT tCt CAc GGA GgG AGT GcT cTG AcG AcA GGT CGT GCC TTT TCA AAT GGA GcA GcA GTt TCC ATT AGt GGT
d) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGA TTG ACA ACT GGC CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
e) GAT ATG AGC ATC TTG CAC CAA GGT TTT TAT CAG GTT GCC CAT GGT ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
f) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
g) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGA GcA GcA GTt TCC ATT AGt GGT
h) GAT ATG AGt ATc TTg CAc CAa GGA TTT TAc CAa GTa GcT CAT GgA ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGt GGT
i) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGt GGA
j) GAT ATG AGC ATc TTg CAc CAa GGT TTT TAT CAG GTT GCC CAT GGT ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
k) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGC TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
l) GAT gTt tCc ATc cTc CAa CAa GGC TTT TAT CAa GTg tCa CAT GGT ggt AGT GcT TTG ACA Acc GGT CGT GCC TTT TCA AAT GGC GCC TTG GTA TcG ATT AGt GGT
m) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgA ACT AGc GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
n) GAT ATG AGC ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
o) GAT ATG AGC ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
p) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
q) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGT GGC TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
r) GAT ATG AGt ATc TTg CAc CAa GGT TTT TAT CAG GTT GcT CAT GgG ACT AGc GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA
s) GAT ATG AGC ATc TTg CAc CAa GGT TTT TAT CAG GTT GCC CAT GGT ACT AGT GGA TTG ACA ACT GGA CGT GCC TTT TCA AAT GGT GCC TTG GTA TCC ATT AGC GGA

FIG. 1—Continued.

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 a) lys thr gly thr ala glu ser tyr val ala asp gly gln gln ala thr asn thr asn ala val ala tyr ala pro ser asp asn pro gln ile ala val ala val
 AAA ACA GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT ACC AAT ACC AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTC GCA GTG 1365
 b) AAA ACA GGT Act GCC GAA AGT TAT GTt GCG Gya GGT Caa GAA Gct Aac AAT ACC AAT Gct GTG GCC TAT Gca CCA TCa GAT AAT CCC CAA ATC GCT GTc GCA GTT
 c) AAA ACA GGT Act GCC GAA AGT TAT GTt GCG Gya GGT Caa GAA Gct Aac AAT ACC AAT Gct GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTC GCA GTG
 d) AAA Acg GGT ACA GCC GAA AGT TAT GTG GCA GAT GGT CAG CAA GCA ACT AAT ACC AAT GCG GTG GCC TAT Gca CCA TCa GAT AAT Cct CAA ATC GCT GTg GCA GTG
 e) AAA ACA GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT ACC AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTC GCA GTG
 f) AAA Acg GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT ACC AAT GCG GTG GCC TAT Gca CCA TCT GAT AAT CCC CAA ATC GCT GTt GCA GTG
 g) AAA ACA GGT Act GCC GAA AGT TAT GTt Gcc Gya GGT CAG CAA GCA ACC AAT ACC AAT Gct GTG GCC TAT Gca CCA TCa GAT AAT Cct CAA ATC GCT GTa Gct GTt
 h) Aag Acc GGT ACA Ggt GAA AGC TAT GTa Gct Ggt GGT Caa gaa Gct Aat AAT ACC AAT Gcc GTG GCC TAT Gct CCA acc Gaa AAT Cct CAA AtT GCT GTt GCA GTa
 i) AAA ACA GGT Acc GCC GAA AGT TAT GTa GCA Ggt GGT Caa gaa Gct Aac AAT Act AAT Gct GTa Gcc TAT Gct CCA TCT GAT AAT CCC CAA ATC GCT GTt GCA GTG
 j) AAA ACA GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT ACC AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTC GCA GTG
 k) AAA Acg GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT Act AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTt GCA GTG
 l) AAA ACA GGT Act GCC GAA AGT TAT GTt Gaa Ggt GGT Caa gaa Gct Aac AAT Act AAT Gct GTG GCC TAT Gca CCA TCa GAT AAT Cct CAA ATC GCT GTa Gct GTG
 m) AAA Acg GGT Act Gct GAA AGT TAT GTt GCA Ggt GGT Caa Gaa Gct Aac AAC ACC AAT Gct GTG GCC TAT Gca CCA TCa GAT AAT Cct CAA ATC GCT GTy Gct GTt
 n) AAA ACA GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT ACC AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTC GCA GTG
 o) AAA ACA GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT ACC AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTC GCA GTG
 p) AAA Acg GGT ACA GCC GAA AGC TAT GTG GCA GAT Gga CAG CAA GCA ACC AAT Act AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTt GCA GTG
 q) AAA Act GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT ACC AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTC GCA GTG
 r) AAA Act GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT ACC AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTC GCA GTG
 s) AAA ACA GGT ACA GCC GAA AGC TAT GTG GCA GAT GGT CAG CAA GCA ACC AAT ACC AAT GCG GTG GCC TAT GCC CCA TCT GAT AAT CCC CAA ATC GCT GTt GCA GTG

490
 a) val phe pro his asn thr asn leu thr asn gly val gly pro ser ile ala arg asp ile ile asn leu tyr gln lys tyr his pro met asn STOP
 GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT AAC CTC TAT AAT AAc CAA TAC CAT CCA ATG AAC TAG AAA GGA AAT 1470
 b) GTC TTT CCT CAT AAc ACC AAC CTT ACA AAT GGT GTc GGA CCT TCC ATT GCG CGT GAT ATT ATC AAC CTC TAT AAT AAc CAA TAC CAT CCA ATG AAT TAG AAA GGA Aca
 c) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTg TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 d) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT Ggc GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTy TAT CAA AAA TAC CAT CCA ATG AAT TAG AAA GGA Act
 e) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTC TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 f) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTy TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 g) GTC TTT CCT CAT AAc ACC AAC CTT ACA AAT GGT GTc GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTC TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 h) GTC TTT CCT CAT AAT ACC AAT CTA ACC Aaa Ggt GGT GTC GGA CCT TCC ATT GCG CGT GAT ATT ATC AAT CTA TAT sac CAA CAC CAT CCA ATG AAT TAG AAA GGA Agc
 i) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAT ATT ATC AAT CTg TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 j) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAT ATT ATC AAT CTC TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 k) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAT ATT ATC AAT CTg TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 l) GTC TTT CCT CAT AAc ACC AAC CTT ACA AAT GGT GTc GGA CCT TCC ATT GCG CGT GAT ATT ATC AAC CTC TAT sac CAA CAT CAT CCA ATG AAT TAG AAA GGA Act
 m) GTC TTT Ccg CAT AAc ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAT ATT ATC AAT CTy TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AtT
 n) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTy TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 o) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTy TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 p) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTy TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 q) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTy TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 r) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTy TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT
 s) GTC TTT CCT CAT AAT ACC AAT CTA ACA AAT GGT GTA GGA CCT TCC ATT GCG CGT GAC ATT ATC AAT CTC TAT CAA AAA TAC CAT CCA ATG AAC TAG AAA GGA AAT

- a) TAT GCT TTA TCC AAC ACC TAT TGC TAA GCT AAT TG
- b) TAT GCT TTA T
- c) TAT GCT TTA T
- d) TAT GCT TTA T
- e) TAT GCT TTA T
- f) TAT GCT TTA T
- g) TAT GCT TTA T
- h) TAT GCT TTA T
- i) TAT GCT TTA T
- j) TAT GCT TTA T
- k) TAT GCT TTA T
- l) TAT GCT TTA T
- m) TAT GCT TTA T
- n) TAT GCT TTA T
- o) TAT GCT TTA T
- p) TAT GCT TTA T
- q) TAT GCT TTA T
- r) TAT GCT TTA T
- s) TAT GCT TTA T

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% CO₂ 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- μ l PCR mixture contained 1 μ g of chromosomal DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8), 1.5 mM MgCl₂, 0.01% gelatin, 0.1% Triton X-100, 1 μ M (each) primer, 200 μ M 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 μ l with deionized H₂O, 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 \pm 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 μ g/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 μ g/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 μ g/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 μ g/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 (\geq 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 μ g/ml) has nucleotide alterations occurring only within a \pm 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 \pm 0.25 μ g/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).

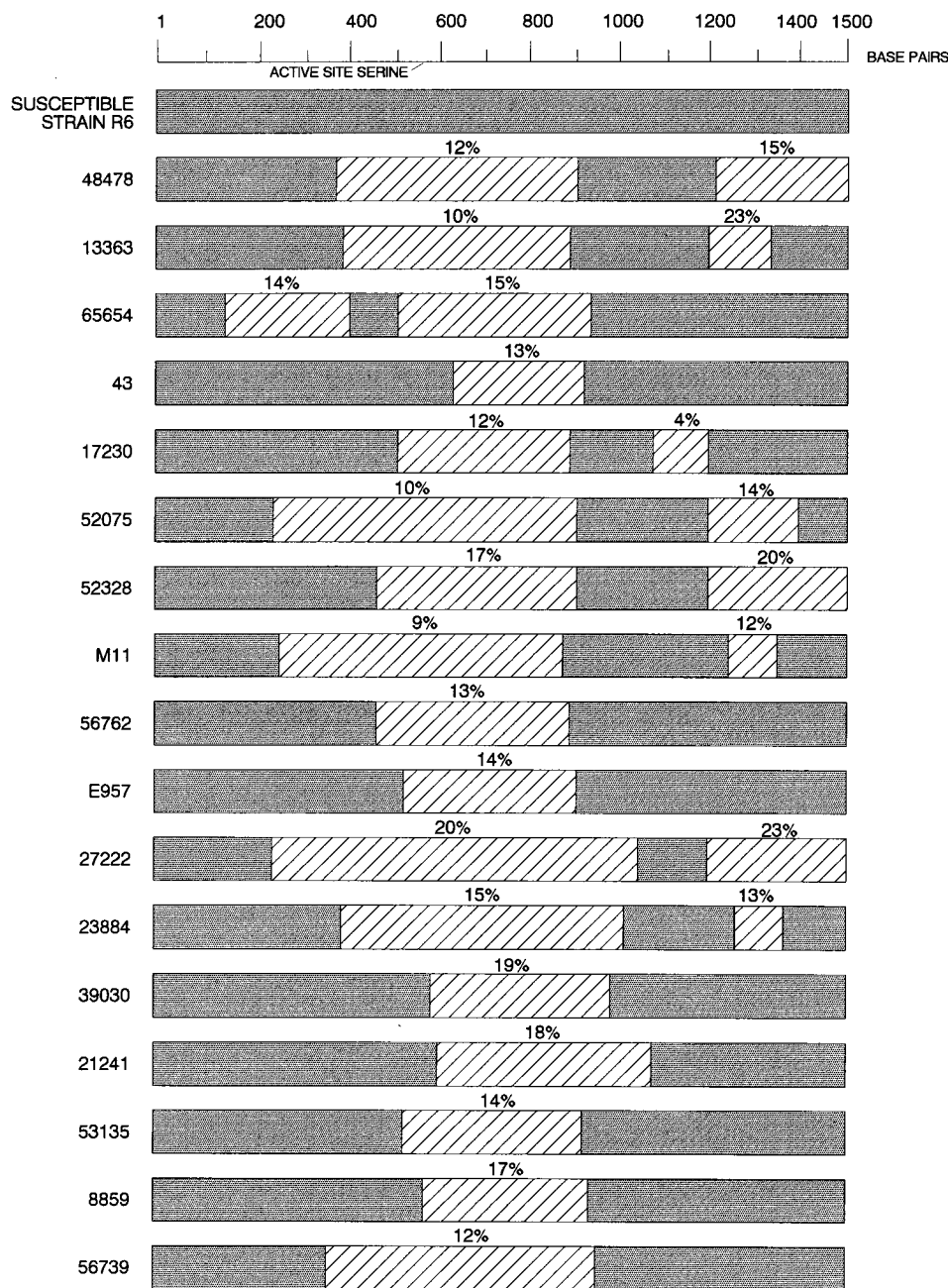


FIG. 2. Sequence diversity of the PBP 2B TERs from penicillin-resistant pneumococci (Table 1) compared with the susceptible strain R6 (isolate 22012 is not included since it has only seven nucleotide substitutions across the 1.5-kb region, which does not alter the amino acid sequence of the protein). 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 pre-

viously 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 μ g/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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