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Naturally Occurring Peptidoglycan Variants of *Streptococcus pneumoniae*

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Analysis by high-performance liquid chromatography of the stem peptide composition of cell walls purified from a large number of pneumococcal strains indicates that these bacteria produce a highly conserved species-specific peptidoglycan independent of serotype, isolation date, and geographic origin. Characteristic features of this highly reproducible peptide pattern are the dominance of linear stem peptides with a monomeric tripeptide, a tri-tetra linear dimer, and two indirectly cross-linked tri-tetra dimers being the most abundant components. Screening of strains with the high-performance liquid chromatography technique has identified two naturally occurring peptidoglycan variants in which the species-specific stem peptide composition was replaced by two drastically different and distinct stem peptide patterns, each unique to the particular clone of pneumococci producing it. Both isolates were multidrug resistant, including resistance to penicillin. In one of these clones—defined by multilocus enzyme analysis and pulsed-field gel electrophoresis of the chromosomal DNAs—the linear stem peptides were replaced by branched peptides that most frequently carried an alanyl-alanine substituent on the ε amino group of the diamino acid residue. In the second clone, the predominant stem peptide species replacing the linear stem peptides carried a seryl-alanine substituent. The abnormal peptidoglycans may be related to the altered substrate preference of transpeptidases (penicillin-binding proteins) in the pneumococcal variants.

Peptidoglycans surround the bacterial cell with an uninterrupted network of covalently interlinked muropeptides which is essential for bacterial survival in most environments. While all eubacterial peptidoglycans are constructed on the basis of similar structural principles, the amino acid composition and cross-linking mode of peptidoglycans show sufficient species-to-species variation to be used for taxonomic purposes (25, 26). In the system of Schleifer and Kandler (25), the nature of the cross-linking peptide bond, the amino acid residues connected, and the chemical nature of the diamino acid component of muropeptides were used as the basis of taxonomic classification. The complexity of peptidoglycan structure has only been fully recognized after the recent introduction of the high-resolution analytical technique of high-performance liquid chromatography (HPLC) (17), which demonstrated the unexpectedly large number of distinct muropeptides present in the peptidoglycans of both gram-negative (1, 14, 23, 28) and gram-positive (4, 16) species. To what extent and with what degree of precision the unique composition of these complex supramolecular structures is reproduced in each cell division are not known. Testing this was one of the main purposes of the studies described here. We found that pneumococcal strains of various serotypes, different isolation dates, and geographic origins produced a species-specific peptidoglycan characterized by highly conserved molar ratios of 18 different muropeptides, resolvable by the HPLC technique. In the process of examining the muropeptide composition of a large number of *Streptococcus pneumoniae* isolates, we have also identified what appear to be naturally occurring peptidoglycan variants of this bacterium with muropeptide compositions that differ drastically from the species-specific pattern exhibited by the great majority of pneumococcal isolates.

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MATERIALS AND METHODS

Strains and growth conditions. The *S. pneumoniae* strains used in this study were from a collection housed at the Laboratory of Microbiology at Rockefeller University. Bacterial cultures were grown in a casein-based semisynthetic medium at 37°C without aeration, as described previously (16).

Cell wall preparation. Pneumococcal cell walls were prepared by a published procedure (15, 20) with the following modifications. After the cells were harvested by centrifugation, they were suspended in ice-cold phosphate-buffered saline and quickly dropped into boiling sodium dodecyl sulfate (final concentration, 4%) to inactivate any wall-modifying enzymes. Walls were mechanically broken by shaking with acid-washed glass beads in a Vortex mixer operating at top speed or in a Bead Beater apparatus (Biospec Products, Inc., Bartlesville, Okla.). After removal of unbroken cells and glass debris by low-speed centrifugation (2,000 × *g*, 7 min), the purification of cell walls was done according to previously described procedures.

Enzymatic digestion of cell walls. For the liberation of stem peptides, 1 to 5 mg of cell walls was suspended in 50 mM potassium phosphate buffer, pH 7.0, and treated with affinity-purified pneumococcal autolysin (12) (40 mU) at 37°C for 18 h. The products of amidase digestion were dried, the precipitate was washed with acetone, and the peptides were extracted with acetonitrile-isopropanol-water (25:25:50) containing 0.1% trifluoroacetic acid (TFA). This procedure extracted 90 to 96% of the lysine-labeled material. After removal of the solvents by evaporation, the peptides were redissolved in 0.1% TFA for HPLC analysis.

HPLC. The chromatographic system was made up of a 721 system controller, two S10 pumps, a U6K injector, and a 730 data module from Waters Associates, Inc., Milford, Mass. The detector was a variable-wavelength ISCO V4 detector with a 6-mm path length and a 3.5-μl illuminated volume flow cell (ISCO, Lincoln, Nebr.). The column was a Vydac 218TP54 (The Separations Group, Hesperia, Calif.), and the eluting solvent was an 80-min linear gradient from 0.1% TFA (Pierce Chemical Co., Rockford, Ill.) to 15% acetonitrile (Burdick & Jackson, Muskegon, Mich.) in 0.1% TFA pumped at a flow rate of 0.5 ml/min (13). The eluted fractions were detected and quantitated by their A_{210}.

Analysis of stem peptides VII, VIII, and IX. These new stem peptides generated by the enzymatic hydrolysis of cell walls of strain Hun 665 were eluted from the HPLC column (see Fig. 2), and the pooled fractions were analyzed for amino acid composition and molecular mass by mass spectrometric analysis, as previ-

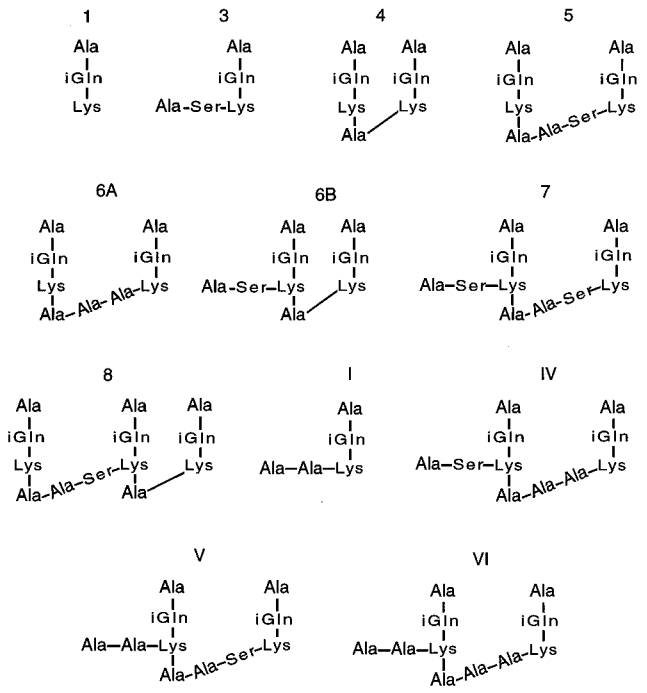

FIG. 1. Structures of pneumococcal cell wall stem peptides (16).

ously described (4). Structures for the other 15 stem peptides identified in the pneumococcal peptidoglycan have already been proposed (16).

RESULTS

Uniform peptidoglycan pattern in clinical isolates of *S. pneumoniae*. In order to obtain information about the species specificity of peptidoglycan stem peptide composition on the level of resolution offered by HPLC, it was important to test the reproducibility of the technique. Five independent preparations of cell walls isolated and purified from the unencapsulated *S. pneumoniae* strain R36A were analyzed by the HPLC technique. Data summarized in Table 1 show that the HPLC method was capable of reproducing with a high degree of precision the proportions of 18 stem peptides in the peptidoglycan of this bacterial strain. The data also show that the strain has reproduced its complex peptidoglycan structure with precision through each one of the five cultures.

The most outstanding, characteristic common features of this highly reproducible peptide pattern were the relative abundances of peptide 1, the major monomeric tripeptide, amounting to 22.3% ± 1.55% (mean ± standard deviation [SD]) of peptide material of the 18 individual stem peptides identified; peptide 4, the most frequent tri-tetra dimer (31.2% ± 1.55% [mean ± SD]); and peptides 5 and 6 (14.4 and 5.7%, respectively), dimers composed of a linear tetrapeptide cross-linked to a tripeptide through either a seryl-alanine or an alanyl-alanine cross bridge (Fig. 1). Peptides IV, V, and VI were present in only minute quantities (sum total of 1.4%). The dominant building blocks of peptidoglycan were linear stem peptides (70%), and the ratio of monomeric to dimeric plus trimeric peptides was low (0.4 ± 0.04 [mean ± SD]).

Analysis of peptidoglycan was next extended to additional *S. pneumoniae* isolates: seven strains of capsular type 6B isolated between 1986 and 1989 in the United States and five capsular type 6B pneumococci isolated between 1991 and 1992 in north-

TABLE 1. Reproducibility of cell wall stem peptide composition in five separate cell wall preparations of *S. pneumoniae* R36A

R36A prepn no.	Peptide material (%)													Recovery (%)	Monomer (%)	Dimer and trimer (%)	Total peptide (%)		B/L
	1	2	3	I	II	4	III	5	6	7	IV	V	8	VI	9	VII	VIII	XI				Linear (L)	Branched (B)	
1	21.1	6.5	2.6	1.7	—[a]	31.2	2.5	13.3	6.2	5.1	0.7	0.7	6.1	—	2.1	—	—	—	68	34	66	72	28	0.4
2	20.5	5.7	2.6	1.7	—	33.7	2.1	12.3	6.2	4.5	0.5	0.5	5.1	—	2.1	—	—	—	79	32	68	72	28	0.4
3	23.6	3.6	4.5	1.3	—	30.9	2.2	15.8	5.4	4.2	0.6	0.6	5.2	—	1.7	—	—	—	72	35	65	69	31	0.45
4	24.1	3.6	4.6	1.6	—	31.0	2.2	16.1	4.6	4.4	0.7	0.7	5.4	—	1.9	—	—	—	71	36	64	69	31	0.45
5	22.4	3.3	4.2	1.3	—	29.4	2.0	14.4	6.1	4.4	1.1	1.1	6.9	—	3.1	—	—	—	61	34	66	68	32	0.5
Mean	22.3	4.5	3.7	1.5	—	31.2	2.2	14.4	5.7	4.5	0.7	0.7	5.7	—	2.2	—	—	—		34	66	70	30	0.4
SD	1.55	1.46	1.01	0.20	—	1.55	0.19	1.62	0.70	0.34	0.23	0.23	0.76	—	0.54	—	—	—		1.48	1.48	1.87	1.87	0.04

[a] —, not present.

TABLE 2. Cell wall stem peptide composition of *S. pneumoniae* and two of its natural peptidoglycan variants

Strain and/or origin	No. of isolates	Serotype	Peptide material (%) [mean (SD)]									
			1	2	3	I	II	4	III	5	6[a]	7
R36A, United States	1		22.3 (1.55)	4.5 (1.46)	3.7 (1.01)	1.5 (0.20)	—[b]	31.2 (1.55)	2.2 (0.19)	14.4 (1.62)	5.7 (0.70)	4.5 (0.34)
United States	7	6B	24.6 (1.33)	1.6 (0.63)	4.6 (1.02)	2.9 (0.61)	0.9 (0.48)	28.6 (4.31)	2.4 (0.79)	9.6 (2.26)	7.7 (0.95)	5.8 (0.61)
Northern Europe	5	6B	28.3 (1.24)	2.0 (0.86)	5.0 (0.98)	3.5 (0.49)	0.4 (0.06)	28.0 (2.33)	0.2 (0.04)	12.0 (1.96)	10.2 (1.63)	1.9 (0.62)
Hungary (PG variant 1)[c]	1	19A	1.9 (0.16)	0.3 (0.10)	8.1 (0.19)	23.6 (0.41)	4.4 (0.32)	0.9 (0.05)	1.2 (0.20)	0.5 (0.06)	1.5 (0.17)	2.6 (0.22)
Czech 2 (PG variant 2)	6	14	16.0 (2.07)	0.4 (0.12)	16.4 (1.40)	5.6 (0.67)	0.6 (0.12)	7.7 (0.89)	1.0 (0.19)	15.4 (0.78)	8.3 (1.01)	14.6 (1.55)

[a] Peptides 6A and 6B were not separated.
[b] —, not present.
[c] PG, peptidoglycan.

ern Europe. The close similarity of peptide patterns with that shown for strain R36A is evident from Table 2.

***S. pneumoniae* isolates with altered peptidoglycan composition: peptidoglycan variant 1 (strain Hun 663).** Table 3 shows the stem peptide composition of a serotype 19A pneumococcal isolate that belonged to a distinct genetic lineage first identified in Hungary (21). The proportions of the 18 stem peptides were completely different from those seen in strains R36A or the strains examined in Table 2. As in the case of the composition of strain R36A, for this Hungarian clone too the muropeptide composition was precisely reproduced in the five independent peptidoglycan preparations shown in Table 3.

In contrast to the peptidoglycan composition of the 13 strains described above (Tables 1 and 2), the major monomeric and dimeric peptides (peptides 1 and 4) were dramatically reduced in the Hun 663 isolate: from 22.3 to 1.9% in the case of peptide 1, from 31.2 to 0.9% in the case of peptide 4, and from 14.4 to 0.5% and 5.7 to 1.5% in the cases of peptides 5 and 6, respectively. These components were replaced by stem peptides that were minor or undetectable components of the peptidoglycans of the 13 strains described in Tables 1 and 2. The major monomeric component of the Hungarian strains was peptide I (Fig. 1) (23.6% ± 0.41% [mean ± SD] of all peptide species separated), a tripeptide carrying an alanyl-alanine substituent on the ε amino group of lysine, and peptide VI, a tri-tetra dimer of peptide I, has become the most abundant dimer (23.3% ± 0.41% [mean ± SD]). The peptidoglycan also contained elevated amounts of peptides IV and V (6.3 and 9.1%, compared with the low proportion of these compounds—0.7% each—in strain R36A). Peptides VII, VIII, and IX are newly identified and prominent components of the peptidoglycans of the Hun isolates (see Fig. 3), which have not been seen in any of the other pneumococcal peptidoglycans. Chemical and mass spectrometric analyses suggest that these components are trimeric stem peptides cross-linked indirectly either via alanyl-alanine or seryl-alanine dipeptide substituents. In assigning tentative chemical structures for these compounds (Table 4 and Fig. 2), we assumed amino acid sequences analogous to the ones shown for peptides IV, V, and VI. The precise sequences of amino acid residues in the branches of the trimeric stem peptides VI, VIII, and IX have not been determined yet. However, in the particular structural assignments, we used as analogies the known sequences of dimeric peptides IV, V, and VI (Fig. 1), which elute from the HPLC column in the same order as the new trimeric peptides VII, VIII, and IX. The combined amounts of these novel peptides represented as much as 15.6% of all stem peptides in the Hun strain.

The amounts of monomeric and dimeric/trimeric peptides in the peptidoglycan of Hun 663 were 40 and 60%, respectively, suggesting little or no change in the degree of cross-linking. The overwhelming majority of stem peptides had a branched structure, with a ratio of branched to linear peptides as high as 24.0.

Stability of the abnormal stem peptide composition of peptidoglycan variant 1. The composition of peptidoglycan is known to depend on the growth rate of bacteria and also to be influenced by the composition of growth medium. For this reason, it was important to test the growth rates of strains showing anomalous peptidoglycan composition. There were no significant differences observed in the mass doubling times (measured as optical density at 620 nm) of any of the strains grown in the synthetic medium (doubling times, 35 to 45 min). Nevertheless, because of the extremely different wall composition in strain Hun 663, we also tested the stability of the unusual peptidoglycan pattern after extensive serial subculture of the bacterium, both *in vitro* and *in vivo* (in an infected mouse) as well.

Bacteria were passaged in synthetic medium in the laboratory through 300 cell generations, after which cell walls were prepared and analyzed. In the second test of stability, the same bacterial strain was inoculated into mice intraperitoneally. Bacteria recovered 2 days later through autopsy of the spleens of dead animals were grown to cultures, and cell walls were prepared and analyzed with the HPLC technique. Bacteria exposed to these *in vitro* and *in vivo* passages have produced peptidoglycan with exactly the same stem peptide composition as the original strain (Fig. 3). Figure 3 also shows the HPLC profile of two additional pneumococcal isolates (Hun 665 and

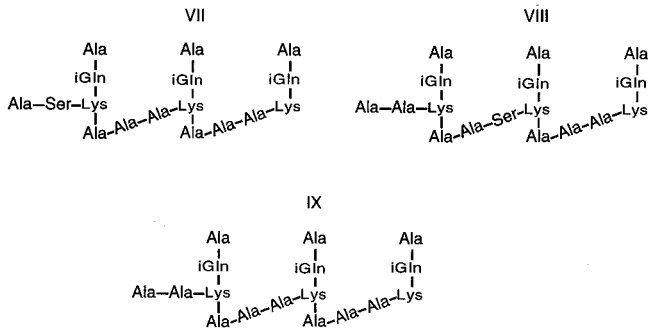

FIG. 2. Tentative structures of stem peptides VII, VIII, and IX isolated from peptidoglycan hydrolysate of strain Hun 665.

TABLE 2—*Continued*

Peptide material (%) [mean (SD)]								Monomer (%)	Dimer and trimer (%)	Total peptide (%)		B/L
IV	V	8	VI	9	VII	VIII	IX			Linear (L)	Branched (B)	
0.7 (0.23)	0.7 (0.23)	5.7 (0.76)	—	2.2 (0.54)	—	—	—	34 (1.48)	66 (1.48)	70 (1.87)	30 (1.8	0.4 (0.04)
0.8 (0.37)	2.2 (0.89)	3.7 (0.56)	2.9 (0.52)	1.4 (0.44)	—	—	—	37 (1.58)	63 (1.89)	64 (4.36)	36 (4.36)	0.6 (0.11)
0.6 (0.19)	0.8 (0.14)	3.5 (0.10)	2.3 (0.73)	1.9 (0.31)	—	—	—	39 (1.17)	61 (1.17)	69 (1.64)	31 (1.64)	0.5 (0.03)
6.3 (0.32)	9.1 (0.25)	0.6 (0.10)	23.3 (0.41)	0.3 (0.11)	2.2 (0.13)	5.4 (0.16)	8.0 (0.28)	40 (0.55)	60 (0.55)	4 (0)	96 (0)	24.0 (0)
3.0 (0.46)	2.8 (0.44)	2.0 (0.63)	1.9 (0.28)	4.4 (0.56)	—	—	—	40 (2.23)	60 (2.23)	35 (2.90)	65 (2.90)	1.9 (0.24)

Hun 963) that belong to the same clone as strain Hun 663, as defined by the multilocus enzyme analysis, penicillin-binding protein (PBP) type, and pulsed-field gel electrophoresis pattern (21). The virtual identity of HPLC elution profiles of strains Hun 663, Hun 665, and Hun 963 is apparent from the figure.

***S. pneumoniae* isolates with altered peptidoglycan composition: peptidoglycan variant 2 (Czech 2).** A second, distinct and altered peptidoglycan composition was identified in another group of six *S. pneumoniae* isolates originating from the Czech Republic (Czech 2). This group of isolates expressed capsular type 14 and also shared a common macrorestriction pattern (after *Sma*I digestion and pulsed-field gel electrophoresis) and common multilocus enzyme type (11). As in isolate Hun 663, in Czech 2 as well (Table 2), the proportions of peptide 1 and 4 were reduced, albeit less drastically than in Hun 663 (from 22.3 to 16.0% and from 31.2 to 7.7%, respectively). These components have been replaced by elevated amounts of peptide 3 (16.4%) (a monomeric tripeptide subunit with an added seryl-alanine branch on the ε amino group of lysine) and peptide 7 (14.6%), a dimer composed of a tetra- and a tri-peptide subunit each carrying the seryl-alanine substituent and cross-linked through the seryl-alanine dipeptide. The peptidoglycan of Czech 2 also contained increased proportions of peptides IV, V, and VI, and the representation of peptides 5 and 6 remained high (15.4 and 8.3%, respectively). The amounts of monomeric and dimeric/trimeric peptides were 40 and 60%, respectively, and the ratio of branched to linear peptides was 1.9, in contrast to 0.4 in the species-specific pneumococcal peptidoglycan and 24.0 in the peptidoglycan variant Hun 663.

DISCUSSION

Pneumococcal clinical isolates share a common cell wall stem peptide pattern which appears to be independent of geographic origin, date of isolation, and serotype. The characteristic features of this pattern are a high proportion of peptides 1, 4, and 5 containing linear subunits and a very low representation of branched peptides (particularly peptides IV through IX). This pattern has been identified in over 30 pneumococcal strains expressing nine different serotypes (2, 3, 4, 6A, 6B, 10, 15B, 19, and 23F) and isolated between 1916 and 1995 in Scandinavia, Spain, South Africa, and the United States. The results described in this paper suggest that the conservation of this species-specific peptidoglycan extends to a remarkably precise reproduction of the proportions of 18 cell wall stem peptides resolved by our technique. Our data confirm the validity of the biochemical classification system proposed by Schleifer and Kandler (25) and extend it to the high level of resolution provided by the HPLC analysis.

The two groups of pneumococcal isolates expressing the peptidoglycan structural variants 1 and 2 have grossly different stem peptide compositions. The mechanism(s) that is responsible for these drastic alterations in composition is not understood. Several conceivable mechanisms may be considered.

Changes in peptidoglycan composition (on the level of resolution allowed by the HPLC technique) have been observed as a function of growth rate and/or growth phase (10), and peptidoglycan composition can also vary under the influence of certain compounds added to the medium (for instance, high

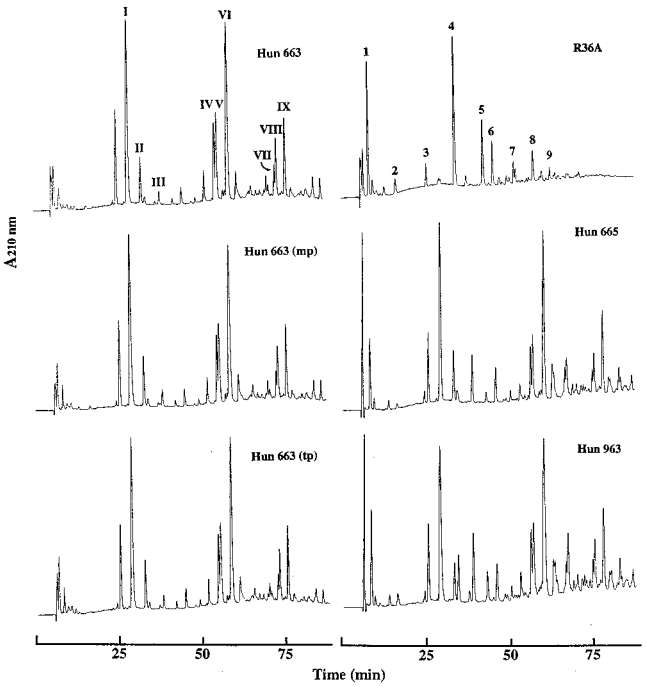

FIG. 3. Stability of stem peptide HPLC fingerprints. Cultures of the penicillin-resistant strain HUN 663 were passaged in vitro in drug-free medium [Hun 663 (tp)] or in vivo in the mouse [Hun 663 (mp)], as described in the text. Peptidoglycans were prepared (as described in Materials and Methods) from strain Hun 663 before passage, after the in vitro and in vivo passages, and also from the resistant strains Hun 665, Hun 963, and the penicillin-susceptible laboratory strain R36A. The HPLC fingerprints were prepared as described in Materials and Methods. Roman numerals refer to some branched stem peptides frequently seen in the resistant pneumococci; arabic numerals indicate the positions of linear peptides in the HPLC elution profiles.

TABLE 3. Reproducibility of cell wall stem peptide composition in five separate cell wall preparations of *S. pneumoniae* Hun 663

Hun 663 prepn no.	Peptide material (%)																Recovery (%)	Monomer (%)	Dimer and trimer (%)	Total peptide (%)		B/L
	1	2	3	I	II	4	III	5	6	7	IV	V	8	VI	9	VII	VIII	IX				Linear (L)	Branched (B)	
1	2.0	0.1	7.9	23.4	4.8	0.8	1.3	0.6	1.9	2.3	6.2	9.0	0.6	23.8	0.1	2.1	5.2	8.0	79	40	60	4	96	24.0
2	1.7	0.4	7.9	24.1	4.7	0.9	1.4	0.5	1.5	2.4	5.9	8.8	0.7	22.8	0.4	2.3	5.6	8.4	77	40	60	4	96	24.0
3	1.9	0.3	8.1	23.0	4.2	0.9	1.3	0.5	1.5	2.6	6.5	9.4	0.8	23.5	0.3	2.3	5.5	7.6	79	39	61	4	96	24.0
4	2.1	0.4	8.3	23.9	4.3	0.8	1.1	0.5	1.4	2.7	6.4	9.3	0.5	22.9	0.4	2.0	5.4	7.8	83	40	60	4	96	24.0
5	1.8	0.3	8.3	23.6	4.0	0.7	1.0	0.5	1.4	2.7	6.6	9.1	0.6	23.5	0.1	2.2	5.5	8.2	75	39	61	4	96	24.0
Mean	1.9	0.3	8.1	23.6	4.4	0.9	1.2	0.5	1.5	2.6	6.3	9.1	0.6	23.3	0.3	2.2	5.4	8.0		40	60	4	96	24.0
SD	0.16	0.10	0.19	0.41	0.32	0.05	0.20	0.06	0.17	0.22	0.32	0.25	0.10	0.41	0.11	0.13	0.16	0.28		0.55	0.55	0	0	0

TABLE 4. Analysis of stem peptides VII, VIII, and IX isolated from peptidoglycan hydrolysate of strain Hun 665

Peptide	Residue (mol/mol) normalized to Gln				M_r (M + H)$^+$		HPLC retention time (min)	
	Ala	Gln	Lys	Ser	Observed	Calculated	Hun 665	Hun 663
VII	3.6	1.0	1.0	0.3	1584.60	1584.88	69.39	69.25
VIII	3.3	1.0	1.1	0.3	1584.90	1584.88	69.82	69.73
IX	3.6	1.0	1.0	0.1	1568.80	1568.89	72.11	72.16

concentrations of glycine [19], D-amino acids [2], etc.). These conditions cause alterations in the degrees of O acetylation or de-N-acetylation (3) or may act through the perturbation of steps in peptidoglycan biosynthesis. Nevertheless, such phenomena do not explain the observations concerning the pneumococcal peptidoglycan variants, since all strains were grown under identical conditions and with comparable growth rates.

A more likely mechanism for the drastically different peptidoglycan patterns observed in the pneumococcal peptidoglycan variants may be that they reflect differences in the composition of the cell wall precursor pools of these strains. While all strains seemed to be capable of producing most of the structurally different stem peptides, the drastic and unique differences in the proportion of these may be the result of alteration in the regulation or the rate of production of these compounds. It is possible that such regulatory abnormalities (observed in peptidoglycan variants 1 and 2) are related to the acquisition of heterologous genetic elements, similar to the mechanism postulated for the origin of mosaic PBP genes in penicillin-resistant strains of pneumococci (6, 8). A precedent for mutationally altered muropeptide precursors replacing "normal" muropeptide components and generating stable and unique peptidoglycan types has been documented in the case of some of the so-called auxiliary mutants of methicillin-resistant *Staphylococcus aureus*. In these bacteria, transposon mutants blocked partially at various stages of muropeptide biosynthesis were shown to incorporate incomplete (abnormal) muropeptides into their peptidoglycan (5, 22). Detection of changes in the proportion of linear versus branched cell wall precursor muropeptides in the pneumococcal peptidoglycan variants would require analysis of the lipid-soluble cell fractions, since the substitution reactions on the diaminoacid residue occur in the bactoprenol-linked form of muropeptides in other bacteria (24).

Both groups of pneumococci expressing the abnormal peptidoglycan compositions were penicillin-resistant isolates, and the peptidoglycan changes may reflect alterations in the properties of peptidoglycan transpeptidases (PBPs) of these bacteria (9, 18, 29). While there is general agreement concerning the involvement of PBPs in terminal states of peptidoglycan assembly, PBP mutants of *E. coli* showed no compositional alteration in their peptidoglycan (16a). On the other hand, drastic alterations were observed in the peptidoglycan composition of a pneumococcal laboratory mutant carrying a truncated PBP3 gene (27). All the pneumococcal isolates representing the two peptidoglycan structural variants contained altered PBPs with reduced affinities for the antibiotic (11, 21). It has been suggested that such proteins may also have suboptimal catalytic activities with their normal, physiological substrates (16). It is possible that stem peptides with different dipeptide substituents on the ε amino group of the diaminoacid residue may have superior binding properties to some domain in the structurally altered PBPs. In

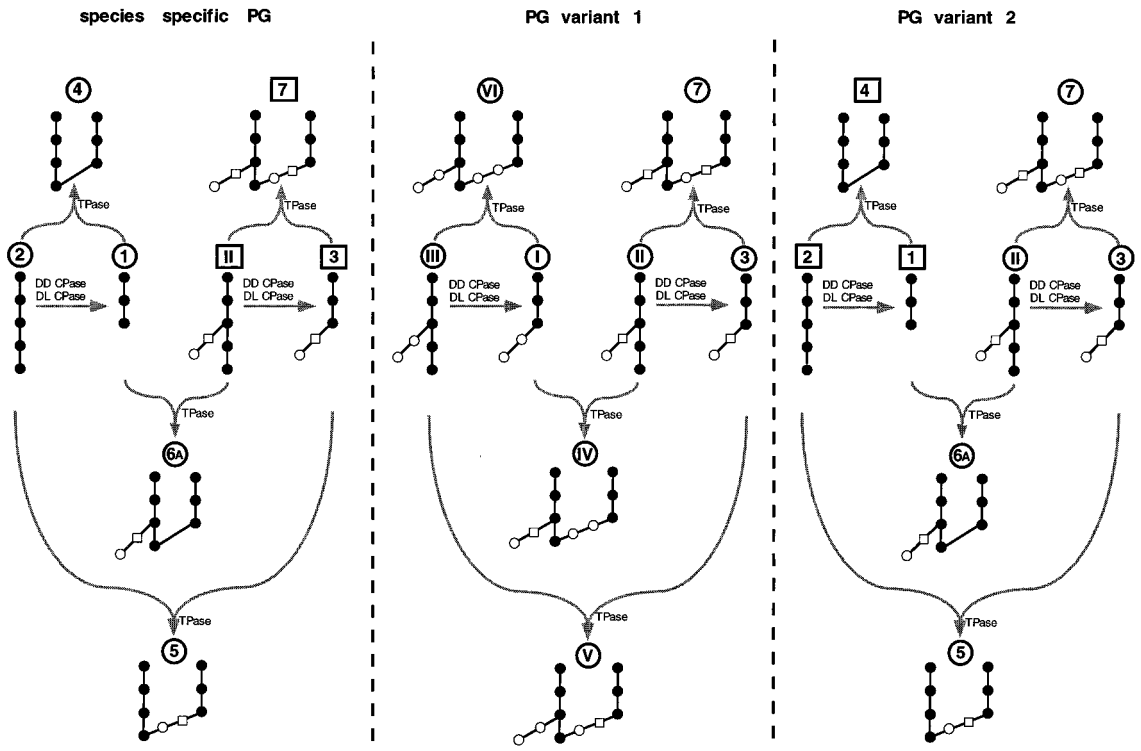

FIG. 4. Model for the altered substrate preference in the peptidoglycan transpeptidases of pneumococcal peptidoglycan (PG) variants. Solid circles represent the amino acid residues in the stem peptides (in the vertical sequence, from top to bottom, L-alanine, D-isoglutamine, lysine, and D-alanine, and D-alanine). Empty squares stand for serine, and empty circles stand for L-alanine residues. Arabic and roman numerals refer to various stem peptides (see structures in Fig. 1). Such peptide numbers inside a square symbol indicate that the particular peptide and its biosynthetic pathway are relatively minor in the particular peptidoglycan type. TPase, transpeptidase; CPase, carboxypeptidase.

the production of the species-specific pneumococcal peptidoglycan, the "normal" PBPs would cross-link peptides 2 and 1 to generate the major dimeric component (peptide 4), while transpeptidation of peptides 2 and 3 or 1 and II would lead to the formation of dimeric components 5 and 6A, respectively. Cross-linking of II and 3 would yield peptide 7, a component less abundant in this peptidoglycan type (Fig. 4). In contrast, the abnormal PBPs of strain Hun 663 may preferentially utilize as donors in the transpeptidation reaction pentapeptide subunits with alanyl-alanine substituents, while PBPs of the Czech 2 clone producing the peptidoglycan variant 2 may prefer seryl-alanine-substituted pentapeptides for this reaction (Fig. 4).

In current thinking, the evolution of altered PBP genes in pneumococci involved heterologous recombinational events leading to remodeled (mosaic) PBP genes encoding PBPs of reduced affinities for the penicillin molecule (7). It is conceivable that the acquisition of new genetic elements also included determinants of stem peptide branching enzymes or mutational events in regulatory genes that control the rate of production of branched-structured muropeptides (Fig. 5).

How the unique peptidoglycans with precise proportions of a large number of stem peptide building blocks are reproduced in each cell division in pneumococci and other bacteria is a question of fundamental interest. The availability of the naturally occurring peptidoglycan mutants of pneumococci as described in this communication should provide experimental systems to explore this question.

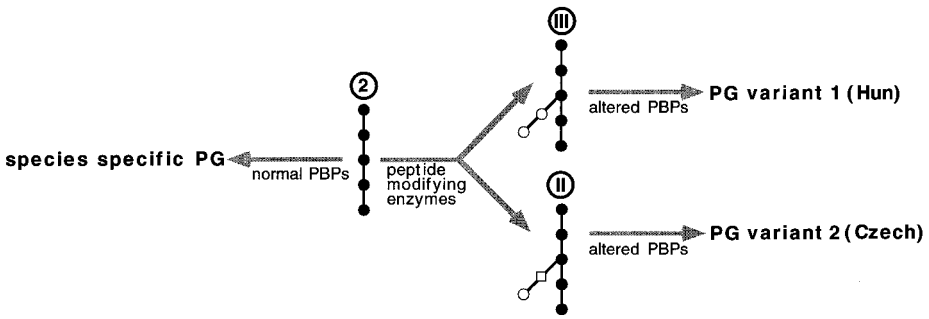

FIG. 5. Model for the genetic changes underlying the peptidoglycan alterations observed in the pneumococcal peptidoglycan (PG) variants 1 and 2. Symbols are as described in the legend to Fig. 4.

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REFERENCES

1. **Burroughs, M. H., Y. S. Chang, D. A. Gage, and E. I. Tuomanen.** 1993. Composition of the peptidoglycan of *Haemophilus influenzae*. J. Biol. Chem. **268:**11594–11598.
2. **Caparros, M., J. C. Quintela, J. I. Leguina, and M. A. de Pedro.** 1993. Amino acids as useful tools in the study of murein metabolism in *Escherichia coli*, p. 147–160. *In* M. A. De Pedro, J.-V. Holtje, and W. Loffelhardt (ed.), Bacterial growth and lysis: metabolism and structure of the bacterial sacculus. Plenum Press, New York.
3. **Clarke, H. J., and C. Dupont.** 1992. D-Acetylated peptidoglycan: its occurrence, pathobiological significance, and biosynthesis. Can. J. Microbiol. **38:** 85–91.
4. **De Jonge, B. L. M., Y.-S. Chang, D. Gage, and A. Tomasz.** 1992. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain: the role of penicillin binding protein 2A. J. Biol. Chem. **267:**11248–11254.
5. **De Jonge, B. L. M., T. Sidow, Y.-S. Chang, H. Labischinski, B. Berger-Bachi, D. A. Gage, and A. Tomasz.** 1993. Altered muropeptide composition in *Staphylococcus aureus* strains with an inactivated *femA* locus. J. Bacteriol. **175:**2779–2782.
6. **Dowson, C. G., T. J. Coffey, C. Kell, and R. A. Whiley.** 1993. Evolution of penicillin resistance in *Streptococcus pneumoniae*; the role of *Streptococcus mitis* in the formation of a low affinity PBP2B in *S. pneumoniae*. Mol. Microbiol. **9:**635–643.
7. **Dowson, C. G., T. J. Coffey, and B. G. Spratt.** 1994. Origin and molecular epidemiology of penicillin-binding protein-mediated resistance to β-lactam antibiotics. Trends Microbiol. **2:**361–366.
8. **Dowson, C. G., A. Hutchison, J. A. Brannigan, R. C. George, D. Hansman, J. Linares, A. Tomasz, J. Maynard Smith, and B. G. Spratt.** 1989. Horizontal transfer of penicillin-binding protein genes in penicillin resistant clinical isolates of *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **86:**8842–8846.
9. **Dowson, C. G., A. Hutchison, and B. G. Spratt.** 1989. Extensive remodelling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of *Streptococcus pneumoniae*. Mol. Microbiol. **3:**95–102.
10. **Driehuis, F., and J. T. M. Wouters.** 1987. Effect of growth rate and cell shape on the peptidoglycan composition of *Escherichia coli*. J. Bacteriol. **169:**97–101.
11. **Figueiredo, A. M. S., R. Austrian, P. Urbaskova, L. A. Teixeira, and A. Tomasz.** 1995. Novel penicillin-resistant clones of *Streptococcus pneumoniae* in the Czech Republic and in Slovakia. Microb. Drug Resist. **1:**71–78.
12. **Garcia, E., J.-L. Garcia, C. Ronda, P. Garcia, and R. Lopez.** 1985. Cloning and expression of the pneumococcal autolysin gene in *Escherichia coli*. Mol. Gen. Genet. **201:**225–230.
13. **Garcia-Bustos, J. F., B. T. Chait, and A. Tomasz.** 1987. Structure of the peptide network of pneumococcal peptidoglycan. J. Biol. Chem. **262:**15400–15405.
14. **Garcia-Bustos, J. F., and T. J. Dougherty.** 1987. Alterations in peptidoglycan of *Neisseria gonorrhoeae* induced by sub-MICs of β-lactam antibiotics. Antimicrob. Agents Chemother. **31:**178–182.
15. **Garcia-Bustos, J. F., and A. Tomasz.** 1987. Teichoic acid-containing muropeptides from *Streptococcus pneumoniae* as substrates for the pneumococcal autolysin. J. Bacteriol. **169:**447–453.
16. **Garcia-Bustos, J., and A. Tomasz.** 1990. A biological price of antibiotic resistance: major changes in the peptidoglycan structure of penicillin-resistant pneumococci. Proc. Natl. Acad. Sci. USA **87:**5414–5419.

16a. **Glauner, B.** 1986. Ph.D. dissertation. Das Murein von *E. coli.* Eberhard-Karls-Universität Tübingen, Tübingen, Germany.

17. **Glauner, B.** 1988. Separation and quantification of muropeptides with high performance liquid chromatography. Anal. Biochem. **172:**451–464.
18. **Hakenbeck, R., M. Tarpay, and A. Tomasz.** 1980. Multiple changes of penicillin-binding proteins in penicillin-resistant clinical isolates of *S. pneumoniae*. Antimicrob. Agents Chemother. **17:**364–371.
19. **Hammes, W., K. H. Schleifer, and O. Kandler.** 1973. Mode of action of glycine on the biosynthesis of peptidoglycan. J. Bacteriol. **116:**1029–1053.
20. **Mosser, J. L., and A. Tomasz.** 1970. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. **245:**287–298.
21. **Munoz, R., J. M. Musser, M. Crain, D. E. Briles, A. Marton, A. J. Parkinson, U. Sorensen, and A. Tomasz.** 1992. Geographic distribution of penicillin resistant clones of *Streptococcus pneumoniae*: characterization by penicillin binding protein profile, surface protein A typing and multilocus enzyme analysis. Clin. Infect. Dis. **15:**112–118.
22. **Ornelas-Soares, A., H. de Lencastre, B. L. M. de Jonge, and A. Tomasz.** 1994. Reduced methicillin resistance in a new *Staphylococcus aureus* transposon mutant that incorporates muramyl dipeptides into the cell wall peptidoglycan. J. Biol. Chem. **269:**27246–27250.
23. **Quintela, J. C., M. Caparrós, and M. A. de Pedro.** 1995. Variability of peptidoglycan structural parameters in gram-negative bacteria. FEMS Microbiol. Lett. **125:**95–100.
24. **Rogers, H. J., H. R. Perkins, and J. B. Ward.** 1980. Biosynthesis of peptidoglycan, p. 239–297. *In* L. Leive (ed.), Microbial cell walls and membranes. Chapman & Hall, Ltd., London.
25. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36:**407–477.
26. **Schleifer, K. H., and P. H. Seidl.** 1985. Chemical composition and structure of murein, p. 201–219. *In* M. Goodfellow and D. E. Minnikin (ed.), Chemical methods in bacterial systematics. Academic Press, New York.
27. **Severin, A., C. Schuster, R. Hakenbeck, and A. Tomasz.** 1992. Altered murein composition in a DD-carboxypeptidase mutant of *Streptococcus pneumoniae*. J. Bacteriol. **174:**5152–5155.
28. **Tuomanen, E., J. Schwartz, S. Sande, K. Light, and D. Gage.** 1989. Unusual composition of peptidoglycan in *Bordetella pertussis*. J. Biol. Chem. **264:** 11093–11098.
29. **Zighelboim, S., and A. Tomasz.** 1980. Penicillin-binding proteins of the multiply antibiotic-resistant South African strains of *S. pneumoniae*. Antimicrob. Agents Chemother. **17:**434–442.