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Structure of the Peptide Network of Pneumococcal Peptidoglycan*

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The peptide network of *Streptococcus pneumoniae* cell walls was solubilized using the pneumococcal autolytic amidase (*N*-acetylmuramoyl-L-alanine amidase, EC 3.5.1.28). The peptide material was fractionated into size classes by gel filtration followed by reverse-phase high-performance liquid chromatography which resolved the peptide population into over 40 fractions. About 40% of the lysines present participate in cross-links between stem peptides. The main components (3 monomers, 5 dimers, and 2 trimers), accounting for 77% of all the wall peptides, were purified. Their structures were determined using a combination of amino acid and end-group analysis, mass spectrometry, and gas-phase sequencing. Two different types of cross-links between stem peptides were found. In the most abundant type there is an alanylserine cross-bridge between the alanine in position 4 of the donor stem peptide and the lysine at position 3 of the acceptor peptide, as in type A3 peptidoglycan. In the second type of cross-link there is no intervening cross-bridge, as in the type A1 peptidoglycan of Gram-negative bacteria. The data indicate that pneumococcal peptidoglycan has a structural complexity comparable to that recently shown in some Gram-negative species.

The bacterial cell wall is a continuous covalent network that determines the shape of the cell and gives it the mechanical strength necessary to survive in a hypotonic environment. The peptidoglycan, or murein, is the common component of all eubacterial cell walls, and its assembly is the target of the penicillin and cephalosporin family of antibiotics.

In Gram-positive bacteria the wall is in direct contact with the external medium, and in *Streptococcus pneumoniae* it interacts with the complement system (1) and induces some of the symptoms of bacterial disease (2, 3).

The pneumococcal cell wall is composed of peptidoglycan and teichoic acid (4), the latter being a complex phosphorylated polysaccharide sometimes referred to as C-polysaccharide (5).

Until recently the analytical techniques available presented a relatively monotonous view of the bacterial peptidoglycan: the structural units were disaccharide tetrapeptide monomers, a variable portion of which were interlinked by peptide bonds to form dimers or oligomers (6). This structural simplicity became increasingly difficult to reconcile with accumulating evidence indicating the complexity of the steps in wall biosynthesis, the unexpectedly large number of penicillin-sensitive enzymes (penicillin binding proteins, PBPs) present in bacteria, and the multiplicity of biological activities exhibited by cell wall components. The recent introduction of high performance liquid chromatography (HPLC)[1] for the analysis of enzymatic hydrolysates of Gram-negative *Escherichia coli* murein by Glauner and Schwarz (7) has radically changed this situation. The new methodology has allowed the resolution of such hydrolysates into about 35 reproducible disaccharide peptide components, many of which are of novel chemical structure. The introduction of this technique for the analysis of cell wall biosynthesis, in normal (7, 8) and antibiotic-inhibited cells (9), in *E. coli* growing at different rates (10) or in cells undergoing autolysis (11), has begun to shed light on the unique physiological functions of these newly identified wall components.

No method of comparable resolving power has so far been introduced for the analysis of Gram-positive cell walls. Our attempt to apply directly the technique of Glauner and Schwarz (7) to the analysis of pneumococcal peptidoglycan resulted in poor recoveries, presumably because of differences in chemical composition. In addition, this technique is difficult to apply to Gram-positive cell walls that commonly contain covalently linked non-murein polymers. Much of the structural complexity in the bacterial peptidoglycan resides in the peptide component. The purpose of the studies described here was to investigate the structure of the peptide network of pneumococcal peptidoglycan and to design a high resolution method that would allow the rapid and quantitative enumeration of the various peptide components of the pneumococcal cell wall solubilized by the autologous *N*-acetylmuramoyl-L-alanine amidase. Many features of this method should also be applicable for the analysis of other Gram-positive cell walls.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Enzymatic Solubilization and Size Fractionation of the Peptide Network of Pneumococcal Cell Walls—Pneumococcal cell walls labeled biosynthetically with radioactive lysine in the

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[1] The abbreviations used are: HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 87 M-2025, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

stem peptides of the murein were solubilized by treatment with purified pneumococcal autolysin (*N*-acetylmuramoyl-L-alanine amidase). After digestion, over 95% of the radioactive label became nonsedimentable by centrifugation at 25,000 × *g* for 15 min, indicating virtually complete solubilization of the walls. The hydrolysate was then passed through a gel filtration system and separated according to size. Eluted fractions were assayed both for radioactivity and for fluorescamine-reacting material. These two detection techniques each revealed five distinct size fractions, and the elution profiles obtained on the basis of radioactivity and amino-reactive material were identical (Fig. 1).

Material from each of the five peaks was pooled and designated as fractions Ex (107–128 ml), Ol (128–160 ml), Tr (160–174 ml), Di (174–196 ml), and Mo (196–250 ml). These fractions were concentrated and analyzed for amino acid and amino sugar composition (Table I) and portions were also used to determine average molecular masses by mass spectrometry. As expected, none of the fractions except the excluded one contained amino sugars.

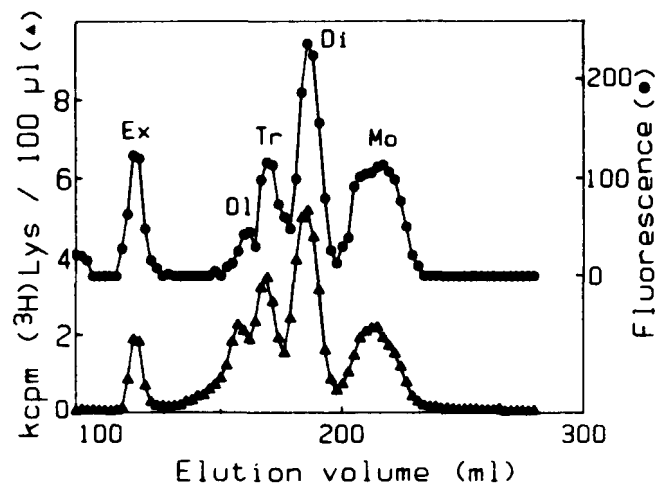

FIG. 1. **Size fractionation of L-alanine amidase digest of pneumococcal cell walls by gel filtration.** The cell wall digest was chromatographed on two coupled gel filtration columns (Ultrogel AcA 202 followed by Trisacryl GF05) as described in the text.[2] The fluorescence measured after fluorescamine derivatization of the samples is expressed in arbitrary units and it is linearly related to the concentration of free amino groups in each fraction. The nomenclature *Ex*, *Ol*, *Tr*, *Di*, and *Mo* for the different peaks was chosen to indicate that they contain material which was excluded, oligomeric, trimeric, dimeric, and monomeric, respectively.

TABLE I

Analysis of the main peptide size fractions in the pneumococcal cell wall

Undigested cell walls and the wall fractions obtained in Fig. 1 were analyzed for amino acids and amino sugars as detailed in the text.[2] The values listed are molar proportions relative to Glx. The results of the amino sugar analyses were not corrected for deacetylation and decomposition during hydrolysis.

Component	Walls	Fraction				
		Excluded	Oligomeric	Trimeric	Dimeric	Monomeric
Ser	0.3	0.4	0.4	0.4	0.2	0.2
Glx	1.0	1.0	1.0	1.0	1.0	1.0
Ala	1.9	1.8	2.2	2.2	1.8	1.2
Lys	0.9	0.8	0.8	0.8	0.8	0.8
Glucosamine	0.9	13.1	0.0	0.0	0.0	0.0
Galactosamine	1.3	13.3	0.0	0.0	0.0	0.0
Muramic acid	0.6	6.3	0.0	0.0	0.0	0.0
Percent Glx	100	7	13	18	36	26

The amino sugars present in the excluded peak appear to represent a mixture of the high molecular size glycan and teichoic acid components of the cell wall, plus peptidoglycan material that was not hydrolyzed during the enzyme treatment. These radiolabeled peptides were apparently attached to teichoic acid-containing glycan chains, since affinity chromatography of the excluded fraction on a myeloma-agarose column that binds phosphorylcholine completely retained the radioactive label (data not documented). No further work was done on this material.

Mass spectrometry has allowed the identification of the fractions labeled *Mo*, *Di*, and *Tr* in Fig. 1 as size classes containing one (monomers), two (dimers), or three (trimers) stem peptides, respectively. The average amino acid compositions of the oligomeric, trimer, dimer, and monomer fractions were comparable, except for a small degree of enrichment in the relative amounts of serine and alanine in all the fractions over that of the monomeric fraction (Table I).

The relative proportions of the different size classes allowed the estimation that approximately 40% of the lysine residues in the pneumococcal cell wall are in cross-links. This figure is derived from the fact that one-half of the lysines in the dimers, two-thirds in the trimers, and approximately three-fourths in the oligomers are involved in cross-linking.

HPLC Analysis of Cell Wall Peptides—Material from the peaks analyzed for composition was also injected into an HPLC system for further resolution. Another portion of the enzymatic digest was injected into the HPLC system without prefractionation. Eluates were assayed both for absorbance at 210 nm and radioactivity. Nine major and about 40 minor peaks were reproducibly obtained in over 10 independent runs (Fig. 2). Of the material eluting in the first 30 min, only *peaks*

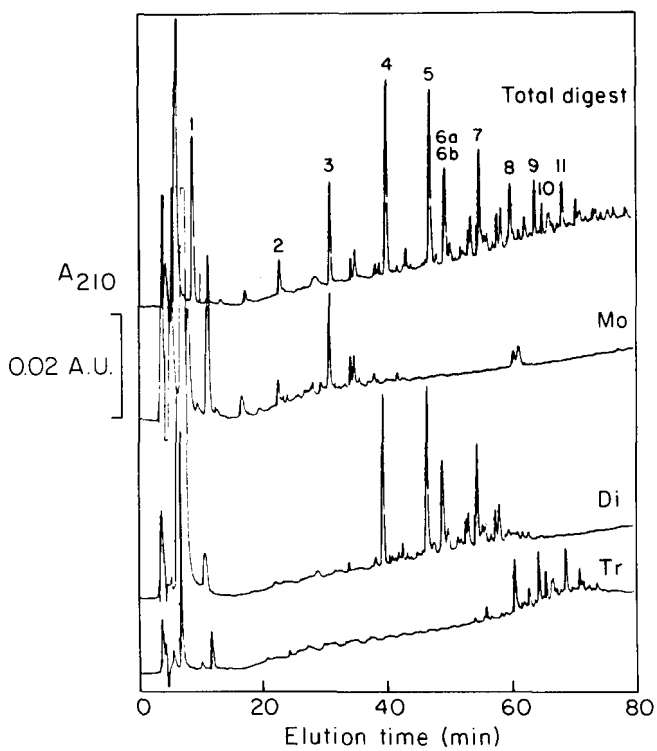

FIG. 2. **Reverse-phase HPLC of L-alanine amidase digest of pneumococcal cell wall and its main component size fractions.** Approximately 80, 20, 40, and 20 nmol of stem peptides from the total digest and from the Mo, Di, and Tr size fractions (Fig. 1), respectively, were loaded onto a Vydac column and eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid as described in the text.[2] *A.U.*, absorbance units.

1 and *2* contained both radiolabel and amino acids. The other peaks between 1 and 2 contained neither radiolabel nor amino acids or amino sugars; these peaks most likely represent buffer components. All the UV-absorbing fractions eluted after 30 min were radiolabeled. A comparison of the elution profiles in Figs. 1 and 2 clearly allows the identification of monomeric, dimeric, and trimeric peptides.

Structural Analysis of Wall Peptides—HPLC resolved these families of peptides into a number of individual components, except in the case of *peaks 6*, *10*, and *11*. The mass spectrum of *peak 6* showed the presence of two different dimeric peptides, with relative molecular masses 16 units apart (Fig. 3). Reverse-phase HPLC at a different pH failed to resolve the two components, but they were readily separated as their dansyl derivatives: *peaks 6a* and *6b* (Fig. 4). Although dansylation precluded accurate amino acid analysis, we determined that *peak 6a* contains Ser (0.6 mol/mol Glx) and that *peak 6b* does not (<0.1 mol/mol Glx). This information and the mass spectrum of each dansyl peptide (Fig. 5) led us to conclude

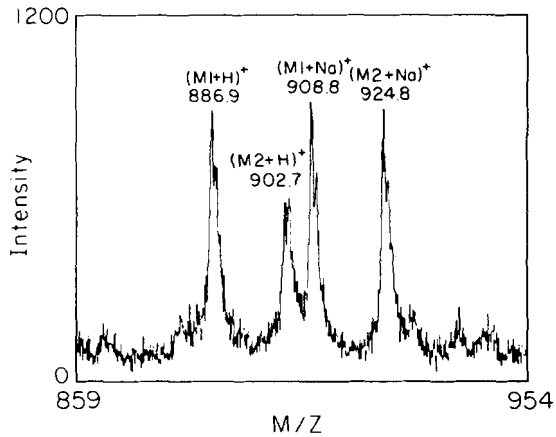

FIG. 3. **Partial mass spectrum of *peak 6* from Fig. 2.** A 3-nmol sample of *peak 6* from the HPLC separation illustrated in Fig. 2 was used to obtain this time-of-flight mass spectrum as described in the text.[2] Intensity refers to the number of ions detected. *M1* and *M2* designate the two components of HPLC *peak 6* and *M/Z* symbolizes the mass-to-charge ratio. The numbers over the peaks are the M_r calculated from the signal of the most abundant isotopic species. Spectrum acquisition time was 40 min.

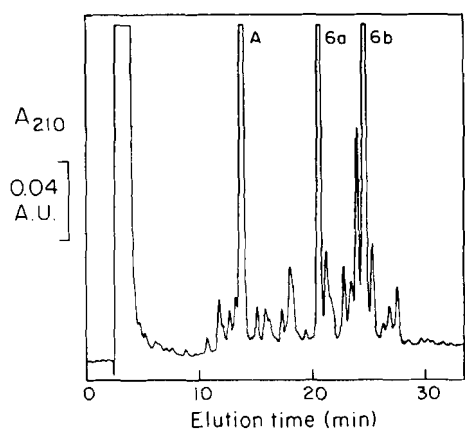

FIG. 4. **Reverse-phase HPLC of the dansylation mixture of *peak 6* from Fig. 2.** The dansylation products of a 30-nmol sample of *peak 6* were applied to an Aquapore column and eluted with a linear gradient from 16 to 32% acetonitrile in 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min for 30 min. Only the fractions labeled *6a* and *6b* contained a significant amount of radiolabel and yielded dansyl amino acids after acid hydrolysis. *Peak A* was identified as dansylamide by thin layer chromatography. *A.U.*, absorbance units.

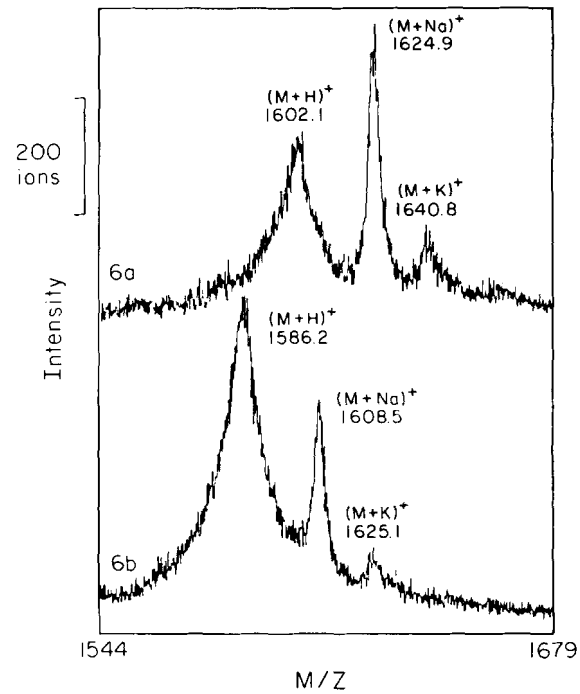

FIG. 5. **Partial mass spectra of *fractions 6a* and *6b* from Fig. 4.** Approximately 2 nmol of dansylated peptides *6a* and *6b* from Fig. 4 were used to obtain the time-of-flight mass spectra illustrated. *M* refers to the dansylated peptide. The numbers above the peaks correspond to the M_r calculated from the theoretical distribution of signals for the four most abundant isotopic species. This averaging was performed due to the broadening of the bands caused by the instability of the dansyl moiety under the conditions employed. Intensity refers to the number of ions detected and *M/Z* symbolizes the mass-to-charge ratio. Spectrum acquisition time was 15 min. Other details as explained in the text.[2]

that the serine residue in *peak 6a* had been replaced by alanine in *peak 6b*, thereby explaining the 16-unit difference in molecular weight.

No structures are proposed for the peptides in *peaks 10* and *11*, since they were not homogeneous and the dansylated trimers were not eluted from the reverse-phase columns, even when butylsilica was used as stationary phase and mixtures of acetonitrile and isopropanol as mobile phases.

All the purified peptides were analyzed for amino acid composition, the nature and number of amino-terminal amino acids, free amide groups, and molecular weight (mass spectrometry). A combination of these analytical data (summarized in Table II) has allowed a tentative assignment of structures to 10 of the major fractions (representing about 77% of the total peptide material), and these are listed in Fig. 6. The structures assigned to *fractions 3* and *7* in Fig. 6 should allow partial sequencing by Edman degradation. Such an experiment was performed (Table III) and the results confirmed the structures proposed.

The mass spectrometry data (Table II) showed that there could be at most only one free carboxyl group per stem peptide, suggesting that the α-carboxyls of the glutamic acid residues or any other amino acid substituting them is amidated (there is 1 mass unit difference between a carboxyl and an amide group). After treatment with bis-(I,I-trifluoroacetoxy)iodobenzene, which eliminates carboxamido groups (17), all the wall peptides tested lost their Glx residues, indicating that the α-carboxyls on those residues were indeed all amidated.

DISCUSSION

The combination of enzymatic hydrolysis and HPLC has allowed us to resolve the pneumococcal peptidoglycan into an

TABLE II

Analysis of the major peptides in pneumococcal peptidoglycan

The analytical data obtained from the HPLC-purified peptides of Figs. 2 and 4 are summarized. The number and type of free amino groups were established by purifying the dansyl peptides and analyzing them by mass spectrometry and thin layer chromatography after acid hydrolysis, as detailed in the text.[2] The molecular weights were obtained from mass spectra like the one illustrated in Fig. 3. The proportion of stem peptides contributed to the wall by each peptide species was estimated by integrating the areas under the chromatogram in Fig. 2.

	Monomers			Dimers					Trimers			
	1	2	3	4	5	6a	6b	7	8	9	10[a]	11[a]
Amino acid composition												
Ser	0.0	0.0	0.8	0.0	0.3	0.3		0.7	0.3	0.4	0.5	0.7
Glx	1.0	1.0	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0
Ala	0.9	2.6	1.6	1.4	2.2	1.8		1.9	1.9	2.3	2.0	2.4
Lys	0.8	0.7	0.8	0.9	0.9	0.8		0.9	0.8	0.8	0.8	1.0
Free amino termini												
Total number	2	2	2	3	3	3	3	3	ND[b]	ND	ND	ND
Type	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	ND	ND
	ε-Lys	ε-Lys		ε-Lys	ε-Lys		ε-Lys		ε-Lys	ε-Lys		
Molecular weight[c]												
Experimental	345.2	487.3	503.4	743.5	901.5	901.8	885.8	1059.8	1300.8	1458.7	1283.6	1442.8
											1459.0	1616.8
												1699.3
Calculated	345.2	487.3	503.3	743.4	901.5	901.5	885.5	1059.6	1300.5	1458.6	ND	ND
Percent total stem peptides	15	2	7	16	14	9		7	3	4	1	3

[a] Fractions 10 and 11 were not homogeneous and experimental M_r values for the molecular weight species detected in the mass spectra are listed.

[b] ND, not determined.

[c] For M_r above 1100 the values shown are the average of the four most abundant isotopic species, because the peaks of the mass spectra were not resolved for the different isotopes.

TABLE III

Edman degradation of pneumococcal wall peptides

Peptide monomer 3 (20 nmol) and peptide dimer 7 (2 nmol) from Fig. 2 and Table II were applied to a gas-phase sequenator and subjected to 5 cycles of Edman degradation. The nmoles of phenylthiohydantoin derivatives obtained in each cycle are listed.

PTH[a]-amino acid	Cycle									
	Peptide 3					Peptide 7				
	1	2	3	4	5	1	2	3	4	5
Ala	27.2	1.7	0.0	0.0	0.0	2.3	0.7	0.0	0.0	0.0
Ser[b]	0.0	3.7	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0

[a] PTH, phenylthiohydantoin.

[b] Values given for PTH-Ser do not include PTH-dehydroserine, a prominent decomposition product present in the second sequencing cycle.

unexpectedly large number of peptide components. The method is reproducible, rapid, and quantitative and should be applicable for the analysis of other cell wall preparations as well. To our knowledge, this is the first example of a method capable of high-resolution analysis of a Gram-positive cell wall and the data obtained represent the first insight into the considerable structural complexity of the peptide network in such walls. Additional structural complexity undoubtedly exists in the length of the glycan chains and in the distribution and structure of the teichoic acid units. Another method capable of assessing at least some of these aspects of the pneumococcal cell wall has been described recently (13). Our findings indicate that in complexity of peptide structure, the pneumococcal cell wall is clearly comparable to that of the peptidoglycan in *E. coli* (7).

Several of the observations require additional comment. The enzymatic digestion used to solubilize the wall peptides seems to result in a quantitative hydrolysis of *N*-acetylmuramyl-L-alanine bonds, since none of the peptide fractions contain hexosamines and one of the amino termini in each stem peptide is Ala. Additional free amino groups are located either on the Lys side chain or on an additional Ala residue. None of the Ser residues are in an amino-terminal position and all the Glu α-carboxyls are amidated.

The combination of chemical analysis and a precise determination of molecular mass by mass spectrometry has allowed us to assign plausible molecular structures to the individual peptides. In assigning these structures we assumed that the amino acid sequence of the stem peptides is that of a typical bacterial peptidoglycan, which is always composed of alternating L and D amino acids except at the carboxyl end, where positions 4 and 5 of the peptide chain are occupied by two consecutive D-Ala residues (6). The amino acid residue at position 1 is usually L-Ala, as is the case in pneumococcus (4). Position 2 is occupied by D-Glu and position 3 by a diamino acid (Lys in pneumococcus) with its L-center bound to the γ-carboxyl of D-Glu. The α-carboxyl of D-Glu is amidated when it does not participate in cross-linking (as in type B peptidoglycans) (21).

Examination of the data in Table II shows that in all peptides except 2, all the Ala residues are accounted for in terms of amino termini and the Ala present in the cross-links connecting the stem peptides of dimers and trimers. The lack of carboxyl-terminal Ala in the peptides analyzed (except 2) is an interesting feature of the pneumococcal cell wall which may be ascribed to the presence of active D,D-, and D,L-carboxypeptidases. In fact, penicillin binding protein 3 of this bacterium has been shown to possess D,D-carboxypeptidase activity (22).

Given the data presented in Tables II and III, the structural assignments for the monomeric peptides seem unambiguous. Interestingly, the major monomeric peptide (peak 1) representing 15% of all stem peptides is a tripeptide, rather than the tetrapeptides most frequently described in other Gram-

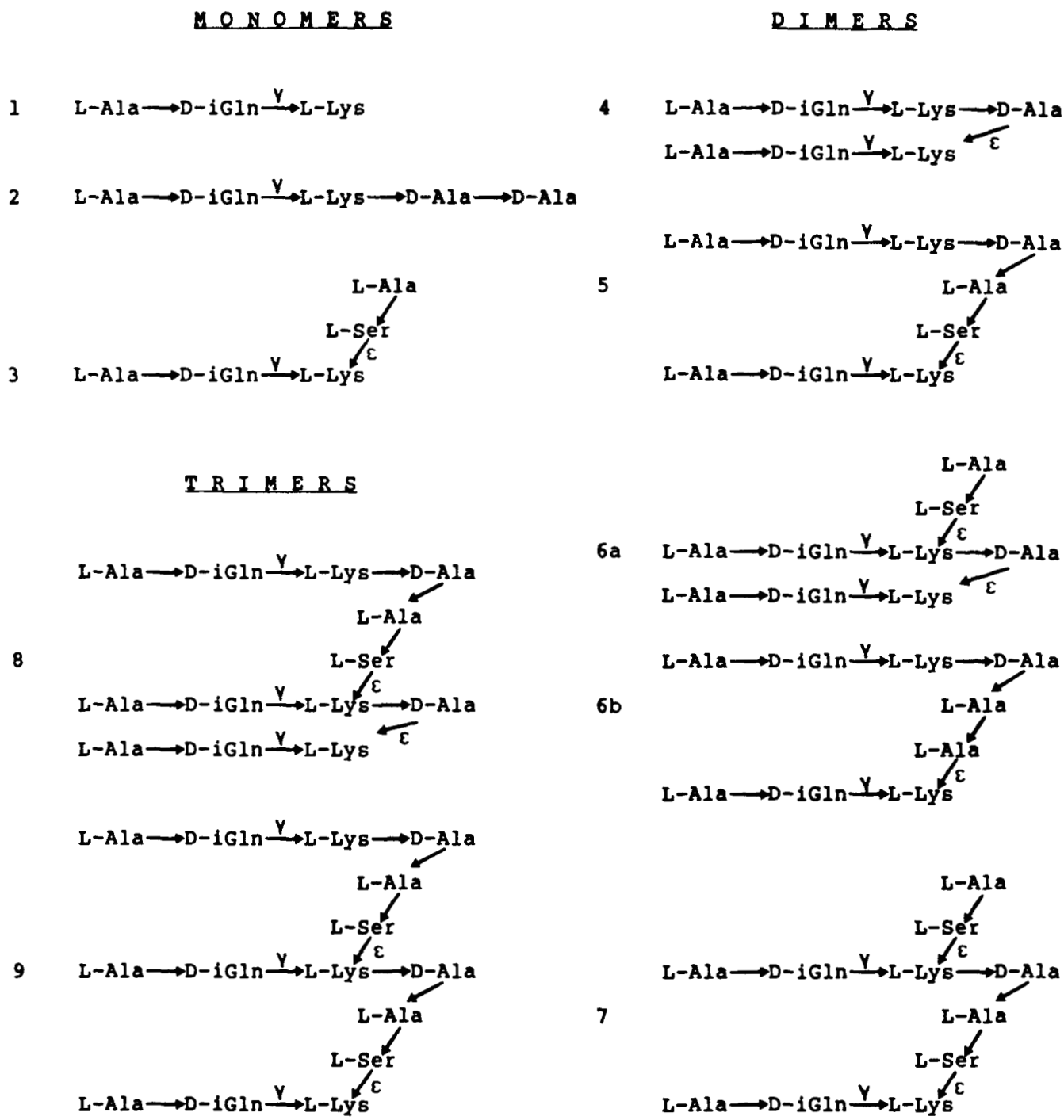

FIG. 6. **Structures of the main pneumococcal wall peptides.** The peptide structures assigned to the numbered fractions in Fig. 2 and Table II are listed. The *arrows* symbolizing the peptide bonds point from the carboxyl to the amino group. Those bonds involving the γ-carboxyl of *iGln* or the ε-amino of Lys are indicated by the corresponding greek letter. The chirality of the amino acid residues was not determined but it was assumed to be as in the other eubacterial peptidoglycans. *iGln*, isoglutamine (α-amidoglutamic acid).

positive cell walls. The second most abundant monomer (peak 3, 7% of the total) has the same basic tripeptide structure, with addition of an alanylserine side chain to the ε-amino group of the Lys residue. For the dimeric and trimeric peptides we used the monomers as guidelines for choosing the structures compatible with all the analytical data. The only real ambiguity in Fig. 6 is whether peptide 6b has a dialanyl cross-bridge (in place of an alanylserine) or contains a carboxyl-terminal alanine. We have chosen the first possibility because of the lack of carboxyl-terminal Ala in the major monomers and the interchange of Ser and Ala in other types of peptidoglycan (23).

There is a second isomeric structure for trimer 8 (Fig. 6) that also fits the analytical data, one in which the cross-bridge is located between the second and third stem peptides.

It is also relevant to point out that the two largest dimer fractions, *peaks 4* and *5*, show two different types of cross-linkages, with and without a cross-bridge, coexisting in the same bacterial strain. Thus, in terms of cross-linking mode, the pneumococcal peptidoglycan may be alternatively as-

signed to groups A1α or A3α (23), depending on which dimer one chooses as representative.

The analytical technique described here should allow detection of structural alterations in the pneumococcal cell wall under interesting physiological conditions and in mutants with altered wall degradative or wall synthetic enzymes. In addition, the method used to determine the status of the glutamic acid carboxyl groups should be useful for the routine characterization of other bacterial cell walls as well, and it should allow a rapid classification of the peptidoglycan as type A or type B (23).

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Supplementary Material to

Structure of the Peptide Network of Pneumococcal Peptidoglycan

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Experimental Procedures

Pneumococcal strains and growth conditions. Strain R6St is a streptomycin-resistant transformant of the pneumococcal laboratory strain R6 (12). Bacteria were grown at 37°C in a chemically defined medium, Cden (A. Tomasz, Bacteriol. Proc., p. 29, 1964), modified by reducing the concentration of lysine to 10 µg/ml. To introduce a radioactive label into the cell walls, L-[4,5-^{3}H(N)]lysine (New England Nuclear, Boston, Mass.) was added to the growth medium (5 µCi and 10 µg/ml final concentration). The cultures were allowed to grow until the middle of the exponential growth phase ($O.D._{620}$ between 0.3 and 0.4); they were then rapidly chilled in an ice-ethanol bath and the cells were harvested by centrifugation at 4°C.

Amidase digestion of cell walls. Pneumococcal cell walls (5 to 50 mg, approximately 0.4 µmoles Lys/mg) were purified as described (13), except that trypsin was used to deproteinize them in place of proteinase K, which was found to decrease the yield of oligomeric peptides. The walls that had been labeled by growth in [^{3}H]lysine-containing medium (final specific activity between 500 and 1,000 cpm/nmole) were suspended at concentrations between 5 and 20 mg/ml in 50 mM HEPES buffer (pH 7.0) containing 0.1% Brij-35 and 0.05% NaN_3 and digested with 20 U/mg of the amidase (170 U/mg protein), purified as previously described (13). One unit (U) of amidase activity was defined as the amount of enzyme capable of solubilizing 1 µg of pneumococcal cell walls in 10 min at 37°C. The digestion was allowed to proceed at 37°C for 18 h under constant stirring and the mixture was then centrifuged at 25,000 x g for 15 min. The supernatant was carefully aspirated, the pellet was washed once with water and the combined supernatants were counted to determine the degree of solubilization of the lysine label.

The soluble material was passed through a column packed with the octadecyl silica extracted from several SepPak C-18 microcolumns (Waters Associates, Milford, Mass.) (2 g silica/ml sample), equilibrated and eluted with a mobile phase composed of 15% acetonitrile, 1% trifluoroacetic acid and 1% N-methylmorpholine. The column retained most of the detergent present in the digestion mixture and allowed the recovery of 97% of the labeled sample in the flow through fraction. Removal of the detergent was important for the subsequent chromatographic steps.

Size fractionation of the amidase digest. The largely detergent-free digestion mixture was reduced in volume to between 0.5 and 1 ml under reduced pressure, filtered through a 0.45 µm-pore size Millex HV filter (Millipore Corp., Bedford, Mass.) and loaded on the gel filtration system. This system was composed of two 1.6 by 90 cm columns connected in series, the first packed with Ultrogel AcA 202 and the second with Trisacryl GF05 (LKB Instruments Inc., Paramus, N.J.), equilibrated with 1 N acetic acid and eluted with the same solvent at a flow rate of 6 ml/h. The void and total volumes of the coupled column system were approximately 116 and 386 ml respectively.

Fractions of 2 to 3 ml were collected. 100 µl samples from each fraction were taken for scintillation counting and 50 µl samples for free amino group detection, using a fluorescamine-based published procedure with minor modifications (14).

Analytical HPLC. The HPLC system consisted of two 510 pumps, a 721 system controller, a U6K injector and a 730 data module (all from Waters), and an ISCO V4 detector equipped with a heat exchanger flow cell of 6 mm path length and 3.5 µl illuminated volume (ISCO, Lincoln, Ne.).

The chromatographic columns used were ODS-Hypersil (4.6 x 250 mm) with a 120 Å pore size, 5 µm in diameter spherical packing (Shandon Southern Instruments Inc., Sewickley, Penn.); an Aquapore octyl, RP-300 (4.6 x 220 mm) with a 300 Å pore size, 10 µm in diameter spherical packing (Pierce Chemical Co., Rockford, Ill.) and a Vydac 218TP54 (4.6 x 250 mm) with a 300 Å pore size, 5 µm in diameter spheroidal packing (The Separations Group, Hesperia, Cal.).

For the analytical chromatographies the samples were applied to the Vydac column and eluted from it using a linear 0 to 15% acetonitrile gradient in 0.1% trifluoroacetic acid, at a flow rate of 0.5 ml/min during 100 min. The eluted fractions were detected by their UV absorbance at 210 nm. At this wavelength the amide bonds are the main chromophores.

Semi-preparative peptide purification by HPLC. Peptide monomers were purified in a semi-preparative scale on the Hypersyl column. Up to 40 nmoles of peptides were applied and eluted using a gradient from 0 to 8% acetonitrile in 0.1% trifluoroacetic acid as the eluent, at a flow rate of 1 ml/min during 15 min. This column is more retentive than the one used to obtain the chromatogram illustrated in Fig. 2 and allowed a better separation of peptide # 1 from the salt peaks.

Peptide-dimers were pre-fractionated first in the Aquapore column because it showed a larger sample capacity. A maximum of 400 nmoles were loaded and eluted with a gradient from 0 to 8% acetonitrile in 0.1% trifluoroacetic acid at 0.5 ml/min for 1 h. The major fractions were purified 30 nmoles at a time in a second step, using the Vydac column and the same gradient slope.

The trimers were also purified using the two-column strategy, but the samples in the Vydac column were eluted at 1 ml/min with a linear gradient from 4 to 8% acetonitrile in 0.1% N-methylmorpholine, adjusted to pH 6 with trifluoroacetic acid.

Amino group analysis. About 20 nmoles (in terms of stem peptides) of each purified cell wall peptide were dansylated as described (15). The dansylation mixture was fractionated using the Aquapore column and the radiolabeled fractions were collected. Elution was performed with linear gradients of acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min with a slope of 1% per min. Approximately 5 nmoles of the purified dansyl peptides were hydrolyzed with 6 N HCl at 110°C for 4 h and the hydrolysis products were analyzed on polyamide thin layer plates as described (16). This procedure allowed the identification of the amino acid residues with free amino groups. To establish the total number of dansylated groups per peptide molecule, approximately 2 nmoles of dansylated peptides were analyzed by mass spectrometry as described below. The difference in mass between the native and dansylated form of a given peptide established the number of dansyl residues attached to each molecule.

Amide group analysis. The amino acid residues with free amide groups were identified by their reactivity towards the reagent bis-(I,I-trifluoroacetoxy)iodobenzene, using a published procedure (17) with the following modifications. Samples containing 10 nmoles of stem peptides were dissolved in 25 µl of 0.1% trifluoroacetic acid and mixed with 25 µl of 10 mg/ml of the reagent in acetonitrile. The mixture was incubated in the dark at 60°C for 4h and it was then dried and hydrolyzed with 200 µl of 6 N HCl at 110°C for 18 h. Amino acid analysis of the hydrolysate showed the loss of the amino acid residues that carried free amide groups. The commercially available pentapeptides L-Ala-D-iGln-L-Lys-D-Ala-D-Ala and L-Ala-D-Glu-L-Lys-D-Ala-D-Ala were used as positive and negative controls respectively. In contrast to the experience with peptides derived from protein material (17), we found that in the case of cell wall peptides and the pentapeptide standards a variable proportion of the amino terminal alanine was also lost.

Time-of-flight mass spectrometry. From 2 to 8 nmoles of native and dansylated peptides in 0.1% trifluoroacetic acid were applied to a thin layer of nitrocellulose, prepared by electrospraying 50 µg of nitrocellulose (1 µg/ul in acetone) onto an aluminized polyester support with 1 cm^2 of surface area (18). The sample was thoroughly dried on the nitrocellulose layer under reduced pressure and its mass spectrum was obtained using a ^{252}Cf fission fragment ionization time-of-flight mass spectrometer constructed at The Rockefeller University (19,20). The molecular weights of the samples were determined with an accuracy of better than 300 ppm, using the mass spectra that exhibited peaks corresponding to the protonated and sodium cationized intact peptide ion species.

Amino acid and amino sugar analyses. Samples (5 to 20 nmoles) were hydrolyzed in evacuated and sealed glass tubes. For amino acid and galactosamine determinations hydrolyses were performed in 6 N HCl for 18 h. For other amino sugars, 4 N HCl and 4 h of hydrolysis were used. Control hydrolyses using amino sugar standards showed that decomposition was negligible under these conditions, but recoveries of galactosamine from cell walls was higher in the first set of hydrolyses. A Durrum D-500 autoanalyzer was used for quantitation in all cases.

Peptide sequencing. Approximately 20 nmoles of the HPLC peak # 3 and 2 nmoles of peak # 7 (Fig. 2) were sequenced using the manufacturer's program in a model 470 A gas-phase sequencer (Applied Biosystems, Inc., Foster City, Cal.). The phenylthiohydantoin amino acids were identified by on-line HPLC.

Chemicals. HPLC solvents were purchased from Burdick & Jackson (Muskegon, Mich.). N-methylmorpholine, trifluoroacetic acid and 6 N HCl were sequanal grade from Pierce Chemical Co. (Rockford, Ill.). Dansyl chloride was obtained from Eastman Kodak Chemical Co. (Rochester, N.Y.) and the peptidoglycan pentapeptides used as standards from Sigma Chemical Co. (St. Louis, Mo.). Acetic acid "gold label grade" was from Aldrich Chemical Co. (Milwaukee, Wi.) and the fluorescamine (Fluram) from Roche Diagnostics (Nutley, N.J.). All other reagents were commercially available analytical grade products.