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Altered Peptidoglycan Structure in a Pneumococcal Transformant Resistant to Penicillin

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A series of isogenic pneumococcal transformants differing in their levels of penicillin resistance and containing altered penicillin-binding proteins were compared for their cell wall structures by using a recently developed technique that can resolve the peptidoglycan stem peptides of *Pneumococcus* strains to over 40 components (J. F. Garcia-Bustos, B. T. Chait, and A. Tomasz, J. Biol. Chem. 32:15400–15405). The stem peptides from the highly resistant transformants differed strikingly from those of the susceptible recipient strain, and the peptide patterns were almost identical to that of the DNA donor. Four peptides representing the major components in the walls of susceptible cells were replaced by six new peptides that were only minor components of susceptible cell walls. A remarkable common feature of these new species was their high alanine content. Amino acid analysis, sequencing, and mass spectrometry allowed the assignment of the extra alanine residues to dialanine or alanylserine cross bridges in the six new stem peptides. The common feature of the four peptide species that were present as major components in the susceptible walls, but became minor species in the resistant cells, was the absence of a cross bridge in at least one of the stem peptide components. We suggest that the extensive remodelling of cell wall synthetic enzymes that accompanies acquisition of penicillin resistance eventually also alters the reactivity of these proteins towards their natural substrates in cell wall synthesis. As a result, highly penicillin-resistant pneumococci will shift from the use of wall precursors with linear stem peptides to a preferential use of precursors containing the more-hydrophobic peptides carrying dialanyl or alanylserine cross bridges.

Bacterial peptidoglycan is synthesized, at least in part, by penicillin-sensitive enzymes recognized as penicillin-binding proteins (PBPs) (7). Clinical isolates intrinsically resistant to beta-lactam antibiotics show altered PBPs with decreased affinity for penicillin when compared with susceptible strains (for a review, see reference 6). This class of antibiotics is thought to be an active-site inhibitor (7), with close structural analogy to the carboxy-terminal D-alanyl-D-alanine residue of the natural substrate in wall synthesis. It is conceptually difficult to envision how bacteria can modify their wall-making enzymes in order to elude the inhibitory action of the substrate analog (antibiotic) but still retain normal reactivity for the natural substrate.

It is possible to genetically transform a susceptible pneumococcal strain to increasing levels of penicillin resistance by using DNA from a highly resistant clinical isolate (8). The acquisition of resistance determinants in the transformants is accompanied by an altered PBP pattern resembling that of the DNA donor (3, 8). The purpose of the present work was to investigate if these changes in PBP size and affinity for penicillin may not also affect the normal activity of these enzymes in vivo. This question was approached by analyzing the composition of the peptide network of pneumococcal peptidoglycan in a series of isogenic transformants with different levels of resistance to penicillin and different PBP patterns. The results indicate that the structure of the peptidoglycan portion of the cell wall in the transformant with the highest resistance level underwent a major change, compared with the pattern typical of a susceptible pneumococcus. The new, altered peptide pattern was very similar to that of the penicillin-resistant DNA donor cells.

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MATERIALS AND METHODS

Strains and growth conditions. The penicillin-sensitive (for which the MIC is 6 ng/ml), unencapsulated pneumococcal strain R6 was used as the DNA recipient in the transformation experiments as previously described (8). The DNA donor was strain 8249, a type 19A encapsulated, penicillin-resistant (for which the MIC is 6 μg/ml) clinical isolate obtained from H. Koornhof (Johannesburg, South Africa). Strains Pen 0.05, Pen 1.6, and Pen 6.0 are the results of the stepwise transformation of R6 to low-, intermediate-, and high-level penicillin resistances, respectively (3, 8). The transformants did not show evidence of a capsule.

Bacteria were grown in a casein-based semisynthetic medium (C+Y) (5) at 37°C without aeration to the mid-logarithmic phase of growth (optical density at 620 nm, between 0.3 and 0.4), after which they were rapidly chilled in a mixture of ice and ethanol and harvested by centrifugation at 4°C (5,000 × *g*, 15 min). To introduce a radioactive label into the cell wall, bacteria were grown for 2 h in a chemically defined medium (Cden [A. Tomasz, Bacteriol. Proc., p. 29, 1964]) containing L-[4,5-^{3}H(N)]lysine (New England Nuclear Corp., Boston, Mass.) at a final concentration of 5 μCi and 10 μg/ml.

Cell wall preparation. Cell walls from exponentially growing cultures were purified and digested with muramidase M1 (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) or the pneumococcal amidase as previously described (2), except that a 50 mM N-methylmorpholine–acetate buffer (pH 7.0) with 0.1% Brij-35 was used with the latter enzyme. Detergent was used to improve the stability of the enzyme.

For the high-performance liquid chromatography (HPLC) analyses, the products of amidase digestion were dried, and the residual buffer was extracted with acetone. The precipitate was dried, and the peptides were extracted with acetonitrile-isopropanol-water (25:25:50) containing 0.1% trifluo-

TABLE 1. Characterization of pneumococcal strains used

Strain[a]	Penicillin MIC (μg/ml)	Doubling time (min)	% Teichoic acid substitution[b]	Amino acid composition (mol/mol of Glx) in cell wall			
				Ala	Glx	Lys	Ser
R6	0.006	31	47	2.0	1.0	0.9	0.6
Pen 0.05	0.05	32	45	1.9	1.0	0.8	0.3
Pen 1.6	1.6	34	46	1.7	1.0	0.7	0.4
Pen 6.0	6.0	31	44	2.7	1.0	0.8	0.5
8249	6.0	26	36	2.3	1.0	0.9	0.5

[a] The strains used were the DNA donor (8249), the DNA recipient (R6), and the penicillin-resistant transformants obtained as described earlier (3, 8).
[b] Percentage of wall lysine residues covalently attached to teichoic acid chains after muramidase digestion (see Materials and Methods).

roacetic acid (TFA). Of the lysine-labeled material, 73 to 96% was extracted. The solvents were removed by evaporation, and the peptides were redissolved in 0.1% TFA for analysis.

Isolation of teichoic acid-containing muropeptides. The muramidase-digested cell walls containing about 1,000 cpm/nmol of [^{3}H]lysine-labeled material as a tracer were fractionated on a myeloma affinity column as described earlier (2). The myeloma protein used, TEPC-15, specifically recognizes the phosphocholine determinants present in pneumococcal teichoic acid. The ratio of label bound to the column to the total label recovered was taken as the degree of teichoic acid substitution of the cell wall (2).

HPLC. The chromatographic system was made up of a 721 system controller, two 510 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 used was a Vydac 218TP54 (The Separations Group, Hesperia, Calif.), and the eluting solvent was a 100-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. The eluted fractions were detected by their A_{210}, at which wavelength the amide bonds are the main chromophores.

Mass spectrometry. The peptides (2 to 8 nmol) were applied in 0.1% TFA to a thin layer of nitrocellulose electrosprayed onto an aluminized polyester support. The sample was thoroughly dried on the nitrocellulose under low pressure, and its mass spectrum was obtained by using a ^{252}Cf fission fragment ionization, time-of-flight mass spectrometer as previously described (1).

Amino acid analysis. Samples were analyzed by either of two methods. Large samples (5 to 20 nmol) were hydrolyzed with 200 μl of 6 N HCl at 110°C for 18 h and analyzed in an autoanalyzer (Durrum D-500; Dionex Corp., Sunnyvale, Calif.). Small samples (1 to 3 nmol) were hydrolyzed with 6 N HCl in the gas phase at 110°C for 22 h, and they were analyzed after derivatization with phenylisothiocyanate by an HPLC amino acid analysis system (PICO TAG; Waters).

Peptide sequencing. Approximately 1.5 nmol of each sample was used to obtain the sequence of the part of the stem peptide amenable to Edman degradation. The procedure was carried out by using a gas-phase sequencer (470A; Applied Biosystems, Inc., Foster City, Calif.) and the manufacturer programs. The phenylthiohydantoin amino acids were identified by on-line HPLC.

RESULTS

Composition of cell walls of pneumococcal strains with different levels of penicillin resistance. Cell walls from five isogenic strains of pneumococci with low, intermediate, and high levels of resistance to penicillin were analyzed. The penicillin MICs, doubling times, and analytical results are summarized in Table 1. There were no detectable quantitative differences between the penicillin-susceptible recipient and the isogenic, highly resistant transformant in the degree of teichoic acid substitution. The most striking difference between the overall cell wall compositions was the high alanine content of the cell walls prepared from the highly resistant transformant Pen 6.

HPLC analysis of wall peptide patterns in pneumococcal strains with different levels of penicillin resistance. Cell wall preparations were hydrolyzed by pneumococcal *N*-acetylmuramoyl-L-alanine amidase (to be referred to from here on as an amidase), and the resultant mixture of soluble peptides was analyzed by a recently developed high-resolution technique on HPLC (1; Fig. 1).

A comparison of the HPLC patterns reveals three observations: (i) the peptide patterns of the penicillin-susceptible recipient strain R6 and the penicillin-resistant DNA donor strain 8249 were strikingly different; (ii) the highly resistant genetic transformant (Pen 6), for which the high MIC is the same as that for DNA donor 8249, had a peptide pattern very similar to that of strain 8249; and (iii) the different peptide patterns of the walls of resistant strains represented alterations in the amounts of existing peptides rather than the appearance of new peptides.

Table 2 provides a quantitative comparison of the data from Fig. 1, expressing the distribution of the 13 major peptide fractions resolved by the HPLC method as a percentage of the total stem peptides. Peaks 1 to 9 represent major peptide components of the walls from penicillin-susceptible cells, the chemical structures of which have been determined recently (1). Peaks I to VI represent peptides that together composed a major fraction of the wall stem peptides in the resistant bacteria Pen 6 and 8249 but which were only present as minor peptide species in the other strains. Peptides 1, 4, 5, and 6, representing 15, 16, 13, and 9%, respectively, of the total peptides in strain R6, were each decreased dramatically in the wall of strain Pen 6 (from 15 to 2%, in the case of peptide 1, and to less than 1%, in the cases of peptides 4, 5, and 6). On the other hand, peptide 3 increased from 6% in R6 to 11% in Pen 6. Peptides I through VI, each present as less than 1% of total peptide material in the wall of strain R6, became major peptide components of the walls of strain Pen 6, together making up up to 37% of the total peptides. No significant change was observed in the amount of peptide 7. Some of the quantitative changes (e.g., the increase in peptides 3 and I with increasing resistance level) appeared to be gradual, while others seemed to occur abruptly, as the resistance level increased from a penicillin MIC of 1.6 to 6.0 μg/ml.

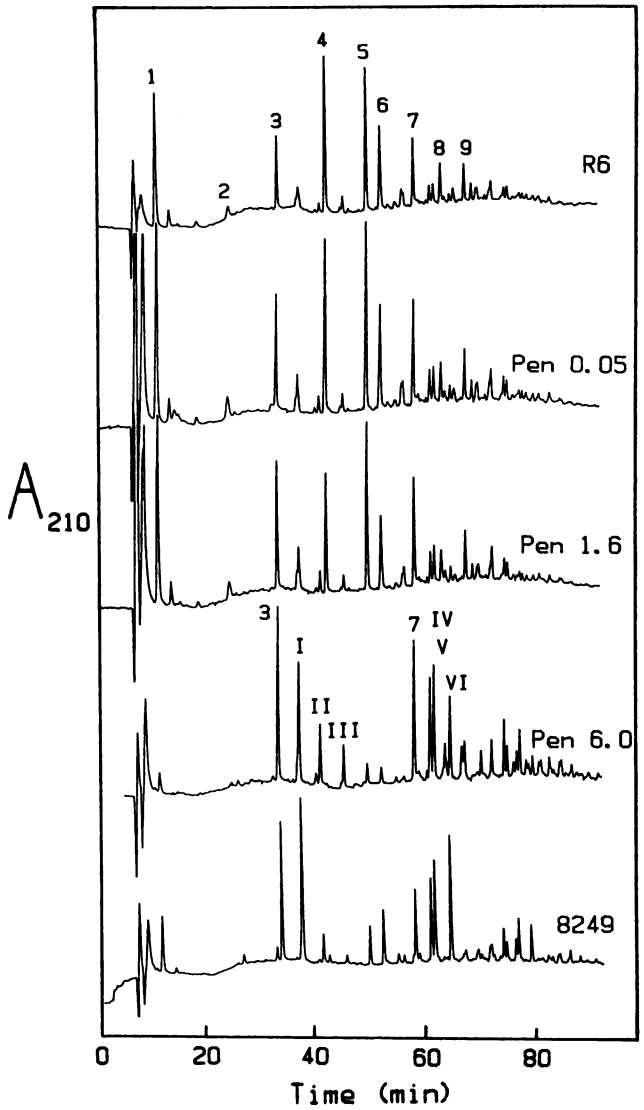

FIG. 1. HPLC chromatograms of cell wall stem peptides from the pneumococcal strains listed in Table 1. Approximately 15 nmol of stem peptides from strain R6 and 20 nmol from the other strains were applied to the column and eluted as described in the text. The individual strains are indicated at the end of the corresponding chromatograms. Peaks 1 to 9 represent peptides whose structures have been previously described (1); peaks I to VI are characteristic of the highly resistant strains and are described in the text. These peptide patterns were completely reproducible, and the same results were obtained with two independent cell wall preparations from strain Pen 6.0 (over 10 analyses) and five independent preparations from strain R6 (over 15 analyses).

Amino acid composition and structure of the major peptide components of the cell walls of strain Pen 6. Fractions I through VI from HPLC analysis were isolated and analyzed for amino acid composition. Molecular masses were determined by time-of-flight mass spectrometry to a precision of better than 0.5 mass unit, placing severe constraints on the number of possible structures. The results confirmed that one of the carboxyl groups in each stem peptide was amidated (1-mass-unit difference). The amides were determined to be in the Glu residues in strain R6 (1). Table 3 summarizes these results as well as the corresponding analytical data for peptides 3 and 7, two peptides present in substantial amounts both in penicillin-susceptible R6 and in highly penicillin-resistant Pen 6 strains. The sequences of the peptide cross bridges containing a free amino terminus were identified by gas-phase Edman degradation (Table 4). Molecular structures proposed for peptides I through VI are shown in Fig. 2.

TABLE 2. Area percent of peptide fractions that show the greatest variations between strains[a]

Fraction	Area % of fraction in strain				
	R6	Pen 0.05	Pen 1.6	Pen 6.0	8249
Monomers					
1	15	19	18	2	5
3	6	7	9	11	11
I	—	—	—	10	15
II	—	—	—	4	2
4	16	14	11	—	—
III	—	—	—	4	—
Dimers					
5	13	14	14	—	—
6	9	9	7	—	—
IV	—	—	—	6	7
V	—	—	—	7	9
VI	—	—	—	6	11

[a] The areas under the peaks were obtained from chromatograms like those shown in Fig. 1. —, Component present in too small a quantity to allow unambiguous identification and integration. Fraction nomenclature is as described in the legend to Fig. 1.

DISCUSSION

Penicillin resistance in clinical isolates of pneumococci is related to mutational alterations in the PBPs that result in decreased reactivity of these proteins with the antibiotic (4). Highly penicillin-resistant pneumococci have a PBP pattern strikingly different from that of susceptible cells. During genetic transformation of penicillin resistance with DNA isolated from such highly resistant cells, the PBP pattern and penicillin reactivity of transformants undergo gradual shifts in the direction of the properties of the PBPs of the DNA donor until, at a certain level of penicillin resistance, the PBP pattern of the transformants becomes indistinguishable from that of the DNA donor cells (8).

In the studies described in this communication, we examined the structure of the peptidoglycan of the same set of

TABLE 3. Analysis of the main peptide fraction in the peptidoglycan of strain Pen 6.0

Fraction[a]	Amino acid composition (mol/mol of Glx)				Mol wt	% of total stem peptides[b]
	Ala	Glx	Lys	Ser		
Monomers						
3	2.1	1.0	0.9	0.9	503.4	11
I	2.9	1.0	1.0	0.2	487.4	10
II	4.7	1.0	1.1	0.6	645.6	4
III	4.5	1.0	1.0	0.0	629.4	4
Dimers						
7	2.6	1.0	1.0	0.8	1,060.2	8
IV	3.0	1.0	1.1	0.6	1,044.3	6
V	3.0	1.0	1.0	0.5	1,044.3	7
VI	3.5	1.0	0.9	0.2	1,028.1	6

[a] Fraction nomenclature is described in the legend to Fig. 1.
[b] Estimated by integrating the areas of the corresponding peaks in the chromatograms shown in Fig. 1.

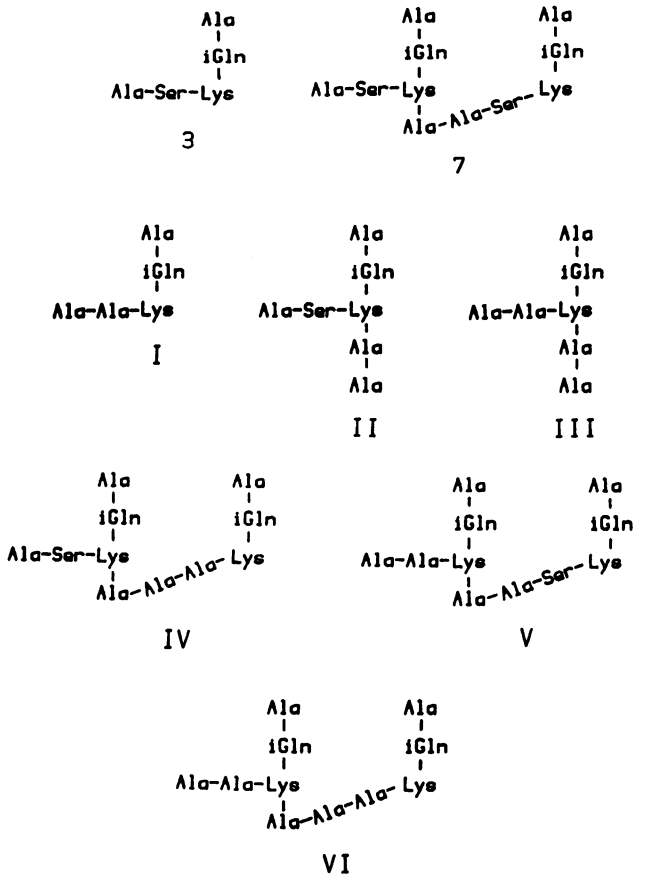

FIG. 2. Proposed structures for the main wall peptides of strain Pen 6.0. Peptides 3 and 7 have already been described for strain R6 (1); peptides I to VI have novel structures. Certain assumptions have been made in the assignment of these structures (see the text for a detailed discussion).

isogenic penicillin-resistant transformants. The major finding we report here is that the cell wall stem peptides of penicillin-susceptible and highly penicillin-resistant cells were completely different; and the wall peptide pattern of the transformant strain with the highest (donor level) penicillin resistance became practically identical to the pattern found in the DNA donor strain. This finding represents the first clear biochemical documentation of a genetic transfer of an altered peptidoglycan structure.

The isolation and chemical analysis of the new peptides that became major components of the walls of the highly resistant cells revealed that the peptides all contained greatly increased proportions of alanine. The nearly one extra mole of alanine observable even by a gross compositional analysis of the resistant cell walls was recovered from peptides I to VI. The structural assignments for the peptide dimers (Fig. 2) are tentative and require comment. The structures (Fig. 2) have been chosen to agree with all the analytical data obtained. In cases for which more than one structure was theoretically possible, the structural formula most closely related to the structures recently identified in strain R6 were chosen (1). More-precise structural assignments for the dimeric peptides will require additional analytical data.

A comparison of the structures of the peptide components that decreased with those that increased in the walls of resistant cells suggests that the peptides with decreased representation were primarily monomers without side chains or peptide dimers in which one of the stem peptides had no dipeptide cross bridge. All the peptides which were increased in the resistant walls had extra alanine residues which, according to the sequencing data (Table 4), were in the cross bridges. Comparable sequencing data could not be obtained for peptide VI. It is interesting to note that the presence of two types of cross-links (with or without cross bridges) characteristic of the penicillin-susceptible pneumococcal strains (1) was changed in the highly resistant bacterium. This highly resistant strain primarily contained the cross bridges typical of the A3 alpha peptidoglycan. A general consequence of the increased alanine content in the major peptides of the penicillin-resistant cell wall was an increase in the general hydrophobicity of the peptide network, as seen by the shift in retention times on the reverse-phase HPLC column (Fig. 1).

In the cell wall of the penicillin-susceptible cells, peptides 1, 4, 5, and 6 make up 53% of the total peptide material. The same peptides represent less than 6% of the peptide material in the resistant bacterium. The loss of those peptides from the wall of resistant cells appeared to be compensated for by the great increase in the representation of peptides I to VI, which together make up at least 37% of the wall stem peptides in the penicillin-resistant pneumococci (Table 2). While some of the structural shifts occurred gradually and could already be seen in the two transformants of intermediate resistance level (e.g., the increase in peptides 3 and I or the decline in peptide 4; Fig. 1), the most striking structural changes occurred abruptly and only in the high-resistance-level transformant (Pen 6.0). Interestingly, the PBP pattern of the transformant for which MIC was 1.6 μg/ml was already identical to that of the DNA donor strain 8249 (for which MIC is 6 μg/ml) (8), and yet, the cell wall peptide pattern of the former resembled more that of the penicillin-susceptible strain. The dramatic shift in peptide patterns accompanied the final increase in MIC from 1.6 to 6 μg/ml. Whether concomitant changes in the affinity of PBPs for penicillin also occur is not known at present. At high resistance levels, some alteration in the polypeptide structure of one or more of the PBPs may change the microenvironment within some relevant domain of these proteins, such that the domain now favors the utilization of substrates with the more-hydrophobic (alanine-rich) stem peptides. The

TABLE 4. Edman degradation sequencing of the main peptide fractions in the peptidoglycan of strain Pen 6.0

Fraction[a]	Phenylthiohydantoin amino acid (amt in pmol) from sequencing cycle[b]:	
	1	2
Monomers		
3	Ala (975)	Ser (18) + dSer
I	Ala (1,632)	Ala (810)
II	Ala (3,316)	Ser (167) + dSer
III	Ala (1,755)	Ala (763)
Dimers		
7	Ala (1,662)	Ser (89) + dSer
IV	Ala (4,474)	Ser (196) + dSer
V	Ala (2,733)	Ala (826)
VI	Ala (1,228)	NI

[a] The fraction nomenclature is as described in the legend to Fig. 1.

[b] Five sequencing cycles were performed, but only the first two yielded significant amounts of phenylthiohydantoin amino acids. Most of the Ser residues decomposed to dehydroserine (dSer), which was not quantified. NI, Not identifiable.

abundance of such peptides in the cell wall of highly resistant pneumococci suggests that the successful expression of resistance at this level may also require the acquisition of a mutation(s) which assures adequate supplies of cross bridge-bearing stem peptides, for instance, through derepression of some biosynthetic step. Thus, this particular set of strains may have the novel feature that high-level penicillin resistance requires not only an alteration of penicillin-sensitive enzymes, but a change in some step(s) in cell wall precursor synthesis as well.

Both of the major types of penicillin-sensitive enzymes, D,D-carboxypeptidases and transpeptidases, remained active in the resistant cells, as evidenced by a stable degree of cross-linking in all the cell walls, even though the chemical nature of the cross-links changed. It is remarkable that the altered peptidoglycan structure of the highly penicillin-resistant strains appeared to be fully compatible with the normal physiological function of the cells; there was no observable decline in growth rate, and the degree of teichoic acid substitution appeared normal in the walls of resistant cells. In the DNA donor strain, the altered wall structure also allowed a stable retention of the polysaccharide capsule.

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