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Separation of Abnormal Cell Wall Composition from Penicillin Resistance through Genetic Transformation of *Streptococcus pneumoniae*

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Compared with most penicillin-susceptible isolates of *Streptococcus pneumoniae*, penicillin-resistant clinical isolate Hun 663 contains mosaic penicillin-binding protein (PBP) genes encoding PBPs with reduced penicillin affinities, anomalous molecular sizes, and also cell walls of unusual chemical composition. Chromosomal DNA prepared from Hun 663 was used to transform susceptible recipient cells to donor level penicillin resistance, and a resistant transformant was used next as the source of DNA in the construction of a second round of penicillin-resistant transformants. The greatly reduced penicillin affinity of the high-molecular-weight PBPs was retained in all transformants through both genetic crosses. On the other hand, PBP pattern and abnormal cell wall composition, both of which are stable, clone-specific properties of strain Hun 663, were changed: individual transformants showed a variety of new, abnormal PBP patterns. Furthermore, while the composition of cell walls resembled that of the DNA donor in the first-round transformants, it became virtually identical to that of susceptible pneumococci in the second-round transformants. The findings indicate that genetic elements encoding the low affinity of PBPs and the penicillin resistance of the bacteria are separable from determinants that are responsible for the abnormal cell wall composition that often accompanies penicillin resistance in clinical strains of pneumococci.

All of the penicillin-resistant *Streptococcus pneumoniae* strains examined so far contained mosaic penicillin-binding protein (PBP) genes with highly divergent DNA sequences (1) encoding PBPs with reduced antibiotic-binding capacity (5, 6, 15). Most interestingly, many clinical isolates of resistant pneumococci also showed grossly altered chemical composition of their cell wall peptidoglycan (4): several muropeptides with a linear stem peptide structure that were major components of susceptible cell walls were replaced by branched peptides that were either minor elements or not detectable in the cell walls of susceptible bacteria. The physiological activity of PBPs is believed to be in the catalysis of terminal stages in peptidoglycan assembly, and it has been suggested that alterations in the chemical structure of peptidoglycan of resistant cells may be related to the reduced or abnormal reactivity of these low-affinity proteins with their natural substrates (3). Experimental support for this proposal came from two sources. Parallel changes in PBP affinities and wall structure have been observed in penicillin-resistant step mutants isolated in the laboratory under conditions that excluded the potential source of exogenous DNA (13). A second type of support came from the finding that during genetic transformation of penicillin resistance from a highly resistant South African strain, resistant transformants also acquired several of the abnormal features of the peptidoglycan of the DNA donor (4). In these experiments, high-level resistance was built up gradually by several consecutive steps of genetic transformation in which transformants with low and intermediate resistance levels served as recipients of the same donor DNA from the clinical strain. A shift in the direction of the abnormal peptidoglycan structure of the DNA donor has begun to occur in transformants with a penicillin MIC of 1 μg/ml or higher. While these experiments were consistent with a common mechanism for resistance and cell wall abnormality, such an association could also be the consequence of the independent uptake of separate resistance-related genes and wall structure-related genes during the multiple exposures of recipients to DNA from donor cells. A recent examination of the peptidoglycan of a large number of penicillin-resistant isolates by a high-resolution analytical technique showed that deviation from the species-specific peptidoglycan composition was related not to the level of antibiotic resistance but rather to the genetic background of the particular resistant clone (12). For these reasons, it was important to reexamine the relationship between penicillin resistance and peptidoglycan structure in genetic transformants. As a DNA donor we chose a multidrug-resistant clinical isolate of the "Hungarian" clone of penicillin-resistant *S. pneumoniae* (10), which also possesses a most unusual cell wall composition (12).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Penicillin-sensitive (MIC, 0.01 μg/ml), unencapsulated pneumococcal strain R6 (Hex^-) was used as the recipient in transformation experiments. The DNA donor was strain Hun 663, a serotype 19A, penicillin-resistant (MIC, 1.6 μg/ml) clinical isolate that is also resistant to tetracycline (MIC, 100 μg/ml), erythromycin (MIC, >8 μg/ml), sulfamethoxazole-trimethoprim (MICs, 100 and 20 μg/ml, respectively), and chloramphenicol (MIC, 12 μg/ml).

Bacterial cultures were grown in a casein-based semisynthetic medium (C+Y) at pH 8.0 supplemented with yeast extract (1 mg/ml of growth medium) (8). The MICs of penicillin and other antibiotics were determined by agar dilution as recommended by the National Committee for Clinical Laboratory Standards (11).

Genetic transformation. DNA was isolated by a previously described method (15). Competent, penicillin-susceptible recipient R6 (Hex^-) cells (4×10^7 viable

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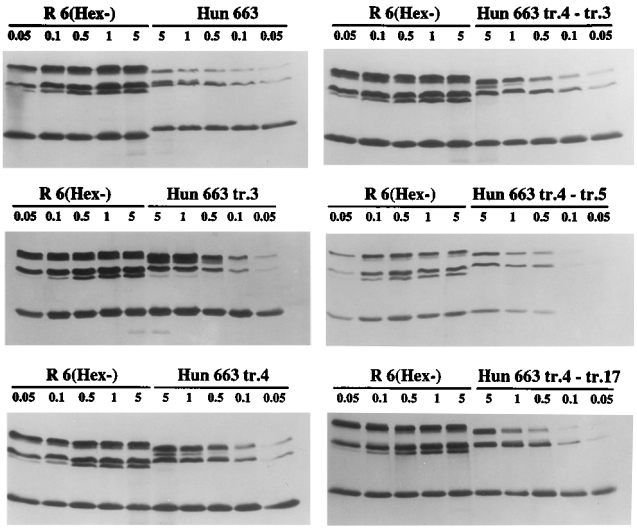

FIG. 1. PBP patterns of penicillin-resistant transformants. Penicillin-resistant clinical strain Hun 663, penicillin-susceptible strain R6 (Hex$^-$), and genetic transformants selected for donor level penicillin resistance were grown, and their PBPs were assayed for penicillin-binding capacity as described in Materials and Methods. The values above the lanes are the concentrations of [^{3}H]penicillin (in nanograms per milliliter) used for labeling.

U/ml) were treated with DNA (0.85 μg/ml) at 30°C for 15 min. After this, 2 ml of C+Y containing albumin (0.8 mg/ml) and pancreatic DNase (1 μg/ml) was added and the suspension was incubated at 37°C for 90 min. Fifty-microliter portions of the suspension were plated on blood agar plates containing the following concentrations (in micrograms per milliliter) of benzylpenicillin (Marsam Pharmaceuticals, Inc., Cherry Hill, N.J.): 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 2.0, and 2.4. The frequency of penicillin-resistant transformants was 10^{-5}. Single transformant colonies picked from the penicillin-containing agar plates were inoculated into nonselective medium, grown to cultures, and used for determination of penicillin resistance levels. Penicillin MICs were determined by agar dilution with approximately 10^6 CFU delivered onto antibiotic-containing agar plates by a Steers Replicator. The MIC was defined as the lowest penicillin concentration at which confluent bacterial growth was reduced to a few (up to 10 to 20) countable microcolonies. With some transformants, MICs were confirmed by population analysis in which dilutions of cultures were plated on agar containing a series of concentrations of the antibiotic. All of the genetic transformants selected for penicillin resistance had full susceptibility to the other antibiotics, for which resistance traits were present in the DNA donor.

In vivo labeling of PBPs. PBPs were identified after treatment of whole cells with radioactive penicillin. Exponential-phase microorganisms (1 ml) were incubated with [^{3}H]benzylpenicillin at concentrations ranging from 0.05 to 5 μg of antibiotic per ml for 10 min at 37°C. An excess of unlabeled benzylpenicillin was then added, and the samples were immediately chilled on ice. The cells were recovered by centrifugation at 12,000 × *g* for 5 min at 4°C, suspended in 50 μl of 0.005 M sodium phosphate buffer (pH 7.0) with 1% Sarkosyl (Sigma, St. Louis, Mo.), and incubated for 15 min at 37°C. This treatment resulted in dissolution of the microorganisms. The techniques used for discontinuous gel electrophoresis, staining, and detection of PBPs with fluorography have already been described (14).

Cell wall preparation. Pneumococcal cell walls were prepared by a previously published procedure (4), with the following modifications. Exponentially growing cultures were rapidly chilled by immersing the flasks in an ethanol-ice bath until the temperature reached 4 to 0°C. After the cells were harvested by centrifugation at 4°C, they were suspended in iced 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., Barltesville, Okla.). Unbroken cells and glass debris were sedimented by low-speed centrifugation (5,000 × *g*, 10 min). Cell wall fragments were pelleted at 25,000 × *g*, suspended in buffered saline with 0.05% sodium azide, and digested with DNase, RNase, and protease as previously described (4), except that proteinase K was substituted for trypsin. Peptides from protease digestion and remaining lipids were extracted with boiling 1% sodium dodecyl sulfate, and the wall fragments were washed twice with water and incubated for 15 min at 37°C, first with 8 M LiCl and then with 100 mM EDTA, to remove material bound by ionic interactions. After another water wash, the fragments were treated with acetone, suspended in water, and lyophilized.

Enzymatic digestion of cell walls. For liberation of stem peptides, 1 to 5 mg of cell wall material was suspended in 50 mM potassium phosphate buffer, pH 7.0, and treated with affinity-purified pneumococcal autolysin (40 mU) (2) 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. 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% trifluoroacetic acid for high-performance liquid chromatography (HPLC) analysis.

HPLC. Peptides were separated with a Shimadzu LC-10A HPLC system as described previously (3). The column was a Vydac 218TP54 (The Separations Group, Hesperia, Calif.), and the eluting solvent was an 80-min linear gradient from 0.1% trifluoroacetic acid (Pierce Chemical Co., Rockford, Ill.) to 15% acetonitrile (Burdick & Jackson, Muskegon, Mich.) in 0.1% trifluoroacetic acid pumped at a flow rate of 0.5 ml/min. The eluted fractions were detected and quantitated by determination of their A_{210}.

RESULTS

Isolation of penicillin-resistant transformants. A competent culture of antibiotic-susceptible strain R6 (Hex$^-$) was exposed to saturating concentrations of DNA from multidrug-resistant clinical isolate Hun 663 to obtain transformants with donor level resistance. Four such independent transformant colonies, for each of which the penicillin MIC was 1.6 μg/ml, were purified and grown to cultures, and their PBP patterns, i.e., the penicillin-binding capacity (affinity) of their PBPs, and the stem peptide composition of their cell walls were determined.

Figure 1 compares the PBPs of the DNA donor (Hun 663) and recipient [R6 (Hex$^-$)] strains and two of the transformant cultures (Hun 663 tr.3 and Hun 663 tr.4). The PBP patterns of the transformants showed several interesting features. As expected, PBP 3 of the transformants showed a molecular size characteristic of PBP 3 of the recipient strain since this PBP is known to remain unaltered during acquisition of penicillin resistance in pneumococci (10, 15). The PBP pattern of the two transformants also differed from that of the DNA donor (Fig. 2) in the relative size of one of the three high-molecular-weight PBPs (the PBP with the second-highest molecular weight in Fig. 1). All high-molecular-weight PBPs showed reduced antibiotic-binding capacity (affinity) compared with that seen in the PBPs of recipient strain R6 (Hex$^-$). In one of the transformants (Hun 663 tr.3), faint bands of PBP 2B were also detectable at high penicillin concentrations (1 and 5 μg of [^{3}H]penicillin per ml), in contrast to the case of the DNA donor strain.

Figure 3 shows HPLC elution profiles of cell wall stem peptides from the DNA donor and recipient strains and the four transformants. The similarity of donor and transformant

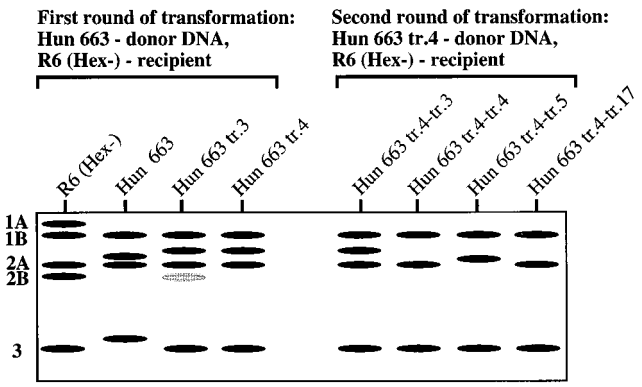

FIG. 2. Variation in the PBP patterns of penicillin-resistant transformants. This sketch, generated on the basis of the fluorograms in Fig. 1, indicates the molecular sizes and numbers of PBPs observed in penicillin-resistant transformants.

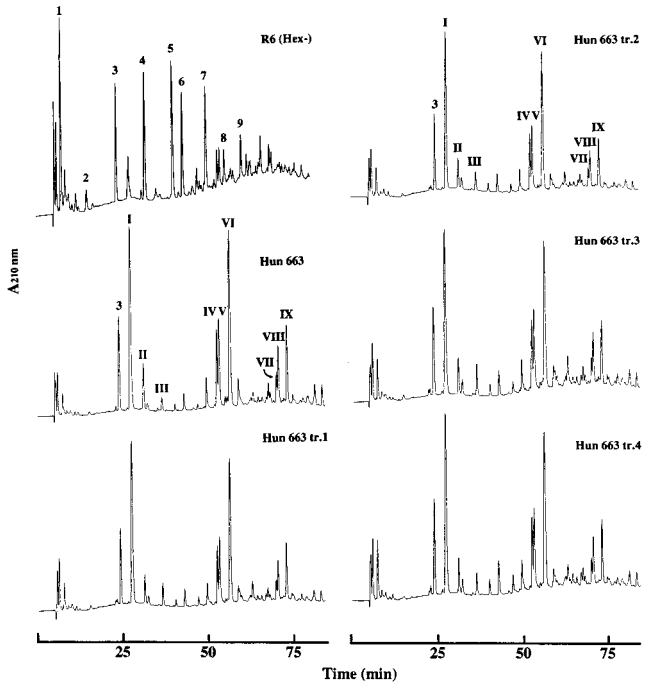

FIG. 3. Similarity in cell wall stem peptide composition between first-round penicillin-resistant transformants and penicillin-resistant donor strain Hun 663. Cell wall stem peptides were isolated from the bacterial strains, and their HPLC column elution profiles were determined as described in Materials and Methods.

profiles is apparent. The cell walls of the four transformants showed a high proportion of branched peptides (Table 1). For instance, in Hun 663 tr.1, peptides IV through IX represented close to 50% of the total stem peptides. In contrast, peptides 1, 4, and 5 were present in low concentrations: 2.8, 1.3, and 0.7%, respectively. Nevertheless, the amounts of these peptides were higher than their representation in DNA donor strain Hun 663 (1.9, 0.9, and 0.4%, respectively), suggesting a shift in the direction of the composition of the recipient cell wall. As a result of these modest but significant increases in the proportion of linear stem peptides, the ratio of branched to linear peptides (B/L) was reduced to 10 (in Hun 663 tr.4), compared with 24 in DNA donor strain Hun 663 (Table 1).

Second-round transformants. DNA isolated from transformant Hun 663 tr.4 was next chosen to generate a group of second-round penicillin-resistant transformants by using again susceptible strain R6 (Hex^-) as the recipient. Of the four transformants picked, the penicillin MIC for three was the same as for the donor strain (1.6 μg/ml); that for the fourth strain (Hun 663 tr.4-tr.17) was 0.2 μg/ml. Each transformant had high-molecular-weight PBPs with reduced affinity, but the second-highest-molecular-weight PBP characteristic of DNA donor Hun 663 tr.4 was present in only one of the four transformants (Hun 663 tr.4-tr.3). The stem peptide HPLC patterns of transformants closely resembled that of the penicillin-susceptible recipient strain (Fig. 4). Table 2 shows, in quantitative terms, reconstitution of the normal (i.e., recipient type) peptidoglycan pattern in these second-round penicillin resistance transformants. The high B/L ratio characteristic of the DNA donor Hun 663 tr.4 (B/L, 10) dropped to 1.3 to 1.6, i.e., a value in the range of the susceptible recipient strain, and there was a large increase in the amounts of linear peptides. For instance, in Hun 663 tr.4-tr.3, the proportions of stem peptides 1, 4, and 5 were 17.4, 11.9, and 12.3%, respectively, similar to the values

TABLE 1. Cell wall stem peptide composition of *S. pneumoniae* during first round of genetic transformation of penicillin resistance

Strain	MIC (μg/ml)	% of peptide material															% Recovery	% Monomer	% Dimer and trimer	% of total peptides		B/L ratio
		1	2	3	I	II	4	III	5	6[a]	7	IV	V	8	VI	9	VII	VIII	IX				Linear (L)	Branched (B)	
R6 (Hex^-) (recipient)	0.01	15.0	2.4	10.1	5.3	0.8	12.5	0.8	15.0	10.3	10.7	3.0	4.0	4.2	1.7	4.2	—[b]	—	—	70	34	66	42	58	1.4
Hun 663 (DNA donor)	1.6	1.9	0.3	8.1	23.1	4.2	0.9	1.3	0.5	1.5	2.6	6.6	9.5	0.2	23.6	0.3	2.3	5.5	7.7	79	39	61	4	96	24.0
Hun 663 tr.1	1.6	2.8	0.5	8.1	24.6	3.5	1.3	2.6	0.7	2.0	3.1	6.2	9.1	0.5	22.0	0.2	1.9	4.6	6.5	79	42	58	6	94	15.7
Hun 663 tr.2	1.6	3.1	0.5	8.5	23.6	3.7	1.8	2.3	0.9	2.3	3.0	6.2	8.3	0.4	21.9	0.2	1.9	4.7	6.9	77	42	58	7	93	13.3
Hun 663 tr.3	1.6	3.0	0.6	8.6	22.6	3.3	1.5	3.0	0.9	2.4	3.5	6.2	9.1	0.5	20.8	1.3	1.9	4.8	5.7	78	41	59	7	93	13.3
Hun 663 tr.4	1.6	4.6	0.6	7.9	22.1	3.3	1.4	1.8	1.2	3.2	2.2	5.5	8.6	0.8	19.8	2.0	2.2	5.2	7.7	70	40	60	9	91	10.0

[a] Peptides 6A and 6B were not separated.
[b] —, not detectable.

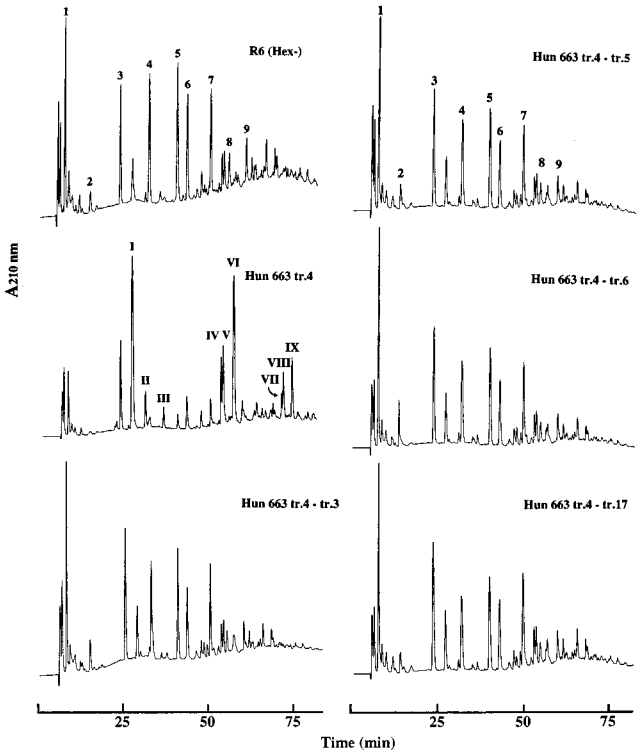

FIG. 4. Similarity in cell wall stem peptide composition between second-round penicillin-resistant transformants and penicillin-susceptible recipient strain R6 (Hex$^-$).

for strain R6 (Hex$^-$) and very different from the proportions of the same peptides in strain Hun 663 tr.4 (4.6, 1.4, and 1.2%, respectively). Branched peptides IV through IX, which were major components in DNA donor strain Hun 663 tr.4 (representing a total of 49% of the total stem peptides), were either present in reduced proportions (sum of peptides IV, V, and VI, 9.8%) or undetectable (peptides VII through IX) (Fig. 5).

DISCUSSION

Previous studies have demonstrated that the penicillin resistance level, PBP type (i.e., the number and molecular sizes of PBPs), and unique and abnormal peptidoglycan pattern characteristic of clinical strain Hun 663 were all clone-specific fingerprints which remained linked and were reproduced with precision during hundreds of generations of growth of these bacteria under nonselective conditions, both in vitro and in vivo (7, 12). On the other hand, the experiments described in this communication show that penicillin resistance and abnormal peptidoglycan patterns could be separated in two consecutive rounds of genetic transformation in which selection was for the antibiotic resistance trait. The PBP pattern characteristic of clinical isolates of the Hungarian clone of penicillin-resistant pneumococci (10) was also lost in the transformants, and the number and molecular sizes of PBPs underwent changes and showed variation from one transformant to another, reminiscent of what was shown in the case of laboratory isolates of low-level cefotaxime-resistant pneumococcal mutants (9). While a close association between abnormal wall composition and resistance was largely retained during the first round of transformation, in which the transforming DNA was derived from the clinical strain, the second round of transfor-

TABLE 2. Cell wall stem peptide composition of *S. pneumoniae* during second round of genetic transformation of penicillin resistance

Strain	MIC (μg/ml)	% of peptide material															% Monomer	% Dimer and trimer	% of total peptides		
		1	2	3	I	II	4	III	5	6[a]	7	IV	V	8	VI	9	VII[b]	VIII[b]	IX[b]	% Recovery			Linear (L)	Branched (B)	B/L ratio
R6 (Hex$^-$) (recipient)	0.01	15.0	2.4	10.1	5.3	0.8	12.5	0.8	15.0	10.3	10.7	3.0	4.0	4.2	1.7	4.2				70	34	66	42	58	1.4
Hun 663 tr.4 (DNA donor)	1.6	4.6	0.6	7.9	22.1	3.3	1.4	1.8	1.2	3.2	2.2	5.5	8.6	0.8	19.8	2.0	2.2	5.2	7.7	70	40	60	9	91	10.0
Hun 663 tr.4-tr.3	1.6	17.4	3.3	14.0	5.9	1.3	11.9	0.7	12.3	8.7	9.5	2.6	3.2	2.5	4.0	2.8				72	43	57	42	58	1.4
Hun 663 tr.4-tr.5	1.6	18.8	2.7	13.9	5.7	1.0	11.6	0.5	12.7	8.8	9.5	2.5	3.2	2.5	2.7	4.0				74	43	57	43	57	1.3
Hun 663 tr.4-tr.6	1.6	17.9	4.3	14.0	5.8	1.1	11.3	0.2	12.6	8.5	9.5	2.5	3.1	2.3	2.6	4.4				69	43	57	44	56	1.3
Hun 663 tr.4-tr.17	0.2	16.6	2.2	15.7	6.3	0.9	9.4	0.6	11.4	8.2	11.6	3.1	3.8	2.7	4.0	3.8				75	42	58	38	62	1.6

[a] Peptides 6A and 6B were not separated.
[b] The absence of a number indicates an undetectable amount of peptide.

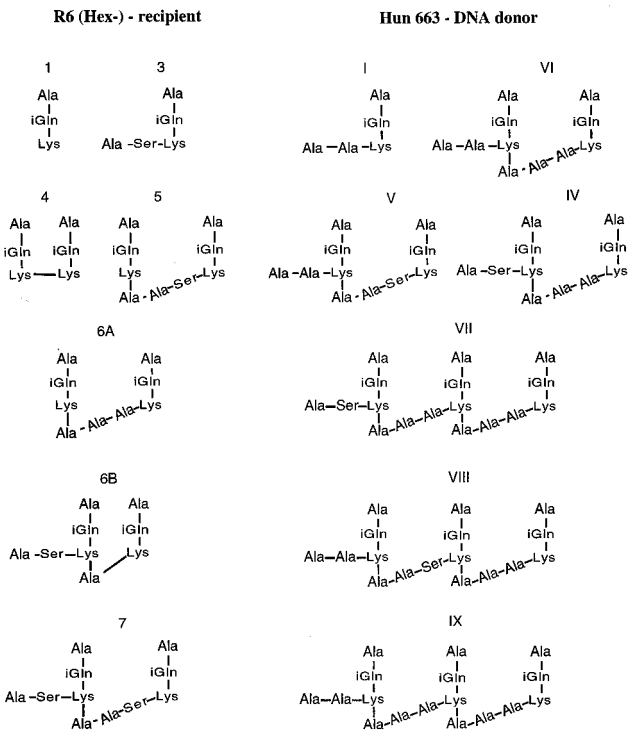

FIG. 5. Chemical structures of stem peptides identified in penicillin-susceptible and penicillin-resistant pneumococcal cell walls. Structures are from references 3 and 12.

mation (with donor DNA from Hun 663 tr.4) generated transformants in which the peptidoglycan composition was virtually identical to that of the susceptible recipient. The findings show that the structural alterations at sites in the resistant PBPs responsible for the reduced penicillin affinity of these proteins (and for the increased MIC for the cells) are not sufficient to account for the distorted muropeptide composition of the cell walls in these bacteria. We propose two alternative models to explain our findings.

In current models, the origin of penicillin resistance in clinical strains of pneumococci is assumed to involve import of heterologous DNA sequences (most likely through the process of in vivo genetic transformation) and the formation of mosaic PBP genes (1). It is conceivable that in some of these recombinational events, genetic determinants other than PBP genes were also taken up by the ancestral pneumococcal recipient of Hun 663, including branched muropeptide structure determinants possibly genetically linked to heterologous PBP genes. One should remember, however, that several muropeptides with branched-stem structures that have become predominant components of the cell walls of resistant bacteria are also detectable, albeit in very low concentrations, in the peptidoglycans of susceptible pneumococci as well. Therefore, the greatly increased representation of these compounds may be, in part, the result of mutations in pneumococcal genes that control the synthesis of branched-stem peptides, resulting in increased production of these molecules, which then may be utilized for wall synthesis in proportion with their increased cellular concentration.

Alternatively, the molecular events that have led to the formation of mosaic PBP genes in resistant clinical strain Hun 663 may have produced alterations not only at the catalytic site (needed for reduced penicillin binding) but also at some other domain(s) in these proteins which may be involved with the selection of branched muropeptides as preferred substrates for the transpeptidase reaction. These hypothetical altered secondary sites on PBP genes may have been trimmed ("crossed out") during the two consecutive laboratory transformations in which selection was only for the penicillin resistance phenotype.

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