

Characterization of the *murMN* Operon Involved in the Synthesis of Branched Peptidoglycan Peptides in *Streptococcus pneumoniae**

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The *murMN* operon, recently identified in the genome of *Streptococcus pneumoniae*, encodes for enzymes involved in the synthesis of branched structured muropeptides in the pneumococcal peptidoglycan; inactivation of *murMN* causes production of a peptidoglycan composed exclusively of linear muropeptides and a virtually complete loss of resistance in penicillin-resistant strains (Filipe, S. R., and Tomasz, A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 4891–4896). The experiments described in this paper follow up these observations. Primer extension analysis was used to identify the putative promoter region of the *murMN* operon in penicillin-susceptible and -resistant strains. Selective inactivation of the *murN* gene in the penicillin-resistant strain Pen6 caused production of an unusual peptidoglycan that contained only single amino acid residues in the muropeptide branches, indicating that the product of *murN* was involved with the addition of the second amino acid and the product of *murM* was involved with the addition of the first amino acid (alanine or serine) to the peptidoglycan cross-bridge. Allelic replacement of the mosaic *murM* gene of strain Pen6 with *murM* of the penicillin-susceptible laboratory strain caused enrichment of the peptidoglycan in linear muropeptides. The findings suggest that the genetic determinant primarily controlling the synthesis of branched muropeptides in the pneumococcal peptidoglycan is *murM*.

The cell wall of *Streptococcus pneumoniae* is a heterogeneous polymer composed of peptidoglycan and polysaccharides of different nature (capsular polysaccharides and teichoic acids) and proteins. The peptidoglycan itself is a highly complex molecule composed of a glycan (polymers of *N*-acetylated and nonacetylated (1) glucosamine and *N*-acetylmuramic acid residues) with short stem peptides attached to the glycan chains. These peptides, when cross-linked by penicillin-binding

proteins (PBPs),¹ interconnect different glycan chains enabling the bacteria to resist high osmotic pressures. The complexity of the peptidoglycan structure has been fully recognized after the introduction of a high resolution analytical technique, high pressure liquid chromatography (HPLC) (2). Use of this technique for the analysis of stem peptide composition of the peptidoglycan of clinical and laboratory strains of *S. pneumoniae* showed a species-specific peptidoglycan characterized by highly conserved molar ratios of 18 different muropeptides (3). A peculiar feature of the pneumococcal peptidoglycan is the simultaneous presence of both directly and indirectly cross-linked (branched) components. In the latter, alanyl-serine or alanyl-alanine dipeptides form the cross-bridge between neighboring muropeptides (3, 4). In the species-specific peptidoglycan, the percentage of these branched peptides, although detectable, is very small (3–5). Cell wall analysis by HPLC of the first high level penicillin-resistant clinical isolates, from South Africa and Hungary, revealed that in the peptidoglycan of these strains the proportion of branched muropeptides was greatly increased, and this abnormality of wall structure was to a significant degree co-transferred with resistance to penicillin during genetic transformation (6). It has been suggested that the abnormally high proportion of branched muropeptides in the cell wall of the resistant strains may provide the bacteria with a set of cell wall precursors, the branched structure of which has a better “fit” into the altered, low affinity active site(s) (7) of the remodeled PBPs of the resistant pneumococcus (6). However, examination of a larger number of penicillin-resistant isolates showed that the abnormally high proportion of branched wall peptides was not always associated with resistance to penicillin. In fact, the abnormality of wall composition detected in several isolates appeared to be related to the particular genetic lineage rather than being an obligatory correlate of resistance itself (4, 5).

The molecular mechanism of penicillin resistance in *S. pneumoniae* involves remodeling of the β -lactam target enzymes, the PBPs, in such a way that their affinity toward the antibiotic molecule is greatly reduced (7). This is achieved by the construction of *pbp* mosaic genes that are believed to be the result of heterologous recombinational events in the case of clinical isolates (8–10) or mutations in the *pbp* genes in the case of laboratory mutants (11). The recent identification of the genetic determinants of the cell wall branching system *murMN* (12) has allowed a reexamination of the relationship between muropeptide structure and penicillin resistance. It was shown that inactivation of the *murMN* operon resulted in the production of a peptidoglycan, both in penicillin-sensitive and penicillin-resistant strains, from which all branched muropeptide components were missing, and, concomitantly, there was a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ250766 (Pen6) and AJ250764 (R36A).

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¹ The abbreviations used are: PBP, penicillin-binding protein; HPLC, high pressure liquid chromatography.

TABLE I
Relevant properties of the strains and plasmids used in this study

Strains	Relevant characteristics	Pen MIC $\mu\text{g/ml}$	Source
<i>S. pneumoniae</i>			
R36A	Pen ^S laboratory strain	0.01	Ref. 13
R6Hex	Pen ^S laboratory strain	0.01	Ref. 14
Pen6	R6Hex transformant with chromosomal DNA from penicillin-resistant clinical isolate 8249 and selected for Pen ^R	6.0	Ref. 7
Pen6 <i>murN</i>	Pen6 strain with the <i>murN</i> gene inactivated	2	This study
Pen6 <i>murMN</i>	Pen6 strain with the <i>murMN</i> operon inactivated	0.032	Ref. 12
Pen6 <i>murMR36A</i>	Pen6 strain with the <i>murM</i> allele from R36A		This study
<i>E. coli</i>			
DH5 α	RecA endA1 gyrA96 thi-1 hsdR17 supE44relA1 ϕ 80 dlac Z Δ M15		Life Technologies, Inc.
Plasmids	Relevant characteristics		Source
pJDC9	<i>E. coli</i> plasmid Ery ^R		Ref. 15
pZOO3	pJDC9/0.7kb encoding amino acids 73–296 of <i>murN</i> from R36A		This study

complete loss of penicillin resistance in each one of several penicillin-resistant strains examined (12). The mechanistic connection between the functioning of *murMN* and the expression of the penicillin-resistant phenotype remains to be elucidated.

Here we report the identification of the sites of action of the MurM and MurN proteins in the assembly of the dipeptide branches and describe the impacts of selective inactivation of *murN* and allelic replacement of *murM* on the composition of peptidoglycan.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—All strains and plasmids used in this study are listed in Table I. *S. pneumoniae* strains were grown in a casein-based semisynthetic medium (C + Y) at 37 °C without aeration, as described previously (6). *S. pneumoniae* and *Escherichia coli* strains containing pJDC9 or its derivatives were grown in the presence of 1 $\mu\text{g/ml}$ and 1 mg/ml erythromycin (Sigma), respectively. Growth rates of the insertionally inactivated mutants of Pen6 were determined with cultures first grown in C + Y containing erythromycin at 1 $\mu\text{g/ml}$ and then diluted 100-fold in fresh C + Y. The OD was then measured at 590 nm over the time.

DNA and RNA Techniques—All routine DNA manipulations were performed using standard methods (16, 17). The chromosomal DNA from *S. pneumoniae* was isolated as described previously (18). Plasmids were isolated using the Wizard Plus Minipreps DNA Purification System (Promega), and polymerase chain reaction products were purified using the Wizard PCR Preps DNA Purification System (Promega). Oligonucleotides were purchased from Life Technologies, Inc. Nucleotide and derived amino acid sequences were analyzed using DNASTAR software. RNA was prepared from exponentially growing cultures at A_{590} of 0.5 and was extracted by using the FastRNA isolation kit (Bio101) according to the recommendations of the manufacturer.

Primer Extension Analysis—Primer extension analysis was performed by using primer ZOO36 (5'-TGTTCTTTGACAACTGATC-3') (Fig. 1), which was end-labeled with [γ -³²P]ATP and purified with the AGTC Gel Filtration Cartridges (Edge BioSystems). RNA from Pen6, R6Hex, and R36A (50 μg) was hybridized with the primer at 65 °C for 90 min and slowly cooled to room temperature. Reverse transcription was carried out by using SuperScript RT (Life Technologies, Inc.) at 42 °C for 90 min, and the reaction mixture was heated at 65 °C for 10 min to inactivate the enzyme. The reaction product was incubated with RNase H (3 units) at 37 °C for 30 min, ethanol-precipitated, resuspended in 10 μl of Sequenase stop solution, denatured, and applied to a 6% sequencing gel. Sequencing reaction mixtures prepared by using the T7 Sequenase Kit version 2.0 (Amersham Pharmacia Biotech) primed by ZOO36 were also applied to the gel.

Transformation and Population Analysis Profiles—*S. pneumoniae* strains were transformed according to published procedures (5). To induce competence, synthetic CSP α was added to the medium at a concentration of 250 ng/ml. The competent cells were then incubated for 30 min at 30 °C in the presence of plasmid DNA followed by the addition of 2 ml of C + Y and a 2-h incubation at 37 °C. Transformants were selected on blood agar plates (tryptic soy agar plus 3% (v/v) sheep blood) containing 1 $\mu\text{g/ml}$ erythromycin. Population analysis profiles were determined by plating serial dilutions of early stationary phase cultures

on plates of tryptic soy agar containing 5% (v/v) of sheep blood (Micro-pure Medical Inc., White Bear Lake, MN) and different concentrations of penicillin G (Sigma) (0, 0.01, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 $\mu\text{g/ml}$). The population analysis profiles were done with and without the presence of erythromycin 1 $\mu\text{g/ml}$ in the medium. Plates were incubated at 37 °C in a 5% CO₂ in air atmosphere for 24 h, and the number of bacteria capable of forming colonies in the presence of various penicillin concentrations was plotted against the concentration of penicillin in the agar medium.

Inactivation of the *murN* Gene—For gene disruption experiments by insertion-duplication mutagenesis, an internal fragment of *murN* was amplified by polymerase chain reaction using as template chromosomal DNA from R36A and cloned into pJDC9, a plasmid that does not replicate in *S. pneumoniae* (15). The following primers were used: ZOO1KP (5'-TATGGTACCGCCGATTATACCCAACAAG-3') and ZOO2BM (5'-TATGGATCCAGTCTCGCGCTTCTGCTTTTC-3'), giving origin to the plasmid pZOO3. Plasmid pZOO3 was used to inactivate the *murN* gene in Pen6 by transforming competent cells.

Allelic Replacement of the *murM* Gene—The abnormal *murM* gene from penicillin-resistant Pen6 strain was replaced by the *murM* gene from R36A laboratory strain by transforming competent cells from Pen6 with chromosomal DNA from R36A*murMN* (R36A with inactivated *murMN*). Transformants were selected with erythromycin (1 $\mu\text{g/ml}$), and their penicillin susceptibility was confirmed. Revertants from these transformants that result from the loss of the plasmid inactivating *murMN* were selected by their penicillin resistance and erythromycin susceptibility. The excision of the plasmid carrying the erythromycin resistance marker allowed the reconstruction of active *murM* allele from R36A. The allelic replacement of the *murM* gene was confirmed by polymerase chain reaction amplification using primers ZOO7 (5'-CAT-AGCGCTGGAACCTCAC-3') and ZOO30 (5'-ATATTCTCTACGTTCA-GAGG-3') followed by restriction with *Pst*I and *Hind*III, two enzymes that cut the Pen6 allele but not the R36A allele.

Cell Wall Preparation—Pneumococcal cell walls were prepared by a previously published method (4, 19) except for the process of breaking the cells, which was done by shaking with acid-washed glass beads with the help of FastPrep FP120 (Bio 101).

Enzymatic Digestion of Cell Walls—Cell wall material (2 mg) was suspended in 25 mM sodium phosphate buffer, pH 7.4, and treated with affinity-purified pneumococcal amidase (5 μg) at 37 °C for 18–24 h with constant stirring. The products were dried, the precipitate was washed with acetone, and the peptides were extracted with acetonitrile/isopropyl alcohol/water (25:25:50) containing 0.1% trifluoroacetic acid as already described (4, 19, 20). After removal of the solvents by evaporation in a SpeedVac, the peptides were dissolved in 0.1% trifluoroacetic acid.

Separation and Analysis of the Cell Wall Stem Peptides—Peptides were separated with a Shimadzu LC-10AVP HPLC system, as described previously (4). The column used was a Vydac 218TP54 (The Separations Group). The peptides were eluted with an 80-min linear gradient from 0 to 15% acetonitrile (Fisher) in 0.1% trifluoroacetic acid (Pierce) pumped at a flow rate of 0.5 ml/min. The eluted fractions were detected and quantified by determination of their ultraviolet absorption at 210 nm (A_{210}).

Characterization of Stem Peptides—The stem peptides generated by enzymatic hydrolysis of cell walls of mutants Pen6*murN* and Pen6*murMN* were recovered from the HPLC column and dried in the SpeedVac. The amino acid composition (21) and the peptides' molecular

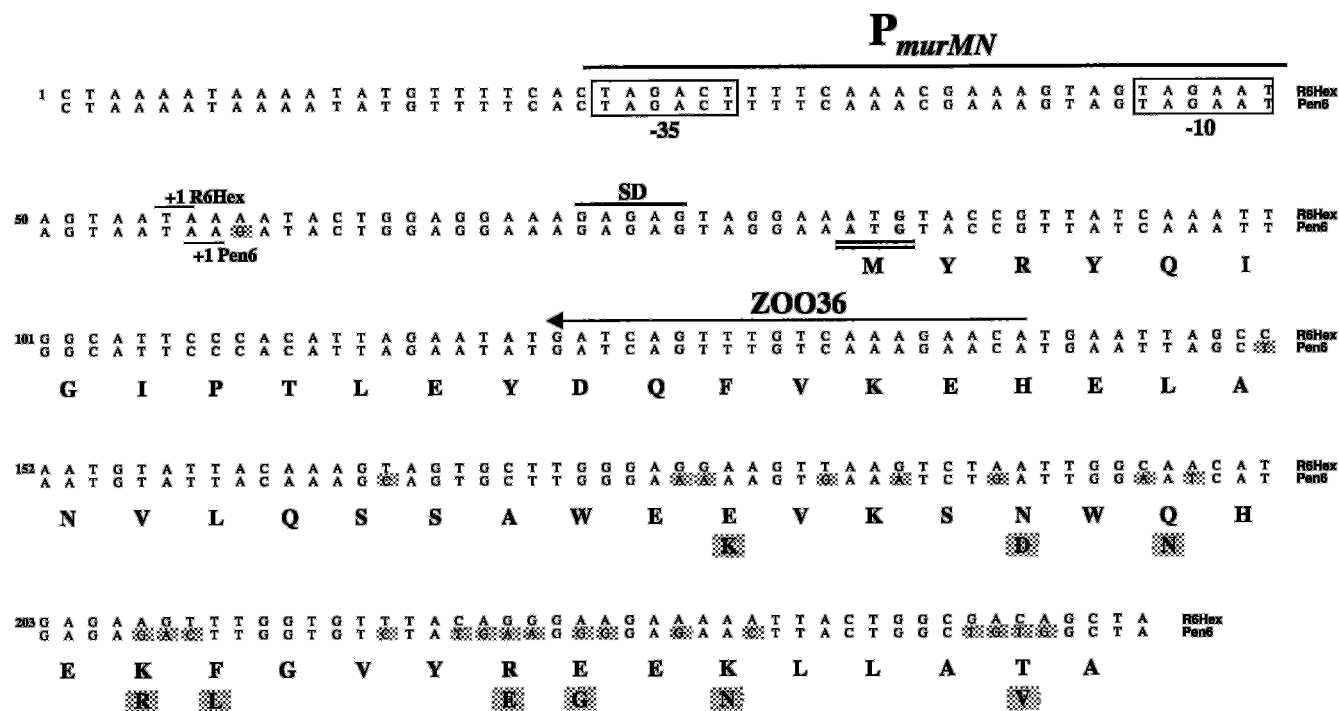


FIG. 1. Nucleotide sequence of the region upstream of *murM* (GenBank™ data base accession numbers AJ250766 (Pen6) and AJ250764 (R36A)). The putative promoter region is highlighted by boxed sequences and labeled -10 and -35. The promoter is designated P_{murMN} . The putative ribosome-binding site is underlined and labeled SD. The 5'-ends of the RNA determined by primer extension are labeled +1. The start codon is in boldface type and double underlined. The primer ZOO36 is indicated by an arrow. Part of the deduced amino acid sequence of *murM* from R36A or Pen are aligned under the DNA sequence.

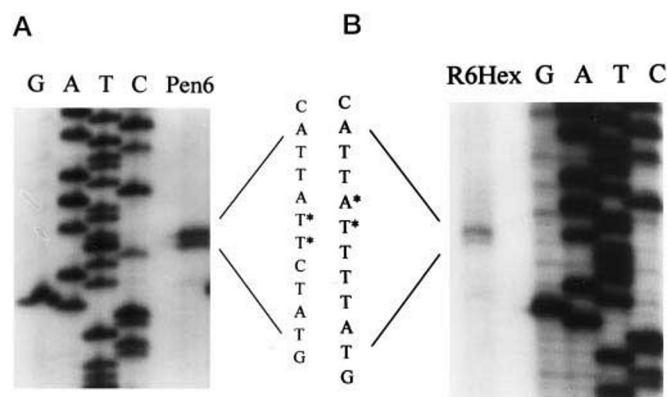


FIG. 2. Mapping of the 5'-end of the *murMN* transcript by primer extension from Pen6 (A) and R6Hex (B). The sequencing encompassing the transcription start site (marked by asterisks) is enlarged.

mass were determined at the Rockefeller University Protein/DNA Technology Center. Approximately 1.1 nmol of sample from the stem peptides 3a, 1a, and 7a were used to obtain the sequence of the part of the stem peptide amenable to Edman degradation. The procedure was performed as recommended by the manufacturer's program in a Hewlett-Packard G-1000A protein sequencer using chemistry 3.5, and the phenylthiohydantoin-derivatives were identified by on-line HPLC at the Rockefeller University Protein/DNA Technology Center.

RESULTS

Determination of the transcription initiation site. Primer extension analysis was performed to determine the transcription start site using the primer ZOO36 that hybridizes with the *murMN* transcript of Pen6 and R6Hex (Fig. 1). Based on this analysis, it was determined that the transcript that includes *murM* can initiate at two different adenine residues (Fig. 2A) located 25 and 26 base pairs upstream of the *murM* start codon in the case of Pen6 (Fig. 1). In R6Hex, the transcript can

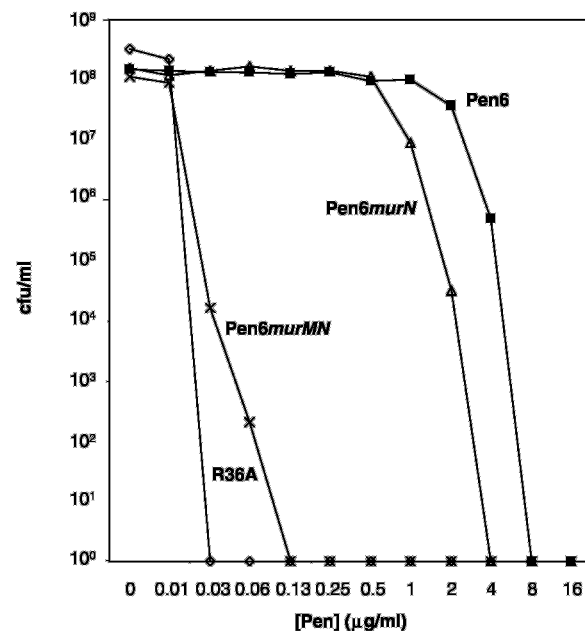


FIG. 3. The effect of inactivation of *murMN* and *murN* on the expression of penicillin resistance. Overnight cultures of strains R36A (◇), Pen6 (■), mutant Pen6*murMN* (×), and mutant Pen6*murN* (△) were plated at different cell concentrations on blood agar plates containing different concentrations of penicillin, and the number of bacterial colonies were counted, as described for population analysis under "Experimental Procedures."

initiate at an adenine or a thymine residue located 26 and 27 base pairs, respectively, upstream of the *murM* start codon (Figs. 1 and 2B). These results indicate that the same region contains the promoter in both the penicillin-resistant strain Pen6 and in the susceptible strain R6Hex (Fig. 1).

Gene Disruption and Characterization of the murMN and

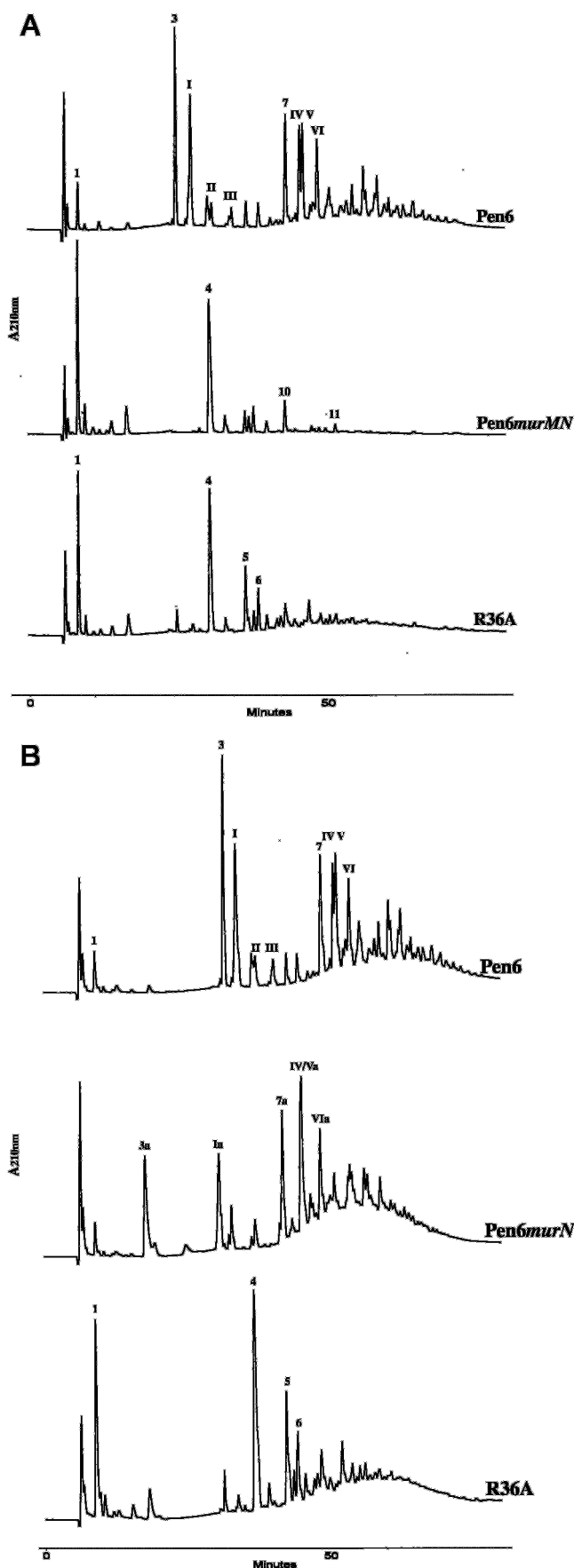


FIG. 4. The effect of disruption of *murMN* (A) and *murN* (B) genes on the composition of peptidoglycan. Cell walls prepared from the penicillin-susceptible strain R36A, the penicillin-resistant Pen6, and Pen6 with inactivated *murMN* or inactivated *murN*

TABLE II
Cell wall peptide composition of *S. pneumoniae* R36A, Pen6, Pen6*murMN*, and Pen6*murN*

Structurally similar stem peptides differing only in the number of amino acid residues in the peptide branches and/or cross-links (*i.e.* peptide 3 and 3a, or 1 and 1a) are grouped together for easier comparison.

Peptides	R6Hex	Pen6- <i>murMR36A</i>	Pen6	Pen6- <i>murN</i>	Pen6- <i>murMN</i>	R36A
1	8.2	17.5	2.5	1.6	23.3	12.0
2	0.9	3.1	0.7	2.3	4.3	3.1
3	10.3	8.1	13.8	2.7	0.1	2.5
3a	0.4	0.7	0.1	13.1	0.8	0.4
I	6.0	3.6	13.9	0.7	0.0	1.6
Ia	1.8	1.5	0.7	9.4	0.4	1.6
II	2.1	1.6	2.6	0.9	1.3	3.1
4	7.0	10.5	3.1	2.3	30.7	18.9
III	1.7	1.4	3.4	0.7	3.8	1.2
5	8.6	12.6	2.1	1.0	7.4	11.4
6	6.4	6.5	2.4	1.0	11.7	6.4
7	12.1	9.1	10.7	5.7	7.9	6.7
7a	0.5	0.3	0.2	12.6	0.0	1.0
IV	3.3	1.8	6.3	3.7	0.1	2.3
V	6.2	3.3	11.9	1.7	0.3	2.5
IV/Va	1.9	1.4	1.6	16.9	2.4	3.6
8	4.3	4.3	2.4	3.9	2.8	7.0
VI	5.2	1.7	9.8	4.4	2.2	5.9
VIa	3.4	5.7	1.2	8.0	0.2	3.9
9	9.8	5.1	11.2	7.8	0.3	5.4
Total	100	100	100	100	100	100.0
Monomers	31	38	37	31	34	26
Larger than monomers	69	62	63	69	66	74
Linear peptides	26	42	11	10	67	45
Branched peptides	74	58	89	90	33	55
B/L peptides	2.9	1.4	8.0	8.5	0.5	1.3

murN Mutants—Inactivation of the *murMN* operon by insertion duplication mutagenesis in the penicillin-resistant strain Pen6 did not cause any significant change in growth rate of the cultures; the mass doubling times of the parental strain Pen6 and its Pen6*murMN* mutant were 34.0 ± 0.5 and 32.5 ± 0.3 min, respectively. However, when *murN* alone was inactivated the doubling time of the mutant increased to 48.5 ± 0.9 min. The rates of autolysis in the stationary phase of growth were the same for Pen6*murMN* and for the parental strain Pen6 ($1.7 \pm 0.3 \times 10^{-3}$ and $2.3 \pm 0.5 \times 10^{-3} \text{ min}^{-1}$, respectively), although the mutant culture started lysing sooner than the parental strain Pen6.

While inactivation of *murMN* caused a virtually complete block in the expression of penicillin resistance (12), inactivation of *murN* resulted only in a modest (2-fold) decrease of the MIC to penicillin (Fig. 3) despite the major impact of the inactivation on the composition of the peptidoglycan (see below).

*Composition of the Peptidoglycan in the Pen6*murMN* and Pen6*murN* Strains*—The cell wall of the Pen6*murMN* and Pen6*murN* was analyzed by HPLC (Fig. 4, A and B). The stem peptide composition of the strains shown as a percentage of the total peptides of the peptidoglycan is presented in Table II, and the corresponding chemical structures are shown in Fig. 6. As was shown before (12), disruption of the *murMN* operon led to the disappearance of all branched mucopeptide monomers and dimers accompanied by a parallel increase in the percentage of linear structured stem peptides and in the appearance of novel peptide structures (peptides 10 and 11, Fig. 4A).

were enzymatically hydrolyzed and analyzed by HPLC for the composition of stem peptides (see "Experimental Procedures").

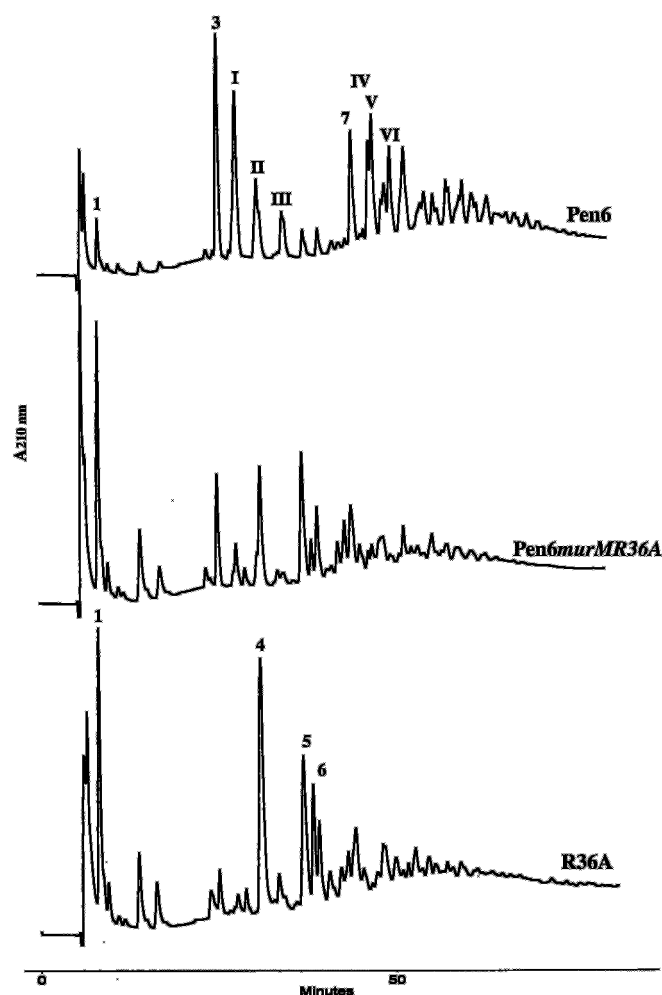


FIG. 5. The effect of allelic replacement of *murM* on the stem peptide composition of pneumococcal cell wall. The mosaic-structured *murM* gene carried by the penicillin-resistant strain Pen6 was replaced by *murM* of the penicillin-susceptible strain R36A, as described in the "Experimental Procedures." The composition of cell walls prepared from Pen6, R36A, and Pen6murMR36A (i.e. Pen6 in which the *murM* gene was replaced by the allele carried in strain R36A) was analyzed by HPLC.

HPLC analysis of the cell walls of strain Pen6 in which the *murN* gene was inactivated showed major changes in the elution profile of stem peptides (Fig. 5); the branched components found in the peptidoglycan of Pen6 disappeared, but in this case there was no increase of the linear peptides. Instead, inactivation of the *murN* led to the appearance of novel peptide components that were not seen before in pneumococcal cell wall preparations. Similarly, these novel stem peptides with anomalous retention times were also detected in the cell walls of the penicillin-susceptible strain R6Hex with inactivated *murN* (data not shown). Nevertheless, there was no change in the degree of cross-linking of the peptidoglycan in the Pen6murMN and Pen6murN mutants (as seen from the unchanged percentage of monomers relative to the total of peptides in Table II).

Characterization of the Novel Stem Peptides in the *murN* and *murMN* Mutants—Analysis of the peptide composition of the peptidoglycan from the *murN* mutant of Pen6 showed an accumulation of two novel peptides (3a and Ia; see Fig. 6). Based on the results of molecular weight determination and amino acid composition, peptides 3a and Ia are proposed to be monomers with only one serine or one alanine, respectively, attached to the ϵ -NH₂-terminal of the stem peptide lysine residue (Table III). Components 7a, IV/Va, and VIa appear to be di-

meric peptides that would result from the transpeptidation reaction of peptides 3a and Ia.

The structures of the peptides 3a, Ia, and 7a were confirmed by Edman degradation. According to the proposed structures (Table III), the only sources of free amino termini in stem peptide 3a would be two alanine residues, and in stem peptide Ia one alanine and one serine. The results of Edman degradation confirmed these predictions; only one alanine residue was released from peptide 3a, and similar amounts of alanine and serine were released from peptide Ia. Edman degradation of peptide 7a resulted in the release of the same number of serine residues as in peptide Ia but twice the amount of alanine, consistent with the proposed structure.

The two new components detected in the peptidoglycan of cells with inactivated *murMN* operon (peptides 10 and 11) were tentatively identified (on the basis of amino acid composition and molecular mass) as trimers and tetramers composed of linear peptide units (Table III).

Allelic Replacement of *murM*—The *murM* alleles from R36A and Pen6 encode proteins that differ by 15% at the amino acid level (12). To determine if the different peptidoglycan types of these two strains were related to the two different MurM proteins, we introduced the R36A *murM* allele into the Pen6 background by genetic transformation, in order to generate the construct Pen6murMR36A. Confirmation of efficient allelic replacement was obtained by sequencing the *murMN* operon of this mutant (data not shown).

The cell wall composition of the mutant Pen6murMR36A was analyzed by HPLC (Fig. 5). The introduction of the *murM* allele from strain R36A caused a large increase in the representation of the monomeric linear peptide 1 (from 2.5% in the parental strain Pen6 to 17.5% in the allelic replacement mutant) and an increased proportion of the directly cross-linked tri-tetra dimer (from 3.1 to 10.5%). The percentage of branched peptide 3 was decreased from 13.8% (in Pen6) to around 8.1%, a value similar to that found in R6Hex (10.3%). (This value was considerably higher than the value found in strain R36A, 2.5%). There was a considerable reduction in the proportion of the branched peptide I (from 13.9 to 3.6%) more pronounced than the reduction in peptide 3.

Despite the extensive variation in the ratio of branched to linear peptides (from 8.0 in Pen6 to 1.4 in the mutant Pen6murMR36A) the level of cross-linking, determined by the percentage of all cross-linked muropeptide species, was similar in all mutants and in their parental strains (62% in the Pen6murMR36A and 63% in Pen6).

DISCUSSION

The enzymes involved in the addition of lateral peptides to the ϵ -amino group of lysine have been identified in the *Staphylococcus* family (22–24) and recently in *S. pneumoniae* (12). In the case of *Staphylococcus aureus*, the synthesis of the pentaglycine bridge involves three enzymes: FmhB, responsible for the addition of the first glycine to the linear pentapeptide precursor (25), and FemA and FemB, involved in the addition of the glycyl residues 2 and 3 (26–27) and glycyl residues 4 and 5 (28), respectively. In *S. aureus*, the *fmbB* gene product was shown to be essential, which may be related to the fact that dimers of linear muropeptides are extremely rare in this bacterial species. In contrast to *S. aureus*, *S. pneumoniae* appears to be able to use either linear or branched cell wall precursors as substrates for the PBPs in the cross-linking reaction of peptidoglycan. The disruption of the *murMN* operon did not impair growth, indicating that the *murMN* genes are not essential in *S. pneumoniae*. However, the growth rate of the mutant Pen6murN was significantly reduced, suggesting that the semibranched peptides of this mutant may not be used as

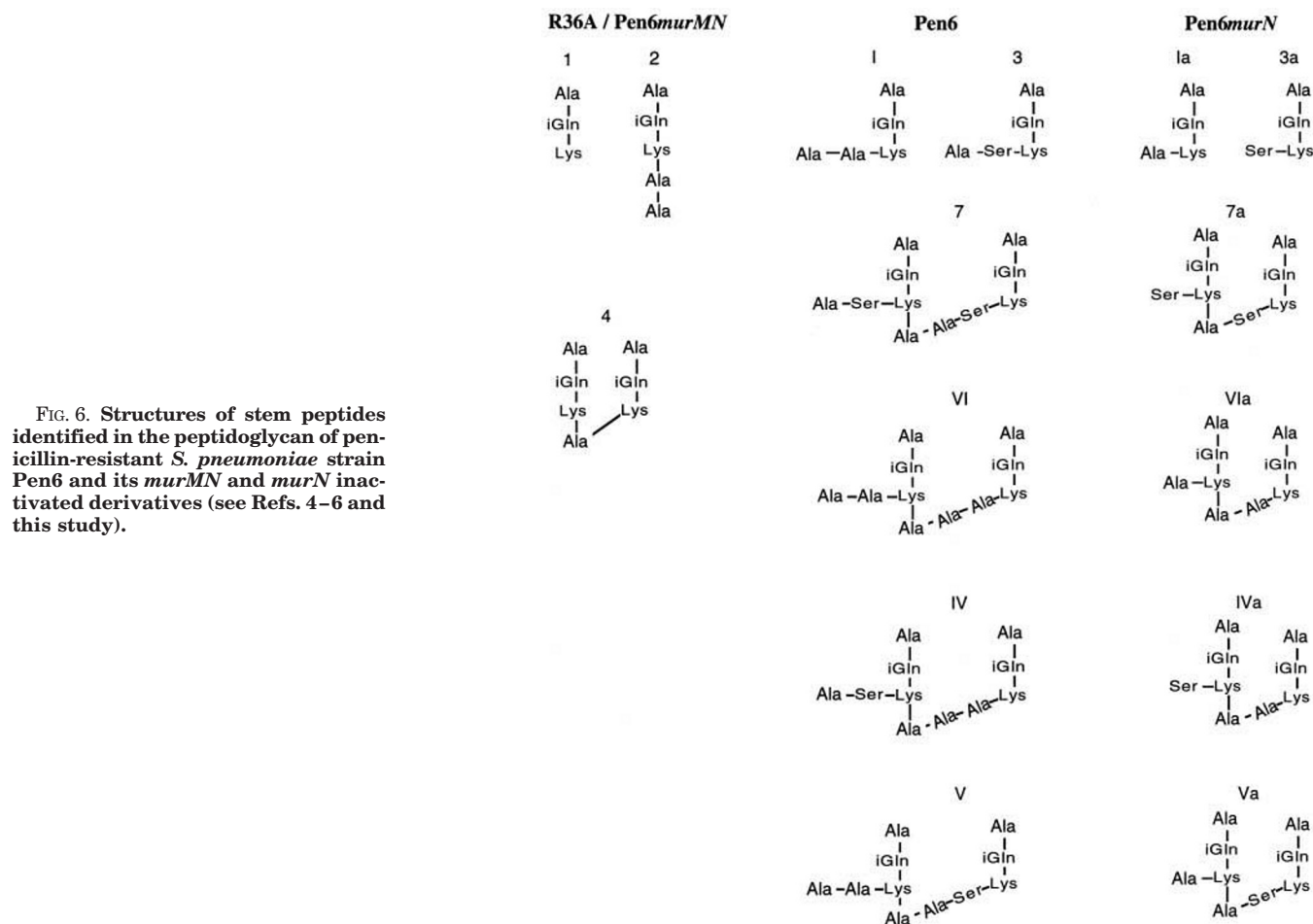


FIG. 6. Structures of stem peptides identified in the peptidoglycan of penicillin-resistant *S. pneumoniae* strain Pen6 and its *murMN* and *murN* inactivated derivatives (see Refs. 4–6 and this study).

efficiently for some growth-limiting function in *S. pneumoniae*.

A combination of genetic and biochemical studies described in this communication has allowed the clarification of the roles of the *murM* and *murN* gene products in the assembly of the branched muropeptides.

Inactivation of the *murMN* operon in *S. pneumoniae* abolished the addition of any amino acid residue to the ϵ -amino group of the stem peptide lysine, resulting in the production of a peptidoglycan that completely lacked muropeptides of branched structure (12). Selective inactivation of *murN* caused the formation of an unusual peptidoglycan in which all dipeptide branches were replaced by branches composed of only one seryl or alanyl residue. These results suggest that the MurN protein is involved with the addition of the second amino acid residue (alanine) and that the MurM protein is involved with the addition of the first amino acid (alanine or serine) to the cross-bridge.

Interestingly, replacement of the dipeptide bridges by branches composed of single amino acids did not alter the overwhelmingly branched muropeptide composition of strain Pen6; the proportion of branched peptides was 89% in Pen6 and 90% in Pen6murN (see Table II). Furthermore, if one considers all branched muropeptides irrespective of whether they contain one or two amino acid residues, the characteristic proportions of the various specific branched muropeptide species also appeared to be retained in the Pen6murN mutant. These observations suggest that the amount of branched muropeptides in the pneumococcal peptidoglycan is primarily determined by the activity of the *murM* gene product.

Additional evidence for the dominant role of *murM* is provided by the changes observed in the peptidoglycan composi-

tion of the penicillin-resistant strain Pen6 in which the mosaic *murM* of this strain (12) was replaced by *murM* of the penicillin-susceptible strain R36A. The *murM* alleles from R36A and Pen6 differ by 15% at the amino acid level (12). Comparison of the peptidoglycan of the two strains shows that Pen6 has a much higher percentage of branched peptides and can add an alanine or a serine as the first amino acid of the cross-bridge, whereas R36A only adds a serine residue efficiently. These differences may be related to the observed 15% difference at the amino acid level of the MurM. In order to determine if there were in fact any differences in the activity of the MurM from Pen6 and R36A, we transformed the R36A *murM* allele into Pen6. This mutant Pen6murMR36A only diverges from the parental strain in the sequence of *murM* allele. Data in Table II show that the introduction of the R36A allele of *murM* shifted the peptidoglycan composition in the direction characteristic of the penicillin-susceptible strain: the proportion of total branched muropeptides was reduced from 89% (in Pen6) to 58% (in Pen6murMR36A), which is close to the proportion of branched peptides (55%) in strain R36A. The ratio of branched to linear peptides also changed from 8.0 (in Pen6) to 1.4 (in Pen6murMR36A), which is comparable with the ratio (1.3) seen in strain R36A. These results suggest that the MurM from R36A is not as efficient as the one from Pen6 in the addition of the first amino acid to the ϵ -NH₂ group of the lysine residue.

It is conceivable that these differences are related to different rates of transcription of the two kinds of *murM* genes. Based on the result obtained by primer extension analysis for the determination of the transcription initiation sites, we proposed a virtually identical promoter region for the *murM* genes in strains Pen6 and R36A/R6Hex. The fact that R6Hex has a

TABLE III

Amino acid composition, molecular weight, and proposed structures of peptides isolated from the mutants Pen6murMN and Pen6murN

Peak	Amino acid composition	Experimental [M+H] ⁺	Calculated [M+H] ⁺	Proposed structure
3a	Glx 1.0 Lys 1.0 Ala 1.1 Ser 1.1	434.3	432.5	Ala-Gln-Lys Ser
Ia	Glx 1.0 Lys 1.0 Ala 2.1 Ser 0.0	418.3	416.5	Ala-Gln-Lys Ala
7a	Glx 1.0 Lys 1.0 Ala 1.5 Ser 0.9	918.5	918.0	Ala-Gln-Lys Ser Ala-Gln-Lys-Ala Ser
IV/Va	Glx 1.0 Lys 1.0 Ala 2.0 Ser 0.5	902.4	902.0	Ala-Gln-Lys Ala Ala-Gln-Lys-Ala Ser
VIa	Glx 0.8 Lys 1.0 Ala 2.4 Ser 0.4	886.4	886.0	Ala-Gln-Lys Ala Ala-Gln-Lys-Ala Ala
10	Glx 0.9 Lys 1.0 Ala 1.8 Ser 0.0	1142.6	1142.3	Ala-Gln-Lys Ala-Gln-Lys-Ala Ala-Gln-Lys-Ala
11	Glx 0.9 Lys 1.0 Ala 1.8 Ser 0.1	1540.8	1540.8	Ala-Gln-Lys Ala-Gln-Lys-Ala Ala-Gln-Lys-Ala Ala-Gln-Lys-Ala

murM allele identical to R36A but has a higher percentage of branched peptides indicates that additional factors may also contribute to the regulation of the amount of branched peptides in the peptidoglycan. However, when the *murM* allele from R36A is introduced in the background of Pen6 and therefore subject to the same regulation, the observed difference in the peptidoglycan composition should be due only to the differences in the protein. Therefore, compositional differences between the peptidoglycans of resistant and susceptible strains most likely reflect differences in the specific activities of the two types of MurM proteins. This possibility is currently under investigation.

Analysis of the composition of the peptidoglycan from Pen6 and its *murN* and *murMN* mutants showed similar proportions of cross-linked species in the peptidoglycan, suggesting that the PBPs from strain Pen6 are not very specific regarding their substrates. In the absence of antibiotic, PBPs seem to be able to use either linear, branched or semibranched peptides as substrates for the transpeptidation reaction in the synthesis of peptidoglycan. On the other hand, when a β -lactam antibiotic

is present in the medium, the bacteria seem to depend on the availability of branched or at least semibranched peptides for the expression of penicillin resistance. It should be noticed that in strain R6Hex 74% of all stem peptides have a branched structure, yet this strain is not resistant to penicillin, indicating that the presence of branched peptides alone is not sufficient for the expression of penicillin resistance.

The fact that PBPs can efficiently cross-link either linear, branched, or semibranched peptides taken together with the fact that substituting the *murM* allele of Pen6 by the allele from R36A results in drastic changes in the peptidoglycan composition indicates that the primary determinants of the type of peptidoglycan stem peptide structure (linear *versus* branched peptides) must be MurM and MurN.

Addendum—Confirmatory evidence for *murM* and *murN* and the role(s) of these genes in peptidoglycan structure and expression of β -lactam resistance, first described in Ref. 12, has appeared recently by Weber *et al.* (29), who identified the same genes but named them *fibA* (same as *murM*) and *fibB* (same as *murN*).

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