The recently identified murMN operon of Streptococcus pneumoniae encodes enzymes involved in the synthesis of branched muropeptides of the pneumococcal cell wall peptidoglycan. Its inactivation was shown to cause production of a peptidoglycan composed exclusively of linear muropeptides and a virtually complete loss of resistance in penicillin-resistant strains. The studies described in this communication follow up these observations in several directions. The substrates of the MurM-catalyzed reaction (addition of alanine or serine) was identified as the lipid-linked N-acetylmuramylpentapeptide. Different murM alleles from several penicillin-resistant S. pneumoniae strains, each with a characteristic branched peptide pattern, were cloned into pLS578, a pneumococcal plasmid capable of replicating in S. pneumoniae, and transformed into the penicillin-susceptible laboratory strain R36A. All transformants remained penicillin-susceptible; however, their cell wall composition changed in directions corresponding to the muropeptide pattern of the strain from which the murM allele was derived. This suggests that the muropeptide composition of the pneumococcal cell wall is determined by the particular murM allele carried by the cells. A 30-amino acid long sequence within the MurM protein was shown to be the main determinant of the specificity of the reaction (addition of alanine versus serine).

The synthesis of the peptidoglycan in bacteria may be divided into three stages: in the first, cytoplasmic, stage several consecutive enzymatic reactions synthesize the peptidoglycan building block, the UDP-MurNAc-pentapeptide. In the case of Streptococcus pneumoniae and several other bacteria, the pentapeptide chain is composed of L-alanine, D-isoglutamine, L-lysine, D-alanine, D-alanine, with the latter representing the C-terminal residue. The second stage of synthesis occurs in the plasma membrane where the peptidoglycan building block is linked to a bactoprenol residue and may undergo a “maturation process” by several as yet not well understood enzymatic reactions. These may include addition of short peptides to the lysine epsilon amino group in some or all of the stem peptides; and amidation of the second stem peptide residue, usually a glutamate, to glutamine. After addition of an N-acetyl glucosamine residue, the bactoprenol-linked and structurally completed disaccharide pentapeptide peptidoglycan precursor is transferred to the outer side of the plasma membrane for presentation to proteins (monofunctional transglycosylases, penicillin binding proteins) that take part in the assembly of the macromolecular peptidoglycan.

The pneumococcal peptidoglycan is composed of both linear and branched muropeptides, the latter of which carry short dipeptide branches. The chemical nature of the branched peptides, seryl “alanine or alanyl” alanine, i.e. the type of muropeptide they are attached to and the proportion of muropeptides carrying branches vary from strain to strain (1–3). The identification of the murMN operon opened up new experimental approaches for the study of the mechanism of synthesis and physiological role of branched peptides. Most interestingly, inactivation of the murMN was shown to cause not only the production of cell wall peptidoglycan composed exclusively of linear muropeptides but also a complete loss of the resistant phenotype in penicillin-resistant strains (4). Several penicillin-resistant isolates were shown to carry murM genes with unique polymorphic regions, which seem to correlate with the preponderance of seryl-alanine or alanyl-alanine branches in the peptidoglycan (5). Selective inactivation of murM and murN showed that the protein products of these genes act in sequence: murM being involved with the addition of the first amino acid (serine or alanine) and murN involved with the addition of second amino acid of the bridge (alanine) (6).

The main purpose of the studies described here was to identify the substrate of the MurM-catalyzed reaction and to better define the roles of various murM alleles in defining cell wall composition and penicillin resistance.

**EXPERIMENTAL PROCEDURES**

**Strains, Growth Conditions, and Antibiotic Susceptibility Tests**—All strains and plasmids used in this study are listed below in Table I. S. pneumoniae strains were grown in a casein-based semisynthetic medium at 37 °C without aeration, as previously described (1). S. pneumoniae strains containing pLS578 or its derivatives were grown in the presence of 1 μg/ml tetracycline, and Escherichia coli strains containing pGEM-3Z plasmid or its derivatives were grown in the presence of 100 μg/ml ampicillin (Sigma Chemical Co.). Penicillin-resistance levels (minimal inhibitory concentration, MIC)

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were determined by the E test following manufacturer’s guidelines (AB Biodisk, Solna, Sweden).

DNA Techniques—All routine DNA manipulations were performed using standard methods (11, 12). Chromosomal DNA from S. pneumoniae was isolated as described previously (13). Plasmids were isolated using Qiagen MiniPreps or MIDPrep DNA purification system (Promega), and PCR products were purified using the Wizard PCR Prep DNA purification system (Promega). Oligonucleotides were purchased from Life Technologies, Inc. DNA sequencing was done at the Rockefeller University Protein/DNA Technology Center with the BigDye terminator cycle sequencing method and either the 3700 DNA analyzer for capillary electrophoresis or the ABI Prism 377 DNA sequencer for slab-gel electrophoresis. Nucleotide and derived amino acid sequences were analyzed using DNASTAR software.

Construction of S. pneumoniae Lacking murM—To isolate a mutant of the penicillin-resistant strain PenF6 lacking murM gene, we transformed Pen6 with a PCR fragment that included the 2-kb fragment from pJDC9, containing the erm marker, flanked by the regions upstream and downstream of murM.

The upstream and downstream segments of murM were amplified from chromosomal DNA of strain Pen6 by PCR with PfuTurbo DNA polymerase (Stratagene). First, the 0.46-kb fragment containing the upstream region of murM was amplified with primers ZOO45EC (5’-AGCGAATACTGGTTTTGACTAC-TACAGGCGC-3’) and ZOO46BM (5’-ATAGTAGCTTCTTTCCAGTAG-TGCCATCGC-3’), digested with EcoRI and BamHI and cloned into plasmid pSF25 (see above). The resulting plasmid was named pSF26. Next, the full murM gene was transferred by cloning in this plasmid (digested with HindIII and XbaI) the product obtained from the chromosomal DNA of strain R36A with the primers ZOO46HI and ZOO48NC (see above). The fragment was cloned into the HindIII/XbaI-digested E. coli plasmid pSF22 (pGEM-3Z carrying a promoterless murN and an Nol site restriction site (see Table I below), yielding plasmid pSF24. Next, the full murM gene was reconstructed by cloning in this plasmid (digested with HindIII and XbaI) the PCR product obtained from the chromosomal DNA of strain R36A with the primers ZOO46HI (see above) and ZOO48NC (see above) and cloned into the HindIII/XbaI-digested pSF25 plasmid. This pneumococcal plasmid, carrying the murM gene from the penicillin-resistant strain R36A and DE1 was named pDE1.

The second chimeric plasmid pM10 included a PCR fragment that encoded for the 240 amino acid residues from the N terminus of the penicillin-resistant strain KY17 (from 1 to 240) fused with the remaining 166 amino acid residues from another penicillin-resistant strain DE1 (from 241 to 406). Plasmid pM10 was constructed in a way similar to that used in the construction of pM9. A PCR fragment encoding for the 240 amino acid residues from the position 1–240 was amplified with the primers ZOO46HI and MMUT9 (see above) and cloned into the HindIII/XbaI-digested pSF24 plasmid, generating the plasmid pSF26. Procedures similar to the ones used in the construction of pM9 were employed for the transfer of the murM chimeras from the E. coli plasmid pSF26 to the pneumococcal plasmid pLS578 generated from pM9.

An additional set of plasmids carrying chimeric murM mutants were constructed to more precisely define the region controlling the specificity of the reaction catalyzed by the MurM protein.

Plasmid pM11 carried a murM allele that was identical to that from strain KY17 (the MurM of which mainly adds serine residues to the muropeptide cross-bridges) except for the position between residues 244 and 245, which was replaced by two adjacent residues from the murM of strain DE1 (the MurM of which mainly adds alanine residues to the muropeptide cross-bridges).

Plasmid pM11 was constructed in several steps. A PCR fragment comprising the sequence that encodes for the residues from position 300 to the end of the MurM protein (the 106 amino acid residues from the C terminus) of strain KY17 was amplified with primers ZOO48HI (see above) and MMUT15 (5’-TGACTTACTGTCTACATTCAGCGGCGCG-3’). The PCR product was cloned into the HindIII/XhoI-digested pSF25 plasmid, yielding plasmid pM12. Next, the full gene was reconstructed by cloning in pM12 the PCR product obtained from the chromosomal DNA of strain KY17 using primers ZOO46HI and MMUT15 (5’-TGACTTACTGTCTACATTCAGCGGCGCG-3’). The finally reconstructed plasmid pM13 was used as a template for the amplification of the remaining 26 nucleotides from both ends of the plasmid, using primers ZOO46HI and MMUT15 (5’-TGACTTACTGTCTACATTCAGCGGCGCG-3’). The resulting plasmid, pM14, was used to transform E. coli DH5α to obtain a corresponding sequence from the murM of strain DE1 (the MurM of which mainly adds serine residues to the muropeptide cross-bridges).

Plasmid pM11 was constructed in several steps. A PCR fragment comprising the sequence that encodes for the residues from position 300 to the end of the MurM protein (the 106 amino acid residues from the C terminus) of strain KY17 was amplified with primers ZOO48HI (see above) and MMUT15 (5’-TGACTTACTGTCTACATTCAGCGGCGCG-3’). The PCR product was cloned into the HindIII/XhoI-digested pSF25 plasmid, yielding plasmid pM12. Next, the full gene was reconstructed by cloning in pM12 the PCR product obtained from the chromosomal DNA of strain KY17 using primers ZOO46HI and MMUT15 (5’-TGACTTACTGTCTACATTCAGCGGCGCG-3’). The finally reconstructed plasmid pM13 was used as a template for the amplification of the remaining 26 nucleotides from both ends of the plasmid, using primers ZOO46HI and MMUT15 (5’-TGACTTACTGTCTACATTCAGCGGCGCG-3’). The resulting plasmid, pM14, was used to transform E. coli DH5α to obtain a corresponding sequence from the murM of strain DE1 (the MurM of which mainly adds serine residues to the muropeptide cross-bridges).

As plasmid pM11, here too, the chimera mutant was initially constructed in an E. coli plasmid. The PCR product obtained from plasmid pM14 with primers ZOO48NC and MMUT15 (5’-TGACTTACTGTCTACATTCAGCGGCGCG-3’) was subcloned into the HindIII/XhoI-digested E. coli plasmid pSF25 (pGEM-3Z carrying a promoterless murN). The final plasmid pM15 was used to transform the pneumococcal strain JS578, carrying the wild-type murM allele, to obtain a corresponding sequence from the murM of strain DE1 (the MurM of which mainly adds serine residues to the muropeptide cross-bridges).
Structure and Function of the MurM Protein in S. pneumoniae

TCTCGAGAATCTTCGATGCAGG-3', where the HindIII and XhoI restriction sites are underlined), was first cloned into the HindIII/XhoI-digested pSF22 plasmid. The resulting plasmid, pSF30, carried a chimeric MurM protein that was deleted by cloning into the pLS578 plasmid a PCR fragment amplified from chromosomal DNA of strain KY17 (double-underlined). The resulting plasmid, pSF31, carried a chimeric MurM protein that was equal to the KY17 MurM except for the position between residues 240 and 275, which came from the murM of strain DE1. After amplification from plasmid pSF31 with primers ZOO48HII and ZOO49NC (see above), the murM chimera was cloned into the HindIII/XhoI-digested pLS578 plasmid, yielding the plasmid pM12.

MurM deletion Mutants—Several simple variants of plasmid pM3, each carrying incomplete murM genes from strain KY17, were also constructed. In plasmid pM13, 50 residues of the C terminus of MurM were deleted by cloning into the pLS578 plasmid a PCR fragment amplified by the following pair of primers: ZOO48HII (see above) and MDC3535N (5'-AACGCAATTATGGATTTACATCCCA-3') that includes an NcoI restriction site (underlined) and a premature stop codon (double-underlined).

In plasmid pM14, 50 residues of the N terminus of MurM were deleted by cloning into the pLS578 plasmid a PCR fragment amplified by the following pair of primers, ZOO49NC (see above) and MNDN68 (5'-ATCAAGCTTAAAGGAGGCTATATGCAGC-3'), which includes an NcoI restriction site (underlined) and an initiation codon (double-underlined).

In plasmid pM15, 10 residues were deleted from the C terminus by cloning into the pLS578 plasmid the PCR product from the KY17 chromosomal DNA using primers ZOO48HII and MDC3956F (5'-CATGCTCAGTGAATATCAAGGACCTGTAACACACCC-3'), which includes an NcoI restriction site (underlined) and a premature stop codon (double-underlined).

In plasmid pM16, the segment between residues 240 and 275 was deleted. To construct this murM deletion mutant we cloned into the E. coli HindIII/XhoI-digested plasmid pSF50 (that carries a DNA fragment encoding the last 131 residues of the C-terminal from KY17 MurM) the PCR product obtained with primers ZOO48HII and MMT3 from the KY17 chromosomal DNA. This PCR product encodes the first 240 amino acid residues from the N terminus. The resulting plasmid, pSF32, carried a murM gene that coded for a protein equal to KY17 except for the deletion between residues 240 and 275. This plasmid with the deletion was then amplified with primers ZOO48HII and ZOO49NC (see above) and cloned into the pneumococcal plasmid pLS578, yielding the plasmid pM16.

Single Residue MurM Mutants—The murM allele from KY17 was also mutated to test the possible importance of some amino acid residues for the specificity of the MurM-catalyzed reaction. The particular residues to be replaced were threonine in position 260 and glutamine in position 27 of the MurM protein. In plasmid pM17 the threonine residue at position 260 of the KY17 murM was mutated to lysine, whereas in plasmids pM18 and pM19 the glutamine residues at position 27 were mutated to either glutamate (pM18) or to threonine (pM19).

The following procedures were used. First, the murM gene was amplified from chromosomal DNA of strain KY17 using primers ZOO48HII and ZOO49NC (see above) and cloned into the HindIII/NcoI site of pSF22, yielding the plasmid pSF23. Then the threonine in the position 260 of the cloned murM was mutated to lysine by using the QuikChange site-directed mutagenesis kit (Stratagene) and the primer PRT260K (5'-GGACCTTGAGAAGTCTGACTGCGG-3') and its reverse PRT260K, yielding the plasmid pSF33 (the mutated codon is in boldface). The glutamine residue in position 27 was also mutated by the same procedure to a glutamate with the primer PDQ27E (5'-GGAATTACATGTTAAGTAGTTGCTGGAAG-3') and its reverse sequence PRT27E, and to a threonine with the primer PDQ27T (5'-AGTAATGCTGAATGTATGCTGGAAG-3') and its reverse, yielding the plasmids pSF34 and pSF35, respectively (the mutated codons are in boldface). Both mutations were confirmed by sequencing. Eventually the mutated murM genes were then transferred to the pneumococcal plasmid pLS578.

Cell Wall Preparation and Enzymatic Digestion—Pneumococcal cell walls were prepared by a previously published method (2, 15) except for the process of breaking the cells that was done by shaking with acid-washed glass beads with the help of FastPrep FP120 (Bio 101 Inc.). Cell wall material (2 mg) was suspended in 25 mM sodium phosphate buffer, pH 7.4, and treated with affinity-purified pneumococcal amidasase (5–10 μg) at 37 °C for 12–18 h with constant stirring. The products were dried, the precipitate was washed with acetone, and the peptides were extracted with acetonitrile-isopropanol-water (25:25:50, v/v) containing 0.1% trifluoroacetic acid as already described (2, 15, 16). After removal of the organic phase by SpeedVac, the peptides were dissolved in 0.1% trifluoroacetic acid.

Separation and Analysis of the Cell Wall Stem Peptides—Peptides were separated with a Shimazu LC-10AVP HPLC system, as described previously (2). The column used was a Vydac model 218TP54 (Separations Group). Peptides were eluted with an 80-min linear gradient from 0 to 10% of 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 UV absorption at 210 nm (A210).

Isolation and Analysis of S. pneumoniae Peptidoglycan Precursors—For analysis of cytoplasmic (UDP-linked) peptidoglycan precursors of S. pneumoniae cultures were grown in C+Y medium to an A590 of 0.3 at which time vancomycin was added at a final concentration of 5 μg/ml (10X MIC). The cultures were incubated for 60 min, harvested by centrifugation, and washed with 50 mM Tris-HCl, pH 8.0. The UDP-linked cell wall precursors were extracted by cold 5% trichloroacetic acid for 30 min. The extract containing the pool of precursors was separated by gel filtration on a Sephadex G-25 column (Amersham Pharmacia Biotech) and washed twice with 1 volume of water. The UDP-linked precursors were then applied to a 3.9×30-mm reverse-phase column (μBondapack C18, Waters) as previously described (17) except that the UDP-linked precursors were eluted with an 80-min linear gradient from 0 to 15% of 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 UV absorption at 254 nm.

Lipid-linked cell wall precursors were prepared essentially as described by Kohlrausch and Holtje (18). Cultures were grown in C+Y medium to an A590 of 0.3 at which time vancomycin at a final concentration of 5 μg/ml (10X MIC) was added. The cultures were incubated for one additional generation period, harvested by centrifugation, and washed with 50 mM Tris-HCl buffer, pH 8.0. The lipid-linked precursors were extracted with ice-cold n-butanol/6% pyridine at 4 °C overnight. The organic phase was collected, dried twice with 1 volume of water, and dried in a SpeedVac. The lipid precursors in the organic extraction phase were hydrolyzed with 0.1 M HCl for 15 min in a boiling water bath to yield GlcNAcβ-1,4-MurNAc peptide derivatives. Before HPLC separation, one volume of 0.5 M borate buffer, pH 9.0, was added to this muramyl residue-containing fraction. The muramyl residues were then reduced to muramyl tetrapeptides with sodium borohydride and analyzed by reverse-phase HPLC, as previously described (19). The amino acid composition of the eluted peaks was determined at the Rockefeller University Protein/DNA Technology Center.

RESULTS

The Addition of Peptide Branches to the S. pneumoniae Muropeptides Occurs in the Plasma Membrane Fraction—To identify the step of the cell wall biosynthetic pathway in which serine or alanine are added to the ε-amino group of the muropeptide lysine residues, we determined the composition of the cytoplasmic and lipid-linked precursors in the penicillin-resistant S. pneumoniae strain Pen6 and its murM deletion mutant (Table I). Both cultures were grown to early exponential phase, at which time vancomycin was added to cause accumulation of UDP-linked (cytoplasmic) or lipid-linked (plasma membrane) cell wall precursors. After removal of the lipid carrier by mild acid hydrolysis, followed by sodium borohydride reduction of the released muramyl peptide component(s), these were analyzed by RP-HPLC and their amino acid compositions were determined. In the lipid extract of the Pen6 murM null mutant, one major peak, c, was identified (Fig. 4A). Two small additional peaks were also consistently detected. The chemical nature of these is unknown. Amino acid analysis showed the presence of glutamic acid, lysine, and alanine in the molar ratio of 1.0, 0.8, and 2.75 suggesting an unsubstituted disaccharide pentapeptide (Glu 1.0, Lys 1.0, Ala 3.0). Peak c had a retention time on the RP-HPLC column identical to that of the unsubstituted

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disaccharide pentapeptide component of *Staphylococcus aureus* peptidoglycan (19).

The lipid extract of strain Pen6 showed accumulation of 2 major peaks (Fig. 1A). Several additional minor peaks were also present. The nature of these is not known at the present time. Amino acid analysis of peak a showed the presence of glutamic acid, lysine, alanine, and serine in the molar ratios of 1:0.8, 3.8, and 1.1, which is consistent with the structure of a disaccharide pentapeptide carrying a seryl-alanine branch (Glu 1.0, Lys 1.0, Ala 4.0, Ser 1.0). Amino acid analysis of the second peak b showed the presence of glutamic acid (1.0), lysine (0.8), and alanine (4.4) with small amounts of contaminating serine consistent with the structure of peak b as the disaccharide pentapeptide containing an alanyl-alanine branch. The small deficit in alanine content (4.4 mol instead of the expected 5.0) together with the presence of small amounts of serine indicate peak b also contains small amounts of seryl-alanine-containing muropeptide. The relative amounts of peak a to peak b was 59 and 41%, respectively, similar to the ratio of seryl-alanine (55%)- and alanyl-alanine (45%)-containing muropeptides in the peptidoglycan of strain Pen6 (Table II) (4).

To rule out that the reaction catalyzed by MurM (addition of serine or alanine to the ε-amino group of the lysine residue) may also occur in the cytoplasmic fraction, we analyzed the composition of the UDP-linked precursors in the Pen6 and its murM null mutant. Extracts from both strains showed accumulation of a single major peak a and two smaller peaks (Fig. 1B). Peak a had the same retention time on the RP-HPLC column as the UDP-linked disaccharide pentapeptide identi-
fied in the cytoplasmic cell wall precursor fraction of several bacteria (17). The nature of the smaller peaks is not known.

**Alterations in the Cell Wall Composition of S. pneumoniae Strain R36A Carrying Different murM Alleles on Plasmids**

Three murM alleles described previously (5) were cloned in the pLS578 plasmid and transformed into strain R36A (a penicillin-susceptible laboratory strain with a relatively low percentage of branched stem peptides). Two of the murM alleles cloned into pLS758 had mosaic structure and came from the penicillin-resistant clinical isolates DE1 and KY17 (5). The third murM came from strain R36A. The peptidoglycan of the two penicillin-resistant strains had a high proportion of branched muropeptides: 77% in strain DE1 and 84% in KY17, as compared with strain R36A in which only 41% of muropeptides had branched structure. The two penicillin-resistant strains also had distinct cell wall stem peptide composition that differed from the composition of strain R36A (see the HPLC chromatograms of Figs. 2 and 3; see also Table III). Strain DE1 had a high percentage of branched peptides with alanine as the first residue of the cross-bridge (monomeric peptide I, 23.9%; dimeric peptide VI, 28.8%). The peptidoglycan of strain KY17 had an increased percentage of branched peptides with serine as the first residue of the cross-bridge (monomeric peptide 3, 29.5%; dimeric peptide 7, 21%). Introduction of the cloned murM alleles in plasmid pLS578 into strain R36A caused a significant increase in the proportion of branched peptides in the peptidoglycan of the transformants, from 41% in the recipient strain to 93 and 90% in transformants R36ApM1 and R36ApM2, respectively. Furthermore, the shifts in peptidoglycan composition observed in the transformants closely paralleled the composition of peptidoglycan in strains DE1 and KY17. In R36ApM2 carrying the murM allele from DE1, the amount of peptides with alanyl-alanine branches increased from 1.7 to 24.7% (peptide I) and from 4.0 to 40.8% (peptide VI). In the peptidoglycan of R36ApM3 carrying the murM allele from KY17, the amount of peptides with seryl-alanine branches increased from 2.7 to 19.9% (peptide III) and from 6.5 to 23.3% (peptide 7).

Although the peptidoglycan composition of the R36A transformants and the respective clinical isolates from which the two murM alleles were amplified was very similar, there were also several differences. For instance, strain DE1 had a higher percentage of linear monomeric peptide 1 and 6 compared to the transformant. This is also suggested by the fact that, when the murM allele from R36A was introduced through

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**Fig. 1.** Analysis of the cell wall precursors by RP-HPLC of Pen6 and its murM deletion mutant. Lipid-linked precursors (A) and UDP-linked precursors (B) from Pen6 and its murM deletion mutant were prepared, and its composition was determined by HPLC as described under “Experimental Procedures.”
R36ApM1, the peptidoglycan of the transformant showed a major increase in the percentage of branched peptides from 41 to 93% whereas the proportion of the branched peptide, containing serine and alanine or only alanine in the cross-bridge composition, remained unaltered (Table III and Fig. 4). In the peptidoglycan of R36ApM1 carrying the murM allele from strain R36A in the plasmid (in addition to the chromosomal encoded), the amount of peptides with seryl-alanine branches increased from 2.7 to 20.2% (peptide III) and from 6.5 to 24.5% (peptide 7).

These results show that, although there are two copies of murM (one in the chromosome and one in the pLS578 plasmid) in the R36A transformants, the composition of the peptidoglycan of these strains is determined by the plasmid-encoded murM, present in a much higher copy number or under the control of a stronger promoter. Additionally, the overexpression of the murM protein did not alter the specificity of the reaction. The strain R36A, with only a copy of the murM protein did not alter the specificity of the reaction. The mutant alleles that resulted from permuting different regions of the MurM protein from strains R36A, KY17, and DE1 were cloned into plasmid pLS578, which was then introduced into strain R36A by genetic transformation, and the peptidoglycan composition of the transformants was analyzed.

Plasmid pM9 was constructed to carry a murM allele that encoded a protein with residues 1 through 240 from the R36A MurM, and residues 241 through 406 were from strain DE1. The peptidoglycan of transformant R36ApM9 was enriched for branched peptides with alanine as the first residue of the cross-bridge (81% of all branched peptides), and this was similar to the composition of peptidoglycan in strain DE1 and in R36ApM2. Virtually identical results were obtained with construct pM10 in which the cloned MurM had residues 1 through 240 that were derived from strain KY17 and residues 241 through 406 came from strain DE1. In transformant R36ApM10, the percentage of branched peptides with alanine as the first residue of the cross-bridge was 80%. These results suggested that the control of specificity of the MurM-catalyzed reaction was within the 241–406 sequence of the protein.

To further define this region two additional fusion mutants were constructed. Plasmid pM11 had a cloned MurM with the entire amino acid sequence derived from strain R36A except for the sequence between residues 244 and 298, which was identical to that of the MurM from strain DE1. The peptidoglycan of transformant R36ApM11 was enriched for branched peptides with alanine residues in the cross-bridge (82%). These results narrowed down the region responsible for the reaction specificity to the sequence between residues 244 and 298. In the second fusion mutant pM12, the entire cloned MurM was from strain KY17 except for the sequence between residues 244 and 274, which came from strain DE1. The peptidoglycan composition of transformant R36ApM12 was again very similar to that of strain DE1 or transformants R36ApM11 and R36ApM2: 81% of the branched reaction resides in the polymorphic regions of murM, specifically within the amino acid residues 229–300 (see Fig. 4C). To more precisely locate this region, chimeric mutants of murM were constructed from segments of the murM gene from strains DE1, KY17, and R36A (Fig. 3).

| Peptide | Pen6 | R36A | Pen6ΔmurM | Pen6ΔmurM pM3N | R36ApM3 | Pen6ΔmurM pM9N | R36ApM9 |
|---------|------|------|-----------|----------------|---------|----------------|---------|
| 1       | 3.0  | 15.9 | 41.4      | 1.0            | 3.2     | 1.0            | 0.9     |
| 2       | 0.8  | 4.0  | 4.9       | 0.7            | 1.0     | 0.1            | 0.0     |
| 3       | 16.1 | 2.7  | 0.1       | 19.3           | 19.9    | 0.7            | 1.3     |
| 4       | 15.1 | 1.7  | 0.1       | 3.1            | 7.5     | 24.3           | 26.0    |
| 5       | 2.8  | 1.7  | 0.2       | 1.8            | 2.4     | 5.6            | 4.1     |
| 6       | 2.8  | 25.4 | 45.4      | 3.5            | 0.6     | 0.8            | 0.8     |
| 7       | 2.8  | 2.1  | 0.4       | 0.9            | 1.1     | 6.8            | 1.4     |
| 8       | 2.2  | 11.8 | 3.7       | 0.9            | 2.0     | 0.4            | 0.1     |
| 9       | 2.7  | 6.9  | 0.1       | 2.9            | 1.4     | 1.7            | 1.1     |
| 10      | 11.6 | 6.5  | 0.4       | 25.0           | 23.3    | 3.6            | 0.5     |
| 11      | 8.2  | 2.0  | 0.3       | 4.9            | 9.3     | 4.1            | 1.2     |
| 12      | 12.1 | 2.4  | 0.5       | 9.4            | 10.1    | 12.7           | 2.7     |
| 13      | 2.0  | 7.9  | 0.2       | 9.7            | 4.0     | 1.6            | 2.6     |
| 14      | 10.8 | 4.0  | 0.6       | 9.4            | 5.2     | 33.2           | 45.8    |
| 15      | 7.1  | 4.9  | 1.6       | 7.4            | 9.0     | 3.2            | 11.5    |

**TABLE II**

Cell wall peptide composition of several strains of S. pneumoniae
peptides of this transformant were composed of alanyl-alanine.

Attempts to pinpoint individual amino acid residues within the 30-amino acid sequence between residues 244 and 274 were only partially successful. In the MurM from strain KY17 (which incorporates mainly serine) the amino acid residue at position 260 is threonine, whereas in the MurM from strain DE1 (which incorporates mainly alanine) position 260 is a lysine. In plasmid pM17, carrying an intact murM allele from strain KY17, the codon encoding the threonine at position 260 was mutated by site-directed mutagenesis to one that encoded lysine. In the corresponding transformant R36ApM17, the proportion of branched peptides increased from 41 to 92% indicating that the mutant protein was highly active. The increased amount of branched peptides is very similar to the value of 93% in the R36ApM1 transformant. However, the percentage of the branched peptides that had alanine as the first residue of the cross-bridge was only increased from 27% (in strain R36ApM3) to 46% in R36ApM17. In transformants carrying the entire 30-amino acid sequence between residues 244 and 274 from strain DE1, the corresponding increase in alanine containing cross-bridges was much larger: from 27 to 81% (see Fig. 5). These results indicate that residues other than lysine in position 260 are also involved with defining the specificity of MurM.

In two other MurM mutants cloned into pLS578, the glutamine residue in position 27 of the MurM of strain KY17 was mutated to glutamate (in pM18) or to threonine (in pM19). This amino acid has been suggested as being involved in the cata-

**Fig. 2. Analysis of cell wall stem peptides by RP-HPLC of several strains.** Cell walls were prepared from strains R36A and DE1 and R36A transformant with the murM from DE1 (R36ApM2); strain KY17 and the R36A transformant with the KY17 murM allele (R36ApM3). Peptidoglycan compositions were analyzed by RP-HPLC.
lytic site of a homologous protein in *Lactobacillus viridescens* (21). The proportion of branched peptides was found to be normally increased in transformants carrying these mutant forms of the KY17 *murM* allele, but there was no change in the chemical composition of the branches.

**Analysis of MurM Deletion Mutants**—Several deletion mutants were constructed from the *murM* allele of strain KY17, and the deleted *murMs* cloned into pLS578 were introduced into strain R36A by genetic transformation. No activity could be detected by plasmids carrying *murM* genes from which 50 amino acid residues were removed at the C terminus (pM13), the N terminus (pM14), or 10 amino acids removed at the C terminus (pM15) or in plasmids in which the sequence of MurM between residues 240 and 274 were deleted. The peptidoglycan of such transformants showed no increase in the percentage of branched structured stem peptides (see Fig. 5).

**Construction of a Pen6 murM Null Mutant and Complementation with murM Encoded in a Plasmid**—To rule out the possibility that the R36A chromosome encoded MurM may have a significant contribution in the alteration in the cell wall structure and function of the MurM protein in *S. pneumoniae*.
composition, we constructed a mutant in which the murM gene was replaced by the erm marker from the pJDC9 plasmid (22). Confirmation of the erythromycin-resistant Pen6 murM null mutant was obtained by sequencing the PCR fragment amplified with primers ZOO30 and ZOO45EC (including the murMN region). Cell wall analysis showed no branched structured stem peptides in the Pen6/H9004 murM mutant. The composition of the peptidoglycan was identical to that of Pen6 murMN, a mutant obtained by insertion of a suicide plasmid in the murM gene (4).

We cloned two different MurM constructs with different specificities for alanine and serine in the murM null mutant. One of these constructs was the MurM from the penicillin-resistant strain KY17, which has serine-rich branched peptides. Analysis of the cell wall composition showed an accumulation of a peptide with retention time similar to that of the semi-branched peptide 3a (data not shown), carrying only a single serine residue in the cross-bridge (6). This result suggests that the expression of murN gene in the chromosome with the erm promoter was not sufficient to fully recover the cell wall composition. To compensate for this, both genes (the murM allele from KY17 and the murN gene from R36A strain) were cloned in pLS578 under the control of the conserved plasmid promoter and were transformed into Pen6 murM. The composition of the peptidoglycan of this transformant (Pen6 murM pM3N) was rich in serine-containing branched peptides, similar to the composition found in the KY17 strain (Table II). There was a decrease in the percentage of linear stem peptide 1 from 41.4% in Pen6 murM to 1% in the transformant. This value was similar to the 3.2% found in R36ApM3. There was also a decrease in the linear dimeric peptide 4 from 45.4% in

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Role of MurM alleles in defining the chemical composition of branched cell wall stem peptides. A, peptidoglycan composition of strains carrying different murM alleles. B, schematic representation of the mosaic regions in the murM genes. The black region indicates more than 10% divergence relatively to the MurM from the penicillin-susceptible reference strain R36A. C, distribution of polymorphic sites among the different murM proteins. The murM alleles were sequenced from the penicillin-susceptible laboratory strain R36A and from the penicillin-resistant strains listed (5). Numbers above the sequences (beginning at the first residue of MurM: residue 10) identify positions at which an amino acid alteration was detected in one of the MurM proteins. Only positions with altered residues are shown; residues identical to those in strain R36A are indicated by dots.

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Regions in the MurM proteins that define the chemical composition of branched stem peptides. The sequence of murM alleles characteristic of strains R36A (cross-hatched), DE1 (black), and KY17 (white), and the murM chimera mutants and murM deletions, are indicated by the drawings. The percentages of branched peptides that have serine or alanine in the first position of the cross-bridge are also tabulated for the various strains and murM constructs.
Pen6αmurM to 3.5% in the transformant. This value was similar to the 0.6% found in R36ApM3. The proportion of branched peptide 3 increased from 0.1 to 19.3% and so did the corresponding dimeric peptide 7, from 0.4 to 25% (Table III). The corresponding values for peptides 3 and 7 in the R36ApM3 transformants were 19.9 and 23.3%, respectively. The percentage of all branched peptides changed from 6% in the Pen6αmurM mutant to 86% in the transformant carrying the pM3N plasmid. Of these branched peptides, 75% had serine as the first residue of the cross-bridge whereas in the remaining 25% the first residue was alanine. This proportion is very similar to that found in R36ApM3 (73 and 27%, respectively).

Additionally, we cloned a derivative from the DE1 murM allele, a chimeric murM mutant in which residues 1–240 came from the MurM of R36A, and residues 241–406 from the MurM of strain DE1. This construct specifically incorporated alanine when cloned into strain R36A (R36ApM9) (Fig. 5). This murM allele was cloned together with the murN gene in pLS578 (pM9N) and transformed into the Pen6 murM null mutant. The peptidoglycan of this construct was enriched in branched peptides with alanine as the first residue of the cross-bridge, as expected from the cell wall composition of DE1 strain. The percentage of linear peptides 1 and 4 was reduced to 1.0 and 0.8%, respectively. Similar values, 0.9 and 0.8, were found in R36ApM9. Concomitantly, there was accumulation of branched stem peptides with only alanine in the cross-bridge. The proportion of peptide I increased from 0.1 to 24.3% and peptide VI from 0.6 to 33.2%. The corresponding values in R36ApM9 were 26 and 45.8%, respectively. The total amount of branched peptides increased from 6% in the Pen6αmurM mutant to 96% in the transformant with plasmid pM9N.

These results make it unlikely that the chromosomally encoded MurM from R36A has made a significant contribution to the cell wall structure in R36A transformants carrying the plasmid encoded MurM.

**DISCUSSION**

A unique feature of the peptidoglycan of *S. pneumoniae* is that it contains both linear as well as branched stem peptide residues, and dimeric and higher oligomeric components are cross-linked both directly as well as indirectly through short cross-bridges composed of dipeptides of either alanyl-alanine or seryl-alanine residues. This feature of the pneumococcal cell wall positions it as a mixed A3 alpha and A1 alpha type in the taxonomic scheme proposed by Schleifer and Kandler (23). The introduction of the RP-HPLC method for the analysis of pneumococcal cell walls (2, 24, 25) has resolved the pneumococcal peptidoglycan to a large number (over 25) of muropeptide components allowing the identification of the chemical structures of these linear and branched cell wall components. Examination of a large number of *S. pneumoniae* clinical isolates recovered from different isolation and geographic sites, at different time periods and expressing a variety of different capsular serotypes, showed that the muropeptide composition of the cell walls was preserved with remarkable accuracy from strain to strain, suggesting the existence of a cell wall composition that is specific for the species of *S. pneumoniae* (1–3). The notion of precise genetic control over cell wall composition has received new support through the examination of cell wall composition in penicillin-resistant *S. pneumoniae* clinical isolates. Several genetic lineages of these resistant bacteria showed an unusually large proportion of branched structured muropeptides in their cell walls, and the cell wall composition also appeared to be specific for the particular clone in terms of the chemical nature of the branched peptides: In some of the clones the amino acid serine predominated as the first amino acid of the dipeptide branch, whereas in other clones the first amino acid was alanine.

What made this structure variation particularly interesting was the observation made in 1990 that the abnormal, highly branched, cell wall composition of a penicillin-resistant South African isolate of *S. pneumoniae* was co-transferred along with the penicillin resistance trait during genetic transformation in which resistance to penicillin was selected for (8, 15). This finding suggested some functional connection between the abundance of branched stem peptides in the cell wall and resistance to penicillin.

The recent identification of the murMN operon in *S. pneumoniae* has opened up this area for new exploration. Inactivation of the murMN operon was reported to cause a disappearance of branched stem peptides from the cell wall and also a complete suppression of the penicillin-resistant phenotype. This indicated to us that further exploration of the mode of action and regulation of this operon would yield valuable new insights both into the mechanism of control of cell wall structure and also into the mechanism of expression of penicillin resistance (4).

Comparison of the DNA sequences of murM and murN genes was determined in a large number of penicillin-susceptible and penicillin-resistant clinical isolates of *S. pneumoniae*. Although the structure of murN genes appeared to be highly conserved showing little sequence variation, a considerable amount of polymorphism was noted in murM genes identified in several penicillin-resistant *S. pneumoniae* isolates (5). The site of involvement of these two genes in the production of the dipeptide branches was clarified (6). Selective inactivation of murN produced a unique type of peptidoglycan not seen among clinical isolates: Such bacteria contained branched stem peptides made up of only a single amino acid residue, the chemical nature of which remained the same as in the parental strain. Besides reduction of the dipeptide branches to a single amino acid, there was no other change in the nature and/or proportion of various structurally distinct muropeptides or in the degree of peptidoglycan cross-linking in these murN mutants. These observations identified the role of murN as the determinant involved with the addition of the second amino acid residue to the dipeptide branches. In concordance with the highly conserved sequence of murN, the nature of the second amino acid residue in the branched peptides was invariably alanine in all the large number of *S. pneumoniae* isolates examined.

The results of these studies suggested that the primary determinant responsible for the clone-specific variation in the structure of the pneumococcal cell walls was the murM gene, and the results of experiments described in this communication provide unequivocal evidence for this proposition.

The evidence identifying murM as the gene primarily responsible for the clone-specific variation in the pneumococcal cell wall was based on an experimental system in which the various murM alleles identified in *S. pneumoniae* isolates were cloned in the pneumococcal plasmid pLS578, which is capable of independent replication in pneumococci (10). These recombinant plasmids were then introduced into the isogenic background of the penicillin-susceptible *S. pneumoniae* strain R36A, and the activity and specificity of the particular murM allele were assessed by determining the impact of the plasmid-borne murM genes on the composition of the peptidoglycan of such transformants. The particular murM alleles used in most of these genetic crosses were murMA, carried by penicillin-susceptible strains of *S. pneumoniae*, murMB3 carried by the penicillin-resistant strain DE1, and murMB5 carried by the penicillin-resistant strain KY17 (5). Strains carrying murMA produce branched peptides the majority of which (71%) have
serine as the first amino acid residue. Strains carrying the murMB3 allele have branched peptides in which most of the amino acid residues (82%) in the first position of the branches is alanine. In strains that carry the murMB5 allele, the first amino酸 residue in the branches (78%) is serine. Analysis of these genetic crosses demonstrated the importance of the particular murM allele both for the percentage representation of branched peptides in the cell wall and also for their chemical nature. The experiments documented in Fig. 5 indicate that the abundance of branched muropeptides in the cell wall depends on the rate of transcription and/or copy number of murM. Introduction of each one of the murM alleles on plasmid pLS578, even the allele murMA identical to the one resident on the chromosome of the recipient cell R36A, caused a massive increase in the percentage of branched peptides in the cell wall. The most likely interpretation of this finding is that the well documented activity of the powerful promoter present on plasmid pLS578 (10) causes extensive transcription of murM in the transformants.

The experiments with the genetic crosses also provide clear documentation that the chemical nature of the branched peptides, i.e. whether the first amino acid residue is serine or alanine, is primarily determined by the structure of the MurM protein. The composition of the peptidoglycan of strain R36A that was the common recipient in all the genetic crosses has undergone extensive changes from serine-rich to alanine-rich branched peptides and also in the percentage of total branched peptides by the introduction of the appropriate murM alleles.

Experiments with the murM deletion mutants showed that these activities of the MurM protein depended on intact C and N termini of the protein. Deletion of 10 or 50 amino acid residues from the C terminus or removal of 50 amino acids from the N terminus caused inactivation of the MurM protein.

The chemical specificity of murM, i.e. whether the protein catalyzed the introduction of serine or alanine as the first residue of the branched peptides, was localized to a 30-amino acid sequence between residues 244 and 274 of the protein.

Biochemical analysis of the cell wall precursor pools has identified the lipid II as the substrate of the MurM protein. This is in contrast to the recently described case for Lactobacillus viridescens in which cell wall branched peptides were shown to be attached to the disaccharide pentapeptide precursor in the cytoplasmic stage of cell wall biosynthesis (21, 26). Our data show that in S. pneumoniae this reaction takes place in the plasma membrane.

The large variation in peptidoglycan composition identified in the plasmid transformants did not seem to cause any perceptible growth defect in the transformants nor in the morphology of the bacteria. Despite the radical changes in the proportion of branched peptides and also in the chemical nature of the branches, other aspects of the structure of peptidoglycan such as the proportion of monomeric and oligomeric muropeptides or the degree of cross-linking remained unchanged in the genetic transformants. In the plasmid transformants R36ApM2 and R36ApM3, the total number of branched muropeptides increased from 41% in strain R36A to 90 and 59%, respectively, in the transformants. Despite this radical change in wall composition, bacterial growth was not perceptively affected indicating that the penicillin binding proteins of the penicillin-susceptible strain R36A were able to utilize either linear or branched peptides with equal facility for cell wall biosynthesis.

It is also clear from the results that the introduction of murM alleles from penicillin-resistant bacteria to the penicillin-susceptible recipient R36A did not cause any increase in penicillin resistance. It seems that the presence of low affinity penicillin binding proteins remains an indispensable prerequisite for the resistant phenotype. The mechanism responsible for the dramatic loss of penicillin resistance in S. pneumoniae with inactivated murMN is the subject of a forthcoming communication. 2

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Functional Analysis of *Streptococcus pneumoniae* MurM Reveals the Region Responsible for Its Specificity in the Synthesis of Branched Cell Wall Peptides

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