Inhibition of the expression of penicillin resistance in *Streptococcus pneumoniae* by inactivation of cell wall muropeptide branching genes

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Penicillin-resistant strains of *Streptococcus pneumoniae* contain low affinity penicillin-binding proteins and often also produce abnormal indirectly crosslinked cell walls. However the relationship between cell wall abnormality and penicillin resistance has remained obscure. We now show that the genome of *S. pneumoniae* contains an operon composed of two genes (*murM* and *murN*) that encode enzymes involved with the biosynthesis of branched structured cell wall muropeptides. The sequences of *murMN* were compared in two strains: the penicillin-susceptible strain R36A producing the species-specific pneumococcal cell wall peptidoglycan in which branched stem peptides are rare, and the highly penicillin-resistant transformant strain Pen6, the cell wall of which is enriched for branched-structured stem peptides. The two strains carried different *murM* alleles: *murM* of the penicillin-resistant strain Pen6 had a ''mosaic'' structure encoding a protein that was only 86.5% identical to the product of *murM* identified in the isogenic penicillin-susceptible strain R36A. Mutants of R36A and Pen6 in which the *murMN* operon was interrupted by insertion-duplication mutagenesis produced peptidoglycan from which all branched muropeptide components were missing. The insertional mutant of Pen6 carried a *pbp2x* gene with the same ''mosaic'' sequence found in Pen6. On the other hand, inactivation of *murMN* in strain Pen6 and other resistant strains caused a virtually complete loss of penicillin resistance. Our observations indicate that the capacity to produce branched cell wall precursors plays a critical role in the expression of penicillin resistance in *S. pneumoniae*.

During the three decades after their first detection in clinical specimens in the late l960s, penicillin-resistant and multiresistant strains of *Streptococcus pneumoniae* have achieved a global spread and have become a major public health concern. The molecular mechanism of penicillin resistance in this pathogen was shown to involve remodeling of the β lactam target enzymes: the penicillin binding proteins (PBPs) in such a way that their affinity is greatly reduced toward the antibiotic molecule (1). The physiological function(s) of PBPs is in terminal stages of bacterial cell wall peptidoglycan assembly, and it was suggested that the reduced affinity of penicillin-resistant PBPs also may affect their catalytic performance (2) with their natural substrates—the cell wall precursor muropeptides—the D-alanyl-D-alanine carboxyl terminal that has close structural analogy to the β-lactam ring. This proposal was based on the intriguing observation that penicillin-resistant clones of pneumococci often were found to produce cell wall peptidoglycans of grossly abnormal muropeptide composition (2, 3). A common feature of this structural abnormality is the replacement of linear structured muropeptides typical of the peptidoglycan of penicillin-susceptible strains with branched structured muropeptides carrying short alanyl- or seryl-alanine substituents on the epsilon amino group of the lysine residues; such branched components are rare in the cell walls of penicillin-susceptible strains (2–4). The frequent occurrence of such a distorted cell wall composition among penicillin-resistant strains suggested some association between the mechanism of penicillin resistance and the chemical abnormality of cell walls, but the precise nature of this relationship has remained unclear. In this paper, we describe our search for and identification of the genetic determinants *murM* and *murN*, which are involved with the biosynthesis of branched cell wall peptides. We report that the inactivation of *murMN* in penicillin-resistant strains results in the ''correction'' of cell wall abnormality and a virtually complete loss of penicillin resistance, indicating that the functioning of these two non-PBP genes is an integral component of the penicillin-resistance mechanism in pneumococci.

Experimental Procedures

Strains and Growth Conditions. All strains and plasmids used in this study are listed in Table 1. The *S. pneumoniae* strains were grown in a casein-based semisynthetic medium C + Y at 37°C without aeration as described (3). *S. pneumoniae* and *Escherichia coli* strains containing the plasmid pJDC9 (9) or its derivatives were grown in the presence of 1 μg/ml and 1 mg/ml of erythromycin (Sigma), respectively.

Identification and Sequencing of *murMN* Region. To find the proteins responsible for the formation of the branched peptides, we searched the *S. pneumoniae* incomplete genome database obtained from The Institute for Genomic Research for homologous proteins of the FmhB of *Staphylococcus aureus* (GenBank accession no. AF106850). Based on the preliminary sequence obtained, we amplified by PCR the chromosomal region containing the *murMN* operon from the strains R36A and Pen6. The following primer pairs were used: ZOO3 (5′-AGCGCAGAAGAAGGAAAAAGAAC-3′) and ZOO4 (5′-TAAAGGCGATGGATGGTAACG-3′); ZOO6 (5′-TATGCCTCAGGAAACGACTTATCT-3′) and ZOO5 (5′-CCCCCATCAATCACAATCA-3′); ZOO7 (5′-CATAGCGCTGGAACTCAC-3′) and ZOO8 (5′-GCAGGGGCATAGAACTTA-3′). The following conditions were used: 94°C for 5 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 2 min; and one final extension step of 72°C for 5 min. The PCR program was used essentially for all amplifications except the extension time at 72°C that was different depending on the size of the PCR fragment to be amplified. DNA sequencing was done at the Rockefeller University Protein/DNA Technology Center with the

Abbreviations: PBP, penicillin binding protein; MIC, minimal inhibitory concentration.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AJ250764 (R36A), AJ250766 (Pen6), and AJ250767 (8249)].

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Table 1. Relevant properties of the strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Pen MIC, μg/ml	Reference
S. pneumoniae			
R36A	Pen^S laboratory strain	0.01	(5)
8249	Penicillin-resistant clinical isolate from South Africa	6.0	(1)
Pen6	R6Hex transformant with chromosomal DNA from penicillin-resistant clinical isolate 8249 and selected for Pen^R	6.0	(1)
Sp2150	Penicillin-resistant clinical isolate belonging to the international serotype 9/14 French/Spanish clone	1.0	(6)
Ala1	Penicillin-resistant serotype 6B clinical isolate from Alaska	0.12	(7)
Clev2	Penicillin-resistant serotype 23F clinical isolate belonging to the multiresistant Spanish/USA clone	1.6	(7)
Hun663	Penicillin-resistant serotype 19A clinical isolate from Hungary	1.6	(7)
Hun663Tr4Tr5	R6Hex transformant with chromosomal DNA from penicillin-resistant transformant Hun663Tr4 selected for Pen^R	1.6	(8)
E. coli DH5α	RecA endA1 gyrA96 thi-1 hsdR17 supE44relA1 φ80 dlac ZΔ M15		BRL
pJDC9	*E. coli* plasmid Ery^R		(9)
pZOO3	pJDC9/0.7 kb encoding amino acids 73–296 of *murN* from R36A		This study
pZOO5	pJDC9/0.4 kb encoding amino acids 20–179 of *murM* from R36A		This study
pZOO6	pJDC9/0.4 kb encoding amino acids 48–184 of *murM1* from Pen6		This study

Taq fluorescent dye terminator sequencing method using a Perkin–Elmer/Applied Biosystems model 377 automated sequencer.

DNA and RNA Methods. All routine DNA manipulations were performed by using standard methods (10, 11). DNA from *S. pneumoniae* was isolated as described (12). RNA was prepared from exponentially growing cultures of *S. pneumoniae* at OD_{590} of 0.5 and was extracted by using the FastRNA isolation kit (Bio 101) according to the recommendations of the manufacturer. RNA samples of 2.5 μg were eletrophoresed through a 1.2% agarose, 0.66 M formaldehyde gel in morpholinepropanesulfonic acid (Mops) running buffer (20 mM Mops/10 mM sodium acetate/2 mM EDTA, pH 7.0). Blotting of RNA onto Hybond N^+ membrane (Amersham Pharmacia) was performed with the Turbo Blotter Neutral Transfer System (Schleicher & Schuell).

A DNA probe corresponding to an internal fragment of the *murN* gene was amplified by using the GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (Perkin–Elmer) and primers ZOO12BM (5′-TATGGATCCGGTTTCTTCTCGTTCCT-3′) and ZOO13EC (5′-GCCGAATTCACCTGTTGTTAAGC-CATCA-3′). The DNA probe was radiolabeled with [α-^{32}P]dCTP (Amersham Pharmacia) by the random prime method using a Ready-to-Go labeling kit (Amersham Pharmacia) and hybridized under high-stringency conditions. The size of the transcript was estimated by comparison to RNA Markers G3191 (Promega).

Plasmids were isolated by using the Wizard Plus Minipreps DNA Purification System (Promega), and PCR products were purified by using the Wizard PCR Preps DNA Purification System (Promega).

Oligonucleotides were purchased from GIBCO/BRL. Nucleotide and derived amino acid sequences were analyzed by using DNASTAR software, and comparisons to the European Molecular Biology Laboratory/GenBank databases were done by using the BLAST algorithm.

Inactivation of the *murMN* Operon. For gene disruption experiments, internal fragments of *murM* (from R36A and Pen6) were amplified by PCR and cloned into pJDC9 (9). For amplification of the internal fragment of *murM* from R36A the following primers were used: ZOO14BM (5′-GCTGGATCCCAT-GAGAAGTTTGGTGTTTA-3′) and ZOO15EC (5′-GCT-GAATTCCTGTTCGAATAGCCTGTT-3′), giving origin to the plasmid pZOO5. Amplification of the internal fragment of *murM* from Pen6 was carried out by using the primers ZOO16BM (5′-TATGGATCCAGGGGAGAACTTACTG-GCTGTGG-3′) and ZOO17EC (5′-GCTGAATTCCTTTGTT-TCGTGCTGTTCGGATAG-3′), giving origin to the plasmid pZOO6. For inactivation of the *murMN* operon in R36A and Pen6, competent cells of R36A and Pen6 were transformed, respectively, with plasmids pZOO5 and pZOO6 (Fig. 1*B*).

Transformation, Population Analysis Profiles, and Determination of Penicillin-Resistance Level. *S. pneumoniae* R36A and Pen6 were transformed essentially according to published procedures (8). To induce competence, synthetic CSPα was added to the medium at a concentration of 250 ng/ml. The competent cells then were incubated for 30 min at 30°C in the presence of plasmidic DNA followed by the addition of 2 ml of C + Y medium and a 2-h incubation at 37°C. Transformants were selected on blood agar plates [tryptic soy agar (TSA) + 3% (vol/vol) sheep blood] containing 1 μg/ml erythromycin. Population analysis profiles (PAPs) were determined by plating serial dilutions of early stationary phase cultures on plates of TSA containing 5% (vol/vol) of sheep blood (Micropure Medical, 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 μg/ml). The PAPs were done with and without the presence of 1 μg/ml erythro-

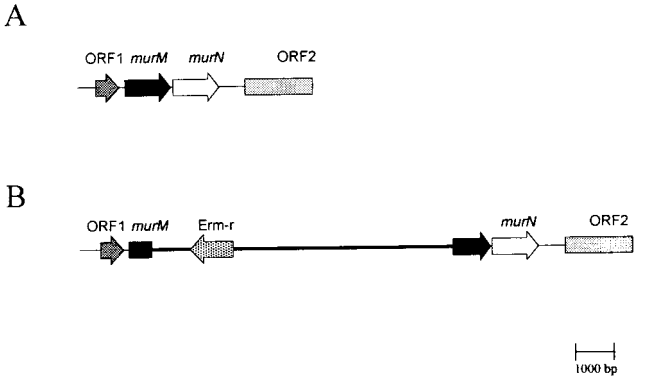

Fig. 1. Genetic organization of the *murMN* operon in *S. pneumoniae* (*A*) and inactivation of *murMN* by insertion duplication mutagenesis (*B*). *Erm*-r, erythromycin resistance gene from the plasmid pJDC9.

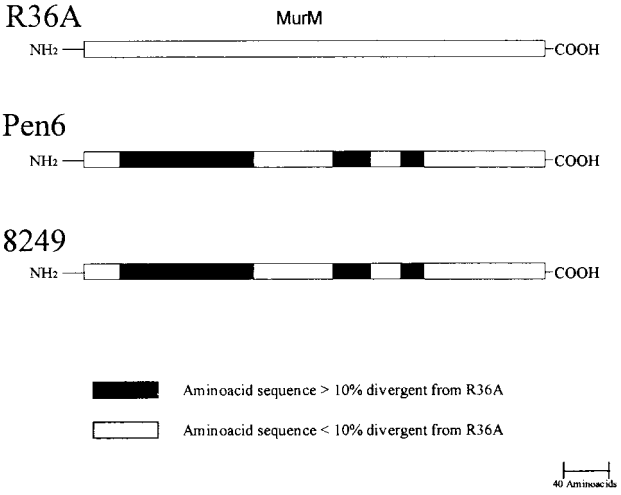

Fig. 2. Comparison of the primary structure of the murM protein in penicillin-susceptible and -resistant pneumococcal strains. The *murM* genes were sequenced from the penicillin-susceptible laboratory strain R36A, the penicillin-resistant South African strain 8249, and its transformant-derivative Pen6. Parts of the *murM* of the resistant strain showing divergence from the corresponding sequences in strain R36A are shown in black.

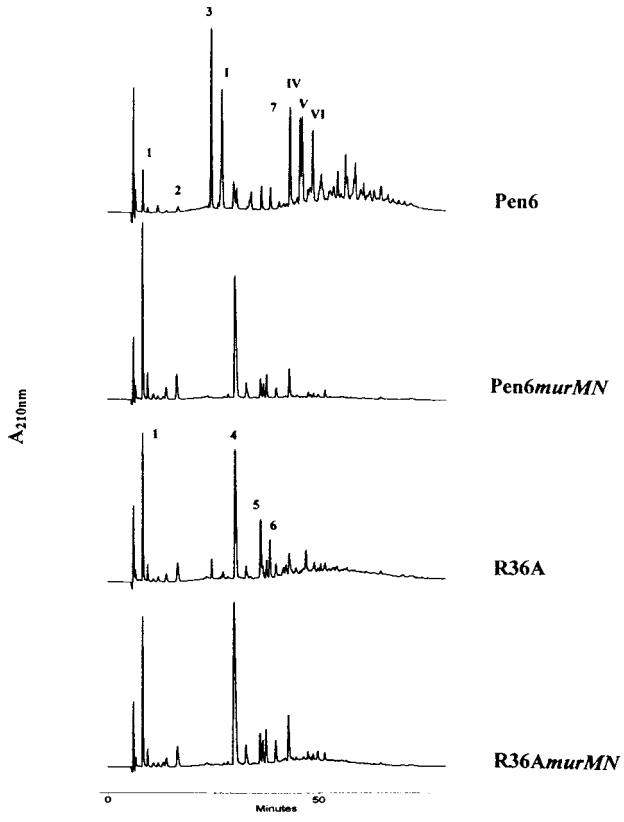

Fig. 3. HPLC profiles of the stem peptide components of the peptidoglycan from the penicillin- susceptible strain R36A and the penicillin-resistant strain Pen6 and their murMN-inactivated mutant derivatives. Cell wall peptidoglycans were prepared from strains R36A, Pen6, and their mutant derivatives with insertionally inactivated *murMN*, and the stem peptide composition was determined by HPLC analysis as described in *Experimental Procedures.*

mycin in the medium. Plates were incubated at 37°C in a 5% CO_2 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. Penicillin-resistance levels (minimal inhibitory concentration, MIC) were determined by the E-test following the manufacturer's guidelines (AB Bidosk, Solna, Sweden).

Cell Wall Preparation. Pneumococcal cell walls were prepared by a previously published method (3, 4) except that breaking the cells was done by shaking the bacterial suspension 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 solubilized wall material was washed with acetone, and the peptides were extracted with acetonitrile-isopropanol-water (25:25:50) containing 0.1% trifluoroacetic acid as described (3, 4, 13). After removal of the solvents by evaporation in a Speed Vac, 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 on a Vydac 218TP54 column (The Separations Group) as described (4). 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 UV absorption at 210 nm (A_{210}).

Results

Identification and *murMN* Region. The incomplete genome database of *S. pneumoniae* from The Institute for Genomic Research showed two genes (*murM* and *murN*) homologous to the *fmhB* gene from *S. aureus*, encoding FmhB, which is involved with the addition of the first glycine residue of the pentaglycine cross-bridge in the *S. aureus* peptidoglycan (14). The proteins encoded by the two pneumococcal genes have similar sizes: 407 and 411 aa in MurM and MurN, respectively. MurM has 44% similarity and 25% of amino acid identity to the FmhB of *S. aureus*. MurN has 65% similarity and 48% of amino acid identity to the zoocin A immunity factor of *Streptococcus zooepidemicus* and 47% similarity and 29% of amino acid identity to the FemA protein of *Staphylococcus simulans*. The *murMN* operon lies between an upstream gene (ORF1 in Fig. 1) with high homology (32% of identity) to *xynC* from *Caldicellulosiruptor saccharolyticus* (GenBank accession no. P23553) that codes for an acetylxylosidase. Downstream of the *murMN* operon there is a gene (ORF2 in Fig. 1) with high homology (56% of amino acid identity) to the *uvrC* gene, that codes for the subunit C of an excinuclease ABC (GenBank accession no. CAA11405) from *S. aureus*. Comparison of the 3.5-kb DNA fragment that includes *murMN* and some sequence upstream and downstream revealed 10 nucleotide residues that were different between the R36A and the pneumococcal TIGR (The Institute for Genomic Research) sequence. Half of these mutations lie in the coding region of the *murM* gene but only two of them result in change of amino acids. The other mutations are in the coding region of *murN* but all are silent substitutions.

Genetic Analysis of the *murMN* Operon. Hybridization of RNA from R36A and Pen6 with an internal fragment from *murN* identified a 2.7-kb band (data not shown). This result suggests that *murM* and *murN* are transcribed together, forming the *murMN* operon. Sequencing of *murMN* showed that the *murN* genes of strains R36A and Pen6 were very similar (99.3% of amino acid level

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Peptides	R36A	R36A*murMN*	Pen6	Pen6*murMN*
1	16.4	17.0	3.3	29.7
2	4.2	4.9	0.9	7.0
3	2.4	0.0	17.9	0.2
I	1.4	0.3	15.0	0.0
II	0.0	0.0	3.9	0.0
4	28.3	47.6	2.3	42.9
III	0.6	0.3	1.9	0.2
5	10.0	5.2	2.4	4.3
6	6.5	0.9	2.6	0.7
7	1.5	1.9	12.3	1.1
IV	1.9	1.2	10.0	0.7
V	2.9	3.8	12.0	1.3
8	8.2	2.8	1.1	1.8
VI	6.4	2.7	11.2	1.7
9	3.2	1.2	2.5	0.7
10	6.2	10.2	0.9	7.8
Total	100	100	100	100
LINEAR PEPTIDES	66	84	10	90
BRANCHED PEPTIDES	34	16	90	10
B/L PEPTIDES	0.5	0.2	8.7	0.1

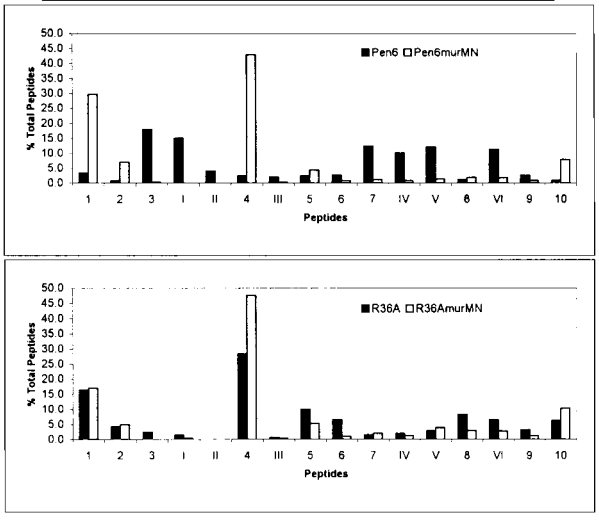

Fig. 4. Effect of the inactivation of *murMN* on the stem peptide composition of peptidoglycan. The amounts of individual stem peptides in the peptidoglycans isolated from the *S. pneumoniae* strains analyzed in Fig. 3 were determined by integration of the UV absorbance peaks in the HPLC profiles. Data are expressed as % of total stem peptides.

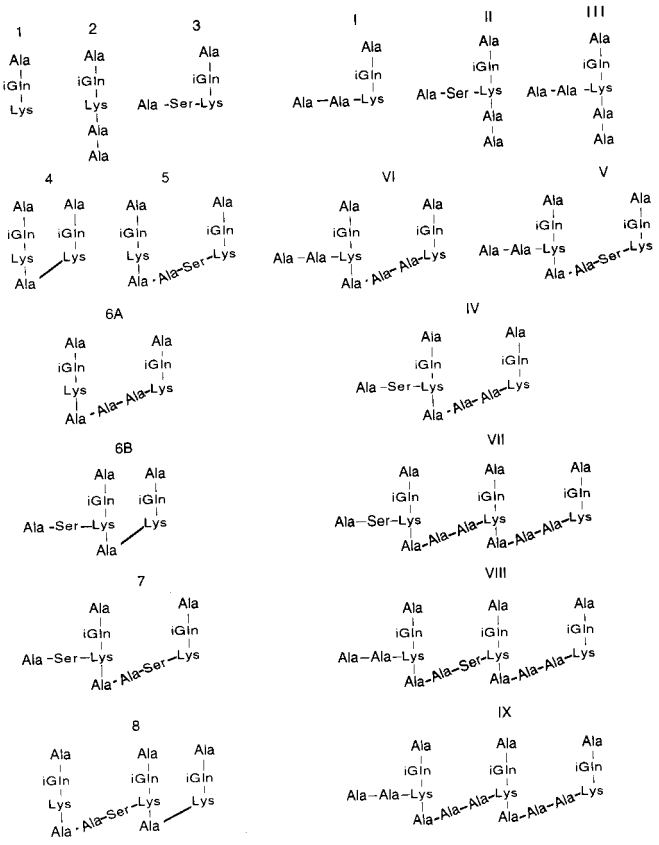

Fig. 5. Structures of the cell wall stem peptides identified in the pneumococcal peptidoglycan of penicillin-susceptible and -resistant strains of pneumococci. Structural assignments were based on methods described earlier (2, 4).

identity) whereas these two strains carried two different alleles of *murM.* One of these alleles identified in the penicillin-resistant strain Pen6 showed only 86.5% identity on the amino acid level to MurM in the penicillin-susceptible strain R36A (Fig. 2).

Gene Disruption and Characterization of Mutants with Inactivated *murMN*. To inactivate the *murMN* operon, internal fragments of *murM* were cloned into pJDC9 and integrated by insertion duplication mutagenesis into the chromosomal DNA of R36A and Pen6, respectively (see Fig. 1*B*). Inactivation of the *murMN* operon in R36A and Pen6 did not cause any significant change of the growth rate, cell morphology, or stationary phase autolysis rates (data not shown).

Two transformants with inactivated *murMN* were selected for cell wall analysis and some other tests. As shown by Figs. 3 and 4, inactivation of *murMN* caused major changes in the peptidoglycan composition of both susceptible and resistant strains. These changes were most prominent in Pen6. The chemical structures of the muropeptides affected are shown in Fig. 5. The major monomeric components of Pen6 (the branched peptides 3 and I) that constitute approximately 32.9% of the peptides in the peptidoglycan of this strain virtually disappeared from the peptidoglycan of the mutant with inactivated *murMN*. Concomitantly there was an increase (from 3.3% to 29.7%) in the linear monomeric component (peptide 1) of the peptidoglycan. The representation of several dimeric stem peptides with branched structure (peptides 6, 7, 9, and IV-VI) also greatly decreased in the peptidoglycan of the mutant whereas the proportion of the linear dimeric component (peptide 4) increased (see Fig. 4). Inactivation of *murMN* in the penicillin-sensitive R36A strain caused similar changes in peptidoglycan composition but these were less noticeable because the branched stem peptides are present only in small amounts in the cell wall of this strain.

Effect of the Inactivation of *murMN* on the Expression of Penicillin Resistance. Cultures of strain Pen6 and its *murMN*-inactivated derivative were plated at serial dilutions on blood agar plates containing various concentrations of penicillin. The population analysis profiles in Fig. 6 show that inactivation of *murMN* caused a striking decrease in the penicillin MIC from 6.0 in strain Pen6 to 0.032 μg/ml in the insertionally inactivated derivative.

The effect of disruption of *murMN* on penicillin resistance also was tested in several other resistant *S. pneumoniae* isolates belonging to distinct clonal lineages. These included strain SP2150, a representative of the serotype 9/14 multiresistant French/Spanish clone (6); strain Clev2, a representative of the serotype 23F multiresistant Spanish/USA clone (7); strain HUN663tr4tr5, a derivative of the multiresistant serotype 19A Hungarian clone (7, 8); and a penicillin-resistant serotype 6B isolate from Alaska (7). Inactivation of *murMN* caused reduction of the penicillin MIC values (determined by the E-test and expressed in μg/ml) in each one of these strains—from 1.0 to 0.032 in strain SP2150; from 1.6 to 0.016 in strain Clev2; from 1.6 to 0.032 in strain HUN663tr4tr5; and from 0.12 to 0.064 in the Alaskan isolate (Table 2).

The *pbp2x* genes of Pen6 and its *murMN*-inactivated derivative

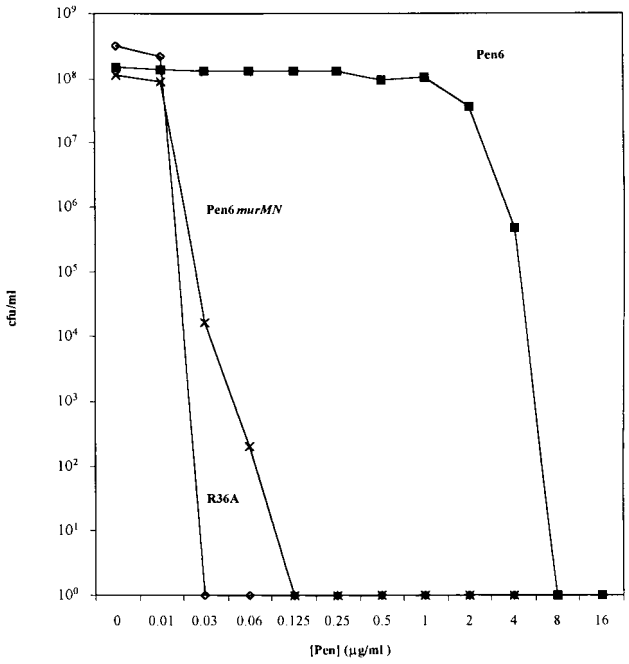

Fig. 6. Inhibition of the expression of penicillin resistance of strain Pen6 by interruption of the *murMN* operon. Cultures of strains R36A, Pen6, and its mutant derivative carrying an inactivated *murMN* operon were plated at various cell concentrations on agar containing serial dilutions of penicillin, and the population analysis profiles were determined as described in *Experimental Procedures*. Strain R36A, ◇; Pen6, ■, and mutant Pen6*murMN*, X.

were amplified and sequenced; no changes were detectable (data not shown), indicating that interruption of *murMN* did not affect the primary resistance mechanism.

Discussion

The major purpose of these investigations was to better understand the genetic determinants of the cell wall peptidoglycan structure in *S. pneumoniae* and its possible relationship to the mechanism of penicillin resistance. Of particular interest were genetic determinants of the branched structured muropeptides that appear as minor components in the species-specific peptidoglycan of penicillin-susceptible pneumococci but become major building blocks of the cell wall in penicillin-resistant strains (2–4). The frequent association between the increased proportion of branched cell wall components and penicillin resistance—both in laboratory mutants (15) and clinical isolates (2–4)—suggested a mechanistic connection between antibiotic resistance and wall structure (2). Support for such a possibility came from genetic crosses in which the abnormal branched-peptide-rich wall structure of the penicillin-resistant South African DNA donor strain 8249 was found to be transferred along with the antibiotic resistance trait into a penicillin-susceptible recipient during genetic transformation (2). On the other hand, more recent genetic experiments of a somewhat different design showed that the abnormal wall structure and penicillin resistance may be separated, implying that the genetic determinants of branched muropeptides are distinct from the low affinity PBPs, which are known to be the primary genetic determinants of penicillin resistance in these bacteria (8).

The results of studies described in this paper provide clarification of several of these issues. Our studies describe identification of the genetic determinants of branched wall structure—the *murM* and *murN* genes—which are indeed separate from the PBP genes and which control the addition of the short dipeptide units (seryl- or alanyl-alanine) to the epsilon amino group of the stem peptide lysine. Data shown in Figs. 3 and 4 demonstrate that interruption of *murMN* causes the marked reduction of branched muropeptide monomers, as well as dimers and oligomers, from the peptidoglycan of both penicillin- resistant and -susceptible strains.

Sequencing of *murM* from the highly penicillin-resistant South African strain 8249 or its transformant derivative Pen6 and comparing the sequence to that of *murM* from the penicillin-susceptible laboratory strain R36A allowed the identification of stretches in the penicillin-resistant strain's *murM* gene that were more than 10% divergent on the amino acid level from the corresponding sequences in the susceptible bacteria. These observations suggest that the *murM* gene of resistant pneumococci may be the product of heterologous recombinational event(s)—in analogy with the case of PBP genes in resistant clinical isolates (16–18).

Although the inactivation of *murMN* had no effect on growth rate and cell morphology, interruption of these genes caused a virtually complete inhibition of the expression of penicillin resistance. The penicillin MIC value of bacteria with inactivated *murMN* was reduced to the vicinity of the MIC characteristic of susceptible strains. This effect was not related to a change in the primary resistance mechanism because there was no change in the DNA sequence of the "mosaic" *pbp2x* gene nor in the molecular size and reduced penicillin affinity of PBPs as measured by the fluorographic assay (data not shown). The same massive inhibition of penicillin resistance was demonstrated in a number of *S. pneumoniae* isolates belonging to different genetic lineages and exhibiting different levels of penicillin resistance (see Table 2).

Table 2. Comparison of several isolates of *S. pneumoniae* and their murMN mutants

Strains	Serotype	"Clonal type"	Peptidoglycan composition	MurM gene	Penicillin MIC, μg/ml: Intact *murMN* operon	Penicillin MIC, μg/ml: Disrupted *murMN* operon
R36A	—	Laboratory strain	Normal	Normal	0.023	0.023
8249	19A	South African	Abnormal	Mosaic	6.0	ND
Pen6	—	Transformant	Abnormal	Mosaic	6.0	0.032
Sp2150	9/14	French/Spanish	Normal	Normal	1.0	0.032
Clev2	23F	Spanish/USA	Normal	Normal	1.6	0.016
Ala1	6B	Alaskan	Normal	Normal	0.12	0.064
Hun663	19A	Hungarian	Abnormal	Mosaic	1.6	ND
Hun663Tr4Tr5	—	Transformant	Normal	Normal	1.6	0.032

ND, not determined.

We can only speculate about the mechanism by which *murMN* contributes to the expression of penicillin-resistant phenotype. It is conceivable that wall precursors with branched structure are superior competitors against the β-lactam molecule for some site on the resistant PBPs; a preference for branched cell wall precursors over linear muropeptides already has been suggested as a possible consequence of the restructuring of PBPs in resistant bacteria (2). Alternatively, the branched muropeptides may perform some as-yet-undefined signaling function in cell wall synthesis or occupy some strategic sites within the cell wall structure, which become critical for the continued cell division and growth of the bacteria in the presence of penicillin. Whatever the precise mechanism of this effect will turn out to be, our observations clearly demonstrate that the expression of penicillin-resistant phenotype in pneumococci requires not only the low affinity PBPs but intact *murMN* genes as well. These genes appear to be the first major non-PBP determinants of β-lactam antibiotic resistance in pneumococci and the MurMN proteins represent potential targets for the design of new antibacterial agents against penicillin-resistant *S. pneumoniae*.

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