

The *murMN* operon: A functional link between antibiotic resistance and antibiotic tolerance in *Streptococcus pneumoniae*

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Inactivation of the recently identified *murMN* operon in penicillin-resistant strains of *Streptococcus pneumoniae* was shown already to cause two major effects: elimination of branched-structured muropeptides from the cell wall and complete loss of penicillin resistance. We now show that cells with inactivated *murMN* also have a third phenotype: an increased susceptibility to lysis when exposed to low concentrations of fosfomicin, D-cycloserine, vancomycin, and nisin, indicating a wide-spectrum hypersensitivity to inhibitors of both early and late stages of cell wall biosynthesis. Mutants of *murMN* also lysed faster than the parental strain when treated with the detergent deoxycholate. Several different alleles of *murM* cloned in plasmid pLS578 and introduced into a *murM* deletion mutant of the penicillin-resistant strain Pen6 were able to reconstitute each one of the three mutant phenotypes: the highly branched cell wall structure, original high level of penicillin resistance, and normal sensitivity to lysis. In a penicillin-susceptible strain the same experiments caused increased concentration of cell wall branched peptides and suppression of sensitivity to antibiotic induced lysis. The observations suggest that the *murMN* operon plays a key role in the regulation of a stress-response pathway that can be triggered by perturbation of cell wall biosynthesis in *S. pneumoniae*.

Several penicillin-resistant clones of *Streptococcus pneumoniae* produce abnormal cell walls in which a high proportion of the stem peptide lysine residues carry short (seryl alanine or alanyl alanine) branches (1). The genetic determinants involved with the branching process, the *murMN* operon, were identified recently (2). Penicillin-resistant isolates with highly branched cell walls were shown to carry *murM* alleles that contained divergent sequences as compared with the *murM* genes of penicillin-susceptible pneumococci (3). The two proteins, MurM (407 amino acids) and MurN (411 amino acids), are involved with the addition of the first and second amino acid residues, respectively, to the cell wall precursor stem peptide at the lipid II stage of biosynthesis (4, 5). Inactivation of *murMN* by insertion-duplication mutagenesis did not interfere with growth of the bacteria but caused two phenotypes: elimination of branched peptides from the cell wall and complete loss of penicillin resistance (2). The purpose of the studies described in this communication was to follow up these earlier findings by the use of a deletion mutant of *murM* and complementation experiments. The surprising observation made was that inactivation of *murM* also caused a third phenotype: increased sensitivity of the bacteria to lysis by cell wall inhibitors.

Experimental Procedures

Strains and Growth Conditions. All strains and plasmids used in this study are listed in Table 1. *S. pneumoniae* strains were grown in a casein-based semisynthetic medium (C + Y) at 37°C without aeration as described (11). *S. pneumoniae* strains containing pLS578 (10) or its derivatives were grown in the presence of 1 µg/ml tetracycline. *Escherichia coli* strains containing the pGEM-3Z plasmid or its derivatives were grown in LB medium in the presence of 100 µg/ml ampicillin (Sigma). *S. pneumoniae*

and *E. coli* strains containing the pJDC9 plasmid (9) or the *erm* marker were grown in the presence of 1 µg/ml and 1 mg/ml erythromycin, respectively.

In the experiments with antibiotic-induced or detergent-induced lysis, the strains were grown in C + Y until early exponential phase (OD₅₉₀ of 0.1–0.2), at which time different concentrations of antibiotics were added, and the optical density of the cultures was followed.

The ability of lysozyme to trigger lysis prematurely in the stationary phase was tested by adding lysozyme at different concentrations to cultures of Pen6 and its *murMN* mutant at an OD₅₉₀ of 0.2 and following the rate of decline of optical densities after the cultures entered the stationary phase.

DNA Techniques. All routine DNA manipulations were performed by using standard methods (12, 13). Chromosomal DNA from *S. pneumoniae* was isolated as described (14). Plasmids were isolated by using the Wizard Plus Minipreps or Midipreps 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 Life Technologies. 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 sequencers for slab-gel electrophoresis. Nucleotide and derived amino acid sequences were analyzed by using DNASTAR software.

Construction of *S. pneumoniae* Lacking *murM*. To isolate a mutant of the penicillin-resistant strain Pen6 lacking the *murM* gene, we transformed Pen6 with a PCR fragment that included the 2-kb fragment from pJDC9, carrying the *erm* marker, flanked by the upstream and downstream regions of *murM*. The details of the preparation of this *murM* null mutant were described in a recent publication (5).

The transforming DNA used to prepare the *murM* null mutant of strain Pen6 was a PCR fragment of plasmid pZOO11, which contains the *erm* marker flanked by the upstream and downstream regions of *murM*, amplified with primers ZOO30 (5'-ATATTCTC-TACGTTTCAGAGG-3') and ZOO45EC (5'-AGCGAATTTCG-GTTTTGACTACTACACGGC-3') (5). The conditions used for the PCR reactions were: 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. This PCR program was used for all amplifications

Abbreviations: MIC, minimum inhibitory concentration; DOC, deoxycholate.

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

	Relevant characteristics	Reference
<i>S. pneumoniae</i> strains		
R36A	Penicillin-susceptible laboratory strain	(6)
Pen6	Penicillin-resistant transformant of R6Hex with donor DNA from the penicillin-resistant strain 8249	(7)
KY17	Penicillin-resistant nontypable clinical isolate from a U.S. collection	(3, 8)
Pen6 <i>murMN</i>	Pen6 mutant with <i>murMN</i> operon interrupted by insertion-duplication mutagenesis with pJDC9	(2)
Pen6Δ <i>murM</i>	Pen6 with <i>murM</i> gene deleted and replaced by <i>erm</i> gene from pJDC9	(5)
Pen6 <i>murMR36A</i>	Pen6 carrying <i>murM</i> allele of R36A in the chromosome	(4)
Pen6Δ <i>murM</i> pM3N through pM18	Transformants of Pen6Δ <i>murM</i> carrying various constructs cloned into pLS578	(5)
R36ApM1 through pM3N	Transformants of R36A carrying various constructs cloned into pLS578	(5)
<i>Escherichia coli</i> strain		
DH5α	RecA endA1 gyrA96 thi-1 hsdR17 supE44relA1 φ80 dlac ZΔ M15	BRL
Plasmids		
pJDC9	<i>E. coli</i> plasmid Ery ^R	(9)
pLS578	<i>S. pneumoniae</i> plasmid Tet ^R	(10)
pM1 through pM9N:	Plasmid pLS578, into which various <i>murM</i> alleles and constructs were cloned	(5)
pM1	<i>murM</i> allele from strain R36A	(5)
pM2	<i>murM</i> allele from strain DE1	(5)
pM3	<i>murM</i> allele from strain KY17	(5)
pM3N	<i>murM</i> allele from strain KY17 plus the <i>murN</i> gene from strain R36A	(5)
pM9N	<i>murM</i> chimeric allele constructed from strains DE1 and R36A plus the <i>murN</i> gene from R36A	(5)
pM18	<i>murM</i> mutant allele from strain KY17 with the residue at position 27 mutated from a glutamine to a glutamate, plus the <i>murN</i> gene from strain R36A	(5)

except the extension time at 72°C, which was different depending on the size of the PCR fragment to be amplified.

The deletion of *murM* in the erythromycin-resistant Pen6Δ*murM* mutant was confirmed by PCR amplification and subsequent sequencing.

Transformation of the *murM* Null Mutant (Pen6Δ*murM*) and the Penicillin-Susceptible Strain R36A with Plasmid-Borne *murM* Alleles.

Several *murM* constructs were prepared (5), each in the *S. pneumoniae* plasmid pLS578, which is a plasmid with a strong promoter that can replicate in *S. pneumoniae* (10). Three *S. pneumoniae* strains were used as the source of *murM* alleles: the penicillin-susceptible laboratory strain R36A and two penicillin-resistant isolates, strain KY17 producing serine-rich branches in the cell wall and strain DE1 producing alanine-rich branches (5).

The *murM* alleles cloned in the pneumococcal plasmid pLS578 were introduced into the Pen6 *murM* null mutant (Pen6Δ*murM*) or into R36A by genetic transformation by using previously described methods (15).

Cell wall preparation and analysis of the cell wall stem peptides were done by using a published method (16, 17) after mechanical breaking of the cells by glass beads (5). Cell wall material (2 mg) was suspended in 25 mM sodium phosphate buffer (pH 7.4) and treated with affinity-purified pneumococcal amidase (5–10 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/isopropanol/water (25:25:50) containing 0.1% trifluoroacetic acid (16, 17). After removal of the solvents by evaporation in a SpeedVac, the peptides were dissolved in 0.1% trifluoroacetic acid and separated on a Shimadzu LC-10AVP HPLC system (17).

Results

Recovery of High Level Penicillin Resistance in the *murM* Null Mutant of Strain Pen6 by Complementation with Various *murM* Alleles Cloned in Plasmid pLS578. Loss of high level penicillin resistance in strain Pen6 upon inactivation of *murMN* by insertion-duplication mu-

tagenesis has been described already (2). To rule out possible indirect and/or polar effects by the inserted plasmid (18, 19), a deletion mutant Pen6Δ*murM* was constructed (5) to allow complementation. In Pen6Δ*murM*, the *murM* gene was replaced by the *erm* marker from the plasmid pJDC9 (9) to prevent recombination with the plasmid-borne *murM* alleles introduced in the course of complementation experiments (18). Confirmation of the erythromycin-resistant Pen6 *murM* null mutant was obtained by sequencing the PCR fragment amplified with primers ZOO30 and ZOO45EC (which includes the *murMN* region). The penicillin minimum inhibitory concentration (MIC) of the parental strain Pen6 (6 μg/ml) was reduced to 0.032 μg/ml in both Pen6Δ*murM* and the insertionally inactivated *murMN* mutant. Deletion of *murM* caused no effect on the growth rate or morphology of the cells. Complementation experiments were done to prove that the loss of penicillin resistance was caused by the inactivation of *murM*. The *murMB5* allele from the penicillin-resistant strain KY17 (3, 4) was cloned together with the *murN* of R36A both under the control of the conserved promoter of plasmid pLS578, and the resulting construct pM3N was transformed into the Pen6Δ*murM* mutant. After introduction of this plasmid into the deletion mutant the MIC increased to 6 μg/ml, indicating full recovery of parental level penicillin resistance (Fig. 1). Full recovery of penicillin resistance also was obtained in the chromosomally complemented Pen6*murM36* and with pM9N in which the *murM* gene was a chimera: the first 250 amino acids were derived from the *murM* of the penicillin-susceptible strain R36A and the next 166 amino acids from the *murM* of the penicillin-resistant strain DE1 (5). The construct also carried *murN* from R36A. Complementation with pM18, a variant of pM3N in which the glutamine residue at position 27 in the *murM* gene was replaced by glutamic acid through site-directed mutagenesis, also resulted in full recovery of penicillin resistance. This substitution has no effect on the activity of *murM* (5). No increase in the penicillin MIC occurred when plasmid-borne *murM* alleles were introduced into the penicillin-susceptible strain R36A.

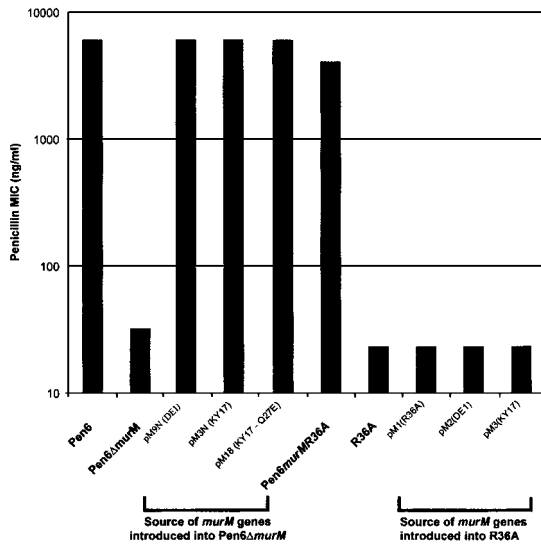


Fig. 1. Recovery of penicillin resistance in Pen6 Δ murM complemented with different murM alleles. The strain Pen6 Δ murM was transformed with plasmid pLS578 carrying different murM alleles and constructs. Several of the plasmid-borne murM alleles also were introduced into the penicillin-susceptible strain R36A. Penicillin MIC values were determined by the E test.

Analysis of the cell wall composition of Pen6 Δ murM showed a virtually complete disappearance of branched structured stem peptides from the cell wall peptidoglycan (Fig. 2). The compo-

sition of the peptidoglycan was indistinguishable from that of the already described Pen6murMN, which was obtained by insertion of a suicide plasmid in the murM gene (2). Cell wall analysis of the pM3N transformant showed a peptidoglycan rich in serine containing branched peptides, similar in composition to that of strain KY17 (5), the source of the murM allele in the plasmid (Fig. 2 and Table 2, which is published as supporting information on the PNAS web site, www.pnas.org). The peptidoglycan of transformants carrying plasmid pM9N was rich in branched peptides in which alanine was the predominant first residue of the crossbridge, as expected from the specificity of the murM in strain DE1 (5).

Increased Sensitivity of murMN Mutants to Lysis by Inhibitors of Cell Wall Synthesis Other Than Penicillin. A careful scrutiny of the optical density profiles of cultures of Pen6 and its deletion mutant in the stationary phase of growth showed that spontaneous autolysis in the stationary phase, a characteristic property of *S. pneumoniae*, began earlier in the murM deletion mutant as compared with the parental strain Pen6. We followed up this observation by testing the lytic effect of a number of different cell wall inhibitors on cultures of strain Pen6 and its deletion mutant. Fig. 3 shows that each one of the five cell wall inhibitors tested and the detergent deoxycholate (DOC) caused unusually rapid lysis of cultures of the murM deletion mutant. In the case of cefotaxime, this lysis was related to the different MIC values of strain Pen6 (2.0 μ g/ml) and its deletion mutant (0.03 μ g/ml). However, the MIC values of Pen6 and Pen6 Δ murM for the rest of the drugs and DOC were either identical or differed only by a factor of 2. The abnormal lysis pattern was reversed in each

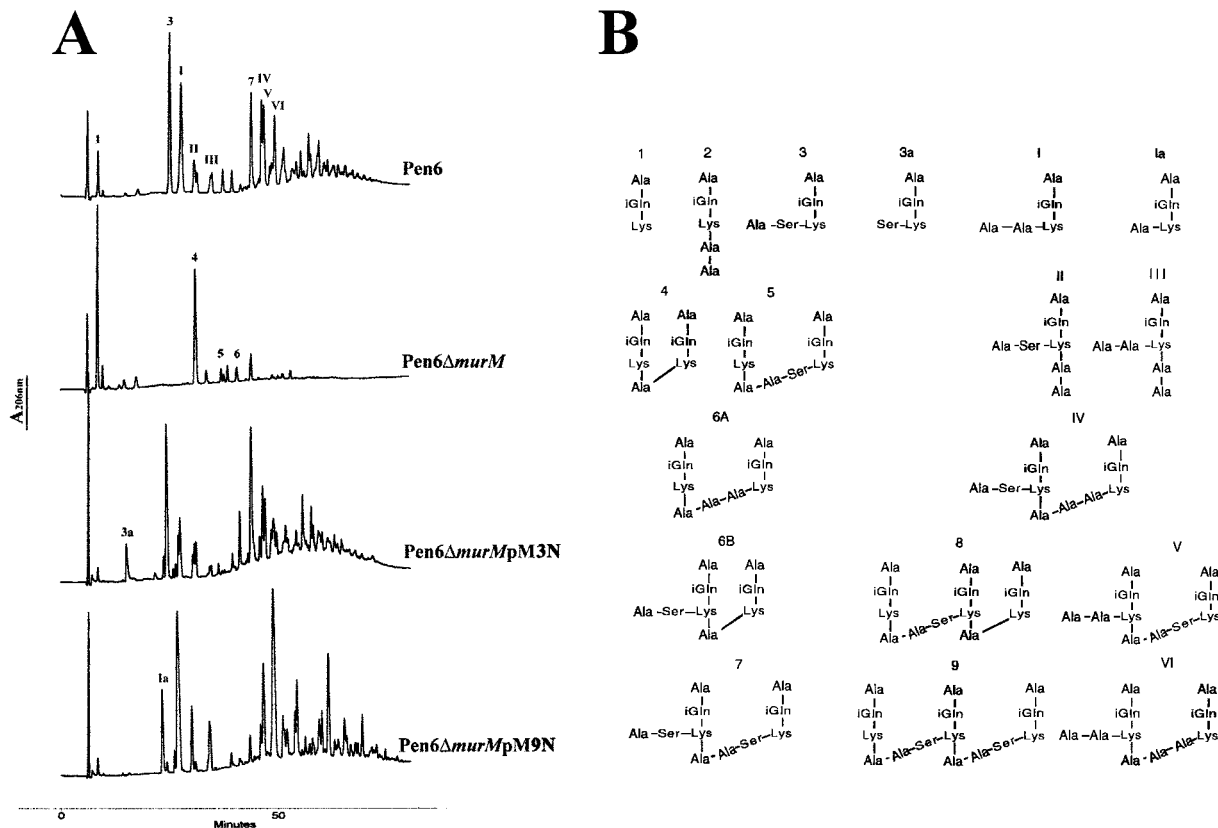


Fig. 2. Cell wall composition of Pen6 Δ murM complemented with different murM alleles. (A) Cell walls from strain Pen6, its murM deletion mutant (Pen6 Δ murM), and two Pen6 Δ murM transformants, Pen6 Δ murMpM3N and Pen6 Δ murMpM9N complemented with murM alleles, were analyzed for peptidoglycan composition by HPLC as described in Experimental Procedures. (B) Structural assignments for the pneumococcal stem peptides. For determination of chemical structures see refs. 4 and 11.

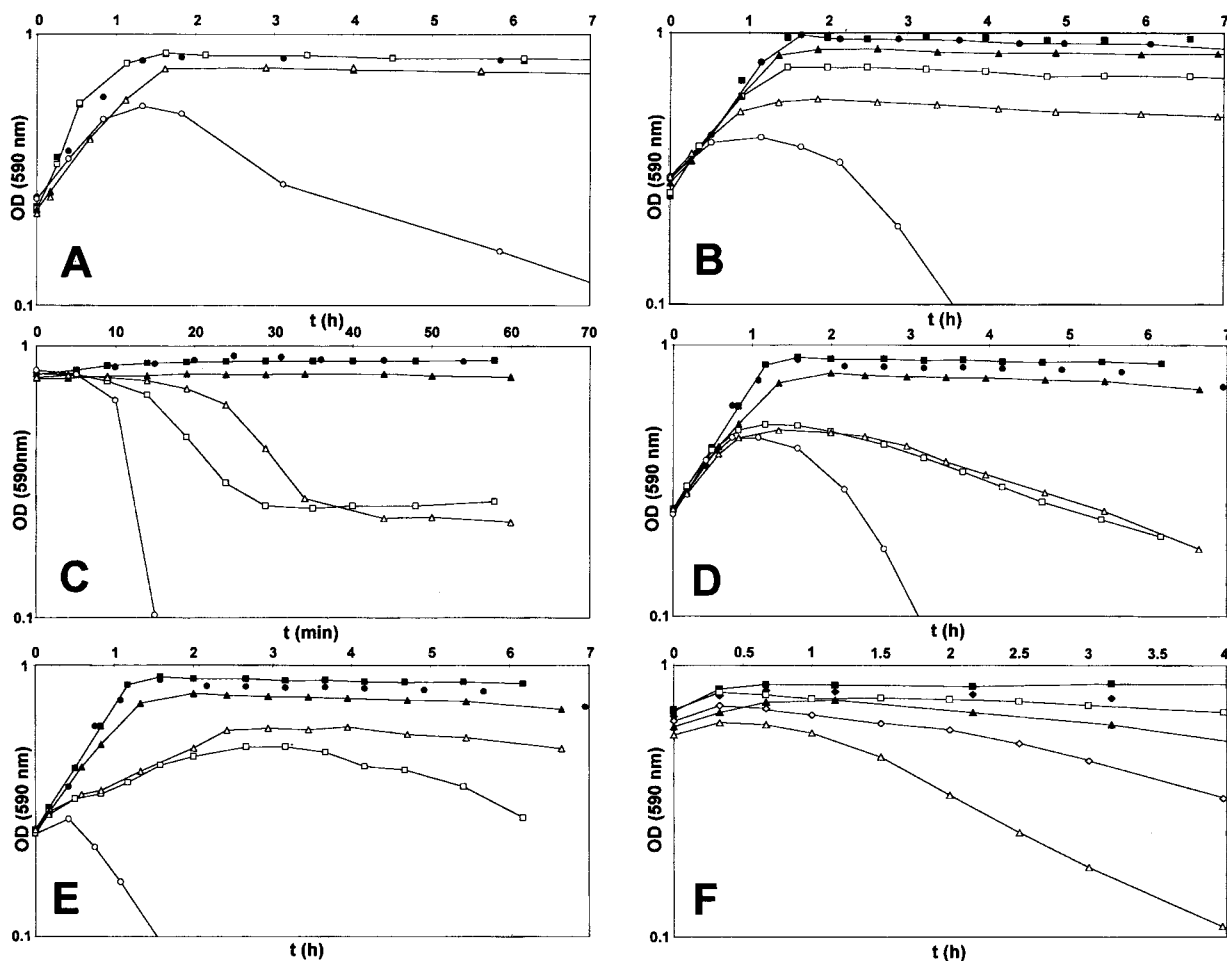


Fig. 3. (A–F) Hypersensitivity of the *murM* deletion mutant Pen6 Δ *murM* to the lytic effect of cell wall inhibitors. Inhibitors were added to cultures of strain Pen6, Pen6 Δ *murM*, and Pen6 Δ *murM* carrying plasmid pM18 in the early exponential phase (OD₅₉₀ of 0.2), and their effects on growth and lysis were followed by monitoring the optical density. Of the antimicrobial agents tested, only cefotaxime had different MIC values for the three strains used; the MIC was 2 μ g/ml for Pen6 and Pen6 Δ *murM*pM18 and 0.03 μ g/ml for Pen6 Δ *murM*. For the rest of the agents the MIC values for the three strains were the same or differed by only a factor of 2. The concentration of various drugs used was as follows: A, cefotaxime (0.05 μ g/ml); B, D-cycloserine (50 μ g/ml, 1 \times MIC); C, nisin (1 μ g/ml, 2 \times MIC); D, fosfomicin (50 μ g/ml, 1 \times MIC); E, vancomycin (0.4 μ g/ml, 1 \times MIC); F, DOC (50 μ g/ml and 100 μ g/ml, 1 and 2 \times MIC). The symbols used for A–E are as follows. Cultures with antibiotic: \square , Pen6; \circ , Pen6 Δ *murM*; \triangle , Pen6 Δ *murM*pM18. Controls without antibiotics: \blacksquare , Pen6; \bullet , Pen6 Δ *murM*; \blacktriangle , Pen6 Δ *murM*pM18. Symbols for F are as follows. \blacksquare and \square , Pen6 and Pen6 Δ *murM* without antibiotics, respectively; \blacklozenge and \lozenge , Pen6 and Pen6 Δ *murM* with 50 μ g/ml DOC, respectively; \blacktriangle and \triangle , Pen6 and Pen6 Δ *murM* with 100 μ g/ml DOC, respectively.

case in complementation experiments in which the plasmid-borne *murMN* operon was introduced into the Pen6 deletion mutant (Fig. 3). Because in such plasmid transformants the majority of cell wall stem peptides had a branched structure (5), the results of the complementation experiments suggested a correlation between the proportion of branched peptides in the cell wall and the susceptibility of the bacteria to lysis during treatment with cell wall inhibitors. To test this correlation further, the experiments were repeated with the penicillin-susceptible strain R36A, which is exquisitely sensitive to the lytic and bactericidal effect of cell wall inhibitors (20) and the cell wall of which is composed mainly of linear stem peptides (1, 17). Cultures of strain R36A and a transformant derivative carrying the plasmid pM18 were treated with fosfomicin, D-cycloserine, and nisin at concentrations close to their respective MIC values. In contrast to the rapid lysis of R36A, the plasmid transformants showed suppression or delay (nisin) of the lytic response to the antibiotics (Fig. 4). Analysis of the cell wall of the plasmid transformants showed that introduction of the plasmid-borne *murMN* gene caused major changes in the composition of the cell wall, which became highly enriched in stem peptides with a

branched structure (Fig. 6 and Table 3, which are published as supporting information on the PNAS web site).

Discussion

In a previous communication we described loss of antibiotic resistance in penicillin-resistant isolates of *S. pneumoniae* in which the *murMN* operon was inactivated by insertion-duplication mutagenesis (2, 4). The observations described in this communication confirm this finding; the penicillin MIC of the *murMN* deletion mutant Pen6 Δ *murM* was reduced from 6.0 μ g/ml to 0.03 μ g/ml, i.e., to the MIC range for penicillin-susceptible pneumococci. Normal levels of resistance were recovered in transformants of Pen6 Δ *murM* that received functional *murM* alleles cloned into a plasmid that can replicate in pneumococci. These experiments together provide unequivocal evidence for the critical importance of the *murMN* operon for penicillin resistance.

In the complementation experiments with the particular constructs used, full recovery of penicillin resistance was accompanied also by an increase in the proportion of branched peptides in the cell wall of transformants. On the other hand,

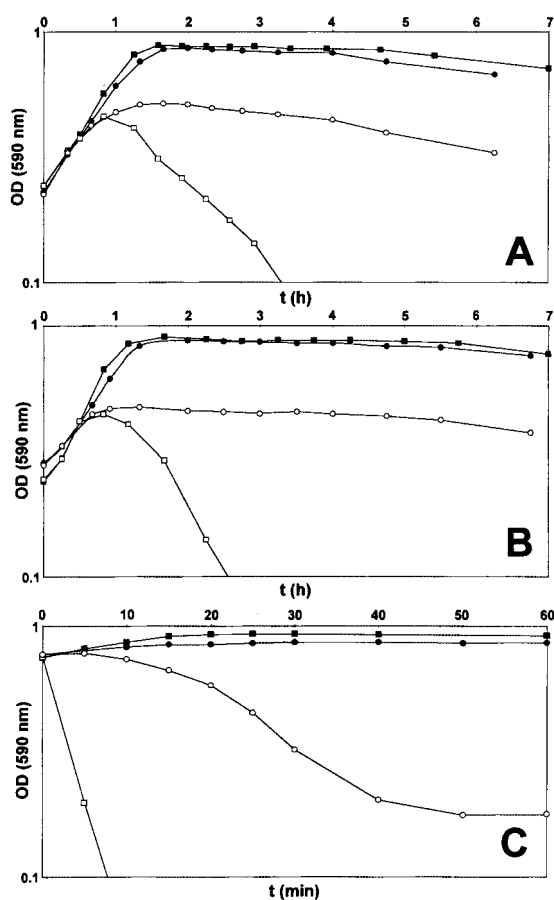


Fig. 4. Suppression of the lytic response to various cell wall inhibitors in strain R36A overexpressing *murMN*. Antibiotics were added to early exponential phase cultures of strain R36A and its plasmid transformant R36ApM18, and the effects of antibiotics on growth and lysis were followed by optical density determinations. Antibiotics were used at concentrations close to their MIC values. **A**, penicillin (0.025 µg/ml, 1–2 × MIC); **B**, fosfomycin (50 µg/ml, 1 × MIC); **C**, nisin (2 µg/ml, 4 × MIC). The symbols used are as follows. Cultures with antibiotics: □, R36A; ○, R36ApM18. Control cultures without antibiotics: ■, R36A; ●, R36ApM18.

the chemical composition of the branched peptides in various transformants differed depending on the source of the particular *murM* allele (3, 5). Serine-rich branched peptides were produced in transformants that received *murM* from strain KY17, whereas transformants with the *murM* strain from DE1 produced alanine-rich branches. It therefore seems that recovery of optimal penicillin resistance, although requiring functional *murM* (and *murN*) genes, does not depend on the reintroduction of the same *murM* allele that was resident originally in the resistant strain Pen6. The situation may be different in the case of strains with a very high level of resistance. In recent communications, Smith and Klugman (21, 22) confirmed and extended the role of *murM* in conferring penicillin resistance in a strain of *S. pneumoniae* that had an extremely high MIC value of 12 µg/ml. They found that transformation of penicillin resistance above a certain high MIC value was not possible by using only the cloned resistant penicillin-binding protein genes as donor DNA but also needed a portion of the *murM* gene from the resistant bacteria.

Our results also make it clear that the *murMN* operon, although necessary, is not sufficient to cause resistance to penicillin, because strain R36A transformed with the *murM* alleles from resistant strains remained fully susceptible to pen-

icillin despite the fact that the cell walls of the transformants became greatly enriched for branched muropeptides (Fig. 1).

An unexpected property of *murMN* mutants described in this communication is the hypersensitivity of the bacterium to a variety of cell wall inhibitors. The most striking feature of this new phenotype was its lack of specificity; inhibitors of both early stages in cell wall synthesis (fosfomycin and D-cycloserine) as well as inhibitors of the late stages (vancomycin and nisin) induced a rapid loss of optical density in cultures of Pen6Δ*murM* at antibiotic concentrations close to the MIC values. The agents also included DOC, a detergent that can induce autolysis of pneumococci by an unknown mechanism.

Rapid and extensive loss of viability followed by lysis is the most frequent response of *S. pneumoniae* and other bacteria to treatment with β-lactam antibiotics and other wall inhibitors. In the case of β-lactams the lethal pathway is initiated by the acylation of penicillin-binding proteins and terminates in the autolytic suicide of the cells. Earlier work from our laboratory has shown clearly that the mechanism of the killing and lytic effects of penicillin involved bacterial factors *beyond* the primary targets of penicillin (i.e., the penicillin-binding proteins; ref. 23). One of these factors is LytA (24), a pneumococcal murein hydrolase primarily responsible for autolytic phenomena in this bacteria. A second factor(s) that seems to control sensitivity of pneumococci to the cidal effect of penicillin has also been described (20). Unique bacterial mutants, so-called “penicillin-tolerant mutants” in which the lytic action alone or together with the bactericidal action of penicillin were suppressed, have been isolated in the laboratory from pneumococci (25) and also from *E. coli* (26) and *Staphylococcus aureus* (27).

Pen6 and Pen6Δ*murM* were grown in the presence of sub MIC concentrations of penicillin. Little if any difference was observed in the susceptibility of cell walls isolated from these bacteria to hydrolysis by LytA *in vitro* (data not shown). Furthermore, inactivation of *murMN* also caused rapid loss of viability of the penicillin-treated bacteria under conditions when the activity of LytA was inhibited (20, 28).

The profound influence of cell wall branching on lytic phenomena described in this communication suggests that the *murMN* operon occupies a critical position in the stress-response pathway triggered by perturbation of cell wall synthesis in *S. pneumoniae* (23). As a model we propose that the relevant activity of *murMN* modulating the lytic phenotype is the production of Lipid II carrying the dipeptide substituents on the ε amino group of the lysine residues in the cell wall precursor peptide (Fig. 5). The presence of the extra dipeptide residues in lipid II should increase the hydrophilic nature of the molecule, and the accumulation of these heptapeptides in the plasma membrane may suppress the assembly of the hypothetical holin-like proteins that were postulated to initiate the irreversible effects of cell wall inhibitors in pneumococci (20) and other bacteria (29–31).

In a previous communication it was proposed that the mechanism of irreversible effects (killing and lysis) by cell wall inhibitors involved some injury to the plasma membrane, the integrity of which is essential for the survival of the bacterium. The same injury then would allow the release of autolytic enzymes to the cell wall and initiate lysis of the bacteria. This model is formally analogous to models proposed for the mechanism of lysis by bacteriophages in which the release of phage progeny is preceded by membrane injury through the insertion of a holin protein into the bacterial membrane (32, 33). Our model also is analogous to the proposed mode of action of nisin at the level of lipid II (34).

It is interesting that the activity of the same *murMN* operon seems to be involved not only in penicillin resistance but in antibiotic tolerance as well. It is conceivable that the step that

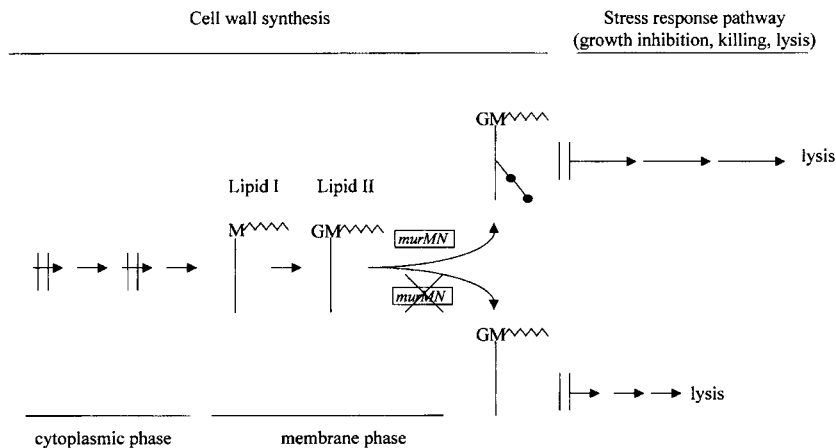


Fig. 5. Model for the postulated role of the *murMN* operon in the regulation of bacterial response to perturbation of cell wall biosynthesis. The model assumes that the critical regulatory component that influences the lytic/cidal response of pneumococci to inhibitors of cell wall biosynthesis is the structure of lipid II. In bacteria with functional *murMN*, lipid II is composed of bactoprenol-linked disaccharide heptapeptides that cause a delay in the hypothetical membrane injury caused by interruption of cell wall biosynthesis (see double vertical lines in model). In bacteria with inhibited *murMN* the structure of lipid II is a bactoprenol-linked linear disaccharide pentapeptide that is unable to delay the membrane injury that initiates the bacterial stress response leading to the suicidal activation of factors including LytA.

defines the MIC of a cell wall active agent may not only depend on the affinity of the drug targets for the drug but also on the status of a response pathway that translates the inhibition of target molecules to its cell biological consequences, i.e., the inhibition of the target cell. Lowering the sensitivity threshold of response to the perturbation of wall synthesis may become the rate-limiting step in drug susceptibility. The evidence about the involvement of *murMN* with both the penicillin MIC and sen-

sitivity to lysis by a variety of wall inhibitors suggests that *murMN* may be a key component of such a sensory circuit.

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