Alterations in MurM, a Cell Wall Muropeptide Branching Enzyme, Increase High-Level Penicillin and Cephalosporin Resistance in *Streptococcus pneumoniae*

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We report that alteration in MurM, an enzyme involved in the biosynthesis of branched-stem cell wall muropeptides, is required for maximal expression of penicillin and cefotaxime resistance in the pneumococcus. Hungarian isolate 3191 (penicillin MIC, 16 μg/ml; cefotaxime MIC, 4 μg/ml) was a source of donor DNA in transformation experiments. Penicillin-binding protein DNA was insufficient to transform recipient strain R6 to full resistance. Further transformation with altered *murM* DNA was required for full expression of donor penicillin and cefotaxime resistance.

Beta-lactam antibiotics inhibit the growth of pneumococci by the inactivation of penicillin-binding proteins (PBPs). PBPs are serine peptidases which catalyze polymerization of peptidoglycan precursors during cell wall synthesis (15). Pneumococcal resistance to β-lactams is the result of altered PBPs with decreased antibiotic affinities (8, 9, 16). Pneumococci contain a set of six PBPs (7). High-level penicillin resistance can be established by alteration in only three of these PBPs, that is, PBPs 2X, 2B, and 1A (2), while only altered PBPs 2X and 1A are required for high-level cefotaxime resistance (10). The role of PBPs in mediating β-lactam resistance in pneumococci was first described in the early 1980s (8, 16). More recently, further mechanisms for β-lactam resistance in pneumococci have been described, i.e., mutations in the histidine protein kinase CiaH (6) and mutations in the glycosyltransferase CpoA (5). These non-PBP mechanisms have been identified only in laboratory mutants and account for very low-level resistance.

In this paper we report a non-PBP resistance determinant that is essential for the complete development of high-level penicillin and cephalosporin resistance in pneumococci isolates. This resistance mechanism involves alteration in MurM, an enzyme involved in the biosynthesis of branched-stem cell wall muropeptides. The major peptide species in susceptible cell walls are of a linear-stem structure, compared to an abnormal branched-stem structure found in resistant cell walls (4). Branched-stem peptides presumably have superior binding to structurally altered PBPs and therefore become the preferred substrate for cell wall synthesis in resistant bacteria. Filipe and Tomasz (3) recently described the *murMN* operon in the pneumococcus that codes for the MurM and MurN proteins, which control the biosynthesis of branched-stem-structured cell wall muropeptides. They showed that a functional *murMN* operon is critical for the expression of penicillin resistance. We extend their findings by showing that alterations in MurM contribute to development of high-level penicillin and cephalosporin resistance in the pneumococcus.

Properties of the pneumococcal strains studied are shown in Table 1. Chromosomal DNAs were extracted from bacterial cells, and genes were amplified from the chromosomal DNAs by PCR using methods that have been described previously (13). For *pbp* gene PCR, primers have been described previously (11, 12). For *murMN* gene PCR, the following primer pairs were used: (i) *murMN*-up (TTCAAACGAAAGTAGTGA GAATAG) and *murMN*-down3 (CCTATCAAACGAAAA GCCAGCGCA) and (ii) *murMN*-up2 (TTTATAATGGAAC CACTATTATAG) and *MurMN*-down (GCATGTCCTCC ACCTTTTCTAG). PCR products were sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) and an Applied Biosystems model 310 automated DNA sequencer. Pneumococcal strain R6 was used as the recipient in transformation studies.

Chromosomal DNA and cloned genes were used as transforming DNA. Pneumococcal strains were made competent as follows. Bacteria were cultured in C medium (14) until the mid-exponential phase (optical density at 620 nm, 0.15) and, after the addition of glycerol to 10%, were frozen at −70°C in 500-μl aliquots. For transformation, 1 μg of DNA was added to 500 μl of competent cells, which were then incubated at 30°C for 45 min and at 37°C for 90 min. Eighty-microliter aliquots were then plated onto Mueller-Hinton blood agar containing increasing concentrations of antibiotic, and the plates were incubated at 37°C for 48 h. Transformants were picked from the plates containing the highest antibiotic concentration. Transformation frequencies were on the order of 10−4 to 10−5.

Our study is based on isolate 3191, a representative of a Hungarian pneumococcal clone, isolated during the period 1997 to 1998, that was found to have notably high levels of penicillin (MIC, 16 μg/ml) and cefotaxime (MIC, 4 μg/ml) resistance (12). In that study, transformation of susceptible strain

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R6 with combinations of all six pbp genes from isolate 3191 resulted in transformants for which the maximum MICs were 4 µg of penicillin per ml and 2 µg of cefotaxime per ml. Resistance in these R6^3191/2X/2B/1A transformants was due to altered PBPs 2X, 2B, and 1A. The full MICs for the donor (penicillin MIC, 16 µg/ml; cefotaxime MIC, 4 µg/ml) could be reached only by further transformation of R6^3191/2X/2B/1A strains with chromosomal 3191 DNA, demonstrating the involvement of a non-PBP resistance determinant.

Our present study has now identified this resistance determinant. Experiments were initiated along the lines of the methods described by Adrian and coworkers (1). The following steps were taken. The chromosomal DNA was digested, and fragments of DNA with transforming ability were identified. In the process of identifying open reading frames with transforming ability, Filipe and Tomasz (3) described the murMN operon in the pneumococcus and proved that a functional murMN operon was critical for the expression of penicillin resistance. We therefore decided to investigate whether the product of this murMN operon was our non-PBP resistance determinant. PCR primers were designed, and the murMN operon was amplified from isolate 3191. The murMN DNA was shown to successfully transform R6^3191/2X/2B/1A to requiring the full MICs of the donor (penicillin MIC, 16 µg/ml; cefotaxime MIC, 4 µg/ml). R6^3191/2X/2B/1A/mur transformants could be selected with either penicillin or cefotaxime.

The murMN genes from isolate 3191 and from R6^3191/2X/2B/1A/mur transformants were then sequenced. The nucleotide sequence of the genes from susceptible strain R6 was used as the basis for comparison with resistant strains. The murMN genes from isolate 3191 displayed a mosaic pattern with 9.5% nucleotide sequence divergence from the genes of strain R6. The murMN operon is divided into the murM and murN genes, with the major sequence divergence occurring in the murM gene. The murM gene revealed 16.2% nucleotide sequence divergence, resulting in 74 amino acid mutations in the 406-amino-acid MurM protein, while the murN gene revealed a nucleotide sequence diversity of only 2.9%, which resulted in only 6 mutations in the 410-amino-acid MurN protein. Sequence analysis of the murMN genes from R6^3191/2X/2B/1A/mur transformants showed that altered MurM was the resistance determinant. Figure 1 schematically illustrates murMN genes from six R6^3191/2X/2B/1A/mur transformants, compared to the genes from donor isolate 3191, and indicates the regions of the genes where altered DNA from isolate 3191 has been introduced. The common area of alteration (nucleotides 208 to 474) could be identified among all transformants, which indicated that this area (located in the murM gene) housed the alterations leading to the development of resistance. This area corresponds to amino acid positions 42 to 131 of the MurM protein (Fig. 2), which account for 20 of the 74 mutations in the altered protein.

We then investigated whether the role played by altered MurM in the development of high-level penicillin and cefotaxime resistance was a unique characteristic of MurM from the Hungarian clone or whether murM genes from other resistant strains were also able to increase β-lactam MICs.

### Table 1. Properties of pneumococci

<table>
<thead>
<tr>
<th>Strain</th>
<th>Penicillin MIC (µg/ml)</th>
<th>Cefotaxime MIC (µg/ml)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6</td>
<td>0.015</td>
<td>0.015</td>
<td>United States (1930s)</td>
</tr>
<tr>
<td>149193</td>
<td>2</td>
<td>2</td>
<td>South Africa (1982)</td>
</tr>
<tr>
<td>50012</td>
<td>2</td>
<td>2</td>
<td>South Africa (1982)</td>
</tr>
<tr>
<td>8303</td>
<td>4</td>
<td>1</td>
<td>South Africa (1995)</td>
</tr>
<tr>
<td>20475</td>
<td>1</td>
<td>1</td>
<td>South Africa (1996)</td>
</tr>
<tr>
<td>806</td>
<td>1</td>
<td>1</td>
<td>South Africa (1998)</td>
</tr>
</tbody>
</table>

![FIG. 1. Schematic representation of murMN genes from six R6^3191/2X/2B/1A/mur transformants, compared to the genes from donor isolate 3191. The hatched areas in the transformants indicate the regions of the genes where altered DNA from isolate 3191 has been introduced. The nucleotide positions are numbered according to the sequence published by Filipe and Tomasz (3).](http://aac.asm.org/DownloadedFrom)
For this analysis, we selected two South African isolates (149193 and 50012) with high-level resistance to both penicillin and cefotaxime (MICs, 2 μg/ml). Transformation experiments with \textit{murMN} DNAs from these South African strains successfully transformed R63191/2X/2B/1A to full levels of resistance, in the same manner as did \textit{murMN} DNA from Hungarian isolate 3191. This indicated that these South African strains may also have the requirement of altered MurM for high-level resistance development and that this resistance determinant may be widespread among pneumococci. Comparison of MurM sequences from isolates 3191, 149193, and 50012 revealed very similar patterns of alteration, and many common amino acid mutations were identified (Fig. 2). MurM genes from three other isolates (8303, 20475, and 806) with lower levels of penicillin and cefotaxime resistance were then analyzed, all of which revealed MurM proteins with only a single V101A substitution. The MurM genes from these isolates showed no resistance-transforming ability; therefore, in effect these isolates had unaltered MurM proteins. This surprisingly also included isolate 8303, for which the penicillin MIC was 4 μg/ml.

Our data show that for the pneumococcus to resist β-lactam antibiotics at extreme concentrations, a functional but altered MurM is required. The branched-stem peptide precursors produced by an unaltered MurM may be adequate for cells with the capacity to resist penicillin at concentrations of up to 4 μg/ml and cefotaxime at concentrations of up to 1 μg/ml, but at higher levels of resistance, an alteration in MurM is required. Altered MurM presumably results in a new species of branched-stem peptides, with superior binding to restructured PBPs in resistant bacteria, compared to the binding of the normal branched-stem peptides. Previous studies (2, 10) have shown that high-level penicillin and cefotaxime resistance can be transferred to susceptible strains using only altered \textit{pbp} DNA; therefore, it is clear that high-level resistance is obtainable in the absence of altered MurM. Because our data have shown a requirement for altered MurM in the development of resistance, we have revealed an alternative pathway in the development of high-level penicillin and cefotaxime resistance.

In conclusion, we have shown that in conjunction with altered PBPs, a non-PBP resistance determinant (altered MurM) can be used as an alternative pathway in the development of high-level penicillin and cephalosporin resistance in the pneumococcus. Alteration of MurM is a mechanism particularly essential for full resistance development in a Hungarian clone.

Nucleotide sequence accession numbers. \textit{murMN} sequence data appear in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under the following accession numbers: AJ250764 (strain R6), AF319941 (strain 3191), and AF319942 (strains 149193 and 50012).

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\textbf{REFERENCES}

3. Filipe, S. R., and A. Tomasz. 2000. Inhibition of the expression of penicillin resistance in \textit{Streptococcus pneumoniae} by inactivation of cell wall muropeps...