Molecular Characterization of \textit{rpoB} Mutations Conferring Cross-Resistance to Rifamycins on Methicillin-Resistant \textit{Staphylococcus aureus}

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Mutations of the \textit{rpoB} gene conferring resistance to rifampin were analyzed in 40 methicillin-resistant \textit{Staphylococcus aureus} isolates obtained from six countries. Interestingly, the majority of clinical isolates showed multiple mutations within \textit{rpoB}. The amino acid substitution 481His\textrightarrow\text{Asn} was the most prevalent one, capable of conferring low-level resistance on its own. Cross-resistance to rifampin, rifabutin, and rifapentine was demonstrated for all mutants identified. The level of resistance to rifamycins correlated with both the mutation position and type of amino acid substitution.

Multiresistance as a common feature in methicillin-resistant \textit{Staphylococcus aureus} (MRSA) is a growing problem not only in hospital settings (5, 10, 22). Glycopeptides are the antibiotics of choice in the treatment of infections caused by MRSA (15). A combination therapy, however, with an antibiotic such as rifampin that reveals strong activity and good tissue penetration often is required to reach deep-seated infections effectively (6, 9, 16). Rifampin acts by inhibiting bacterial RNA polymerase (23). Previous studies on other bacteria provide evidence that mutations in \textit{rpoB}, the gene which encodes theβ subunit of RNA polymerase, are responsible for rifampin resistance (Rifr) (2, 8, 17, 18, 20). By using pairs of isogenic \textit{S. aureus} isolates gathered before and after acquisition of resistance under rifampin therapy, Aubry-Damon et al. recently demonstrated the occurrence of amino acid substitutions within a short conserved region of the β subunit and correlated the level of rifampin resistance with the mutations involved (2).

The present study was aimed at determining the distribution of mutations in the \textit{rpoB} gene in clinical isolates of rifampin-resistant MRSA as well as correlating the MICs of rifampin, rifabutin, and rifapentine with the locations and nature of the amino acid substitutions.

A total of 35 Rifr MRSA clinical isolates and five in vitro mutants generated from two rifampin-susceptible (Rif\textsuperscript{s}) epidemic MRSA strains were analyzed in this study. Fifteen of the Rifr MRSA isolates were furnished by laboratories in the United States, France, Italy, Poland, and Slovenia, whereas 20 of the Rif\textsuperscript{i} initial isolates were obtained between 1993 and 1998 from German hospitals. All MRSA isolates were analyzed by pulsed-field gel electrophoresis as described previously (24). Resistance to rifampin (Sigma, Deisenhofen, Germany), rifabutin (Pharmacia-Upjohn, Milan, Italy), and rifapentine (Hoechst Marion Roussel, Kansas, Mo.) was determined by the agar dilution method with Mueller-Hinton agar (Oxoid, Basingstoke, England) with an inoculum of 10\textsuperscript{8} CFU per spot. Two oligonucleotide primers (Life Technologies, Eggenstein, Germany), rpoB1 (5\textsuperscript{'}-ACC GTC GTT TAC GTT CTG TA) and rpoB2 (5\textsuperscript{'}-TCA GTG ATA GCA TGT GTA TC), were designed to amplify and sequence a 460-bp PCR fragment encompassing clusters I and II of the rifampin resistance mutation sites of the \textit{S. aureus} \textit{rpoB} gene (1). Amplification was performed on a Gene Amp PCR system 2400 (Perkin-Elmer, Weiterstadt, Germany) under standard conditions. Direct sequencing of purified PCR products was carried out by using a dye reaction terminator cycle sequencing kit (PE Applied Biosystems, Weiterstadt, Germany) according to the protocol described by the manufacturer. Amplified DNA was sequenced in both directions by using the 310 genetic analyzer (Perkin-Elmer).

All MRSA isolates were characterized with regard to their genotypes and the MICs of rifampin, rifabutin, and rifapentine as shown in Table 1. Comparable MICs of rifampin and rifapentine could be demonstrated for all MRSA isolates, whereas the MICs of rifabutin generally were two to eight times lower. The degree of resistance allowed classification of the strains in the categories of low-level resistance (MICs, 1 to 4 μg/ml) and high-level resistance (MICs ≥8 μg/ml). Restriction analysis of chromosomal DNA demonstrated the unrelatedness of the majority of isolates and revealed 19 different genotypes, with MRSA T38 and T23 representing the southern and northern German epidemic strains, respectively (Fig. 1). Sequence analysis of 40 MRSA isolates from six countries revealed missense mutations in a short region of the \textit{rpoB} gene equivalent to clusters I and II of \textit{Escherichia coli} (18) (Fig. 2). Twelve mutational changes at 10 positions were identified, with 473Ala\textrightarrow\text{Thr} representing a new mutation site. New amino acid substitutions, 465Gln\textrightarrow\text{Arg}, 466Leu\textrightarrow\text{Ser}, 468Gln\textrightarrow\text{Lys}, and 477Ala\textrightarrow\text{Thr} in cluster I and 527Ile\textrightarrow\text{Met} and 529Ser\textrightarrow\text{Leu} in cluster II, were described, thereby emphasizing the high variability of these amino acid positions (2, 4, 7, 8, 18–21). Sequence findings allowed the categorization of all of the rifampin-resistant MRSA isolates into 12 different genotypes with respect to the mutations involved (Table 2). Despite the different geographical origins of the isolates (Table 1), codon 481 was mutated on 32 separate occasions, which indicated a central role of this amino acid. All in vivo isolates that demonstrated two or three amino acid changes exhibited high-level resistance. Interestingly enough, all of these isolates showed the mutational change 481His\textrightarrow\text{Asn}, which is capable of conferring low-level resistance on its own, thereby indicating...
The single amino acid substitution 468Gln was not demonstrated to occur through multiple mutations within these isolates. High-level resistance in vivo, however, was not demonstrated to occur through multiple mutations alone. The single amino acid substitution 468Gln of rifampin-supplemented agar per ml revealed a new low-level resistant mutant with the amino acid substitution 471Asp on October 13, 2017 by guest http://aac.asm.org/ Downloaded from
With respect to the pharmacokinetics of rifampin (27) and the findings presented in this study, we agree with the suggestion made by Aubry-Damon et al. (2) to revise the breakpoints for rifampin set by the National Committee for Clinical Laboratory Standards (13). Accordingly, new breakpoints of $\leq 0.5$ and $\geq 8 \mu g/ml$ for the categorization of *S. aureus* relative to rifampin seem reasonable.

The goal of the present study was to elucidate the distribu-

![Pulsed-field gel electrophoresis patterns of *Sma*I digests of total DNA from representatives of each pulsotype (indicated by capital letters above lanes). Lanes m, size markers. Molecular sizes (in kilobases) are indicated on the right.](image1)

**FIG. 1.** Pulsed-field gel electrophoresis patterns of *Sma*I digests of total DNA from representatives of each pulsotype (indicated by capital letters above lanes). Lanes m, size markers. Molecular sizes (in kilobases) are indicated on the right.

<table>
<thead>
<tr>
<th>Cluster I</th>
<th>E. coli</th>
<th>507</th>
<th>G S H Q L S Q F M V H N P L T H K R R I S B L 533</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. tuberculosis</em></td>
<td>432</td>
<td><em>T</em> * * * * * * * * * * * * * * * * G L * * * * L * * * 458</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>462</td>
<td>* * * * * * * * * * * * A * * * * L * * * L * * * 488</td>
</tr>
</tbody>
</table>

**Cluster II**

<table>
<thead>
<tr>
<th>E. coli</th>
<th>560</th>
<th>P I E T E G P N I L I N S L 575</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em></th>
<th>485</th>
<th>* * * * * * * * * * * * * * * G * * 500</th>
</tr>
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</table>

| *S. aureus* | 515 | * * * * * * * * * * * * * * * M L 530 |

**FIG. 2.** Alignment of *E. coli*, *Mycobacterium tuberculosis*, and *S. aureus rpoB* sequences representing clusters I and II (1–3, 11, 14, 25, 26). The amino acid alignment is presented in a single-letter code. Asterisks symbolize identity to *E. coli* sequence. Positions involved in rifampin resistance are marked; mutations are indicated by downward-pointing arrows; insertions are indicated by upside-down triangles; and deletions are underlined. The new RifR mutation site found in this study is indicated by a double downward-pointing arrow. Amino acid substitutions involved in RifR found in this study are presented in italics. Amino acid substitutions not previously described are indicated in boldface.
tion of mutations within the rpoB gene in rifampin-resistant MRSA and to assess the in vitro effectiveness of different rifamycins against these isolates. By presenting seven new mutations, the study confirms that rpoB mutations are responsible for the common Rif’ phenotype in MRSA and that the level of resistance to any rifampin is dependent on both the type and the location of the mutation within the rpoB gene. The occurrence of multiple mutations within rpoB for S. aureus is first described in this study. Their presence might be explained by the epidemic nature of many MRSA strains and, consequently, the frequent exposure of these strains to rifampin chemotherapy. Finally, we were able to demonstrate that the mutations involved confer cross-resistance to rifampin, rifabutin, and rifapentine, thereby indicating that these antibiotics are likely to exhibit comparable effectiveness in the treatment of S. aureus infection.

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REFERENCES