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Molecular Characterization of *rpoB* Mutations Conferring Cross-Resistance to Rifamycins on Methicillin-Resistant *Staphylococcus aureus*

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Mutations of the *rpoB* gene conferring resistance to rifampin were analyzed in 40 methicillin-resistant *Staphylococcus aureus* isolates obtained from six countries. Interestingly, the majority of clinical isolates showed multiple mutations within *rpoB*. The amino acid substitution 481His→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 *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 *rpoB*, the gene which encodes the β subunit of RNA polymerase, are responsible for rifampin resistance (Rif^r) (2, 8, 12, 17, 18, 20). By using pairs of isogenic *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 *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 Rif^r MRSA clinical isolates and five in vitro mutants generated from two rifampin-susceptible (Rif^s) epidemic MRSA strains were analyzed in this study. Fifteen of the Rif^r MRSA isolates were furnished by laboratories in the United States, France, Italy, Poland, and Slovenia, whereas 20 of the Rif^r 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 10^4 CFU per spot. Two oligonucleotide primers (Life Technologies, Eggenstein, Germany), rpoB1 (5′-ACC GTC GTT TAC GTT CTG TA) and rpoB2 (5′-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 *S. aureus 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 *rpoB* gene equivalent to clusters I and II of *Escherichia coli* (18) (Fig. 2). Twelve mutational changes at 10 positions were identified, with 473Ala→Thr representing a new mutation site. New amino acid substitutions, 465Gln→Arg, 466Leu→Ser, 468Gln→Lys, and 477Ala→Thr in cluster I and 527Ile→Met and 529Ser→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 indicates 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→Asn, which is capable of conferring low-level resistance on its own, thereby indicating

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TABLE 1. Strain characterization

Strain	Origin	Genotype	MIC (μg/ml) of:		
			Rifampin	Rifabutin	Rifapentine
T236	United States	K	2	1	2
T248	France	C	256	32	256
T249	France	B	256	32	256
T250	France	B	256	32	256
T262	Slovenia	I	512	64	512
T264	Poland	J	512	64	256
T295	Poland	J	256	64	256
T296	Poland	J	256	64	256
T297	Poland	J	256	64	256
T397	Italy	S	512	512	256
T398	Italy	S	128	64	128
T399	Italy	Q	256	64	256
T400	Italy	R	256	128	256
T401	Italy	B	512	64	512
T402	Italy	B	128	32	128
T4	Germany	A	2	1	2
T20	Germany	B	512	64	512
T25	Germany	B	1	1	2
T36	Germany	L	512	64	512
T46	Germany	E	1,024	128	512
T59	Germany	M	512	128	1,024
T66	Germany	H	512	128	512
T109	Germany	D	>1,024	512	512
T112	Germany	B	256	64	256
T113	Germany	D	512	512	256
T115	Germany	N	2	1	2
T118	Germany	G	256	64	256
T124	Germany	F	512	256	256
T161	Germany	O	256	32	256
T166	Germany	H	512	128	512
T211	Germany	P	2	1	2
T212	Germany	B	256	32	256
T382	Germany	D	2	1	2
T403	Germany	D	2	1	2
T404	Germany	D	2	1	2
T38	Germany	D	≤0.008	≤0.008	≤0.008
T38a[a]	This study	D	512	256	512
T38b[b]	This study	D	512	512	512
T23	Germany	B	≤0.008	≤0.008	≤0.008
T23a[a]	This study	B	4	2	4
T23aa[c]	This study	B	>1,024	512	512
T23b[b]	This study	B	1,024	256	512

[a] Rifampin concentration of 0.0625 μg/ml used for selection of mutants from Rif[s] MRSA.
[b] Rifampin concentration of 64 μg/ml used for selection of mutants from Rif[s] MRSA.
[c] Rifampin concentration of 64 μg/ml used for selection of mutants from T23a.

a two-step resistance mechanism in vivo to high-level resistance within these isolates. High-level resistance in vivo, however, was not demonstrated to occur through multiple mutations alone. The single amino acid substitution 468Gln→Lys also causes high-level resistance.

Generation of Rif[r] mutants in vitro resulted in both of the phenotypes observed. Low-level resistance followed by a second mutation leading to high-level resistance also was demonstrated in vitro (Table 2). Rif[s] strain T23 cultured on 0.0625 μg of rifampin-supplemented agar per ml revealed a new low-level resistant mutant with the amino acid substitution 471Asp→Tyr (T23a). When, in turn, T23a was plated on 64 μg of rifampin per ml, a new mutant readily acquired a second mutation, 486Ser→Leu, resulting in high-level resistance (T23aa). Rif[s] strain T38 was cultured on rifampin-supplemented agar at concentrations of 0.0625 and 64 μg/ml, and two single high-level resistance mutations were found with 468Gln→Lys (T38a) and 481His→Tyr (T38b), respectively. A substitution at amino acid position 481 within isolates T38b and T382 conferred low- or high-level resistance depending on the nature of the new amino acid (Table 2).

TABLE 2. Correlation of mutations in the *rpoB* gene and the level of resistance to rifampin

Strain	Rifampin MIC (μg/ml)	Nucleotide change[a]	Amino acid change[b]
T4	2		
T25	1		
T115	2		
T211	2	CAT→AAT	481His→Asn
T236	2		
T382	2		
T403	2		
T404	2		
T36	512		
T59	512		
T66	512	CAT→AAT TTA→TCA	481His→Asn 466Leu→Ser
T118	256		
T166	512		
T112	256		
T161	256		
T212	256		
T248	256		
T249	256		
T250	256		
T262	512		
T264	512	CAT→AAT TCA→TTA	481His→Asn 529Ser→Leu
T295	256		
T296	256		
T297	256		
T398	128		
T399	256		
T401	512		
T402	128		
T397	512	CAT→AAT	481His→Asn
T400	256	ATT→ATG	527Ile→Met
T20	512	CAT→AAT CAA→CGA TCA→TTA	481His→Asn 465Gln→Arg 529Ser→Leu
T46	1,024	CAT→AAT GCA→ACA GCT→ACT	481His→Asn 473Ala→Thr 477Ala→Thr
T109	>1,024		
T113	512	CAA→AAA	468Gln→Lys
T124	512		
T38a	512	CAA→CTT	468Gln→Leu
T38b	512	CAT→TAT	481His→Tyr
T23a	4	GAC→TAC	471Asp→Tyr
T23aa	>1,024	GAC→TAC TCA→TTA	471Asp→Tyr 486Ser→Leu
T23b	1,024	TCA→TTA	486Ser→Leu

[a] The mutations listed are for all strains within a grouping. Base changes are underlined.
[b] The amino acid changes listed are for all strains within a grouping.

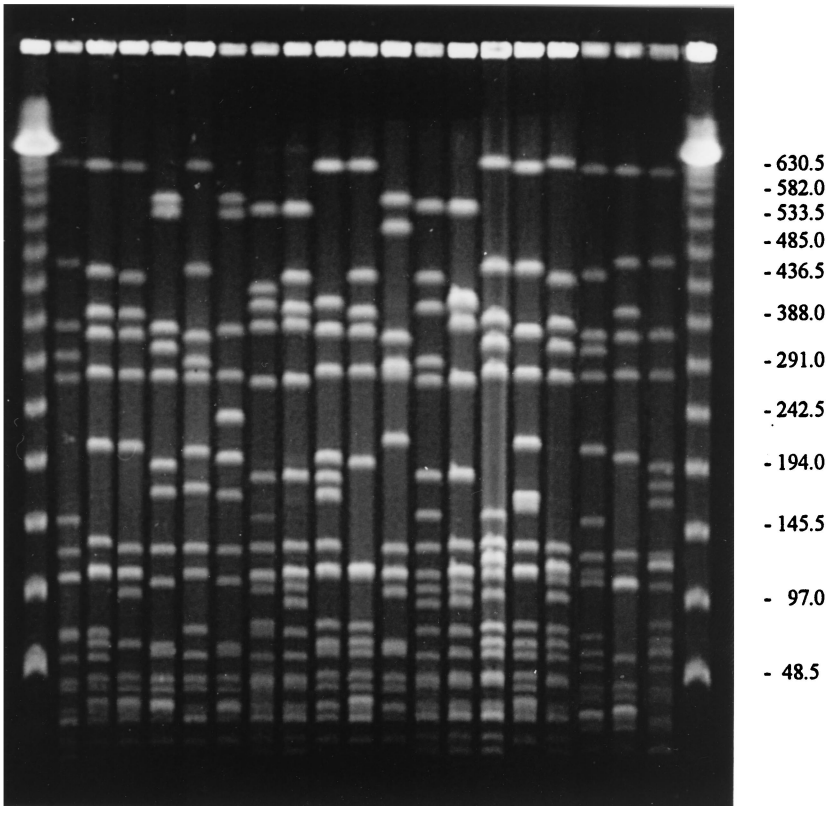

FIG. 1. Pulsed-field gel electrophoresis patterns of *Sma*I digests of total DNA from representatives of each pulsotype (indicated by capital letters above lanes). Lanes m, size markers. Molecular sizes (in kilobases) are indicated on the right.

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 ≤0.5 and ≥8 μg/ml for the categorization of *S. aureus* relative to rifampin seem reasonable.

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

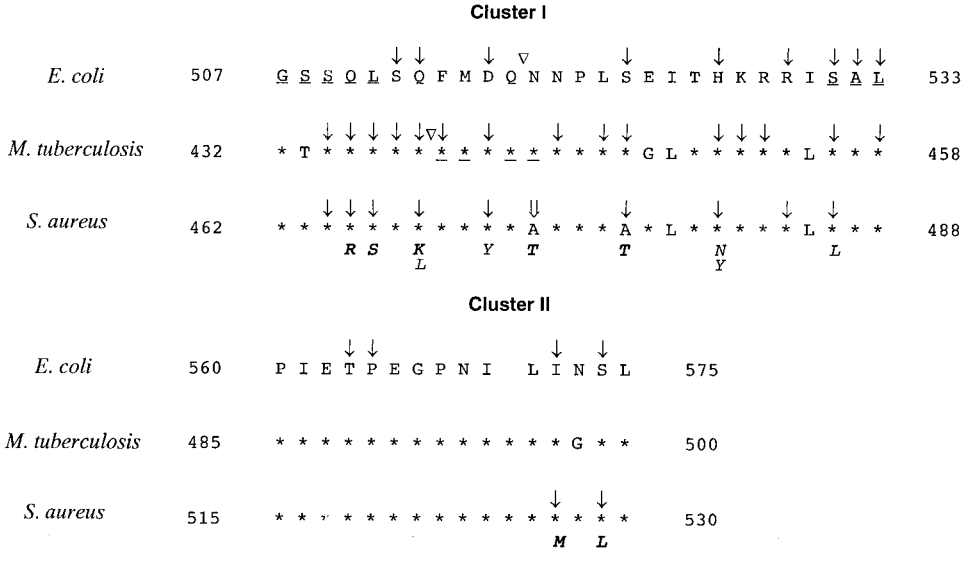

FIG. 2. Alignment of *E. coli*, *Mycobacterium tuberculosis*, and *S. aureus rpoB* sequences representing clusters I and II (1–3, 11, 14, 25, 26). The amino acid alignment is presented in a single-letter code. Asterisks symbolize identity to *E. coli* sequence. Positions involved in rifampin resistance are marked; mutations are indicated by downward-pointing arrows; insertions are indicated by upside-down triangles; and deletions are underlined. The new Rif^r mutation site found in this study is indicated by a double downward-pointing arrow. Amino acid substitutions involved in Rif^r 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^r phenotype in MRSA and that the level of resistance to any rifamycin 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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