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Mutations in the Beginning of the *rpoB* Gene Can Induce Resistance to Rifamycins in both *Helicobacter pylori* and *Mycobacterium tuberculosis*

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A clinical isolate of *Helicobacter pylori* that developed resistance to rifabutin during therapy carried an *rpoB* gene that retained a wild-type cluster region sequence but had acquired a novel codon 149 (V149F) mutation. In transformation experiments, the mutation was shown to confer high-level rifabutin resistance. The equivalent mutation (V176F) was present in several resistant isolates of *Mycobacterium tuberculosis*.

Rifabutin and other derivatives of rifampin are inhibitory against *Helicobacter pylori* at very low concentrations in vitro (1, 4). Triple therapy including rifabutin, amoxicillin, and a proton pump inhibitor has been effective in the eradication of *H. pylori* after failure of other therapies and in spite of resistance to other antibiotics (10). Resistance to rifampin and rifabutin is caused by amino acid exchanges in the β subunit of the DNA-directed RNA polymerase (RpoB). Mutations at codons 146, 507 to 533, 563 to 572, and 687 of the *rpoB* gene in *Escherichia coli* (11) or at codons 507 to 533 (cluster region) in *Mycobacterium tuberculosis* (8, 9) have been shown to induce resistance.

All resistant mutants of *H. pylori* ATCC 43504 selected in vitro in a previous investigation (4) showed mutations in the cluster region encompassing codons 525 to 545 and codon 586.

Here we describe a clinical isolate of *H. pylori* that developed resistance during therapy. The patient was treated with lanzoprazole and rifabutin, but amoxicillin was discontinued due to intolerance. Isolates of *H. pylori* before (DR62a) and after (DR62n) treatment were available for evaluation. Culture, storage, E-test, agar dilution assay, PCR, and sequencing of the cluster region were performed as described recently (4). Table 1 shows the primers for the amplification and evaluation of large and small segments of the respective *rpoB* regions. Sequencing was performed in both directions (Perkin-Elmer Applied Biosystems).

DR62n was resistant to rifampin (E-test, MIC of >256 μg/ml) and rifabutin but, in contrast to previous findings (4), showed no difference from the published wild-type sequence (14) in the cluster region. The MICs of rifabutin for *H. pylori* DR62a and DR62n were 0.002 and 8 μg/ml, respectively (Table 2). Both strains yielded homologous patterns when typed by arbitrarily primed PCR (2; unpublished work). Amplification and sequencing of DR62n *rpoB* (rifabutin resistant) from bp 387 to bp 916 revealed a codon exchange, GTC→TTC (V149F). DR62a (rifabutin susceptible) showed wild-type sequence in both the V149 region and the cluster region.

In order to confirm that the amino acid mutation V149F is responsible for high-level resistance, large fragments of the *rpoB* gene (bp 54 to 916 harboring codon 149 and bp 1271 to 2106 harboring the cluster region) from DR62n and DR62a were amplified and sequenced. The amino acid mutation V149F was the only difference between DR62n and DR62a or the published wild-type sequence (14). All fragments were transformed into a competent, rifabutin-susceptible strain, *H. pylori* 2802A. Transformation of *H. pylori* 2802A was done, using a dense suspension of a fresh culture adjusted to a McFarland standard of >4 in 1 ml at an optical density value at

TABLE 1. PCR primers for amplification and sequencing of the respective *H. pylori* and *M. tuberculosis rpoB* segments

Sp. and region	Primer	Nucleotide sequence (5′ to 3′)	Position in the *rpoB* gene (bp)
H. pylori V149	RpoB-ri 1[a]	CCCAACAGATTTAGAAGT	54–71 (sense)
	RpoB-ri-F	GATCCCTTTGATGACAGAAC	387–406 (sense)
	RpoB-ri-R[a]	TACCATAACAGGCTCAGC	916–899 (antisense)
Cluster I + II	RpoB-5	AAATGATCACAAGCACCATC	1530–1549 (sense)
	RpoB-CL	ACCTTGCCATCCACAACC	1839–1822 (antisense)
	RpoB-CLF[a]	ATGTGCCTGATTACATCACGAC	1271–1292 (sense)
	RpoB-CLR[a]	TTGGCGCTGCATGTTAGTCC	2106–2087 (antisense)
M. tuberculosis V176	Tb176-f	CTTCTCCGGGTCGATGTCGTTG	294–315 (sense)
	Tb176-R	CGCGCTTGTCGACGTCAAACTC	658–637 (antisense)
Cluster I + II	TbRif-1	CAGACGTTGATCAACATCCG	1243–1262 (sense)
	TbRif-2	TACGGCGTTTCGATGAAC	1547–1530 (antisense)

[a] Primers for amplification of the large fragments used for transformation.

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TABLE 2. MICs (agar dilution) of rifampin and rifabutin for *H. pylori* with wild-type *rpoB* sequence (DR62a and 2802A) and for *H. pylori* harboring a novel mutation, V149F (DR62n and 2802A-R)[a]

Strain	Codon no. (V149/V176) and amino acid	MIC (μg/ml)	
		Rifampin	Rifabutin
H. pylori			
DR62a		0.25	0.002
DR62n	V149F	16	8
2802A		0.25	0.008
2802A-R	V149F	16	16
M. tuberculosis[b]			
69		1	0.03
71		2	0.5
72		4	1
75	V176F	8	1
83	V176F	32	2
84	V176F	32	2

[a] The equivalent mutation (V176F) was seen for three of six *M. tuberculosis* samples with the rifampin resistance phenotype but the wild-type cluster region.
[b] *M. tuberculosis* strain designations are from the work of Yang et al. (17). The data for *M. tuberculosis* come from 6 samples out of 100 of a collection of *M. tuberculosis* isolates for which MICs of rifamycin are elevated.

550 nm (OD_{550}) of 10, diluted to an OD_{550} value of 0.2 in brucella broth with 8% fetal calf serum. One milliliter of a suspension with an OD_{550} value of 0.2 (in a 24-well plate) was incubated at 36°C with 3 to 5% CO_2 for 6 h. At 0, 2, and 4 h, 3 to 10 μl of a PCR product (1 to 3 μg of DNA) was added. Cells were cultured on Wilkins-Chalgren agar containing rifabutin (0.03 μg/ml) under microaerophilic conditions for 3 to 5 days. Single colonies were picked and grown on selective agar for MIC determination, storage, DNA amplification, and sequencing.

Repeated transformation of *H. pylori* 2802A with PCR products harboring the V149F mutation (bp 54 to 916 from DR62n *rpoB*) generated 10^5 to 10^6 CFU/well (2802A-R) showing the same level of resistance as seen in the donor strain (Table 2). Transformation with PCR products of the wild-type 5′ region (bp 54 to 916) or cluster region (bp 1371 to 2106) of *H. pylori rpoB* generated fewer than five resistant colonies per well.

Positive controls for the cluster region (e.g., Q527R) were amplified from the DNA of rifabutin-resistant *H. pylori* ATCC 43504 variants (4) and generated 10^5 to 10^6 CFU/well (data not shown).

After we had the confirmation of a newly described mutation outside the cluster region conferring high-level resistance in *H. pylori*, we wondered whether this arrangement might also occur in *M. tuberculosis*. PCR-based systems for the detection of mutations in the cluster region are of outstanding importance for the determination of rifampin susceptibility of slow-growing organisms like *M. tuberculosis* but sometimes fail to match with a resistant phenotype. Reported failure rates range from 3 to 6% (3, 5, 7, 9, 13, 16, 17). DNA samples of six *M. tuberculosis* isolates from Japan for which MICs of rifampin were elevated but with wild-type sequence in the *rpoB* cluster region were provided by the authors of reference 17.

Primers for PCR and sequencing are shown in Table 1. Three out of six samples showed the mutation GTC→TTC (V176F), consistent with our findings in DR62n. The amino acid changes detected are shown in Table 2 and mapped in Fig. 1. There is no report in the literature so far of a mutation in this region in mycobacteria.

Rudi Rossau et al. (Innogenetics) recently presented data at a meeting (R. Rossau, W. Mus, G. Jannes, K. de Smet, H. van Heuverswijn, H. Traore, and F. Portaels, 20th Annu. Congr. Eur. Soc. Mycobacteriol., abstr. OC27, p. 44, 1999) which are in strong agreement with ours and emphasize the importance of our findings. They investigated 203 resistant *M. tuberculosis* isolates, finding discrepant results in four isolates. Three out of four samples showed the mutation V176F, induced by the same base exchange. Their collection contained no isolates from Japan. However, no comparable mutation in eight resistant *M. tuberculosis* strains with the wild-type cluster region was found by Telenti et al. (8; A. Telenti, personal communication; no MIC given).

The amino acid mutation V146F conferring high-level resistance in *E. coli* was first observed by Lisitsyn et al. in 1984 (6) and verified by Severinov et al. (12) by site-directed mutagenesis (Fig. 1). Recently, three mutations associated with resistance were described for a *Rickettsia typhi* isolate (15). Mutations were observed at codons 151 (F151L), 201, and 271. Codon 151 is located next to the region which has been shown to confer resistance and is possibly the one responsible for the resistant phenotype. Out of six *M. tuberculosis* isolates, three with the higher level of resistance to rifampin (8 to 32 μg/ml) carried the V176F mutation, while the low-level resistance (1 to 4 μg/ml) remains unexplained.

Mutations in the 5′ region of the *rpoB* gene inducing rifamycin resistance in *H. pylori* and possibly in *M. tuberculosis* have not been sufficiently examined. Resistance can be caused by a single base exchange outside the cluster region. This should be considered in future developments of molecular biology-based testing for mycobacteria.

The high homology of this region on the amino acid level (Fig. 1) indicates that there might be mutations conferring resistance in other species like *Legionella pneumophila* or *Staphylococcus aureus* as well.

Nucleotide sequence accession numbers. The relevant sequence fragments of *M. tuberculosis* strain 84 and *H. pylori* DR62n have been deposited in the GenBank database under accession no. AF 177294 and AF 177295, respectively.

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gb|AAD08242.1 H. pylori 26695   EIPLMTERTSFIINGVERVVVNQLHRSPGVIF   (V149)
              H. pylori DR62n   ....................F...........   (V149F)
gi|396326     E. coli           ......DNGT.V...T...IVS.........F.   (V146)
gi|468334     M. tuberculosis   DF.M...KGT.....T....VS..V.....Y.   (V176)
gi|2668777    R. typhi          D.....KNGTF....T.....S.M......F.   (F151L)
gi|3549149    L. pneumophila    ......DVGT.VV..T.....S..........   (V159)
sp|P47768     S. aureus         DF....DTGT.V...A...I.S..V...S.Y.   (V135)
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FIG. 1. Alignment of the homologous amino acid sequences of the N-terminal region of RpoB of different species. The figure shows mapping of codons associated with the rifamycin resistance phenotype (boxes).

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