ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2001, p. 962–965 Vol. 45, No. 3
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.3.962–965.2001

Mutations of the *Helicobacter pylori* Genes *rdxA* and *pbp1* Cause Resistance against Metronidazole and Amoxicillin

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Received 9 June 2000/Returned for modification 17 August 2000/Accepted 25 November 2000

To investigate amoxicillin and metronidazole resistance of *Helicobacter pylori*, we compared putative resistance genes between resistant strains obtained in vitro and their sensitive parent strain. All metronidazole-resistant strains had *rdxA* mutations, and an amoxicillin-resistant strain had *pbp1* and *pbp2* mutations. By transforming PCR products of these mutated genes into antibiotic-sensitive strains, we showed that *rdxA* null mutations were sufficient for metronidazole resistance, while *pbp1* mutations contributed to amoxicillin resistance of *H. pylori*.

Although most infections with *Helicobacter pylori* are asymptomatic, and some might even be beneficial for the host, the pathogen is usually eradicated with antibiotics such as amoxicillin and metronidazole in order to cure gastritis and peptic ulcer diseases (3, 4). Resistance against metronidazole is common among *H. pylori* strains, while there are only a few reports of amoxicillin-resistant *H. pylori* strains (6, 8; M. Guslandi, Letter, Lancet **353:**241–242, 1999; A. A. van Zwet, C. M. Vandenbroucke-Grauls, J. C. E. Thijs, J. van der Wouden, M. M. Gerrits, J. G. Kusters, and C. M. Vandenbrouke-Grauls, Letter, Lancet **352:**1595, 1998).

Metronidazole resistance of *H. pylori* was shown to be due to the mutational inactivation of *rdxA* (7, 11). In turn, a metronidazole-resistant strain (Mtz^r) was rendered sensitive when complemented with the *rdxA* gene of a metronidazole-sensitive strain (Mtz^s). It was concluded that RdxA functions as a metronidazole-reducing nitroreductase (11). Resistance against β-lactam antibiotics like amoxicillin is generally due to hydrolysis by a β-lactamase (5) or by mutational modification of the penicillin binding proteins (13).

To investigate the molecular basis for antibiotic resistance in *H. pylori*, we did an in vitro selection for amoxicillin and metronidazole resistance on the strains 503 (ATCC 43503) and 69A (69A and 888–0: clinical isolates obtained from R. Haas, Max-von Pettenkofer-Institut, Munich, Germany). In contrast to other investigators (12, 15), we performed the selection not on agar plates, but in liquid culture (brain heart infusion medium [BHI; Difco-BD Biosciences, Md.] plus 5% fetal calf serum [FCS; Eurobio, Les Ulis, France]) at 37°C with microaerobic incubation (5 to 6% O_2, 8 to 10% CO_2) in the presence of these antibiotics. The MIC was determined by inoculating logarithmically growing *H. pylori* cells with an optical density at 578 nm (OD_{578}) of 0.04 in 10 ml of BHI–5% FCS in 50-ml cell culture flasks (Greiner) on a shaker incubator at 90 rpm. The MIC of metronidazole was defined as no OD_{578} increase in 10 to 14 days, and that of amoxicillin was defined as growth to an OD_{578} lower than 0.4, with no increase after the first 24 h of incubation. We obtained stable metronidazole resistance after three serial passages over the course of 8 to 10 days with increasing metronidazole concentration in the growth medium (from 2 μg/ml to 25 μg/ml). The metronidazole MIC for nine independently selected 69A/Mtz^r and 503/Mtz^r strains was >25 μg/ml, while that for strains 503 and 69A was <5 μg/ml (data not shown). These results correspond to the observation of metronidazole resistance developing de novo during the course of a typical antibiotic therapy (1). In vitro selection of amoxicillin-resistant *H. pylori* strains was done similarly. Since the amoxicillin MIC for strains 503 and 69A was 0.02 to 0.05 μg/ml, we began selection at an amoxicillin concentration of 0.01 μg/ml. After 11 passages and 35 days under the permanent selective pressure of amoxicillin, the amoxicillin MIC for strain 69A reached 0.5 to 1 μg/ml, and after 35 passages and 89 days, an amoxicillin MIC of 15 μg/ml for the highly resistant strain 69A/Amx^r was obtained. Comparable selection results were obtained for the strain 503 (data not shown). In contrast to other Amx^r strains described in the literature (9), the resistance was stable after cultivation in the absence of antibiotics and storage at −80°C. The induction of resistance was specific for the antibiotic used for selection. Amoxicillin-resistant strains remained sensitive to metronidazole and vice versa.

To investigate the molecular mechanisms for resistance, we sequenced putative resistance genes from 69A strains: five independently selected 69A/Mtz^r strains and one 69A/Amx^r strain. The *rdxA* gene of each 69A/Mtz^r strain had at least one mutation compared to the copy of the wild-type 69A strain (Fig. 1A). In three cases, the mutations caused stop codons in the *rdxA* open reading frame, which is also common for metronidazole-resistant clinical isolates (Fig. 1B). In the remaining two strains, the mutations caused either one or two amino acid

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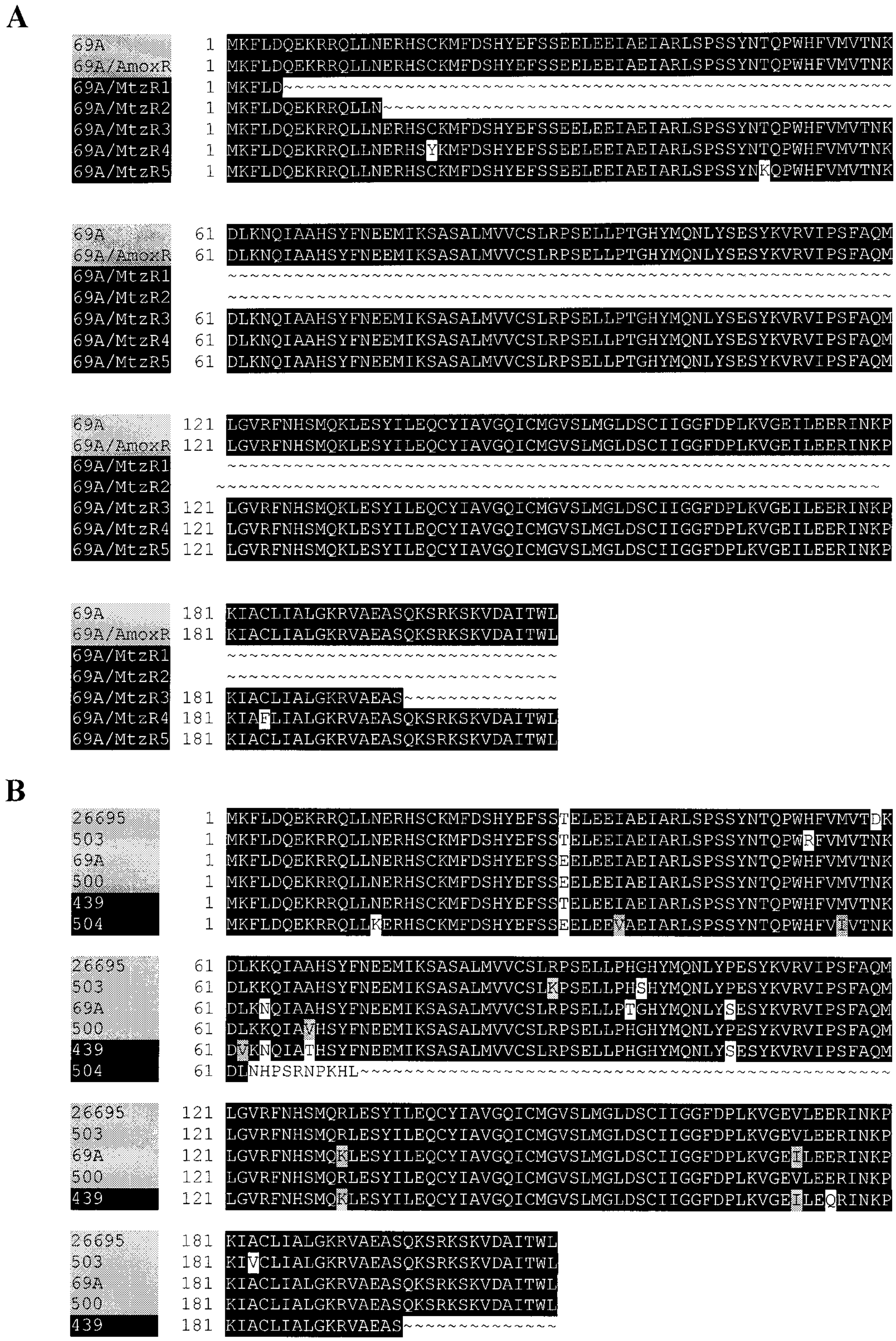

FIG. 1. Alignment of RdxA proteins from *H. pylori* 69A strains selected for antibiotic resistance (A) and those of *H. pylori* wild-type strains (B). (A) The strains 69A and 69A/Amx^{r} were metronidazole sensitive, and the 69A/Mtz^{r}1 to -5 strains were metronidazole resistant. (B) *H. pylori* strains 26695, 503, 69A, and 500 were metronidazole sensitive, and *H. pylori* strains 439 and 504 (ATCC 43504) were metronidazole resistant. A black background shows the conservation of amino acid residues. *H. pylori* amino acid sequences are given as follows: 26695, reference 16; strains 500 and 439, reference 11; strain 503, GenBank accession no. AF316109; strain 504, accession no. AF315501; and strain 69A, accession no. AF315502.

TABLE 1. Transformation of mutated genes into antibiotic-sensitive *H. pylori* with metronidazole or amoxicillin selection medium[a]

H. pylori strain	Metronidazole concn (μg/ml)	Result for transformed gene[b]					Amoxicillin concn (μg/ml)	Result for transformed gene[b]			
		rdxA/Mtz^{r}1	*rdxA*/Mtz^{r}3	*rdxA*/Mtz^{r}4	*rdxA*/Mtz^{r}5	Control (no DNA added)		*pbp1*	*pbp2*	*pbp1* + *pbp2*	Control (no DNA added)
69A	5	+	+	+	+	−	0.2	+	−	+	−
69A	25	+	+	+	+	−	0.5	+	ND	+	−
26695	5	+	+	+	+	−	0.2	+	−	+	−
26695	25	+	+	+	+	−	0.5	+	ND	+	−
888-0	5	+	+	+	+	−	0.2	+	−	+	−
888-0	25	+	+	+	+	−	0.5	+	ND	+	−

[a] For transformation with mutated *rdxA* genes, the *rdxA* genes of the indicated 69A/Mtz^r strains were amplified by PCR with primers 5′*rdx*A1 (ATGGGTTGCTG ATTGTGGTTTATGG) and 3′*rdx*A2 (GCTTGAAAACACCCCTAAAAGAGCG) and purified by gel electrophoresis. For transformation, the DNA (250 to 500 ng) was added to logarithmically growing metronidazole-sensitive *H. pylori* strains. After 16 h of incubation, the bacteria were diluted in medium containing metronidazole to select for transformants. For transformation with mutated *pbp1* and *pbp2* genes, the mutated *pbp* genes of the *H. pylori* 69A/Amx^r strain were amplified by PCR with 5′*pbp*1-1 primers (AATCAAGCGGTGAGTATCCTTGTGG), 3′*pbp*1-2 (CTACGGTTTCTAAACCCCTTTTACG), 5′*pbp*2-1 (GTTATAAGCGGTGGAATGAGT TGG), and 3′*pbp*2-2 (TGACGGCTTTTTATTCAAAACTTTGC), purified by gel electrophoresis, and transformed into logarithmically growing amoxicillin-sensitive *H. pylori* strains. Transformants were selected for by dilution in medium containing amoxicillin.

[b] +, growth of bacteria after 3 to 5 days of incubation; −, no growth after 14 days of incubation; ND, not determined.

changes at positions conserved in metronidazole-sensitive strains (Fig. 1B). The 69A/Amx^r strain had no *rdxA* mutation (Fig. 1A), but had four *pbp1* mutations (S414R, Y484C, T541I, and P600T) and one *pbp2* mutation (T498I). All of these mutations cause amino acid changes at positions conserved in the Amx^s strains 26695 (16), J99 (2), and 69A (GenBank accession no. AF315503 and AF315504) and were located in the putative transpeptidase domains of the proteins (10).

To prove that these mutations were indeed responsible for antibiotic resistance, we amplified these genes by PCR, purified the DNA by gel electrophoresis, and transformed it into antibiotic-sensitive strains. To do this, we used a simplified protocol for transformation of *H. pylori*, in which we added linear PCR fragments without an additional resistance marker to logarithmically growing *H. pylori* and then selected for transformants with amoxicillin or metronidazole, respectively. After transformation with the mutated *rdxA* genes from four 69A/Mtz^r strains, bacteria of three metronidazole-sensitive *H. pylori* strains (26695, 69A, and 888–0) were rendered resistant (Table 1). Transformation with the mutated *pbp1* gene from the 69A/Amx^r strain rendered bacteria of these strains (26695, 69A, and 888–0) moderately amoxicillin resistant (MIC of 0.5 to 1 μg/ml). Transformation with the *pbp2* gene from 69A/Amx^r caused no amoxicillin resistance. The cotransformation of *pbp1* and *pbp2* did not show increased resistance compared to transformation with *pbp1* alone (Table 1).

To exclude the possibility that antibiotic resistance after transformation was due to a different spontaneous mutation, we sequenced the *rdxA* genes from six transformed metronidazole-resistant strains and the *pbp1* genes from two transformed amoxicillin-resistant strains. In all but one case, we found the same mutations as in the respective donor strain (Table 2). The only exception was observed for transformation with the *rdxA* gene of 69A/Mtz^{r}4: The *rdxA* gene from 69A/Mtz^{r}4 contained two mutations (C19Y and C184F), while *H. pylori* transformed with this gene acquired only the C19Y mutation (Table 2). We therefore do not know if the C184F mutation is involved in metronidazole resistance.

Our findings independently confirm previous results (7, 11, 14) and expand their data in the sense that not only stop codons and extensive deletions in the *rdxA* open reading frame cause metronidazole resistance, but so do single amino acid changes. We have proven that two alterations of the RdxA protein, C19Y and T49K, have the same effect on the phenotype of *H. pylori* (Mtz^s→Mtz^r) as *rdxA* null mutations and conclude that they severely affect RdxA function. The same is true for deletion of the C-terminal 14 amino acids of RdxA. By systematically transforming *rdxA* genes with single mutations into metronidazole-sensitive *H. pylori* strains by the simplified protocol, we have illustrated how it would be possible to identify additional residues essential for RdxA function. We have also shown that *pbp1* mutations can affect amoxicillin resistance, but are not sufficient for the high-level amoxicillin resistance of 69A/Amx^r. This indicates that mutations in more than one gene are probably required to render *H. pylori* amoxicillin resistant. This could explain the many cycles necessary for

TABLE 2. Sequences of *rdxA* and *pbp1* genes from antibiotic-resistant *H. pylori* strains gained by transformation with mutated *rdxA* and *pbp1* genes

Donor strain	Gene	Amino acid change(s) in donor strain	Acceptor strain	Amino acid change(s) in acceptor strain
69A/Mtz^{r}1	*rdxA*	6 Q → stop	69A	6 Q → stop
69A/Mtz^{r}3	*rdxA*	197 Q → stop	69A	197 Q → stop
69A/Mtz^{r}4	*rdxA*	19 C → Y, 184C → F	69A	19 C → Y
69A/Mtz^{r}5	*rdxA*	49 T → K	69A	49 T → K
69A/Mtz^{r}4	*rdxA*	19 C → Y, 184C → F	26695	19 C → Y
69A/Mtz^{r}5	*rdxA*	49 T → K	26695	49 T → K
69A/Amx^r	*pbp1*	414 S → R, 484 Y → C, 541 T → I, 600 P → T	69A	484 Y → C, 541 T → I, 600 P → T[a]
69A/Amx^r	*pbp1*	414 S → R, 484 Y → C, 541 T → I, 600 P → T	26695	484 Y → C, 541 T → I, 600 P → T[a]

[a] Codon 414 of *pbp1* was not sequenced in these strains.

the in vitro selection of amoxicillin-resistant *H. pylori* strains and their low occurrence in vivo.

The work described here was supported by a grant from the German Ministry of Education and Research (BMBF; BEO/22 031 0777).

We thank Klaus Bensch and Wolfram Steinhilber for discussions.

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