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Effects of 16S rRNA Gene Mutations on Tetracycline Resistance in *Helicobacter pylori*

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The triple-base-pair 16S rDNA mutation $AGA_{926-928}$→TTC mediates high-level tetracycline resistance in *Helicobacter pylori*. In contrast, single- and double-base-pair mutations mediated only low-level tetracycline resistance and decreased growth rates in the presence of tetracycline, explaining the preference for the TTC mutation in tetracycline-resistant *H. pylori* isolates.

Tetracycline is a cheap and effective antibiotic for the treatment of *Helicobacter pylori* infections (7, 8), but in the past few years the incidence of tetracycline resistance has significantly increased (1, 5, 6, 9, 13). The only known mechanism mediating tetracycline resistance in *H. pylori* involves mutations at positions 926 to 928 in both the 16S rRNA genes (2, 4, 12). *H. pylori* strains with high-level tetracycline resistance (Tet^r) carried the triple-base-pair mutation $AGA_{926-928}$→TTC in both copies of the 16S rRNA genes (4, 12), whereas strains with low-level Tet^r contained only single- and double-base-pair mutations in the exact same region (2). As the different mutations were present in unrelated strains, it is still unclear whether high-level tetracycline resistance requires the $AGA_{926-928}$→TTC mutation or whether single- or double-base-pair mutations at these positions may suffice for high-level tetracycline resistance. Therefore, we have created all possible combinations of single, double, and triple mutations at the 16S rRNA gene positions 926 to 928 in *H. pylori* strain 26695 and have determined the effects of the mutations on levels of tetracycline resistance, stability, and growth rate.

Site-directed mutagenesis at positions 926 to 928 was carried out by using a three-step PCR approach (3, 10) with primers listed in Table 1, followed by natural transformation to *H. pylori* reference strain 26695 (4). Tet^r *H. pylori* colonies were selected on plates containing tetracycline (1 μg/ml). For each possible base pair mutation or combination thereof, eight Tet^r transformants from at least two independent transformation experiments were selected. Both alleles of the 16S rRNA genes were amplified by PCR (4) to confirm the presence of the desired base pair mutations. With the exception of the AGC mutants, all mutants contained the desired mutations in both alleles of the 16S rRNA genes. All AGC mutants were heterozygous and contained the AGC mutation in the *rrnA* gene and a TTC mutation in the *rrnB* gene.

The effects of the 16S rRNA mutations on both the stability and the level of tetracycline resistance were determined by subculturing two mutants of each type for 20 passages on Columbia agar plates supplemented with 7% lysed horse blood (BioTrading, Mijdrecht, The Netherlands) in either the presence or absence of tetracycline (1 μg/ml). After each five rounds of subculturing, the MIC of tetracycline was determined by using E-test (AB Biodisk, Solna, Sweden) (4). In addition, for all mutants at time point zero (t_0) and after passage 20 (t_{20}), the 16S rRNA genes were sequenced. The stability of the various types of mutations and their effects on the levels of resistance are summarized in Table 2.

At t_0, the single- and double-base-pair mutations did not result in tetracycline resistance with levels of clinical relevance, as the MIC of tetracycline was below 4 μg/ml. With the exception of those for the AGC mutants, the MICs of tetracycline at t_{20} were similar to those at t_0 (Table 2). The MIC of tetracycline for the AGC mutants increased from 1.5 to 6.0 μg/ml. Subsequent analysis of the 16S rRNA gene sequences of all mutants at t_0 and t_{20} revealed that the TGA and AGC mutations were unstable. Already during the two subculturing steps needed for propagation of the colonies to obtain sufficient material for storage at −80°C, the sequences of both 16S rRNA genes of the TGA mutants had changed to TGC, and while stable during the initial propagation steps, after 20 rounds of subculturing both AGC mutants contained a TTC sequence at positions 926 to 928 within both 16S rRNA genes instead of only in the *rrnB* gene. Apart from these TGC and AGC mutations, all other mutations were stable as the complete 16S rRNA gene sequences did not reveal any other sequence changes except for the desired mutations.

To determine whether the 16S rRNA mutations affected the growth rates of the mutants in the presence or absence of tetracycline, all mutants from t_0 were cultured in duplicate in brucella broth supplemented with 3% newborn calf serum (Life Technologies Ltd., Auckland, New Zealand). One culture of each mutant was supplemented with tetracycline (1 μg/ml), whereas the other culture was kept unsupplemented. Growth was monitored by measuring the optical density at 600 nm each 24 h for a period of 72 h. In the absence of tetracycline, the growth of the mutants did not differ from that of the *H. pylori* wild-type strain 26695 (Table 2). However, in the presence of tetracycline the growth of the wild-type strain and

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TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)[a]	Position relative to 16S rRNA gene[b]	16S rRNA mutation[c]
Sequencing, cloning, and PCR			
Hp16S-F1	CTGACGCTGATTGCGCGAAA	711–730, forward	NA
Hp16S-F2	CCTGCTGGAACATTACTGAC	696–715, forward	NA
Hp16S-F3	TTTATGGAGAGTTTGATCCT	1–20, forward	NA
Hp16S-F4	TAGTTGTTGGAGGGCTTAGT	789–808, forward	NA
Hp16S-R1	TCGTTGCGGGACTTAACCCA	1071–1052, reverse	NA
Hp16S-R2	TGGCTCCACTTCGCAGTATT	1245–1226, reverse	NA
Hp16S-R3	AGGAGGTGATCCAACCGCA	1499–1480, reverse	NA
Hp16S-*rrnA*	CCCAAATCCTTGAGCGTTTA	1840–1821, reverse[d]	NA
Hp16S-*rrnB*	CGCATTCATAATCAGCTCAG	1826–1807, reverse[e]	NA
Mutagenesis			
Hp16S-mutF1	TAATTCGATGATACACGAAG	918–937, forward	TGA
Hp16S-mutF2	TAATTCGAATATACACGAAG	918–937, forward	ATA
Hp16S-mutF3	TAATTCGAAGCTACACGAAG	918–937, forward	AGC
Hp16S-mutF4	TAATTCGATTATACACGAAG	918–937, forward	TTA
Hp16S-mutF5	TAATTCGATGCTACACGAAG	918–937, forward	TGC
Hp16S-mutF6	TAATTCGAATCTACACGAAG	918–937, forward	ATC
Hp16S-mutF7	TAATTCGATTCTACACGAAG	918–937, forward	TTC
Hp16S-mutR1	CTTCGTGTATCATCGAATTA	937–918, reverse	TGA
Hp16S-mutR2	CTTCGTGTATATTCGAATTA	937–918, reverse	ATA
Hp16S-mutR3	CTTCGTGTAGCTTCGAATTA	937–918, reverse	AGC
Hp16S-mutR4	CTTCGTGTATAATCGAATTA	937–918, reverse	TTA
Hp16S-mutR5	CTTCGTGTAGCATCGAATTA	937–918, reverse	TGC
Hp16S-mutR6	CTTCGTGTAGATTCGAATTA	937–918, reverse	ATC
Hp16S-mutR7	CTTCGTGTAGAATCGAATTA	937–918, reverse	TTC

[a] Primers used for amplification were based on the published genome sequence of *H. pylori* strain 26695 (11). The substituted residues are underlined.
[b] Positions of oligonucleotides are given relative to the start point of the *rrnA* gene of *H. pylori* strain 26695 (11).
[c] 16S rRNA mutations at positions 926 to 928 (numbering according to sequence of the *rrnA* gene of *H. pylori* strain 26695) (11). The substituted residues are underlined. NA, not applicable.
[d] The *rrnA*-specific oligonucleotide is located outside the 16S rRNA gene at positions 1207242 to 1207261 of the *H. pylori* 26695 genome sequence (4, 11).
[e] The *rrnB*-specific oligonucleotide is located outside the 16S rRNA gene at positions 1510809 to 1510828 of the *H. pylori* 26695 genome sequence (4, 11).

the mutants with single- and double-base-pair mutations was clearly reduced compared to that of the TTC mutant. The reduction was most pronounced in the wild-type strain and the AGC, ATA, and TGC mutants (Table 2).

From this study it is apparent that for high-level tetracycline resistance, *H. pylori* requires the triple-base-pair mutation $AGA_{926-928} \rightarrow TTC$ in both copies of the 16S rRNA genes. Such high-level tetracycline resistance is most likely generated by a stepwise process that is driven by selection that depends on both the duration of exposure to and the dose of tetracycline. For this reason, it was not surprising that the previously described *H. pylori* strains with low-level Tet^r contained only single- or double-base-pair mutations in the exact same region as the TTC mutation (2). In this report (2) it was also sug-

TABLE 2. Characterization of mutants construction by using site-directed mutagenesis

Strain or mutant[a]	MIC (μg/ml) of TET at[b]:			Growth rate	
	t_0[c]	t_{20}			
		On TET-free medium	On TET (1μg/ml)	On TET-free medium[d]	On TET (1 μg/ml)[e]
Wild-type strain 26695	0.19	0.19	0.19	+++	No growth
TGA[f]	ND	ND	ND	ND	ND
ATA	1.5	1.5	2.0	+++	+
AGC[g]	1.5	6.0	6.0	+++	+
TTA	1.5	2.0	2.0	+++	++
TGC	1.0	1.5	2.0	+++	+
ATC	2.0	2.0	3.0	+++	++
TTC	6.0	6.0	8.0	+++	+++

[a] The substituted residues are underlined.
[b] MIC were determined by using E-test for two independent mutants. MIC shown are means of results from four to six independent experiments. TET, tetracycline; ND, not determined.
[c] At t_0 all mutants were already subcultured twice in order to obtain sufficient bacteria for storage at −80°C.
[d] Growth rates of the isogenic mutants relative to the growth rate of the wild-type strain 26695 (100%). +++, 95 to 100%; ++, 70 to 95%; +, 50 to 70%.
[e] Growth rates of the isogenic mutants containing single- and double-base-pair mutations relative to the growth rate of the isogenic mutants containing the triple-base-pair mutation TTC (100%). +++, 95 to 100%; ++, 70 to 95%; +, 50 to 70%.
[f] The TGA sequence had already changed into the TGC sequence during subculturing before storage.
[g] Each of the tested mutants contained an AGC mutation in the *rrnA* gene and a TTC mutation in the *rrnB* gene.

gested that mutant *H. pylori* strains with even a small increase in tetracycline resistance have an advantage whenever inhibitory concentrations of tetracycline are encountered. However, it could not be excluded that the observed differences were due to strain differences or secondary mutations. The data presented here have been obtained from mutants with identical genetic backgrounds, thus excluding the effect of strain differences. Taken together, the data indicate that the preference in *H. pylori* for particular 16S rRNA gene mutations mediating tetracycline resistance results not only from the differences in MICs but also from the differences in growth rates in the presence of tetracycline and from the stability of the mutations.

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