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Explaining the Bias in the 23S rRNA Gene Mutations Associated with Clarithromycin Resistance in Clinical Isolates of *Helicobacter pylori*

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A single point mutation in the 23S rRNA gene of *Helicobacter pylori* is known to confer resistance to clarithromycin. Most prevalent among clarithromycin-resistant clinical *H. pylori* isolates are the mutations from A-2142 to G and A-2143 to G in the 23S rRNA gene. The bias in the 23S rRNA gene mutations conferring clarithromycin resistance may result from the higher MIC, stability of resistance, and growth rate found for the strains with the above-mentioned mutations.

Helicobacter pylori is the principal cause of chronic active gastritis and peptic ulcer disease (7). Clarithromycin is frequently used with success in eradication therapy (3, 7, 12); the predominant reason for eradication failure in patients treated with monotherapy clarithromycin is the development of resistance during treatment due to mutations in the 23S rRNA (2, 4, 9–11). There seems to be a bias towards the A-2142-to-G and the A-2143-to-G mutations, but the A-2142-to-C mutation is also found, albeit with a low frequency (9). The nucleotide numbering is in accordance with that of Taylor et al. (10) and differs from that used in our previous study (2). The aim of the present study was to explain the observed bias.

We used site-directed mutagenesis to create mutants that contained either a mutation from A-2142 to G, C, or T or a mutation from A-2143 to G, C, or T. These mutants were derived from a clarithromycin-sensitive (Cla^s) wild-type (WT) strain (NCTC 11637). We measured the MIC, stability of resistance, and growth rate for the WT strain, for its isogenic clarithromycin-resistant (Cla^r) laboratory mutants, and for *H. pylori* clinical isolates (20 paired clinical isolates and 20 additional Cla^r clinical isolates).

H. pylori strains were grown and stored as previously described (2). MICs were determined with the E test as described before (2). Cla^r mutants of *H. pylori* NCTC 11637 were created by site-directed PCR mutagenesis. PCR amplifications were performed with the Primezym DNA polymerase kit (Biometra, Gottingen, Germany) according to the manufacturer's instructions. *H. pylori* NCTC 11637 was mutagenized by natural transformation with a PCR product that contained the desired mutation and selected for clarithromycin resistance. These PCR products were generated with the primers listed in Table 1, essentially as described by Shimada (8). Transformation by natural competence was performed as described by Wang et al. (13). As a control, recipient cells were mutagenized with a PCR mixture that contained either WT DNA or no DNA at all. Transformants were selected for clarithromycin resistance. Clarithromycin was added to a final concentration of either 2 or 0.1 μg/ml to Columbia agar (Becton Dickinson, Cockeysville, Md.) supplemented with 10% lysed horse blood (CA) (Bio Trading, Mijdrecht, The Netherlands). After 2 days of incubation, single colonies were restreaked on clarithromycin-containing plates and then genomic DNA was isolated for sequencing of 23S ribosomal DNA. Extraction of genomic DNA was performed as described by Ausubel et al. (1). For nucleotide sequence analysis of all strains, an 850-bp fragment of the 23S rRNA gene was amplified with the oligonucleotide primers 5′-GCG TTG AAT TGA AGC CCG AGT AAA C-3′ and 5′-TGT GTG CTA CCC AGC GAT GCT C-3′. The obtained PCR product was used as template DNA in a sequencing reaction with the Thermo-Sequenase premixed cycle sequencing kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Sequencing was performed on an Amersham Vistra 725 DNA sequencer, and data were analyzed with Lasergene software (DNASTAR Inc., Madison, Wis.).

Growth rate experiments were performed with paired pretreatment (Cla^s) and posttreatment (Cla^r) clinical isolates and strain NCTC 11637 (Cla^s) and its isogenic, laboratory-derived mutants. Strains were incubated for 2 days on CA plates and then harvested. A 10-fold serial dilution was prepared in brucella broth (Difco Laboratories, Detroit, Mich.). CA plates and CA plates containing clarithromycin at a final concentration of 2 or 0.1 μg/ml were inoculated with 50 μl of these dilutions. Plates were incubated for 3 to 5 days. Plates with approximately the same colony density (10 to 30 colonies/plate) were selected, and at preset time intervals the diameters of the same 20 colonies were measured with a calibrated ocular micrometer in a Stemi SV II stereomicroscope (Zeiss, Oberkochen, Germany).

The stability of resistance of the clinical isolates and the in vitro mutants was tested by repeated subculture of multiple colonies of each strain on clarithromycin-free agar. MICs were determined after every four passages. When selection was performed on plates containing 2 μg of clarithromycin per ml, no transformants of *H. pylori* NCTC 11637 were obtained with WT DNA, with the negative control without template DNA, or with the PCR product containing the A-2143-to-T mutation. All other PCR products generated transformants at a frequency of $\sim 10^4$/μg of DNA. Selection on plates containing 0.1 μg of clarithromycin per ml yielded the same results except that now transformants were also obtained for the PCR product containing the A-2143-to-T mutation, at a frequency of

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TABLE 1. Nucleotide sequences of the oligonucleotide primers used

Oligonucleotide primer	Positions[a]	Sequence (5′→3′)[b]	Direction
MUT 1	2121–2142	TCCTACCCGCGGCAAGACGGA	Forward
MUT 2	2121–2142	TCCTACCCGCGGCAAGACGG**C**	Forward
MUT 3	2121–2142	TCCTACCCGCGGCAAGACGG**G**	Forward
MUT 4	2121–2142	TCCTACCCGCGGCAAGACGG**T**	Forward
MUT 5	2122–2143	CCTACCCGCGGCAAGACGGAA	Forward
MUT 6	2122–2143	CCTACCCGCGGCAAGACGGA**C**	Forward
MUT 7	2122–2143	CCTACCCGCGGCAAGACGGA**G**	Forward
MUT 8	2122–2143	CCTACCCGCGGCAAGACGGA**T**	Forward
MUT 9	2742–2762	CACGTCCATCCCGGGCCTCTC	Reverse
R1	1962–1986	GCGTTGAATTGAAGCCCGAGTAAAC	Forward
R2	2789–2810	TGTGTGCTACCCAGCGATGCTC	Reverse

[a] The numbering used is that of Taylor et al. (10), which differs from the numbering used in previous publications (2, 4, 9).
[b] Primers MUT 2 to MUT 4 and MUT 6 to MUT 8 differ from the WT 23S rRNA at the underlined positions.

$\sim 10^4$/μg of DNA. Four transformants (Cla^r) of each type were selected for further analysis, and sequencing showed that they all contained the desired point mutation. Strikingly, all mutants were homozygous with respect to the 23S rRNA gene mutations; i.e., both copies of the 23S ribosomal DNA were mutated.

Of the 20 Cla^r clinical isolates, 13 contained an A-2142-to-G mutation and 7 contained an A-2143-to-G mutation.

The effects of the various types of mutations on the MIC, stability of resistance, and growth rate measured for the in vitro mutants and the Cla^r clinical isolates are summarized in Table 2. The clarithromycin MICs for strains with a mutation from A-2142 to G or C were high, while those for strains with a mutation from A-2142 to T were low. A mutation at position 2143 had a smaller effect on the MIC than a mutation at position 2142. The smallest increase in MIC was observed for the A-to-T mutation at position 2143.

Mutations from A-2142 to G or C and from A-2143 to G were stable. The MICs for strains with a mutation from A-2142 to T or from A-2143 to C or T showed a gradual decrease with increasing numbers of passages on clarithromycin-free media. Since resistance is due to a point mutation, this was unexpected, unless the observed MIC was the result of a mixed population of resistant and sensitive cells. Therefore, we investigated individual colonies obtained after 21 passages on clarithromycin-free media. Individual colonies were either sensitive (MIC < 0.016 μg/ml) or resistant (MIC ≥ 64 μg/ml); this indicated that the intermediate MICs were the result of heterogeneous cell populations. Colonies for which the MICs were <0.016 μg/ml had WT sequence 23S rRNA, while those for which the MICs were high had the mutant sequence (data not shown).

Growth rates of the mutants containing a mutation from A-2142 to G or C or from A-2143 to G did not differ from that of the parental strain (NCTC 11637). However, the mutants containing a mutation from A-2142 to T or from A-2143 to C or T showed a marked reduction in growth rate. This reduction was most pronounced in the strains containing a mutation from A-2142 or A-2143 to T. The results of the growth rate experiments were comparable for the clinical isolates and the in vitro mutants.

Most striking is that in all clarithromycin-resistant clinical isolates tested in our laboratory, the point mutation is from A to G (in the 20 strains used in this study and 10 posttreatment strains described in our previous study). The mutation from A-2142 to C has been described (9), but it occurs at an ex-

TABLE 2. Growth rate and MIC results

Group and mutation	Growth rate		CLA[a] MIC (μg/ml)		
			Initial	After subculture on CLA-free media	
	On CLA-free media[b]	On CLA (2 μg/ml)[c]		4 passages	21 passages
In vitro mutants					
No mutation (WT)	+++	No growth	<0.016	<0.016	<0.016
A-2142 to G	+++	+++	>256	>256	>256
A-2143 to G	+++	+++	64	32	48
A-2142 to C	+++	+++	>256	>256	>256
A-2143 to C	++	++	64	16	4
A-2142 to T	++	++	64	16	4
A-2143 to T	+	No growth	0.5	<0.016	<0.016
Clinical isolates					
No mutation (pretreatment)	+++	ND	<0.016	<0.016	<0.016
A-2142 to G	+++	ND	>256	>256	>256
A-2143 to G	+++	ND	>256	>256	>256

[a] CLA, clarithromycin.
[b] For in vitro mutants, relative to the growth rate for the WT (NCTC 11637) (100%); for clinical isolates, relative to the growth rate for the paired pretreatment Cla^s isolate (100%). +++, 95 to 100%; ++, 95 to 70%; +, 50 to 70%.
[c] Relative to the growth rate for the Cla^r NCTC 11637 mutant with a mutation from A-2142 to G (100%). +++, 90 to 100%; ++, 70 to 90%. ND, not done.

tremely low frequency. The apparently high frequency of the A-to-G mutation among clinical isolates might be the result of biological features generated by this mutation. Nash and Inderlied (6) observe that mutations in the peptidyltransferase loop of the 23S rRNA of *Mycobacterium avium* change the thermodynamics of the region. These changes in free energy cause a conformational change within the ribosome. It is hypothesized that mutations from A-2142 to T or C and A-2143 to T or C have a greater effect on the structure of the assembled ribosome than mutations from A-2142 to G and A-2143 to G and therefore on the growth rate of the strain.

The strain with an A-2142-to-C mutation behaved essentially the same as the strains with an A-2142-to-G or an A-2143-to-G mutation. This explains why this mutation can be found among Cla^r clinical isolates (9) but does not explain why it is found at such a low frequency. It is possible that this mutation leads to a minor alteration in the ribosome structure, with a minimal effect on growth rate, and that this was not detectable during the relatively short time span of our in vitro experiments. Xia et al. (14) studied the prevalence and in vitro stability of primary clarithromycin resistance in *H. pylori*. Patients who had previously received clarithromycin were excluded from their study. Of the 444 strains studied, 20 were Cla^r. In nine of these the resistance was unstable. This is in contrast to our findings and those of Hultén and coworkers (4), which showed that resistance to clarithromycin was stable in all clinical *H. pylori* isolates. Our Cla^r isolates and those from the study of Hultén et al. contained one of the two A-to-G transitional mutations. The nucleotide sequences of the strains used in the study of Xia et al. (14) are unknown. We conclude that the apparent preferential mutation, A-2142 to G or A-2143 to G, observed among Cla^r clinical isolates is due not to a preference for this particular mutation but to selection. This selection results from the higher growth rates, higher MICs, and more stable resistance found for the strains with these mutations than for strains with mutations from A-2142 to T and A-2143 to C or T.

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