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Molecular Patchwork: Chromosomal Recombination between Two *Helicobacter pylori* Strains during Natural Colonization

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Genetic analysis of two *Helicobacter pylori* strains isolated from a single gastric biopsy showed evidence of extensive horizontal gene transfer. Several large recombinations were identified in the *rdxA* gene, which is involved in metronidazole resistance.

The gastric pathogen *Helicobacter pylori* displays considerable genetic diversity (2). This diversity can be attributed to genetic drift, which was observed in clinical isolates obtained at intervals of several years from one patient (7, 10, 14), and to horizontal gene transfer. Several studies have demonstrated that recombination between *H. pylori* strains occurs frequently enough to virtually eliminate the effect of clonal descent on the population structure of *H. pylori* and to generate a linkage equilibrium between alleles at different loci (1, 8, 17, 19). Furthermore, some *H. pylori* strains contain a 37-kb genomic region, the *cag* pathogenicity island (PAI), which is located in the glutamate racemase gene between two short direct repeats. The *cag* PAI is not a stable genomic region, since it can be readily lost from the chromosomes of *cag*⁺ strains by recombination with strains lacking the *cag* gene (13, 22). The variability between strains can be significant for the outcome of *H. pylori* infection. For instance, the *cag* PAI and certain alleles of the *vacA* and *iceA* genes are more frequent in strains that cause peptic ulceration or gastric cancer (4, 24).

In *H. pylori*, random mutations that inactivate the *rdxA* gene cause metronidazole resistance (9). *rdxA* mutations that have been described for resistant isolates include frameshifts, premature stop codons, codon changes resulting in amino acid changes, and promoter alterations (9, 11, 12, 15, 20). In a previous study, we reported that in stomach biopsies with both metronidazole-sensitive (Mtz^s) and metronidazole-resistant (Mtz^r) bacteria, usually a single *H. pylori* strain is present, which suggested that a subpopulation of the original, susceptible strain had become resistant by mutation (3). However, in one biopsy, random amplified polymorphic DNA and restriction fragment length polymorphism genotyping showed that two different strains were present. Interestingly, both strains contained Mtz^s as well as Mtz^r subpopulations. In the present study we determined whether horizontal gene transfer has

occurred between these two strains. The results show that extensive recombination has taken place in the *rdxA* locus.

Strain identification. Twelve isolates were subcultured from biopsy BH9809-109 and named L1 to L12 according to increasing Mtz resistance (Table 1). Seven isolates (L1 to L7) were Mtz^s (MIC, <8 mg/liter) (Table 1), and five (L8 to L12) were Mtz^r (MIC, ≥8 mg/liter). Six Mtz^s isolates (L1 to L4, L6, and L8) and three Mtz^r isolates (L9, L11, and L12) belonged to the first genotype (strain 1). The second genotype (strain 2) included two Mtz^s isolates (L5 and L7) and one Mtz^r isolate (L10). *cag* and *vacA* statuses were assessed by line probe assay. The line probe assay types two variable regions of the *vacA* gene, the S region and M region. Furthermore, it determines the presence or absence of the *cag* PAI (23). Strain 1 isolates contained *vacA* type S1a/M2 and were *cag*⁺; strain 2 isolates had *vacA* type S2/M2 and lacked *cag* (Table 1). Neither strain showed variation in *vacA* or *cagA* status among individual isolates; this indicated that no recombination that affected the S/M type of the *vacA* gene and the presence of the *cag* PAI had taken place.

The *rdxA* alleles. Because both strains included Mtz^s as well as Mtz^r isolates and reversion of nonsense mutations is unlikely, resistant subpopulations appeared during colonization from the Mtz^s ancestors of either strain (with intact *rdxA* alleles). To demonstrate a possible transfer of a resistant *rdxA* allele from one strain to the other, we sequenced the *rdxA* genes in all 12 isolates. The results are shown in Fig. 1. Two different alleles of the *rdxA* gene (allele A and allele B), which differed from each other at 22 base positions in the 630-bp open reading frame (ORF), were present in the set of isolates. However, the distribution of the *rdxA* alleles did not correspond to the genotypes of the isolates. Strain 1 included isolates with *rdxA* allele A (L3, L4, L6, L8, L9, L11, and L12) as well as isolates with *rdxA* allele B (L1 and L2), which indicates that one of the alleles must have been acquired by horizontal DNA transfer. Of the isolates with the genetic background of strain 2, L10 had allele A but the other two (L5 and L7) possessed a mixed allele. L5 had an allele that was predominantly type A, with a single polymorphism at base position 549. This change was consistent with allele B, which suggests the

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TABLE 1. Metronidazole susceptibilities, genotypes, *rdxA* alleles, presence of the *cag* PAI, and *vacA* type of isolates L1 to L12

Isolate	Metronidazole MIC (mg/liter) ^a	Genotype ^b	<i>rdxA</i> allele	<i>cagA</i> gene present	VacA type
L1	0.5	1	B	+	S1a/M2
L2	0.5	1	B	+	S1a/M2
L3	0.38	1	A	+	S1a/M2
L4	0.75	1	A	+	S1a/M2
L5	1.5	2	A	-	S2/M2
L6	1.5	1	A	+	S1a/M2
L7	1.5	2	A/B	-	S2/M2
L8	48	1	A	+	S1a/M2
L9	>256	1	A	+	S1a/M2
L10	>256	2	A	-	S2/M2
L11	>256	1	A	+	S1a/M2
L12	>256	1	A	+	S1a/M2

^a Sensitive, <8 mg/liter; resistant, ≥8 mg/liter.
^b According to random amplified polymorphic DNA typing and restriction fragment length polymorphism of the *ureC* gene.

transfer of a very small DNA fragment. However, this change could also result from a single point mutation which happened to match the sequence of allele B.

In L7, of the 22 polymorphic base pairs that distinguish

allele A from allele B, the 3 polymorphic sites at the 5'-end region of the ORF were identical to those in the allele B group, whereas the other 19 in the middle and at the 3' end were concordant with the allele A group. Between the allele A and allele B regions of L7, there was a gene fragment of 77 bases with perfect homology between allele A and allele B. These data indicate that a recombination event introduced part of the *rdxA* locus of strain 1 into the L7 genome.

Origin of metronidazole resistance. All resistant isolates contained *rdxA* allele A, which suggests transfer of the resistance mutation. However, there was a difference between the resistant isolate of strain 2 (L10) and the resistant isolates of strain 1 (L8, L9, L11, and L12). In L10, a single-base-pair deletion was found at base position 192 of the ORF, causing a frameshift that interrupts the reading frame. This frameshift occurred in a homopolymeric stretch of 10 adenine residues interrupted by one cytosine. An identical deletion was observed earlier in an unrelated strain (11). Apparently, this locus is vulnerable to slipped-strand mispairing. The resistant isolates of strain 1 (L8, L9, L11, and L12) did not possess this frameshift but showed a C-to-T mutation at position 200 of the ORF, which causes an amino acid change from alanine to valine. To confirm that this minor difference causes metroni-

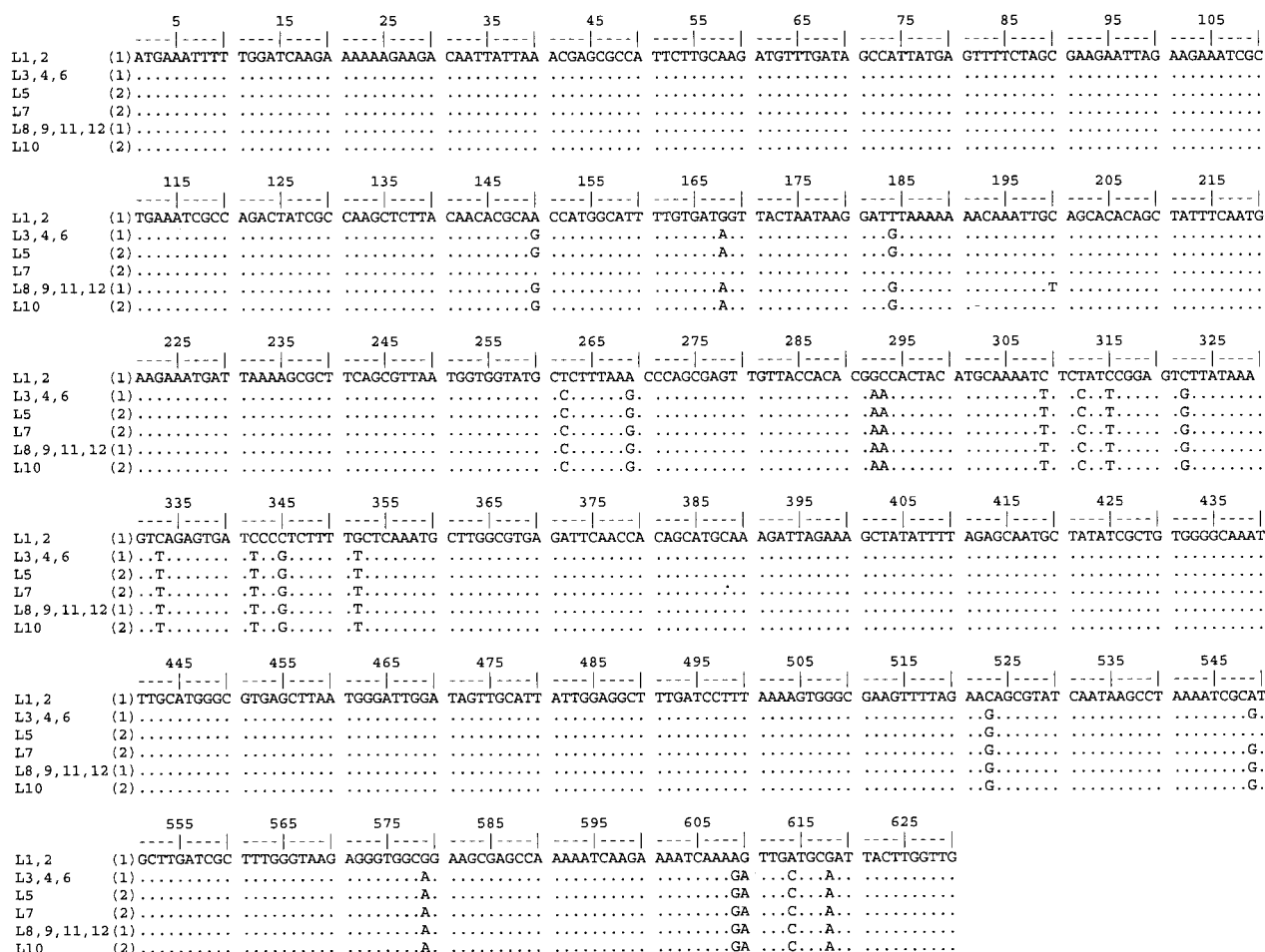


FIG. 1. Alignment of the *rdxA* ORFs of isolates L1 to L12. Numbers in parentheses indicate the strain type (1 or 2).

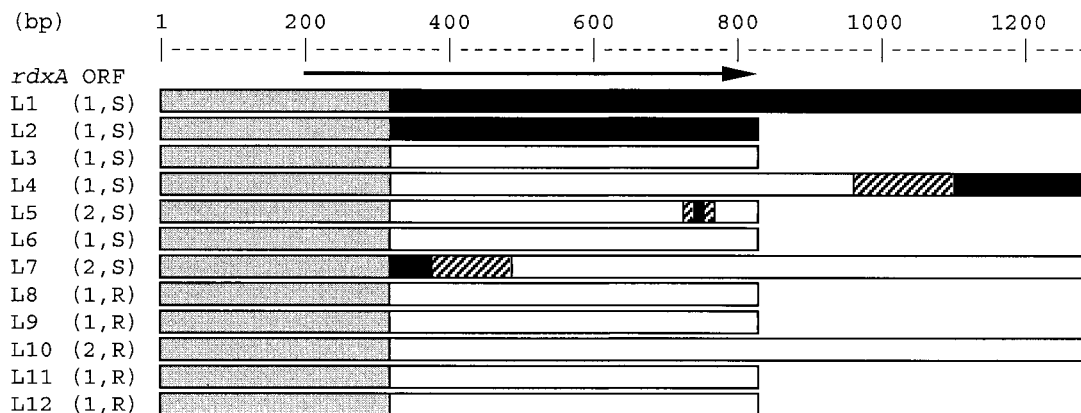


FIG. 2. Distribution of alleles A and B in isolates L1 to L12. Numbers in parentheses indicate the genotype (1 or 2). S and R, metronidazole susceptibility and resistance, respectively. The arrow shows the *rdxA* ORF. Open bars, allele A; solid bars, allele B; hatched bars, crossover sequence; gray bars, identical sequence. Strain L4 and strain L7 contain recombined sequences. The point mutations that cause resistance in the resistant isolates L8 to L12 are not shown.

dazole resistance, we used a PCR amplicon of the L8 *rdxA* allele for natural transformation (25) to susceptible *H. pylori* strains. This yielded Mtz^r transformants with a frequency of 10^{-3} per recipient cell, which indicated that this amino acid change is sufficient to cause metronidazole resistance. We conclude that resistance in both strains arose by an independent mutation of the *rdxA* gene after transfer of an intact *rdxA* allele A had taken place. Despite the high frequency of horizontal gene transfer between strain 1 and strain 2, an *rdxA* mutation arose independently in both strains.

Region around *rdxA*. In order to determine the length of the recombinations between strain 1 and strain 2, the DNA sequence on both sides of the *rdxA* ORF was determined for two isolates from strain 1 (L1 with allele B and L4 with allele A), as well as two isolates from strain 2 (L7, which contains the 5' end of allele B and the complementing part of allele A, and isolate L10, which contains allele A). The results are shown in Fig. 2. Downstream, another crossover between the two alleles was identified: after a perfect homology of 145 bp between the four sequences, the sequences of the strain 1 isolates L4 and L1 were identical over a length of at least 205 bp, with eight mismatches between strain 1 (L1 and L4) and strain 2 (L7 and L10). This is concordant with ancestral combinations of strain 1 with allele B and strain 2 with allele A. Upstream of the *rdxA* ORF, however, the sequences of L1, L4, L7, and L10 were identical over a length of >1.7 kbp (results not shown). It is unlikely that the original sequences of strain 1 and 2 were already identical before their cohabitation, because in this region there is only 94% conservation between these strains and the published genome sequences (2, 21). Apparently, a previous, major recombination event had replaced the original sequence in one of the strains, which results in an identical sequence in all four isolates. In an attempt to find a copy of the other original sequence, from the remaining eight isolates a fragment of 800 bp upstream of *rdxA* was sequenced. Their sequences were identical to the one in L1, L4, L7, and L10. Thus, the second ancestral sequence is not present in our set of 12 isolates.

Most observations on natural horizontal gene transfers concern transfer of genetic elements that are mobile by nature,

such as plasmids or transposons. Horizontal gene transfer of nonmobile elements is only rarely observed outside experimental settings (16). It is usually inferred from sequence analysis of unrelated isolates of mucosal pathogens that are competent for DNA exchange by natural transformation: *Streptococcus pneumoniae*, *Neisseria* spp., *Haemophilus influenzae*, and *H. pylori*. In some cases, the recombining sequences must have been over 1 kb long (5, 6). For *H. pylori*, its isolated habitat in the gastric mucosa with prolonged contact between different strains provides the unique opportunity to isolate recombining strains together. Kersulyte et al. identified recombination events between two *H. pylori* strains from the same patient that involve up to 400 bp (13). Falush et al. investigated genetic relationships of sequential isolates of *H. pylori* from a single patient and estimated the mean size of the recombination fragments to be 417 bp (7).

Here, we demonstrate that multiple homologous recombination events occur in a single locus during cohabitation of two strains and that these events result in a molecular patchwork between two ancestral sequences. Since the duration of the contact is not known, we can make no estimate of the frequency of recombination. The sequences upstream of the *rdxA* ORF are identical for at least 1.7 kb, which suggests that a large fragment was transferred and integrated in the ancestors of all isolates and that *H. pylori* can exchange DNA fragments long enough to contain one or more complete genes. The lack of polymorphic sites for over 1.7 kb in this area obscures the second recombination site for two of the isolates, L4 and L7 (Fig. 2). Two crossovers had taken place, at 145 and 77 bases of perfect homology. This is in accordance with experimental data which indicate that recombination efficiency is highly dependent on the presence of a perfect homology of sufficient length (18). The molecular patchwork that is described here illustrates the frequent recombination during cocolonization of *H. pylori* strains that leads to the panmycotic population structure of this species.

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