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Evolutionary History of *hrgA*, Which Replaces the Restriction Gene *hpy*IIIR in the *hpy*III Locus of *Helicobacter pylori*

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A recently identified *Helicobacter pylori* gene, *hrgA*, was previously reported to be present in 70 (33%) of 208 strains examined (T. Ando, T. M. Wassenaar, R. M. Peek, R. A. Aras, A. I. Tschumi, L.-J. Van Doorn, K. Kusugami, and M. J. Blaser, Cancer Res. 62:2385-2389, 2002). Sequence analysis of nine such strains indicated that in each strain *hrgA* replaced *hpy*IIIR, which encodes a restriction endonuclease and which, together with the gene for its cognate methyltransferase, constitutes the *hpy*III locus. As a consequence of either the *hrgA* insertion or independent mutations, *hpy*IIIM function was lost in 11 (5%) of the 208 strains examined, rendering chromosomal DNA sensitive to *Mbo*I digestion. The evolutionary history of the locus containing either *hpy*III or *hrgA* was reconstructed. By homologous recombination involving flanking sequences, *hrgA* and *hpy*IIIR can replace one another in the *hpy*III locus, and there is simultaneous replacement of several flanking genes. These findings, combined with the *hpy*IM/*iceA2* locus discovered previously, suggest that the two most strongly conserved methylase genes of *H. pylori*, *hpy*IIIM and *hpy*IM, are both preceded by alternative genes that compete for presence at their loci.

Type II restriction-modification (R-M) systems are comprised of paired enzymes, a restriction endonuclease that cleaves DNA within a specific 4- to 8-bp sequence and a methyltransferase that specifically methylates the DNA within the same sequence, protecting the sequence from cleavage (4, 5, 14). *Helicobacter pylori*, a gram-negative bacterium that colonizes the human stomach, affects the risk of getting upper gastrointestinal tract diseases, including gastric cancer (13). *H. pylori* strains are highly heterogeneous in terms of the number and nature of the R-M systems that they carry (2, 10, 11, 18, 19, 21, 23). During characterization of the *hpy*IIIR-*hpy*IIIM locus in Asian and Western strains, we found numerous strains with a novel gene that we designated *hrgA* in place of *hpy*IIIR (encoding an isoschizomer of *Moraxella bovis Mbo*I). The presence of *hrgA* appears to have predictive value for virulence in *cagA*-positive strains from Asia (3). Neither gene is essential, but since no strain that lacks or contains both genes has been identified thus far, it is hypothesized that there is selection for the presence of either gene. The work described here addressed the following questions. How conserved is *hrgA*? How did the *hrgA-hpy*III locus evolve? And are *hrgA* and *hpy*IIIR functional, and can the two genes be exchanged by natural transformation? Mutants bearing antibiotic resistance cassettes were constructed to investigate exchange of the genes between strains by natural transformation. The results indicate that exchange of these genes is possible and may involve transfer of a DNA fragment containing substantial flanking sequences, thus increasing the potential for genomic plasticity in *H. pylori*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The *H. pylori* strains used in this study are summarized in Table 1. *Escherichia coli* was routinely grown at 37°C in Luria-Bertani broth or agar supplemented with ampicillin (100 μg/ml) and/or chloramphenicol (30 μg/ml), when appropriate. *H. pylori* strains were grown on Trypticase soy agar (TSA) with 5% sheep blood (BBL) or brucella serum (BBL) agar with 10% newborn calf serum (Intergen) at 37°C in an atmosphere containing 5% CO_2.

DNA and protein techniques. Standard molecular techniques were used (15). *H. pylori* chromosomal DNA was prepared from cells of each strain after 48 h of growth on two agar plates, as described previously (3). Plasmid DNA was prepared from *E. coli* after overnight culture by using a midi-prep protocol (Qiagen Inc., Valencia, Calif.) according to the manufacturer's instructions. PCRs were performed in 50-μl mixtures containing 0.5 U of *Taq* polymerase (Qiagen), 1.5 mM $MgCl_2$, and 200 ng of each primer. The PCR protocol (30 cycles) included a denaturation step at 94°C for 1 min, annealing at 5°C below the predicted melting temperature of the primers for 1 min, and extension at 72°C for 1 min/kb of amplification product. The primers reflecting conserved sequences in the *hpy*III locus, hpRf, hpRr, hpMf, hpMr, hrgAf, hrgAr, locf, and locr, are described elsewhere (3). Other primers used in this study are listed in Table 2. Sequence analysis was performed as described elsewhere (3).

Phylogenetic analysis and Ka/Ks ratios for *hrgA* and *hpy*IIIM. *hrgA* nucleotide sequences were aligned by using GCG Pileup (Wisconsin Package, version 9.1), and a phylogram was constructed by using Paup 4.0b2 (Sinauer Associates, Sunderland, Mass.) and was displayed by using midpoint rooting with Paup 3.1 (Illinois Natural History Survey, Champaign). To determine the ratios of the rate of nonsynonymous substitution (Ka) to the rate of synonymous substitution (Ks), multiple-sequence alignments were created with ClustalW and were analyzed by using SWAAP 1.0.0 (distributed by D. T. Pride and available at http://www.bacteriamuseum.org/SWAAP).

Disruption of *hpy*IIIR or *hrgA* in *H. pylori* strain 26695 or JP26. Insertion of a chloramphenicol resistance gene (*cat*) in *hrgA* has been described previously (3). The same procedure was used to introduce *cat* into *hpy*IIIR of strain 26695 with primers NthpRf and XhhpMr (Table 2). The product was cloned into pBluescript by using *E. coli* DH5α. A unique *Eco*RI site was created in *hpy*IIIR by performing inverse PCR with primers hpRinr and hpRinf. *cat* was amplified from pBSC103 (22) by using primers that added *Eco*RI restriction sites, as described previously (3). This cassette was ligated with the inverse PCR product, thereby disrupting *hpy*IIIR. *H. pylori* strains 26695, J99, J188, and B146 were transformed to chloramphenicol resistance with this construct to create 26695-*hpy*IIIR::*cat*, J99-*hpy*IIIR::*cat*, J188-*hpy*IIIR::*cat*, and B146-*hpy*IIIR::*cat*.