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Evolution of the *Helicobacter pylori* Vacuolating Cytotoxin in a Human Stomach

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We describe two subclones of *Helicobacter pylori*, isolated contemporaneously from a human stomach, which differ markedly in the vacuolating cytotoxin gene, *vacA*, but whose near identity in sequences outside this locus implies a very recent common origin. The differences are consistent with homologous recombination with DNA from another strain and result in a changed *vacA* midregion and, importantly, in changed toxicity.

The stomach-colonizing bacterium *Helicobacter pylori* is the main cause of peptic ulceration and gastric cancer, although most colonized people remain asymptomatic. One important virulence factor is the vacuolating cytotoxin, VacA, a secreted pore-forming toxin that causes epithelial cell vacuolation. Virtually all *H. pylori* strains express VacA, but those expressing active forms are more frequently associated with disease (8). The basis of differential toxicity is that the toxin gene, *vacA,* is polymorphic (4). *vacA* varies most markedly in its midregion, which encodes the toxin-cell binding domain (4, 23): type m1 VacA binds more extensively to cells and is more closely associated with disease than is type m2 (14, 21, 23). *vacA* also varies in its signal region, encoding the signal peptide and the N terminus of the mature toxin (4, 6): type s1 VacA is toxic, but type s2 has a short N-terminal extension on the mature toxin which abolishes vacuolating activity (16, 18). Strains with all four possible combinations of the *vacA* signal and midregions have been found, implying previous homologous recombination within *vacA* between *H. pylori* strains (4, 17).

H. pylori exhibits pronounced genetic diversity, and nucleotide sequence comparisons between loci from different strains show that a common source of variation in *vacA* and other genes is past recombination events between homologous genes from different strains (25). Indeed, *H. pylori* exhibits evidence of more frequent recombination events with heterologous strains than any other known bacterial species (25), and mathematical analysis and microarray and nucleotide sequence analysis of strains isolated longitudinally from the same patient imply that this recombination is ongoing (7, 11, 15). A previous study has shown that otherwise identical isolates in a single stomach differed in the presence of another locus that is important in *H. pylori* virulence, the *cag* pathogenicity island, and sequence analysis showed that this difference likely arose through recombination with another strain (13). A different study, in which *H. pylori* was reisolated from a series of patients after a period of time, showed recombination in several genes including, in one case, *vacA* (7). The recombination in *vacA* resulted in a stop codon and lack of VacA expression—a rare finding. From these data, it might be predicted that rapid evolution of *vacA* may occur to rearrange the gene's mosaic structure and result in the different commonly observed signal and midregion combinations (and thus changed toxicity). However, despite the important implications this occurrence would have for pathogenicity and patient management, it has not previously been demonstrated.

As part of another study looking at the association between *H. pylori vacA* types and virulence in Mexico, we identified both s1/m1 and s1/m2 *vacA* isolates from a single stomach. Cocolonization by different *H. pylori* strains is not rare (12, 24), and it has been shown previously that it is particularly common in Mexico (10, 22). We and others have repeatedly shown that such strains are easily distinguished by a variety of DNA fingerprinting methods. However, to our surprise, on this occasion the s1/m1 and s1/m2 isolates had identical fingerprints on initial testing, implying a very recent common origin. In the present study, we definitively confirm the near identity of these isolates, show that the sequence of *vacA* alleles suggests recent homologous recombination with another strain, and show that this has resulted in a marked difference in toxicity between the isolates. If this phenomenon proves to be widespread, it has potentially important implications for *H. pylori* virulence and for infection management strategies.

In a local ethically approved study, *H. pylori* was cultured from endoscopic biopsy specimens from the stomach of a 71-year-old Mexican male with duodenal ulceration. DNA was extracted from six single-colony isolates from the gastric antrum and five from the corpus. PCR-based typing, performed as previously described (5) (Table 1), showed that all of the corpus isolates were midregion type m2, but that four antral isolates were m1 and two were m2. All of the isolates were signal region type s1.

To assess whether isolates with different *vacA* types represented different strains of *H. pylori*, we compared the genomic

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TABLE 1. Primers used in this study

Primer	Sequence (5′ to 3′)	Use	Reference
VA1F	ATGGAAATACAACAAACACAC	*vacA* signal typing	4
VA1R	CTGCTTGAATGCGCCAAAC	*vacA* signal typing	4
VAGF	CAATCTGTCCAATCAAGCGAG	*vacA* midregion typing	5
VAGR	CTAGCGTCAAAATAATTCCAAGG	*vacA* midregion typing	5
1254	CCGCAGCCAA	RAPD-PCR	2
ADH1	ACGGTATGCGACAG	AFLP	9
ADH2	AGCTCTGTCGCATACCGTGAG	AFLP	9
HI-A	GGTATGCGACAGAGCTTA	AFLP	9
mutY 101	AGCGAAGTGATGAGCCAACAAAC	*mutY* sequencing	1
mutY 102	AAAGGGCAAATCGCACATTTGGG	*mutY* sequencing	1
yphC F1	CACTATTACCACGCCTATTTTTTTGAG	*yphC* sequencing	1
yphC R4	AAGCAGCTGGTTGTGATCACGGGGGC	*yphC* sequencing	1
DL1	GCTTTGATGGACACCCCACAAGG	*vacA* sequencing	
VacF1	GTTGGGATTGGGGGAATGCCG	*vacA* sequencing	
DL2	GTCATTATGCAAAAAGCCAC	*vacA* sequencing	
JR1F	GATGGGCCGTTTGCAATACGTG	*vacA* sequencing	
VacR4	ATCAATCAATAAGGTTTGTAAGA	*vacA* sequencing	
VacR5	CATGCTTTGATTGCCGATAGC	*vacA* sequencing	

fingerprints of the m1 and m2 isolates by two methods, random amplified polymorphic DNA (RAPD)-PCR and amplified fragment length polymorphism (AFLP) analysis, as previously described (2, 9) (Table 1). Both methods gave identical fingerprints for all isolates, implying a single clonal origin (Fig. 1). Isolates from six other Mexican patients all gave obviously different fingerprints by the two methods. To assess more sensitively whether the isolates represented different genomic types, we performed a microarray analysis (11) of these isolates. As expected, and exactly as has been shown previously for single-colony isolates of the same strain from another patient (11), this analysis showed some differences at other loci (data not shown). However, hierarchical cluster analysis revealed that m1 and m2 isolates were related to each other just as closely as m1 isolates were related to other m1 isolates and m2 isolates were related to other m2 isolates, showing that these were not distinct, separate genomic groupings (Pearson correlation coefficients were 0.81 to 0.82 for m1 versus m2, 0.81 to 0.82 for m1 versus m1, and 0.82 to 0.88 for m2 versus m2). Finally, we selected one isolate each from *vacA* types m1 and m2 and PCR amplified and sequenced regions from two unrelated genes, 471 bp of HP0142 (*mutY,* an adenine glycosylase gene) and 1,051 bp of HP0834 (*yphC*, a GTPase gene) (26). In published comparisons, the mean nucleotide substitution rates between alleles from different strains at these loci were 24 and 19%, respectively, with minimum rates of 7 and 6% (1). For our isolates, *mutY* homologues were identical and *yphC* homologues had a single base substitution, confirming the recent clonal origins of our isolates.

To analyze differences in *vacA* between the essentially clonal m1 and m2 isolates, we sequenced the *vacA* genes of isolates of each type by automated sequencing of overlapping PCR products. Differences were found in two regions, one of 439 bp (with 35 different base pair substitutions) and one of 378 bp (with 48 bp substitutions and a 75-bp insertion) (Fig. 1). The second was in the midregion, explaining the difference in PCR-based *vacA* typing. Outside these regions, the *vacA* sequences were identical, except for a single base substitution in the untranslated mRNA leader (bp 719), again confirming the sequences' near clonality. A partial *vacA* sequence analysis of one additional isolate each of types m1 and m2 showed complete identity with the originals.

To show that the m1 and m2 *vacA* sequences were not acquired from elsewhere in the genome, we designed specific PCR primers internal to the differing m1 and m2 midregions. Using the m1-specific primers, as expected, we amplified a product of the predicted size from chromosomal DNA from the m1 isolates but not from the m2 isolates. We found the converse to be true when we used the m2-specific primers. We did not isolate plasmid DNA from isolates despite successful isolation from a control strain with a plasmid. This result implies that the midregion was acquired from another strain either simultaneously or previously colonizing the stomach. Extensive searching, by picking individual colonies from frozen stored biopsy specimens, failed to identify this parent strain, implying either that it had been selected against or that it colonized a nonsampled part of the stomach.

To assess whether the evolution of *vacA* had resulted in a changed toxin phenotype, we next assessed the vacuolating cytotoxin activity of broth culture supernatants from the *vacA* m1 and m2 isolates, as previously described (4, 6, 19). We found that *vacA* m1 isolates caused extensive vacuolation of the gastric epithelial cell line AGS, whereas m2 isolates caused no vacuolation (Fig. 1); this finding was expected, since other m2 strains do not induce AGS cell vacuolation (18). For a control, we assessed the vacuolation of RK13 cells which bind both m1 and m2 VacA (23), and these cells were vacuolated with both isolates. We confirmed that different effects on AGS cells were not due to different levels of VacA by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and VacA immunoblotting with culture supernatants. These results, showing a changed phenotype, were as we would predict based on previous mechanistic studies of the *vacA* sequence-function relationship: the exchange of the *vacA* m1 midregion for an m2 midregion confers cell line specificity on the vacuolating phenotype of *vacA*, and the opposite exchange, replacing an m2 midregion with an m1 midregion, removes that cell line specificity (18). Thus, it seems likely that in the strain we describe here, the recombination in the midregion rather than

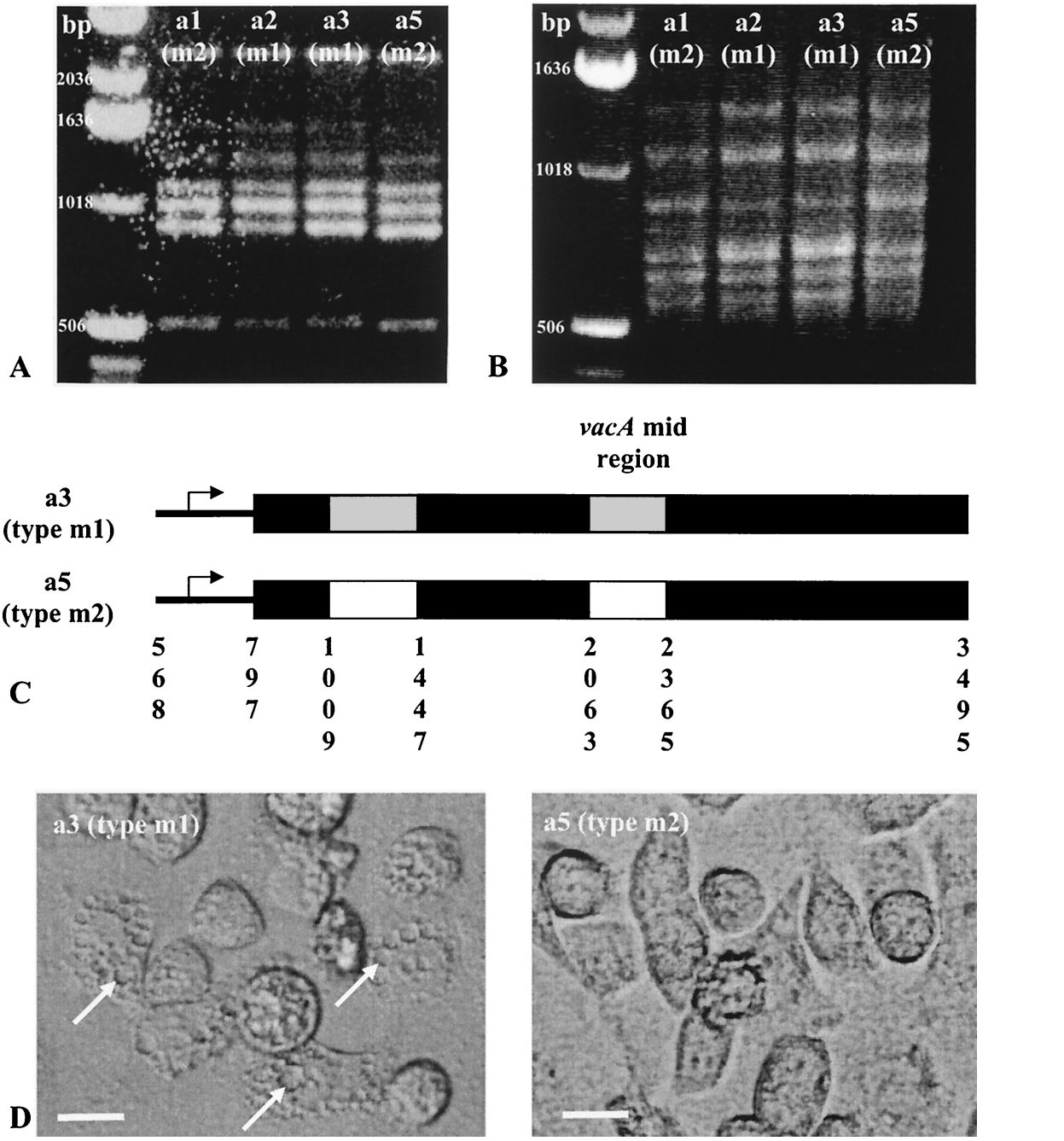

FIG. 1. (A and B) Genomic fingerprints of *H. pylori* single colonies a1 (*vacA* type m1), a2 (*vacA* type m2), a3 (*vacA* type m1), and a5 (*vacA* type m2) obtained by RAPD-PCR (A) or AFLP (B), showing that all colonies have the same clonal origin. (C) Diagram of *vacA* from colonies a3 (*vacA* type m1) and a5 (*vacA* type m2). Identical regions of the nucleotide sequences are shown in black, and nonidentical regions are shown in grey and white. The numbers refer to the published nucleotide sequence of strain 60190 (GenBank accession number U05676). (D) Vacuolating cytotoxin activity of culture supernatants on AGS cells. The m1 clone (a3) caused extensive vacuolation (indicated by arrows, left panel) whereas the m2 clone (a5) was inactive (right panel). Scale bar, 30 μm.

that in the more-5′ region is responsible for the change in toxin phenotype.

We have shown that the VacA toxin can evolve in vivo to alter its toxicity, presumably through recombination with another, unidentified, *H. pylori* strain. Because only two strains were identified, we cannot be certain which is the daughter, but both acquisition and loss of toxin activity within the stomach have important potential implications for pathogenesis and future clinical management strategies. For example, if *H. pylori* pathogenicity changes, disease expression may change, conceivably contributing to phenomena such as the waxing and waning of ulcers. One reason for developing typing systems for *H. pylori* based on virulence determinants such as *vacA* has been the hope that such strains could be identified and treated

before they cause disease (3, 20). If rapid evolution in vivo as demonstrated here is widespread, such a strategy would be illogical. One challenge now is to assess whether the evolution of virulence determinants such as *vacA* and *cag* is a common phenomenon, as would be predicted from the observed extent and pattern of DNA sequence diversity at other loci (1, 7, 25). That it has been demonstrated by chance in *vacA* in this study and in *cag* in a previous study (13) would imply that such evolution is not rare.

Nucleotide sequence accession numbers. GenBank accession numbers for new DNA sequence data referred to in this paper are AY663830 to AY663835.

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