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Helicobacter acinonychis: Genetic and Rodent Infection Studies of a *Helicobacter pylori*-Like Gastric Pathogen of Cheetahs and Other Big Cats

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Insights into bacterium-host interactions and genome evolution can emerge from comparisons among related species. Here we studied *Helicobacter acinonychis* (formerly *H. acinonyx*), a species closely related to the human gastric pathogen *Helicobacter pylori*. Two groups of strains were identified by randomly amplified polymorphic DNA fingerprinting and gene sequencing: one group from six cheetahs in a U.S. zoo and two lions in a European circus, and the other group from a tiger and a lion-tiger hybrid in the same circus. PCR and DNA sequencing showed that each strain lacked the *cag* pathogenicity island and contained a degenerate vacuolating cytotoxin (*vacA*) gene. Analyses of nine other genes (*glmM*, *recA*, *hp519*, *glr*, *cysS*, *ppa*, *flaB*, *flaA*, and *atpA*) revealed a ~2% base substitution difference, on average, between the two *H. acinonychis* groups and a ~8% difference between these genes and their homologs in *H. pylori* reference strains such as 26695. *H. acinonychis* derivatives that could chronically infect mice were selected and were found to be capable of persistent mixed infection with certain *H. pylori* strains. Several variants, due variously to recombination or new mutation, were found after 2 months of mixed infection. *H. acinonychis* ' modest genetic distance from *H. pylori*, its ability to infect mice, and its ability to coexist and recombine with certain *H. pylori* strains in vivo should be useful in studies of *Helicobacter* infection and virulence mechanisms and studies of genome evolution.

Functional and sequence comparisons among related bacterial strains and species can provide insights into evolutionary mechanisms and help identify factors that contribute to the virulence of pathogens (37, 51). Here we report studies of strains of *Helicobacter acinonychis* (formerly *H. acinonyx*), which chronically infects the gastric mucosa of cheetahs and other big cats and that, based on 16S rRNA sequence data, seems to be the most closely related of known helicobacters to the human gastric pathogen *Helicobacter pylori* (12, 13, 45). Chronic infection of cheetahs by *H. acinonyx* is thought to contribute to the development of severe gastritis, a frequent cause of their death in captivity (12, 35).

H. pylori itself is a most genetically diverse species: independent clinical isolates are usually distinguishable by DNA fingerprinting (4) and typically differ from one another by some 2% or more in sequences of essential housekeeping genes and 5% or more in gene content (1, 3, 5, 43). This diversity probably stems from a combination of factors, including (i) mutation (50); (ii) recombination between divergent strains and species (1, 5, 16, 46, 47); (iii) selection for host-specific adaptation during chronic infection, which reflects differences between people and also within individual stomachs in traits that can be important to *H. pylori* (2, 11, 25, 33); and (iv) a highly localized (preferentially intrafamilial) pattern of transmission (22, 38), which promotes genetic drift and minimizes the chance of selection for just one or a few potentially most-fit genotypes.

It is not known when *H. pylori* became human adapted. One theory proposes that its association with humans is truly ancient, that *H. pylori* infection has been near universal in humans and in our nonhuman primate ancestors for perhaps millions of years (6). This proposal was used in developing a controversial idea that chronic *H. pylori* infection and the gastritis accompanying it might be quite normal and, thus, it bears on discussions of whether *H. pylori* eradication should or should not be a societal goal (6). Our alternative theory (29) proposes that *H. pylori* infection became widespread in humans more recently, perhaps in early agricultural societies, some 10,000 years ago. As with the jumps of other pathogens in humans, this might have been promoted by the increased contact with animals, the higher population density, and the poorer sanitation in agricultural communities than in bands of hunter-gatherers (10, 29). The potential of *H. pylori* to surmount barriers between host species is illustrated by the many reports of human *H. pylori* strains adapted to mice and other mammals (11, 18, 19, 31, 42). The present study of *H. pylori*'s close relative, *H. acinonychis*, was motivated by interest in

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understanding the control and specificity of infection, of how and when *H. pylori* may have become widespread in humans, and by the potential value of comparing related *Helicobacter* species in this context.

Earlier studies had shown that *H. acinonychis* could infect domestic cats (13), as can certain *H. pylori* strains (39), although an attempt to infect BALB/c mice was not successful (13). Part of a putative adhesin gene of *H. acinonychis* (*hxaA*) was 83% matched to that of *H. pylori* (*hpaA* [14]), and point mutations could be moved between *H. pylori* and *H. acinonychis* by DNA transformation in culture (40). Here we characterize sequence relationships of *H. acinonychis* isolates from captive big cats from North America and Europe to each other and to human *H. pylori*, identify two distinct groups of strains, and select *H. acinonychis* derivatives that can chronically infect mice either alone or in combination with certain *H. pylori* strains.

MATERIALS AND METHODS

***Helicobacter* strains and culture.** Ten veterinary isolates of *H. acinonychis* were studied here. Six, named 89-2579, 90-119, 90-548, 90-624, 90-736, and 90-788, were from cheetahs with gastritis in the Columbus (Ohio) Zoo. *Hin*dIII digest genomic DNA profiling had indicated that these isolates were closely related to one another (12, 13). Four additional *H. acinonychis* strains were from animals in a European Circus: two from lions (named Sheeba and Mac), one from a tiger (named India), and one from a lion-tiger hybrid (named Sheena) (8, 40; G. Cattoli and J. G. Kusters, unpublished data). Each of these big cats was born in captivity. The six zoo animals may have been in contact with one another, directly and/or via handlers, utensils, etc., as may have been the four circus animals. To our knowledge, however, there had been no contact between the big cats in the United States and those in Europe, and it is not known when their ancestors were captured in the wild.

Five mouse-adapted strains of *H. pylori* were used here: SS1 (31, 36); X47 (also known as X47-2AL [2, 15]); 88-3887, a close relative of strain 26695 (24, 27, 34), whose genome has been fully sequenced (47); and AM1 from India and AL10103 from Alaska (D. Dailidiene, A. K. Mukhopadhyay, M. Zhang, and D. E. Berg, unpublished data).

Helicobacter strains were grown in a gas-controlled incubator under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C, usually on brain heart infusion agar (Difco) supplemented with 7% horse blood, 0.4% IsoVitaleX, and the antibiotics amphotericin B (8 μg per ml), trimethoprim (5 μg per ml), and vancomycin (6 μg per ml). Nalidixic acid (10 μg per ml), polymixin B (10 μg per ml), and bacitracin (200 μg per ml) were added to this medium when culturing *Helicobacter* isolates from mouse stomachs. *H. acinonychis* isolates were tested for susceptibility to metronidazole (MTZ) by spotting aliquots of diluted cultures containing, variously, 10^3 to 10^6 cells (10-fold dilutions) on media with fixed concentrations of antibiotics, as described elsewhere (9, 26). Tests for susceptibility to other antibiotics (tetracycline [Tet], clarithromycin [Cla], and chloramphenicol [Cam]) were carried out similarly but by spotting only about 10^6 cells on drug-containing media.

Strains carrying rRNA resistance mutations were constructed by transformation with 16S ribosomal DNA (rDNA) containing TTC in place of AGA at position 965 to 967 for Tet resistance (9) and 23S rDNA containing G in place of A at position 2144 for Cla resistance (49). Strains carrying *vacA*::*cat* (K. Ogura and D. E. Berg, unpublished data) and *rdxA*::*cat* (26) (chloramphenicol resistance) mutations were similarly generated by transformation (26) as needed.

Mice. Mice of three inbred lines were used here: C57BL/6J wild type; the congenic C57BL/6J interleukin-12β (IL-12β; p40 large subunit) homozygous mutant knockout line; and BALB/cJ (all from Jackson Laboratories [hence, "J" designation], Bar Harbor, Maine). These mice were maintained in the Washington University Medical School Animal Quarters (Animal Welfare Assurance A-3381-01) with water and standard mouse chow ad libitum and used in protocols approved by the Washington University Animal Studies Committee (approval 20010039).

Experimental infection. *Helicobacter* cultures were grown overnight on brain heart infusion agar and suspended in phosphate-buffered saline at densities of approximately 2×10^9 CFU per ml. A 0.5-ml aliquot of this suspension was used for each inoculation. In cases of mice inoculated with two strains, the 0.5-ml suspension contained an equal amount of each strain (final concentration, 2×10^9 CFU per ml). To score colonization, mice were sacrificed by CO_2 asphyxiation and cut open with clean sterile scissors immediately after; their stomachs were removed and cut longitudinally along the lesser curvature, and any gastric contents were removed with clean, sterile forceps. The forestomach (not a major site of *H. pylori* colonization), which was identified as a rather thin structure that is separated from the corpus by a white line, was removed and discarded. The remainder of the stomach was homogenized, and the homogenate or dilutions of it were spread on agar medium.

DNA methods. *Helicobacter* genomic DNAs were isolated from confluent cultures grown on agar medium using a QIAamp DNA mini kit (Qiagen Corporation, Chatsworth, Calif.). Randomly amplified polymorphic DNA (RAPD) fingerprint analysis was carried out essentially as described previously (4) in 25-μl reaction mixtures containing either 5 or 20 ng of genomic DNA (to assess reproducibility of patterns), 5 mM $MgCl_2$, 20 pmol of each of four arbitrary primers (Table 1), a 0.25 mM concentration of each deoxynucleoside triphosphate, and 1 U of Biolase thermostable DNA polymerase (Midwest Scientific, St. Louis, Mo.) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl under the following cycling conditions: 45 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min.

Gene-specific PCR was carried out in 20-μl volumes containing 5 to 10 ng of DNA, 0.25 to 0.5 U of *Taq* polymerase (Biolase; Midwest Scientific), 2.5 pmol of each primer (Table 1), and a 0.25 mM concentration of each deoxynucleoside triphosphate, in a standard buffer for 30 cycles with the following cycling parameters: denaturation at 94°C for 30 s, annealing generally at 52°C (low stringency, to compensate for possible mismatches with *H. acinonychis* sequences) for 30 s, and DNA synthesis at 72°C for an appropriate time (1 min per kb). PCR products for sequencing were purified with a PCR purification kit (Qiagen) or extracted from agarose by centrifugation with Ultrafree-DA (Amicon, Millipore). DNA sequencing was carried out using a Big Dye Terminator DNA sequencing kit (Perkin-Elmer) and ABI automated sequencers. Direct sequencing of PCR products was done with 5 μl of PCR fragment (about 100 ng of DNA), 1 μl of primer (1.6 pM), and 4 μl of Big Dye under the following conditions: 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min under oil-free conditions (Perkin-Elmer 2400). DNA sequence editing, alignment, and analysis were performed with the Vector NTI suite of programs (Informax, Bethesda, Md.) and with programs and data in the *H. pylori* Genome Sequence Databases (5, 47) and Blast and pfam (version 5.3) homology search programs (http://www.ncbi.nlm.nih.gov/blast/blast.cgi; http://pfam.wustl.edu/hmmsearch.shtml). Diversity within and between taxa were analyzed using MEGA 2.1 (30). Phylogenetic analysis was performed using the neighbor-joining approach as implemented in PAUP version 4b10 (D. Swofford, Sinauer Associates). To determine the significance of observed groupings in the phylogeny, bootstrap analysis (PHYLIP Phylogeny Inference Package, version 3.573c; J. Felsenstein, Department of Genetics, University of Washington, 1993) was performed with 1,000 replicates in a neighbor-joining (41) environment, with Jukes-Cantor two-parameter distances as implemented in PAUP version 4b10 or/and PHYLIP version 3.573c.

Nucleotide sequence accession numbers. The nucleotide sequences analyzed in this study were deposited in the NCBI GenBank database under accession numbers AY269142 to AY269185. The primers used for PCR and sequencing are listed in Table 1.

RESULTS

Phylogenetic relationships. We studied *H. acinonychis* isolates from six cheetahs from a zoo in Ohio and from two lions, a tiger, and a lion-tiger hybrid from a European circus. Two *H. acinonychis* groups were identified by RAPD fingerprinting (Fig. 1). Group I contained all isolates from the cheetahs from the Ohio zoo and also two lions from the European circus; group II contained isolates from the tiger and the lion-tiger hybrid from the same circus. Two variants were found among group I isolates, differing reproducibly in 3 of 34 bands that were generated with four RAPD primers (Fig. 1). The two group II isolates also differed slightly but reproducibly from one another (Fig. 1).

Lack of *cag* PAI. Two sets of PCR tests indicated that *H. acinonychis* strains lack the *cag* pathogenicity island (PAI). First, no amplification was obtained with DNAs from group I

TABLE 1. Primers for PCR and sequencing

Gene	Primer name	Sequence (5′-3′)[a]	Length of PCR fragment (bp)
glmM (ureC)	glmM F	5′-TTTGGGACTGATGGCGTGAGGG	1,303
	glmM R	5′-TCTTTTAATTCTTGCATTTTGGATTCTA	
recA	recA1 F	5′-GCGTTGGTACGCCTTGGGGATAAGCAA	833
	recA4 R	5′-GCCTTGCCCTAGCTTTTTATCCTGGT	
hp519	519 F1	5′-GGCTTTTTCATAGCCAAATTCTGCG	740
	519 R1	5′-GTTGCCGTTTTCRCTTTGTATAGCT	
picB	cagE-F	5′-CACTCTCAATGAACCCGTTATG	700
	cagE-R	5′-GACGCATTCCTTAACGCTTTGT	
picA	cagD-F	5′-CATAAGAATTGAATACGGCCAATA	1,000
	cagAR-0429	5′-TAGTGGTCTATGGAGTTG	
glr	5733	5′-CACATCGCCGCTCGCATGA	800
	6544	5′-AAGCTTTGTGTATTCTAAAATGCAAC	
ppa	ppa8	5′-CCCCTAGAAAATCCTATTTTGATAATC	902
	ppa9	5′-AGTGGTGAGCTTTAGCGACGCTC	
cysS	cysS-F	5′-CTACGGTGTATGATGACGCTCA	1,287
	cysS-R	5′-CCTTGTGGGGTGTCCATCAAAG	
atpA	atpA1	5′-GCTTAAATGGTGTGATGTCG	1,200
	atpA6	5′-CTTATTCGCCCTTGCCCATT	
vacA	cysS-F2[b]	5′-TGATGGACACCCCACAAGG	530
	va1-R	5′-CTGCTTGAATGCGCCAAAC	
	2579-F2[b]	5′-AGGTGTCGCTTCAAGAACAGCCGG	1,580
	2579-R2	5′-GAGCATTTTCCCGCACTCATACCATG	
	2579-F3[b]	5′-GTGTGGATGGGCCGTTTGCAATAT	1,000
	2579-R2	5′-GAGCATTTTCCCGCACTCATACCATG	
	Vam-F[b]	5′-GGCCCCAATGCAGTCAGTGAT	706
	Vam-R	5′-GCTGTTAGTGCCTAAAGAAGCAT	
	vac6-F[b]	5′-TAATAGAGCAATTCAAAGAGCGCC	790
	vac6-R	5′-CCAAAHCCDCCYACAATRGCTT	
	vac7-F[b]	5′-CCAATGTTTGGGCTAACGCTATTGG	950
	vac7-R	5′-GCRYGGKTTTAAGACCGGTATTT	
vacAs	va1-F	5′-ATGGAAATACAACAAACACAC	*s1*,259
	va1-R	5′-CTGCTTGAATGCGCCAAAC	*s2*,286
vacAs	vac5-F[c]	5′-GTGTCGCTTCAAGAACAGC	100
	vac5-R	5′-CCCAACCCTAATCTCYTTG	
vacAm[d]	vam-F	5′-GGCCCCAATGCAGTCAGTGAT	706
	vam-R	5′-GCTGTTAGTGCCTAAAGAAGCAT	
vacAm2[e]	va4-F	5′-GGAGCCCCAGGAAACATTG	352
	va4-R	5′-CATAACTAGCGCCTTGCAC	
flaA	flaA-F	5′-AAGAATTYCAAGTDGGKGCTTATTYTAAC	870
	flaA-R	5′-TTTTTGCACAGAACCYAARTCAGAKCGSAC	
flaB	flaB-F	5′-TTTWCTAAYAAAGAATTTCAAATYGGHGCG	675
	flaB-R	5′-CTGAARTTCACVCCGCTCACRATRATGTC	
cag empty site	luni1	5′-ACATTTTGGCTAAATAAACGCTG	535
	r5280	5′-GGTTGCACGCATTTTCCCTTAATC	
cag empty site	luni 1	5′-ACATTTTGGCTAAATAAACGCTG	850
	P4	5′-GCTTTGGATTTTTTCAAACCGCA	
16s rDNA	16s-F	5′-CGGTTACCTTGTTACGACTTCAC	1,400
	16s-R	5′-TATGGAGAGTTTGATCCTGGCTC	
	RAPD primers		
	1254	5′-CCGCAGCCAA	
	1281	5′-AACGCGCAAC	
	1283	5′-GCGATCCCCA	
	1290	5′-GTGGATGCGA	

[a] For mixed bases, the following code was used: A/G = R, A/C = M, A/T = W, G/O = S, G/T = K, C/T = Y, A/G/O = V, A/G/T = D, A/C/T = H, G/C/T = B, A/G/C/T = N. Not shown here are sequences of additional standard *cag* PAI primers, designed from known *H. pylori* sequences (5, 47) and used to test for the possible presence of any *cag* genes (but for which no amplification was obtained), as detailed in the text. Sequences are available from authors on request.

[b] Series of primer pairs used for *vacA H. acinonychis* sequencing.

[c] Primer specific for *H. acinonychis.*

[d] For PCR amplification of *vacA* mid-region; each primer was used for sequencing.

[e] For *vacA m2.*

or group II strains with sets of primers specific for the *cagA* gene nor for any of 11 other *cag* PAI genes (*picA* and *picB*, also near the right end, and *hp520*, *hp522*, *hp524*, and *hp526-hp531* at or near the left end [47]). Although the *cagA* gene is so diverse in *H. pylori* populations that a lack of PCR amplification might be considered inconclusive (48, 52), an equivalent lack of amplification with all other *cag* PAI genes tested seemed definitive. This reasoning is based primarily on the

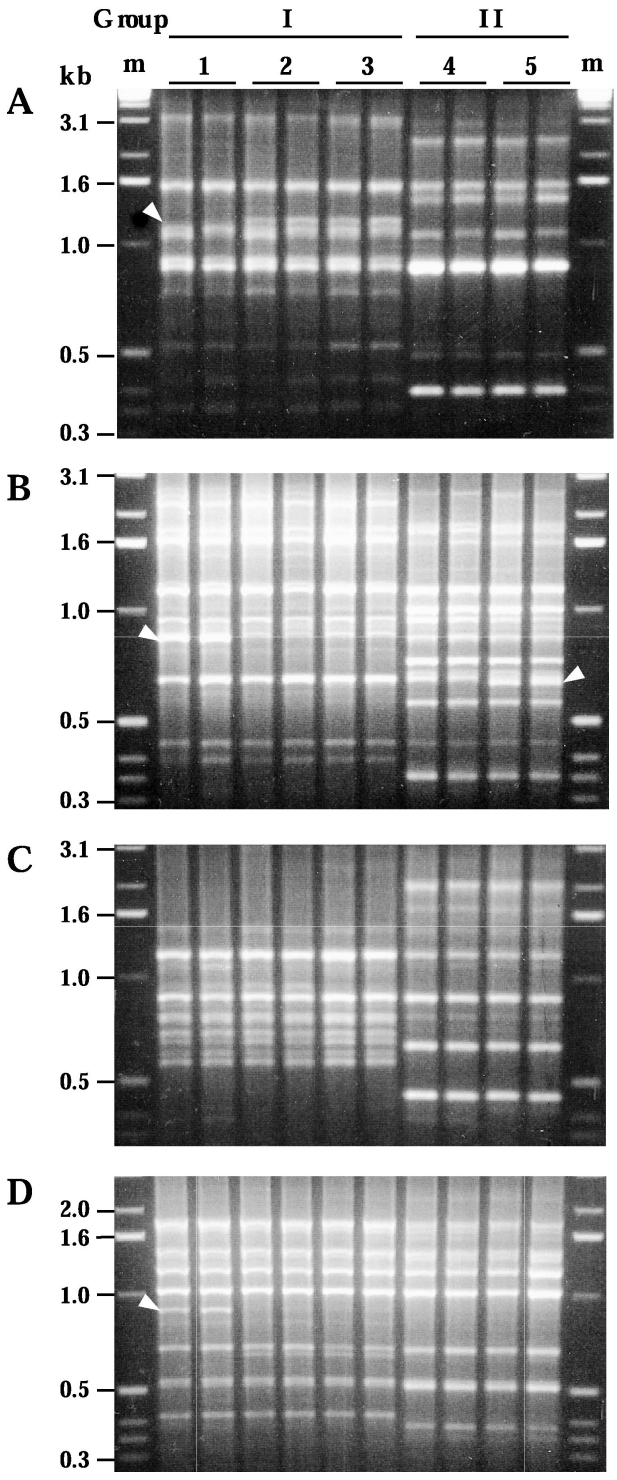

FIG. 1. RAPD fingerprinting identified two groups of *H. acinonychis* strains. Profiles shown were obtained with RAPD primers 1247 (A), 1254 (B), 1281 (C), and 1283 (D). With each DNA sample, RAPD tests were run with 5 ng (left lane) and with 20 ng (right lane) of template DNA to identify subtle differences that are reproducible and thus informative. Arrowheads identify bands that distinguish different strains of the same group. Lane pairs 1, 2, and 3 contain profiles of group I isolates (cheetah strain 89-2579, Mac, and Sheebah, respectively); lane pairs 4 and 5 contain profiles of group II isolates (Sheena and India, respectively). The profiles of cheetah strain 89-2579 shown here are representative of those obtained from other cheetah isolates, except for strain 90-548, which reproducibly yielded one extra band (1.1 kb) with primer 1254. m, marker DNA.

following: (i) our sequence analyses of three other *cag* PAI genes (*hp520*, *picA*, and *picB*) in a global *H. pylori* strain collection and the finding that they were no more diverse than housekeeping (metabolic) genes (G. Dailide, M. Ogura, and D. E. Berg, unpublished data), which were readily amplified from *H. acinonychis* DNA (see below); and (ii) a sense that most genes whose proteins act internally in bacterial cells, unlike *cagA*, should not have been subject to diversifying selection. Second, as independent evidence that *H. acinonychis* does not contain the *cag* PAI, PCR products of sizes expected for *cag* PAI empty sites were obtained using primers specific for flanking genes (*hp519* and *glr*; 0.53 and 0.85 kb, depending on primers used). Sequences from these products (GenBank accession numbers AY269155 and AY269157) were 91% matched to one another and 93% matched (group I) and 86% matched (group II) to corresponding empty sites of *H. pylori* clinical isolates that also lack the *cag* PAI (GenBank accession nos. AF084492 and AF084493, respectively). We conclude that these *H. acinonychis* strains lack a *cag* PAI.

***vacA* status.** PCR products were obtained from both groups of *H. acinonychis* isolates with primers that are specific for relatively conserved sites in the middle region of the *vacA* gene (vam-F and vam-R; Table 1). Products were also obtained with primers specific for the 5′ end (signal sequence region; va1-F and va1-R), although these products were 240 and 121 bp long (group I and II isolates, respectively), not 259 or 286 bp, which are obtained with *H. pylori vacA s1* or *s2* alleles, respectively (Table 1).

The sequence of a 4,006-bp DNA fragment containing the *vacA* gene from a group I strain (89-2579, from a cheetah) was determined (GenBank accession no. AY269171). It was 84% identical to the most closely related of currently available (as of July 2003) *H. pylori vacA* sequences (GenBank accession no. AF050327; strain CHN5114a). It differed from this *H. pylori* sequence by nine insertions and eight deletions ranging from 3 to 59 bp and ~43 translational stops (due variously to out-of-frame insertions and deletions and base substitutions [nonsense codons]). The first 2.2 kb of *vacA* from a group II strain (Sheena) was also sequenced (GenBank accession no. AY269176). It differed from the corresponding part of the group I *vacA* sequence by 5.8% base substitutions and 11 insertions and deletions and from the corresponding part of the *vacA* sequence of *H. pylori* strain CHN5114a by 13 insertions and deletions and 22% base substitutions. The many disruptions of *vacA* open reading frames indicated that these *vacA* genes would not encode an active vacuolating cytotoxin or a full-length VacA protein.

Additional sequencing of *vacA*-containing segments from the two group I strains from lions in Europe (724 bp of the *vacAs* region from Sheeba; 996 bp of *vacAs* and 664 bp of the *vacAm* regions from Mac) identified only a 1-bp difference from the sequence of the U.S. cheetah strain (in the *vacAs* segment). This near-identity suggests that *vacA*-null alleles existed while these *H. acinonychis* strains infected big cats and were not artifacts of laboratory culture.

TABLE 2. DNA sequence relationships of group I[a] and group II[a] *H. acinonychis* strains to each other and to a reference *H. pylori* strain[a]

Comparison	% Identity with gene (sequence length [bp])									Avg
	glmM (648)	*flaB* (615)	*recA* (642)	*hp519* (726)	*glr* (768)	*flaA* (645)	*cysS* (659)	*atpA* (1,080)	*ppa* (427)	
Cheetah (I) vs 26695	91.8	89.8	93.6	91.9	91.7	91.3	93.4	93.7	93.7	92.4
Sheena (II) vs 26695	91.9	88.8	93.6	91.2	91.1	91.3	93.6	93.5	92.7	
Cheetah[b] (I) vs Sheena (II)	97.9	97.7	98.7	95.6	97.3	100	97.5	99.3[c]	97.2	98.2

[a] Group I and group II refer to sets of *H. acinonychis* strains with the different RAPD profiles illustrated in Fig. 1 and described in the text; 26695 is a reference strain of *H. pylori* whose genome has been fully sequenced.
[b] Cheetah refers to strain 89-2579 from a cheetah. However, where tested, identical corresponding sequences were found in isolates from other cheetahs as well.
[c] Divergence in *atpA* consists of 7 nt substitutions in 142 bp of the 1,080 bp sequenced.

Relatedness assessed with functional genes. Further sequence analyses were carried out using nine genes that are probably needed in vivo and thus likely to be intact, not inactivated by mutation: six housekeeping genes (*glr*, just to the right of the *cag* PAI; *cysS*, just upstream of *vacA*; and *glmM*, *recA*, *atpA*, and *ppa*), the *flaA* and *flaB* flagellin genes, and *hp519*, a putative regulatory gene just to the left of the *cag* PAI. PCR products of sizes expected based on *H. pylori* sequences were obtained for each gene from both groups of *H. acinonychis* strains. Because the primers used had been designed from *H. pylori* sequences, all amplification was carried out with low-stringency (52°C) annealing. The sequences of PCR products obtained from group I and II isolates differed from one another by about 1.8%, on average (Table 2). However, identical sequences were found in 645 bp of *flaA* and in all but 142 of the 1,080 bp of *atpA* (Table 2). These matches were noteworthy because identical sequences are only rarely found in independent *H. pylori* isolates. In addition, sequences identical to those of the U.S. cheetah strain (group I) were found in all genes sequenced from the European group I strains (*glmM*, *recA*, *hp519*, *glr*, *cysS*, and *atpA* from Mac; *glmM* and *atpA* from Sheeba). Similarly, the two group II strains were identical at all but 1 bp in the five genes sequenced from both of them (*glmM*, *recA*, *hp519*, *glr*, and *cysS*). Thus, these sequence and RAPD profile data (Fig. 1) each showed that these 10 *H. acinonychis* isolates belong to just two major lineages.

The *H. acinonychis* genes, other than *vacA*, that were analyzed were about 8% divergent from homologs in *H. pylori* reference strains such as 26695 (47) and J99 (5). The close, but distinct, species relationship between *H. acinonychis* and *H. pylori* is further illustrated by comparison of *flaA* and *flaB* gene sequences (Fig. 2). We note that two of the genes analyzed here, *ppa* and *atpA*, had also been studied in an unusual outgroup of *H. pylori* strains from South Africa (17). The *ppa* and *atpA* sequences from *H. acinonychis* were more closely related to those of the South African *H. pylori* outgroup (4.7% DNA divergence, on average) than either the *H. acinonychis* or outgroup sequences were to those of most other known *H. pylori* strains, including reference strains 26695 and J99 (divergences of 6.6 and 7.6% between sequences from reference *H. pylori* strains versus outgroup *H. pylori* strains and versus *H. acinonychis*, respectively).

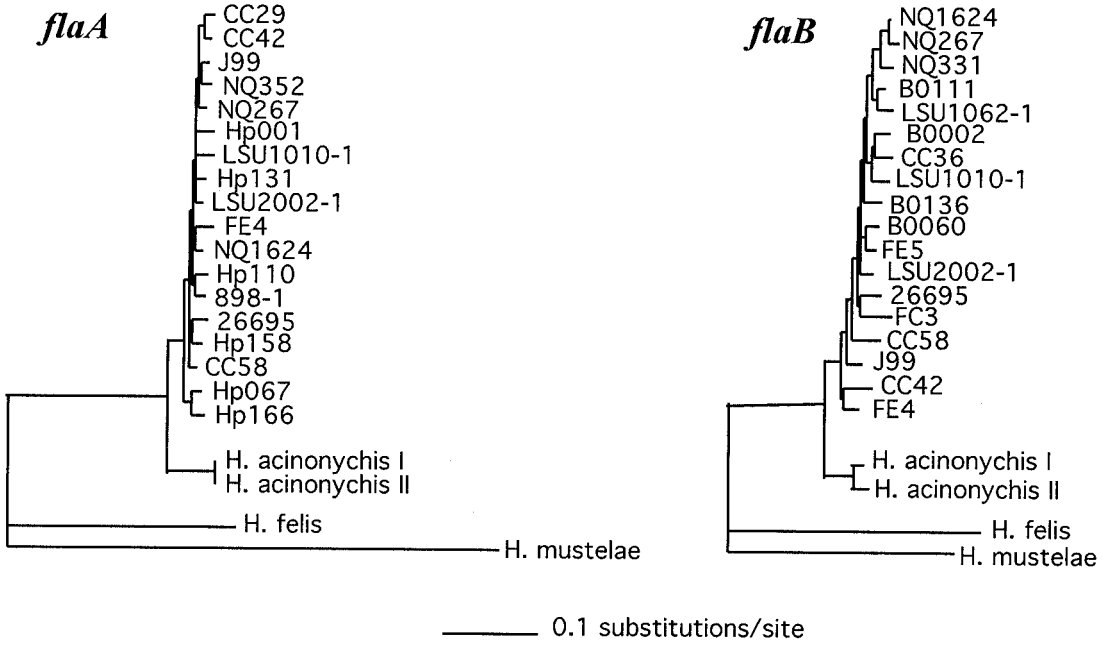

FIG. 2. The neighbor-joining tree of *Helicobacter* flagellin genes inferred from DNA sequences confirmed the separate species groupings of *H. acinonychis* strains. The *H. acinonychis* sequences were determined here. All other sequences are from the GenBank public database. Left, FlaA; right, FlaB.

TABLE 3. MTZ susceptibility profiles of *H. acinonychis* strains

Strain	RAPD[a] group	MIC (μg of MTZ/ml)[b] for:	
		WT	*rdxA*::*cat* (null)
89-2579	I	1.5	32
90-624	I	1.5	32
90-736	I	16	NT
90-548	I	32	NT
90-119	I	128	NT
90-788	I	128	NT
Mac	IA	1.5	32
Sheeba	IA	1.5	NT
Sheena	II	3	16
India	II	8	16

[a] RAPD groups are defined in the legend for Fig. 1. The subtle differences in RAPD profiles between group I strains from cheetahs in a U.S. zoo and the strains from lions in a European circus (here designated group IA) are also illustrated in Fig. 1.

[b] In the present usage, MIC indicates the lowest concentration of MTZ used that resulted in at least a 100-fold reduction in efficiency of colony formation. WT, wild type; NT, not tested.

Susceptibility to MTZ. Resistance to the important anti-*Helicobacter* drug MTZ is common among strains of both *H. acinonychis* and *H. pylori* (8, 13, 26), probably in part because it is also much used against anaerobic and parasitic infections. The susceptibility or resistance of each *H. acinonychis* isolate to MTZ was characterized, in part, to help choose Mtz^s strains for mouse infection studies (below). Two of the six Ohio zoo isolates and three European circus isolates were MTZ sensitive (MIC = 1.5 μg of MTZ/ml [or in one case, 3 μg of MTZ/ml]), and the other five isolates were moderately or highly resistant (MIC range from 8 to 128 μg of MTZ/ml) (Table 3). Two types of Mtz^s *H. pylori* are known and can be distinguished by the ease of mutation to resistance: type I requires inactivation of just the *rdxA* nitroreductase gene (because the related *frxA* gene is quiescent), and type II requires inactivation of both *rdxA* and *frxA* (26, 34). The three mouse-colonizing strains of *H. pylori* characterized to date (SS1, X47, and 88-3887) are each type II (26, 34). Cam^r transformants of Mtz^s *H. acinonychis* isolates were generated using an *rdxA*::*cat* (null) allele from *H. pylori*. Each Cam^r transformant was Mtz^r, with MTZ MICs of 32 and 16 μg per ml in group I and II strains, respectively (Table 3), suggesting that the *frxA* nitroreductase gene is either quiescent or absent from these strains. The small differences in MICs were reproducible and suggested quantitative differences in parameters such as basal levels of other nitroreductases, of MTZ uptake, or of repair of MTZ-induced DNA damage (see references 26 and 34).

Adaptation to mice. An earlier effort to achieve *H. acinonychis* infection of BALB/c mice was not successful (13). Here, we also attempted to isolate mouse-colonizing *H. acinonychis* strains, but this time we used IL-12β-deficient C57BL/6J mice, which seem more permissive than congenic wild-type C57BL/6J or BALB/c mice for *H. pylori* (19, 24, 36), and pools of isolates, rather than just a single strain, to avoid possible problems of strain attenuation in culture. *H. acinonychis* organisms were recovered 2 weeks after inoculation from each of four mice that had received Mtz^s group I strains (89-2579 and 90-624; Sheeba and Mac) (20 to 500 CFU per stomach) and also from two of four mice that had received group II strains (India and Sheena) (about 2,000 CFU per stomach). These pools of recovered *H. acinonychis* organisms were used in a second inoculation of IL-12β-deficient mice; 1,000 to 3,000 CFU were recovered 2 weeks later from each of 10 mice (5 inoculated with each *H. acinonychis* group). No further increase in bacterial yield was seen after a third cycle of infection of IL-12β-deficient mice. RAPD fingerprinting, as shown in Fig. 1, suggested that these mouse-adapted strains were derived from a cheetah isolate (group I) and from Sheena (group II). These strains, now adapted to IL-12β-deficient mice, were used to inoculate wild-type C57BL/6J and BALB/cJ mice: 1,000 to 3,000 CFU were obtained per C57BL/6J mouse stomach at 2 weeks and also at 12 weeks after inoculation (five mice per time point per strain); 1,000 to 3,000 CFU and 500 to 1,000 CFU were obtained per BALB/cJ mouse stomach inoculated with group I and group II strains (five mice in each group). Thus, *H. acinonychis* strains selected initially for colonization of C57BL/6J IL-12β-deficient mice were also well suited for infection of two other wild-type lines (C57BL/6J and BALB/cJ).

***H. acinonychis-H. pylori* mixed infection.** The similar genetic distances of *H. acinonychis* and the African *H. pylori* outgroup to other *H. pylori* strains, the ease of DNA transformation between the two species in culture, and interest in evolutionary consequences of interspecies gene transfer led us to test for mixed infection in vivo. In the first test, mice were inoculated with *H. acinonychis* and also SS1 or X47, *H. pylori* strains that colonize mice at high density but at different preferred gastric sites (SS1 in the antrum, X47 in the corpus) (2). Mice were sacrificed 2 weeks later, gastric contents were cultured, and single colonies were tested by PCR or by susceptibility when SS1 was marked genetically (Tet^r) to distinguish the two species. Based on these tests, only 6 of 336 colonies from the mixed inoculation with SS1 were of the *H. acinonychis* type; similarly, just 1 of 96 colonies from the mixed inoculation with X47 was of the *H. acinonychis* type (Table 4). A sequential inoculation protocol was used next, to assess if the low yield of *H. acinonychis* might be due primarily to inefficient initiation of infection. A *vacA*-null (Cam^r) derivative of *H. pylori* strain SS1 was used because *vacA* is needed by this strain to initiate infection efficiently but not to maintain it after the first few critical days (42). Mice were inoculated with group II *H. acinonychis* first and then with *H. pylori* 1 week later; the mice were sacrificed and *Helicobacter* was cultured from them 2 weeks after superinfection. All but 6 of 230 single colonies tested (at least 20 per mouse) was resistant to chloramphenicol, indicating that most were of the SS1 *vacA*-null type (Table 4). Equivalent sequential inoculation tests were carried out using a Cla^r derivative of strain X47; all but 14 of 284 colonies tested was similarly of the Cla^r X47 type (Table 4). These results emphasized that *H. pylori* strains SS1 and X47 can each outcompete *H. acinonychis*, even if inoculated a week after the *H. acinonychis* infection has started.

Given *H. pylori*'s genetic diversity, it seemed that other mouse-adapted strains might be less vigorous or differ in tissue tropism from strain SS1 or X47 and, therefore, be able to establish a more balanced mixed infection with *H. acinonychis*. This was tested first by inoculating mice with genetically marked derivatives of *H. acinonychis* (Tet^r group I, Cla^r group II) and of *H. pylori* strain 88-3887 (Cam^r; *rdxA*::*cat*) and scoring the types of helicobacters recovered 2 weeks later by drug

TABLE 4. *H. pylori* strains SS1 and X47 outcompete *H. acinonychis*

Input		No. of colonies recovered		No. of mice	Single-colony test method
H. acinonychis	*H. pylori*	*H. acinonychis*	*H. pylori*		
Tests of single colonies					
I	SS1 WT[a]	0[b]	48	4	PCR
I	SS1 Tet^r	2	118	4	Phenotype
II	SS1 WT	1[b]	47	4	PCR
II	SS1 Tet^r	3	117	4	Phenotype
I	X47 WT	0[b]	48	4	PCR
II	X47 WT	1[b]	47	5	PCR
Sequential infection[c]					
II	SS1 *vacA*::*cat*	6	230	9	Phenotype
II	X47 Cla^r	14	284	9	Phenotype

[a] WT, wild type.
[b] PCR tests using pools of >1,000 colonies and primers vac5-1-F and vac5-1-R indicated that *H. acinonychis* was also present in each mixed infection.
[c] Mice received *H. acinonychis* first and then *H. pylori* superinfection 1 week later.

resistance patterns. Figure 3 (left panel) shows that *H. acinonychis* was recovered from 14 of 20 mice coinoculated with these two species. Similar mixed infections were obtained after coinoculation with *H. acinonychis* group I and either of two other mouse-adapted *H. pylori* strains (AM1 and AL10103) (data not shown), indicating that the ability to coexist with *H. acinonychis* is not unique to strain 88-3887.

A final set of coinfection studies was carried out by first inoculating mice with Tet^r or Cla^r *H. acinonychis*, superinfecting them with Cam^r 88-3887 1 week later, and scoring the types of helicobacters recovered at 1, 3, and 8 weeks after superinfection. Much as with simultaneous inoculations, persistent mixed infections were found in just over half of the mice examined (Fig. 3, right): 5 of 9 mice scored at 1 week, 5 of 10 scored at 3 weeks, and 6 of 10 scored at 8 weeks after superinfection.

Variants accumulated during 8 weeks of mixed infection. One Tet^s derivative of *H. acinonychis* group I and one Cam^s

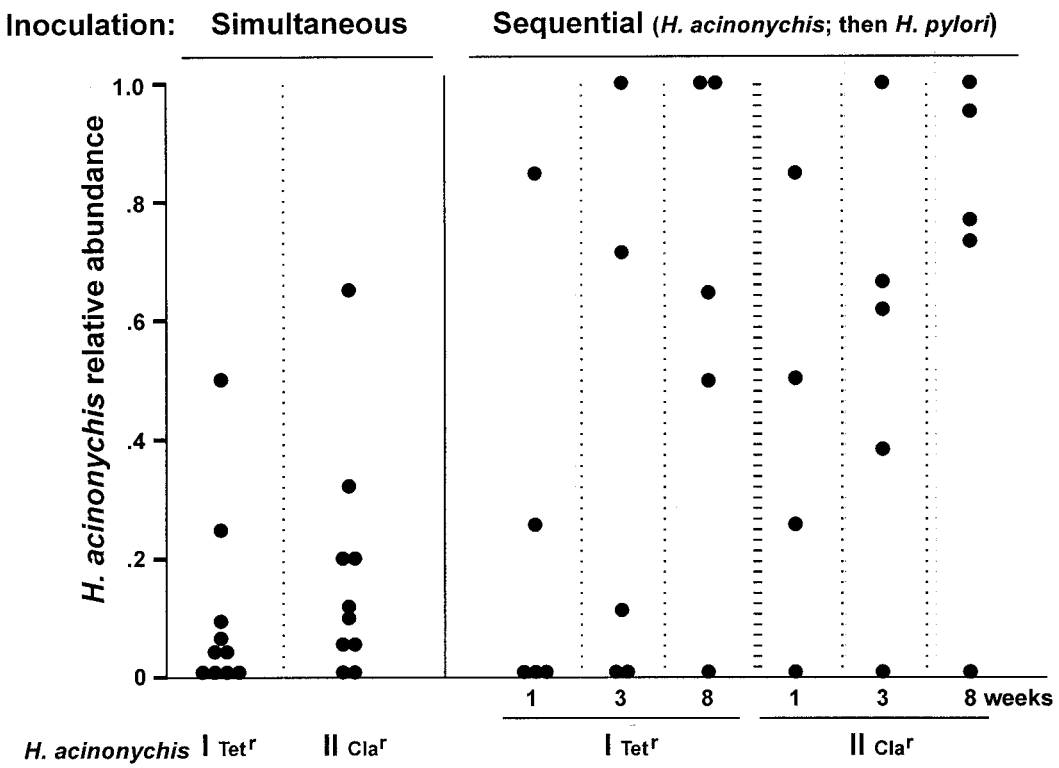

FIG. 3. Mixed infections resulting from simultaneous and sequential inoculations with genetically marked *H. acinonychis* and *H. pylori*. The mutations conferring resistance to Tet and to Cla are in 16S and 23S rDNAs, respectively (9, 49). Cam resistance is conferred by a chloramphenicol acetyltransferase gene (*cat*) inserted in the *rdxA* nitroreductase gene of *H. pylori* strain 88-3887 (25, 34). Frequencies of each strain type were estimated by testing ≥20 single colonies per mouse for antibiotic susceptibility and also by colony counts on selective agar. Weeks refers to time between superinfection and mouse sacrifice and culturing of *Helicobacters* that they harbored.

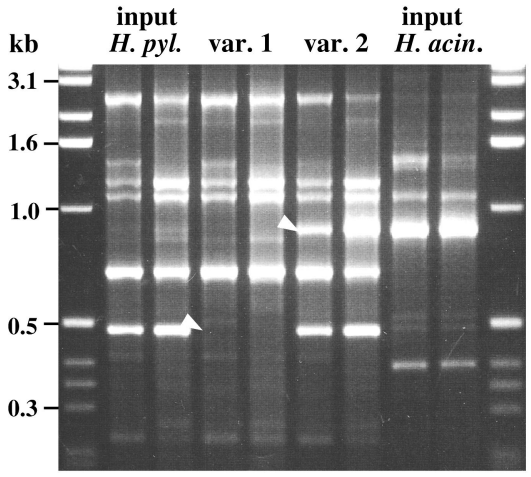

FIG. 4. RAPD fingerprinting (primer 1247) identified two variant *H. pylori* strains isolated after 2 months of mixed infection with *H. acinonychis* in C57BL/6J IL-12β knockout mice. With each DNA sample, RAPD tests were run with 5 ng (left lane) and with 20 ng (right lane) of template DNA, as for Fig. 1. White arrowheads identify bands that distinguish variants. m, marker DNA.

derivative of *H. pylori* were found among 337 *Helicobacter* colonies recovered 8 weeks after superinfection and screened for drug susceptibility (66 group I and 72 group II *H. acinonychis*; 199 *H. pylori*) (experiment in Fig. 3, right) (species of two susceptible isolates were identified by RAPD test). Analysis of the 16S rDNA sequence of the Tet^s isolate indicated that it arose by interstrain recombination involving the 16S rDNA genes: a replacement of a short patch in *H. acinonychis* (less than 137 bp) containing TTC (resistant allele) by AGA (sensitive allele) at positions 965 to 967. In contrast, PCR tests of the Cam^s *H. pylori* variant with *rdxA*- and *cat*-specific primers revealed only a normal-length *rdxA*::*cat* insertion allele, not an intact *rdxA* allele. In addition, Cam^r revertants of this Cam^s *rdxA*::*cat* strain were obtained at frequencies of about 10^{-6}. No equivalent Cam^r mutants were detected among 10^8 cells of an isogenic control strain that lacks *cat* gene sequences. We therefore infer that this variant arose by mutation, not by replacement of the *rdxA*::*cat* allele with the intact *rdxA* gene of *H. acinonychis*.

Two other variants, both *H. pylori*, were found by RAPD fingerprinting of 39 isolates (23 *H. pylori*; 6 group I and 10 group II *H. acinonychis*; Fig. 3, right); the primer 1247 profile of one variant lacked a characteristic ~0.5-kb RAPD band, and that of the other contained an extra ~0.9-kb band that comigrated with a characteristic *H. acinonychis* band (Fig. 4). No other difference from the input *H. pylori* strain was found in RAPD tests with any of four RAPD primers.

DISCUSSION

Two groups of *H. acinonychis* strains were identified: one group consisting of isolates from six cheetahs from a U.S. zoo and two isolates from lions from a European circus, and the other group consisting of isolates from two other felines (a tiger and a lion-tiger hybrid) from the same circus. The two groups differed from one another by about 2% in gene sequence, on average, but were identical in one gene (*flaA*) and in most of another (*atpA*), a pattern suggesting recombination between lineages. Such exchange might have occurred during mixed infection in captivity, perhaps following direct contact between infected animals or transmission by human handlers. More remarkable, from an *H. pylori* perspective, was the near-identity of *H. acinonychis* isolates from the United States and Europe, since any given *H. pylori* isolate is usually easily distinguished from other independent isolates by the DNA tests used here (4, 17). Having so few *H. acinonychis* genotypes implies disproportionate contributions from very few index cases (a genetic bottleneck) and/or a far more epidemic mode of transmission of *H. acinonychis* in captive big cats than of *H. pylori* in humans.

Derivatives that could chronically infect mice were readily obtained from each *H. acinonychis* group using C57BL/6J IL-12β knockout mice as initial hosts and C57BL/6J and BALB/cJ wild-type mice later. *H. acinonychis* was so-named because it was first isolated from cheetahs (12, 13), and it is associated with severe gastritis, a frequent cause of their death in captivity (8, 12, 13, 35). *H. acinonychis'* ability to infect other felids (illustrated by the present lion and tiger isolates) and mice raises questions about its host range in nature. Are cheetahs or other big cats necessarily its only, or even most common, natural host? Or, might *H. acinonychis* also often infect other carnivores and/or even the herbivores on which they prey?

Any flexibility in *Helicobacter* host range bears on discussions of how and when *H. pylori* became a human pathogen (7). The popular ancient-origins theory envisions near-universal *H. pylori* infection in hominids for perhaps millions of years (6, 17). Our alternative theory envisions *H. pylori* infection of humans becoming widespread more recently, perhaps in early agricultural societies (29), facilitated by close animal-human contact and increased chances for person-to-person spread (10). The ease of adapting *H. pylori* to mice and other animals (11, 18, 19, 21, 31, 39) illustrates again that potential host species barriers are easily surmounted. Also noteworthy is an unusual outgroup of *H. pylori* strains from Africa, some 7% divergent from the more-abundant groups of *H. pylori* strains in housekeeping gene sequences (17). Although initially interpreted as representing an ancient *H. pylori* lineage sequestered until recently in a very isolated group of humans (17), this *H. pylori* outgroup seemed more closely related to *H. acinonychis* than either it or *H. acinonychis* were to predominant *H. pylori* groups. Thus, the data also fit with a model in which the ancestors of this *H. pylori* outgroup jumped from animals to people recently during human evolution. By extrapolation, the more-abundant groups of human-adapted *H. pylori* strains might also have been acquired quite recently by humans.

H. pylori strains SS1 and X47 were far more fit than *H. acinonychis* in mice: even after *H. acinonychis* had begun to establish itself, it was displaced soon after superinfection by these stronger *H. pylori* strains. In accord with this finding are preliminary observations that these two strains each also outcompete strain 88-3887 (M. Zhang, D. Dailidiene, and D. E. Berg, unpublished data). In further tests using strains that were genetically marked (for efficiency in scoring many colonies), derivatives of *H. acinonychis* (Tet^r or Cla^r) were able to establish mixed infections with derivatives of *H. pylori* 88-3887 (*rdxA*::*cat*; Cam^r) and also with two other mouse-adapted *H.*

pylori strains (AM1 and AL10103). In a sequential-infection experiment, half of the mice inoculated first with *H. acinonychis* and then *H. pylori* 88-3887 a week later harbored quite similar levels of the two species 8 weeks after superinfection. We suggest that such experimental mixed infections may provide good models for understanding the human condition, especially in many developing countries, where risks of infection are high for children and also for adults (23, 44).

Two cases of genetic change were detected among 337 single-colony isolates that were tested for drug resistance markers: a loss of tetracycline resistance from *H. acinonychis* by interstrain recombination and a loss of chloramphenicol resistance from *H. pylori*, but by mutation not recombination. This one case of mutation (among only 199 *H. pylori* isolates) was unexpected, but it is in accord with other indications that mutation can be frequent in this species (50). Two changes in the RAPD profile were also found in the screening of 39 isolates: one gain of an *H. acinonychis*-like RAPD band and one loss of a characteristic *H. pylori* band. Precedent suggests that these two variants may have arisen by interstrain recombination (28), although the possibility of a mutational origin also merits consideration.

People, like other mammalian hosts, are diverse in traits that can be important to individual *H. pylori* strains—for example, in distribution or abundance of carbohydrate structures that *H. pylori* uses for adherence, in gastric acidity, in the repertoire of host defenses, and in the history of other infections that in turn affect host responses to *H. pylori* (11, 20, 25, 33). *H. pylori*, in turn, is extraordinarily diverse genetically, in part probably because of legacies of diversifying selection in a succession of hosts and because of transmission patterns that minimize chances of population-wide selection for any one or a few most-fit genotypes. Given *H. pylori*'s great genetic diversity, an important challenge will be to identify those polymorphic determinants in helicobacters that contribute to colonization and disease—a bacterial counterpart of the quantitative trait loci that determine many aspects of the phenotypes of humans and other higher organisms (32). We suggest that *H. acinonychis* may have just the right mix of moderate genetic distance from and similarity in physiology and gastric tropism to *H. pylori* for such studies. Mouse-adapted *H. acinonychis* should be valuable as a resource for analysis of the interplay between *Helicobacter* and its host that shapes the specificity and vigor of infection, the risks of various types of disease, and the evolutionary trajectories that may result.

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