

Who Ate Whom? Adaptive *Helicobacter* Genomic Changes That Accompanied a Host Jump from Early Humans to Large Felines

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***Helicobacter pylori* infection of humans is so old that its population genetic structure reflects that of ancient human migrations. A closely related species, *Helicobacter acinonychis*, is specific for large felines, including cheetahs, lions, and tigers, whereas hosts more closely related to humans harbor more distantly related *Helicobacter* species. This observation suggests a jump between host species. But who ate whom and when did it happen? In order to resolve this question, we determined the genomic sequence of *H. acinonychis* strain Sheeba and compared it to genomes from *H. pylori*. The conserved core genes between the genomes are so similar that the host jump probably occurred within the last 200,000 (range 50,000–400,000) years. However, the Sheeba genome also possesses unique features that indicate the direction of the host jump, namely from early humans to cats. Sheeba possesses an unusually large number of highly fragmented genes, many encoding outer membrane proteins, which may have been destroyed in order to bypass deleterious responses from the feline host immune system. In addition, the few Sheeba-specific genes that were found include a cluster of genes encoding sialylation of the bacterial cell surface carbohydrates, which were imported by horizontal genetic exchange and might also help to evade host immune defenses. These results provide a genomic basis for elucidating molecular events that allow bacteria to adapt to novel animal hosts.**

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Introduction

The stomachs of half of the global human population are colonized by the Gram-negative bacterium *Helicobacter pylori*, which results in chronic gastritis and can cause peptic ulcers and gastric cancer [1]. Since the original description of *H. pylori* in 1984 [2], various *Helicobacter* species have been identified in a wide range of vertebrate hosts, possibly reflecting long-term co-evolution of microbe and host [3]. However, the length of association between *H. pylori* and humans is controversial and was even suggested to reflect a recent host jump from domesticated animals [4,5]. Domestication occurred during the last 10,000 years, the Neolithic period, and this interpretation would imply that *H. pylori* has only been associated with humans for less than 10,000 years. Instead, recent data show that *H. pylori* with East Asian genotypes can be isolated from Amerinds in North and South America [6–8], and thus presumably accompanied human migrations across the Bering Strait, prior to the Neolithic period. Furthermore, the population genetics of *H. pylori* mimics that of humans, and seems to reflect ancient human migrations [8]. Therefore, humans probably acquired *H. pylori* quite early in their history, long before the migrations of modern humans out of Africa [9].

Other than humans, the only natural hosts for *H. pylori* seem to be non-human primates [10], but *H. pylori* can infect animals such as mice, dogs, and gerbils in laboratory experiments. The stomachs of carnivores (dogs, cats, and cheetahs) are also frequently naturally colonized with

Helicobacter, but these belong to *Helicobacter felis*, *Helicobacter bizzozeronii*, and other species that are quite different from *H. pylori* [3,11,12]. Unusually, one *Helicobacter* species, *Helicobacter acinonychis*, that is closely related to *H. pylori* according to the sequences of ribosomal RNA and multiple other genes [5,13–15] (Figure 1), colonizes the stomachs of large felines,

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Abbreviations: CDS, coding sequence; D_N, pair-wise non-synonymous genetic distance; D_S, pair-wise synonymous genetic distance; HGT, horizontal gene transfer; IS, insertion element; LCA, last common ancestor; OMP, outer membrane protein

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Synopsis

Little is known about the mechanisms that allow jumps of parasites from one host species to a second. We describe the genomic sequence of strain Sheeba of *Helicobacter acinonychis*, a bacterial parasite that can cause severe gastritis of tigers, lions, and cheetahs. The genomic sequence possesses an unusually high number of defective genes that have been fragmented through frameshift mutations, stop codons, and small insertions and deletions. Together with phylogenetic analyses, these observations indicate that *H. acinonychis* arose 200,000 years ago via a host jump of *Helicobacter pylori* from early humans. *H. acinonychis* also possesses five genes involved in sialylation of cell surface carbohydrates that were probably acquired by horizontal gene transfer from unrelated bacteria. Epitopes on the bacterial cell surface were diminished by gene fragmentation (inactivation of outer membrane proteins) and sialylation (masking of carbohydrates), which should facilitate the evasion of feline immune defenses, and may have been crucial for the host jump.

including cheetahs, tigers, and lions. The existence of *H. acinonychis* led to the suggestion that *H. pylori* resulted from a host jump to humans from a non-domesticated zoonotic source [5], such as large felines. However, neutral sequence variation alone cannot indicate the direction of a host jump, which leaves the question of the origins of *H. acinonychis*

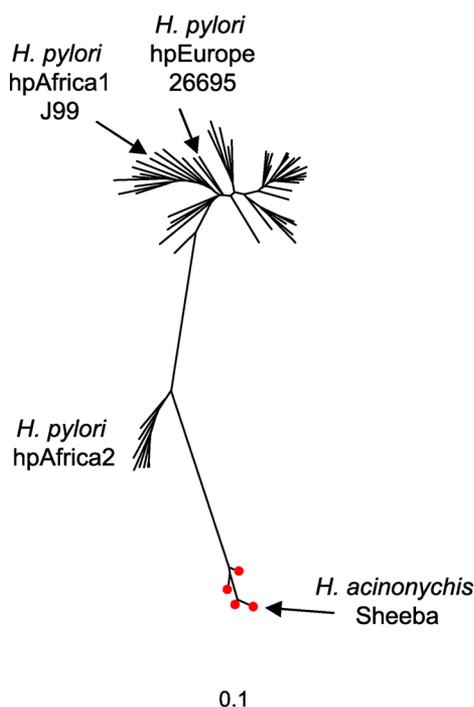


Figure 1. Neighbor-Joining Tree Based on the GTR+G+I Evolutionary Model for 3,406 bp Sequences from 58 Strains of *H. pylori* and Four Strains of *H. acinonychis*

The tree shows the phylogenetic relationships between *H. acinonychis* and populations within *H. pylori*, and arrows indicate the three strains, J99, 26695, and Sheeba, from which genome sequences are currently available. This phylogenetic tree indicates that *H. pylori* (lines) and *H. acinonychis* (lines plus red dots) are closely related but cannot resolve the direction of ancestor-descendent relationships. Genetic distance scale bar at bottom.

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unresolved, namely, “who ate whom?” We have therefore determined the genome sequence of *H. acinonychis* strain Sheeba (Figure S1) and performed comparative genomic analyses with the genomes of *H. pylori* strains 26695 [16] and J99 [17], as well as other Campylobacterales, to identify signatures of adaptation that might have accompanied a recent host jump.

Results

Relatedness between *H. acinonychis* and *H. pylori*

The size and GC content of the Sheeba genome are very similar to those of 26695 and J99 (Table 1), and all three genomes share numerous orthologous coding sequences (CDSs) (Figure 2). The proteins encoded by the 612 orthologous CDSs that are present in all three genomes and do not have internal gaps differ at only few of their amino acids, as indicated by pair-wise estimates of 3–4% for D_N , the frequency of non-synonymous nucleotide polymorphisms (Table S1). Similarly, for most CDSs in *H. acinonychis*, normalized Blast scores against either of the two *H. pylori* genomes were very high (Figure 2C), whereas much lower scores were found in pair-wise comparisons against *Helicobacter hepaticus*, a distantly related *Helicobacter* species from rodents [18]. *H. acinonychis* is thus very closely related to *H. pylori*, almost as closely related as are the two *H. pylori* genomes to each other.

D_S , the synonymous genetic distance, can be used to estimate the elapsed time since divergence from a last common ancestor (LCA) of the three genomes. D_S was calculated among the 612 orthologs described above by two different estimators, that of Li et al. [19] and the modified Nei-Gojobori method [20], which is based on the ratio of

Table 1. General Features of *Helicobacter* Genomes

Category	Species			
	<i>H. acinonychis</i>	<i>H. pylori</i>	<i>H. pylori</i>	<i>H. hepaticus</i>
Strain	Sheeba	26695	J99	ATCC 51449
Hosts	Large felines	Humans	Humans	Rodents
Genome Size (bp)	1,553,928	1,667,867	1,643,831	1,799,146
G+C content (%)	38.2	39.0	39.0	35.9
Coding Sequences				
Predicted Number	1,611	1,590 (1,552 ^a)	1,459	1,875
Coding Area (%)	89.7	91.0	90.8	93.0
Average Length (bp)	865	945	998	1,082
Flexible Genome Pool				
Plasmids	pHac1	None ^b	None ^b	None
Bacteriophage genes	41	None	None	3
Insertion Sequence (IS)	ISHa1675 (1)	IS605	IS605 (partial)	None
Elements (number of copies)	ISHa1942 (1) ISHa1152 (2)	IS606 — ^c	IS606 — ^c	
Genomic Islands	HacGI (integron)	cagPAI	cagPAI	HHGI1

^aRevised number of predicted *H. pylori* CDSs in 26695 according to Boneca et al. [89].

^bSequences from nine plasmids of *H. pylori* were found in GenBank (pHE14 and pHE15 [30], pHPM8 [29], pHPM180 [90], pHP489 [91], pHP51 [92], pHP100, pHPM186, pAL202) but no plasmids are present in 26695 or J99.

^cIShp608 is present in numerous *H. pylori* strains [93] but is lacking in 26695 and J99. cagPAI, cytotoxin associated pathogenicity associated island; HHGI1, *H. hepaticus* Genomic Island.

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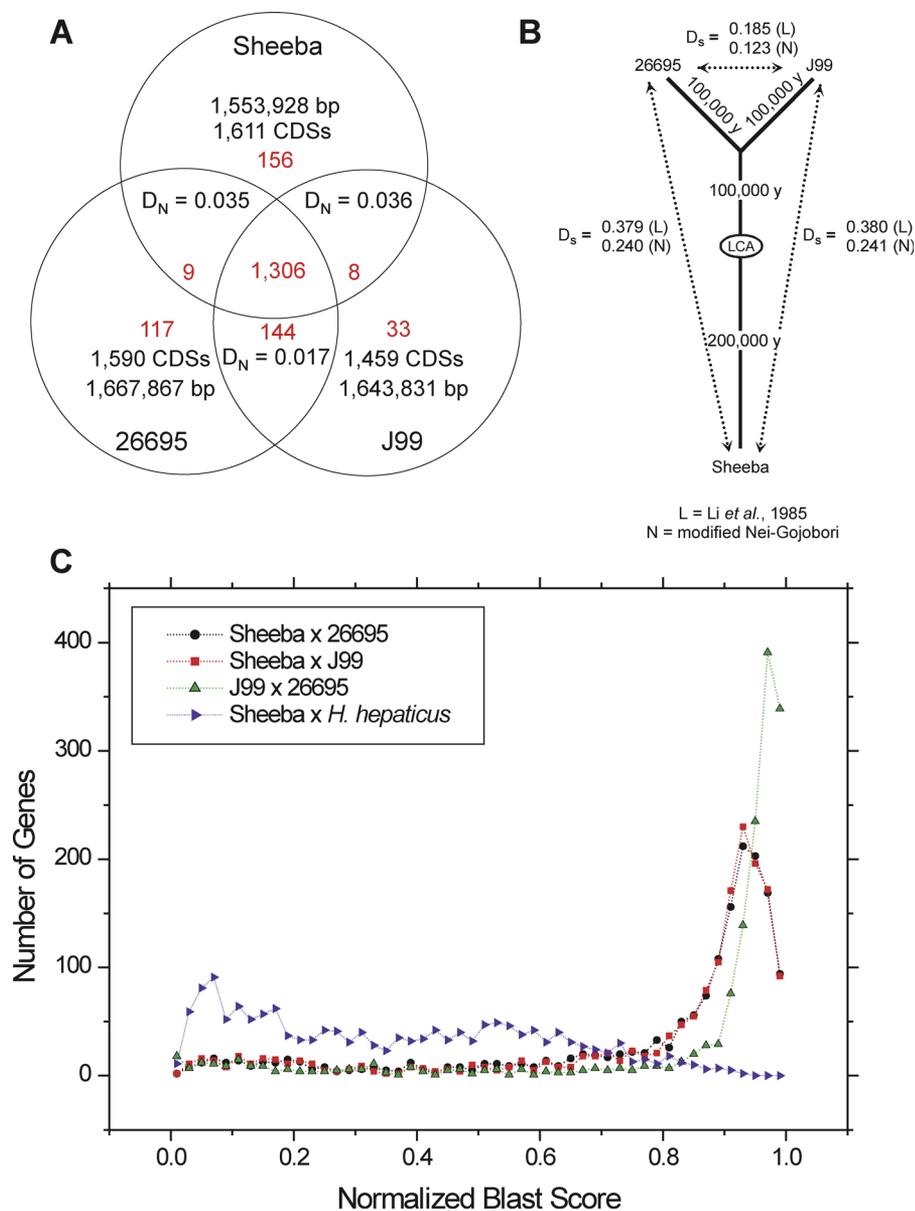


Figure 2. Similarities between the Genomes of *H. acinonychis* Sheeba and *H. pylori* 26695 and J99

(A) Venn diagram of genomic properties. Numbers in red within each arc represent numbers of genes, each of which may contain multiple CDSs if the corresponding gene is fragmented.

(B) Age calculations since a common ancestor (LCA) based on synonymous pair-wise distances for 612 conserved genes according to the methods of Li *et al.*, 1985 and the modified Nei-Gojobori method.

(C) Frequencies of normalized blast scores in pair-wise comparisons between Sheeba and three other genomes.

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transitions to transversions. Each method yielded different estimates but both methods indicated that D_s between Sheeba and either 26695 or J99 is approximately twice as high (2.0 ± 0.05) as D_s between 26695 and J99 (Figure 2B). This indicates that Sheeba diverged from the LCA two-fold as long ago as did 26695 and J99 from their last common ancestor. Thus, an estimate of the time of divergence between 26695 and J99 would automatically also provide an estimate of the divergence time for Sheeba from the LCA. Strain 26695 belongs to the hpEurope population of *H. pylori*, which is found throughout Europe and countries colonized by Europeans, while J99 belongs to hpAfrica1, which is found in Africa and countries affected by the slave trade [8,15]. The

accumulation of sequence differences between hpEurope and hpAfrica1 has probably occurred over the same time scale as divergence within modern humans from their LCA, or approximately 100,000 y (100 ky) [21,22] (range based on molecular data: 50–200 ky [23–26]). This interpretation implies that 200 ky (range 100–400 ky) has elapsed since Sheeba diverged from the LCA (Figure 2B; see Materials and Methods).

Genomic Differences between *H. acinonychis* and *H. pylori*

Having demonstrated that the core genomes of *H. pylori* and *H. acinonychis* are very similar, we now focus on genomic differences that might be relevant to a host jump. Gene

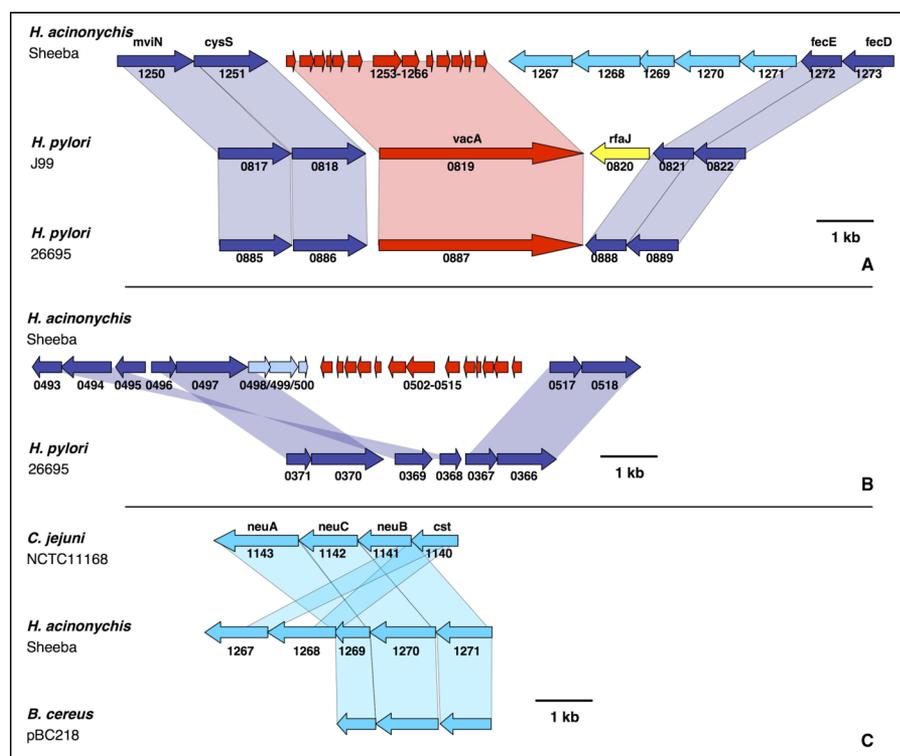


Figure 3. Gene Fragmentation, Duplication, and Import

(A) Fragmentation of the *vacA* (vacuolating cytotoxin) gene (red) into 13 fragments and import of *neuACB*, *cst*, *cst* (blue-green) within Sheeba. *neuACB* encode acylneuraminate cytidyltransferases and *cst* encodes a sialyltransferase.

(B) Translocation of a duplicate of the fragmented *vacA* gene to a different genomic location. The duplicated *vacA* gene (red) contains the same fragmentation pattern and differs by only one sequence polymorphism in 3,815 bp from that in part A, indicating that the duplication is recent and occurred after the fragmentation event. Next to the duplicated *vacA* gene are located three genes (light blue) that are unique to *H. acinonychis*.

(C) Homologies of the *neuACB*, *cst*, *cst* gene cluster from part A with syntenic clusters in *C. jejuni* NCTC11168 and the *B. cereus* virulence plasmid pBC218. DOI: 10.1371/journal.pgen.0020120.g003

products that become unimportant or even deleterious after a host jump might be lost or inactivated. Alternatively, a host jump may be triggered or facilitated by the acquisition of novel genes by horizontal gene transfer (HGT). In both cases, the absence or presence of such genes should be uniform within the newly evolved species, whereas genes that are variably present after a host jump probably reflect events that were not essential for that host jump because they arose subsequently. Therefore, we searched for genes that were specifically inactivated or novel in the genome of Sheeba versus 26695 and J99. To this end, when CDSs in one of the genomes were markedly shorter than their orthologs in the other genomes, the length of the longest orthologous CDS among the genomes was used to mark the boundaries of a “gene” and genes that contain one or more markedly shorter CDSs will be referred to as “fragmented.”

The frequency of fragmented genes clearly indicates that the host jump proceeded from primates to large felines. Scattered around the Sheeba genome (Table S2) are 255 CDSs derived from 92 fragmented genes. Gene fragmentation accounts for more predicted CDSs, less coding area, and smaller average CDS size within the Sheeba genome than within 26695 or J99 (Table 1). Gene fragmentation was due to frameshift mutations and stop codons and only one of the 255 fragmented CDSs was associated with the transposition of insertion elements (ISs). Conserved hypothetical proteins, whose relevance to a host jump is uncertain, are encoded by

45 fragmented genes. The other frequent classes (Figure S2) encode 12 outer membrane proteins (OMPs), 11 restriction or modification enzymes, eight transport systems, six transposases, three fucosyl transferases, and two VacA vacuolating cytotoxins, that arose by a duplication event after the *vacA* gene was shredded into 13 fragments (Figure 3).

The frequency of fragmented genes is particularly high in Sheeba compared to the *H. pylori* genomes, even among the 1,306 CDSs that are common to all three genomes (Table 2): almost three times as many Sheeba genes are fragmented as in 26695 and over ten times as many as in J99. Indeed, 45 genes are fragmented in Sheeba but intact in both 26695 and J99, whereas only one gene is fragmented in both 26695 and J99 but intact in Sheeba.

Table 2. Numbers of Fragmented Genes among 1,306 Common Genes in Sheeba, 26695, and J99

Comparison	Fragmented Genes				
	Genome 1	Genome 2	Genome 1	Genome 1 and 2	Genome 2
Sheeba	26695		54	7	21
Sheeba	J99		50	11	4
26695	J99		27	1	14

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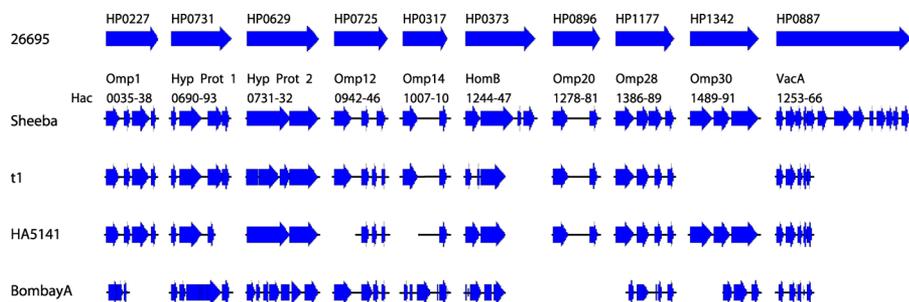


Figure 4. Fragmentation Patterns in Ten Genes among Three *H. acinonychis* Strains

Ten genes that are intact in 26695 but are fragmented in the Sheeba genome (subgroup B) were re-sequenced from strains t1 and HA5141 of subgroup B and BombayA of subgroup A. Black lines indicate sequenced fragments and thick blue arrows indicate CDSs of ≥ 140 bp. Designations at the top indicate CDS designations in 26695 whereas designations above the Sheeba sequences indicate both the protein name and the CDS designations in Sheeba (Hac0035, Hac0036, etc.).

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We tested whether fragmentation is uniform within *H. acinonychis* by re-sequencing ten fragmented genes from three additional strains of *H. acinonychis*, except for two PCR products that could not be amplified. All these sequences were fragmented (Figure 4), but they differed by single nucleotide polymorphisms and/or small deletions (Table S3). Two differing patterns of fragmentation were found that correspond to sub-groupings within *H. acinonychis* that were revealed by microarray analysis (see below). Thus, isolates of *H. acinonychis* from lions, tigers, and cheetahs all contain multiple fragmented genes that probably arose early after a host jump to large felines.

Similar to fragmented genes, the deletion of entire genes might possibly also have been associated with the host jump, especially because 144 genes are lacking in Sheeba that are present in both 26695 and J99 (Figure 2). However, according to DNA microarray analyses, hundreds of CDSs have been lost from at least some of 56 representative strains of *H. pylori* [15]. Thus, the absence of 144 genes within Sheeba versus 26695 and J99 need not reflect events that are relevant to or occurred soon after the host jump. Of the 1,150 genes that are uniformly present within *H. pylori*, only ten, encoding six hypothetical proteins, two OMPs (*omp6*, *omp19*) and other functions (*icfA*, *thiM*) were absent in all four strains of *H. acinonychis* that were tested [15]. Thus, if a loss of gene function accompanied the host jump, most such events are probably represented among the 92 fragmented genes in Sheeba.

Unique Genes and HGT

Was the host jump to large felines facilitated by the acquisition of novel genes via HGT? Sheeba possesses 156 CDSs that are absent in 26695 and J99 (Figure 2), many of which were possibly imported by HGT (Figure S3). Prophages are unknown in *H. pylori* but 41 of the imported CDSs in Sheeba are within two prophages, called prophage I (11.6 kb) and prophage II (28.4 kb) (Figure S1, Table S4). Multiple CDSs in prophage II are orthologs of genes within a prophage of *Campylobacter upsaliensis* RM3195 that was isolated from a patient suffering from Guillain-Barré syndrome [27]. Prophages have been implicated in the acquisition of virulence [28] and genes in prophages I and II might potentially be relevant to host adaptation and specificity. However, most of the CDSs in these prophages encode hypothetical proteins whose relevance to infection by *H. acinonychis* cannot currently be evaluated.

The flexible gene pool in Sheeba includes one or two copies each of three distinct ISs, with homologies to *IS605*, *IS606*, and *ISHp608*, respectively (Table 1). Sheeba also contains a 3,661 bp plasmid, pHac1 (Figure S4), with homologies to *H. pylori* plasmids pHPM8 [29] and pHel4 [30]. And three genes, *mccB*, *mccC*, and *repA*, that are present in *H. pylori* on plasmids pHPM8 and pHel4, form the “HacGI” integron that is flanked by two ISs within the Sheeba chromosome (*mccC* is intact but *mccB* and *repA* are fragmented).

We tested whether these and other unique CDSs in Sheeba were universal within and specific to *H. acinonychis* by hybridization of six strains of *H. acinonychis* and 21 representative strains of *H. pylori* against DNA microarrays containing 99 PCR products (Figure 5). Only 37 CDSs were universally present within *H. acinonychis* and lacking in all *H. pylori* (group I). Others (group III) were also found within some strains of *H. pylori* and are not specific to *H. acinonychis*. Group III probably corresponds to genes that were present in a common ancestor and were subsequently lost in some *H. pylori* strains such as J99 and 26695 [15]. Still other CDSs (group II), including most of the prophage genes, were present within one subgroup of *H. acinonychis* but were lacking from the second. Group II genes are also unlikely to have been essential for the host jump to large felines and probably represent lysogenization and HGT that first occurred after genetic differentiation into subgroups.

The 37 CDSs in group I were probably also acquired by HGT. Most of them encode hypothetical proteins or are related to plasmid genes; the possible relevance of such genes for a host jump remains uncertain. However, group I does include a five-gene cluster, consisting of *neuACB* followed by two *cst* genes (Figure 3), all of which are in the same orientation. These are syntenic to homologous clusters of genes on the virulence plasmid pBC218 of *Bacillus cereus* [31] and the chromosome of *Campylobacter jejuni* NCTC11168 [32,33]. In both *B. cereus* and *C. jejuni*, these genes have been implicated in interactions with the host’s immune response through sialic acid synthesis (*neuACB*, CMP-NANA synthetase, biosynthesis of N-acetyl-D-mannoseamine, NANA synthetase [34]) and transfer (*cst* , a bi-functional $-2,3\text{-}l\text{-}2,8\text{-}$ sialyltransferase [33]) to carbohydrates on the bacterial cell envelope. Group I also included one Omp, *mccB*, and three genes from prophage I, all of which might be relevant to the host jump.

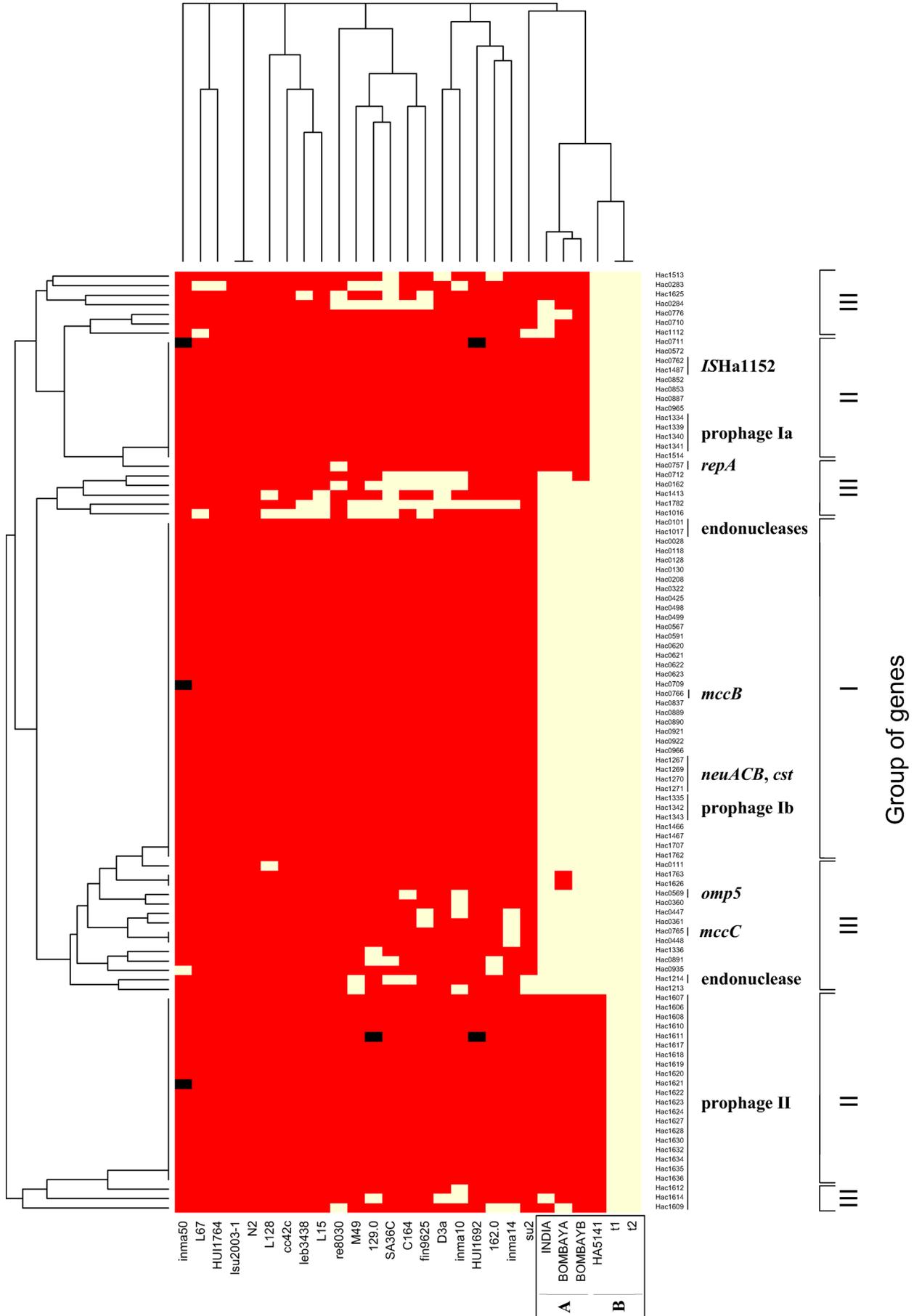


Figure 5. Hybridization of a DNA Microarray Chip Containing 99 PCR Products against Representative Strains of *H. acinonychis* and *H. pylori*

Six strains of *H. acinonychis* from subgroups A and B and 21 strains that represent the genetic diversity of *H. pylori* (Table S5) were tested for hybridization (yellow) or lack of hybridization (red) with 99 PCR products (Table S6) from genes that are present in Sheeba and lacking in 26695 and J99. The results were clustered according to genes (left) and strains (top). Genes that hybridize exclusively with all *H. acinonychis* strains are summarized as group I (right), genes hybridizing only with some *H. acinonychis* are in group II and genes hybridizing with some *H. pylori* are in group III. Where gene functions were attributed, they are indicated at the right and other genes encode hypothetical proteins. black, missing data.

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Synteny and Genomic Rearrangements

Large parts of the Sheeba genome are not only homologous to genes in 26695 and/or J99 (Figures 2 and S5) but are also arranged in syntenic order (Figures 6 and S6). Minor loss of synteny is due to the two prophages, ISs and plasmid-borne sequences that are specific to Sheeba (Table 1). Synteny is also disrupted by the absence of the 40 kb *cag* pathogenicity island (Figure S7), which apparently was acquired by *H. pylori* after the host jump to large felines that led to *H. acinonychis* [15].

The genomes of 26695 and J99 are largely colinear, with few exceptions [17], but only little colinearity is apparent in comparisons of *H. pylori* with the distantly related species *H. hepaticus*, *Wolinella succinogenes*, and *C. jejuni* [35]. Similarly, little obvious colinearity was detected when the Sheeba genome was compared to these distantly related species (Figures 6 and S6). In contrast, large genomic stretches were colinear between Sheeba and 26695 or J99, but these have been interrupted by at least 91 distinct genomic rearrangements.

Are these genomic rearrangements relevant to and did they possibly contribute to genomic adaptation during the host jump? In this case, one might expect to find a higher frequency of fragmented CDSs flanking the genomic rearrangements than the global frequency of fragmented CDSs within Sheeba. Alternatively, some or all of the rearrangements might have occurred within the lineage leading to *H. pylori* after the host jump. Most of the genomic rearrangements were flanked by intact housekeeping or hypothetical genes (Table 3) and we could not discern any obvious features of these flanking genes that could have provided an evolutionary or mechanistic basis for the rearrangements. However, 20 of the 182 flanking genes were fragmented within *H. acinonychis*, mostly corresponding to OMPs or restriction/modification enzymes. These genomic rearrangements may then have provided a mechanism for those instances of fragmentation and might be related to the host jump. Other genomic events such as duplication and gene conversion may also have resulted in several novel OMPs of the Hop and Hor family (Figure S8), which seem to represent mosaics between OMPs that are present in 26695 and/or J99. Thus, genomic rearrangements may have been an important mechanism for the inactivation of OMPs and their structural diversity. However, the overall frequency of fragmented CDSs flanking genomic rearrangements (11.6%) is lower than the frequency of fragmented CDSs within the Sheeba genome (16%), indicating that genomic rearrangements are not a major cause of gene fragmentation. Thus, many of the rearrangements are probably irrelevant to the host jump and may have occurred within the *H. pylori* lineage after its separation from the lineage leading to *H. acinonychis*.

Discussion

Evolution of *H. acinonychis*

Based on its ability to infect laboratory and captive animals, *H. pylori* infection can be thought of as an

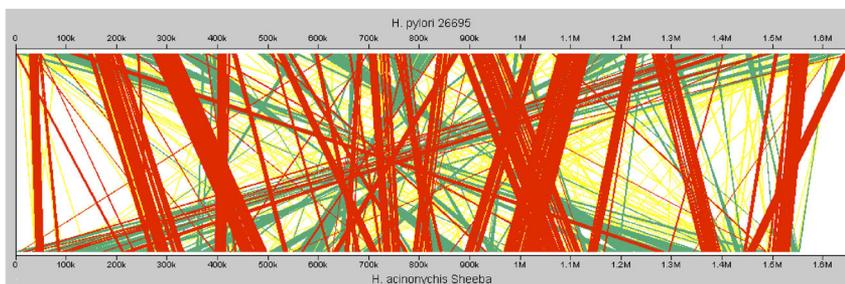
anthroponosis, whereby human infections are a source of disease for animals. We propose that just such an anthroponotic infection resulted in a host jump followed by speciation of *H. acinonychis*. According to this interpretation, *H. acinonychis* probably arose after a large feline became infected by eating an early human whose stomach was colonized by *H. pylori*. Such transmission events will only rarely result in host jumps, due to inefficient transmission of the pathogen between individuals of the novel host [36]. However, the particular transmission event that resulted in *H. acinonychis* was successful because the bacteria had imported novel genes and adapted to their feline host by gene inactivation. With time, the predecessor of *H. acinonychis* became so strongly adapted to infection of large felines that *H. acinonychis* now colonizes mice only poorly [5], and is not known to naturally infect any host species other than large felines. Random genetic drift after the host jump has also resulted in sub-differentiation into two genetic sub-groups within this species.

A single host jump has been the source of all modern isolates of *H. acinonychis*. Otherwise, gene fragmentation patterns and the presence of imported genes would not have been as uniform as was observed. This conclusion contrasts with HIV viruses, where multiple host jumps from distinct species of chimpanzees have been inferred from phylogenetic analyses [37]. Similarly, multiple host jumps to humans are thought to have occurred with dengue viruses [38] and the possibility that additional host jumps from avians to humans may result in new influenza pandemics has been a recent cause of concern [36]. If *H. pylori* jumped from humans or other primates to large felines on multiple occasions, those bacteria are not included within *H. acinonychis*, which is monophyletic, but might instead be found among the non-cultivable *Helicobacter* that have been described in the stomachs of large felines [12].

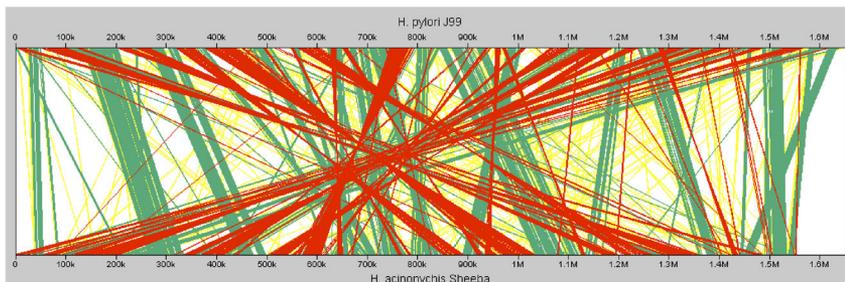
When Did It Happen?

Several bacterial pathogens are thought to have evolved recently. These include *Yersinia pestis*, a clone of *Yersinia pseudotuberculosis* which has differentiated within the last 20 ky [39], *Mycobacterium tuberculosis*, a clone of smooth tubercle bacilli that diversified over approximately the same time period [40,41] and *Salmonella enterica* Typhi, a clone of *S. enterica* that is thought to be approximately 50 ky old [42]. All of these dates are based on a molecular clock rate (5×10^{-9} per year) for synonymous mutations that is calibrated by the 140 My since *Escherichia coli* and *S. enterica* shared a common ancestor [43], an estimate that is now almost 20 y old. Unfortunately, the synonymous molecular clock rate is not necessarily constant for different bacterial species and its ratio to the 16S rRNA clock rate can vary by an order of magnitude [44]. The currently most reliable bacterial synonymous clock rate is probably that for the obligate intracellular bacteria, *Buchnera aphidicola* (8×10^{-9}) [44], which was calibrated by the dates of separation of their insect hosts

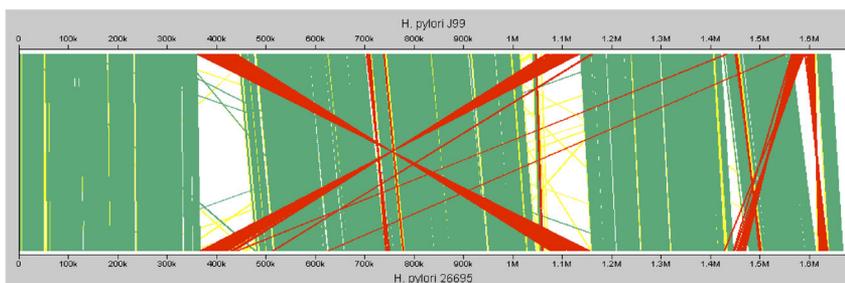
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 1334 pairs
 Total 349 segments
 66 direct
 70 opposite
 213 singletons



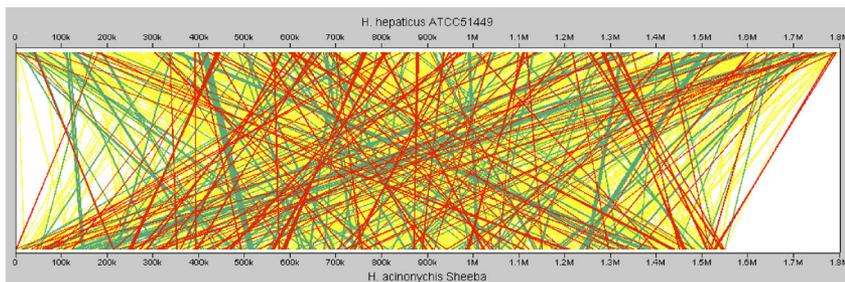
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 60 opposite
 207 singletons



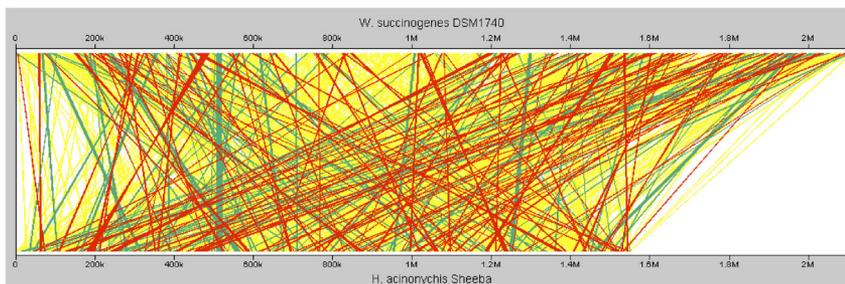
C
 1402 pairs
 Total 61 segments
 39 direct
 11 opposite
 11 singletons



D
 952 pairs
 Total 673 segments
 103 direct
 79 opposite
 491 singletons



E
 873 pairs
 Total 600 segments
 75 direct
 93 opposite
 432 singletons



F
 845 pairs
 Total 670 segments
 73 direct
 75 opposite
 522 singletons

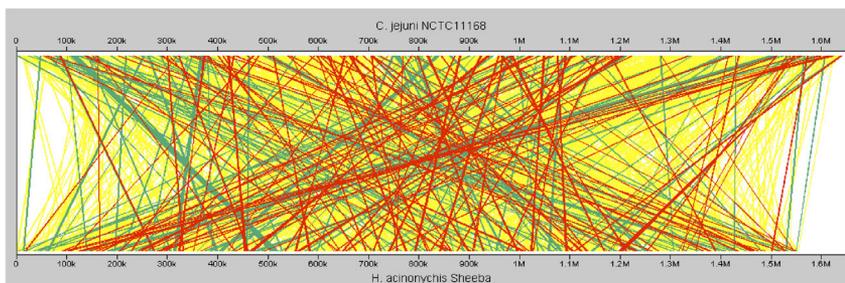


Figure 6. Genomic Rearrangements in Pair-Wise Genomic Comparisons between Sheeba and other *Helicobacteraceae* [16–18,85] or Campylobacteraceae [86] (A–B, D–F) or between 26695 and J99 (C)

Stretches consisting of two or more syntenic orthologs are indicated in green for orthologs in the same orientation relative to the origin and in red for orthologs in inverted orientation. Single orthologous CDSs are indicated in yellow. Ortholog matches were plotted using CGViz (<http://www-ab.informatik.uni-tuebingen.de/software/cgviz>).

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based on fossil data. Not only are all other clock rate estimates almost certainly much less reliable, but it is also not clear whether clock rates based on times of separations of 50–150 My can even be extrapolated to much more recent events.

Based on synonymous differences over 612 conserved genes that are present in all three genomes, *H. acinonychis* diverged from *H. pylori* twice as long ago as did hpEurope from hpAfrica1. Our interpretation that hpEurope separated from hpAfrica1 100 ky (range: 50–200 ky) ago then extrapolates to an estimate of 200 ky (range: 100–400 ky) for the age of *H. acinonychis*. The time of separation between hpEurope and hpAfrica1 was equated with the time of separation between modern Africans and Europeans [21–26], which is justified by the close correlations between genetic diversity in *H. pylori* and ancient human migrations [8]. However, the time of separation between Africans and Europeans is not known with certainty, and may well be modified as additional evidence becomes available. Furthermore, rather than using the distance between one representative of each of the bacterial populations, it would have been preferable to base time estimates on genetic distances between the entire hpEurope and hpAfrica1 populations, after correcting for admixture and drift. It would also have been desirable to calibrate the clock rates after incorporating additional milestones in human history that can be associated with separation into distinct bacterial populations. Because neither of these improvements is currently feasible, we calculated an upper limit on the time since the most recent common ancestor of *H. pylori* plus *H. acinonychis* based on the 3,406-bp concatenated sequences in Figure 1. The Bayesian Skyline coalescent model [45] that is incorporated in BEAST indicated a maximal age of 395 ky (95% confidence limits: 347–447 ky), which supports our initial estimate of the age of *H. acinonychis*. However, we note that coalescent analysis is inappropriate for recombining sequences, such as in *H. pylori*,

and therefore feel that these upper limits should be treated with caution.

When taken at face value, our estimate of 100 ky for the separation of hpEurope from hpAfrica1 indicates that the synonymous molecular clock rate is 6.2×10^{-7} [19] to 9.2×10^{-7} [20], depending on the algorithm used to calculate synonymous distances, values that are 50-fold higher than the synonymous clock rate for *E. coli* – *S. enterica*. Higher mutation rates in *H. pylori* than within enteric bacteria are not necessarily surprising: the MutS mismatch repair system that reduces the mutation frequency in *E. coli* is not functional in *H. pylori* [46,47] and unlike *E. coli*, where mutators in mismatch repair are rare among natural isolates [48], many natural isolates of *H. pylori* have high mutation rates [47]. These considerations also support the inference by Ochman et al. [44] that different bacterial species may possess very different synonymous clock rates.

Genomic Changes Related to the Host Jump

Comparative genomic analyses of several pathogenic bacteria have demonstrated that niche changes and host specialization can be accompanied by an explosive multiplication of insertion elements plus an accumulation of frameshift mutations and stop codons, which result in genome degradation [49–52]. However, in almost all cases, it is very difficult to distinguish genomic differences that accompanied the niche change from events that reflect subsequent microevolution within a species. For example, microevolution within *Y. pestis* has resulted in genomic rearrangements [53] that are extensive even in comparison with the genomic differences between *Y. pestis* and *Y. pseudotuberculosis* [51]. Similarly, major differences in genomic content were found by comparisons of multiple strains within *E. coli* [54] and *Streptococcus agalactiae* [55]. Given these observations, we attempted to identify genomic changes related to the host jump by focusing on differences between *H. pylori* and *H. acinonychis* that were unique to and ubiquitous throughout the latter. We therefore ignored genes that are specifically lacking in the Sheeba genome relative to 26695 and J99, because almost all these genes are also lacking in some strains of *H. pylori* [15]. Similarly, we ignored prophages and other genes that are present in some isolates of *H. acinonychis* but lacking in others. Such genetic changes may be interesting for an understanding of microevolution within *H. acinonychis*, but they are almost certainly unrelated to a host jump that only happened once. Finally, many of the genomic rearrangements that distinguish *H. pylori* from *H. acinonychis* may have occurred during the evolution of *H. pylori* 100–200 ky ago, before the separation of hpEurope from hpAfrica1, rather than within *H. acinonychis*. Instead, our focus on the host jump identified a limited number of genes that fulfilled our criteria: similar to *Chlamydomophila abortus* [52], genes in Sheeba were fragmented due to frameshifts and/or stop codons (but not insertion elements as in *Bordetella pertussis* [50] or *Y. pestis* strain CO92 [56]) or had been imported from

Table 3. Genes in the Sheeba Genome Flanking 91 Genomic Rearrangements

Gene Function (Number)	Gene Status (Number)	
	Intact	Fragmented
Housekeeping	76	
Hypothetical	58	7
OMP	11	6
R/M	6	4
tRNA	5	
Ribosomal protein	3	
Fucosyl-transferase		3
16S rRNA	2	
23S rRNA	1	
Total (182)	162	20

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unrelated bacteria by HGT. A small fraction of the fragmented genes were also associated with genomic rearrangements. The frequency of fragmented genes in Sheeba is unusually high: 16% of the CDSs in the Sheeba genome were derived from fragmented genes (Table 1), whereas lower frequencies were observed in *B. pertussis* (9.4%), *Y. pestis* (3.7%), and *Cp. abortus* (3.0%), which are also thought to have undergone gene fragmentation due to a change in niche specificity.

Most of the genes putatively associated with the host jump lacked known homologs (“hypothetical proteins”). It is quite likely that some of these are relevant to niche adaptation, as documented on several occasions in other microbes [57]. However, it was particularly striking that genes that are potentially linked to changes in the bacterial cell surface were so prominent among the fragmented and imported genes, suggesting that changes of the bacterial cell surface were essential for the success of the host jump to large felines. *H. acinonychis* contains an imported *neuACB,cst,cst* cluster of genes that can potentially sialylate the bacterial cell surface polysaccharides and evade host immune defenses. Sialylation of lipopolysaccharide renders *Neisseria gonorrhoeae* resistant to complement-mediated phagocytosis [58] and has been invoked as a virulence and host-specificity determinant for pathogenic *Neisseria* and *Haemophilus influenzae* [59]. Similarly, 12 OMPs are fragmented and two others are specifically lacking within the Sheeba genome. Host immunity drives sequence variation and lack of expression of OMPs in *Neisseria meningitidis* [60,61] and variable expression of OMP lectins is important for the host-specific adaptation of *H. pylori* [62,63]. Thus, fragmentation and novel combinations of OMPs in *H. acinonychis* may have eliminated cell-surface targets for immune defenses and provided novel adhesins that were important for the adaptation to its new host.

Subsequent Genomic Changes

Once adaptation to its initial host had succeeded, *H. acinonychis* spread globally within and between distinct feline species. Today, *H. acinonychis* can be isolated from cheetahs, lions and tigers, who differentiated from their LCA 11 million y ago [64], long before the host jump. During its global spread, two subgroups evolved within *H. acinonychis* with distinct gene content (Figure 5), sequence polymorphisms [5,15] and fragmentation patterns [5] (Figure 4). Possibly, the two subgroups are specific for different species of large felines in nature, but this question cannot currently be resolved because all currently available isolates of *H. acinonychis* are from captive animals. Subgroup B, including Sheeba, carries two prophages that are largely absent from subgroup A. We note that the acquisition of prophages and HGT may have been facilitated by changes in the complement of restriction/modification enzyme systems that can defend against the acquisition of foreign DNA. Due to extensive gene fragmentation, Sheeba contains only six potentially functional restriction/modification enzyme systems, consisting of three orthologs of the eleven that are found in *H. pylori* 26695 [16] plus three novel restriction endonucleases (CDSs Hac0101, Hac1017, Hac1214).

Concluding Remarks

Zoonoses resulting in potential human epidemics have recently received considerable attention [36,37,65]. Possibly

due to our anthropomorphic bias, the contrary phenomenon of an anthroponosis resulting in a host jump to an animal has received much less attention. The natural infection of armadillos by *Mycobacterium leprae* probably represents one such example of an anthroponotic host jump [66] and the infection of large felines by *H. acinonychis* provides a second. In both cases, isolates from humans strongly resemble those from the novel host, indicating that only very few changes may be needed for such a host jump to succeed. DNA can readily be transferred by transformation between *H. pylori* and *H. acinonychis* [67], and *H. acinonychis* can infect laboratory animals [5]. The genomic sequence described here now provides the prerequisites that can allow the dissection of the molecular basis of host jumps in bacterial pathogens.

Materials and Methods

Bacterial strains. Bacterial strains are listed in Table S5. Strain Sheeba was isolated from a biopsy of a Russian circus lion with gastroenteritis. It was chosen for sequencing because its genome was the largest among the seven isolates of *H. acinonychis* in Table S5 according to pulsed-field gel electrophoresis.

Genome sequencing and annotation. Genomic DNA from *H. acinonychis* Sheeba was isolated using the Qiagen Genomic DNA Kit (Qiagen, Hilden, Germany). Shotgun DNA libraries with insert sizes of 1–2 kb, 3–5 kb (TOPO Shotgun Subcloning Kit, Invitrogen, United States) and 40 kb (EpiFOS, Epicentre, Madison, United States) were end-sequenced to 7-fold coverage and remaining gaps were closed by direct sequencing, using genomic DNA as a template. The final sequencing error rate was estimated to be $<2 \times 10^{-6}$ using the PHRED/PHRAP/CONSED software package [68–71].

Curation and annotation of the genome was performed using the annotation package GENDB [72]. Protein coding genes were predicted with the programs GLIMMER [73] and CRITICA [74], which are integrated into the GenDB package, and annotated on the basis of similarity searches against public databases using BLASTX and manual curation. The remaining ORFs were post-processed with RBSFINDER [75], which identifies ribosomal binding sites. Functional classification to COG categories [76] was performed with INTERPRO and metabolic pathways were constructed with reference to KEGG [77]. The origin of DNA replication was localized by identification of known motifs of the primosome as well as the GC skew of the genome. tRNA and rRNA genes were identified by tRNA-SCAN and BLASTN, respectively.

All predicted CDSs of the three species were tested in reciprocal pair-wise BLASTP comparisons using a cut-off e-value of $\leq 1 \times 10^{-15}$, followed by manual editing. Fragmented, paralogous or duplicated CDSs were each assigned a distinct CDS number (Hac0001, Hac0002, etc.). As a result, Sheeba contains more CDSs than the total numbers of shared and species specific genes in Figure 2. A list of the unique genes within Sheeba can be found in Table S4.

Sequence homologies between genomes and age calculations. BLASTP analyses were performed for each translated CDS within the Sheeba genome against itself and against a query genome. The normalized blast score is the ratio of the score for the query genome divided by the self score.

In order to calculate pair-wise genetic distances for age calculations, we compiled a list of 1,058 unfragmented orthologs with reciprocal best FASTA hits of $>80\%$ homology that were present within Sheeba, 26695 and J99. 612 of these orthologs contained no internal gaps and were used to calculate synonymous differences. After excluding the start and stop codons, the number of potential synonymous sites and non-synonymous sites were calculated separately according to the algorithms of Li et al. [19] and the modified Nei-Gojobori algorithm, which accounts for R, the ratio of transitions to transversions (see pages 57–58 of Nei and Kumar [20]) (Table S1). The number of synonymous changes in pair-wise comparisons was calculated as described on pages 52–54 of Nei and Kumar. Synonymous distances were calculated after applying the Jukes and Cantor correction for forward and reverse mutations [78] by the formula

$$D_S = -(3/4) \times \ln(1 - \frac{4}{3}p) \quad (1)$$

where p is the observed proportion of synonymous changes and D_S is the corrected synonymous distance.

In order to calculate the time since a last common ancestor, we calibrated the synonymous molecular clock rate on the basis that genomes 26695 and J99 belong to the hpEurope and hpAfrica1 populations, respectively, whose human hosts diverged ~100 ky ago [21,22], for a total separation time of ~200 ky. This calibration results in a synonymous clock rate (rate) of 9.23×10^{-7} [19] to 6.13×10^{-7} (modified Nei-Gojobori) per year. Assuming a constant clock rate, D_S between Sheeba and 26695 or J99 can be used to calculate the time since their last common ancestor (*age*) as

$$age = \frac{D_S}{2 * rate} \quad (2)$$

age is $205,471 \pm 271$ y according to Li et al. [19] and $196,169 \pm 244$ y according to the modified Nei-Gojobori method, for a mean value of $200,820 \pm 258$.

DNA microarray analyses. Microarrays were constructed and analyzed as described [15], with the following differences. The microarray chip contained six copies of 99 unique CDSs plus seven housekeeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, and *yphC*), amplified from two strains each as a positive control, and printing buffer as a negative control. PCR products corresponding to at least 80% of the target CDS were amplified using specific amplification primers that were designed using Primer3 [79] (Table S6). The microarrays were hybridized against fluorescently labeled (Cy3/Cy5) genomic DNA from six strains of *H. acinonychis* and 21 strains that represent the global diversity of *H. pylori* (Table S5) with dye-swapping. Local background values were subtracted from each spot, the intensities for each fluorescent signal were normalized to the geometric mean intensities of the positive controls, and the geometric means of the intensities of the six copies per chip were calculated. Individual fluorescence values that deviated from the geometric mean by more than three-fold were excluded from the analysis and only geometric means based on at least two values were retained. These were then normalized to the geometric means of data from the housekeeping genes to yield a normalized ratio. The normalized means from the two dye-swap experiments were averaged, except in those few cases where only one normalized mean was available. Experiments in which both normalized means were lacking were treated as missing data. A histogram of the normalized ratios was used to determine a cut-off value of 0.4, above which hybridization with CDSs was scored as being present. This cut-off value reflects a local minimum within the histograms as well as the results of comparisons of the microarray data to manual inspection of the (unpublished data) genome sequence of *H. pylori* strain 162.0. Clustering of genes and strains was performed using the “heatmap.2” function of the package “gplots”, that is implemented in R [80].

Re-sequencing of ten fragmented genes. The universality of fragmentation of genes within Sheeba was tested by re-sequencing ten genes from three additional isolates of *H. acinonychis* from PCR products that were amplified with the oligonucleotide primers in Table S7. The fragmentation patterns in Figure 4 are described in detail in Table S3. Strain BombayA (subgroup A) showed fragmentation patterns that differed extensively from those of Sheeba (subgroup B) while the fragmentation patterns of strains t1 and HA5141 (subgroup B) were similar to those of Sheeba.

Phylogenetic analyses. The sequence data in Figure 1, consisting of concatenated fragments of the *atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, and *yphC* housekeeping genes (<http://pubmlst.org/helicobacter>) from 62 strains, have been described by Gressmann et al. [15]. MODELTEST 1.05 [81] was used to determine that the optimal evolutionary model was GTR+G+I (parameters: gamma [shape]=0.52; I [PInvar]=0.62), which was then used to construct a neighbor-joining tree in PAUP* [82].

The maximal age of the most recent common ancestor (t_{mrc}) of *H. pylori* plus *H. acinonychis* was calculated on the same data from the 62 strains using BEAST V1.3 (<http://evolve.zoo.ox.ac.uk/beast>) after 10 million Markov chain iterations with sampling at intervals of 1,000. Similar results were obtained with concatenated sequences of all seven housekeeping gene fragments and of only the *atpA*, *efp*, *mutY*, and *ppa* genes. Similar results were also obtained when the t_{mrc} was calculated including *C. jejuni* as an outgroup for concatenated sequences of the latter four gene fragments. It was not possible to test concatenates of more than four housekeeping genes with an outgroup because orthologs of *trpC*, *ureI*, and *yphC* are lacking in *H. hepaticus*, *W. succinogenes*, and *C. jejuni*. Finally, comparisons of the step-wise (“constant”) and linear population growth models also yielded in significantly different log likelihoods for the constant model and comparable results were obtained after specifying either three or ten populations. The results presented here are based on three

populations, the linear growth model, seven housekeeping genes, no outgroup and a molecular clock rate of 2.32×10^{-7} , which was calculated as above on the basis of all polymorphisms between 26695 and J99 in Table S1.

Supporting Information

Figure S1. Circular Representation of the *H. acinonychis* Sheeba Genome

Circular wheels from outside in: 1,2) CDSs on the plus (wheel 1) and minus (2) strands, colored according to COG classifications (see Figure S2); 3) fragmented genes indicated in red; 4) genes that are present in Sheeba but absent in both 26695 and J99. Dark blue, genes with database orthologs (Sept. 2005); light blue, genes without significant database homology; 5) genes associated with genomic mobility: orange = prophages, yellow = plasmid-associated, green = IS elements, light orange = restriction/modification; 6) GC plot, showing that genes in wheels 3–5 often deviate in GC content; 7) cumulative GC skew, (range: white [minimum] to black [maximum]), calculated with GENSKW (<http://mips.gsf.de/services/analysis/genkw>), which was used to show that the origin of replication is near *dnaA*; 8) 36 tRNA genes (brown), two 16S rRNA, and two 23S-5S rRNA ribosomal regions (orange) are indicated by arrowheads.

Found at DOI: 10.1371/journal.pgen.0020120.sg001 (2.3 MB EPS).

Figure S2. Functional Classification of the Complete Genome Inventory of CDSs (A) and Fragmented Genes (B) of *H. acinonychis* Sheeba

The pie charts show the frequency of CDSs by COG category [76], except for 39% of the CDSs that are indicated as “No hits” because hit levels were $<1 \times e^{-15}$ or they matched COG entries that were not yet assigned to a COG category. (A) All CDSs within the Sheeba genome were analyzed, including CDSs that were assigned to a fragmented gene. (B) Fragmented genes after reconstruction by comparisons with 26695 and/or J99. Eight of 31 genes within the “No hits” category represent OMPs, which are not assigned to a distinct COG category.

Found at DOI: 10.1371/journal.pgen.0020120.sg002 (41 KB PDF).

Figure S3. Taxonomic Sources of Closest Orthologs (A), Neighboring Mobile Elements (B) and Predicted Functions (C) of 93 Unique CDSs in Sheeba

The following observations apply to 93 CDSs with database orthologs, and 63 other CDSs encoding hypothetical proteins without database orthologs were excluded. For most CDSs, the best Blast hits were to orthologs within the ϵ -Proteobacteria (A). Most unique CDSs are located next to prophages, plasmid-borne genes or IS elements (B), suggesting that they reflect HGT. However, the “core chromosome” genes neighboring 18 CDSs are homologous to genes in *C. upsaliensis* RM3195, *C. jejuni* NCTC1168, and *H. pylori* 99515 and might have been inherited by vertical descent.

Found at DOI: 10.1371/journal.pgen.0020120.sg003 (44 KB PDF).

Figure S4. The pHac1 Plasmid

Six open reading frames on the plus (outer wheel, facing out) and minus (facing in) strands are color-coded according to COG classification as described in Figure S2. The gene *repA* (pHac06) is homologous to plasmid genes that are responsible for replication initiation. The N-terminal portion of *repA* is also homologous to Hac0757 on the Sheeba chromosome, which is located within the HacGI integron. Genes pHac01 and pHac02 are orthologs of *jhp0828* and *jhp0825*, respectively, that flank an *IS606* transposase (*jhp0826–7*) within the J99 chromosome. No database orthologs were found for three hypothetical genes (pHac03–05). The inner wheel shows cumulative GC skew, with color coding as in Figure S1. The average GC content of plasmid pHac1 is 34.6% versus 38.2% for the core chromosome. Plasmid pHac1 possesses homologies with the *H. pylori* plasmids pHPM8 (7.8 kb) and pHel4 (11.0 kb), including a perfect 22 bp repeat region upstream of *repA* gene, which is characteristic of theta plasmids [30,83].

Found at DOI: 10.1371/journal.pgen.0020120.sg004 (816 KB EPS).

Figure S5. Relatedness of Orthologous Genes between Sheeba and Other Genomes

(A and B) and (D–G) show normalized blast scores of individual CDSs between Sheeba and other genomes, sorted by the CDS order within Sheeba, within which features of particular interest are highlighted

by colors (see color code at the bottom). Cumulative normalized blast scores, sorted in descending order, are shown in (H). The normalized blast scores relative to *H. pylori* genomes in (A and B) were high for most CDSs, e.g. the highly conserved F0F1 ATP synthase or ribosomal proteins involved in translation. CDSs in (A and B) with low normalized blast scores are unique to Sheeba, and were attributed to HGT. These include the *neuACB-cst* cluster, two prophages, plasmid-associated genes and restriction and modification enzymes. Fragmentation within certain genes, e.g., *vacA*, was associated with a series of CDSs with intermediate normalized blast scores. Note that normalized blast scores against other Campylobacterales (D–F) were lower and against the unrelated *Escherichia coli* (G) were much lower than in comparisons with *H. pylori* (A and B). (C) is similar to (A), except that it shows a comparison between two *H. pylori* genomes and that most of the normalized Blast scores are high. Two arrows in (C) indicate hypothetical proteins flanked by the insertion sequence elements IS605, which correspond to regions 1 (left) and 3 of the five distinct G + C regions in 26695 [16].

Found at DOI: 10.1371/journal.pgen.0020120.sg005 (3.7 MB PPT).

Figure S6. Gene Order and Colinearity in *Helicobacteraceae* and *Campylobacteraceae* Genomes

CDSs in genomic order were tested for colinearity between pairs of genomes, as indicated. Each point represents a matching pair of orthologs with an e-value of $<1 \times e^{-15}$ (A–F) or the maximal unique match ("MUM") (G–L) according to MUMmer [84] using 20 bp as the minimum length of a MUM. A summary of the genes flanking breakpoints in colinearity between Sheeba and 26695 or J99 is presented in Table 3. A quantitative co-linearity factor was calculated from the genomic positions (x and y coordinates) of each ortholog pair relative to O, the number of CDSs in the target genome, as follows. For each pair of neighbouring ORFs on the query genome (x_i, x_{i+1}), the position of the corresponding orthologs on the target genome (y_i, y_{i+1}) was used to calculate $D = \text{Min}(|y_{i+1} - y_i|, O - |y_{i+1} - y_i|)$. The colinearity factor $C = \Sigma D/O$. These calculations yielded values for C of 18 for *H. pylori* J99 versus 26695, 41–45 for Sheeba versus 26695 or J99 and 204–238 for Sheeba versus *H. hepaticus* [18], *W. succinogenes* [85] or *C. jejuni* [86].

Found at DOI: 10.1371/journal.pgen.0020120.sg006 (863 KB PDF).

Figure S7. Genomic Content of the Region Flanking the *cagPAI* in *H. pylori* 26695 and J99 versus *H. acinonychis* Sheeba

The *cagPAI* genes of *H. pylori* 26695 and J99 (red) are flanked by pairs of 31 bp repeats [87], as indicated by diamonds. The *cagPAI* region between the repeats is lacking in the Sheeba genome and only one of the repeats is present. The region flanking these repeats is syntenic in all three genomes, as indicated by color coding but has suffered some rearrangements. As part of these rearrangements, the syntenic cluster in yellow has been inverted and transposed downstream of the green cluster and additional sequences have been inserted into the grey region of Sheeba, resulting in a cluster of fragmented genes between Hac0887 and Hac0894, which shows a partial homology to HP0548 in 26695 and the region between jhp0495 and jhp0496 in J99.

Found at DOI: 10.1371/journal.pgen.0020120.sg007 (74 KB PPT).

Figure S8. Phylogenetic Tree of OMPs from the Hop and Hor Families in *H. acinonychis* Sheeba and *H. pylori* 26695 and J99

Omps of the Hop- and Hor- families [88] were assigned sequential numbers based on their genomic positions within 26695 (genes labelled HPxxxx) and J99 (genes labelled jhpxxxx) [16,17], except for HP1066 (HorD), which was designated OmpXX. We assigned independent, sequential numbers to OMPs within Sheeba based on their genomic locations within its genome; these numbers differ from those of orthologs in the *H. pylori* genomes due to genomic rearrangements. Orthologs between OMPs in the three genomes were determined by phylogenetic clustering of predicted proteins.

References

- Suerbaum S, Michetti P (2002) *Helicobacter pylori* infection. *New England J Med* 347: 1175–1186.
- Marshall BJ, Warren JR (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1: 1311–1315.
- Solnick JV, Schauer DB (2001) Emergence of diverse *Helicobacter* species in the pathogenesis of gastric and enterohepatic diseases. *Clin Microbiol Rev* 14: 59–97.
- Kersulyte D, Mukhopadhyay AK, Velapatino B, Su W, Pan Z, et al. (2000) Differences in genotypes of *Helicobacter pylori* from different human populations. *J Bacteriol* 182: 3210–3218.

Where pseudo-genes existed due to fragmentation, the clustering was based on reconstructed genes designated Hacxxxxrc for Sheeba and HPxxxxrc (Omp08, Omp03) for 26695. All Sheeba OMP genes have close orthologs within 26695 and/or J99, except for Omps 6 (Hac0713rc), 7 (Hac0751rc), and 8 (Hac0826) and all OMP genes within 26695 or J99 possess orthologs in Sheeba, except for Omps 1 (HP0009), 2 (HP0025), 6 (HP0229), 9 (HP0317), 13 (HP0638), 19 (HP0896), and 28 (HP1243). Of the Sheeba Omps that were reconstructed from gene fragments, seven (Omps 1, 12, 14, 20, 28, 30, 31) are of the Hop family, one (Omp18) is of the Hor family, and two (Omps 6, 7) are distantly related to both families. Dotted lines represent truncated numbers of nucleotide substitutions.

Found at DOI: 10.1371/journal.pgen.0020120.sg008 (46 KB PDF).

Table S1. Synonymous and Non-Synonymous Distances in Pair-Wise Comparisons of 612 Conserved Genes among Three *Helicobacter* Genomes

Found at DOI: 10.1371/journal.pgen.0020120.st001 (29 KB DOC).

Table S2. Fragmented Genes in Sheeba and Their Orthologs in 26695 and/or J99

Found at DOI: 10.1371/journal.pgen.0020120.st002 (192 KB PDF).

Table S3. Sequence Differences of Ten Fragmented Genes between Sheeba and Three Strains of *H. acinonychis*

Found at DOI: 10.1371/journal.pgen.0020120.st003 (67 KB PDF).

Table S4. Unique Genes in Sheeba

Found at DOI: 10.1371/journal.pgen.0020120.st004 (136 KB PDF).

Table S5. Sources of *H. acinonychis* and *H. pylori*

Found at DOI: 10.1371/journal.pgen.0020120.st005 (56 KB DOC).

Table S6. Primer Pairs for Microarrays

Found at DOI: 10.1371/journal.pgen.0020120.st006 (43 KB PDF).

Table S7. PCR-Primer Pairs

Found at DOI: 10.1371/journal.pgen.0020120.st007 (86 KB DOC).

Accession Numbers

The EMBL Nucleotide Sequence Database (<http://www.ebi.ac.uk/embl>) accession numbers used in this study include: Sheeba genome (AM260522), pHac1 plasmid (AM260523), and the sequences referred to in Figure 4 and Table S3, AM285650–77.

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Author contributions. MA and SCS conceived and designed the experiments. ME, CB, BL, CL, HK, GM, and HG performed the experiments. ME, CB, BL, GR, MA, and SCS analyzed the data. ME, CB, GR, GM, and SCS contributed reagents/materials/analysis tools. MA wrote the paper with the assistance of ME, CB, SCS, and BL.

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- Dailidiene D, Dailide G, Ogura K, Zhang M, Mukhopadhyay AK, et al. (2004) *Helicobacter acinonychis*: Genetic and rodent infection studies of a *Helicobacter pylori*-like gastric pathogen of cheetahs and other big cats. *J Bacteriol* 186: 356–365.
- Ghose C, Perez-Perez GI, Dominguez-Bello MG, Pride DT, Bravi CM, et al. (2002) East Asian genotypes of *Helicobacter pylori* strains in Amerindians provide evidence for its ancient human carriage. *Proc Natl Acad Sci U S A* 99: 15107–15111.
- Yamaoka Y, Orito E, Mizokami M, Gutierrez O, Saitou N, et al. (2002) *Helicobacter pylori* in North and South America before Columbus. *FEBS Letters* 517: 180–184.

8. Falush D, Wirth T, Linz B, Pritchard JK, Stephens M, et al. (2003) Traces of human migrations in *Helicobacter pylori* populations. *Science* 299: 1582–1585.
9. Cavalli-Sforza LL, Feldman MW (2003) The application of molecular genetic approaches to the study of human evolution. *Nat Genet* 33 Suppl: 266–275.
10. Solnick JV, Chang K, Canfield DR, Parsonnet J (2003) Natural acquisition of *Helicobacter pylori* infection in newborn rhesus macaques. *J Clin Microbiol* 41: 5511–5516.
11. Dewhirst FE, Shen Z, Scimeca MS, Stokes LN, Boumenna T, et al. (2005) Discordant 16S and 23S rRNA gene phylogenies for the genus *Helicobacter*: Implications for phylogenetic inference and systematics. *J Bacteriol* 187: 6106–6118.
12. Terio KA, Munson L, Marker L, Aldridge BM, Solnick JV (2005) Comparison of *Helicobacter* spp. in cheetahs (*Acinonyx jubatus*) with and without gastritis. *J Clin Microbiol* 43: 229–234.
13. Eaton KA, Dewhirst FE, Radin MJ, Fox JG, Paster BJ, et al. (1993) *Helicobacter acinonyx* sp. nov., isolated from cheetahs with gastritis. *Int J Syst Bacteriol* 43: 99–106.
14. Schroder HD, Ludwig C, Jakob W, Reischl U, Stolte M, et al. (1998) Chronic gastritis in tigers associated with *Helicobacter acinonyx*. *J Comp Pathol* 119: 67–73.
15. Gressmann H, Linz B, Ghai R, Pleissner KP, Schlapbach R, et al. (2005) Gain and loss of multiple genes during the evolution of *Helicobacter pylori*. *PLoS Genet* 1: e43.
16. Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, et al. (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 388: 539–547.
17. Alm RA, Ling LSL, Moir DT, King BL, Brown ED, et al. (1999) Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* 397: 176–180.
18. Suerbaum S, Josenhans C, Sterzenbach T, Drescher B, Brandt P, et al. (2003) The complete genome sequence of the carcinogenic bacterium *Helicobacter hepaticus*. *Proc Natl Acad Sci U S A* 100: 7901–7906.
19. Li WH, Wu CI, Luo CC (1985) A new method for estimating synonymous and non-synonymous rates of nucleotide substitution considering the relative likelihood of nucleotide and codon changes. *Mol Biol Evol* 2: 150–174.
20. Nei M, Kumar S (2000) *Molecular evolution and phylogenetics*. Oxford: Oxford University Press. 333 p.
21. Stringer CB, Andrews P (1988) Genetic and fossil evidence for the origin of modern humans. *Science* 239: 1263–1268.
22. Stringer C (2002) Modern human origins: Progress and prospects. *Philos Trans R Soc Lond B Biol Sci* 357: 563–579.
23. Underhill PA, Shen P, Lin AA, Jin L, Passarino G, et al. (2000) Y chromosome sequence variation and the history of human populations. *Nat Genet* 26: 358–361.
24. Ingman M, Gyllenstein U (2001) Analysis of the complete human mtDNA genome: Methodology and inferences for human evolution. *J Hered* 92: 454–461.
25. Zhivotovskiy LA, Rosenberg NA, Feldman MW (2003) Features of evolution and expansion of modern humans, inferred from genome-wide microsatellite markers. *Am J Hum Genet* 72: 1171–1186.
26. Liu H, Prugnolle F, Manica A, Balloux F (2006) A geographically explicit genetic model of worldwide human-settlement history. *Am J Hum Genet* 79: 230–237.
27. Fouts DE, Mongodin EF, Mandrell RE, Miller WG, Rasko DA, et al. (2005) Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. *PLoS Biol* 3: e15. DOI: 10.1371/journal.pbio.0030015
28. Brüssow H, Canchaya C, Hardt WD (2004) Phages and the evolution of bacterial pathogens: From genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev* 68: 560–602.
29. Quinones M, Knesek JE, McIntire SA (2001) Sequence and gene expression analyses of plasmid pHPM8 from *Helicobacter pylori* reveal the presence of two operons with putative roles in plasmid replication and antibiotic activity. *Plasmid* 46: 223–228.
30. Hofreuter D, Haas R (2002) Characterization of two cryptic *Helicobacter pylori* plasmids: A putative source for horizontal gene transfer and gene shuffling. *J Bacteriol* 184: 2755–2766.
31. Hoffmaster AR, Ravel J, Rasko DA, Chapman GD, Chute MD, et al. (2004) Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. *Proc Natl Acad Sci U S A* 101: 8449–8454.
32. Linton D, Karlyshev AV, Hitchen PG, Morris HR, Dell A, et al. (2000) Multiple N-acetylneuraminic acid synthetase (*neuB*) genes in *Campylobacter jejuni*: Identification and characterization of the gene involved in sialylation of lipo-oligosaccharide. *Mol Microbiol* 35: 1120–1134.
33. Gilbert M, Karwaski MF, Bernatchez S, Young NM, Taboada E, et al. (2002) The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. *J Biol Chem* 277: 327–337.
34. Vimr ER, Kalivoda KA, Deszo EL, Steenbergen SM (2004) Diversity of microbial sialic acid metabolism. *Microbiol Mol Biol Rev* 68: 132–153.
35. Eppinger M, Baar C, Raddatz G, Huson DH, Schuster SC (2004) Comparative analysis of four *Campylobacterales*. *Nat Rev Microbiol* 2: 872–885.
36. Horimoto T, Kawaoka Y (2005) Influenza: Lessons from past pandemics, warnings from current incidents. *Nat Rev Microbiol* 3: 591–600.
37. Lemey P, Pybus OG, Wang B, Saksena NK, Salemi M, et al. (2003) Tracing the origin and history of the HIV-2 epidemic. *Proc Natl Acad Sci U S A* 100: 6588–6592.
38. Holmes EC, Twiddy SS (2003) The origin, emergence, and evolutionary genetics of dengue virus. *Infect Genet Evol* 3: 19–28.
39. Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, et al. (2004) Microevolution and history of the plague bacillus, *Yersinia pestis*. *Proc Natl Acad Sci U S A* 101: 17837–17842.
40. Sreevatsan S, Pan X, Stockbauer K, Connell ND, Kreiswirth BN, et al. (1997) Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proc Natl Acad Sci U S A* 94: 9869–9874.
41. Gutierrez MC, Brisse S, Brosch R, Fabre M, Omais B, et al. (2005) Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PLoS Pathog* 1: e5. DOI: DOI: 10.1371/journal.ppat.0010005
42. Kidgell C, Reichard U, Wain J, Linz B, Torpdahl M, et al. (2002) *Salmonella typhi*, the causative agent of typhoid fever, is approximately 50,000 years old. *Infect Genet Evol* 2: 39–45.
43. Ochman H, Wilson AC (1987) Evolution in bacteria: Evidence for a universal substitution rate in cellular genomes. *J Mol Evol* 26: 74–86.
44. Ochman H, Elwyn S, Moran NA (1999) Calibrating bacterial evolution. *Proc Natl Acad Sci U S A* 96: 12638–12643.
45. Drummond AJ, Rambaut A, Shapiro B, Pybus OG (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol Biol Evol* 22: 1185–1192.
46. Wang G, Humayun MZ, Taylor DE (1999) Mutation as an origin of genetic variability in *Helicobacter pylori*. *Trends Microbiol* 7: 488–493.
47. Björkholm B, Sjölund M, Falk PG, Berg OG, Engstrand L, et al. (2001) Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. *Proc Natl Acad Sci U S A* 98: 14607–14612.
48. Matic I, Radman M, Taddei F, Picard B, Doit C, et al. (1997) Highly variable mutation rates in commensal and pathogenic *Escherichia coli*. *Science* 277: 1833–1834.
49. Parkhill J, Dougan G, James KD, Thomson NR, Pickard D, et al. (2001) Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* 413: 848–852.
50. Parkhill J, Sebahia M, Preston A, Murphy LD, Thomson N, et al. (2003) Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica*. *Nat Genet* 35: 32–40.
51. Chain PSG, Carniel E, Larimer F, Lamerdin J, Stoutland PO, et al. (2004) Insights into the genome evolution of *Yersinia pestis* through whole genome comparison with *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* 101: 13826–13831.
52. Thomson NR, Yeats C, Bell K, Holden MT, Bentley SD, et al. (2005) The *Chlamydomonada abortus* genome sequence reveals an array of variable proteins that contribute to interspecies variation. *Genome Res* 15: 629–640.
53. Deng W, Burland V, Plunkett G III, Boutin A, Mayhew GF, et al. (2002) Genome sequence of *Yersinia pestis* KIM. *J Bacteriol* 184: 4601–4611.
54. Welch RA, Burland V, Plunkett G III, Redford P, Roesch P, et al. (2002) Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. *Proc Natl Acad Sci U S A* 99: 17020–17024.
55. Tettelin H, Masignani V, Cieslewicz MJ, Donati C, Medini D, et al. (2005) Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: Implications for the microbial “pan-genome.” *Proc Natl Acad Sci U S A* 102: 13950–13955.
56. Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MT, et al. (2001) Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* 413: 523–527.
57. Lawrence JG (2005) Common themes in the genome strategies of pathogens. *Curr Opin Genet Dev* 15: 584–588.
58. Kim JJ, Zhou D, Mandrell RE, Griffiss JM (1992) Effect of exogenous sialylation of the lipooligosaccharide of *Neisseria gonorrhoeae* on opsonophagocytosis. *Infect Immun* 60: 4439–4442.
59. Mandrell RE, Apicella MA (1993) Lipo-oligosaccharides (LOS) of mucosal pathogens: Molecular mimicry and host-modification of LOS. *Immunobiology* 187: 382–402.
60. Smith NH, Maynard Smith J, Spratt BG (1995) Sequence evolution of the *porB* gene of *Neisseria gonorrhoeae* and *Neisseria meningitidis*: Evidence of positive Darwinian selection. *Mol Biol Evol* 12: 363–370.
61. Urwin R, Holmes EC, Fox AJ, Derrick JP, Maiden MC (2002) Phylogenetic evidence for frequent positive selection and recombination in the meningococcal surface antigen PorB. *Mol Biol Evol* 19: 1686–1694.
62. Aspholm-Hurtig M, Dailide G, Lahmann M, Kalia A, Ilver D, et al. (2004) Functional adaptation of BabA, the *H. pylori* ABO blood group antigen binding adhesin. *Science* 305: 519–522.
63. Backstrom A, Lundberg C, Kersulyte D, Berg DE, Boren T, et al. (2004) Metastability of *Helicobacter pylori* bab adhesin genes and dynamics in Lewis b antigen binding. *Proc Natl Acad Sci U S A* 101: 16923–16928.
64. Johnson WE, Eizirik E, Pecon-Slattery J, Murphy WJ, Antunes A, et al. (2006) The late Miocene radiation of modern Felidae: A genetic assessment. *Science* 311: 73–77.

65. Moya A, Holmes EC, Gonzalez-Candelas F (2004) The population genetics and evolutionary epidemiology of RNA viruses. *Nat Rev Microbiol* 2: 279–288.
66. Monot M, Honore N, Garnier T, Araoz R, Coppee JY, et al. (2005) On the origin of leprosy. *Science* 308: 1040–1042.
67. Pot RG, Kusters JG, Smeets LC, van Tongeren W, Vandenbroucke-Grauls CMJE, et al. (2001) Interspecies transfer of antibiotic resistance between *Helicobacter pylori* and *Helicobacter acinonychis*. *Antimicrob Agents Chemother* 45: 2975–2976.
68. Ewing B, Hillier L, Wendl MC, Green P (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8: 175–185.
69. Ewing B, Green P (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 8: 186–194.
70. Gordon D, Abajian C, Green P (1998) Consed: A graphical tool for sequence finishing. *Genome Res* 8: 195–202.
71. Gordon D, Desmarais C, Green P (2001) Automated finishing with autofinish. *Genome Res* 11: 614–625.
72. Meyer F, Goesmann A, McHardy AC, Bartels D, Bekel T, et al. (2003) GenDB—An open source genome annotation system for prokaryote genomes. *Nucleic Acids Res* 31: 2187–2195.
73. Salzberg SL, Delcher AL, Kasif S, White O (1998) Microbial gene identification using interpolated Markov models. *Nucleic Acids Res* 26: 544–548.
74. Badger JH, Olsen GJ (1999) CRITICA: Coding region identification tool invoking comparative analysis. *Mol Biol Evol* 16: 512–524.
75. Suzek BE, Ermolaeva MD, Schreiber M, Salzberg SL (2001) A probabilistic method for identifying start codons in bacterial genomes. *Bioinformatics* 17: 1123–1130.
76. Tatusov RL, Koonin EV, Lipman DJ (1997) A genomic perspective on protein families. *Science* 278: 631–637.
77. Kanehisa M, Goto S, Kawashima S, Nakaya A (2002) The KEGG databases at GenomeNet. *Nucleic Acids Res* 30: 42–46.
78. Jukes TH, Cantor CR. (1969) Evolution of protein molecules. In: Munro HN, editor. *Mammalian protein metabolism*. New York: Academic Press. pp. 21–132.
79. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132: 365–386.
80. Ihaka R, Gentleman R (1996) R: A language for data analysis and graphics. *J Comput Graph Stat* 5: 299–314.
81. Posada D, Crandall KA (1998) MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14: 817–818.
82. Swofford DL (1998) PAUP*: Phylogenetic analysis using parsimony and other methods. Version 4.0 beta. Sunderland (Massachusetts): Sinauer Associates.
83. De Ungria MC, Kolesnikow T, Cox PT, Lee A (1999) Molecular characterization and interstrain variability of pHPS1, a plasmid isolated from the Sydney strain (SS1) of *Helicobacter pylori*. *Plasmid* 41: 97–109.
84. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, et al. (2004) Versatile and open software for comparing large genomes. *Genome Biol* 5: R12.
85. Baar C, Eppinger M, Raddatz G, Simon J, Lanz C, et al. (2003) Complete genome sequence and analysis of *Wolinella succinogenes*. *Proc Natl Acad Sci U S A* 100: 11690–11695.
86. Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, et al. (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403: 665–668.
87. Akopyants NS, Clifton SW, Kersulyte D, Crabtree JE, Youree BE, et al. (1998) Analyses of the *cag* pathogenicity island of *Helicobacter pylori*. *Mol Microbiol* 28: 37–53.
88. Alm RA, Bina J, Andrews BM, Doig P, Hancock RE, et al. (2000) Comparative genomics of *Helicobacter pylori*: Analysis of the outer membrane protein families. *Infect Immun* 68: 4155–4168.
89. Boneca IG, de Reuse H, Epinat JC, Pupin M, Labigne A, et al. (2003) A revised annotation and comparative analysis of *Helicobacter pylori* genomes. *Nucleic Acids Res* 31: 1704–1714.
90. Minnis JA, Taylor TE, Knesek JE, Peterson WL, McIntire SA (1995) Characterization of a 3.5-kbp plasmid from *Helicobacter pylori*. *Plasmid* 34: 22–36.
91. Song JY, Park SG, Kang HL, Lee WK, Cho MJ, et al. (2003) pHP489, a *Helicobacter pylori* small cryptic plasmid, harbors a novel gene coding for a replication initiation protein. *Plasmid* 50: 236–241.
92. Song JY, Choi SH, Byun EY, Lee SG, Park YH, et al. (2003) Characterization of a small cryptic plasmid, pHP51, from a Korean isolate of strain 51 of *Helicobacter pylori*. *Plasmid* 50: 145–151.
93. Kersulyte D, Velapatino B, Dailide G, Mukhopadhyay AK, Ito Y, et al. (2002) Transposable element ISHp608 of *Helicobacter pylori*: Nonrandom geographic distribution, functional organization, and insertion specificity. *J Bacteriol* 184: 992–1002.