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Review

Genome and population dynamics during chronic infection with *Helicobacter pylori*

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Helicobacter pylori is responsible for one of the most prevalent bacterial infections worldwide. Chronic infection typically leads to chronic active gastritis. Clinical sequelae, including peptic ulcers, mucosa-associated lymphoid tissue lymphoma or, most importantly, gastric adenocarcinoma develop in 10–15% of cases. *H. pylori* is characterized by extensive inter-strain diversity which is the result of a high mutation rate, recombination, and a large repertoire of restriction-modification systems. This diversity is thought to be a major contributor to *H. pylori*'s persistence and exceptional aptitude to adapt to the gastric environment and evade the immune system. This review covers efforts in the last decade to characterize and understand the multiple layers of *H. pylori*'s diversity in different biological contexts.

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Introduction

Helicobacter pylori is an important bacterial pathogen of humans. It infects more than one half of the human population, usually during childhood, and establishes a chronic and often lifelong infection [1,2]. *H. pylori* infection invariably induces a chronic inflammatory response of the gastric mucosa. Infections can remain asymptomatic or progress to symptomatic diseases that include gastroduodenal ulcer disease, gastric adenocarcinoma, and gastric lymphoma of the mucosa-associated lymphoid tissue lymphoma [2,3]. *H. pylori* has been recognized as a Class I human carcinogen since the early 1990s [4], and is etiologically responsible for approx. new 900 000 cases of gastric cancer per year, a substantial proportion of all human cancers [5].

H. pylori is a bacterial species with an exceptional degree of genetic diversity and variability. It was acquired by humans in Africa around 100 000 years ago [6,7], and has accompanied modern humans during multiple ancient and more recent migrations out of Africa [6,8,9]. In present times, the common and complex history of humans and *H. pylori* is now reflected by a highly distinctive phylogeographic population structure [7,8,10–12]. The exceptionally high allelic diversity of *H. pylori* results from the combination of a very high mutation rate and a highly efficient DNA uptake and recombination machinery enabling DNA exchange between different *H. pylori* strains during mixed infections [13–15]. Potential roles of other genetic elements in creating genomic diversity, such as commonly occurring prophages and insertion sequences [16], remain to be elucidated. The molecular mechanisms responsible for this unique genetic plasticity of *H. pylori* and the contribution of *H. pylori* genome variation to its success as a global pathogen are the major topics of this review article, which summarizes the most important developments in this field in the last decade.

Mutation and recombination in *H. pylori in vitro*

H. pylori has a very high mutation rate, approx. 100-fold higher than *Escherichia coli* and most other bacterial species [17]. This 'mutator-type' mutation rate is the result of multiple features, including the lack of a mismatch repair pathway (MMR) and a promutagenic activity of its DNA polymerase I [18]. *H. pylori* thus introduces nucleotide polymorphisms into the population at an accelerated rate. Moreover, the lack of MMR is also thought to be responsible for the extremely high mutation rates at repetitive DNA sequences (e.g. homopolymeric tracts or dinucleotide repeats) due to slipped strand mispairing, which are used to reversibly activate and deactivate genes ('contingency genes') and facilitate phase variation [19–21].

H. pylori is able to take up exogenous DNA from the surrounding environment ('natural competence') by means of a highly unusual DNA uptake machinery, the ComB system [22], and to subsequently integrate homologous DNA into its chromosome in a RecA-dependent recombination process [23]. Recombination has been shown to contribute even more to the genetic diversification of *H. pylori* than spontaneous mutations [24]. *H. pylori* typically integrates short fragments of the exogenous DNA into its chromosome ('imports') compared to other competent bacteria [24–26]. The length of the imports after transformation in the laboratory and during chronic infection is similar and follows a bimodal length distribution, displaying longer (macroimports, mean length 1645 bp) and extremely short (microimports, mean length 28 bp) fragments [27]. Quantitative analysis of import patterns obtained from *in vitro* transformants suggests that micro- and macroimports frequently arise from single DNA molecules [28]. Microimports could specifically be involved in the continued reduction of linkage disequilibrium in the *H. pylori* genome, although this hypothesis still needs to be explored further [29]. Nevertheless, the mechanisms responsible for these two types of imports remain incompletely characterized. We could recently show that the formation of microimports depends strongly on the activity of the UvrC nuclease, a component of the nucleotide excision repair pathway [28,30]. A *uvrC* mutant no longer displayed the bimodal distribution pattern characteristic of *H. pylori* wild-type strains. Remarkably, this function of UvrC in microimport formation was independent of its canonical function in the repair of DNA lesions, since site-directed mutations of conserved amino acids involved in these canonical functions did not affect microimports while inactivating the protective role of UvrC against (e.g. UV mediated) DNA damage [28]. Future work will be devoted to identifying the molecular mechanism how *H. pylori* UvrC contributes to microimport formation.

Genome evolution of *H. pylori* during chronic human infection

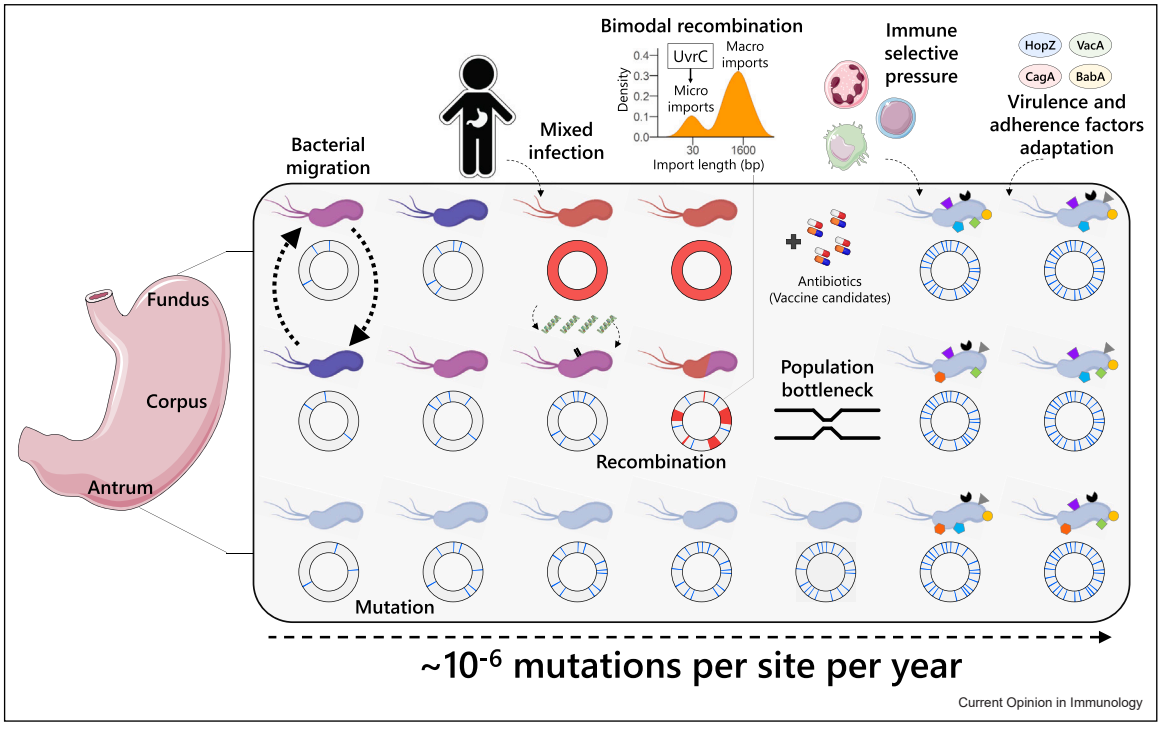
Due to the unique combination of very high overall within species diversity and high mutation and recombination rates, *H. pylori* has become a paradigm for a bacterium that can be observed, almost in real time, to evolve during the infection of individual patients (Figure 1) [15]. This was first shown by multilocus sequence comparisons between single isolates of *H. pylori* cultured from biopsies obtained at different time points [25,31], and was then later expanded to whole-genome analysis of isolates cultured from chronically infected individuals at multiple sequential timepoints [26]. The mutation rate of *H. pylori* during chronic infection *in vivo* has been estimated repeatedly, using different approaches. The first robust estimate of the *in vivo* mutation rate of *H. pylori* during years to decades of infection was based on sequence comparisons of 78 (almost exclusively housekeeping) genes in 34 pairs of sequential isolates from the Netherlands, Colombia and the USA. This study estimated the mutation rate of *H. pylori* at 1.4×10^{-6} mutations per site and year, and also estimated the long-term mutation rate (i.e. evolution over millennia) at 2.6×10^{-7} [31]. Later on, a first whole full genome-based analysis was based on four pairs of sequential isolates from Colombia taken at a three-year interval and yielded a mutation rate of 2.5×10^{-5} [26]. Another estimate derived from paired isolates (antrum and corpus) from South Africa yielded a similar (synonymous) mutation rate of 1.38×10^{-5} [32]. There is currently no evidence that these observations are limited to specific phylogeographic populations of *H. pylori*. However, in light of the large diversity of *H. pylori*, it seems of interest to perform similar studies in patients infected with strains belonging to different phylogeographic populations.

While the mutation rates of *H. pylori* are higher than those of many other bacteria, it is only the unusual combination with a highly efficient recombination machinery that gives *H. pylori* its exceptional genetic plasticity [13]. While mutations are about five times more frequent than recombination events, recombination frequently leads to the import of several hundred to more than 1000 base pairs, likely to contain multiple polymorphisms. As a result, a polymorphism in *H. pylori* is about three times more likely to have been introduced by recombination than mutation [31]. However, it is important to note that the contribution of recombination to the genetic diversity of *H. pylori* varies widely between strains and phylogeographical populations, which likely reflects the availability of DNA released by unrelated *H. pylori* strains during (transient) mixed infections and the individual DNA uptake activity of the resident populations [32,33].

H. pylori genetic plasticity and vaccine evasion

Due to the high prevalence of *H. pylori* infection, the need for antibiotic combination therapy, and rising rates of antibiotic resistance, it is inconceivable to control *H. pylori* infection on a global scale with antibiotics alone. A vaccine is thus widely considered of high medical and societal interest. However, despite intense efforts that began soon after the discovery of *H. pylori*, and multiple vaccine candidates entering clinical trials in humans, no vaccine against *H. pylori* has yet reached the market. The reasons for the failure of most candidates tested so far are likely multifactorial, but the high genetic diversity and variability of *H. pylori* seem likely to have contributed. We tested this hypothesis using *H. pylori* strains obtained from two clinical vaccine trials with

Figure 1



Evolution of *H. pylori* within the human host during chronic infection. The figure schematically depicts important processes relevant to the evolution of the *H. pylori* population within the human stomach as discussed in more detail in the text. The *x*-axis indicates the evolution over time during chronic infection and the *y*-axis represents the fact that the stomach is a large organ with physiologically distinct regions requiring adaptation. Subpopulations of *H. pylori* in a given region and at a given timepoint are represented by a bacterial cell and a corresponding bacterial chromosome. Within-host diversity is represented by different shades of blue while the coinfection is displayed in red. Accumulation of polymorphisms in the chromosome is represented by blue tick marks for spontaneous mutations and red tick marks for imports.

human volunteers [34,35]. In both studies, volunteers received a candidate vaccine (or placebo). The two candidate vaccines were an orally administered *Salmonella* strain expressing *H. pylori* urease [34], or an injectable tricomponent vaccine containing three *H. pylori* virulence factors, CagA, VacA, and NAP [35]. In both studies, volunteers were vaccinated with the respective candidate or placebo and subsequently challenged with a well-characterized *H. pylori* strain (in one case, BCS 100, a *cag*-island deficient strains, in the other case BCM-300, a strain with an intact *cag*PAI), *H. pylori* was then re-isolated from those volunteers who had become infected by the challenge strain. For both studies, we compared the genomes of the challenge strains with the sequences of reisolates. This permitted the calculation of mutation rates during the initial phase of infection and to assess potential changes in the vaccine antigen-encoding genes [36,37]. Mutation rates were 4.5 and 5.2×10^{-6}/site and year, thus very similar to the estimates obtained in the earlier studies based on sequential isolates from chronically infected individuals summarized above. Importantly, there was no evidence for an accelerated mutational rate ('mutational burst') compared with chronic infection, in contrast with a single report of such a mutation burst in one patient [38]. The number of volunteers/reisolates was too small to draw definitive conclusions about the contribution of genetic variability to vaccine failure. However, an inactivation of *cag*PAI activity and/or VacA vacuolating cytotoxin expression was observed in multiple volunteers who had received the tricomponent vaccine (which contained both proteins), suggesting that this was potentially the result of vaccine-induced immune pressure [36]. It thus would appear important that the high genetic variability of *H. pylori* is taken into account when designing future vaccine candidates. In particular, nonessential genes (such as *vacA* and *cagA*) can be rapidly inactivated by multiple mechanisms in *H. pylori*.

Within-host diversity of *H. pylori* and adaptation to different stomach niches

In the first two decades of *H. pylori* research, comparative studies were almost exclusively limited to one or two strains per patient. In order to be able to quantitate the genetic diversity of *H. pylori* within one stomach, we obtained multiple biopsies from two to three different locations in the stomach (corpus, fundus, and antrum) and 10 single colonies of *H. pylori* were cultured from

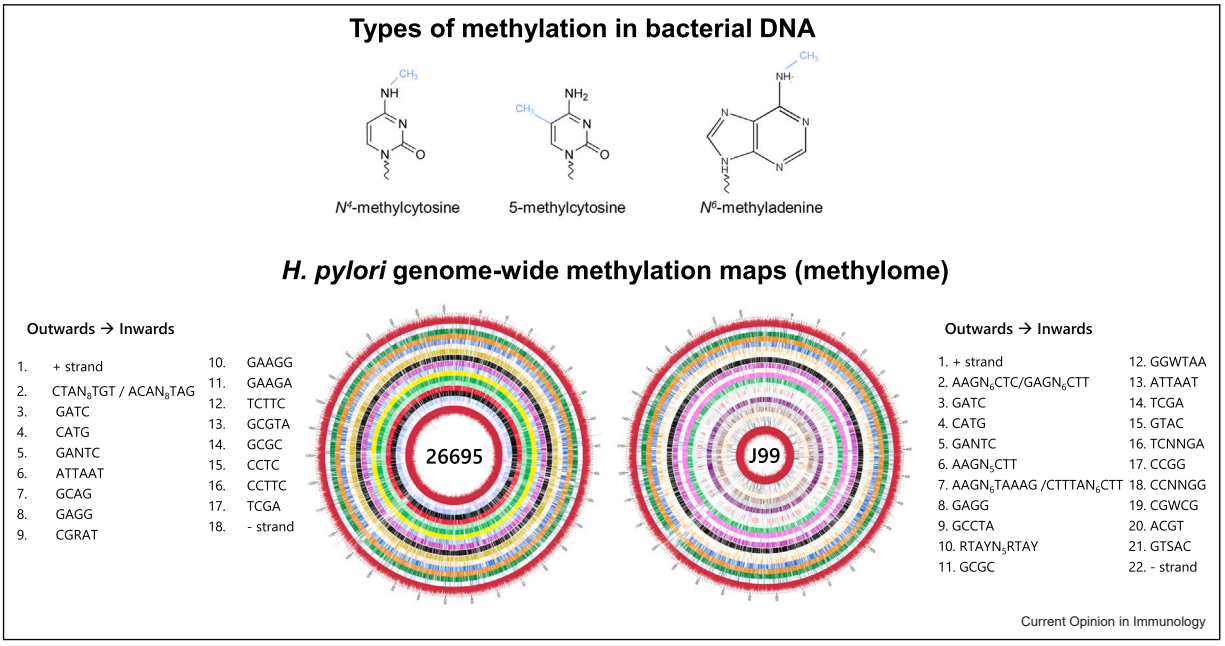
each location [39]. Subsequently, DNA was purified from all isolates and their genomes were sequenced. This sampling was performed for 16 individuals, resulting in a total dataset of 440 genomes. The analysis showed that every patient was host to one unique population of bacteria. Overt mixed infections with multiple unrelated strains were not detected in the dataset, in agreement with earlier studies suggesting that *H. pylori* coinfections are mostly transient. Bacteria sequenced from a given patient were always highly related to all other bacteria from the same patient, and differed extensively from the bacteria isolated from the other patients. Subpopulations specifically associated with each gastric region could be observed and phylogenetic analysis revealed that *H. pylori* can migrate between stomach locations. The degree of sequence variation within an individual patient varied widely, ranging from highly diverse, with thousands of polymorphisms originating from both mutations and recombined imports, to relatively homogeneous, with less than 100 polymorphisms. Our study [39] identified numerous genes showing signals of adaptation to different gastric niches. Strikingly, many of these were involved in motility and chemotaxis, which was highly plausible in light of the known importance of chemotactic flagellar motility for *H. pylori* colonization [40–46]. For each *H. pylori* population derived from an individual host, the time to the most recent common ancestor (TMRCA) could be calculated to quantitate within-host diversity. For the 16 individuals, aged 32–76 years, the TMRCA of their *H. pylori* populations ranged between 4 months and 7.1 years [39]. This confirmed findings of an earlier study, where TMRCA had been calculated using 40 pairs (1 from antrum and 1 from corpus region of the stomach) of *H. pylori* isolates from adult individuals in South Africa, yielding an average TMRCA of 3.6 years and a maximum TMRCA of 11.1 years [32]. Thus, available data concur that TMRCA or *H. pylori* populations within one human host are much lower than the age of the infected person, despite the fact that most *H. pylori* infections are thought to have been acquired in childhood. This is likely the result of multiple mechanisms. There is now substantial evidence that antibiotics play an important role in reducing *H. pylori* diversity. Administration of antibiotics, such as macrolides, tetracycline, metronidazole, or fluoroquinolones, can have an impact on *H. pylori* populations even when not used as part of eradication treatments directed against *H. pylori*. Within the 16 individual populations, seven contained alleles that have been associated with resistance against one or multiple antibiotics, likely indicating previous antibiotic use by the patient. These seven populations had significantly lower TMRCA than the populations where no resistance alleles were found. In three populations, subpopulations from different gastric niches displayed variable antibiotic susceptibilities, indicating that the distribution of *H. pylori* across the stomach regions can contribute to heteroresistance. In one case, a follow-up sampling after two years could be obtained. Strikingly, a population initially devoid of antibiotic resistance alleles had converted to fully clarithromycin resistant, and the initial TMRCA of seven years was reduced to less than one year. These data indicate that antibiotic use can induce substantial selective bottlenecks with massive reductions of population size and loss of diversity [39]. However, it still seems likely that antibiotics are not the only reason for the low TMRCA values, since TMRCA was also much lower than patient ages when no traces of prior antibiotic uses were observed. One second likely contributor is genetic drift, spontaneous loss of diversity with time due to low effective population sizes [32]. Finally, immune selection might also play a role in reducing genetic diversity of the resident *H. pylori* population (see the review article by Faass et al. [47] in this Special Issue for additional references regarding the interaction between *H. pylori* and the immune system).

While there is now strong evidence that *H. pylori* uses its high genetic plasticity to adapt important host interaction molecules to the requirements of a large, physiologically diverse, and changing habitat, there are currently still few examples where this has been functionally validated. The best example is the Lewis b blood group antigen binding adhesion BabA, where allelic variation occurs during chronic infection [48], and has helped to adapt the bacteria to different acidities in the stomach [49]. Another example is the *cag* pathogenicity island protein CagY, which was shown to contribute to the modulation of *cag*PAI-mediated proinflammatory activity *in vivo* [50].

The *H. pylori* 'methylome'

H. pylori possesses an exceptionally high number of Restriction-Modification (R-M) systems, and the portfolio of R-M systems varies widely between strains [51–55]. R-M systems in most cases consist of a sequence-specific methyltransferase (MTase) and an endonuclease that cleaves the same recognition sequence in a methylation-dependent way. Many variations of this basic architecture exist, and MTases can also occur as so-called orphan MTase, without a cognate restriction endonuclease (or together with an inactive endonuclease gene) [56]. The diverse portfolio of MTases translates into a highly diverse methylation pattern of the *H. pylori* chromosome [57,58]. Functional studies on R-M systems on a genome-wide scale have been strongly enabled by the development of the Single Molecule, Real-Time (SMRT) sequencing technology by Pacific Biosciences. The technology permits to detect DNA methylation in parallel to the determination of the nucleotide sequence [59]. In a collaboration that included scientists from Pacific Biosciences and the group of Richard J. Roberts at NEB, we determined the methylomes of the two best

Figure 2



Methylome diversity of *H. pylori*. Three types of DNA methylation detected in *H. pylori*. Circular plots representing the location of methylated motifs determined by SMRT (Pacific Biosciences) sequencing in two strains of *H. pylori*. For each strain, each motif described in the accompanying list is visualized by a separate track. Circular plots are from Ref. [57].

characterized *H. pylori* strains, 26695 and J99 (Figure 2), and assigned all but one methylated target sequences to known or newly characterized MTases [57]. Only the MTase responsible for the methylation of the motif CGRAT remains unknown [57]. Methylome analyses were also performed for the two studies of vaccine evasion described above, showing, in both studies, plasticity of the methylome as an additional layer of variation [36,57]. We also characterized the GCGC-specific MTase JHP1050, the only ^{m5}C MTase invariably present in all strains of *H. pylori*, and showed that GCGC methylation has an important effect on gene transcription when a GCGC motif is located within a promoter sequence [60]. Other groups have obtained related results with other MTases [61–66], and work is in progress to fully understand the role of epigenetic modification of the bacterial chromosome in *H. pylori* transcriptional regulation.

Conclusions

Research performed over the last decade has strongly advanced our understanding of *H. pylori* population dynamics. These studies have been made possible by advances in sequencing technology, bioinformatic, and population genetic analysis methods as well as functional analysis methods. However, there are still many open questions. For example, our analyses of within-host diversity of *H. pylori* have identified a large number of genes likely to be involved in the adaptation of *H. pylori* to specific parts of the stomach, which have not yet been functionally characterized. The same study has identified the most variable genes in the *H. pylori* chromosome during infection, which are likely to be involved in host interaction and/or immune evasion. Many of these are yet uncharacterized proteins awaiting further functional characterization.

Data Availability

No data were used for the research described in the article.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- •• of outstanding interest

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