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Genome and Methylome Variation in *Helicobacter pylori* With a *cag* Pathogenicity Island During Early Stages of Human Infection

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BACKGROUND & AIMS: *Helicobacter pylori* is remarkable for its genetic variation; yet, little is known about its genetic changes during early stages of human infection, as the bacteria adapt to their new environment. We analyzed genome and methylome variations in a fully virulent strain of *H pylori* during experimental infection. **METHODS:** We performed a randomized Phase I/II, observer-blind, placebo-controlled study of 12 healthy, *H pylori*–negative adults in Germany from October 2008 through March 2010. The volunteers were given a prophylactic vaccine candidate (n = 7) or placebo (n = 5) and then challenged with *H pylori* strain BCM-300. Biopsy samples were collected and *H pylori* were isolated. Genomes of the challenge strain and 12 reisolates, obtained 12 weeks after (or in 1 case, 62 weeks after) infection were sequenced by single-molecule, real-time technology, which, in parallel, permitted determination of genome-wide methylation patterns for all strains. Functional effects of genetic changes observed in *H pylori* strains during human infection were assessed by measuring release of interleukin 8 from AGS cells (to detect *cag* pathogenicity island function), neutral red uptake (to detect vacuolating cytotoxin activity), and adhesion assays. **RESULTS:** The observed mutation rate was in agreement with rates previously determined from patients with chronic *H pylori* infections, without evidence of a mutation burst. A loss of *cag* pathogenicity island function was observed in 3 reisolates. In addition, 3 reisolates from the vaccine group acquired mutations in the vacuolating cytotoxin gene *vacA*, resulting in loss of vacuolization activity. We observed interstrain variation in methylomes due to phase variation in genes encoding methyltransferases. **CONCLUSIONS:** We analyzed adaptation of a fully virulent strain of *H pylori* to 12 different volunteers to obtain a robust estimate of the frequency of genetic and epigenetic changes in the absence of interstrain recombination. Our findings indicate that the large amount of genetic variation in *H pylori* poses a challenge to vaccine development. ClinicalTrials.gov no: NCT00736476.

Keywords: Microbe; Stomach; Pathogen; Cancer.

Helicobacter pylori is a highly prevalent bacterial pathogen that infects the human stomach. If untreated, *H pylori* can establish a lifelong infection that can either remain asymptomatic, or lead to severe sequelae, including peptic ulcer disease and gastric cancer.[1] The species *H pylori* is notable for its exceptionally high genetic diversity and variability. Elevated rates of spontaneous mutations are attributed to the lack of a number of classic DNA repair genes,[2–4] in combination with specific mutagenic properties of its DNA polymerase I.[5] In addition, recombination during mixed infections with multiple *H pylori* strains within one stomach was shown to be the dominant driving force of genetic variability.[6–8] The genetic variability of *H pylori* is thought to be important for its adaptation to different individual hosts, and to the constantly changing conditions of the gastric niche.[2] To date, in vivo genome evolution of *H pylori* has been mainly studied in isolates obtained from chronically infected individuals.[6,9–12] These studies showed that outer membrane protein (OMP) encoding genes were more frequently affected by genomic changes than other genes, pointing to a strong selection for the diversification of proteins that interact with the host during chronic infection.[10] Immune evasion is suggested to be one potential driving force for the diversification of *H pylori* in vivo.

H pylori therapy aims at the eradication of infection, yet the increasing spread of antibiotic resistance necessitates the development of alternative approaches for the control of *H pylori* infection. Therefore, since the early 1990s, multiple attempts have been made to develop a vaccine, but to date, no effective therapeutic or prophylactic vaccine is commercially available,[13–16] and the high genetic diversity and variability of *H pylori* may have contributed to this situation.

***Authors share co-first authorship.**

Abbreviations used in this paper: **ANOVA, analysis of variance; *cag*PAI, *cag* pathogenicity island; IL, interleukin; MTases, methyltransferases; OMP, outer membrane protein; PCR, polymerase chain reaction; R-M, Restriction-Modification; SMRT sequencing, single-molecule, real-time sequencing; SNPs, single-nucleotide polymorphisms; T4SS, type IV secretion system; UBT, urea breath test.**

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EDITOR'S NOTES

BACKGROUND AND CONTEXT

Helicobacter pylori bacteria have been shown to evolve and diversify in chronically infected patients. Little has been known about the genetic adaptation of *H pylori* in the early phase infection following the initial acquisition.

NEW FINDINGS

The researchers determined the genome sequences of *H pylori* before challenge and after several months of infection, permitting to precisely determine rates of mutations in early-stage infections, and to identify the bacterial genes affected by the mutations.

LIMITATIONS

Only one bacterial re-isolate clone was available for each volunteer. Thus, an assessment of the variability within the individual stomach was not possible. The number of volunteers in the vaccinated and non-vaccinated subgroups were relatively small.

IMPACT

The findings indicate that the large amount of genetic variation in *H pylori* poses a challenge to vaccine development.

The analysis of strains from chronically infected individuals, as performed previously,[6,8–10,12] does not permit investigation of the in vivo diversification of OMPs and other important virulence genes during early colonization, because infections might have been established for many years and initial changes are most likely masked by subsequent changes and purifying selection. Experimental infections of *H pylori*–negative individuals with a defined strain are better suited to specifically investigate early adaptation to the individual host. So far, only 2 studies have analyzed genomic changes of *H pylori* isolates obtained during the initial phase of an experimental human infection in 1 and 2 volunteers, respectively.[10,17] Although Kennemann and coworkers[10] found only marginal genomic changes and not a single recombination event, Linz and coworkers[17] reported rapid genome evolution through a mutation burst and numerous recombination events. The reason for the discrepancy between the 2 studies was unknown.

Methylation of DNA is an important form of epigenetic modification catalyzed by methyltransferases (MTases). *H pylori* is characterized by a striking abundance and substantial interstrain diversity of MTases and restriction-modification systems.[18–22] Recent studies have taken advantage of the Single-Molecule, Real-Time (SMRT) sequencing technology to characterize the genome-wide DNA methylation in multiple *H pylori* strains.[19,20,23] However, to date, no study has investigated methylation in the context of functional adaptation during human infection in vivo.

In this study, we applied SMRT sequencing to analyze both genome and methylome variation in 12 *H pylori* isolates obtained after experimental infection of human volunteers during a vaccine trial with *H pylori* challenge strain BCM-300. This *babA*-positive strain has the *vacA* s1m1 genotype, carries an intact *cag* pathogenicity island (*cag*PAI) and expresses the effector protein CagA. We identify individual sequence differences in all reisolates, many of which affect virulence and host interaction factors, such as *cagA* and *vacA*. Variations in the methylome were likewise detected, resulting from phase-variable expression of 2 MTase genes.

Materials and Methods

H pylori *Strains and Ethics Statement*

The experimental human infection study was a randomized Phase I/II, observer-blind, placebo-controlled, single-center study performed in healthy *H pylori*–negative adults from October 2008 to March 2010 (ClinicalTrials.gov: NCT00736476). The study was performed at the Clinic of Gastroenterology, Hepatology and Infectious Diseases at the Otto-von-Guericke University of Magdeburg, Germany; it followed all good clinical practice criteria and International Conference on Harmonization guidelines, and received the approval from the local ethical committee and written informed consent from all subjects.

The challenge strain *H pylori* BCM-300 that was used for experimental human infection[24] was originally isolated from an asymptomatic volunteer with mild superficial gastritis (ATCC BAA-1606). Gastric biopsies obtained from study participants were subjected to culture with single colony purification for isolation of *H pylori*. The histological typing and grading of gastritis was performed according to the recommendations of the updated Sydney classification.[25] Inflammation and all other parameters were semiquantitatively scored as either 0 (absent), 1 (mild), 2 (moderate), or 3 (severe).

Microbiological and Molecular Biology Techniques

Details of the culture conditions for *H pylori* isolates and mutants as well as *Escherichia coli* strains, DNA preparation, Sanger sequencing, quantitative polymerase chain reaction (PCR), neutral red uptake assay, IL8 induction, BabA expression, Le(b) binding, insertion mutagenesis in *H pylori*, overexpression of Hpy300XI in *E coli,* and restriction analyses are described in Supplementary Materials and Methods.

SMRT Sequencing and Base Modification Analysis

Total genomic DNA was extracted using QIAGEN Genomic-tip 100/G columns (QIAGEN, Hilden, Germany). BCM-300 and 10 reisolates were genome-sequenced at Pacific Biosciences (Menlo Park, CA). SMRTbell template library construction of 15-kb shotgun libraries was performed as previously described.[26] Genomes were sequenced on the Pacific Biosciences RSII instrument using 1 SMRT Cell per strain applying P4/C2 chemistry. Strains HE134/09 and HE178/09, *H pylori* mutant strains and *E coli* expression strains were sequenced at DSMZ (Braunschweig, Germany) as follows: SMRTbell template libraries were prepared according to the instructions from Pacific Biosciences following the Procedure & Checklist for 10 kb Template Preparation and Sequencing. Sequencing of 2 SMRT Cells per strain was performed using the Pacific Biosciences RSII instrument and P6/C4 chemistry. De novo genome assembly was carried out with HGAP2 (Pacific Biosciences) and HGAP3 (DSMZ)[27] and genome consensus using