Genome-wide mutation analysis of *Helicobacter pylori* after inoculation to Mongolian gerbils

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**Abstract**

**Background:** *Helicobacter pylori* is a pathogenic bacterium that causes various gastrointestinal diseases in the human stomach. *H. pylori* is well adapted to the human stomach but does not easily infect other animals. As a model animal, Mongolian gerbils are often used, however, the genome of the inoculated *H. pylori* may accumulate mutations to adapt to the new host. To investigate mutations occurring in *H. pylori* after infection in Mongolian gerbils, we compared the whole genome sequence of TN2 wild type strain (TN2wt) and next generation sequencing data of retrieved strains from the animals after different lengths of infection.

**Results:** We identified mutations in 21 loci of 17 genes of the post-inoculation strains. Of the 17 genes, five were outer membrane proteins that potentially influence on the colonization and inflammation. Missense and nonsense mutations were observed in 15 and 6 loci, respectively. Multiple mutations were observed in three genes. Mutated genes included *babA*, *tlpB*, and *gltS*, which are known to be associated with adaptation to murine. Other mutations were involved with chemoreceptor, pH regulator, and outer membrane proteins, which also have potential to influence on the adaptation to the new host.

**Conclusions:** We confirmed mutations in genes previously reported to be associated with adaptation to Mongolian gerbils. We also listed up genes that mutated during the infection to the gerbils, though it needs experiments to prove the influence on adaptation.

**Keywords:** *Helicobacter pylori*, Mongolian gerbil, Animal model, Genome comparison, Adaptive mutation, Protein structure

**Background**

*Helicobacter pylori* (*H. pylori*) is known to a risk factor of various gastrointestinal diseases [1–4]. Previous studies investigated genetic diversification of *H. pylori* in the time course of chronic infection or transmission and revealed that the mutation rate of this bacterium is high [5–8]. However, *H. pylori* is well adapted to the human stomach but does not easily infect other animals. In search of a good animal model, experimental infection was attempted in Rhesus monkeys [9], mice [10], and Mongolian gerbils [11–16]. Genetic diversification of *H. pylori* in the infected animals was also studied [9, 10, 16–19].

Model animals are expected to respond to the stimulation in the similar manner to humans and be maintained on reasonable cost and handling efforts. Small rodent Mongolian gerbils develop similar symptoms to human by *H. pylori* infection as gastric inflammation, ulceration and cancer [13, 15, 20, 21]. Thus, they work as the good animal model.

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We also used Mongolian gerbils as the model animal and discovered that babA expression in H. pylori initially increased upon infection but reduced over time, then lost after 6 months [22] and that infection with oipA or babA mutants resulted in significantly reduced cytokine levels but alpAB mutant did not infect Mongolian gerbils [22].

Earlier studies used PCR to investigate changes in genes during animal infection. However, DNA sequencing advancements enabled the extensive exploration of mutations by sequencing bacterial genomes before and after infection [16, 19]. Here, we used the whole genome sequence of TN2 wild type (TN2wt) as a reference and sequenced short reads from three derivative strains to identify genomic mutations during infection in Mongolian gerbils. We detected mutations in agreement with previous studies and identified new mutations that may be associated with adaptation of the bacteria to different hosts.

**Methods**

**Inoculation, euthanasia and isolation of H. pylori**

We inoculated TN2 wild type strain [21] (TNwt) to Mongolian gerbils as described in our previous paper [12]. Six-week-old male Mongolian gerbils (MGS/Sea; Harlan Sprague Dawley) were orogastrically inoculated 3 times (days 0, 1, 2) with 1.0 mL of H. pylori (10⁹ colony-forming units/mL) or sterile brain–heart infusion (BHI) broth using gastric intubation needles after 16 h of fasting [10]. No specific pretreatments were administered prior to orogastric H. pylori inoculation. Inoculated Mongolian gerbils were sacrificed after 1 month (TN2-1M), 3 months (TN2-3M), and 6 months (TN2-6M). At necropsy, an ~1-mm² piece of gastric mucosa from the antrum was collected for culturing of H. pylori and subsequent DNA extraction.

**Bacterial culture and DNA extraction**

Helicobacter pylori were cultured on confluent plates expanded from a single colony under microaerobic conditions (12% CO₂) at 37 °C. Bacterial DNA was extracted from the plates using a commercially available kit (QIAgen Inc., Valencia, CA, USA).

**Sequencing of the genomic DNA**

The whole genome sequence of TN2wt was provided by our collaborator at the Okinawa Institute of Advanced Sciences. The whole-genome sequencing of TN2wt was carried out using the PacBio RS II (Pacific Biosciences, Menlo Park, CA) platform. De-novo assembly was performed using the hierarchical genome assembly process (HGAP) workflow [23], including consensus polishing with Quiver v.2.3.3. By this workflow, the complete genome sequence of TN2wt was obtained. Annotation was performed by MiGap service provided by National Institute of Genetics. The genome DNA of H. pylori strains retrieved from the Mongolian gerbils were sequenced by HiSeq2000 (paired end, 2 × 100 bp). DNA was quantified by Qubit fluorometric method (Thermo Fisher Scientific). DNA purity was assessed by the UV absorbance ratio at 260/280 with 1.8–2.0. Finally, 500 ng of DNA input was used for DNA library preparation. The numbers of reads obtained were 13,574,248, 14,583,596, and 13,938,018 for TN2-1M, TN2-3M, and TN2-6M, respectively; 99.69%, 99.74%, and 99.75% of the reads mapped to the reference TN2wt genome, resulting in average mapping depths of 758.8, 815.7, and 779.6 for TN2-1M, TN2-3M, and TN2-6M, respectively. The coverage of the reference genome was 100% in all strains.

**Results and discussion**

**Non-synonymous mutations in the retrieved strains**

Compared with the original TN2wt genome, strains TN2-1M, TN2-3M, and TN2-6M had 6, 9, and 6 non-synonymous mutations, respectively (Table 1, Fig. 1). These mutations were resided in 17 genes. In accordance with our previous report [10], 5 of the 17 genes were outer membrane proteins that potentially influence on colonization and inflammation.

Some genes had multiple mutations. TN2-1M had two missense mutations in kefB and single missense mutation in other three genes. A nucleotide insertion in hofH of TN2-1M (1290th nucleotide in the gene) caused frameshift, however, it did not cause a premature stop codon. Instead, the frameshift delayed the occurrence of a stop codon and elongated the gene 15 bp. Consequently, mutations observed in TN2-1M were all missense. KefB is a component of potassium ion (K⁺) transportation system that regulates cytoplasmic pH and influence on bacterial growth and survival [27]. UreI is a pH-gated urea channel that enable H. pylori to colonize in acidic
environment [28–30]. Missense mutations in these genes might change reactivity to pH fluctuation. GltS is a Glu-specific transporter and known also to be essential for colonization of H. pylori in Mongolian gerbils [31, 32].

TN2-3M contained seven missense and two nonsense mutations. Nucleotide deletion in oppA that leads to the premature stop codon was observed both in TN2-3M and TN2-6M. OppA is one of the ABC-type transporter genes for oligopeptide transport. Previous in-vitro study reported that disruption of oppA did not significantly change the growth of the mutant from the wild type [33]. This may suggest that the nonsense mutation in oppA was allowed because this gene is not essential for growth. Another possibility is that loss of oppA is neutral in vitro or in the originated human stomach but rather advantageous in the Mongolian gerbil stomach. Considering that the nonsense mutation of oppA was observed both in TN2-3M and TN2-6M, the latter hypothesis is also probable.

TN2-6M contained two missense and four nonsense mutations. In this strain, babA, oppA, tlpB, and outer membrane protein had nonsense mutations. As for tlpB, two missense mutations were also observed in TN2-3M. TlpB and babA are known to be involved with H. pylori adaptation to Mongolian gerbils. Our previous study revealed that infection with mutated babA reduced

### Table 1 Mutations observed in outcome strains

| Strains | Position | Mutation | Depth | Ratio | Gene                        | Amino acid change |
|---------|----------|----------|-------|-------|-----------------------------|-------------------|
| TN2-1M  | (1) 31255 | C → A    | 1025  | 99.7  | Outer membrane protein (hefG) | A61S              |
| (2) 517741 | G → A    | 569     | 99.7  | Glutathione-regulated potassium-efflux system protein (kefB) | N232S             |
| (3) 517792 | T → C    | 676     | 99.7  | Outer membrane protein (hoFH) | Frameshift without stop |
| (4) 1241193 | Insertion | 625     | 96.6  | Glutamate permease (gltS) | W131G             |
| (5) 1297623 | A → G    | 776     | 99.5  | Urease accessory protein (ureI) | H131R             |
| (6) 1496148 | A → C    | 529     | 95.1  | Uncharacterized protein | G201W             |
| TN2-3M  | (2) 112286 | G → A    | 866   | 93.6  | Dinucleoside polyphosphate hydrolase | R139C             |
| (3) 188008 | C → G    | 766     | 100.0 | Type II restriction enzyme R protein (hsdR) | R173T             |
| (4) 194568 | G → T    | 774     | 99.6  | Outer membrane protein (hopB) | T123A             |
| (5) 926807 | Insertion | 773     | 97.0  | cag pathogenicity island protein (cagB) | Stop at 136th codon |
| (6) 1007324 | A → G    | 867     | 99.1  | Outer membrane protein (oppA) | G240V             |
| (7) 1202841 | C → G    | 780     | 99.9  | P-type DNA transfer ATPase (virB11) | H314Y             |
| (8) 989679 | Deletion  | 629     | 95.0  | Outer membrane protein (babA) | Stop at 93th codon |
| (9) 1174908 | C → A    | 570     | 94.4  | Lipopolysaccharide biosynthesis proteins | G154W             |
| (1) 935451 | C → T    | 691     | 99.9  | P-type DNA transfer ATPase (virB11) | Stop at 464th codon |
| (2) 1251850 | Insertion | 605     | 91.8  | Outer membrane protein | Stop at 305th codon |
| TN2-6M  | (2) 87451 | Deletion | 853   | 95.2  | Oligopeptide ABC transporter periplasmic oligopeptide-binding protein (oppA) | Stop at 464th codon |
| (1) 791  |       |         | 96.3  |       |                            |                   |
| TN2-3M  | (3) 175008 | G → T    | 591   | 97.8  | Methyl-accepting chemotaxis protein (tlpB) | G26W              |
| (4) 175755 | G → T    | 795     | 99.9  | Oligopeptide ABC transporter periplasmic oligopeptide-binding protein (oppA) | Stop at 256th codon |
| (2) 175691 | Deletion | 810     | 99.9  | Oligopeptide ABC transporter periplasmic oligopeptide-binding protein (oppA) | Stop at 256th codon |

Position indicates the location of the mutation in the TN2 genome. Depth and ratio represent number of reads that covered the locus and percentage of the mutated reads, respectively. Numbers in the parentheses correspond with those in Fig. 1.
cytokine levels and inflammatory cell infiltrations of the host [22] and that babA expression disappeared 6 months after inoculation to Mongolian gerbils [12]. TlpB is a chemoreceptor that detects acidity and urea [34, 35]. Similar to babA, mutants lacking tlpB colonized as good as wild type but caused less inflammations in the stomach of mice and Mongolian gerbils [36, 37]. TlpB accepts posttranslational regulation by small RNA that targets guanin repeat (G-repeat) upstream of the gene [38]. Because expression of tlpB is affected by the G-repeat length, we counted the G-repeat length of our strain. The lengths were 12 for TN2wt, TN2-1M, and TN2-6M and 11 for TN2-3M, which are associated with low level of tlpB expression [38].

Mutations in oppA and tlpB have also been reported [19] (Table 2), but the inoculated animal in this study was a mouse. There were no genes in common with another genome study using the Mongolian gerbil as a model [16]. Another research group compared the H. pylori genome before (PMSS1) and after (SS1) inoculation [19]. They reported that oppA was disrupted in the original strain; we also observed disruption of this gene in the derived strains. The authors also reported a change at the 443rd amino acid in tlpB. Although the details of the mutations were different, these genes may be associated with the host change, since they were observed in independent studies, which occurs rarely by chance.

We previously performed a PCR-based study [12] wherein we examined 20 samples of Mongolian gerbils inoculated with H. pylori. TM2-6M is one of the strains used in the study. Although the disruption of babA by nucleotide deletion/insertion was observed in half of the samples, the deletion/insertion locations and lengths were different. The frequency of disrupted babA increased over time after inoculation. This suggested a possible advantage to losing babA.

Apart from babA, increasing number of nonsense mutations were observed in the current study. The frequencies of the nonsense mutations were 0/6, 2/9, and 3/6 in TN2-1M, TN2-3M, and TN2-6M. Disruption of a gene will not be desirable for the bacteria in its native environment, but it may be selected for if it is advantageous in a new environment. Gene disruption also occurs more easily than gain of a new function by substitution because genes can be broken in various ways, like in babA.

**Mutated loci on the protein structure**

Protein structure data were available for ureI (3UX4) [39] and virB11 (1NLZ) [40]. We downloaded the data and marked the mutated loci on the structure.

UreI channel consists of six protomers that form a hexametric ring. Figure 2 shows the half of the hexametric ring and the location of H131R in each protomer. H131 is located in periplasmic loop 2 (PL2). Previous

### Table 2 Mutations reported by previous studies

| Gene | TN2-3M | TN2-6M | Reference [19] | Reference [12] |
|------|--------|--------|----------------|----------------|
| oppA | del 1 bp (87451) | del 1 bp (87451) | del 1 bp (1279518 PMSS1) | Deletion 0/6 |
| tlpB | G26W, G275W | del 1 bp (175691) | H443R (PMSS1:SS1) | Insertion 4/20 |
| babA | del 1 bp (989679) | | | Substitution 3/20 |

'del' stands for a deletion at the genomic position specified within parenthesis. Description under Ref. [12] is the number of samples that harbored the mutation among 20 samples studied.
study substituted amino acids of various loci in PL2 and reported that H131R hampered urea transportation in *Xenopus laevis* oocytes [41]. Figure 3 shows the location of H314Y in VirB11. VirB11 also form a hexametric assembly. H314Y is located in a b-sheet near the end of the protomer, however, no function is reported about this locus.

Structure data of TlpB was also available but G26W and G275W were outside of the analyzed region. According to protein domain information, G26W is contained in the tm1 (transmembrane helices 1) and G275W is in HAMP (histidine kinase, adenylly cyclase, methyl-binding protein, phosphatase) domain. Tm1 mediates signal transmission across the membrane by piston-like motion of tm2 relative to tm1. HAMP domain is supposed to constitutes a switch region that translates the piston-like motion into a different type of transition within the distal portions [42]. Therefore, mutations G26W and G275W may influence on the function of the chemoreceptor for acidity and urea.

Conclusions

We compared *H. pylori* genomes between original TN2wt and three strains retrieved after inoculation to Mongolian gerbils. We identified mutations in 21 loci of 17 genes of the post-inoculation strains. Mutated genes included *babA*, *tlpB*, and *gltS*, which is known to be associated with adaptation to murine. Other mutations were involved with chemoreceptor, pH regulator, and outer membrane proteins, which also have potential to influence on the adaptation to the new host.

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Authors’ contributions

RS designed the study, carried out the bioinformatic analysis, and wrote the manuscript. TM and JA performed the DNA extraction. AS, MS, and KT carried out DNA sample preparation for sequencing by PacBio. H5 and TH carried out DNA sequencing by PacBio and assembled the whole genome. YY supervised the study, carried out the animal experiment, and reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Genome sequence data of TN2wt is available from GenBank under the Accession number AP019730.

Ethics approval and consent to participate

The animals used in this study were cared for in accordance with our institutional guidelines. Gerbils had free access to food and drinking water throughout the experiment. The experimental protocol was approved by the Animal Care Committee of the Michael E. DeBakey Veterans Affairs Medical Center, Houston, Texas.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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