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Phylogeny of a Novel "*Helicobacter heilmannii*" Organism from a Japanese Patient with Chronic Gastritis Based on DNA Sequence Analysis of 16S rRNA and Urease Genes

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"*Helicobacter heilmannii*" is an uncultivable spiral-shaped bacterium inhabiting the human gastric mucosa. It is larger and more tightly-coiled than *H. pylori*. We encountered a patient with chronic gastritis infected a "*H. heilmannii*"-like organism (HHLO), designated as SH6. Gastric mucosa derived from the patient was orally ingested by specific pathogen free mice. Colonization of the mice by SH6 was confirmed by electron microscopy of gastric tissue specimens. In an attempt to characterize SH6, 16S rRNA and urease genes were sequenced. The 16S rRNA gene sequence was most similar (99.4%; 1,437/1,445 bp) to HHLO C4E from a cheetah. However, the urease gene sequence displayed low similarity (81.7%; 1,240/1,516 bp) with HHLO C4E. Taxonomic analysis disclosed that SH6 represents a novel strain and should constitute a novel taxon in the phylogenetic trees, being discriminated from any other taxon, with the ability of infecting human gastric mucosa.

***Keywords*:** "*Helicobacter heilmannii*", HHLO, phylogeny, phylogenetic tree, 16S rRNA, urease gene

Since the successful cultivation of *Helicobacter pylori* from humans and the elucidation of its association with gastritis and peptic ulceration (Marshall and Warren, 1984), *Helicobacter* spp. have been isolated from an ever-expanding range of host species (Solnick and Schauer, 2001).

Human gastric infections have been documented in association with not only *H. pylori* but also *H. bizzozeronii*, "*H. heilmannii*", and *H. felis* (Solnick *et al*., 1993; Jalava *et al*., 2001; De Bock *et al*., 2007), as well. Among these, "*H. heilmannii*" has not hitherto been successfully cultivated. This has limited characterization of the biochemical, physiological, and pathogenic aspects of the bacterium. Although "*H. heilmannii*" is present in a variety of animals including primates, dogs, cats, and pigs, it is uncommon in humans as compared with *H. pylori.* It has been suggested that "*H. heilmannii*" infections in humans result from zoonotic transmission (Stolte *et al*., 1994). "*H. heilmannii*" has been reported in association with a spectrum of human gastric diseases (Hilzenrat *et al*., 1995; Stolte *et al*., 1997; Okiyama *et al*., 2005), however, "*H. heilmannii*"-associated gastritis appears to be less significant than *H. pylori*-associated gastritis (Hilzenrat *et al*., 1995; Stolte *et al*., 1997; Okiyama *et al*., 2005). "*H. heilmannii*" human infections may be accompanied by chronic active gastritis that resemble *H. pylori*-associated infections in many respects (Morris *et al*., 1990; Heilmann and Borchard, 1991).

Solnick *et al*. (1993) proposed that "*H. heilmannii*" should be subtyped as "*H. heilmannii*" type 1 and type 2 based on differences in the 16S rRNA gene sequence. Recently, O'Rourke *et al*. (2004) and Matsumoto *et al*. (2006) reported on the phylogenetic analysis by DNA sequencing of 16S rRNA in conjunction with partial urease genes of a "*H. heilmannii*"-like organism (HHLO). As far as we know concerning human infections, these are the only reports of phylogenetic analysis by means of DNA sequencing of both 16S rRNA and urease genes in HHLO.

Presently, we have similarly phylogenetically analyzed a HHLO, designated SH6, which was obtained from gastric tissue of a Japanese patient with chronic gastritis. As well electron microscopy was used to obtain information of the organism's ultrastructure.

Materials and Methods

Patient

The patient was a 70-year-old Japanese male who underwent esophagogastroduodenoscopy for medical checkup at the endoscopy unit of the Maruko General Hospital. The procedure disclosed chronic gastritis due to a characteristic spiral-shaped HHLO.

Culture and electron microscopy

Homogenized whole-antral biopsy specimens were given orally to specific-pathogen-free mice through a stomach tube

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as described previously (Dick *et al*., 1989; Lee *et al*., 1989). Gastric mucus scrapings from infected mice were inoculated onto 5% sheep blood agar (Nippon Becton Dickinson, Japan) and chocolate agar (Nippon Becton Dickinson) with no addition of selective agents, and onto Helicobacter-selective agar (Nissui Pharmaceutical, Japan) containing vancomycin, trimethoprim, polymyxin B, and amphotericin B. All media were incubated for up to 14 days at 37°C microaerobically in a humidified atmosphere of 15% CO_2. For transmission electron microscopy (TEM), gastric samples were prepared from a SH6-infected mouse. Samples were fixed and stored in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4°C. After rinsing in the same buffer, the samples were postfixed in phosphate buffered 1.0% OsO_4, dehydrated through a graded series of alcohol, immersed in propylene oxide, and embedded in an epoxy resin. For histopathological evaluation, thick sections (approximately 1 μm) were prepared and stained with a toluidine blue solution. Ultrathin sections were then prepared from trimmed blocks and collected on single-slot copper grids covered with a Formvar film, and stained with uranyl acetate and lead citrate. The ultra-thin sections were examined using a Jeol JEM1230 transmission electron microscope operating at 80 kV acceleration voltage.

DNA extraction

DNA was extracted directly from the gastric mucosa of infected mice using the Perfect gDNA blood mini kit (Eppendorf AG, Germany). Extracted DNA was preserved at -20°C until use.

Polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA and urease genes

PCR amplification was carried out in a total reaction volume of 20 μl that was comprised of 2 μl of sample DNA and 18 μl of a reaction mixture containing 50 mM KCl, 20 mM Tris-HCl, pH 8.3, 3.0 mM $MgCl_2$, gelatin 0.01% (w/v), 1.0 U AmpliTaq Gold (Applied Biosystems, USA), 0.2 mM dNTPs, and 0.5 μM of each primer. Amplification was performed using a Mastercycler EP gradient S thermal cycler (Eppendorf). PCR products were separated on 3% agarose gels in 10 mM Tris/borate, 1 mM EDTA (TBE) and photographed under ultraviolet transillumination after staining with ethidium bromide. To detect Helicobacter-specific DNA, the near-complete 16S rRNA gene was amplified and sequenced using primers 27F with H676R and H276F with 1494R (O'Rourke *et al*., 2004). PCR was carried out as described previously (O'Rourke *et al*., 2004). PCR amplification of the HHLO *ureA* and *ureB* genes was performed using previously described U430F primers (O'Rourke *et al*., 2004) with UH6R2, which were presently designed in this study (Table 1). The cycling conditions for the *ureA* and *ureB* genes were as follows; initial denaturation at 95°C for 10 min; 35 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1.5 min; followed by a final extension step of 72°C for 5 min. The products were then sequenced with primers as described previously (O'Rourke *et al*., 2004). As tabulated in Table 1, further oligonucleotides for sequencing of this region were designed by primer walking.

DNA sequencing and phylogenetic analysis

PCR products were purified using the Qiaex II gel extraction kit (QIAGEN, USA). In a subsequent study, fluorescent sequencing templates were produced from each PCR product using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing products were then separated using a Model 3100 DNA sequencer (Applied Biosystems). Both strands of DNA were sequenced with contiguous overlaps. DNA sequences of both the 16S rRNA and urease genes from SH6 and from the reference organism were aligned using CLUSTAL W software (Thompson *et al*., 1994) and the resulting alignment was modified to remove regions containing gaps. A phylogenetic tree was constructed according to the neighbor-joining method (Saitou and Nei, 1987) and the Jukes-Cantor model (Jukes and Cantor, 1969). The stability of relationships was assessed by a bootstrap analysis comprising of 1,000 data settings.

Results

Cultural examinations and morphological properties

Endoscopic mucosal biopsies from the gastric antrum of the patient demonstrated chronic gastritis with mild mononuclear cell infiltration (Fig. 1A). The gastric surface mucous cells failed to show epithelial degeneration such as cellular drop out, exfoliation, or budding (Fig. 1B). We were successful in infecting specific-pathogen-free mice by oral inoculation through a stomach tube. However, repeated attempts to cultivate SH6 *in vitro* were unsuccessful. Therefore, SH6 obtained from the human patient was maintained by use of *in vivo* culture technique. In the stomach of SH6-infected mice, the bacteria were present in the mucous gel or on the surface mucous cells without adhesion to gastric epithelial cells (Fig. 2). The helical bacteria observed in the mucus layer were long (5~6 μm×0.5~0.6 μm) and possessed 3~6 polar flagella The presence of long, tightly coiled

Table 1. Oligonucleotide primers used for amplification and sequencing reactions of urease gene

Primer	Gene amplified	Purpose	Sequence (5'→3')	Reference
U430F	urease	PCR and sequencing	GCKGAWTTGATGCAAGAAGG	O'Rourke *et al*. (2004)
U850F	urease	sequencing	CAGCTGTGCGCTTTGAACCT	O'Rourke *et al*. (2004)
U1050R	urease	sequencing	TCTTCGCCATAAGTGGTGC	O'Rourke *et al*. (2004)
UH6F3	urease	sequencing	GCTGATGGTACKAATGCGAC	This study
UH6F4	urease	sequencing	TACGCTTAATGAGGCCTGC	This study
UH6R2	urease	PCR and sequencing	TTATCCAAGTGGTGGCACACC	This study

K corresponds to G or T; W corresponds to A or T

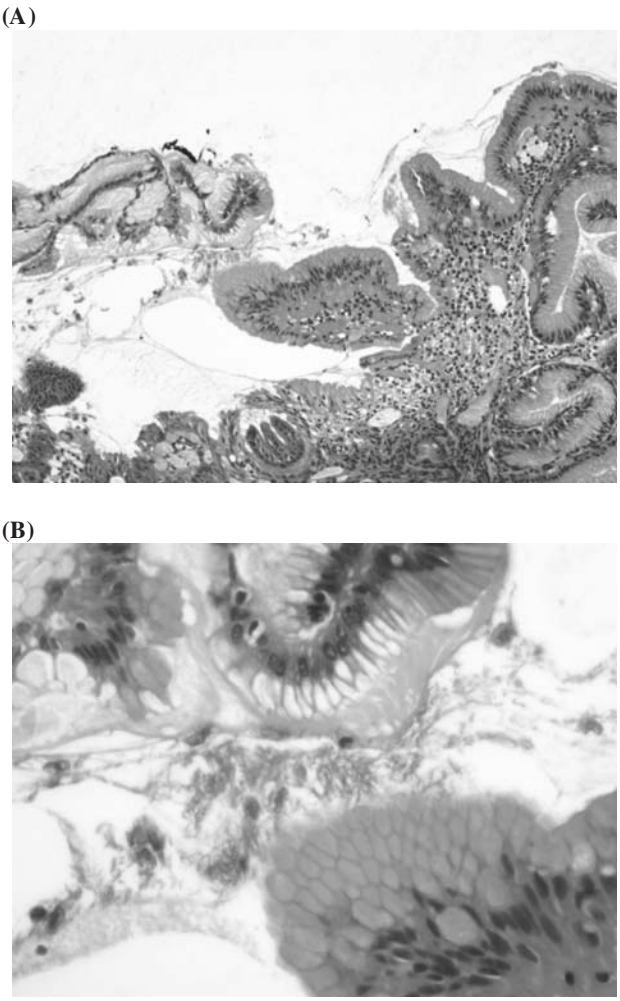

Fig. 1. Histological findings of "*H. heilmannii*" SH6-infected chronic gastritis in a gastric biposy specimen from the patient. (A) Surface mucous cells showed no degenerative changes. Mild mononuclear cells were present in the lamina propria. "*H. heilmannii*" SH6 was present in small groups in gastric pits and (B) not in contact with surface mucous cells.

bacteria within parietal cell canaliculi was observed. The size and shape of the bacteria were much more consistent with HHLO than *H. pylori*. Despite careful TEM observation, periplasmic fibrils were not evident (Fig. 2).

Analysis of 16S rRNA gene

The 16S rRNA gene was amplified by PCR with primers 27F with H676R and H276F with 1494R obtained from SH6-derived DNA isolated from infected mice. The obtained sequence represented virtually the entire 16S rRNA gene with 1,445 bp (GenBank/EMBL/DDBJ accession no. AB462257) of readable sequence determined from the DNA. The SH6 sequence created a distinct sub-group within the *Helicobacter* lineage, together with other gastrospirilla; *H. felis*, *H. bizzozeronii*, *H. salomonis*, and "*H. heilmannii*" types 1 and 2. High sequence similarities (99.4%; 1,437/1,445 bp) were noted with both HHLO C4E AF506778 and C2S AF506775 from the cheetah. In contrast, the similarities to other gastrospirillum-like bacteria were comparatively low; *H. felis* M57398 (98.2%; 1,419/1,445 bp), *H. bizzozeronii* Y09404 (97.7%; 1,391/1,423 bp), *H. salomonis* AY366430 (96.3%; 1,268/1,316 bp), "*H. heilmannii*" type 1 AF506784 (96.6%; 1,396/1,445 bp), and "*H. heilmannii*" type 2 AF506786 (98.1%; 1,418/1,445 bp) repectively. A phylogenetic tree for 16S rRNA gene based on this analysis, shown in Fig. 3, positioned SH6 proximate to *H. felis*, *H. bizzozeronii*, *H. salomonis*, and "*H. heilmannii*" type 2.

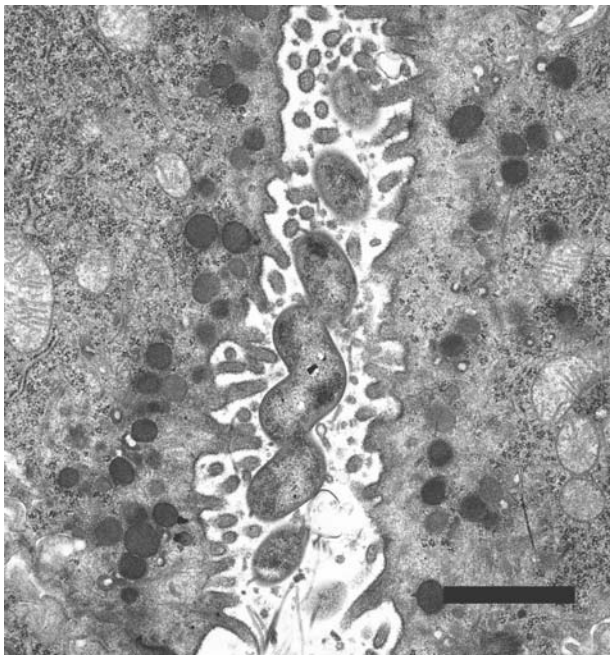

Fig. 2. Ultrastructure of "*H. heilmannii*" SH6 colonized mouse gastric mucosa. "*H. heilmannii*" SH6 displayed a tightly coiled spiral configuration with flagella and were positioned on the apical surface of surface mucous cells. Bar=1.0 μm.

Analysis of urease gene

SH6 urease gene was PCR amplified using primers U430F and UH6R2. The latter primer was designed based on the consensus region in the urease gene sequences for both *H. pylori* and "*H. heilmannii*" type 2 (GenBank/EMBL/DDBJ accession no. AF508016 and AF508012, respectively), which were capable of amplifying the 1,531 bp region of the urease gene complex. The readable 1,483 bp sequence represented a partial urease gene (GenBank accession no. AB462258). Additional oligonucleotide-primers designed by primer walking (Table 1) were used for sequencing of this region as described previously (O'Rourke *et al*., 2004). Comparatively high similarity of the DNA sequence to that of HHLO C4E AF508000 was observed (81.7%; 1,240/1,516 bp). On the contrary, similarities to other gastrospirillum-like bacteria were rather low; 80.0% (1,214/1,512 bp) for *H. felis* CS2 AY368259, 79.6% (1,180/1,482 bp) for *H. bizzozeronii* AF508003, 80.2% (1,204/1,500 bp) for *H. salomonis* AF508005, 76.9% (1,161/1,508 bp) for "*H. heilmannii*" type 1 AF508010, and 78.8% (1,192/1,512 bp) for "*H. heilmannii*" type 2 AF508012. A

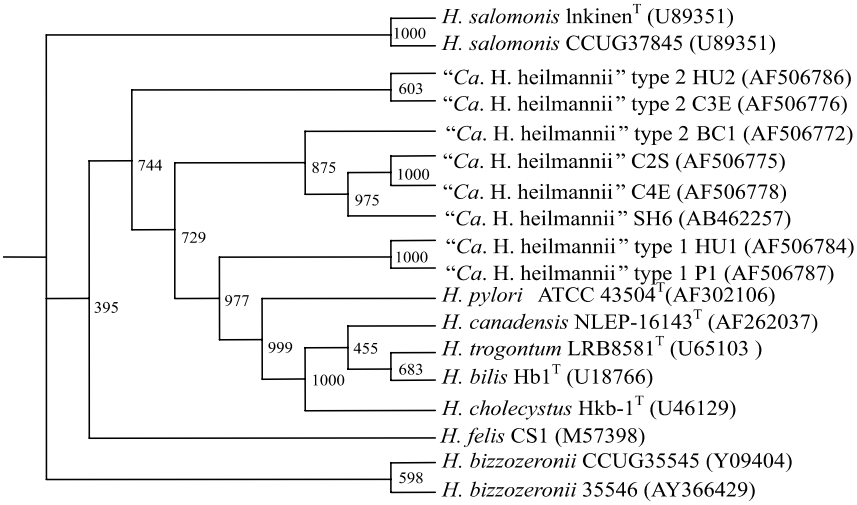

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences demonstrating the relationship between *Helicobacter* spp. from animals and previously validated or provisional *Helicobacter* species. The number in parentheses indicates the GenBank accession no.

phylogenetic tree was constructed on the basis of urease gene analysis (Fig. 4). SH6 possessed an intergenic 3 bp spacer region between *ureA* and *ureB*. At the amino acid sequence level, UreA from SH6 was highly similar to UreA from "*H. heilmannii*" type 1 (81.8%), "*H. heilmannii*" type 2 (86.3%), and *H. felis* CS2 (88.5%). UreB, which contains the putative active site for the enzyme (Labigne *et al*., 1991), showed 92%, 91.3%, and 95% amino acid identity with UreB from "*H. heilmannii*" type 1, "*H. heilmannii*" type 2, and *H. felis* CS2, respectively (Fig. 5).

Discussion

Infection with "*Candidatus* H. heilmannii" in humans is associated with gastritis and mucosa-associated lymphoid tissue lymphoma, and is considered to be communicated by zoonotic transmission from dogs, cats or pigs infected with HHLO (Stolte *et al*., 1994; Yeomans and Kolt, 1996; Meining *et al*., 1998; Svec *et al*., 2000).

The 16S rRNA gene is well-known as an appropriate chronometer for bacterial phylogenetic investigation. However, the sequences of the urease genes *ureA* and *ureB* are much more discriminatory than the 16S rRNA gene sequence, and considered to be just useful for phylogenetic analysis of gastric *Helicobacter* species (O'Rourke *et al.*, 2004). Presently, the SH6 16S rRNA gene sequence exhibited 99.4% (1,437/1,445 bp) sequence similarity to HHLO C4E and C2S from the cheetah. The percent sequence similarity to "*H. heilmannii*" type 2 was higher in comparison with that of "*H. heilmannii*" type 1. In the subsequent phylogenetic analysis based on urease gene sequencing, however, SH6 exhibited a sequence similarity of 81.7% (1,240/1,516 bp) to HHLO C4E from the cheetah. Although the human-derived SH6 was related to *H. felis* CS2 80.0% (1,194/1,492 bp), it is noteworthy that the urease sequence of human SH6 is less to HHLO C4E, *H. felis*, and the other HHLOs. This im-

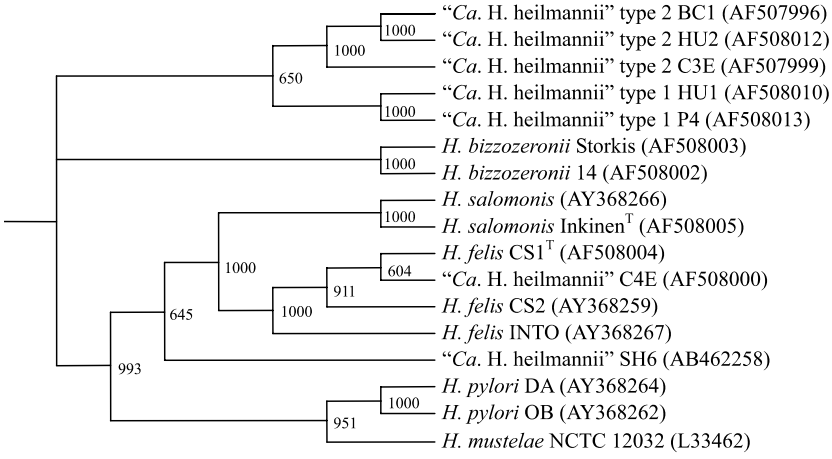

Fig. 4. Phylogenetic tree based on the partial *ureA* and *ureB* gene sequences for the SH6 and other urease-positive species of the genus *Helicobacter*. The number in parentheses indicates the GenBank accession no.

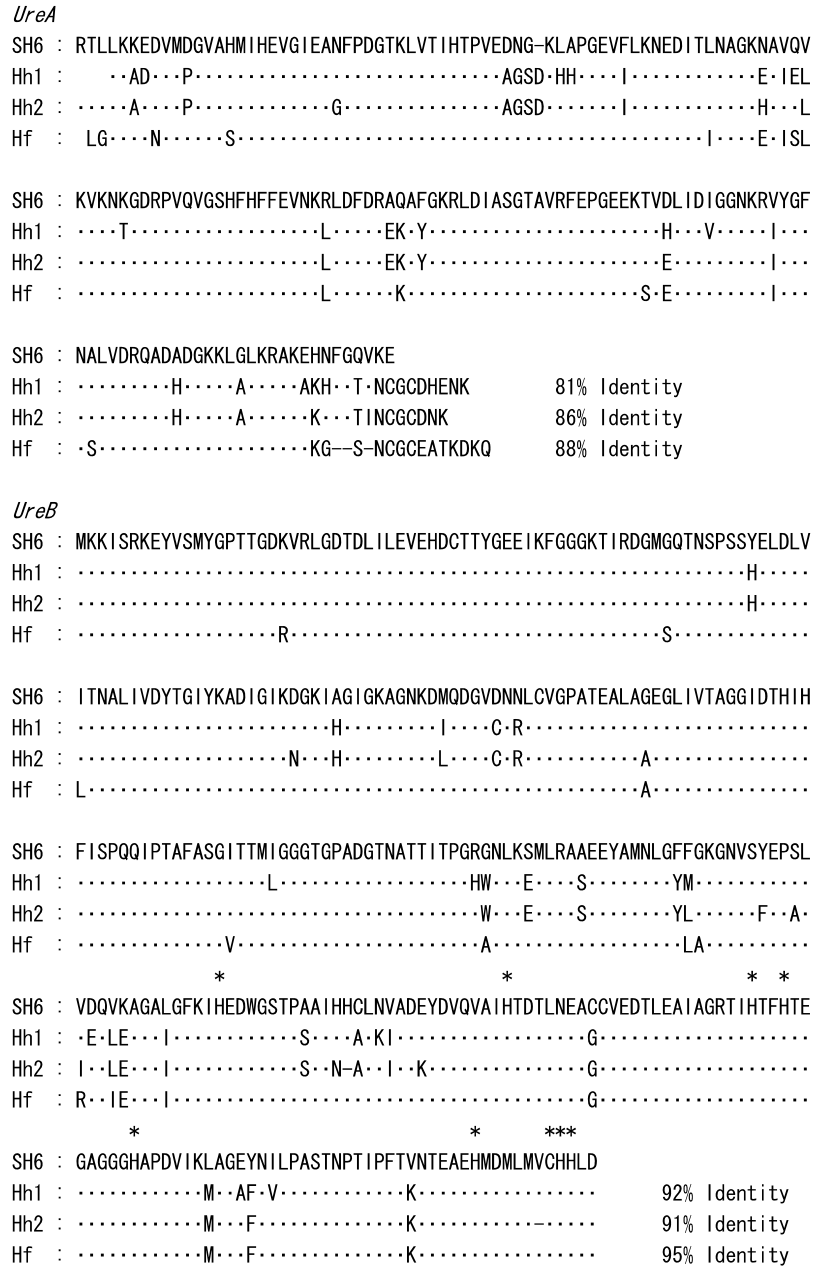

Fig. 5. Predicted amino acid sequences of a partial *ureA* and *ureB* from SH6 aligned with the corresponding predicted sequences from "*H. heilmannii*" type 1 (Hh1), "*H. heilmannii*" type 2 (Hh2) and *H. felis* (Hf). Sequence identity with SH6 strain is indicated by small dots; gaps in sequence to optimize alignment are indicated by dashes. Percentages indicate percent amino acid identity compared with SH6 strain. Asterisk (*) are shown above the conserved histidine and cysteine residues in UreB.

plies that SH6 may be a novel HHLO.

The intergenic spacer regions between *ureA* and *ureB* have been reported to be 3 bp for *H. pylori*, 9 bp for *H. felis*, and 14 bp for "*H. heilmannii*" (Labigne *et al*., 1991; Ferrero and Labigne, 1993; O'Rourke *et al*., 2004). The presently determined *H. pylori* intergenic spacer region of 3 bp agrees well with other reports (Labigne *et al*., 1991; Ferrero and Labigne, 1993). However, it should be noted that the base-composition in the intergenic spacer region of SH6 (GAA) is entirely different from that of *H. pylori* (TGC). Based solely upon the sequence similarity of the urease gene, SH6 strain should possibly be related to HHLO C4E among the *Helicobacter* spp. investigated, which conforms with the result obtained on the basis of 16S rRNA gene sequence analysis. However, considering the low (81.7%) urease gene sequence homology together with the careful analyses of these genetic findings, it seems much more likely that SH6 is unrelated to any other known HHLO, and, therefore, should be considered as a novel species.

Helicobacter species capable of infecting gastric mucosa

of animals unexceptionally possess urease activities, and urease-negative mutants are unable to establish a stomach infection (Tsuda *et al*., 1994; Karita *et al*., 1995). Urease should thus be considered as essential for gastric colonization. Therefore, although the urease sequence of SH6 was obviously different from that of either *H. pylori* or "*H. heilmannii*", there is little doubt that SH6 possesses urease activity, considering that it was capable of colonizing the gastric mucosa of a patient with chronic gastritis.

The eight histidine residues and one cystein residue believed to play an important role in nickel binding and enzymatic activity at the active site (Labigne *et al*., 1991) were well-conserved in the SH6 UreB polypeptide (Fig. 5). The marked conservation of the SH6 histidine residues implies that the expression of urease is a pathogenic factor for gastric *Helicobacter* species. Moreover, the notable differences of both the urease gene sequence and the intergenic spacer region of SH6 is further evidence of its identity as a novel HHLO of human origin.

Despite the histologically confirmed presence of HHLOs, upwards of 40% of paraffin-embedded gastric biopsy specimens were previously demonstrated to be negative by the PCR assays using the 16S rRNA regions (De Groote *et al*., 2005). Our attempts to detect HHLO in SH6-infected gastric samples from mice utilized the same primers. The fact that we did not detect SH6 in gastric samples from SH6-infected mice might not reflect absence of the organism. Indeed, as-yet unknown and undetectable *Helicobacter* species could be involved in HHLO-induced disease (De Groote *et al*., 2005). The present morphological and phylogenetic analyses indicate that SH6 represents a novel "*H. heilmannii*" associated with chronic human gastritis.

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References

De Bock, M., K. Van Den Bulck, A. Hellemans, S. Daminet, J.C. Coche, J.C. Debongnie, A. Decostere, F. Haesebrouck, and R. Ducatelle. 2007. Peptic ulcer disease associated with *Helicobacter felis* in a dog owner. *Eur. J. Gastroenterol. Hepatol.* 19, 79-82.

De Groote, D., L.J. Van Doorn, K. Van den Bulck, P. Vandamme, M. Vieth, M. Stolte, J.C. Debongnie, A. Burette, F. Haesebrouck, and R. Ducatelle. 2005. Detection of non-*pylori Helicobacter* species in "*Helicobacter heilmannii*"-infected humans. *Helicobacter* 10, 398-406.

Dick, E., A. Lee, G. Watson, and J. O'Rourke. 1989. Use of the mouse for the isolation and investigation of stomach-associated, spiral-helical shaped bacteria from man and other animals. *J. Med. Microbiol.* 29, 55-62.

Ferrero, R.L. and A. Labigne. 1993. Cloning, expression and sequencing of *Helicobacter felis* urease genes. *Mol. Microbiol.* 9, 323-333.

Heilmann, K.L. and F. Borchard. 1991. Gastritis due to spiral shaped bacteria other than *Helicobacter pylori*: clinical, histological, and ultrastructural findings. *Gut* 32, 137-140.

Hilzenrat, N., E. Lamoureux, I. Weintrub, E. Alpert, M. Lichter, and L. Alpert. 1995. *Helicobacter heilmannii*-like spiral bacteria in gastric mucosal biopsies. Prevalence and clinical significance. *Arch. Pathol. Lab Med.* 119, 1149-1153.

Jalava, K., S.L.W. On, C.S. Harrington, L.P. Andersen, M.L. Hanninen, and P. Vandamme. 2001. A cultured strain of "*Helicobacter heilmannii*", a human gastric pathogen, identified as H. bizzozeronii: evidence for zoonotic potential of *Helicobacter*. *Emerg. Infect. Dis.* 7, 1036-1038.

Jukes, T.H. and C.R. Cantor. 1969. Evolution of protein molecules, vol. 3. Mammalian protein metabolism, p. 21-132. Academic Press, Inc., New York, N.Y., USA.

Karita, M., M. Tsuda, and T. Nakazawa. 1995. Essential role of urease *in vitro* and *in vivo Helicobacter pylori* colonization study using a wild-type and isogenic urease mutant strain. *J. Clin. Gastroenterol.* 21 Suppl 1, S160-163.

Labigne, A., V. Cussac, and P. Courcoux. 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. *J. Bacteriol.* 173, 1920-1931.

Lee, A., R.P. Eckstein, D.I. Fevre, E. Dick, and J.E. Kellow. 1989. Non *Campylobacter pylori* spiral organisms in the gastric antrum. *Aust. NZJ Med.* 19, 156-158.

Marshall, B.J. and J.R. Warren. 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1, 1311-1315.

Matsumoto, T., M. Kawakubo, H. Ota, M. Shiohara, T. Kumagai, K. Yamauchi, T. Kastuyama, and Y. Kawakami. 2006. Phylogenetic analysis of "*Helicobacter heilmannii*" isolates from different Japanese patients based on DNA sequences of 16S rRNA and urease genes. *Helicobacter* 11, 409.

Meining, A., G. Kroher, and M. Stolte. 1998. Animal reservoirs in the transmission of *Helicobacter heilmannii*. Results of a questionnaire-based study. *Scand. J. Gastroenterol.* 33, 795-798.

Morris, A., M.R. Ali, L. Thomsen, and B. Hollis. 1990. Tightly spiral shaped bacteria in the human stomach: another cause of active chronic gastritis? *Gut* 31, 139-143.

Okiyama, Y., K. Matsuzawa, E. Hidaka, K. Sano, T. Akamatsu, and H. Ota. 2005. *Helicobacter heilmannii* infection: clinical, endoscopic and histopathological features in Japanese patients. *Pathol. Int.* 55, 398-404.

O'Rourke, J.L., J.V. Solnick, B.A. Neilan, K. Seidel, R. Hayter, L.M. Hansen, and A. Lee. 2004. Description of '*Candidatus* Helicobacter heilmannii' based on DNA sequence analysis of 16S rRNA and urease genes. *Int. J. Syst. Evol. Microbiol.* 54, 2203-2211.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406-425.

Solnick, J.V., J. O'Rourke, A. Lee, B.J. Paster, F.E. Dewhirst, and L.S. Tompkins. 1993. An uncultured gastric spiral organism is a newly identified *Helicobacter* in humans. *J. Infect. Dis.* 168, 379-385.

Solnick, J.V. and D.B. Schauer. 2001. Emergence of diverse *Helicobacter* species in the pathogenesis of gastric and enterohepatic diseases. *Clin. Microbiol. Rev.* 14, 59-97.

Stolte, M., G. Kroher, A. Meining, A. Morgner, E. Bayerdorffer, and B. Bethke. 1997. A comparison of *Helicobacter pylori* and *H. heilmannii* gastritis. A matched control study involving 404 patients. *Scand. J. Gastroenterol.* 32, 28-33.

Stolte, M., E. Wellens, B. Bethke, M. Ritter, and H. Eidt. 1994. *Helicobacter heilmannii* (formerly *Gastrospirillum hominis*) gastritis: an infection transmitted by animals? *Scand. J. Gastroenterol.* 29, 1061-1064.

Svec, A., P. Kordas, Z. Pavlis, and J. Novotny. 2000. High prevalence of *Helicobacter heilmannii*-associated gastritis in a small, predominantly rural area: further evidence in support of a zoonosis? *Scand. J. Gastroenterol.* 35, 925-928.

Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673-4680.

Tsuda, M., M. Karita, M.G. Morshed, K. Okita, and T. Nakazawa. 1994. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. *Infect. Immun.* 62, 3586-3589.

Yeomans, N.D. and S.D. Kolt. 1996. *Helicobacter heilmannii* (formerly *Gastrospirillum*): association with pig and human gastric pathology. *Gastroenterology* 111, 244-247.