Phylogeny of Helicobacter felis sp. nov., Helicobacter mustelae, and Related Bacteria


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Strain CS1T (T = type strain) is a gram-negative, microaerophilic, urease-positive, spiral-shaped bacterium that was isolated from the gastric mucosa of a cat. Additional strains which possessed biochemical and ultrastructural characteristics similar to those of strain CS1T were isolated from the gastric mucosa of cats and dogs. The guanine-plus-cytosine content of the DNA of strain CS1T was 42.5 mol%. The 16S rRNA sequences of strain CS1T, strain DS3 (a spiral-shaped isolate from a dog), and Helicobacter mustelae were determined by direct RNA sequencing, using a modified Sanger method. These sequences were compared with the 16S rRNA sequences of Helicobacter pylori, "Flexispira rappini," Wolinella succinogenes, and 11 species of campylobacters. A dendrogram was constructed based upon sequence similarities. Strains CS1T and DS3 were very closely related (level of similarity, 99.3%). Two major phylogenetic groups were formed; one group consisted of strains CS1T and DS3, H. mustelae, H. pylori, "F. rappini," and W. succinogenes, and the other group contained the true campylobacters. The average level of similarity between members of these two groups was 84.9%. Within the first group, strains CS1T and DS3, H. pylori, and H. mustelae formed a cluster of organisms with an interspecies similarity level of 94.5%. The phylogenetic positions of W. succinogenes and "F. rappini" were just outside this cluster. On the basis of the results of this study, we believe that strains CS1T (= ATCC 49179T) and DS3 represent a new species of the genus Helicobacter, for which we propose the name Helicobacter felis.

Campylobacter species commonly colonize the alimentary and genital tracts of mammals and birds, and some are responsible for gastrointestinal diseases (32). It has been proposed that the mucus lining the gut mucosa is the ecological niche inhabited by these bacteria (22, 25). Recently, increased attention has been focused on the species isolated from the stomachs of mammals, including humans (14, 24). The gastric mucus appears to be the natural habitat of Helicobacter pylori, a bacterium associated with histological gastritis in human stomachs (27, 31), and Helicobacter mustelae, an organism associated with gastritis and ulcers in adult ferrets (9, 10). On the basis of 16S rRNA sequencing data and biochemical and phenotypic criteria, these two species were recently transferred from the genus Campylobacter to the genus Helicobacter (13). The overall phylogeny of members of the genus Campylobacter and related bacteria has been studied extensively (21, 34, 36, 40). These bacteria fall into three phylogenetic groups. The first group comprises the true campylobacters, including Campylobacter jejuni, subspecies of Campylobacter fetus, Campylobacter hydrogenotrophus, Campylobacter concisus, Campylobacter mucosalis, Campylobacter sp., Campylobacter coli, Campylobacter lari, Wolinella recta, Wolinella curva, and two misclassified bacteroides, Bacteroides gracilis and Bacteroides ureolyticus. The second group contains H. pylori, H. mustelae, Wolinella succinogenes, and two misclassified campylobacters, Campylobacter cinaedi and Campylobacter fennelliae (13, 40; unpublished data). A third group, which presently consists of only two species, Campylobacter cryaerophilus and Campylobacter nitrofigilis, branches off from the other two groups at a deeper phylogenetic level (40).

Many animal species, such as dogs, cats, and primates, have their own distinctive gastric floras (11, 23). A gram-negative, microaerophilic, urease-positive, spiral-shaped rod (strain CS1T = type strain) was isolated from the gastric mucosa of a cat and has been described previously (28). Strain CS1T is physiologically similar to, but morphologically different from, H. pylori and H. mustelae. The gastric bacteria isolated thus far have different distinctive morphologies with respect to cell shape and number and arrangement of flagella (11, 17).

In this study, six additional bacterial strains were isolated from the gastric mucosa of dogs and cats. These isolates were similar to strain CS1T with respect to ultrastructural and biochemical characteristics. The partial 16S rRNA sequences for the cat gastric spiralum (strain CS1T) and for one dog isolate (strain DS3) were determined in order to establish their phylogenetic position among the campylobacters, H. pylori, and related bacteria. As discussed below, the name Helicobacter felis is proposed for these spirillumlike organisms, and we refer to them below by the proposed name. In addition, we present the 16S rRNA sequence of H. mustelae since the phylogenetic relationship of this organism to other bacteria was uncertain previously.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains of H. felis were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% sterile fetal calf serum (Sigma Chemical Co., St. Louis, Mo.) and an antibiotic combination consisting of vancomycin (10 ng/ml), polymyxin (2.5 µg/ml), trimethoprim (5 µg/ml), and amphotericin (2 µg/ml). Flasks containing liquid media were evacuated once to a level of 26 in. (ca. 66 cm) of Hg and filled with a 5% CO2-5% O2-90% N2 atmosphere in order to give a final O2 concentration of approximately 5%. Flasks were shaken at 150 rpm for 3 to 5 days at 37°C.
H. mustelae ATCC 43772 was grown as previously described by Fox et al. (12).

Isolation of H. felis strains. H. felis CS1, CS2, CS5, and CS6 were isolated from the stomachs of individual cats, and strains DS1, DS2, and DS3 were isolated from the stomachs of individual dogs. Cats and dogs were obtained from the Animal Breeding and Holding Unit at the University of New South Wales. The health status of animals was not determined. The ages, sexes, and breeds of the animals varied. The animals were euthanized, and the stomachs were removed for bacterial culture. The procedures used for isolation and initial cultivation have been described previously (27). Briefly, mucus scrapings of gastric mucosa were streaked onto lysed horse blood agar (blood agar base no 2; Oxoid, Basingstoke, United Kingdom) supplemented with antibiotics as described above. After 4 and 7 days of microaerophilic incubation at 37°C, the plates were examined for spreading colonies on the agar surfaces.

Biochemical characterization. Phenotypic tests commonly used to biotype campylobacters were performed, and in some instances the procedures were modified as described by Benjamin et al. (2). Oxidase and catalase activities were assayed as described previously (2). Urease was tested by the microtiter method as described by Hazell et al. (15). H₂S production was measured by using a modification of the method of Skirrow and Benjamin (39). Briefly, a large loopful of bacterial cells was inoculated as a lump into semisolid agar deeps consisting of nutrient broth no 2, 0.6% (wt/vol) bacteriological agar, 0.1% (wt/vol) yeast extract, 0.05% (wt/vol) ferrous sulfate, 0.05% (wt/vol) sodium metabisulfite, and 0.05% (wt/vol) sodium pyruvate. After 4 h of incubation at 37°C, H₂S production was indicated by the presence of blackening caused by the formation of iron sulfide. The hippurate hydrolysis test was performed by using the protocol of Hwang and Ederer (16). Commercially available kits for enzyme analysis were utilized. The two systems used were AN-DENT strips (API Analytab Products, Plainview, N.Y.) and Rosco Diagnostica tablets (Rosco Diagnostica, Taastrup, Denmark). These kits were used according to the manufacturers' instructions. Rosco Diagnostica tablets were also used for determining carbohydrate utilization.

Cultures were grown under aerobic conditions, microaerophilic conditions (Oxoid type HP11 anaerobic jar equipped with a model BR56 Campylobacter gas-generating kit and catalyst), and anaerobic conditions (Oxoid anaerobic jar equipped with a model BR38 anaerobic gas-generating kit and catalyst) at 37°C. According to the manufacturer, the use of a model BR56 microaerophilic gas-generating kit provides an oxygen concentration of about 6%. Cultures were also incubated at three temperatures (25, 37, and 42°C) in a microaerophilic atmosphere. Growth on lysed horse blood agar plates was determined after 72 h of incubation.

Tolerance to 1.0% (wt/vol) glycine and tolerance to 1.5% (wt/vol) NaCl were determined by cultivating organisms on lysed horse blood agar plates supplemented with each compound. The plates were incubated for 72 h under microaerophilic conditions.

Susceptibility to antimicrobial agents. The surfaces of lyzed horse blood agar plates were inoculated with a swab which had been moistened with a heavy suspension of bacteria (ca. 10⁹ cells per ml). The plates were dried gently in a laminar flow cabinet, and susceptibility disks (Oxoid) containing nalidixic acid (30 μg) and cephalothin (30 μg) were placed onto the agar surfaces. Resistance to these antimicrobial agents was determined by the absence of a clear zone of inhibition after 48 h (for C. jejuni) or 72 h (for the helicobacters tested) of incubation.

Electron microscopy. Negatively stained samples, ultrathin sections, and freeze-etched replicas for transmission electron microscopy were prepared as previously described (28, 35). Specimens were examined by using an Hitachi model 7000 transmission electron microscope.

G+C content of DNA. The guanine-plus-cytosine (G+C) contents of DNA were determined by thermal denaturation analysis as described by Breznak and Canale-Parola (3) and were calculated by using the equation of De Ley (6). DNA isolated from Escherichia coli K-12 was used for control determinations.

Isolation and purification of rRNAs. rRNAs were isolated and partially purified by a modification of the procedure of Puce et al. (33), as previously described (34).

16S rRNA sequencing. rRNA sequences were determined by using a modification of the standard Sanger dideoxy chain termination technique in which primers complementary to conserved regions of the 16S RNA sequences were elongated by the enzyme reverse transcriptase (20). Seven primers were used to obtain nearly complete sequences for H. felis and H. mustelae. Additional modification of these procedures have been described previously (34).

Data analysis. A program set for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and phylogenetic tree construction for 16S rRNA data was written in Microsoft QuickBASIC for use on IBM PC-AT and compatible computers. RNA sequences were entered and aligned as previously described (34). Presently, our data base, which contains RNA sequences for approximately 250 different bacterial strains, comprises sequences determined in our laboratory, previously published sequences, and unpublished sequences provided by other investigators. Similarity matrices were constructed by comparing only those regions that could be unambiguously aligned. Dendrograms were constructed by using the modified unweighted pair group method of Li (29).

GenBank accession numbers. The sequences of the microorganisms which we investigated are available for electronic retrieval from GenBank under accession numbers M37642 (for H. felis CS1), M37643 (for H. felis DS3), and M35048 (for H. mustelae ATCC 43772).

RESULTS AND DISCUSSION

Several additional bacterial strains that were ultrastructurally and physiologically similar to H. felis CS1 were isolated from the gastric mucosa of cats (strains CS2, CS5, and CS6) and dogs (strains DS1, DS2, and DS3). All of the isolates were motile, gram-negative, spiral-shaped bacteria which possessed periplasmic fibers that wrapped around the cell body (Fig. 1). All strains were microaerophilic and asaccharolytic (they did not ferment glucose, maltose, mannitol, lactose, ribose, and D-xylose). Key phenotypic traits that differentiate H. felis from closely related bacteria are shown in Table 1. Phenotypic traits that differentiate H. felis from other Helicobacter species include its tightly helical ultrastructure, its ability to reduce nitrate to nitrite, its resistance to nalidixic acid, its susceptibility to cephalothin, and its growth at 42°C (Table 1). The G+C content of the DNA of strain CS1 was 42.5 ± 0.5 mol%.

We determined approximately 95% of the total sequence for H. felis CS1 and DS3 and H. mustelae. The sequences of H. felis CS1 and DS3 and H. mustelae are shown in Fig. 2 aligned with the sequence of E. coli. Table 2 shows a
FIG. 1. Electron micrographs of pure cultures of *H. felis* sp. nov., showing the characteristic spiral morphology, multiple flagella and periplasmic fibers. (A) Negatively stained cell of strain DS3. Bar = 0.25 μm. (B and C) Thin sections of strain CSIT<sup>T</sup>. Bars = 0.25 μm. (D) Freeze-etched replica of strain CSIT<sup>T</sup>. Bar = 0.1 μm.

similarity matrix derived from approximately 1,200 base comparisons for the 16S rRNA sequences of *H. felis* CSIT<sup>T</sup> and DS3, *H. mustelae*, *Campylobacter* species, and phylogenetically related species that have been described previously (21, 34, 36, 40). The nearly complete sequence for "*Flexispira rappini*" (34a) was included in the phylogenetic analyses for comparative purposes. "*F. rappini*," which has not been named formally (3a), is a gram-negative organism that was first isolated from aborted ovine fetuses (4, 19). A dendrogram constructed from the similarity data is shown in Fig. 3, which is an expanded and updated version of a phylogenetic tree published previously (34). Although only partial sequences (approximately 860 bases [36]) of *C. coli*, *C. jejuni*, and *C. lari* were available for construction of the dendrogram, these organisms were included in the tree to illustrate their phylogenetic positions. The exact branching positions of these organisms may change slightly when more complete sequences become available. The organisms which we investigated were divided into two major phylogenetic groups; one group comprised the gastric bacteria (i.e., *H. felis* CSIT<sup>T</sup> and DS3, *H. pylori*, *H. mustelae*, "*F. rappini*," and *W. succinogenes*), and the other group contained the remaining organisms (the true campylobacters). The average level of similarity between members of these two groups was 84.9%. The phylogenetic relationships between these organisms and distantly related bacterial species, such as *E. coli* and *B. fragilis*, revealed average levels of similarity of only 76.9 and 72.0%, respectively (data not shown).

As Fig. 3 shows, *H. felis* CSIT<sup>T</sup>, *H. pylori*, and *H. mustelae* formed a cluster with an average interspecies similarity level of 94.5%. These data indicate that *H. felis* belongs in the newly formed genus *Helicobacter* (13). *H. felis* CSIT<sup>T</sup>, which was isolated from a cat, and *H. felis* DS3, which was isolated from a dog, were very closely related (level of similarity, 99.3%), indicating that these organisms are two strains of the same species. Previous studies that demonstrated DNA
homology between \textit{H. mustelae} and \textit{H. pylori} either were inconclusive (13) or produced conflicting results (9, 12). The 16S rRNA sequence analysis provided definitive molecular evidence that \textit{H. mustelae} is related to \textit{H. pylori} and thus belongs the genus \textit{Helicobacter}. \textit{W. succinogenes} was more distantly related to this cluster (average level of similarity, 91.8%). As suggested by Goodwin et al. (13), \textit{W. succinogenes} is sufficiently different from \textit{Helicobacter} species with regard to phylogeny, phenotypic characteristics, antibiotic susceptibility, and biochemical traits that a separate genus designation is warranted. However, on the basis of phylogenetic data, \textit{W. succinogenes} does indeed belong in the same family as the helicobacters.

On the basis of the results of this study, the exact phylogenetic position of "\textit{F. rappini}" is unclear. Although this species is most closely related to \textit{H. mustelae} (level of similarity, 95.5%), it is also closely related to \textit{W. succinogenes} (level of similarity, 93.1%). These data suggest that "\textit{F. rappini}" should be included in the genus \textit{Helicobacter}. However, this uncertainty may be resolved by determining the phylogeny of additional unclassified organisms which have many phenotypic traits similar to those of "\textit{F. rappini}". Archer et al. (1) described microaerophilic, fusiform-shaped bacteria that possessed multiple bipolar flagella and a corrugated surface formed by periplasmic fibers. These microorganisms were isolated from two humans suffering from chronic gastroenteritis. An anaerobic bacterium isolated from the mucosal epithelium of a murine large bowel also had a similar ultrastructure (38). This unusual ultrastructure was also observed in spiral-shaped bacteria isolated from the crypts of mice and rat gastrointestinal tracts (35).

In previous studies, comparative analyses of partial 16S rRNA sequences (approximately 650 bases) of \textit{C. cinaedi} and \textit{C. fennelliae} demonstrated that these species are related phylogenetically to the gastric bacteria (40). When these sequences were included in our analyses, similar results were obtained, although both \textit{C. cinaedi} and \textit{C. fennelliae} fell outside the \textit{Helicobacter} cluster (data not shown). When more complete sequences of these two species become available, the true phylogenetic positions of these bacteria can be determined.

Other spiral-shaped bacteria also have been observed in the stomachs of cats and dogs (30, 41) and nonhuman primates (5, 37). These organisms have the same tight helical morphology as \textit{H. felis}, but the periplasmic fibers are not present. However, like \textit{H. felis}, these organisms are found only in the gastric mucosa of animals. Similar bacteria have been found in humans, and it has been suggested they are transmitted zoonotically to humans via animal contact (7, 24, 26). None of these spiral bacteria has been cultured in vitro, but isolates from cats, dogs, monkeys, and humans have been maintained in large numbers in the stomachs of laboratory mice (8).

The clustering of the gastric bacteria as shown in the dendrogram (Fig. 3) presumably has evolutionary significance. It is tempting to speculate that the helicobacters and related bacteria evolved in a distinct ecological habitat, such as the gastric mucus, whereas the true campylobacters and related bacteria evolved in the large bowels and subgingival crevices. Both of these groups may have arisen from a common ancestor that colonized the mucous membranes of the alimentary tracts of primitive mammals. For example, \textit{H. felis} strains are very closely related phylogenetically, and yet they have been isolated from both cats and dogs. Furthermore, it is interesting that the oral species (namely,
Fig. 2. Sequences of *H. felis* CS1 and DS3 and *H. mustelae* (Hm) aligned with the sequence of *E. coli* (Ec). Numbering is relative to *E. coli* base positions. A and a, Adenine; C and c, cytosine; G and g, guanine; U and u, uracil; n, base could not be determined. Lower-case letters indicate some uncertainty in base identification. Dashes indicate gaps that were inserted for sequence alignment, and dots indicate regions that were not sequenced.
TABLE 2. Similarity matrix

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Abbreviations: CS1, H. felis CSIT; DS3, H. felis DS3; Hp, H. pylori; Hm, H. mustelae; Fr, "F. rappini"; Ws, W. succinogenes; Bg, B. gracilis; Wr, W. recta; Wc, W. curva; Cc, C. concisus; Cf, C. fetus subsp. fetus; Ch, C. hyointestinalis; Ci, C. coli; Cj, C. jejuni; Cl, C. lari; Bu, B. ureolyticus; Cs, C. sputorum subsp. bubulus. Sequences for "F. rappini" 1937, C. fetus subsp. fetus ATCC 27374, and C. hyointestinalis ATCC 35217 are unpublished data. Other previously published sequences were obtained elsewhere (34, 36). The numbers above the diagonal represent uncorrected percentages of similarity. The numbers below the diagonal are percentages of difference corrected for multiple base changes by the method of Jukes and Cantor (18).

W. recta, W. curva, C. concisus, and B. gracilis) form a tight subcluster within the true campylobacters (Fig. 3).

Description of Helicobacter felis sp. nov. Helicobacter felis (fe' lis. L. gen. n. felis, of a cat). Rigid, spiral-shaped, gram-negative cells that are 0.4 μm wide and 5 to 7.5 μm long and have five to seven spirals per cell. Spherical forms (diameter, 2 to 4 μm) are present in older cultures. No endospores are produced. Cells are motile with a rapid corkscrewlike motion. Cells have tufts of 10 to 17 polar sheathed flagella (thickness, 25 nm) that are positioned slightly off center at the end of the cell. Cells are surrounded by periplasmic fibers which appear as concentric helical...
ridges, either in pairs, threes, or singly on the surfaces of the cells. Microaerophilic, but can grow anaerobically. Grows at 37 and 42°C but not at 25°C. One strain does not grow at 42°C. Nutritionally fastidious, growing only on media enriched with blood or serum. Ascaccharolytic. No acid is produced from maltose, sucrose, lactose, fructose, xylose, sorbitol, arabinose, raffinose, glucose, and galactose. Urease, oxidase, and catalase positive. Alkaline phosphatase, arginine aminopeptidase, leucine aminopeptidase, and γ-glutamyl transpeptidase activities are detected. Most strains have histidine and leucine aminopeptidase activity. No production of N-acetylglucosaminidase, α-glucosidase, α-arabinosidase, β-glucosidase, α-fucosidase, α-galactosidase, β-galactosidase, indoxylacetate, proline aminopeptidase, pyroglytamic acid amylamidase, tyrosine aminopeptidase, alanine aminopeptidase, phenylalnine aminopeptidase, glycine aminopeptidase, and arginine dihydrolase. Nitrate is reduced to nitrite. Hippurate is not hydrolyzed. Indole and H₂S are not produced. No growth occurs in the presence of 1% glyco and 1.5% NaCl. Susceptible to cephalothin, ampicillin, erythromycin, metronidazole, and bismuth compounds, but resistant to nalidixic acid. Isolated from the gastric mucosa of cats and dogs. Strain CS1 (ATCC 49179), which was isolated from the gastric mucosa of a cat, is the type strain; its G+C content is 42.5 mol% (as determined by the thermal denaturation method).

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