Helicobacter muridarum sp. nov., a Microaerophilic Helical Bacterium with a Novel Ultrastructure Isolated from the Intestinal Mucosa of Rodents

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Helical organisms with novel ultrastructural characteristics were isolated from the intestinal mucosa of rats and mice. These bacteria were characterized by the presence of 9 to 11 periplasmic fibers which appeared as concentric helical ridges on the surface of each cell. The cells were motile with a rapid corkscrewlike motion and had bipolar tufts of 10 to 14 sheathed flagella. The bacteria were microaerophilic, nutritionally fastidious, and physiologically similar to *Helicobacter* species and *Wolinella succinogenes* but could be differentiated from these organisms by their unique cellular ultrastructure. Using 16S rRNA sequencing, we found that strain ST1^T (T = type strain) was related to previously described *Helicobacter* species, "*Flexispira rappini*," and *W. succinogenes*. The closest relatives of strain ST1^T were *Helicobacter mustelae* and "*F. rappini*" (average similarity value, 96%). On the basis of phylogenetic data, strain ST1^T (= ATCC 49282^T) represents a new species of the genus *Helicobacter*, for which we propose the name *Helicobacter muridarum*.

Bacteria with a variety of spiral morphologies are common inhabitants of the gastrointestinal tracts of both humans and animals (8–10, 20, 24–26, 30, 34, 39). Several of these spiral bacteria are helically coiled and possess bipolar tufts of flagella and concentric ridges that are due to periplasmic fibers that run the length of each cell. They have been seen in a variety of gastrointestinal preparations, including ceca (8) and ilea of rats (34), colons of mice (39), and gastric mucosa (26) and fundic glands of dogs and cats (20). Recently, organisms with a similar morphology have been implicated in ovine abortions (5, 21) and in intestinal diseases in animals and humans (1, 37).

Much of the recent work on spiral bacteria has concentrated on *Helicobacter pylori* and other *Campylobacter*-like organisms because of the postulated association of these bacteria with human gastrointestinal disease (6, 18, 28, 29). The taxonomy of this group of organisms is now being clarified. Recent phylogenetic work has resulted in the creation of the genus *Helicobacter*, and new species of this genus are rapidly being recognized (14, 17, 32, 33, 36, 40, 43). Although three of the gastric helicobacters are very similar physiologically and show high degrees of similarity (96%) in their 16S rRNA sequences, these bacteria have very different morphologies (19, 24, 31). *H. pylori* is a relatively short organism with one or two turns in its spiral body, while *Helicobacter mustelae* is a short, slightly curved, rod-shaped organism with an unusual flagellum configuration. In contrast, *Helicobacter felis* is 5 to 7 µm long and has a tightly spiralled body with five or six turns; it is entwined with a distinctive series of periplasmic fibers.

The distinctive periplasmic fibers are not new to us because 10 years ago we observed similar structures on a bacterium isolated from a rat ileum (34). The same organism

has been described previously in the elegant electron microscopic studies of Erlandsen and Chase, who observed spiral microorganisms routinely in the crypts of Lieberkühn in rat small intestines and within the digestive vacuoles of Paneth cells (11). While the natural habitat of this organism is the lower bowel, we have occasionally observed it in the stomachs of normal mice (19a). Thus, we thought that it was possible that this organism is another helicobacter. This possibility was supported by the results obtained with a genus-specific *Helicobacter* DNA probe, which indicated that the organism is indeed another member of the genus *Helicobacter* (16). In this paper we provide data which support this hypothesis and describe the unique ultrastructure of this bacterium.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains ST1^T (T = type strain) and ST2 were isolated from the intestinal mucosa of rodents by Phillips and Lee (34). Other strains were isolated from rat (strain ST4) and from individual mice (strains ST3 and ST5) by using similar methods. Briefly, mucosal scrapings from rodent ileal and cecal tissue were streaked onto moist lysed blood agar (blood agar base no. 2; Oxoid, Basingstoke, England) supplemented with 5% horse blood and antibiotics (10 µg of vancomycin per ml, 2.5 µg of polymyxin B per ml, 5 µg of trimethoprim per ml, and 2 µg of amphotericin per ml). The plates were incubated under microaerophilic conditions at 37°C for 4 to 6 days.

Ultrastructure and phylogeny studies. All strains were stained with 2% phosphotungstic acid and examined with a Hitachi model H7000 transmission electron microscope to determine their morphology. The cell walls were disrupted by resuspending cultures in a nonionic detergent for 10 min before they were prepared for transmission electron microscopy. The detergent used was Teepol (Shell Chemicals,

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FIG. 1. Freeze-dried preparation of *H. muridarum* sp. nov. showing characteristic S shape, tufts of bipolar flagella, and periplasmic fibers. Bar = 1 μ m.

Sydney, Australia) at a concentration of 0.2%. Freeze-dried and freeze-fractured preparations of strain ST1^T were prepared as previously described (24).

Biochemical characterization, tests for susceptibility to antimicrobial agents, and 16S rRNA sequencing and analysis were performed as previously described (33). Dendograms were constructed by using the neighbour joining method of Saitou and Nei (38).

DNA base composition. DNA was extracted by using the method of Marmur (27) except that we added two protease treatments, one after lysis by sodium lauryl sulfate and one after the RNase step (2). The G+C values were determined by the T_i method of Sly et al. (41), using a model 2600 microprocessor-controlled spectrophotometer (Gilford Instruments, Oberlin, Ohio) fitted with a thermal programmer.

GenBank accession number. The sequence for *Helicobacter muridarum* ST1^T is available for electronic retrieval from GenBank under accession number M80205.

RESULTS

Isolation and growth characteristics. The helical bacteria designated strains ST1^T, ST2, ST3, ST4, and ST5 grew

as fine, spreading films on lysed blood agar plates that were incubated under microaerophilic conditions for 2 to 3 days. It was important to use fresh, moist plates and to incubate them with their lids uppermost for the isolation and cultivation of these bacteria. The optimum growth conditions for these organisms have been described previously (34).

Morphology. The bacteria were helical, gram negative, and motile with a rapid corkscrewlike motion. The cells were 3.5 to 5 μ m long and 0.5 to 0.6 μ m wide with two to three spiral turns (Fig. 1).

Ultrastructure. Previous studies (34) of strains ST1^T and ST2 revealed that these organisms have a novel morphology and ultrastructure. They are characterized by the presence of 9 to 11 periplasmic fibers that are coiled around a helical protoplasmic cylinder and by the presence of bipolar tufts of sheathed flagella. Further study elucidated the structure of these features. The detailed ultrastructural analysis described below was performed by using strains ST1^T and ST2; the other strains were examined for characteristic morphological features (i.e., multiple polar flagella and periplasmic fibers).

FIG. 2. Periplasmic fibers of *H. muridarum* sp. nov. (A) Transverse section of strain ST1^T showing circular, hollow periplasmic tubules. Bar = 0.1 μ m. (B) Freeze fracture preparation of strain ST1^T showing arrangement of periplasmic fibers. Bar = 0.2 μ m.

Thin sections examined by transmission electron microscopy showed that the periplasmic fibers, which were cut at a variety of angles, were not solid filaments but were hollow tubules with a diameter of 28 to 32 nm (Fig. 2A). The thickness of the tubule wall was approximately 5 nm. The fibers on the cell surface were coiled around the length of each organism. Observation of many preparations indicated that the arrangement of the periplasmic fibers was not constant; the spacing, distribution, and termination points of the ridges were variable (Fig. 2B).

Sections also revealed that the polar regions of the cells adjacent to the attachment sites of the flagella were electron translucent (Fig. 3A). Associated with each translucent region were electron-dense bands that lined the cytoplasmic

membrane. These bands were 8 to 10 nm wide and 20 nm from the cytoplasmic membrane and contained striated material with a periodicity of 10 nm (Fig. 2A and 3B).

The sheaths on the flagella were a continuation of the cell wall that was loosely arranged proximal to the protoplasmic cylinder, and they contained electron-dense cores having a maximum diameter of 12 to 13 nm. Where the cores of the flagella entered the periplasm, there was a flat, electron-dense band that was below the level of the cell wall and had a diameter of 40 ± 4 nm (Fig. 3B).

Fractures through the polar region of the cells revealed more details of the components associated with insertion of the flagella. Characteristically, this region was free of ridges and was the site of variable numbers (average, 13 to 15) of

FIG. 3. Flagella of *H. muridarum* sp. nov. (A) Thin section of the polar region of strain ST1^T showing the electron-translucent region adjacent to the attachment sites of the flagella (t) and the electron-dense bands lining the cytoplasmic membrane (arrow). Bar = 0.2 μm. (B) Thin section of the polar region of strain ST1^T showing the continuous nature of the cell wall and the sheath of the flagella and the electron-dense material below the insertion of the flagella (arrow). Bar = 0.2 μm. (C) Freeze fracture preparation of strain ST1^T showing the circular complexes (arrow) associated with the flagella. Bar = 0.2 μm.

circular complexes (Fig. 3C). Each flagellum cleaved near the base appeared as a central circular component that was surrounded by 17 to 20 oval particles, each approximately 15 by 7 nm; these particles occurred in a ring with a diameter of approximately 90 nm.

Treatment with the detergent Teepol disrupted the outer membrane without destroying the periplasmic fibers or flagella. The periplasmic fibers unravelled from the protoplasmic cylinder (Fig. 4A), and we observed that they were not inserted into the protoplasmic cylinder at any point. There was no association between the fibers and the polar flagella, which confirmed previous observations. The ends of the fibers had no specialized structures or any internal core material and were of a constant diameter (28 to 30 nm). At a higher magnification the fibers had a striated appearance and a periodicity of 9 nm (Fig. 4B).

Treatment with Teepol also led to breakdown of the sheath on the flagella, leaving a core that was 15 nm in diameter and remained inserted into the protoplasmic cylinder. By briefly sonicating Teepol-treated organisms for 15 s, we ruptured the cytoplasmic membrane, revealing components that were associated with the insertion of the flagella. Many flagella were attached at one end to the center of a small circular body (Fig. 4C). Each circular body had three rings; one of these rings had a diameter of 16 to 20 nm, another had a diameter of 42 to 47 nm, and the external ring had a diameter of 68 to 72 nm.

In older cultures there were many coccoid cells which, when examined by transmission electron microscopy, appeared to be degenerating helical cells. Two types of coccoid cells were observed, those with disrupted cytoplasmic membranes (Fig. 5A) and those with altered cell walls (Fig. 5B and C). Where the cytoplasmic membrane had ruptured, leaving only the outer membrane enclosing the cell contents, the cytoplasm filled the periplasm, swelling the surrounding cell wall. In organisms in which the peptidoglycan was degraded, the cytoplasm was contained in spheroplasts bounded by the outer and cytoplasmic membranes. Although periplasmic fibers were not detected on the altered cell walls, the ridges on the surface remained.

Biochemical and physiological characteristics. The biochemical and physiological characteristics of the five strains which we examined are shown in Table 1 together with the characteristics of the other *Helicobacter* species, *Campylobacter jejuni*, and *Wolinella succinogenes*.

Genetic relatedness. The nucleotide base composition of strain ST1^T DNA was 34.6 ± 0.6 mol % G+C.

Approximately 95% (1,442 bases) of the total 16S rRNA sequence of *H. muridarum* ST1^T was sequenced. This sequence was aligned in our data base, which consists of nearly complete 16S rRNA sequences of more than 300 microorganisms. A similarity matrix was determined from a comparative analysis of the 16S rRNA sequences of strain ST1^T, *Helicobacter* species, *Campylobacter* species, and other related bacteria. Figure 6 shows a phylogenetic tree which was constructed from the similarity data. As this dendrogram shows, strain ST1^T is clearly related to *Helicobacter* species (average level of similarity, approximately 94%).

DISCUSSION

The data described above confirm that the rodent isolates are members of a *Helicobacter* species and deserve a formal name. Previously, we speculated that the helicobacters are a group of related bacteria that have evolved such that they

can survive in gastric environments. In contrast, the natural habitat of *H. muridarum* is the lower bowel, an observation which seems to conflict with the gastric hypothesis. However, we have shown that if conditions in the stomach are modified, *H. muridarum* can colonize gastric pits in large numbers (19a). The bacteria observed in the stomachs of BALB/c mice by Queiroz et al. (35) were probably *H. muridarum*.

As the genus *Helicobacter* expands, it is clear that it includes bacteria other than gastric colonizers. The rectal inhabitants *Campylobacter cinaedi* and *Campylobacter fenelli* have recently been classified as helicobacters (43). Furthermore, *Campylobacter*-like organisms isolated from the feces of wild birds were confirmed as a new *Helicobacter* species by using DNA probes and 16S rRNA sequencing (40).

A comparison between the gastric helicobacters and the new nongastric helicobacters should provide important information that is relevant to our understanding of gastroduodenal disease; i.e., we should be able to identify more features that are essential to survival in the stomach. In a comparative study in which we used *H. pylori*, *H. felis*, and *H. muridarum* we found that the urease of the gastric colonizers has two pH optima and that one of these optima is at an acid pH, in contrast to *H. muridarum*, which has one pH optimum at a neutral pH. We have proposed that urease activity at low pH values is essential for colonization of gastric mucosa (12).

H. muridarum has many ultrastructural features in common with other spiral bacteria. The insertion sites of the flagella resemble similar structures in *H. felis* (24), *Aquaspirillum serpens* (7), *Spirillum voltans* (42), and *W. succinogenes* (22). Furthermore, the insertion region contains features that closely resemble the cytoplasmic flagellum bases observed in *S. volutans* by Swan (42). The cytoplasm near the tip of each cell was found to be electron translucent, and a "polar membrane" similar to that described by Coulton and Murray for other polarly flagellated bacteria, including *C. jejuni* (3, 7), was found lining the cytoplasmic membrane in this region. An examination of negatively stained preparations of disrupted cells revealed circular, concentric ring structures, some of which were attached to flagella. These structures resembled the concentric membrane ring found in *Vibrio cholerae* and *Campylobacter fetus* (13). Freeze fractures of the polar cell wall regions revealed rings of intramembrane particles surrounding the core of the flagella, features also observed in *H. felis* (24) and *A. serpens* (7). From an analysis of the information derived from negative staining, thin sectioning, and the freeze fracture technique, the observations described below were made. The dark band that was observed under the cell wall in thin sections was the same diameter (70 nm) as the circular rings observed in negatively stained preparations, while the central hole was the same width (15 nm) as the appendages in section. The ring of particles observed in the fracture preparations had a diameter similar to the diameter of the circular ring structures and lay immediately above these structures in the cell wall. Coulton and Murray (7) suggested that the most likely role for the concentric membrane ring in spirilla is to stabilize and reinforce the cell wall membrane against the force of the spinning flagella. Such a role could also be applicable to the spiral organisms, especially as the cell walls of these organisms do not enclose the basal complex as they do in bacteria with unsheathed flagella, such as *S. serpens*.

The periplasmic fibers of *H. muridarum* reveal an inter-

FIG. 4. Negatively stained preparations of strain ST1^T after treatment with Teepol. (A) Unravelling of periplasmic fibers showing that they were not inserted into the protoplasmic cylinder and their lack of association with the flagella. Bar = 0.5 μ m. (B) Striated nature of the periplasmic fibers (small arrow) and circular bodies attached to the flagella (large arrow). Bar = 0.1 μ m. (C) Higher magnification showing greater detail of the circular bodies attached to the flagella. Bar = 0.1 μ m.

FIG. 5. Thin sections of older cultures of strain ST1^T. (A) Coccoid cell with a disrupted cytoplasmic membrane. Bar = 0.2 μ m. (B and C) Coccoid cells with disrupted cell walls. Bars = 0.1 μ m.

TABLE 1. Biochemical and physiological characteristics of *H. muridarum* and related bacteria

Characteristic	<i>H. muridarum</i> (five strains)	<i>H. pylori</i> NCTC 11637	<i>H. mustelae</i> ATCC 43773	<i>H. felis</i> ATCC 49179	<i>W. succinogenes</i> NCTC 11488	<i>C. jejuni</i> (two strains)
Urease activity (rapid)	+(5) ^a	+	+	+	—	—
Catalase activity	+(5)	+	+	+	—	+
Oxidase activity	+(5)	+	+	+	+	+
H ₂ S production	—(5)	—	—	—	—	+
G+C content (mol%)	34.6	37	36	42.5	47	30–38
Nitrate reduction	—(5)	—	+	+	+	+
Hippurate hydrolysis	—(5)	—	—	—	—	+
Alkaline phosphatase activity	+(5)	+	+	+	—	—
Arginine aminopeptidase activity	+(5)	+	+	+	+	+
Histidine aminopeptidase activity	—(5)	+	+	+	—	—
Leucine aminopeptidase activity	+(4)	+	+	+	+	+
Glutamyl transpeptidase activity	+(5)	+	+	+	—	+
Resistance to:						
Nalidixic acid (30-μg disk)	+(5)	+	—	+	+	—
Cephalothin (30-μg disk)	+(5)	—	+	—	+	+
Growth in the presence of:						
1% Glycine	—	—	—	—	ND ^b	+
1.5% NaCl	—	—	—	—	—	+
Growth at:						
42°C	—	—	+	+	+ or —	+
37°C	+(5)	+	+	+	+	+
25°C	—	—	—	—	—	—
Growth under:						
Aerobic conditions	—	—	—	—	—	—
Microaerophilic conditions	+(5)	+	+	+	—	+
Anaerobic conditions	—	+	+	+	+	+

^a The numbers in parentheses are the numbers of strains that have the reactions indicated.^b ND, not determined.

esting ultrastructure. The striated structures shown in Fig. 4A and B are reminiscent of myosin and thus are suggestive of a primitive contractile structure. The function of these fibers is unknown, but it is presumably related to the

vigorous corkscrewlike motility of the bacteria. This could be active or passive as the ridges formed by the fibers could increase torque in viscous environments. Given that we have claimed that the spiral bacteria of gastrointestinal tracts have

FIG. 6. Phylogenetic tree for the helicobacters, campylobacters, and related organisms. The scale bar represents a 5% difference in nucleotide sequences (determined by taking the sum of all of the horizontal lines connecting two species). Vertical distance has no meaning.

evolved to survive best in a mucous environment (23), the study of the motility of the *Helicobacter* species which exhibit very distinct morphologies could be very revealing.

Physiologically, as Table 1 shows, the four species which we examined are very similar. Indeed, the only species-specific biochemical tests are the tests for histidine aminopeptidase (negative for *H. muridarum*) and resistance to nalidixic acid (negative for *Helicobacter mustelae*). As expected, the differences between *H. muridarum* and *W. succinogenes* and *C. jejuni* are marked.

The position of "*Flexispira rappini*" (4) still needs clarification, Goodwin (17) having stated that this organism is not a helicobacter. "*F. rappini*" certainly shares the morphological feature of multiple periplasmic fibers, but it does not have a helical morphology. We previously reported, on the basis of 16S rRNA sequencing data, that this organism may represent a species of helicobacter (33), and this hypothesis is supported by our data, which showed that "*F. rappini*" and *H. muridarum* have a level of similarity of 96%. Therefore, the positions of this bacterium and the organism that was described by Archer et al. (1, 36) as being associated with gastritis may become clearer as more *Helicobacter* species are described. The phylogenetic position of *W. succinogenes* still remains uncertain, although this organism is clearly related to the helicobacters.

Thus, the genus *Helicobacter* is expanding, with a number of possible new species waiting in the wings. Characterization of this group is important as it may identify unique properties that may be linked to pathogenicity. Accordingly, below we propose a name for the murine strains, which form a coherent group.

Description of *Helicobacter muridarum* sp. nov. *Helicobacter muridarum* (mu.ri.da'rum. L. n. Muridae, family name for Old World rats and mice; L. gen. n. *muridarum*, of the Muridae). Helical cells that are 0.5 to 0.6 μ m wide and 3.5 to 5.0 μ m long and have two to three spiral turns. Spherical forms 2 to 4 μ m in diameter are present in older cultures. Gram negative. No endospores are produced. Cells are motile with a rapid corkscrewlike motion. Cells have bipolar tufts of 10 to 14 sheathed flagella. Each cell is surrounded by 9 to 11 periplasmic fibers which appear as concentric helical ridges on the surface of the cell. Microaerophilic. Good growth occurs in atmospheres containing 1 to 16% oxygen and 5 to 10% CO₂. No growth occurs under aerobic or anaerobic conditions. Nutritionally fastidious, growing only on media enriched with blood or serum. Optimum growth occurs on a moist agar surface. Grows as a fine, translucent, spreading film on agar after 2 to 3 days. Growth occurs at 37°C but not at 25 or 42°C. Viability is lost rapidly. No acid is produced from arabinose, cellobiose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, raffinose, ribose, salicin, sorbitol, starch, sucrose, or xylose. Urease, oxidase, and catalase positive. Alkaline phosphatase and arginine aminopeptidase are produced. Lipase and lecithinase are not produced. Gelatin, casein, starch, tributyrin, and hippurate are not hydrolyzed. No production of *N*-acetyl-glucosaminidase, α -glucosidase, α -arabinosidase, β -glucosidase, α -fucosidase, α -galactosidase, β -galactosidase, leucine aminopeptidase, proline aminopeptidase, pyroglutamic acid arylamidase, tyrosine aminopeptidase, arginine aminopeptidase, alanine aminopeptidase, histidine aminopeptidase, phenylalanine aminopeptidase, or glycine aminopeptidase. Nitrate is not reduced. Indole and H₂S are not produced. No growth occurs in the presence of 2% bile salts, 1% glycine, or 1.5% NaCl. Resistant to nalidixic acid and cephalothin.

Isolated from the intestinal mucosa of the rats and mice. Type strain ST1 (= ATCC 49282) was isolated from a rat ileum, and its G+C content is 34 mol%.

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