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Helicobacter winghamensis sp. nov., a Novel *Helicobacter* sp. Isolated from Patients with Gastroenteritis

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From 1997 to 1999 seven isolates of *Campylobacter*-like organisms from five patients that were exhibiting symptoms of gastroenteritis, including fever, stomach malaise, and diarrhea, were investigated. The organisms were isolated from stool samples and found to exhibit a diverse colony morphology; hence multiple isolates were submitted from one of the patients. All isolates were found to be identical. The organisms were catalase, urease, alkaline phosphatase, and nitrate negative but oxidase and indoxyl acetate positive. They grew at 37°C but not at 42°C, and three of the isolates from two different patients were sensitive to nalidixic acid and cephalothin. Full 16S rRNA sequence analysis not only grouped these organisms within the *Helicobacter* genus but also differentiated them from previously identified *Helicobacter* species. The closest relative by phylogenetic analysis was *Helicobacter* sp. flexispira taxon 1. Electron microscopy showed that these isolates had one or two bipolar flagella; however, the periplasmic fibers, a characteristic of the known *Helicobacter* sp. flexispira taxa, were not observed. The present isolates also lacked a flagellar sheath, a trait shared with four other *Helicobacter* spp., *H. canadensis*, *H. mesocricetorum*, *H. pullorum*, and *H. rodentium*. On the basis of the unique phenotypic properties of these isolates and 16S rRNA sequence analysis, we propose the classification of a new *Helicobacter* species, *Helicobacter winghamensis* sp. nov.

Helicobacters have emerged as a burgeoning cause of enteric disease in humans. Members of this genus have gained recognition largely as a result of *Helicobacter pylori* (15), which colonizes the stomachs of humans and which has been associated with gastritis, peptic ulcer disease, and most recently with the development of adenocarcinoma and gastric mucosa-associated lymphoma (11, 17, 24, 27). Indeed, *H. pylori* constitutes a significant disease burden for the human population, and extensive investigations have been undertaken to better define the pathogenicity of this organism.

To date, the pathogenesis of many *Helicobacter* species which are isolated from the intestinal contents of humans and animals remains in doubt as often they are isolated in the absence of symptoms. Such is the case with *H. pullorum*, *H. muridarum*, and *H. pametensis* (2, 7, 19, 33). As a consequence, little has been done to elucidate the nature of the disease mechanisms of these intestinal helicobacters. The recent isolation and characterization of the novel *H. canadensis* and its demonstrated link to gastroenteritis have raised the profile of these intestinal helicobacters and have underscored their potential role in human disease (12).

In the past decade the taxonomy of *Helicobacter* has expanded dramatically with an average of two or three new species added to the group each year. Currently, the genus comprises 28 species isolated from mammalian and avian sources. Of these, 19 have been validated in accordance with the international rules of nomenclature, 7 have yet to be validated, and 2 are candidate species (10). Five of the 28 species (*H. canadensis*, *H. canis*, *H. cinaedi*, *H. fennelliae*, and *H. pullorum*) have been isolated from the intestinal contents of humans suffering from diarrhea, while *H. bizzozeronii* has been obtained from a single patient with gastritis (12, 33, 34, 37; K. Jalava, S. L. W. On, C. S. Harrington, L. P. Anderson, M.-L. Hänninen, and P. A. R. Vandamme, Abstr. 10th Int. Workshop *Campylobacter*, *Helicobacter*, Related Organisms, abstr. HD5, 1999). In addition, a *Campylobacter*-like organism with the provisional name "*Flexispira rappini*" was also isolated from the intestinal contents of humans with diarrhea (1). Members of the "*Flexispira rappini*" group, also named *Helicobacter* sp. flexispira (8), represent a collection of organisms with similar morphological and phenotypic properties. Based on 16S rRNA sequence analysis, these have been described as comprising 10 taxa within the *Helicobacter* genus (8).

In this study we describe seven *Campylobacter*-like isolates which, following phenotypic and genotypic analysis, were shown to belong to a new species of *Helicobacter*. All isolates came from the feces of humans with enteric tract symptoms, thus further underscoring the potential for other intestinal helicobacters to induce human disease. Phenotypically, these organisms were similar to *H. canis* but differed in their abilities to grow at 42°C and in their alkaline phosphatase activities. Following phylogenetic analysis using 16S rRNA sequencing, a close relationship to *Helicobacter* sp. flexispira taxon 1 was observed; however, major phenotypic and ultrastructural differences between these two were noted. Based on the unique characteristics of this group of isolates and in keeping with the recently described minimal standards for describing new species of *Helicobacter* (6) we propose a new *Helicobacter* species, *H. winghamensis* sp. nov.

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TABLE 1. *Helicobacter* strains used in this study

Organism	Source no.	GenBank accession no.
H. winghamensis 1	NLEP 97–1090[c]	AF246984
H. winghamensis 2	NLEP 98-2019	AF246985
H. winghamensis 3	NLEP 98-2020	AF246986
H. winghamensis 4	NLEP 98-2021	AF246987
H. winghamensis 5	NLEP 99-4873	AF246988
H. winghamensis 6	NLEP 97-1611	AF363062
H. winghamensis 7	NLEP 98-0305	AF363063
H. aurati	MIT 97-5075c	AF 297868
H. acinonychis	ATCC 51101	M88148
H. bilis	ATCC 51630	U18766
H. bizzozeronii	ATCC 700030	Y09404
H. bizzozeronii	ATCC 700031	AF302107
H. canadensis	ATCC 700968	AF262037
H. canis	ATCC 51401	L13464
H. cinaedi	CCUG 18818	M88150
H. cholecystus	Hkb-1	U46129
H. colifelis	N/A[a]	AF142062
H. felis	ATCC 49179	M37642
H. fennelliae	ATCC 35684	M88154
H. heilmannii	Str 1	AF058768
H. hepaticus	ATCC 51488	AF302103[b]
H. mainz	ATCC 51800	X81028
H. mesocricetorum	MU97-1514	AF072471
H. muridarum	ATCC 49282	AF302104[b]
H. mustelae	ATCC 43772	M35048
H. nemestrinae	ATCC 49396	AF363064[b]
H. pametensis	ATCC 51478	AF302105[b]
H. pullorum	ATCC 51801	L36141
H. pylori	ATCC 43504	AF302106[b]
Helicobacter sp. flexispira	ATCC 43966 taxon 8	M88138
Helicobacter sp. flexispira	ATCC 43968 taxon 1	U96300
H. rodentium	ATCC 700285	U96296
H. salomonis	CCUG 37848	Y09405
H. suncus	Kaz-1	AB006147
H. trogontum	ATCC 700114	U07574
H. typhlonicus	Strain MU	AF061104
H. westmeadii	Taxon 218	U44756

[a] N/A, not available (species unculturable).
[b] GenBank resubmission.
[c] Type strain.

MATERIALS AND METHODS

Bacterial isolates. Over the 3-year period 1997 to 1999, seven isolates of *Campylobacter*-like organisms were submitted from the Provincial Laboratories of Public Health in Alberta, Ontario, and Manitoba to the National Laboratory for Enteric Pathogens for species identification and further characterization. All were clinical isolates from stools derived from two children and three adults all with symptoms of gastroenteritis. The bacterial strains used for comparison are outlined in Table 1.

Phenotypic characterization. Cultures were recovered using Mueller-Hinton (MH) agar supplemented with 10% sheep blood and grown in a microaerobic atmosphere consisting of 3% O_2, 7% H_2, 7% CO_2, and 83% N_2 for 48 h. All phenotypic and biochemical tests requiring growth of the organisms employed the same microaerobic atmosphere. All media and reagents were obtained from Oxoid (Nepean, Ontario, Canada). Morphology was established using phase-contrast microscopy and Gram staining, while further traits were assessed using the biochemical tests for oxidase, catalase, indoxyl acetate hydrolysis (read at 15 min), alkaline phosphatase activity (read at 2 h), urease activity, and nitrate reduction (3, 5, 25, 28). Growth was assayed on MH agar containing 10% sheep blood at 25, 37, and 42°C, and results were read at 72 h. Growth tolerance studies were performed in modified brucella broth supplemented with 1% bile (23, 26). Disk diffusion assays, read at 48 h, using MH agar supplemented with 10% sheep blood were used to evaluate the susceptibility of organisms to nalidixic acid (30 μg) and cephalothin (30 μg) (Becton Dickinson, Cockeysville, Md.). A zone or no zone interpretation was used as the determining factor for resistance and susceptibility criteria as previously described for *Campylobacter* identification (18).

Electron microscopy. The morphology of organisms including the flagellum arrangements and structure as well as the presence or absence of surface projections was investigated by negative-stain transmission electron microscopy (TEM) procedures (29, 30). Bacterial cells were suspended in modified brucella broth (23), added dropwise to carbon-coated 400 mesh TEM grids, drained, and negatively stained using 2% (wt/vol) phosphotungstic acid (Marivae, Halifax, Nova Scotia, Canada). Preparations were examined with a CM120 transmission electron microscope (Philips Electron Optics, Toronto, Ontario, Canada).

Genotypic characterization. Preliminary genotypic analysis was achieved using the 16S rRNA PCR-restriction fragment length polymorphism (RFLP) procedure developed by Marshall et al. (20). In brief, chromosomal DNA was isolated from the organisms and subjected to a PCR procedure that amplified a 1-kb portion of the 16S rRNA gene. The resulting amplicon was then digested with endonucleases *Dde*I and *Bsr*I (New England Biolabs, Mississauga, Ontario, Canada), and the resulting RFLP patterns were visualized after electrophoresis by ethidium bromide staining.

16S rRNA gene sequencing and analysis. For 16S rRNA gene sequencing, chromosomal DNA was first extracted from the isolates using either DNAzol (Molecular Research Center, Inc., Cincinnati, Ohio) or Integrated Separation Systems automated DNA extractor Autogen 540 according to the manufacturer's specifications (Enprotech, Natick, Mass.). Approximately 1.5 kb of DNA from each isolate was amplified using PCR for the16S rRNA gene with primers pA and pHr from Edwards et al. (9). The PCR products were subjected to electrophoresis in low-melting-point agarose (Eclipse Molecular Biologicals, Mississauga, Ontario, Canada), excised, and purified using the Wizard PCR Preps DNA purification system (Promega, Madison, Wis.). The resulting PCR product was sequenced in six fragments using pA, pC, pDr, pE, pFr, and pHr primers also described by Edwards et al. (9). The sequenced fragments were assembled using the software program Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Mich.). The complete sequences from each isolate were then compared to the GenBank database through the National Center for Biotechnology Information (National Institutes of Heath, Bethesda, Md.). The sequences were subsequently aligned with other *Helicobacter* sequences using the ClustalW method incorporated in the MegAlign software of Lasergene (DNASTAR Inc., Madison, Wis.). Finally, phylogenetic analysis was done by first converting the files using ForCon software (Department of Biochemistry, University of Antwerp, Antwerp, Belgium) and drawing phylogenetic relationships using an unrooted neighbor-joining tree, generated from a distance matrix calculated with the Kimura two-parameter model of nucleotide substitution using MEGA 1.01 software (Institute of Molecular Evolutionary Genetics, Pennsylvania State University, University Park, Pa.).

Nucleotide sequence accession numbers. The 16S rRNA gene for the reference strain was submitted to GenBank under the accession number AF246984. Accession numbers for the other *H. winghamensis* isolates are as follows: AF246985, AF246986, AF246987, AF246988, AF363062, and AF363063.

RESULTS

Phenotypic characterization and electron microscopy. A summary of the phenotypic profile and the ultrastructural observations of the seven isolates together with other intestinal *Helicobacter* species is presented in Table 2. All of the clinical isolates possessed similar biochemical properties with the exception that three showed susceptibility to the antibiotics nalidixic acid and cephalothin. Like many other intestinal helicobacters the seven isolates were urease negative. They were oxidase and indoxyl acetate positive and tolerated 1% bile, but, most notably, they were uniformly negative for catalase. *H. canis* and *Helicobacter* sp. flexispira taxon 7 and taxon 8 are the only other *Helicobacter* spp. to share the unusual negative catalase reaction; however, they differ from the presently described isolates by a number of phenotypic traits. Both *H. canis* and the two *Helicobacter* sp. flexispira taxa grow at 42°C, whereas the present isolates do not. Furthermore, *H. canis* exhibits alkaline phosphatase activity and *Helicobacter* sp. flexispira taxon 7 and taxon 8 are urease positive and lack the ability to hydrolyze indoxyl acetate, properties that were not found with our group of isolates. *H. cholecystus* and *H. pametensis*, which were differentiated from each other by their antibiotic sensitivity profiles, differed from our isolates to the greatest degree, with five divergent reactions: catalase, nitrate, indoxyl acetate,

TABLE 2. Phenotypic characteristics of *H. winghamensis* and other intestinal *Helicobacter* spp.

Organism	Result[a] of test for:										Flagella[c] (type, no.)	Presence of		Reference
	Oxidase	Catalase	Urease	Nitrate	Indoxyl acetate	Alkaline phosphatase	Growth at 42°C	Tolerance of 1% bile	Susceptibility to[b]: Na	Susceptibility to[b]: Ceph		Flagellar sheath	Periplasmic fibers	
H. winghamensis	+ (7/7)	− (7/7)	− (7/7)	− (7/7)	+ (7/7)	− (7/7)	− (7/7)	+ (7/7)	R (4/7)	R (4/7)	BP, 1–2	−	−	
H. bilis	+	+	+	+	−	−	+	ND[f]	R	R	BP, 3–14	+	+	13
H. bizzozeronii	+	+	+	+	+	+	+	−	R	S	BP, 10–20	+	−	16
H. canadensis	+	+	−	+/−[e]	+	−	+	+	R	R	BP, 1–2	−	−	12
H. canis	+	−	−	−	+	+	+	+	S	S	BP, 2	+	−	34
H. cinaedi	+	+	−	+	−	−	−	−	S	S	MP, BP, 1–2	+	−	37
H. fennelliae	+	+	−	−	+	+	−	−	S	S	MP, BP, 1–2	+	−	37
H. hepaticus	+	+	+	+	+	−	−	ND	R	R	BP, 2	+	−	14
H. mesocricetorum	+	+	−	+	ND	+	+	ND	R	S	BP, 2	−	−	32
H. muridarum	+	+	+	−	−	+	−	ND	R	R	BP, 10–14	+	+	19
H. pametensis	+	+	−	+	−	+	+	+	S	S	BP, 2	+	−	7
H. pullorum	+	+	−	+	−	−	+	+	S	R	MP, 1	−	−	33
H. pylori	+	+	+	−	−	+	−	−	R	S	BP, 4–8	+	−	15
Helicobacter sp. flexispira taxon 1	+	+	+	−	+	−	+	ND	R	R	BP, 10–20	+	+	8
Helicobacter sp. flexispira taxon 8	+	−	+	−	+	−	+	ND	R	R	BP, 10–20	+	+	8
H. rodentium	+	+	−	+	−	−	+	ND	R	R	BP, 2	−	−	31
H. trogontum	+	+	+	+	−	−	+	ND	R	R	BP, 5–7	+	+	22

[a] +, positive reaction; −, negative reaction; S, sensitive; R, resistant. Numbers in parentheses, number of isolates exhibiting the indicated reaction per number of isolates tested.
[b] Na, nalidixic acid; Ceph, cephalothin.
[c] MP, monopolar, BP, bipolar.
[d] +, presence; −, absence.
[e] +/−, variable trait among identified isolates.
[f] ND, not determined.

alkaline phosphatase, and growth at 42°C. Standard microscopic observation indicated that these organisms, in keeping with most helicobacters, were motile, gram-negative, nonspore-forming, slightly curved bacilli. By TEM these organisms were approximately 2 μm in length and 0.3 to 0.6 μm in diameter with one or two unsheathed bipolar flagella (Fig. 1A). The absence of a flagellar sheath, uncharacteristic of most helicobacters, is shared by four other *Helicobacter* species, *H. canadensis*, *H. mesocricetorum*, *H. pullorum*, and *H. rodentium* (12, 31–33). However, these species differed by several significant traits from this group of isolates, including catalase activity, nitrate reduction, indoxyl acetate hydrolysis, alkaline phosphatase activity, and growth at 42°C. Periplasmic fibers, a structure found by TEM on *H. muridarum* and all *Helicobacter* sp. flexispira taxa (Fig. 1B), were not found on these isolates.

Genotypic characterization. Preliminary genotypic analysis by 16S rRNA PCR-RFLP showed that these isolates had restriction patterns that were identical but that they were different from those described for a range of *Campylobacter*, *Arcobacter*, and *Helicobacter* species by Marshall et al. (20). The present isolates also differed in their 16S rRNA patterns from the other intestinal helicobacters (*H. bizzozeronii*, *H. canadensis*, and *Helicobacter* sp. flexispira taxon 8). The common *Helicobacter* pattern H2 (750 and 230 bp) was produced by restriction with enzyme *Dde*I; however, following digestion with *Bsr*I, a distinctive, species-specific pattern comprising bands of 290, 250, 220, 150, and 110 bp resulted (Fig. 2).

16S rRNA sequence analysis. The phylogenetic relationship of these isolates and of other *Helicobacter* species based on full 16S rRNA sequence data and incorporating the Kimura two-parameter model of nucleotide substitution is shown in Fig. 3. Close analysis of the GenBank sequences for some of the reference strains revealed several gaps and "N" nucleotide designations. The 16S rRNA genes for these reference strains were resequenced and corrected. The *H. winghamensis* organisms formed a distinct group with a bootstrap value of 100 and cluster most closely to *Helicobacter* sp. flexispira taxon 1. By using the MegAlign software, the phylogenetic tree was augmented to give a divergence matrix created from the 16S rRNA sequences. A 2.7% divergence compared to *Helicobacter* sp. flexispira taxon 1 and *H. cholecystus* was observed, a figure considerably lower than those for any of the other *Helicobacter* 16S rRNA gene sequence comparisons. This supported the phylogenetic relationship between these organisms and the *H. winghamensis* isolates as can be seen in Fig. 3. The next most highly divergent species was *H. rodentium* at 2.8%. This species also shared with *H. winghamensis* the presence of unsheathed flagella, and this, together with the 16S rRNA similarity, suggested a phylogenetic relationship between these two species.

DISCUSSION

An increasing number of novel *Helicobacter* and *Helicobacter*-like organisms have been isolated from the stools of humans with gastrointestinal symptoms. Agents such as *H. bizzozeronii*, *H. canadensis*, *H. canis*, *H. pullorum*, *H. cinaedi*, *H. fennelliae*, *Helicobacter* sp. flexispira taxon 8, and now *H. winghamensis* (1, 12, 33, 34, 37; K. Jalava et al., Abstr. 10th Int. Workshop *Campylobacter*, *Helicobacter*, Related Organisms) are often identified as *Campylobacter* species that prove difficult to fully characterize in a routine clinical microbiology laboratory setting. In such situations, unconventional or nonstandard phenotypic markers or complex genotypic identifiers are required, tests for which are often performed in reference facilities. That

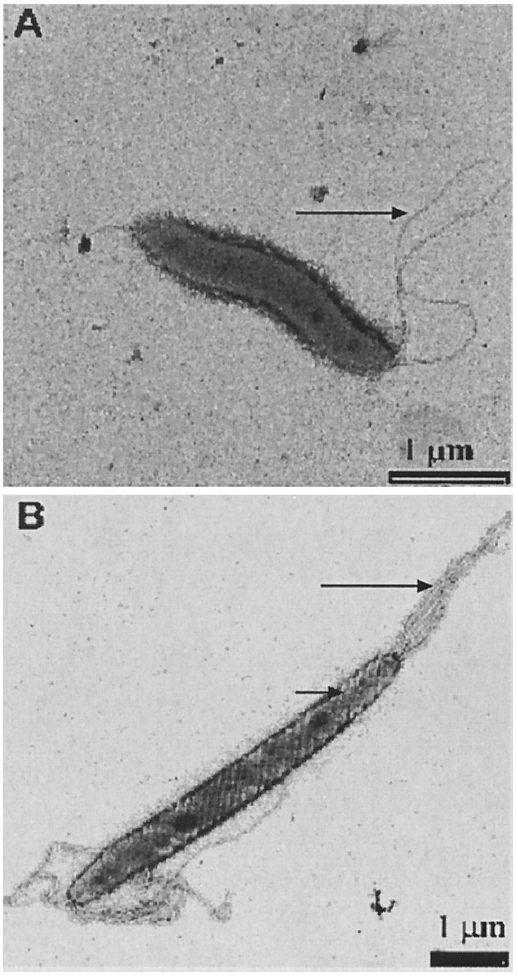

FIG. 1. (A) Electron micrograph of *H. winghamensis.* Note unsheathed flagella (arrow) and a lack of periplasmic fibers on the surface of the organism. (B) Electron micrograph of *Helicobacter* sp. flexispira taxon 1 showing sheathed flagella (long arrow) and periplasmic fibers (short arrow).

this group has expanded rapidly in the past few years strongly suggests that the etiology of diarrhea induced by these *Campylobacter*-like organisms is far from clear.

Campylobacteriosis is the most common bacterial human enteric disease in Canada (D. L. Woodward, Y. D. Yaschuck, L. J. Price, A. Moterassed, J. G. Moses, W. M. Johnson, and F. G. Rodgers, Abstr. 10th Int. Workshop *Campylobacter*, *Helicobacter*, Related Organisms, abstr. CD9, 1999). Despite this, the identification of *Campylobacter* as the causative agent of disease is often determined using a limited phenotypic analysis based on Gram stain, size and shape, microaerobic growth, and catalase, oxidase, and hippuricase activity. This has resulted in erroneous reports of *Campylobacter coli* and *Campylobacter lari* isolates from the stools of patients with gastroenteritis that eventually proved to be *H. pullorum*; indeed, these groups of organisms have proved difficult to differentiate (2, 21). As a result of these problems *H. pullorum* is considered underreported as a human enteric pathogen (4, 21, 35). Like *H. pullorum*, the *H. winghamensis* isolates studied in this investigation are similar to *Campylobacter* in that they are gram negative and have common properties of morphology, oxidase activity, and microaerobic growth. Hence, they too are almost certainly underrepresented in the spectrum of disease agents causing human gastroenteritis. That the five isolates included in the present study were from unrelated individuals and from different geographic locations in Canada supports the potential for underreporting and suggests that *H. winghamensis* might play a more prominent role in gastroenteritis.

H. winghamensis isolates atypically produce a negative catalase reaction, which sets them apart from most *Helicobacter* species except *H. canis* and *Helicobacter* sp. flexispira taxon 7 and taxon 8; however, these organisms may be differentiated by a number of phenotypic traits. Although a negative catalase reaction is not uncommon among campylobacters, they have the common property of nitrate reduction. The negative nitrate reaction for *H. winghamensis* should facilitate the separation of this newly described species from all other *Campylobacter* species, with the exception of *Campylobacter jejuni* subspecies *doylei*. Overall these organisms are relatively biochemically distinct and may be easily identified from related groups of bacteria by applying a more extensive range of biochemical tests including those for nitrate reduction, alkaline phosphatase activity, and indoxyl acetate hydrolysis. The presence of a species-specific 16S rRNA PCR-RFLP pattern (20) also contributes to the accurate identification of these agents. 16S rRNA similarities and differences form the basis for most phylogenetic dendrograms to define bacterial species. It is possible that 16S rRNA similarities may be an indication of a common origin among prokaryotic organisms. The phylogenetic similarity of these isolates to *Helicobacter* sp. flexispira taxon 1 and the low 16S rRNA sequence divergence from *H. cholecystus* and *H. rodentium*, isolated from murine sources, may be indicative of a common origin for these organisms.

The unique phenotypic and genotypic characteristics of these organisms should facilitate the detection of this newly proposed species. These identification traits will provide valuable laboratory-based epidemiological markers to better understand the role that these helicobacters play in gastroen-

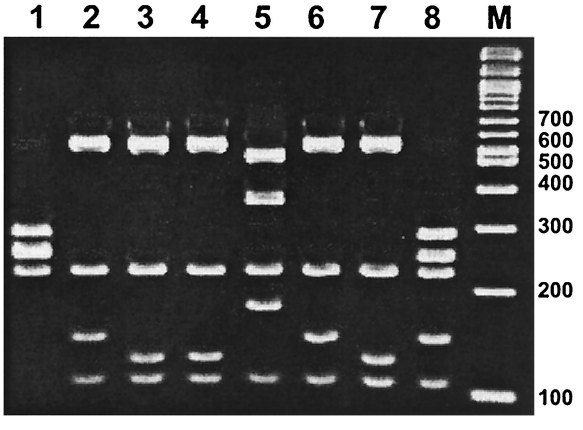

FIG. 2. 16S rRNA PCR-RFLP patterns for intestinal helicobacters generated using restriction enzyme *Bsr*I. Lane 1, *H. bizzozeronii*; lane 2, *H. canadensis*; lane 3, *H. canis*; lane 4, *H. cinaedi*; lane 5, H. *fennelliae*; lane 6, *H. pullorum*; lane 7, *Helicobacter sp.* flexispira taxon 8; lane 8, *H. winghamensis*; lane M, 100-bp molecular weight marker (New England Biolabs)

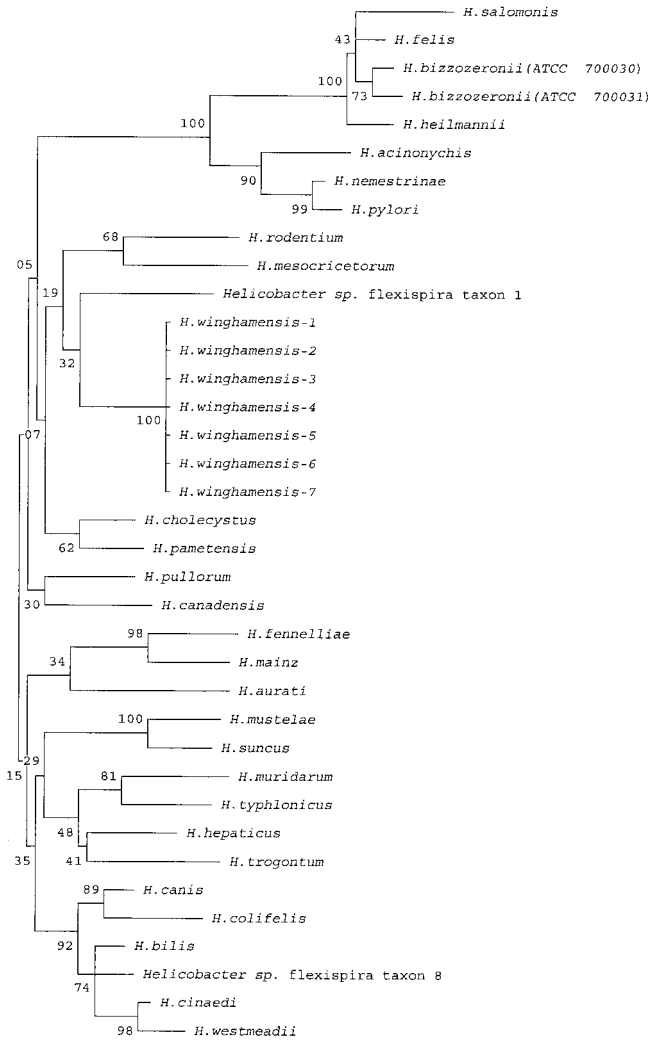

FIG. 3. Phylogenetic dendrogram for *Helicobacter*. Numbers at nodes represent bootstrap support values (percentage of 1,000 resampled data sets that supported the node in the recalculated tree). Scale: 1.6 cm, approximate genetic distance of 0.01 base substitutions per nucleotide pair.

teritis and will facilitate investigations of the virulence mechanisms they employ to induce human disease.

Description of *H. winghamensis* sp. nov. *H. winghamensis* is a gram-negative, slightly curved to spiral, non-spore-forming bacillus. The organism is approximately 2 μm in length by 0.3 to 0.6 μm in width, and it is motile by one or two bipolar, unsheathed flagella. Cultures grow on solid agar media supplemented with 10% sheep blood and exhibit a seemingly diverse colonial morphology of nonspreading and spreading colonies. The organisms grow in a microaerobic atmosphere at 37°C but fail to grow at 42°C or in aerobic or anaerobic atmospheres. This *Helicobacter* is oxidase and indoxyl acetate positive, tolerates 1% bile, and is alkaline phosphatase, catalase, and urease negative. It does not reduce nitrate. All isolates induced similar symptoms of gastroenteritis in humans, and these included general stomach malaise, vomiting, diarrhea, cramping, and mild fever. Currently the recorded host range for this organism does not extend beyond humans. The first isolate fitting the characterized description of this species was from Wingham, Ontario, Canada; thus, we propose the name *Helicobacter winghamensis.* The type strain is NLEP 97–1090 and has GenBank accession no. AF246984.

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