Phylogeny of *Helicobacter* Isolates from Bird and Swine Feces and Description of *Helicobacter pametensis* sp. nov.

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Previously, nine fecal isolates from wild birds and a domestic swine were identified as helicobacters by phenotypic characterization and reaction with a helicobacter genus-specific DNA probe. These isolates fell into three biotypes by analysis of phenotypic traits. To further characterize these isolates, full 16S rRNA sequences were determined for strains representing each biotype, and sequence comparison indicated that the strains represented three novel, phylogenetically defined *Helicobacter* species. Three 16S rRNA-based DNA probes were designed and used to identify the remaining strains. Probe reactivity divided the strains into the same three groups identified phenotypically. Six of the isolates represented a new species of the genus *Helicobacter* for which we propose the name *Helicobacter pametensis* sp. nov. The following phenotypic features distinguished *H. pametensis* from other *Helicobacter* and *Campylobacter* species: positive tests for oxidase, catalase, alkaline phosphatase, nitrate reduction, growth at 42°C, and growth in the presence of 1% glycine; negative tests for urease, gamma glutamyl transpeptidase, indoxyl acetate hydrolysis, and hippurate hydrolysis; and susceptibility to nalidixic acid and cephalothin. *H. pametensis* cells were motile and possessed one subterminal sheathed flagellum at each end. The two additional *Helicobacter* species were similar to *H. pametensis* except that they were urease positive, hydrolyzed indoxyl acetate, and were resistant to cephalothin. Because these two additional species are phenotypically similar and are represented by only two isolates for one species and one isolate for the other, they are not formally named but are referred to as *Helicobacter* sp. "Bird-B" and *Helicobacter* sp. "Bird-C." These three new *Helicobacter* species can easily be confused with *Campylobacter coli*, *Campylobacter lari*, and *Campylobacter jejuni* if only a limited number of phenotypic traits are used for identification. Since it is now known that birds can harbor *Helicobacter* as well as *Campylobacter* species, methods which clearly distinguish these genera should be used to identify bird campylobacter-like isolates or bacterial strains traceable to bird fecal contamination. The zoonotic potential of these new *Helicobacter* species should be examined.

The genera *Helicobacter*, *Wolinella*, *Campylobacter*, and *Archaeobacter* constitute the epsilon division of the *Proteobacteria* (42), also known as RNA superfamily VI (47). The genus *Helicobacter* is currently composed of 10 named species: *Helicobacter pylori* (17, 25), *H. mustelae* (14, 17, 32), *H. felis* (23, 32), *H. muridarum* (24), *H. fennelliae* (12, 46, 47), *H. cinaedi* (12, 46, 47), *H. nemestrinae* (4, 40), *H. acinonyx* (11), *H. canis* (7, 43), and *H. hepaticus* (13). Strains representing additional species have been identified by rRNA-DNA hybridization (47) and 16S rRNA sequence analysis (11, 43). These include "*Flexispira rappini*," (2, 6, 38) and CLO-3 (12, 46, 47). Two uncultivable human gastric spiral organisms, "*Gastrospirillum hominis*" 1 and 2, have been identified as helicobacters by 16S rRNA analysis (41).

H. pylori, *H. acinonyx*, *H. nemestrinae*, *H. mustelae*, and *H. felis* colonize the gastric mucosae of their hosts. All except *H. nemestrinae* produce gastric diseases ranging from mild gastritis or ulcers to gastric carcinoma (4, 11, 14, 15, 25, 26, 29, 32). *H. cinaedi*, *H. fennelliae*, *H. canis*, and *H. muridarum* colonize the intestinal tract and can produce gastroenteritis (2, 7, 12, 46). *H. muridarum* can colonize the stomachs as well as ceca of older rats (24). *H. hepaticus* colonizes the gastrointestinal tracts of mice and has the pathogenic potential to elicit a persistent hepatitis (13). Human infections with *H. cinaedi* and

H. canis may be zoonotic, because the primary hosts are hamsters (16) and dogs (7, 43), respectively.

In this study, we describe nine *Helicobacter* isolates obtained primarily from wild birds (39). Six of these isolates fall within one species, for which we propose the name *Helicobacter pametensis* sp. nov. The remaining three isolates constitute two additional *Helicobacter* species, which are described but which will not be formally named until additional isolates are obtained and characterized.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The initial isolations from feces and characterization of the *Helicobacter* strains examined in this report have been described previously (39). Helicobacters were cultured microaerophilically on Trypticase soy agar II with 5% sheep blood (BBL Becton Dickinson Microbiology Systems) at 37°C. Microaerophilic conditions were established by culture in Brewer jars evacuated to 25 in. (63.50 cm) of Hg and filled with N₂-H₂-CO₂ (80:10:10 [vol/vol]) or by use of a campylobacter gas pack (BBL Becton Dickinson Microbiology Systems).

Biochemical characterization. Partial biochemical characterization of these nine strains was previously reported (31, 39). Alkaline phosphatase and naphthylamide aminopeptidases were determined with An-Ident panels (Analytab Products, Plainville, N.Y.). Cells grown on Trypticase soy agar II with 5% sheep blood were collected and suspended in distilled water (or the diluent required by the commercial tests) to a

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TABLE 1. Sources and accession numbers of strains studied

Organism	Strain examined ^a	Culture collection no. ^b	GenBank no. ^c	Reference ^d
Isolates				
<i>Helicobacter pametensis</i>	Seymour B9 ^T	CCUG 29255 ^T , ATCC 51478 ^T	M88147	
<i>Helicobacter pametensis</i>	Seymour B7	CCUG 29253	M88145	
<i>Helicobacter pametensis</i>	Seymour B12	CCUG 29257, ATCC 51479	M88140	
<i>Helicobacter pametensis</i>	Seymour B13	CCUG 29258	M88141	
<i>Helicobacter pametensis</i>	Seymour B15	CCUG 29259	M88142	
<i>Helicobacter pametensis</i>	Seymour M17	CCUG 29260	M88155	
<i>Helicobacter</i> sp. Bird-B	Seymour B10 ^T	CCUG 29256 ^T , ATCC 51480 ^T	M88139	
<i>Helicobacter</i> sp. Bird-B	Seymour B8	CCUG 29254, ATCC 51481	M88146	
<i>Helicobacter</i> sp. Bird-C	Seymour B52 ^T	CCUG 29561 ^T , ATCC 51482 ^T	M88144	
Reference species				
<i>Arcobacter cryaerophilus</i>	CCUG 17801 ^T	ATCC 43158 ^T	L14624	43
<i>Arcobacter butzleri</i>	CCUG 10373		L14626	43
<i>Arcobacter skirrowii</i>	CCUG 10374 ^T		L16625	43
<i>Campylobacter coli</i>	CCUG 11238 ^T	ATCC 33559 ^T	L04312	11
<i>Campylobacter concisus</i>	Tanner 484 ^T	ATCC 33237 ^T	L04322	11
<i>Campylobacter fetus</i> subsp. <i>fetus</i>	ATCC 27374 ^T		M65012	48
<i>Campylobacter lari</i>	CCUG 23947 ^T	ATCC 35221 ^T	L04316	11
<i>Campylobacter rectus</i>	Tanner 371 ^T	ATCC 33238 ^T	L04317	11
" <i>Flexispira rappini</i> "	NADC 1893 ^T	ATCC 43966 ^T	M88137	11
" <i>Gastrosprillum hominis</i> " 1	Uncultivable		L10079	41
" <i>Gastrosprillum hominis</i> " 2	Uncultivable		L10080	41
<i>Helicobacter acinonyx</i>	Eaton 90-119-3 ^T	ATCC 51101 ^T , CCUG 29263 ^T	M88148	11
<i>Helicobacter canis</i>	NCTC 12739 ^T		L13464	43
<i>Helicobacter cinaedi</i>	CCUG 18818 ^T	ATCC 35683 ^T	M88150	43
<i>Helicobacter felis</i>	Lee CS1 ^T	ATCC 49179 ^T	M37642	32
<i>Helicobacter felis</i>	Lee DS3	Not deposited	M37643	32
<i>Helicobacter fennelliae</i>	CCUG 18820 ^T	ATCC 35684 ^T	M88154	43
<i>Helicobacter mustelae</i>	Fox R85-13-6 ^T	ATCC 43772 ^T	M35048	32
<i>Helicobacter muridarum</i>	Lee ST1 ^T	CCUG 29262 ^T , ATCC 49282 ^T	M80205	23
<i>Helicobacter nemestrinae</i>	ATCC 49396 ^T		X67854	40
<i>Helicobacter pylori</i>	ATCC 43504 ^T		M88157	11
<i>Helicobacter</i> sp. CLO 3	CCUG 14564		M88151	43
<i>Wolinella succinogenes</i>	Tanner 602W ^T	ATCC 29543 ^T	M88159	11

^a Strains from which sequences were determined were obtained from the following individuals or culture collections: K. A. Eaton, Department of Veterinary Pathobiology, Ohio State University, Columbus; J. G. Fox, Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge; A. Lee, Department of Microbiology and Immunology, University of New South Wales, Sydney, Australia; C. Seymour, Department of Microbiology, Boston University School of Medicine, Boston, Mass.; A. Tanner, Department of Microbiology, Forsyth Dental Center, Boston, Mass.; ATCC, American Type Culture Collection, Rockville, Md.; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; NADC, National Animal Disease Center, Ames, Iowa; and NCTC, National Collections of Type Cultures, London, United Kingdom.

^b Alternate culture collection sources for sequenced strains.

^c 16S rRNA sequences for these strains are available for electronic retrieval from GenBank under the accession numbers given above. Through cross-distribution of databases, these sequences should also be available from the EMBL and DDBJ databases.

^d Literature references to sequences not obtained in this paper.

density corresponding to that of a no. 5 McFarland turbidity standard. Following addition of inoculating suspension to each microwell, the strips were incubated at 37°C for 4 h. Amino-peptidase reactions were developed by addition of cinnamaldehyde reagent. Oxidase was determined with DrySlide oxi-

dase slides (Difco). Catalase activity was determined by formation of bubbles upon addition of a loopful of cells to 100 µl of 3% hydrogen peroxide. Gamma glutamyl transpeptidase was determined by adding 100 µl of a no. 5 McFarland density suspension of cells to a microtiter well containing 0.1 mg of L-glutamic acid γ-(4-methoxy-β-naphthylamide) (Sigma). Cells were incubated for 4 h at 37°C, and the reaction was developed by addition of 1 drop of cinnamaldehyde reagent (Innovative Diagnostics, Inc.). Urease activity was determined by using Christensen (8) urease test agar (Remel, Lenexa, Kans.) incubated at 37°C for 4 h. Indoxyl acetate hydrolysis was determined by the impregnated disc method (18) with a 20-min incubation at room temperature. Methods for testing antibiotic sensitivity, hippurate hydrolysis, H₂S production, and growth in the presence of 3.5% NaCl and 1% glycine were described previously (39).

Electron microscopy. Cells grown on Trypticase soy agar-blood plates were gently suspended in 10 mM Tris buffer (pH 7.4) at a concentration of approximately 10⁸ cells per ml. Samples were negatively stained with 1% (wt/vol) phospho-

TABLE 2. DNA probes

Target	Location (bases) ^a	Sequence
All campylobacters	400-423	5'-CGAAAAGTGTTCATCTCCACGCGG-3'
All helicobacters	274-300	5'-TCTCAGGCCGGATACCCGTCATAGCCT-3'
Strain B9	818-845	5'-ACAAGCTCCACAACAACATAGCATTCATC-3'
Strain B10	623-651	5'-GAGTAATAGTTTCAATGCACTTCTGTAG-3'
Strain B52	1010-1033 ^b	5'-GTAACTAGACACTCCCATATCTCT-3'

^a Numbering relative to *E. coli* (5).

^b Note that strain B52 has one extra base relative to *E. coli* in this region and is 25 rather than 24 bases long.

TABLE 3. Differential characteristics of helicobacter isolates^a

Biotype	Strain	Source	Urease activity	Indoxyl acetate hydrolysis	Susceptibility to:		Reactivity with probe specific for:				
					Cephalothin	Nalidixic acid	Campylobacter	Helicobacter	B9	B10	B52
A	B7	Tern	—	—	S	S	—	+	+	—	—
A	B9	Tern	—	—	S	S	—	+	+	—	—
A	B12	Gull	—	—	S	S	—	+	+	—	—
A	B13	Gull	—	—	S	S	—	+	+	—	—
A	B15	Gull	—	—	S	S	—	+	+	—	—
A	M17	Pig	—	—	S	S	—	+	+	—	—
B	B8	Tern	+	+	R	S	—	+	—	+	—
B	B10	Tern	+	+	R	S	—	+	—	+	—
C	B52	House sparrow	+	+	R	MS	—	+	—	—	+

^a —, negative reaction; +, positive reaction; R, resistant, no zone of inhibition; S, sensitive (zone of inhibition, <20 mm); MS, marked sensitivity (zone of inhibition, >25 mm) with 30- μ g antibiotic-impregnated discs.

tungstic acid (pH 6.5) for 20 to 30 s. Specimens were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

G+C content of DNA. The G+C contents of DNAs were determined by thermal denaturation analysis as described by Breznak and Canale-Parola (3) and calculated with the equation of De Ley (9). DNA isolated from *Escherichia coli* K-12 was used for control determinations.

16S rRNA sequencing. rRNA was isolated and partially purified by phenol extraction and ethanol and salt precipitations as previously described (30). rRNA was sequenced by using a modified Sanger dideoxy chain termination technique in which primers complementary to conserved regions were elongated with avian myeloblastosis virus reverse transcriptase (22). The details of our protocol, including special sequencing primers, have been previously described (11, 30).

16S rRNA data analysis. A program set for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and dendrogram construction for 16S rRNA data was written in Microsoft QuickBASIC for use on IBM PC-AT and compatible computers (30). RNA sequences were entered and aligned as previously described (30). Our sequence database contains approximately 300 sequences determined in our laboratory and another 200 sequences

obtained from GenBank or the Ribosomal Database Project (27). Reference strains used in the 16S rRNA analysis are given in Table 1. Similarity matrices were constructed from the aligned sequences by using only those sequence positions for which 90% of the strains had data. The similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor (20). Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (35, 45).

DNA probe design, synthesis, and labeling. DNA probes were designed by locating 24- to 30-base regions of 16S rRNA sequence in which the target organism differed from the sequences of all related organisms as previously described (10). Oligonucleotides were synthesized on an ABI 392 DNA/RNA Synthesizer (Foster City, Calif.). The probes used in this study are given in Table 2. Validation of the all-helicobacter and all-campylobacter probes has been previously reported (15, 39).

DNA probe labeling. Probe oligonucleotides were end labeled with [³²P]ATP by using the T4 polynucleotide kinase method (36). The labeled oligonucleotide probes were purified by chromatography on a Sep-Pak C₁₈ column (37).

Dot blot hybridization. Whole cells were harvested from agar plates and spotted onto Whatman 541 filter paper. Hybridization of probes to partially lysed fixed cells was performed as previously described (10). Bound radioactivity was localized and quantitated with a Betascope 603 (Betagen, Waltham, Mass.).

Nucleotide sequence accession number. The GenBank and culture collection accession numbers for the strains examined in this report are given in Table 1.

RESULTS AND DISCUSSION

The nine isolates were catalase, oxidase, alkaline phosphatase, and nitrate reduction positive. As previously reported, they grew at 42°C in the presence of 1% glycine but not in the presence of 3.5% NaCl (39). They did not hydrolyze hippurate or show gamma glutamyl transpeptidase activity. The naphthylamine aminopeptidase tests were of little value for differentiating among *Helicobacter* and *Campylobacter* species. The isolates were positive for hydrolysis of the arginine beta-naphthylamide but negative for hydrolysis of leucine, proline, pyroglutamate, tyrosine, phenylalanine, and glycine beta-naphthylamides. Alanine and histidine beta-naphthylamide tests were usually negative but sometimes weakly positive and thus were unreliable. The strains differed in their urease, indoxyl acetate hydrolysis, and antibiotic sensitivity tests, and, on the basis of these tests, the strains were divided into three biotypes

FIG. 1. Transmission electron micrograph of *H. pametensis* B9^T. Note that flagella are sheathed. Bar, 0.3 μ m.

TABLE 4. Similarity matrix based on 16S rRNA sequence comparisons^a

	<i>H.pa</i>	<i>H.Sc</i>	<i>H.B</i>	<i>H.ms</i>	<i>H.mr</i>	<i>H.ca</i>	<i>H.ct</i>	<i>F.ra</i>	<i>H.fe</i>	<i>H.S</i>	<i>H.py</i>	<i>H.ne</i>	<i>H.ac</i>	<i>G.h1</i>	<i>G.h2</i>	<i>H.fe</i>	<i>W.su</i>	<i>A.cr</i>	<i>A.sk</i>	<i>A.bu</i>	<i>C.re</i>	<i>C.co</i>	<i>C.jf</i>	<i>C.la</i>	<i>C.je</i>
<i>Helicobacter pametensis</i>	98.1	97.9	97.2	95.7	96.5	95.5	95.5	96.5	95.5	95.1	94.5	94.6	94.0	92.6	93.7	93.9	94.2	86.0	85.6	86.5	85.9	87.6	86.5	87.2	87.7
<i>Helicobacter</i> sp. Bird-C	2.0	98.3	98.0	96.3	96.9	95.6	96.5	94.6	95.5	94.2	94.5	93.5	92.2	92.9	93.2	94.6	85.6	85.1	86.3	85.5	86.8	86.6	87.3	87.7	87.1
<i>Helicobacter</i> sp. Bird-B	2.1	1.8	98.7	96.0	96.8	95.4	95.9	94.6	95.2	93.8	94.0	93.5	92.2	93.0	93.4	94.2	86.1	85.9	86.9	85.1	86.5	86.2	86.9	87.2	86.9
<i>Helicobacter mustelae</i>	2.9	2.1	1.3	95.8	96.4	95.2	95.8	94.4	95.2	93.9	94.0	93.5	92.0	93.2	93.4	93.8	86.0	85.6	86.5	84.8	86.1	85.8	86.5	87.2	86.6
<i>Helicobacter muridarum</i>	4.5	3.8	4.1	4.4	96.5	95.9	96.2	95.1	94.3	93.1	92.5	91.9	92.4	92.6	93.4	93.4	85.0	84.6	85.8	84.8	86.4	85.6	86.4	86.2	85.8
<i>Helicobacter canis</i>	3.6	3.2	3.2	3.7	3.6	97.8	98.1	95.4	96.0	93.9	93.8	93.2	92.3	92.7	93.2	93.4	85.4	85.0	85.9	85.6	87.2	86.5	87.2	87.1	86.6
<i>Helicobacter cinacdi</i>	4.6	4.5	4.8	5.0	4.2	2.3	98.8	95.9	95.3	92.8	93.3	92.4	92.0	92.4	92.6	92.9	85.0	84.6	85.4	85.8	87.2	86.4	86.3	86.5	86.0
" <i>Flexispira rappini</i> "	3.6	3.6	4.2	4.3	3.9	1.9	1.2	95.4	95.6	93.3	93.5	92.5	92.3	92.4	92.7	93.2	85.2	84.8	85.6	86.0	87.5	86.9	87.3	87.3	86.8
<i>Helicobacter fennelliae</i>	4.6	5.6	5.6	5.8	5.1	4.7	4.3	4.7	94.8	93.1	92.8	92.3	92.6	92.8	93.0	92.5	84.5	84.3	85.6	85.4	87.0	86.1	86.0	86.8	86.2
<i>Helicobacter</i> sp. CLO3	5.0	4.7	5.0	4.9	5.9	4.1	4.9	4.5	5.4	93.9	93.4	93.0	92.7	92.8	93.2	92.4	85.5	85.1	86.3	85.2	86.8	85.9	85.9	86.6	85.9
<i>Helicobacter pylori</i>	5.7	6.1	6.5	6.4	7.2	6.4	7.5	7.1	7.3	6.4	98.2	97.4	94.9	95.1	95.4	90.9	84.5	84.0	85.3	84.1	85.1	85.1	85.6	85.6	85.4
<i>Helicobacter nemestrinae</i>	5.6	5.7	6.2	6.2	7.3	6.4	7.0	6.8	7.6	6.9	1.8	96.7	94.7	94.9	95.5	91.0	85.0	84.4	85.6	84.8	86.0	85.7	86.2	86.0	85.7
<i>Helicobacter actinonx</i>	6.3	6.8	6.8	6.8	8.0	7.1	8.0	7.8	8.1	7.4	2.6	3.3	94.7	96.0	96.4	90.6	84.2	83.9	85.1	83.6	84.8	84.6	85.2	85.2	85.0
" <i>Gastropirillum hominis</i> " 1	7.8	8.2	8.2	8.4	8.6	8.1	8.4	8.2	7.8	7.6	5.3	5.5	5.5	96.5	96.6	89.3	83.5	83.2	83.7	83.2	84.6	84.5	85.0	84.9	84.4
" <i>Gastropirillum hominis</i> " 2	6.6	7.5	7.3	7.1	8.0	7.6	8.0	8.0	7.6	7.5	5.1	5.3	4.1	3.6	98.8	90.0	83.7	83.2	84.1	82.9	84.5	84.4	85.0	85.1	84.6
<i>Helicobacter felis</i>	6.3	7.1	6.9	6.9	7.8	7.1	7.8	7.7	7.3	7.1	4.7	4.7	3.7	3.4	1.3	90.2	83.5	83.3	84.2	83.0	84.5	84.5	85.0	85.1	84.6
<i>Wolinella succinogenes</i>	6.1	5.6	6.1	6.4	6.9	6.9	7.4	7.1	7.9	8.0	9.8	9.5	10.1	11.5	10.7	10.5	85.6	85.2	86.1	86.3	86.3	85.9	86.0	86.8	86.4
<i>Arcobacter cryaerophilus</i>	15.4	16.0	15.3	15.4	16.8	16.2	16.7	16.5	17.4	16.1	17.4	16.8	17.7	18.7	18.4	18.7	15.9	99.0	97.4	86.1	87.6	86.1	86.5	87.2	87.3
<i>Arcobacter skirrowii</i>	16.0	16.6	15.6	16.0	17.2	16.7	17.3	17.0	17.6	16.6	18.0	17.5	18.1	19.1	19.0	18.9	16.4	1.0	97.3	85.6	87.6	86.0	86.2	86.9	87.2
<i>Arcobacter butzleri</i>	14.9	15.2	14.4	14.9	15.7	15.6	16.2	15.9	16.0	15.1	16.3	16.0	16.6	18.3	17.9	17.7	15.3	2.6	2.8	86.6	88.0	86.1	86.4	86.7	86.9
<i>Campylobacter rectus</i>	15.6	16.1	16.6	16.9	17.0	16.0	15.8	15.4	16.2	16.5	17.8	17.0	18.5	19.1	19.4	19.3	15.1	15.4	16.0	14.8	96.0	94.0	91.8	92.5	92.9
<i>Campylobacter concisus</i>	13.6	14.5	14.9	15.4	15.1	14.0	14.0	13.7	14.3	14.6	16.6	15.5	17.0	17.2	17.4	17.3	15.1	13.6	13.6	13.1	4.1	95.6	92.3	93.9	93.8
<i>Campylobacter jejens</i> subsp. <i>jejens</i>	14.9	14.7	15.3	15.7	16.0	14.9	15.0	14.4	15.4	15.6	16.6	15.9	17.3	17.4	17.4	17.4	15.6	15.4	15.5	15.3	6.2	4.6	93.3	94.2	94.5
<i>Campylobacter helveticus</i>	14.0	13.9	14.4	14.9	15.0	14.0	15.1	13.9	15.5	15.6	16.0	15.3	16.5	16.7	16.7	16.7	15.5	14.9	15.3	15.0	8.6	8.1	7.0	96.7	97.1
<i>Campylobacter lari</i>	13.4	13.5	14.0	14.1	15.2	14.1	14.8	13.9	14.6	14.8	16.0	15.5	16.4	16.8	16.7	16.6	14.5	14.0	14.4	14.7	7.9	6.4	6.0	3.4	98.7
<i>Campylobacter jejuni</i>	14.0	14.1	14.4	14.7	15.8	14.7	15.5	14.5	15.2	15.7	16.2	15.8	16.7	17.5	17.2	17.2	15.1	13.9	14.1	14.4	7.5	6.5	5.7	3.0	1.3

^a Numbers above the diagonal represent uncorrected percentages of similarity, and those below the diagonal are percentages of difference corrected for multiple base changes by the method of Jukes and Cantor (20).

FIG. 2. Phylogenetic tree for strains representing 26 species of *Helicobacter*, *Arcobacter*, *Campylobacter*, and related bacteria based on 16S rRNA sequence similarity data. The scale bar represents a 5% difference in nucleotide sequences, as determined by measuring the lengths of horizontal lines connecting any two species.

(Table 3). The sole difference between biotypes B and C is that the biotype B strains had moderate sensitivity to nalidixic acid (zone of inhibition, <20 mm) and strain B52 showed marked sensitivity to nalidixic acid (zone of inhibition, >25 mm). The separation of biotypes B and C was subsequently validated by the molecular sequencing and DNA probe data described below.

Phase microscopy of cells representing each of the three biotypes yielded similar results. Cells were curved or, occasionally, short spiral rods. In older cultures (greater than 3 days), coccoid forms predominated. Rapidly motile cells were seen in young cultures (1 to 2 days). Growth often formed as thin films that swarmed over Trypticase soy agar-blood plates. Electron micrographic examination of cells representative of each biotype revealed curved rods 0.4 μ m in diameter by 1.5 μ m in length. An electron micrograph of strain B9 is shown in Fig. 1. Usually a single sheathed flagellum was inserted subterminally at each end of the cell. However, about 10% of cells possessed a third flagellum inserted subterminally adjacent to one of the other two flagella. Other strains representing biotypes A, B, and C appeared identical by electron microscopy, although a third flagellum was not observed in biotypes B and C.

Using eight primers, 95% of the 16S rRNA sequence (approximately 1,450 bases) was determined for one representative strain of each of the three biotypes: B9, B10, and B52 for biotypes A, B, and C, respectively. The sequences for each of the strains were unique. Comparison of the sequences with others in our database indicated that each of the three strains represented new species in the genus *Helicobacter*. DNA probes were designed and synthesized on the basis of comparison of these sequences with all other *Helicobacter* sequences in our database (Table 2). The remaining six isolates were screened for hybridization to these DNA probes (Table 3). Probe reactivity corresponded exactly to the previously determined biotypes. Subsequently, full 16S rRNA sequences were obtained for all isolates. The sequences for the six strains of biotype A differed from one another by three or fewer base

changes. The sequences for the two strains of biotype B were identical.

A similarity matrix for a total of 26 *Helicobacter*, *Campylobacter*, *Arcobacter*, and *Wolinella* species is shown in Table 4. A dendrogram showing the phylogenetic relationships for these species is shown in Fig. 2. The three new species of fecal helicobacters are most closely related to *H. mustelae*.

Bootstrapping with the program MEGA (21a) was performed with 300 resamplings and pairwise elimination of incomplete data. The clade composed of *H. mustelae* and *Helicobacter* sp. Bird-B was present in 98% of the resamplings. The clade composed of *Helicobacter* sp. Bird-C and the two previous species was present in 74% of the resamplings. The clade composed of *H. pametensis* and the three previous species was present in 98% of the resamplings. In separate analyses including the five *H. pametensis* strains and the two *Helicobacter* sp. Bird-B strains, the strains fell with their respective species in 100% of 100 resamplings. Since *Helicobacter* sp. Bird-C is represented by only one strain, nothing can be inferred about its reproducibility at the species level. Bootstrap analysis examines the robustness of sequence data for reproducing a given tree by resampling with replacement but does not indicate the reliability of a given phylogenetic tree. With the above caveat, the bootstrap analysis reported strongly supports (100%) the existence of *H. pametensis* and *Helicobacter* sp. Bird-B as taxa separate from other described species.

On the basis of the sequence and phenotypic data, we propose the name *Helicobacter pametensis* sp. nov. for the six isolates of biotype A. A formal description of this species is given below. Because of the limited number of strains and limited differential characteristics for biotypes B and C, we will not formally name these species. We will refer to them as *Helicobacter* sp. Bird-B and *Helicobacter* sp. Bird-C in accordance with their biotypes.

Differentiation of these new *Helicobacter* species from each other and from *Campylobacter* species such as *C. coli*, *C. jejuni*,

TABLE 5. Characteristics which differentiate *H. pametensis* from other *Helicobacter* and *Campylobacter* species^a

Taxon	Catalase produc- tion	Nitrate reduc- tion	Alkaline phosphatase hydrolysis	Urease	Gamma-2- glutamyl transpepti- dase	Indoxyl acetate hydro- lysis	Growth at 42°C	Growth at 1% glycine	Susceptibility to (30-µg disc):		Peri- plasmic fibers	No. of flagel- la/cell	Distribution of flagella/ cell	G+C content (mol%)
									Nalidixic acid	Cepha- lothin				
<i>H. pametensis</i>	+	+	+	-	-	-	+	+	S	S	-	2	Bipolar	38
<i>Helicobacter</i> sp. Bird-C	+	+	+	+	-	+	+	+	S	R	-	2	Bipolar	30
<i>Helicobacter</i> sp. Bird-B	+	+	+	+	-	+	+	+	S	R	-	2	Bipolar	31
<i>Helicobacter mustelae</i>	+	+	+	+	+	+	+	+	S	R	-	4-8	Peritrichous	36
<i>Helicobacter</i> sp. strain CLO3	+	-	+	+	-	+	+	+	I	R	-	ND	ND	45
<i>Helicobacter fennelliae</i>	+	-	+	+	-	+	+	+	S	S	-	2	Bipolar	35
<i>Helicobacter muridarum</i>	+	-	+	+	-	+	-	-	R	R	+	10-14	Bipolar	34
<i>Helicobacter canis</i>	-	+	+	-	ND	+	+	ND	S	I	-	2	Bipolar	48
<i>Helicobacter cinaedi</i>	+	+	-	+	-	-	+	+	S	I	-	1-2	Bipolar	37-38
" <i>Flexispira rappini</i> "	+	-	-	+	+	ND	+	+	R	R	+	10-20	Bipolar	34
<i>Helicobacter pylori</i>	+	-	+	+	+	-	-	-	R	S	-	4-8	Bipolar	35-37
<i>Helicobacter acinonyx</i>	+	-	+	+	+	-	-	-	R	S	-	2-5	Bipolar	30
<i>Helicobacter nemestrinae</i>	+	-	+	+	ND	-	-	-	R	S	-	4-8	Bipolar	24
<i>Helicobacter felis</i>	+	+	+	+	+	-	+	-	R	S	+	14-20	Bipolar	42
<i>Campylobacter coli</i>	+	+	ND	-	ND	+	+	+	S	R	-	1	Polar	30-33
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i>	+	+	ND	-	ND	+	+	+	S	R	-	1	Polar	30-33
<i>Campylobacter jejuni</i> subsp. <i>doylei</i>	V	-	ND	-	ND	+	+	+	S	S	-	1	Polar	30-31
<i>Campylobacter lari</i>	+	+	ND	V	ND	-	+	+	R	R	-	1	Polar	30-32

^a Data were obtained from Eaton (11), Stanly (43), Vandamme (47), and this study. +, positive reaction; -, negative reaction; V, variable; S, susceptible; R, resistant; I, intermediate; ND, not determined.

and *C. lari* is difficult by using phenotypic traits (Table 5). *H. pametensis*, *Helicobacter* sp. Bird-B, *Helicobacter* sp. Bird-C, *C. coli*, *C. jejuni* subsp. *jejuni*, and *C. lari* are all oxidase, catalase, and nitrate reduction positive; are H₂S production negative; and grow at 42°C and in the presence of 1% glycine but do not grow at 25°C or in the presence of 3.5% NaCl. Resistance to nalidixic acid differentiates *C. lari* from the other five species. Sensitivity to cephalothin distinguishes *H. pametensis* from the remaining four species. *C. lari* and *H. pametensis* are also distinguished from the other four species by a negative test for indoxyl acetate hydrolysis. Thus, even with 11 phenotypic tests, *Helicobacter* sp. Bird-B, *Helicobacter* sp. Bird-C, *C. coli*, and *C. jejuni* subsp. *jejuni* would still be indistinguishable. Hippurate hydrolysis, urease, and alkaline phosphatase tests differentiate these species. However, it is quite possible that bird *Helicobacter* species have been misidentified as *Campylobacter* species in previous studies relying on a limited number of phenotypic traits. In our previous study, we obtained 22 thermotolerant campylobacter-like isolates from birds (39). Thirteen isolates were identified as *C. jejuni*, *C. coli*, or *C. lari* on the basis of phenotypic criteria. Nine isolates differed from these typical *Campylobacter* species in one or more biochemical tests. Eight of the atypical isolates reacted with an all-helicobacter DNA probe. One atypical isolate was a mixed culture, containing *C. jejuni* and *Helicobacter* sp. Bird-C. It was only through reactivity with both the all-campylobacter probe and all-helicobacter probe that we realized the culture was mixed and eventually separated it into pure cultures of each species. With the limited number of reliable phenotypic traits known for these organisms and the number of *Campylobacter*, *Arcobacter*, and *Helicobacter* species approaching 50 (including species not formally named but identified by 16S rRNA sequence analysis), it is becoming difficult to reliably differentiate species by traditional biochemical characterization. Molecular tools have the potential to distinguish each of these species, but almost no DNA probes have been validated against the most recently described species. Because of the limited number of changes in the 16S rRNA between closely related species, we find that it is not always possible to identify a *Helicobacter* species by using a single probe based on 16S rRNA sequences. It may be prudent for laboratories to confirm presumptively identified isolates by a second method such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles.

While the zoonotic potential of the three new *Helicobacter* species is unknown, isolates of *H. pametensis* were recovered from two different species of wild birds and a domestic pig, suggesting that the species has a broad host range. Other intestinal helicobacters such as *H. canis* and *H. cinaedi* may on occasion cause human gastroenteritis by zoonotic transmission (7, 16, 28). Zoonotic transmission of *Campylobacter jejuni* infection by birds is well known, with domestic poultry being the major source of human infection (44). Recent reports implicate wild birds, principally magpies (*Pica pica*) and jackdaws (*Corvus monedula*), as a source of *C. jejuni*-contaminated milk. Birds contaminated milk in bottles placed on porches by pecking through the bottle caps (19, 34). This novel method of zoonotic transmission of *C. jejuni* emphasizes that wild birds are an extensive reservoir of *C. jejuni* (21). During an outbreak of waterborne campylobacteriosis in military personnel, identical serotypes of *C. jejuni* were cultured from affected humans and from a common teal (*Anas crecca*) that may have contaminated the water supply (1). It has been noted that the concentration of campylobacters in surface water is highly dependent upon water temperature and the presence of waterfowl (33). It seems highly probable that humans are exposed

to these newly identified fecal helicobacters as well as the more well-known campylobacters. Further studies are required to ascertain whether humans are colonized with these enteric helicobacters and, importantly, to ascertain if these bacteria can cause diarrheal disease in humans or other mammals.

Description of *H. pametensis* sp. nov. *Helicobacter pametensis* (pa.me.ten'sis. N.L. of the Pamet River, Truro, Mass.). The description is based in part on our previous data (39). Cells are curved gram-negative rods, approximately 0.4 μ m in diameter by 1.5 μ m in length, with rounded ends. Cells are motile and possess one sheathed flagellum inserted subterminally at each end. Occasionally cells possess a third flagellum adjacent to one of the other flagella. No endospores are produced. Growth in a microaerobic environment at 37°C and 42°C. There is weak growth under anaerobic conditions, and there is no growth in air at 37°C. Oxidase, catalase, alkaline phosphatase, and arginine beta-naphthylamide aminopeptidase activities are produced. Proline, pyroglutamate, tyrosine, alanine, phenylalanine, and glycine beta-naphthylamide aminopeptidases and gamma glutamyl transpeptidase activities are not detected. Hippurate, urea, and indoxyl acetate are not hydrolyzed. H₂S is not produced on a triple sugar iron slant. Lysine and ornithine decarboxylase activity are not detected. Nitrate is reduced to nitrite. Growth occurs in the presence of 1% glycine but not in the presence of 3.5% NaCl. *H. pametensis* is sensitive to nalidixic acid but resistant to cephalothin when 30- μ g antibiotic-impregnated discs are used.

H. pametensis has been isolated from tern, gull, and swine feces. Six strains have been isolated (B7, B9, B12, B13, B15, and M17). Strains B9^T and B12 are available from the American Type Culture Collection as ATCC 51478 and ATCC 51479, respectively. All strains are available from the Culture Collection of the University of Göteborg, Göteborg, Sweden (CCUG 29253, 29255, and 29257 to 29260, respectively). The type strain, B9^T (ATCC 51478^T, CCUG 29255^T), has a G+C content of 38 mol%. The essentially complete 16S rRNA sequence of the type strain of *H. pametensis*, B9, is available from the GenBank, EMBL, and DDBJ databases under accession number M88147.

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