***Helicobacter ganmani* sp. nov., a urease-negative anaerobe isolated from the intestines of laboratory mice**

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Spiral bacteria were isolated from the intestines of laboratory mice during a study examining the presence of *Helicobacter* species and other spiral organisms naturally infecting mice maintained at four different animal facilities in Sydney, Australia. One group of 17 isolates, cultured from mice from three of the four facilities, were found to be helicobacters but did not fall within any of the 18 currently recognized species. These isolates were unusual in that they only grew anaerobically at 37 °C and were incapable of growth under microaerobic conditions. Like *Helicobacter rodentium*, isolates possessed single, bipolar, unsheathed flagella and were urease-negative. They were positive for oxidase and reduced nitrate to nitrite but did not hydrolyse hippurate or indoxyl acetate, grew on charcoal agar and were resistant to cephalothin. 16S rDNA sequences from four strains were determined and found to be identical to one another. *H. rodentium* was the most closely related species in terms of 16S rDNA sequence similarity (98.2%). Numerical analysis of whole-cell proteins by SDS-PAGE for nine isolates was carried out with a comparison to all known *Helicobacter* species, including newly determined profiles from three *H. rodentium* strains. The new isolates were clearly differentiated from *H. rodentium* and other *Helicobacter* spp. On the basis of this data, including genetic, biochemical and protein analysis, it is proposed that these isolates belong to *Helicobacter ganmani* sp. nov. (type strain CMRI H02^T = CCUG 43526^T = CIP 106846^T).

Keywords: *Helicobacter ganmani*, *Helicobacter rodentium*, phylogeny, 16S rDNA, SDS-PAGE

INTRODUCTION

The discovery and culture of *Helicobacter pylori* by Marshall *et al.* (1984) reinvigorated the field of gastrointestinal microbiology and led to the establishment of a new genus of bacteria with the transfer of *H. pylori* and *Helicobacter mustelae* from the genus *Campylobacter* in 1989 (Goodwin *et al.*, 1989). Since the establishment of this genus, the number of recog-

nized species has risen dramatically, currently standing at 18 formally named, and two provisional (*Candidatus*), species. The normal site of colonization for all of these bacteria is the gastrointestinal tract, with isolates being divided between gastric and intestinal colonizers. Species have been isolated from a wide variety of mammals and birds with the intestinal colonizers making up well over half of the described species. Phylogenetically, the genus *Helicobacter* is found within the ϵ -subclass of the class *Proteobacteria* (Murray *et al.*, 1990). This separate bacterial lineage has also been defined as rRNA superfamily VI (Vandamme *et al.*, 1991) keeping in line with the distinction of rRNA superfamilies within the *Proteobacteria* (De Ley, 1992). Other genera found within this subclass include *Campylobacter* and *Arcobacter*,

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Abbreviation: SPF, specific pathogen-free.

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which together make up the family *Campylobacteraceae* (Vandamme & De Ley, 1991).

The discovery of several murine helicobacters has led to uncertainties about the suitability of some laboratory mice for helicobacter vaccine studies and the validity of a number of past studies in other areas of biomedical research. In *H. pylori* vaccine research, prior antigen exposure is important in terms of host immune response and the use of mouse models in which animals have been, or still are, naturally infected with other *Helicobacter* species is significant. Furthermore, several murine *Helicobacter* species are themselves associated with disease in some infected animals. Mice infected with *Helicobacter hepaticus* can develop chronic liver inflammation and, in some strains of mice, hepatocellular carcinoma after long-term infection (Fox *et al.*, 1994; Fox & Wang, 1997). *H. hepaticus* infection has been shown to be widespread among commercially available mice in the USA (Shames *et al.*, 1995). *Helicobacter bilis* has also been associated with hepatitis in aged, inbred strains of mice (Fox *et al.*, 1995) and, like *H. hepaticus*, was isolated from commercially available mice (Shames *et al.*, 1995). Other *Helicobacter* species that have been found to colonize the murine intestinal tract include *Helicobacter muridarum* (Lee *et al.*, 1992), *Helicobacter trogontum* (Mendes *et al.*, 1996) and *Helicobacter rodentium* (Shen *et al.*, 1997). The misnamed '*Flexispira rappini*' has also been isolated from the mouse intestinal tract (Schauer *et al.*, 1993). As the mouse represents a convenient and widely used animal model for *H. pylori* infection, workers in the field must be vigilant in detecting natural infections with other *Helicobacter* species in laboratory mice.

During such a survey of spiral bacteria infecting mice obtained from four animal facilities in Sydney, Australia, we cultured a novel species of *Helicobacter*, for which we propose the name *Helicobacter ganmani*. The description takes into account the recent proposal of minimal standards for describing new species of the genus *Helicobacter* (Dewhirst *et al.*, 2000), and pays special attention to a comparison with *H. rodentium*, the most closely related species, also isolated from laboratory mice (Shen *et al.*, 1997). Isolates of the proposed species were characterized using morphological, biochemical, physiological and genetic methods.

METHODS

Bacterial isolation and cultivation. Conventional or Specific Pathogen-Free (SPF) Quackenbush/Swiss mice ($n = 23$) were obtained from four separate animal facilities in Sydney, Australia. Routine screening for spiral bacteria was carried out by culturing intestinal mucus scrapings and homogenized liver samples on blood agar (BA) containing Blood Agar Base No. 2 (Oxoid) plus 5% horse blood, or on Campylobacter selective agar (CSA) containing BA supplemented with amphotericin ($2.5 \mu\text{g}$ Fungizone ml^{-1} ; E. R. Squibb) and Skirrow's selective supplement ($2.5 \mu\text{g}$ polymyxin B ml^{-1} , $10 \mu\text{g}$ vancomycin ml^{-1} , $5 \mu\text{g}$ trimethoprim ml^{-1}). Mouse intestinal mucus scrapings or homo-

genized livers were inoculated directly onto fresh, moist BA and CSA. Alternatively, filters with a pore size of $0.65 \mu\text{m}$ (Millipore) were placed onto the surface of plates and mucus scrapings or homogenized livers were inoculated onto the centre of the filters and plates placed in a 37°C incubator set at 10% CO_2 and 95% humidity for 2 h. After this time the filters were removed. All plates were incubated, lids up-permost, in an anaerobic jar (HP 11; Oxoid) with a microaerobic gas-generating kit (BR 56; Oxoid) or with an anaerobic gas-generating kit (BR 38; Oxoid) at 37°C for 72–96 h. After incubation, plates were examined for growth of spiral bacteria using phase-contrast microscopy. Suspect colonies were subcultured until pure cultures were obtained.

Electron microscopy. Fresh bacterial cultures were fixed in 1% glutaraldehyde for 1 min then transferred to cacodylate buffer (0.1 M sodium cacodylate-HCl, pH 7.4). Bacteria were negatively stained with phosphotungstic acid (1%) and viewed with an Hitachi H7000 transmission electron microscope.

DNA extraction. DNA was extracted from a loopful of cells using a modification of the bacterial lysis method of Tillett & Neilan (2000). Cells were resuspended in 1 ml XS buffer containing 0.01% (w/v) potassium ethyl xanthogenate (Fluka Chemika), 0.8 M ammonium acetate, 0.1 M Tris/HCl (pH 8.0), 20 mM EDTA (pH 8.0) and 1% (w/v) SDS and mixed by vortexing for 10 s. The solution was incubated at 70°C for 30 min, vortexed for 10 s and cooled on ice for 30 min. The tube was centrifuged at 15000 r.p.m. for 10 min at 4°C and the supernatant carefully removed into a new microfuge tube. The supernatant, containing genomic DNA, was precipitated by the addition of an equal volume of 2-propanol and incubated at room temperature for 10 min. The DNA was collected by centrifugation at 15000 r.p.m. for 20 min at 4°C . The resulting pellet was washed with 70% ethanol, dried using a vacuum desiccator then resuspended in 200 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). An aliquot of DNA was diluted 1/10 in distilled water for use in PCR applications.

PCR amplification and sequencing. Typically, amplification reactions included $1 \times$ reaction buffer [67 mM Tris/HCl, 16 mM $(\text{NH}_4)_2\text{SO}_4$, 0.45% Triton X-100, 0.2% gelatin], 1 unit *Taq* DNA polymerase (Biotech International), 200 μM each dNTP, 2 mM MgCl_2 , 10 pmol each oligonucleotide primer and 1 μl diluted DNA (approx. 20–100 ng) in a final volume of 50 μl . Two *Helicobacter* genus-specific PCRs were used to screen isolates using primers F27(UFP) and R274 or primers H276f and H676r (Table 1). Both these reactions were performed as has been described previously (Lee *et al.*, 1997; Riley *et al.*, 1996). PCR amplification of the 16S rDNA was achieved using primers F27(UFP) and R1492(URP) (Table 1). Here, cycling conditions included an initial denaturation at 94°C for 3 min then five cycles of 94°C for 45 s, 53°C for 30 s and 72°C for 1 min, followed by 28 cycles of 94°C for 45 s, 48°C for 30 s and 72°C for 1 min. A final extension step at 72°C for 10 min was carried out at the end of the amplification. Thermocycling was performed in an FTS-320 Thermal Sequencer (Corbett Research). All PCR products were separated on agarose mini-gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and photographed under UV transillumination after staining with ethidium bromide. Whole length 16S rDNA PCR products were purified prior to sequencing using the Prep-A-Gene DNA Purification System (Bio-Rad). Amplified DNA was then directly sequenced using the ABI PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems) and the GeneAmp PCR System 2400

Table 1. 16S rDNA amplification and sequencing primers

Underlined sequences are those of the universal sequencing primers (UFP and URP) that were attached to assist in successive sequencing reactions.

Primer	Sequence (5'–3')	Reference
Amplification		
F27(UFP)	<u>TAGTGTA</u> AAACGACGGCCAGTAGAGTTTGATCCTGGCTCAG	Neilan <i>et al.</i> (1997)
R1492(URP)	TAGCAGGAAACAGCTATGACACGGTTACCTTGTTACGACTT	Neilan <i>et al.</i> (1997)
R274	TCTCAGGCCGGATACCCGTCATAGCCT	Fox <i>et al.</i> (1992)
H276f	CTATGACGGGTATCCGGC	Riley <i>et al.</i> (1996)
H676r	ATTCCACCTACCTCTCCCA	Riley <i>et al.</i> (1996)
D86	GTCCTTAGTTGCTAACTATT	Shen <i>et al.</i> (1997)
D87	AGATTTGCTCCATTTCACAA	Shen <i>et al.</i> (1997)
Sequencing		
27F	AGAGTTTGATCCTGGCTCAG	Neilan <i>et al.</i> (1997), Weisburg <i>et al.</i> (1991)
119F	GTGGCGGACGGGTGAGTA	Weisburg <i>et al.</i> (1985)
341R	CTGCTGCCTCCCGTAGG	Neilan <i>et al.</i> (1997), Weisburg <i>et al.</i> (1985)
530F	GTGCCAGCAGCCGCGG	Neilan <i>et al.</i> (1997), Weisburg <i>et al.</i> (1985)
685R	TCTACG(G/C)ATTT(T/C)ACCGCTAC	Weisburg <i>et al.</i> (1985)
929R	TCC(T/A)CCGCTTGTCGGGG	Neilan <i>et al.</i> (1997), Weisburg <i>et al.</i> (1991)
942F	GGGCCCCGACAAGCGG	Neilan <i>et al.</i> (1997), Weisburg <i>et al.</i> (1985)
1221R	ATTGTAG(T/C)ACGTGTGTAGCC	Neilan <i>et al.</i> (1997), Weisburg <i>et al.</i> (1985)
1241F	GCTACACACGT(A/G)CTACAATG	Neilan <i>et al.</i> (1997), Weisburg <i>et al.</i> (1985)
1494R	TACGGCTACCTTGTTACGAC	Neilan <i>et al.</i> (1997), Weisburg <i>et al.</i> (1991)

(Perkin Elmer) according to the manufacturers' protocols. Sequencing products were separated on model 377 DNA Sequencer machines and analysed using programs contained in the INHERIT package (PE Applied Biosystems). Primers used for sequencing reactions are listed in Table 1. In all cases both strands of DNA were sequenced with contiguous overlaps.

Phylogenetic inferences. Sequences were aligned using the GCG program PILEUP, version 8 (Genetics Computer Group, Program Manual for the Wisconsin Package, 1994) and the multiple-sequence alignment and profile alignment tools in the CLUSTAL x package (Thompson *et al.*, 1994). Aligned sequences were corrected manually and nucleotide positions that contained ambiguities were removed from further analysis. Genetic distances, corrected for multiple base changes by the method of Jukes & Cantor (1969), were calculated using the DNADIST program in the PHYLIP package, version 3.57c (Felsenstein, 1989). The phylogenetic tree was reconstructed by the neighbour-joining method of Saitou & Nei (1987) and the tree plotted using NJPLOT in the CLUSTAL x package. Bootstrap values were obtained from analysis of one hundred resamplings of the corrected alignment created using the program SEQBOOT (Felsenstein, 1985) and analysed using both distance (DNADIST) and parsimony (DNAPARS) algorithms and CONSENSE all from the PHYLIP package.

Biochemical characterization. Sixty-three phenotypic characteristics were determined for isolates using standardized

methods performed as recommended in an extensive identification scheme designed for *Campylobacter*, *Helicobacter* and related bacteria (On *et al.*, 1996). All tests were performed twice on separate occasions. Type and reference strains of *H. rodentium* were also examined for critical comparison with the putative new species. All tests were performed using freshly prepared media, whose quality was assured by appropriate control strains. Growth tolerance tests for both *H. ganmani* and *H. rodentium* were performed under anaerobic conditions.

SDS-PAGE of whole-cell proteins. Whole-cell protein extracts were prepared from isolates and SDS-PAGE was performed as described previously (Pot *et al.*, 1994). The densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed using the GelCompar software package version 4.2 (Applied Maths), and the profiles were recorded and stored on a PC computer. The similarity between all pairs of traces was expressed by the Pearson product-moment correlation coefficient and is presented as percentages of similarity.

RESULTS

Isolation, growth characteristics and ultrastructure

Out of a total of 110 spiral isolates cultured, 17 were isolated on BA in anaerobe jars fitted with anaerobic

Fig. 1. Negative stain of *Helicobacter ganmani* CMRI H02^T showing spiral morphology and unsheathed, single, bipolar flagella. Bar, 0.5 µm.

gas generating kits. These isolates were curved or spiral-shaped when observed in wet mounts using phase-contrast microscopy, were Gram-negative and not capable of growth under microaerobic conditions or on CSA under either microaerobic or anaerobic conditions. After 3–5 d incubation at 37 °C, growth appeared as a thin, transparent, spreading film. Single colonies (< 1 mm diameter) were isolated when the initial inoculation of intestinal mucus scraping was made onto membrane filters with a pore size of 0.65 µm and were characterized by irregular edges, consistent with a spreading, motile organism. No isolates representing this group of bacteria were cultured from mice obtained from one of the animal facilities (conventional), while all spiral isolates cultured from mice from another facility (SPF) were members of this group ($n = 9$). Cultures were isolated from the small bowel (1/17), caecum (11/17), large bowel (3/17) and liver (2/17). Both cultures obtained from liver samples were from SPF animals obtained from a single facility. *H. ganmani* cells were 0.3×2.5 µm and possessed single, bipolar, unsheathed flagella (Fig. 1).

DNA analysis

All 17 isolates gave positive results in *Helicobacter* genus-specific PCRs. Near-complete 16S rDNA sequences were generated from four isolates (Table 2), and these sequences were identical to one another and had 98.2% sequence similarity to the type strain of *H. rodentium* (MIT 95-1707^T) (Shen *et al.*, 1997). They also had 100% sequence identity with a sequence from an unnamed mouse isolate, *Helicobacter* sp. MIT 95-2011 (GenBank no. U96298). DNA from all 17 isolates was subjected to a *H. rodentium*-specific PCR

Table 2. *Helicobacter ganmani* and *Helicobacter rodentium* strains examined

Taxon	Strain/other designation	GenBank no.
<i>Helicobacter ganmani</i>	CMRI H02 ^T , CCUG 43526 ^T , CIP 106846 ^T	AF000221
<i>Helicobacter ganmani</i>	CMRI H03	AF000222
<i>Helicobacter ganmani</i>	CMRI H04	
<i>Helicobacter ganmani</i>	CMRI H09*	
<i>Helicobacter ganmani</i>	ABHU H11, CCUG 43527	AF000223
<i>Helicobacter ganmani</i>	ABHU H13*	
<i>Helicobacter ganmani</i>	ABHU H15	
<i>Helicobacter ganmani</i>	UNSW H16, CCUG 43528	AF000224
<i>Helicobacter ganmani</i>	UNSW H17	
<i>Helicobacter rodentium</i> †	MIT 95-1707 ^T , ATCC 700285 ^T	U96296‡
<i>Helicobacter rodentium</i> †	MIT 95-2178	
<i>Helicobacter rodentium</i> †	MIT 95-2060	

* Strain not examined phenotypically.

† Strain kindly provided by J. G. Fox, Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA, USA.

‡ 16S rRNA sequence determined previously (Shen *et al.*, 1997).

Fig. 2. Phylogenetic tree showing the position of *Helicobacter ganmani* based on analysis of near complete 16S rDNA sequences derived from members of the *Helicobacter*, *Campylobacter* and *Arcobacter* genera with *Escherichia coli* as the outgroup. Scale bar represents 0.02 substitutions per site. Significant bootstrap values (> 50%) from both distance and parsimony analyses are indicated at the nodes (parsimony values shown in parentheses).

using primers D86 and D87 (Table 1) as described by Shen *et al.* (1997). All samples were positive in this reaction. A phylogenetic tree was reconstructed from a multiple alignment of our consensus sequence with 32 other bacterial 16S rDNA sequences including members of the *Helicobacter*, *Campylobacter* and *Arcobacter* genera (Fig. 2). The alignment was 1358

nucleotides in length after positions containing ambiguities in any of the sequences were removed.

Phenotypic characterization

Seven strains of *H. ganmani* were examined (Table 2) and, as expected they bore a close resemblance to *H.*

Table 3. Characteristics that differentiate culturable *Helicobacter ganmani* from other *Helicobacter* species

Taxa: 1, *H. ganmani*; 2, *H. rodentium*; 3, *H. pylori*; 4, *H. nemestrinae*; 5, *H. acinonychis*; 6, *H. felis*; 7, *H. bizzozeronii*; 8, *H. salomonis*; 9, *H. pullorum*; 10, *H. pametensis*; 11, *H. cholecystus*; 12, *H. mustelae*; 13, *H. fennelliae*; 14, *H. muridarum*; 15, *H. hepaticus*; 16, *H. trogonum*; 17, *H. canis*; 18, *H. bilis*; 19, *H. cinaedi*. +, 80–100% strains positive; v, 50–67% strains positive; d, 20–43% strains positive; –, 0–17% strains positive; ND, not determined. TTC, triphenyl-tetrazolium chloride. All biochemical results (except *H. bilis*) obtained using the standardized methods recommended for use with the identification scheme of On *et al.* (1996). Distribution of flagella: Bp, bipolar; Mp, monopolar; Pe, peritrichous. Data taken from this study, Fox *et al.* (1995), On *et al.* (1996), Jalava *et al.* (1998), and unpublished results of S. L. W. On.

Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Catalase production	–/w	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+
Nitrate reduction	+	+	–	–	–	+	+	+	+	+	+	+	–	–	+	+	–	+	+
Alkaline phosphatase hydrolysis	–	–	+	+	+	v	v	v	–	+	+	+	d	+	–	–	+	–	d
Urease	–	–	+	+	+	+	+	+	–	–	–	+	–	+	+	+	–	+	–
Indoxyl acetate hydrolysis	–	–	–	–	d	d	d	–	–	–	–	+	+	+	+	–	+	–	–
Growth at 42 °C	–	+	d	+	d	v	v	–	+	+	+	v	d	–	–	+	+	+	d
Growth on CCDA	+	–	–	–	d	–	–	–	v	–	–	–	–	–	–	–	–	ND	–
Tolerance to 1.0% glycine	–	+	–	–	–	–	–	–	–	v	+	–	–	–	+	+	–	+	–
Tolerance/redn of 0.04% TTC	+	v	d	+	–	–	–	–	d	v	+	+	+	–	+	+	v	ND	+
Periplasmic fibres	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	+	–	+	–
Sheathed flagella	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+
No. flagella	2	2	4–8	4–8	2–5	14–20	10–20	10–23	1	2	1	4–8	2	10–14	2	5–7	2	3–14	1–2
Distribution of flagella	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Mp	Bp	Mp	Pe	Bp	Bp	Bp	Bp	Bp	Bp	Mp/Bp

Fig. 3. Whole-cell protein profiles of a representative selection of *H. ganmani* strains showing the three protein electrophoretic types and of the *H. rodentium* strains examined. The molecular mass markers used (top and bottom lanes) are indicated from left to right: lysozyme (14.5 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), bovine albumin (66 kDa) and β -galactosidase (116 kDa).

rodentium. Nonetheless, these species were differentiated on the basis of α -haemolytic activity and ability to grow at 42 °C and on *Campylobacter* charcoal-deoxycholate agar base medium (CCDA). *H. ganmani* was also distinct from other extant species (Table 3). The phenotype of the new species is described in detail below.

SDS-PAGE of whole-cell proteins

The whole-cell protein profiles of nine *H. ganmani* isolates (Table 2) were compared with those of *H. rodentium* reference strains and other *Helicobacter* species already present in the database. The *H. rodentium* reference strains had virtually identical

whole-cell protein profiles. The nine *H. ganmani* isolates examined were subdivided into three subgroups, characterized by the presence or the absence of dense protein bands with approximate molecular masses of 47 and 43 kDa. One subgroup had both protein bands, a second had only a dense 47 kDa protein band, while the third had only a dense 43 kDa protein band. Fig. 3 illustrates the three different protein electrophoretic types within *H. ganmani* and the whole-cell protein profiles of the *H. rodentium* strains. In a numerical analysis of the whole-cell protein profiles of all strains examined and of *Helicobacter* reference strains, the three different protein electrophoretic types of *H. ganmani* strains formed distinct clusters (data not shown). When the region with these variable dense protein bands was omitted

Fig. 4. Dendrogram derived from the unweighed pair group average linkage of correlation coefficients (shown as a percentage similarity) between the whole-cell protein patterns of strains of *H. ganmani*, *H. rodentium* and *Helicobacter* and *Wolinella* reference species. The region of the protein profiles comprising the variable dense protein bands was omitted from the analysis.

from the numerical comparison, all strains clustered tightly together (Fig. 4).

DISCUSSION

The results from our investigations presented here support the establishment of a novel species of *Helicobacter* for which we propose the name *Helicobacter ganmani*. Isolates were cultured from all sections of the intestinal tract of mice examined, however, the caecum appeared to be the preferred site of colonization. Isolates were obtained from both conventional and SPF mice and were also isolated from liver samples of SPF animals which had no evidence of infection with other *Helicobacter* species. This is the first report of a *Helicobacter* species which grows exclusively in an anaerobic atmosphere provided by standard anaerobic jars and gas-generating kits. Many other *Helicobacter* species are capable of anaerobic growth but are routinely cultured using microaerobic conditions. However, we found only one strain of *H. rodentium* grew under microaerobic conditions provided by the gas-replacement method used here. This result is in contrast with that of Shen *et al.* (1997), but differences in the methods used to generate microaerobic conditions between laboratories may account for this discrepancy.

H. ganmani possesses unsheathed flagella, bringing to three, with *H. rodentium* and *Helicobacter pullorum*, the number of *Helicobacter* species lacking sheathed flagella (Shen *et al.*, 1997; Stanley *et al.*, 1994). 16S rDNA sequence analysis revealed a close phylogenetic relationship between *H. ganmani* and *H. rodentium*

and all of our isolates were amplified in the *H. rodentium*-specific PCR as described by Shen *et al.* (1997). This result was not surprising considering the level of sequence similarity between these two species, and closer examination of the 16S rDNA sequence of four *H. ganmani* isolates revealed that the target sites for primers D86 and D87 were indeed present. It should be noted, therefore, that *H. rodentium* cannot be positively identified using this PCR to the exclusion of *H. ganmani*. A PCR designed to differentiate between these species was not developed. The high level of sequence similarity between *H. rodentium* and *H. ganmani*, and problems encountered with lab-to-lab reproducibility would render such a reaction of limited practical use. While the sequence similarity observed between these two species was above the level that allows discrimination between closely related species (Stackebrandt & Goebel, 1994), data obtained from SDS-PAGE of whole-cell proteins clearly discriminated them as separate species (Figs 3 and 4). Numerical analysis of SDS-PAGE of whole-cell protein extracts has been recommended by the International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Campylobacter* and related bacteria as a suitable alternative to DNA-DNA hybridization studies for discriminating between closely related species of *Helicobacter* (Dewhirst *et al.*, 2000). The nine *H. ganmani* strains examined using this technique clustered into three protein electrophoretic subgroups, each containing three isolates. With one exception, these groups contained strains isolated from animals housed at a single facility (data not shown). Such intraspecies variability has been observed for many *Helicobacter* and *Campylobacter* species, but the

overall pattern was virtually identical for all isolates. Previous studies with other *Campylobacter*-like organisms characterized by similar variable dense protein bands as those observed here have shown that such variable regions must be omitted from the numerical analysis in order to locate the different protein electrophoretic types of a single species into a single cluster (Costas *et al.*, 1987; Owen *et al.*, 1989; Vandamme *et al.*, 1990, 1992). This was confirmed in the present study (Fig. 4). Detailed phenotypic analysis of seven strains revealed that *H. ganmani* and *H. rodentium* shared many biochemical properties, notably a lack of urease and alkaline phosphatase activities, and failure to hydrolyse indoxyl acetate, but were clearly distinguished from each other and from other *Helicobacter* species (Table 3). These differences further support our proposal for a new *Helicobacter* species.

H. ganmani represents the 19th named species of *Helicobacter* and reinforces once again the diversity of species that naturally infect rodents and, significantly, laboratory mice. We strongly support the recommendation of Shen *et al.* (1997) for screening of laboratory mice for indigenous *Helicobacter* spp. in laboratories engaging in any area of *Helicobacter* research, particularly in immunological and vaccine studies.

Description of *Helicobacter ganmani* sp. nov.

Helicobacter ganmani (gan.man'i. N.L. gen. n. *ganmani* arbitrary name derived from *ganman* which, in the language of the Gadigal people, indigenous Australians who live in the Sydney Harbour area, means 'snake finder'. Thus, 'ganman' refers to the spiral or 'snake-like' morphology and the fact that this organism was isolated from mice in Sydney, Australia, the home of the Gadigal people).

Cells are curved to spiral rods ($0.3 \times 2.5 \mu\text{m}$) with two turns per cell and have single, unsheathed flagella in a bipolar arrangement. Single colonies are rarely seen and are $< 1 \text{ mm}$ in diameter, irregular, unpigmented and translucent, after 3–5 d growth on 5% horse blood agar. Anaerobic; no growth obtained in micro-aerobic or aerobic conditions. Pitting of the agar not observed. All strains produce oxidase. Weak catalase activity detected (2/7 strains). Nitrate and triphenyl-tetrazolium chloride (TTC) reduced. No urease, alkaline phosphatase, hippuricase or DNase activity detected. Selenite not reduced. Indoxyl acetate not hydrolysed. Neither hydrogen sulphide nor acid from sugar fermentation produced in triple-sugar iron agar. Non-haemolytic. All strains grow anaerobically at 37°C on *Campylobacter* charcoal-deoxycholate (CCD) agar, and on media containing 0.1% trimethylamine *N*-oxide (TMAO), 1.0 and 2.0% ox-bile, 0.04% TTC, 32 mg cephalothin l^{-1} , 32 mg carbenicillin l^{-1} (BA base), 64 mg cefoperazone l^{-1} (BA base), 5-fluorouracil or 0.1% sodium fluoride. No growth obtained anaerobically at room temperature ($18\text{--}22^\circ\text{C}$), 25 or 42°C , on tyrosine or casein media, or on

media containing 1.0% glycine, 2–4% NaCl, 32 mg nalidixic acid l^{-1} , 0.0005% crystal violet, 0.1% janus green or 0.02% pyronin. Growth on 0.032% methyl orange media (6/7 strains), minimal and 0.005% basic fuchsin media (5/7 strains) and 4 mg metronidazole l^{-1} (BA medium) (3/7 strains). Strains do not always give reproducible results when tested for growth on MacConkey, 0.1% sodium deoxycholate or 0.02–0.05% safranin media. Cells have been isolated from caeca, large bowels, small bowels and livers of mice. The type strain is CMRI H02^T (= CCUG 43526^T = CIP 106846^T). When a difference in phenotype was observed between strains, the description given above applies to the type strain, CMRI H02^T.

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REFERENCES

- Costas, M., Owen, R. J. & Jackman, P. J. H. (1987). Classification of *Campylobacter sputorum* and allied campylobacters based on numerical analysis of electrophoretic protein patterns. *Syst Appl Microbiol* **9**, 125–131.
- De Ley, J. (1992). The Proteobacteria: ribosomal RNA cistron similarities and bacterial taxonomy. In *The Prokaryotes*, pp. 2111–2140. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Dewhirst, F. E., Fox, J. G. & On, S. L. W. (2000). Recommended minimal standards for describing new species of the genus *Helicobacter*. *Int J Syst Evol Microbiol* **50**, 2231–2237.
- Felsenstein, J. (1985). Confidence limits on phylogenetics: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1989). PHYLIP. Phylogeny inference package. *Cladistics* **5**, 164–166.
- Fox, J. G. & Wang, T. C. (1997). *Helicobacter* and liver disease. *Ital J Gastroenterol Hepatol* **29**, 5–12.
- Fox, J. G., Paster, B. J., Dewhirst, F. E., Taylor, N. S., Yan, L. L., Macuch, P. J. & Chmura, L. M. (1992). *Helicobacter mustelae* isolation from feces of ferrets: evidence to support fecal-oral transmission of a gastric helicobacter. *Infect Immun* **60**, 606–611.
- Fox, J. G., Dewhirst, F. E., Tully, J. G., Paster, B. J., Yan, L., Taylor, N. S., Collins, M. J., Gorelick, P. L. & Ward, J. M. (1994). *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. *J Clin Microbiol* **32**, 1238–1245.
- Fox, J. G., Yan, L. L., Dewhirst, F. E., Paster, B. J., Shames, B., Murphy, J. C., Hayward, A., Belcher, J. C. & Mendes, E. N. (1995). *Helicobacter bilis* sp. nov., a novel *Helicobacter* species isolated

- from bile, livers, and intestines of aged, inbred mice. *J Clin Microbiol* **33**, 445–454.
- Goodwin, C. S., Armstrong, J. A., Chilvers, T., Peters, M., Collins, M. D., Sly, L., McConnell, W. & Harper, W. E. (1989). Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. *Int J Syst Bacteriol* **39**, 397–405.
- Jalava, K., On, S. L. W., Vandamme, P. A. R., Happonen, I., Sukura, A. & Hänninen, M.-L. (1998). Isolation and identification of *Helicobacter* spp. from canine and feline gastric mucosa. *Appl Environ Microbiol* **64**, 3998–4006 [erratum **65**, 887 (1999)].
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Lee, A., Phillips, M. W. & O'Rourke, J. L. & 8 other authors (1992). *Helicobacter muridarum* sp. nov., a microaerophilic helical bacterium with a novel ultrastructure isolated from the intestinal mucosa of rodents. *Int J Syst Bacteriol* **42**, 27–36.
- Lee, A., O'Rourke, J., De Ungria, M. C., Robertson, B., Daskalopoulos, G. & Dixon, M. F. (1997). A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* **112**, 1386–1397.
- Marshall, B. J., Royce, H., Annear, D. I., Goodwin, C. S., Pearman, J. W., Warren, J. R. & Armstrong, J. A. (1984). Original isolation of *Campylobacter pyloridis* from human gastric mucosa. *Microbiol Lett* **25**, 83–88.
- Mendes, E. N., Queiroz, D. M. M., Dewhirst, F. E., Paster, B. J., Moura, S. B. & Fox, J. G. (1996). *Helicobacter trogonum* sp. nov., isolated from the rat intestine. *Int J Syst Bacteriol* **46**, 916–921.
- Murray, R. G. E., Brenner, D. J., Colwell, R. R., De Vos, P., Goodfellow, M., Grimont, P. A. D., Pfennig, N., Stackebrandt, E. & Zavarzin, G. A. (1990). International Committee on Systematic Bacteriology. Report of the ad hoc committee on approaches to taxonomy within the Proteobacteria. *Int J Syst Bacteriol* **40**, 213–215.
- Neilan, B. A., Jacobs, D., Del Dot, T., Blackall, L. L., Hawkins, P. R., Cox, P. T. & Goodman, A. E. (1997). rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. *Int J Syst Bacteriol* **47**, 693–697.
- On, S. L. W., Holmes, B. & Sackin, M. J. (1996). A probability matrix for the identification of campylobacters, helicobacters, and allied taxa. *J Appl Bacteriol* **81**, 425–432.
- Owen, R. J., Morgan, D. D., Costas, M. & Lastovica, A. (1989). Identification of “*Campylobacter upsaliensis*” and other catalase-negative campylobacters from pediatric blood cultures by numerical analysis of electrophoretic protein patterns. *FEMS Microbiol Lett* **58**, 145–150.
- Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Modern Microbial Methods (Chemical Methods in Prokaryotic Systematics)*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.
- Riley, L. K., Franklin, C. L., Hook, R. R., Jr & Besch-Williford, C. (1996). Identification of murine helicobacters by PCR and restriction enzyme analyses. *J Clin Microbiol* **34**, 942–946.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schauer, D. B., Ghori, N. & Falkow, S. (1993). Isolation and characterization of “*Flexispira rappini*” from laboratory mice. *J Clin Microbiol* **31**, 2709–2714.
- Shames, B., Fox, J. G., Dewhirst, F., Yan, L., Shen, Z. & Taylor, N. S. (1995). Identification of widespread *Helicobacter hepaticus* infection in feces in commercial mouse colonies by culture and PCR assay. *J Clin Microbiol* **33**, 2968–2972.
- Shen, Z., Fox, J. G., Dewhirst, F. E., Paster, B. J., Foltz, C. J., Yan, L., Shames, B. & Perry, L. (1997). *Helicobacter rodentium* sp. nov., a urease-negative *Helicobacter* species isolated from laboratory mice. *Int J Syst Bacteriol* **47**, 627–634.
- Stackebrandt, E. & Goebel, B. M. (1994). A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stanley, J., Linton, D., Burnens, A. P., Dewhirst, F. E., On, S. L. W., Porter, A., Owen, R. J. & Costas, M. (1994). *Helicobacter pullorum* sp. nov. – genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. *Microbiology* **140**, 3441–3449.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Tillett, D. & Neilan, B. A. (2000). Rapid nucleic acid isolation from cultured and environmental cyanobacteria: novel techniques based on xanthogenate. *J Phycol* **36**, 251–258.
- Vandamme, P. & De Ley, J. (1991). Proposal for a new family, *Campylobacteraceae*. *Int J Syst Bacteriol* **41**, 451–455.
- Vandamme, P., Pot, B., Falsen, E., Kersters, K. & De Ley, J. (1990). Intra- and interspecific relationships of veterinary campylobacters revealed by numerical analysis of electrophoretic protein profiles and DNA:DNA hybridizations. *Syst Appl Microbiol* **13**, 295–303.
- Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. & De Ley, J. (1991). Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* **41**, 88–103.
- Vandamme, P., Dewettinck, D. & Kersters, K. (1992). Application of numerical analysis of electrophoretic protein profiles for the identification of thermophilic campylobacters. *Syst Appl Microbiol* **15**, 402–408.
- Weisburg, W. G., Oyaizu, Y., Oyaizu, H. & Woese, C. R. (1985). Natural relationships between *Bacteroides* and flavobacteria. *J Bacteriol* **164**, 320–326.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.