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'*Flexispira rappini*' strains represent at least 10 *Helicobacter* taxa

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'*Flexispira rappini*' is a provisional name given to Gram-negative, microaerophilic, motile, spindle-shaped micro-organisms with spiral periplasmic fibres and bipolar tufts of sheathed flagella. Several investigators, including Kirkbride, Romero, and Archer isolated strains possessing this morphology. Previously, the phylogenetic position of three '*Flexispira rappini*' strains was determined by 16S rRNA sequencing, which indicated that flexispira were members of the genus *Helicobacter*. As more organisms with '*F. rappini*' morphology were isolated, it became apparent that there were multiple *Helicobacter* taxa with this distinctive morphology. The purpose of this study was to examine a collection of 36 '*F. rappini*' strains from diverse habitats by using 16S rRNA sequence analysis. The strains fell into 10 taxa, each possibly representing a novel *Helicobacter* species. Two of these flexispira taxa were previously named, by us, *Helicobacter bilis* and *Helicobacter trogontum*. Currently, none of the flexispira taxa contains enough phenotypically and genotypically characterized strains to be formally named '*Helicobacter rappinii*'.

Keywords: *Helicobacter*, '*Flexispira rappini*', rRNA, phylogeny

INTRODUCTION

'*Flexispira rappini*' is a provisional name given by Bryner to Gram-negative, microaerophilic, motile, fusiform-shaped organisms with spiral periplasmic fibres and bipolar tufts of sheathed flagella (Bryner *et al*., 1986, 1987). Throughout this paper, the term 'flexispira' will be used to refer to organisms with this distinct morphology. A flexispira strain was first isolated in pure culture, by Kirkbride, from aborted lambs with focal hepatic necrosis (Kirkbride *et al*., 1985). Kirkbride was able to produce abortions in pregnant sheep by inoculating the cultured organism (strain 84-3345), fulfilling Koch's postulates (Kirkbride *et al*., 1986). Bryner demonstrated that the same strain could produce experimental infection, abortion in pregnant guinea pigs and hepatic necrosis in aborted feti (Bryner *et al*., 1987). Flexispira organisms were recovered from stool specimens from two humans, one with and one without gastroenteritis, and from their asymptomatic dog, as well as from a second patient with diarrhoea (Romero *et al*., 1988; Archer *et al*., 1988). Organisms with the flexispira morphology (distinctive periplasmic fibres that have a criss-cross appearance in negatively stained electron micrographs and multiple bipolar sheathed flagella) had been previously described by Lockard as one of three morphotypes seen in the gastric mucosa of dogs (Lockard & Boler, 1970), and by Savage as one of two morphotypes in the murine large bowel (Savage *et al*., 1971).

The phylogenetic position of '*Flexispira rappini*' strains was shown to be closely related to the genus *Helicobacter* by DNA–rRNA hybridization (Vandamme *et al*., 1991) and actually fell within the genus *Helicobacter* according to 16S rRNA sequence analysis (Paster *et al*., 1991). Ongoing 16S rRNA sequencing efforts in our laboratory have indicated that '*F. rappini*' strains represent multiple *Helicobacter* taxa. The purpose of the present study was to examine a large number of '*F. rappini*' strains to determine their phylogenetic diversity and to place the strains in coherent taxa.

Abbreviation: IVS, intervening sequence.

Table 1. Flexispira strains

The leftmost column subheadings are strain group, followed by proposed name. The following abbreviations are used: ATCC, American Type Culture Collection; NADC, National Animal Disease Center. Strains in bold were sequenced; other numbers are alternative designations for the same strain. 'Same' indicates that the sequence is the same (within 2 base differences) as that of the GenBank number above.

Collection accession nos			Sequence accession no.	Isolated by:	Source	Reference
ATCC	NADC	Original designation				
Flexispira taxon 1						
43968	2010	86-13625	U96300	C. E. Gates	Pig	Archer *et al.* (1988)
Flexispira taxon 2						
49314	2012	86-2279	AF225546	C. E. Gates	Sheep	
		87-3845	Same	C. E. Gates	Sheep	
49315	2013	87-161	Same	C. E. Gates	Aborted sheep foetus	
49316	2015	87-14409	Same	C. E. Gates	Aborted sheep foetus	
49319	2039	88-924	Same	C. E. Gates	Aborted sheep foetus	
		89-4925	Same	C. E. Gates	Sheep	
Flexispira taxon 3						
49320	2040	88-2491	AF225547	C. E. Gates	Pig stomach; aborted foetus	
Flexispira taxon 4						
49310	1977	86-1775	AF225548	C. E. Gates	Sheep	
Flexispira taxon 5						
43966/49313	**1893**/2011	**84-3345**	M88137	C. A. Kirkbride	Aborted sheep foetus	Kirkbride *et al.* (1985, 1986) Bryner *et al.* (1987)
Flexispira taxon 6: *Helicobacter trogontum*						
700114^T		**8581**^{T}	U65103	E. N. Mendes	Rat	Mendes *et al.* (1996)
		8718	Same	E. N. Mendes	Rat	Mendes *et al.* (1996)
		9056	Same	E. N. Mendes	Rat	Mendes *et al.* (1996)
Flexispira taxon 7						
		1302/Dog-4	U51874	K. A. Eaton	Dog stomach	Eaton *et al.* (1996)
Flexispira taxon 8						
43879	**1937**	38264	M88138	J. R. Archer	Human stool (Case 1 father)	Romero *et al.* (1988)
49308	1938	39051	Same	J. R. Archer	Dog stool (Case 1 pet)	Romero *et al.* (1988)
49309	1939	39801	Same	J. R. Archer	Human stool (Case 1 daughter)	Romero *et al.* (1988)
43880	2005	14020	AF225549	J. R. Archer	Human stool (Case 2)	Romero *et al.* (1988)
49317	2016	87-4654	AF047851	C. E. Gates	Dog stool	
		87-4551	Same	C. E. Gates	Dog stool	
		87-5733	Same	C. E. Gates	Dog stool	
		Fr-mo*	L12765	D. S. Schauer	Mouse	Schauer *et al.* (1993)
		H3153*	AF118017	P. Sorlin	Human blood	Sorlin *et al.* (1999)
		FH 9702248*	AF034135	W. Tee	Human blood	Tee *et al.* (1998)
		NIH-1*	AF118807	V. J. Gill	Human blood	Weir *et al.* (1999)
Flexispira taxon 9: *Helicobacter bilis*						
51630		**93-1909**/Hb1	U18766	J. G. Fox	Mouse faeces	Fox *et al.* (1995)
51631		**93-1920/Hb2**	U18767	J. G. Fox	Mouse faeces	Fox *et al.* (1995)
51632		**93-1990/Hb3**	U18768	J. G. Fox	Mouse faeces	Fox *et al.* (1995)
		1502/Dog-5	U51873	K. A. Eaton	Dog faeces	Eaton *et al.* (1996)
		96-5983		J. G. Fox	Gerbil faeces	
		96-6119		J. G. Fox	Gerbil faeces	
		97-1034		J. G. Fox	Hamster faeces	
		9615-IN3-1		J. G. Fox	Rat faeces	Haines *et al.* (1998)
		9615-IN3-2		J. G. Fox	Rat faeces	Haines *et al.* (1998)
		9615-IN3-3		J. G. Fox	Rat faeces	Haines *et al.* (1998)
		9615-IN3-4		J. G. Fox	Rat faeces	Haines *et al.* (1998)
		92-7660†	AF0478431	J. G. Fox	Human gall-bladder	Fox *et al.* (1998)
		93-3055†	AF047844	J. G. Fox	Human gall-bladder	Fox *et al.* (1998)
		93-4659†	AF047845	J. G. Fox	Human gall-bladder	Fox *et al.* (1998)
Flexispira taxon 10						
		97-6194-5	AF107494	J. G. Fox	Cotton-top tamarin faeces	Saunders *et al.* (1999)
		97-6194-4	Same	J. G. Fox	Cotton-top tamarin faeces	Saunders *et al.* (1999)
		97-6194-3	Same	J. G. Fox	Cotton-top tamarin faeces	Saunders *et al.* (1999)

* Strain not part of this study; see reference.

† PCR product, not isolate.

METHODS

Bacterial isolation and culture. The strains examined in this report were supplied by a number of investigators, as indicated in Table 1.

Phenotypic characterization. Most of the tests were performed as previously described (Paster *et al.*, 1991). Tests for γ-glutamyl arylamidase, phosphatase, urease and nitrate reduction were performed using the RapID/NH System (Innovative Diagnostic Systems). Indoxyl acetate hydrolysis was performed using indoxyl acetate discs (Remel).

Crude DNA isolation and amplification. Bacteria were cultured on TSA blood agar plates for 48 h under microaerophilic conditions. A small loopful of cells (5 µl) was harvested and lysed by suspension in 15 µl Gene Releaser according to the manufacturer's (BioVentures) microwave protocol. PCRs were performed in thin-walled tubes with a Perkin-Elmer 480 thermal cycler, a GeneAmp PCR reagent kit and an AmpliWax PCR Gem 100s (Promega). Forward primers C71 (*Escherichia coli* position 7–23; 5′-GAG AGT TTG ATC MTG GC-3′) and reverse primer C72 (1509–1492; 5′-GYT ACC TTG TTA CGA CTT-3′) were used (Fox *et al.*, 1995). The Gene Releaser mixture was centrifuged at 14000 ***g*** for 2 min. One microlitre of the clear supernatant was combined with 1 µM each primer and other reagents in the Hot Start protocol suggested by Perkin-Elmer. The following conditions were used for amplification: denaturation at 72 °C for 45 s, annealing at 50 °C for 45 s and elongation at 72 °C for 45 s, with 5 s added for each elongation step. A total of 30 cycles were performed, followed by a final elongation step at 72 °C for 15 min. The purity of the product was determined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide and viewed under long-wavelength UV light.

Purification of PCR products. Amplified DNA was purified by precipitation with PEG 8000 (Kusukawa *et al.*, 1990). After removal of AmpliWax, 0·6 vols 20% PEG 8000 (Sigma) in 2·5 M NaCl were added and the mixture incubated at 37 °C for 10 min. The sample was centrifuged for 15 min at 15000 ***g*** and the pellet was washed with 80% ethanol and pelleted as before. The pellet was air-dried and dissolved in 30 µl distilled water and used for cycle sequencing as described below.

Sequencing methods. The DNA sample from the PCR was directly sequenced using the TAQuence cycle-sequencing kit (US Biochemical). The manufacturer's protocol was followed. The eight sequencing primers used for *Helicobacter* 16S rRNA and for intervening sequences present at *E. coli* position 210 have been described previously (Fox *et al.*, 1995). Primers were end-labelled with ^{33}P (NEN/Dupont) using the manufacturers' protocol. Approximately 100 ng purified DNA from the PCR was used for sequencing. Reaction products were loaded on to 8% polyacrylamide/urea gels, electrophoresed and then detected by exposure to X-ray film for 24 h. Some recent sequences were obtained using a model ABI 377 DNA sequencer with BigDye terminator cycle sequencing as described previously (Dewhirst *et al.*, 1999).

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 Quick BASIC for use on PCs (Paster & Dewhirst, 1988). RNA sequences were entered and aligned as described previously. Our sequence database contains approximately 1000 sequences determined in our laboratory and another 500 obtained from GenBank or the Ribosomal Database Project (Maidak *et al.*, 1999). 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 at single positions by the method of Jukes & Cantor (1969). Phylogenetic trees were constructed using the neighbour-joining method of Saitou & Nei (1987).

GenBank accession numbers. The GenBank and culture collection accession numbers for the strains examined in this study are given in Table 1.

RESULTS AND DISCUSSION

Thirty-six strains of flexispira were examined by 16S rRNA sequence analysis. The essentially complete (1450–1620 bases) sequence was determined for each strain. The GenBank and other accession numbers are given in Table 1. Where multiple sequences differed by less than five bases, only one representative sequence was deposited with GenBank, but all of the sequences are available from the corresponding author. Comparison with sequences in our database indicated that all of the strains fell within the genus *Helicobacter*. The 36 strains fell into 10 distinct groups (some represented by only a single member), each of which probably represents a novel *Helicobacter* taxon (Table 1). The sequences of strains within each group differed by seven or fewer bases out of 1450. A phylogenetic tree based on one strain from each group and 34 representative *Campylobacter* and *Helicobacter* species is shown in Fig. 1. This tree is based on an analysis of 1409 nucleotide positions. The flexispira taxa, while not monophyletic, do cluster in the helicobacter tree. They occur in the large central branch, away from the *Helicobacter pylori–Helicobacter felis–'Gastrospirillum'* branch, the *Helicobacter pametensis–Helicobacter mustelae* branch and the *Helicobacter rodentium* branch.

We have previously named two of the 10 taxa: *Helicobacter bilis* (flexispira taxon 9; Fox *et al.*, 1995) and *Helicobacter trogontum* (flexispira taxon 6; Mendes *et al.*, 1996). *H. bilis*, originally isolated from diseased livers of laboratory mice, has recently been isolated from dogs, hamsters, gerbils and rats, and the DNA of *H. bilis* has been detected in human bile and gall-bladder tissues in subjects with chronic cholecystitis (Eaton *et al.*, 1996; Fox *et al.*, 1998; Haines *et al.*, 1998). This organism appears to have the broadest host range of any helicobacter described to date. Other than *Helicobacter pylori*, *H. bilis* may be the flexispira species with the greatest human pathogenic potential and therefore its role in hepatic disease is currently under intense scrutiny by several laboratories.

The flexispira taxon with the next highest number of isolates after *H. bilis* is flexispira taxon 8. This group includes the original Archer human and dog isolates (Archer *et al.*, 1988; Romero *et al.*, 1988) as well as additional dog and mouse isolates. This taxon of isolates differs from other flexispira taxa (except flexispira taxon 7) in that most of the isolates are

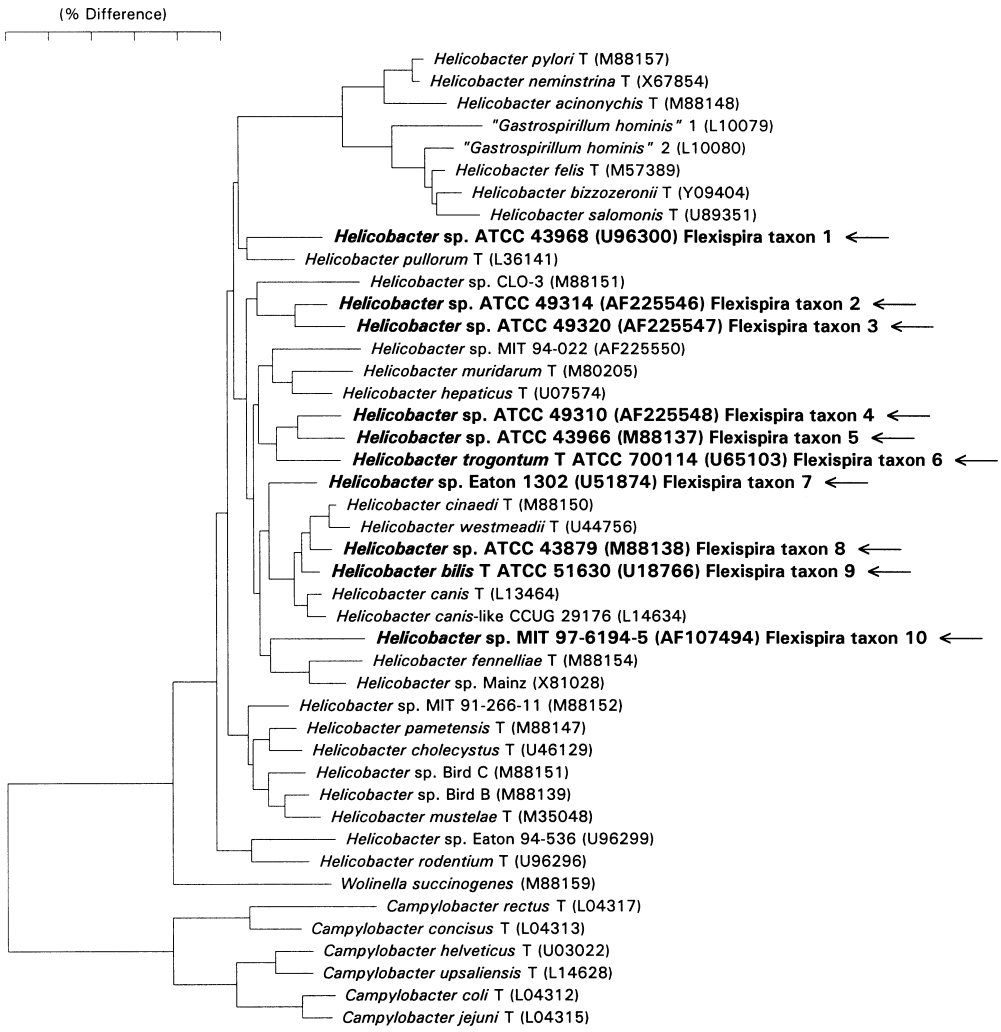

Fig. 1. Neighbour-joining phylogenetic tree for flexispira taxa and representative *Helicobacter* and *Campylobacter* species, based on 16S rRNA sequence comparisons of 1409 base positions. GenBank accession numbers are given in parentheses. Strains without culture collection numbers are type strains.

catalase-negative. Recently, three strains of flexispira taxon 8 have been recovered from patients with bacteraemia (Tee *et al.*, 1998; Sorlin *et al.*, 1999; Weir *et al.*, 1999).

Flexispira taxon 2 includes six sheep isolates from aborted foetuses. Although strains of this taxon were isolated in South Dakota in the late 1980s, their worldwide role in association with animal diseases has not been examined.

Flexispira taxon 10 includes three isolates from cotton-top tamarins (*Saguinus oedipus*). These helicobacter isolates have been described, but they have not been formally named because of the limited number of isolates obtained from a single colony of tamarins (Saunders *et al.*, 1999). The tamarin colony has a high incidence of chronic colitis. The relationship between infection with this helicobacter and ulcerative colitis is under investigation.

The remaining flexispira taxa (1, 3, 4, 5 and 7) contain only one or two isolates per group. Strain ATCC 43966 is the sole representative of flexispira taxon 5, but it is the strain used to fulfil Koch's Postulates by producing abortions in sheep and guinea pigs (Kirkbride *et al.*, 1985; Bryner *et al.*, 1987). This particularly virulent strain caused placentitis and fetal hepatic necrosis. While abortions in domestic animals due to campylobacter infections have been well documented (Véron & Chatelain, 1973), it is not clear whether earlier (and even subsequent) studies could clearly differentiate among the >80 taxa of *Campylobacter*, *Arcobacter* and *Helicobacter* that can now be differentiated by 16S rRNA sequence analysis. Flexispira isolates have been recovered from aborted foetuses in sheep, pigs and dogs. The role of helicobacters in abortions in domestic animals needs to be examined. Even in sheep, it is not known whether transmission of helicobacters is faecal–oral or if helicobacters can

Table 2. Characteristics that differentiate *Helicobacter* species

Data were obtained from Fox *et al*. (1994, 1995), Mendes *et al*. (1996), Saunders *et al*. (1999), Shen *et al*. (1997), On *et al*. (2000) and this study. +, 80–100 % strains positive; [+], 50–66 % strains positive; [−], 20–43 % strains positive; −, 0–17 % strains positive; s, susceptible; r, resistant; i, intermediate; W, weak; Nal, nalidixic acid (30 μg disk); Ceph, Cephalothin (30 μg disk)

Taxon	Catalase production	Nitrate reduction	Alkaline phosphatase hydrolysis	Urease	Indoxyl acetate hydrolysis	γ-Glutamyl transpeptidase	Growth at 42 °C	Growth with 1 % glycine	Susceptibility to:		Periplasmic fibres	No. flagella	Distribution of flagella	G+C content (mol %)
									Nal	Ceph				
H. pylori	+	−	+	+	−	+	[−]	−	r	s	−	4–8	Bipolar	35–37
H. nemestrinae	+	−	+	+	−		+	−	r	s	−	4–8	Bipolar	24
H. acinonychis	+	−	+	+	−	+	[−]	−	r	s	−	2–5	Bipolar	30
H. felis	+	+	[+]	+	[−]	+	[+]	−	r	s	+	14–20	Bipolar	42
H. bizzozeronii	+	+	[+]	+	[−]	+	[+]		r	s	−		Bipolar	
H. salomonis	+	+	[+]	+	+	+	−		r	s	−		Bipolar	
Flexispira taxon 1	+	−	−	+	−	+	+	−	r	r	+	10–20	Bipolar	
H. pullorum	+	+	−	−	−		+	−	r	s	−	1	Monopolar	34–35
Helicobacter sp. CLO-3	+	−	+	−	+	−	+	+	i	r	−		Bipolar	45
Flexispira taxon 2	+	−	−	+	−	+	+	−	r	r	+		Bipolar	
Flexispira taxon 3	+	−	−	+	−	+	+	−	r	r	+		Bipolar	
H. muridarum	+	−	+	+	+	+	−	−	r	r	+	10–14	Bipolar	34
H. hepaticus	+	+	−	+	+	−	−	+	r	r	−	2	Bipolar	
Flexispira taxon 4	+	−	−	+	−	+	+	−	r	r	+		Bipolar	
Flexispira taxon 5	+	−	−	+	−	+	+	W	r	r	+		Bipolar	
Flexispira taxon 6: *H. trogontum*	+	+	−	+	−	+	+		r	r	+	5–7	Bipolar	
Flexispira taxon 7	−	−	−	+	−	+	+/−	−	r	r	+		Bipolar	
H. cinaedi	+	+	[−]	−	−	−	[−]	+	s	i	−	1–2	Bipolar	37–38
H. westmeadii	+	+	+	−			−		s	r			Bipolar	
Flexispira taxon 8	−	−	−	+	−	+	+	−	r	r	+	3–14	Bipolar	
Flexispira taxon 9: *H. bilis*	+	+	−	+	−	+	+	+	r	r	+	3–14	Bipolar	
H. canis	−	−	+	−	+	+	+		s	i	−	2	Bipolar	48
Flexispira taxon 10	+	−	−	−	−	−	+	+	r	r	+	6–12	Bipolar	
H. fennelliae	+	−	[+]	−	+	−	[−]	+	s	s	−	2	Bipolar	35
H. pametensis	+	+	+	−	−	−	+	[+]	s	s	−	2	Bipolar	38
H. cholecystus	+		+	−	−	−	+	+	i	r	−		Bipolar	
Helicobacter sp. Bird C	+	+	+	+	+	−	+	+	s	r	−	2	Bipolar	30
Helicobacter sp. Bird B	+	+	+	+	−	+	+	+	s	r	−	2	Bipolar	31
H. mustelae	+	+	+	+	[+]	+	+	−	s	r	−	4–8	Peritrichous	36
H. rodentium	+	+	−	−	−	−	+	+	r	r	−	2	Bipolar	

inhabit the male and female reproductive tracts and be transmitted sexually. *Helicobacter hepaticus*, isolated from diseased livers of mice (Fox *et al*., 1994), has been recovered from foetuses of near-term SCID mice, implying transplacental transmission (Li *et al*., 1998). Whilst the role of helicobacters in human abortions is probably minor in developed countries, it has never been examined. Indeed, helicobacters may play a significant role in some populations, particularly in developing countries in which people and animals share living spaces.

In sequencing the strains Bryner deposited in ATCC (provided to us by Phyllis Pienta of the American Type Culture Collection, ATCC), we noted a discrepancy between the sequence for strain ATCC 43966 and our previous sequence for strain NADC 1893 (supposedly the same strain). We received NADC 1893 from Bryner in 1991 and deposited its sequence in GenBank as M88137. The discrepancy was resolved by sequencing the original Kirkbride strain, 84-3345. Strain ATCC 43966 corresponds to the original Kirkbride strain and so GenBank entry M88137 (Eaton *et al*., 1993) has been corrected. Unfortunately, this strain sequencing error has misled other investigators into believing that the Kirkbride strain, ATCC 43966 (NADC 1893), is closely related to the Archer strain, ATCC 43879 (NADC 1937). In the recent study by Weir *et al*. (1999), based on the 99·8 % 16S rRNA sequence similarity between their '*Helicobacter rappinii*' isolate and sequences M88137 (uncorrected) and M88138, they performed DNA–DNA hybridization but found only 24 % DNA binding of their flexispira taxon 8 isolate to DNA from strain ATCC 43966 (a flexispira taxon 5 organism). Had the sequence M88137 for strain ATCC 43966 been correct at the time of their study, Weir *et al*. (1999) would have correctly chosen to use strain ATCC 43879 for their DNA–DNA hybridization studies and, as a result, would probably have demonstrated much higher binding.

The name '*F. rappini*' was proposed in two abstracts from meetings (Bryner *et al*., 1986; Bryner, 1987), but the name was never approved. Now that it is know that flexispira belong to the genus *Helicobacter*, it is desirable to apply the combined name '*H. rappinii*' to the appropriate flexispira taxon. Bryner proposed Kirkbride strain 84-3345 as the type strain for '*F. rappini*'. It is not appropriate to name a species based upon a single isolate, but, unfortunately, strain 84-

3345 is the only isolate representing the flexispira taxon 5 identified since 1984.

Flexispira taxon 8 could be a candidate for the name '*H. rappinii*' because it includes the four original Archer strains plus several recent isolates. This taxon has the 16S rRNA sequence that has been associated with '*F. rappini*' since 1993 (Eaton *et al.*, 1993). What precludes us from naming flexispira taxon 8 '*H. rappinii*' is that taxon 8 appears to be phenotypically heterogeneous. Half of the flexispira taxon 8 strains have an intervening sequence (IVS) of 187 bases replacing the 8-base helix that is normally present at position 198–219 (*E. coli* numbering). The Archer strains ATCC 43879, 43880, 49308 and 49309 and the recently reported human bacteraemia strains (Tee *et al.*, 1998; Sorlin *et al.*, 1999; Weir *et al.*, 1999) do not have the IVS, whereas the remaining strains do. IVSs at this position have been found in several *Campylobacter* and *Helicobacter* species (Linton *et al.*, 1994). The IVS, when present, is essentially identical to that found in *H. bilis* and differs by only approximately 6 bases from that found in flexispira taxon 2 strains. The Archer strains and a majority of remaining flexispira taxon 8 strains are catalase-negative. We have subsequently identified a helicobacter from mice (MIT 97-5078C; our unpublished results) that has essentially the same 16S rRNA sequence as our taxon 8 isolates but lacks the flexispira morphology. The flexispira taxon 8 isolates may represent more than one species and therefore require further study before being named. Thus, we are currently in the unfortunate taxonomic position of being unable to name any of the eight unnamed flexispira taxa '*H. rappinii*'. As shown in Table 2, several of the flexispira taxa cannot be distinguished by phenotypic criteria. We recommend that the name '*H. rappinii*' (ra.pin′ni.i. N.L. gen. n. *rappinii* in honour of the 19th century microbiologist J. P. Rappin) be applied eventually to either flexispira taxon 5 or taxon 8 as these contain the initial isolates of Kirkbride and Archer, respectively. Until these remaining taxa are formally named, it may be useful to refer to these 16S rRNA-sequence-defined taxa using the names given in Table 1, i.e. *Helicobacter* sp. flexispira taxon 1.

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