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Recommended minimal standards for describing new species of the genus *Helicobacter*

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The International Committee of Systematic Bacteriology Subcommittee on the taxonomy of *Campylobacter* and related bacteria has agreed in principle on minimum requirements for the description of new species of the genus *Helicobacter*. These requirements include the recommendation that the description of a putative new species or subspecies be based on examination of at least five strains in order to provide a measure of phenotypic and genotypic variation. Recommendations for required phenotypic and molecular data are presented. The requirements are consistent with the polyphasic approach to bacterial systematics.

Keywords: *Helicobacter*, minimal standards

INTRODUCTION

Members of the genus *Helicobacter* are curved, spiral, or fusiform bacteria with or without helical periplasmic fibres. The type species of the genus, *Helicobacter pylori*, was first described as a species of the genus *Campylobacter* (Marshall & Warren, 1984; Marshall *et al*., 1984), but was subsequently placed in its own genus based on marked 16S rRNA sequence divergence from other *Campylobacter* species (Romaniuk *et al*., 1987; Paster & Dewhirst, 1988; Goodwin *et al*., 1989). There are currently 18 validly published *Helicobacter* species in the genus (Table 1). Two '*Candidatus* Helicobacter' species have been validly published. Three putative additional helicobacter species have been effectively published and are included in Table 1. Furthermore, 16S rRNA sequence analysis of over 225 helicobacter isolates from mammals and birds indicates the genus to be phylogenetically diverse, potentially containing over 30 additional taxa of species status (Dewhirst *et al*., 1999). Members of this genus have been isolated from the gastrointestinal tracts of humans, non-human primates, cats, dogs, cheetahs, ferrets, rodents, cows, sheep, pigs, dolphins and birds.

This proposal was prepared and approved by members of the ICSB Subcommittee on *Campylobacter* and related organisms.

General features of *Helicobacter* spp.

Helical, curved or fusiform rods of width 0·3–0·6 μm and length 1–5 μm. Cells may become spherical or coccoid, especially in old cultures. Non-spore forming. Gram-negative. Motile by means of single or multiple flagella. In most species the flagella are sheathed (Table 2). Optimum temperature for growth is 37–42 °C. Usually microaerophilic and have a respiratory type of metabolism. Oxidase-producing; most strains produce catalase. All gastric *Helicobacter* spp. described to date produce copious amounts of urease. The G+C content of the DNA is 24–48 mol%. Although strains of several species are capable of growth on simple nutritional agar media, the majority require media supplemented with blood or serum for culture. The growth conditions for some species may be exacting (e.g. *Helicobacter bizzozeronii*, *Helicobacter felis* and *Helicobacter salomonis*; Jalava *et al*., 1998). The detailed biochemical, morphological and physiological aspects have been previously reported for the named species (Tables 1 and 2). Most *Helicobacter* spp. can be discriminated from the neighbouring genera *Campylobacter*, *Arcobacter*, *Wolinella*, *Sulfurospirillum and Thiovulum* by the presence of sheathing around the flagellar protein. However, two *Helicobacter* species (*Helicobacter pullorum* and *Helicobacter rodentium*) possess unsheathed flagella and therefore resemble species assigned to the other related genera. Similarly, although resistance to polymixin B has been shown to

Table 1. Accession numbers and references for named *Helicobacter* species

Taxon	Type strain	16S rRNA GenBank no.	Effective publication	Valid publication*
H. pylori	ATCC 43504^T	M88157		Goodwin *et al.* (1989)
H. mustelae	ATCC 43772^T	M35048		Goodwin *et al.* (1989)
H. felis	ATCC 49179^T	M57389		Paster *et al.* (1991)
H. fennelliae	ATCC 35684^T	M88154		Vandamme *et al.* (1991)
H. cinaedi	ATCC 35683^T	M88150		Vandamme *et al.* (1991)
H. nemestrinae	ATCC 49396^T	X67854		Bronsdon *et al.* (1991)
H. muridarum	ATCC 49282^T	M80205		Lee *et al.* (1992)
H. acinonychis	ATCC 51101^T	M88148		Eaton *et al.* (1993)
H. canis	NCTC 12739^T	L13464	Stanley *et al.* (1993)	Stanley *et al.* (1994a)
H. hepaticus	ATCC 51448^T	U07574	Fox *et al.* (1994a)	Fox *et al.* (1994b)
H. pametensis	ATCC 51478^T	M88147		Dewhirst *et al.* (1994)
H. pullorum	NCTC 12824^T	L36141	Stanley *et al.* (1994b)	Stanley *et al.* (1995)
H. bilis	ATCC 51630^T	U18766	Fox *et al.* (1995)	Fox *et al.* (1997)
H. bizzozeronii	ATCC 700030^T	Y09404		Hänninen *et al.* (1996)
H. trogontum	ATCC 700114^T	U65103		Mendes *et al.* (1996)
H. cholecystus	ATCC 700242^T	U46129	Franklin *et al.* (1996)	Franklin *et al.* (1997)
H. rodentium	ATCC 700285^T	U96296		Shen *et al.* (1997)
H. salomonis	CCUG 37845^T	U89351		Jalava *et al.* (1997)
'*Candidatus* H. suis'	V2BXA†	AF127028		De Groote *et al.* (1999a)
'*Candidatus* H. bovis'	R2XA†	AF127027		De Groote *et al.* (1999b)
'*H. westmeadii*'	None designated	U44756	Trivett-Moore *et al.* (1997)	
'*H. suncus*'	None designated	AB006147	Goto *et al.* (1998)	
'*H. typhlonicus*'	MIT 97-6810^T	AF127912	Franklin *et al.* (1999)	

* Articles published in IJSB or effectively published elsewhere that appear on a Validation List.

† Clone designation, not a cultivable strain.

be a useful marker for many *Helicobacter* spp. (Burnens & Nicolet, 1993), *H. pullorum* is known to be sensitive. At present there is no single phenotypic feature that unequivocally differentiates *Helicobacter* spp. from closely related genera. Genotypic data, such as 16S rRNA sequence analysis, does however unequivocally differentiate the genus *Helicobacter* from all other genera. A phylogenetic tree based on neighbour-joining analysis of 16S rRNA sequences is shown in Fig. 1. The sequence of the entire genome of the type species, *H. pylori*, has been determined for strains 26695 (ATCC 700392) and J99 (Tomb *et al.*, 1997; Alm *et al.*, 1999). Using common laboratory methods (e.g. Hugh and Leifson test for oxidation and/or fermentation of glucose) (Hugh & Leifson, 1953), *Helicobacter* spp. appear asaccharolytic. However, recent studies suggest that *H. pylori* possesses highly specific monosaccharide kinases, has the ability to phosphorylate glucose, and may oxidize glucose via the Entner–Doudoroff pathway (Mendz & Hazell, 1993; Mendz *et al.*, 1993; Chalk *et al.*, 1994; Tomb *et al.*, 1997). Similar investigations have not yet been undertaken on other helicobacters.

General comments

The description of a new species or subspecies of *Helicobacter* should be based on characteristics necessary for assigning the new taxon to the genus, and on characteristics that serve to differentiate the new taxon from existing taxa of the genus. The description should be based on as many strains as possible, preferably not fewer than five isolates from different sources. Where too few strains are available for formal naming, a description can be usefully published. For example, in the original publication naming (as campylobacters) *Helicobacter cinaedi* and *Helicobacter fennelliae*, an isolate representing a third species, CLO-3, was described but not named (Totten *et al.*, 1985), and the publication naming *Helicobacter pametensis* describes strains that represent two additional species, Bird-B and Bird-C (Dewhirst *et al.*, 1994).

For critical comparisons with other species, controls consisting of type or reference strains of the appropriate taxa should be tested. For all phenotypic test procedures, the inoculum size, composition of the gaseous atmosphere, period of incubation, and composition of the basal growth medium should be stated. The use of standardized, well-described tests and methods is recommended (Mégraud *et al.*, 1985; On & Holmes, 1991a, b, 1992, 1995; Paster *et al.*, 1991; Barrow & Feltham, 1993).

Putative new species of uncultured organisms for which molecular sequence data (such as 16S rRNA

Table 2. Characteristics which differentiate *Helicobacter* species

Data were obtained from references in Table 1 and from results of standardized phenotypic tests (On *et al*., 1996, 2000; Jalava *et al*., 1998; Atabay *et al*., 1998). Abbreviations: +, 100% of strains positive; −, 0% strains positive; (+), 80–94% strains positive; (−), 7–33% strains positive; V, 42–66% strains positive. S, sensitive; R, resistant; I, intermediate; ND, not determined; Bp, bipolar; Mp, monopolar; Po, polar; Pt, peritrichous. Taxa: 1, *H. pylori*; 2, *H. mustelae*; 3, *H. felis*; 4, *H. fennelliae*; 5, *H. cinaedi*; 6, *H. nemestrinae*; 7, *H. muridarum*; 8, *H. acinonychis*; 9, *H. canis*; 10, *H. hepaticus*; 11, *H. pametensis*; 12, *H. pullorum*; 13, *H. bilis*; 14, *H. bizzozeronii*; 15, *H. trogontum*; 16, *H. cholecystis*; 17, *H. rodentium*; 18, *H. salomonis*.

Character	Taxon:																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Catalase production	+	+	+	(+)	(+)	+	+	+	−	+	(+)	+	+	+	+	+	+	+
Nitrate reduction	−	+	+	−	+	−	−	−	−	+	+	+	+	+	+	+	+	+
Alkaline phosphatase hydrolysis	+	+	V	(−)	(−)	+	+	+	+	−	(+)	−	−	V	−	+	−	V
Urease	+*	+*	(+)	−	−	+	+	+	−	+	−	−	+	(+)	+	−	−	+
Indoxyl acetate hydrolysis	(−)	+	(−)	+	(−)	−	−	(−)	+	+	−	−	−	(−)	−	−	−	(−)
γ-Glutamyl transpeptidase	+	+	+	−	−	ND	+	+	+	−	−	ND	+	+	+	−	−	+
Growth at 42 °C	(−)	V	V	(−)	V	+	−	(−)	+	−	+	+	+	V	+	+	+	−
Growth with 1% glycine	−	−	−	−	−	−	−	−	−	+	V	−	+	(−)	ND	+	+	−
Sensitivity to 30 μg disc:																		
Nalidixic acid	R	S	R	S	S	R	R	R	S	R	S	R	R	R	R	I	R	R
Cephalothin	S	R	S	S	I	S	R	S	I	R	S	S	R	S	R	R	R	S
Periplasmic fibres	−	−	+*	−	−	−	+	−	−	−	−	−	+	−	+	−	−	−
Sheathed flagella	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+
No. of flagella	4–8	4–8	14–20	2	1–2	4–8	10–14	2–5	2	2	2	1	3–14	10–20	5–7	1	2	10–23
Distribution of flagella	Bp	Pt	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Mp	Bp	Bp	Bp	Po	Bp	Bp
G+C content (mol%)	35–37	36	42	35	37–38	24	34	30	48	ND	38	34–35	ND	ND	ND	ND	ND	ND

* Atypical strains and/or spontaneous mutants giving a negative result have been described previously (Costas *et al*., 1991; Eaton *et al*., 1996; Jalava *et al*., 1998; Majewski & Goodwin, 1988).

sequence) are available may qualify for assignment to the provisional taxonomic status *Candidatus* (Murray & Schleifer, 1994) in accordance with the proposals of Murray & Stackebrandt (1995). An important requirement of the *Candidatus* status is that a DNA probe, based upon the molecular data, identify the proposed organism by *in situ* hybridization. 16S rRNA sequence data by itself is not sufficient for *Candidatus* status, and in addition, morphotype, Gram reaction and other preliminary metabolic data are required. As with culturable helicobacters, it is preferred that five examples (typified, for example, by 16S rRNA gene sequences and DNA probe results) from different sources are studied, and that multiple sequences of the putative new '*Candidatus* Helicobacter' species cluster together in phylogenetic analyses (see below). This convention has been used for '*Candidatus* Helicobacter suis' (De Groote *et al*., 1999a) and '*Candidatus* Helicobacter bovis' (De Groote *et al*., 1999b), and may be appropriate for other uncultured helicobacters such as other gastric spiral organisms described by Solnick *et al*. (1993).

Cell morphology

The shape, size and spiral wavelength (where appropriate) of bacterial cells should be reported. The tendency to undergo transformation to coccoid forms on exposure to air or in older cultures should be noted. The number and arrangement of flagella should be determined by electron microscopy, as well as the presence or absence of flagellar sheaths and periplasmic fibres.

Staining behaviour

The behaviour of the cells in the Gram stain must be stated.

Motility

Cells should be observed by microscopic examination of wet mounts or hanging-drop preparations of young cultures in buffered saline or broth. Cells in old cultures, or under certain culture conditions, may lose motility. There are currently no known non-motile *Helicobacter* species.

Colony morphology

The size, shape and colour of colonies should be described for optimal growth conditions on solid media. The presence of swarming on solid media should be noted.

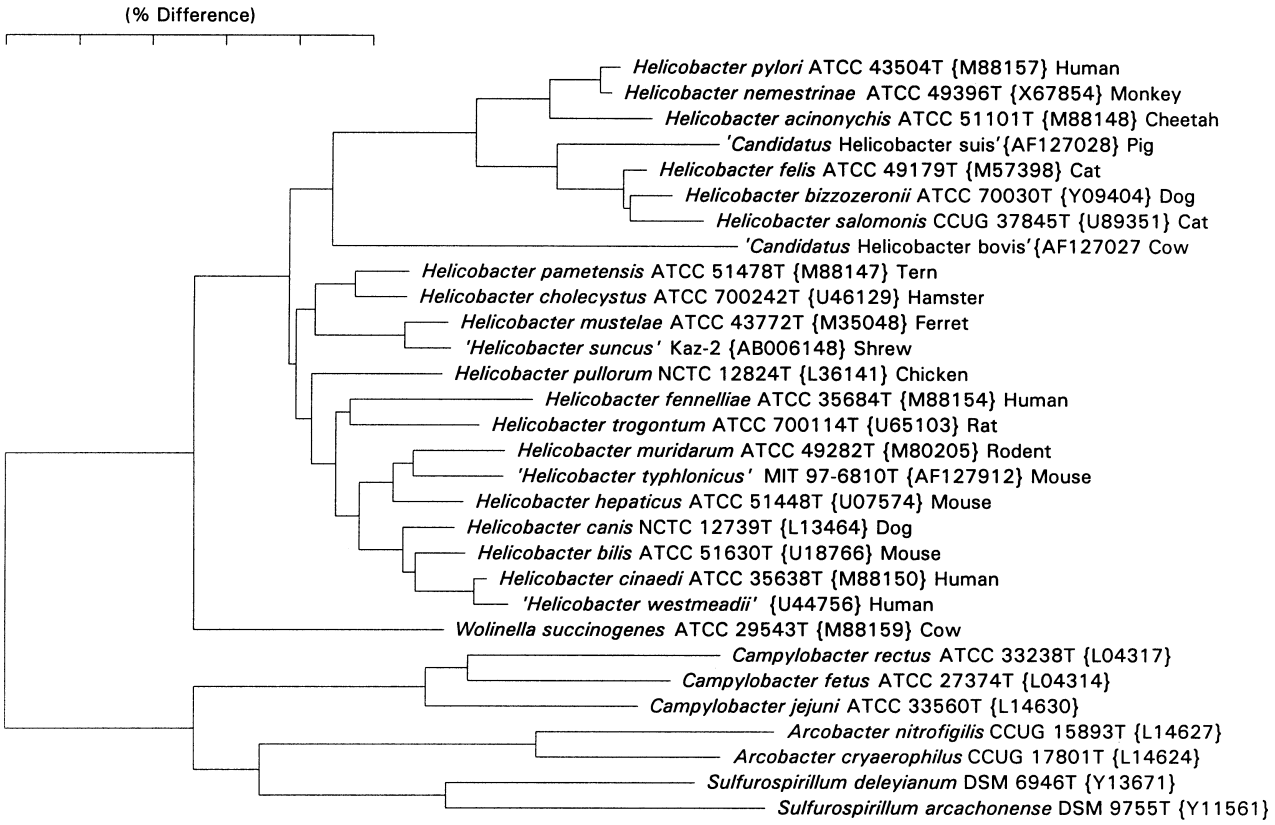

Fig. 1. Phylogenetic tree of the genus *Helicobacter* and closely related genera based upon 16S rRNA sequence comparisons. The scale bar represents a 5% difference in nucleotide sequence. Distance is determined by measuring the lengths of all horizontal lines connecting two species.

Growth conditions

Factors affecting growth should be tested under conditions that are near optimal unless stated otherwise.

Culture medium

The type of nutrient agar base medium used [brain-heart infusion (BHI), Mueller–Hinton, etc.] and the manufacturer from which it was obtained should be stated. The type and percentage of blood used to supplement the base medium should be given. The presence or absence of haemolytic activity should be recorded.

(ii) Temperature range. The duration of incubation and ability to grow in specified broth or agar media from standardized inocula at various temperatures should be reported. The following temperatures should be used: 25, 35 or 37, and 42 or 43 °C.

(iii) Gaseous requirements. The ability of the strains to grow under aerobic, microaerobic and anaerobic conditions should be reported. The oxygen and hydrogen content should be specified for microaerobic conditions.

Biochemical properties

Results for the following tests are required: (i) oxidase activity, by use of any conventional method (for example, Kovács, 1956); (ii) catalase activity, with percentage of reagent solution and time of observation given; (iii) acid production from glucose, for example by the method of Hugh & Leifson (1953); (iv) nitrate reduction, preferably by the plate method of Cook (1950); (v) indoxyl acetate hydrolysis, preferably using a disk method (Mills & Gherna, 1987; On & Holmes, 1992) with percentage of reagent solution, volume of impregnation and time of observation given; (vi) urease activity, using a rapid method (e.g. Owen *et al.*, 1985); (vii) alkaline phosphatase activity (e.g. Itoh *et al.*, 1987), with time of observation given; and (viii), γ-glutamyl transpeptidase (e.g. Mégraud *et al.*, 1985).

Other tests

Test results for the following are desirable: selenite reduction (e.g. On & Holmes, 1992); growth on media containing 1% glycine, 1% ox-bile, 0·032% methyl orange and 0·04% triphenyl-tetrazolium chloride (e.g. On & Holmes, 1991b, 1995). The ability of bacteria to reduce the latter compound should also be stated (On & Holmes, 1991a).

Resistance to antimicrobial agents

Susceptibility to nalidixic acid (30 μg) and cephalothin (30 μg) should be determined either by diffusion (Goodwin *et al*., 1989) or by plate MIC tests (On & Holmes, 1991b). For the former, the absence of a clear zone of inhibition should be recorded as resistance; for sensitivity strains, the inhibition zone sizes should be stated. The type of base medium used should be stated. BHI agar with added 7% lysed blood is recommended. Brucella Agar and other media with bisulfite have been shown to inhibit the growth of *H. pylori* and therefore should be avoided (Hawrylik *et al*., 1994)

Molecular data

Standard methods (melting temperature or enzymic) for the determination of G+C content of the DNA may be used (De Ley, 1970). Reference DNA, such as *Escherichia coli* ATCC 11775^T (51 mol% G+C) or *H. pylori* ATCC 700392 (39 mol% G+C) should be analysed at the same time. The strain used and its estimated G+C content (mol%) expressed relative to the reference DNA should be reported.

The essentially complete (greater than 1450 bases) 16S rRNA sequence should be determined for the type strain. Specific methods for sequencing 16S rRNA from *Helicobacter* species, including intervening sequences (IVS), have been described (Fox *et al*., 1995). Intervening sequences in the 16S rRNA gene should be fully sequenced and the 16S rRNA gene sequence, including any IVS, deposited in nucleic acid databases as a single sequence. The 16S rRNA sequence of two additional independent isolates of the putative new taxon also should be obtained to determine the sequence heterogeneity within the species. Phylogenetic tree construction should demonstrate that the novel sequence branches within the cluster of validly described *Helicobacter* spp. A full description of alignment and treeing methods, including software, algorithms, treatment of gap penalties, and treatment of IVS sequences should be included. Currently, *Wolinella succinogenes* is the most closely related species in a neighbouring genus.

Relationships to neighbouring *Helicobacter* spp. should be determined by quantitative DNA–DNA hybridization (Owen & Pitcher, 1985). Several methods have been applied specifically to *Helicobacter* spp. (Fox *et al*., 1988, 1989; Stanley *et al*., 1992; Hänninen *et al*., 1996). It should be noted that estimates of the degree of relatedness may vary significantly depending on the method and experimental conditions used (Ezaki *et al*., 1988; Fox *et al*., 1989), and that the accurate resolution of close genomic relationships can be difficult where semi-quantitative assays (e.g. dot-blot assays) are employed (Ezaki *et al*., 1988). A good correlation between classical DNA–DNA hybridization studies, and the results of numerical analysis of whole-cell protein profiles has been observed (Pot *et al*., 1994); the latter may be considered to be an alternative or adjunctive method for taxonomic discrimination at the species or defined subspecies level. Other alternative methods for determining whole-genome relatedness between strains, such as multigene sequence analysis (a logical extension of the work of Maiden *et al*., 1998), or numerical analysis of high-resolution amplified fragment length polymorphism (AFLP)-based fingerprints (Janssen *et al*., 1996), could also be considered when their efficacy is validated.

Ecology

The natural habitat(s) of the proposed species should be detailed as much as possible, and its pathogenicity and host range, if known, should be reported.

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