Ultrastructure of the bacteria *Campylobacter* and *Helicobacter*: Implications for the phylogeny of mammal gastric bacteria

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Abstract: Negative staining, thin sections, and surface replica methods were used to produce ultrastructural descriptions of curved-shaped, flagelated bacteria *Campylobacter jejuni*, *Helicobacter pylori*, and *H. muridarum*, the last one from the ileon of mice. The former has nude monotrichous flagella, the others have the sheated lobotrichous type. *H. muridarum* presents 10-12 periplasmic fibers. A cladogram of some gastric bacteria, based on the evolutionary history of the mammal hosts, hypothesizes that (1) the genus *Helicobacter* evolved about 65 million years ago and that (2) primate parasites such as *H. pylori* and *H. nemestrinae* are close relatives, as are parasites of carnivores which include *H. mustelae*, *H. felis* and *H. acynonyx*.

Key words: *Campylobacter jejuni*, *Helicobacter pylori*, *Helicobacter muridarum*, ultrastructure, electron microscopy, cladism, phylogeny.

Bacilli in gastric mucosa from several animals, including humans, were sporadically reported since 1880; nevertheless, the systematic study of these microorganisms was neglected for almost a century. Recently, the finding of a high prevalence of *Helicobacter pylori* in gastric biopsies from patients with chronic gastritis or peptic ulcers, focused atention on this microniche (Lee & Hazell 1988).

Other species of *Helicobacter* were described in mammals: *H. mustelae*, *H. nemestrinae*, *H. felis*, *H. muridarum*, and *H. acynonyx;* from ferrets *(Mustela putoris furo)*, monkeys *(Macaca nemestrina)* cats, mice, and cheetahs *(Acynonyx jubatus)*, respectively (Lee and Hazell 1988, Lee and O'Rourke 1993, Goodwin 1990). Two additional *Campylobacter* species were included in the genus *Helicobacter: H. cinaedi*, and *H. fennelliae*, both associated with diarrhea in homosexual men (Vandamme *et al.* 1991). Except for the last two species cited, *Helicobacter* represent a highly specialized group of bacteria that colonize the gastric mucosa of some mammals.

At least one other bacteria has been described, but not isolated, from the gastric mucosa of humans with gastritis: "*Gastrospirillum hominis*" (McNulty *et al.* 1989). Disturbances on the gastric niche, such as intestinal metaplasia or antimicrobial treatment, may allow the establishment of enteric Gram-negative rods and oropharingeal Gram-positive coccus (Sjosted *et al.* 1988, 1989; Mikelsaar *et al.* 1990). Other bacteria from the abiotic environment or from animals can also colonize the human gastric mucosa (Taylor *et al.* 1991).

At present, it is accepted that the gastric mucus from mammals, including humans, is the natural habitat of specially adapted bacteria (Lee & Hazel 1988, Goodwin 1990). These gastric microorganisms share several characteristics: (a) all are Gram-negative spiral or curved shaped; (b) they exhibit a strong motility and are able to cross through the viscous layer of mucus; (c) this movement is due to sheathed flagella; some species have a single mono or bipolar flagellum and others present a polar or bipolar tuft; (d) they are mi-

croaerophilic or anaerobic; (e) their adaptation to the stomach environment (pH < 4) is caused by a strong urease activity. This enzyme breaks the gastric urea in carbon dioxide and ammmonia, producing a "neutralizing cloud" that surrounds the bacteria and protects them from acid (Hazell 1991).

Other curved-shape bacteria, genetically related with *Helicobacter*, inhabit the mouth and small intestine of mammals. These agents include *Wollinella*, an agent of periodontal infections in humans, *Campylobacter* spp. associated with zoonotic diarrhea, and *Arcobacter* spp. previously called "Aerotolerant *Campylobacter*" (Vandamme *et al.* 1991).

The characteristics shared by curved-shape bacteria from the gastrointestinal tract of several mammals suggest that they had a common ancestor with adaptations to different niches, such as mouth, stomach, and intestine (Lee & Hazel 1988, Goodwin 1990, Paster *et al.* 1991).

This paper describes the ultrastructure of three curved-shape bacteria (*Campylobacter jejuni, Helicobacter pylori*, and *H. muridarum*) using scanning electron miccroscopy (SEM) and transmission electron microscopy (TEM) and presents a cladistic phylogenetic analysis of some gastric bacteria.

MATERIAL AND METHODS

Bacterial strains: *Campylobacter jejuni* was isolated from stool samples of children with acute diarrhea from the National Children Hospital (San José, Costa Rica). The samples were inoculated on Skirrow medium and incubated under a microaerophilic atmosphere for 2 days and identified by their biochemical reactions.

Helicobacter pylori was isolated from antral mucosal biopsy specimens at the endoscopic examination as was previously described (Hernández *et al.* 1991). The biopsy tissue was inoculated on blood agar plates without supplements and incubated under a microaerophilic atmosphere at 37 °C for 5 days. The cultured organisms were identified as *H. pylori* from colonial morphology, Gram-staining characteristics, and by determination of oxidase, catalase, and urease activities.

H. muridarum were obtained from the small intestine content of adult mice (Hernández *et al.* 1985). Samples of murine ileum and its con-

tents were processed for electron microscopy and inoculated on Skirrow and blood agar plates incubated as was indicated by Phillips and Lee (1983).

Thin sections: Samples of bacterial suspensions, bacterial colonies and mice tissues were fixed in Karnovsky (1965) solution, washed with phosphate buffer (PBS), post-fixed with Os O4, dehydrated by an increasing ethanol series, and embeded in resin (Spurr). The thin sections were double-stained with uranyl acetate and lead citrate and analysed under a transmission electron microscope.

Scanning electron microscopy (SEM): Samples -bacterial suspensions and tissues- were fixed and dehydrated as described above for thin sections. Then, the specimens were immersed in amyl-acetate, critical point dried, ion sputter covered with *ca* 200 A° thickness of gold, and analysed by scanning electron microscopy.

Surface replicas: Samples -bacterial suspensions and tissues-were processed as described above for SEM; but, instead of sputter coating they were shadowed with platinum-carbon in a metal evaporator. The shadowed surface was covered with a plastic gum (Cementidine CR); then the organic matter was digested with sodium hypochloride (domestic bleach) and the plastic dissolved in acetone in order to liberate the replica, that was picked-up with uncovered grids, and analysed under TEM. This technique was designed by Akahori (personal communication) and was described in detail by Hernández *et al.* (1986). For stereopairs analyses two consecutive electron micrographs were taken with a tilt angle of 10° between both.

Negative staining: Bacterial suspensions and small-intestinal contents were applied to formvar-covered grids, negative stained with 1% uranil acetate or 0.5% phosphotungstic acid and analysed by TEM.

RESULTS AND DISCUSION

Campylobacter has a rough surface, sharp-pointed ends, and a single polar or bipolar unsheathed flagellum, that emerges from a terminal pit (Figs. 1 and 2). Abnormal shapes were observed in old cultures as spiral forms, coccoid bodies, or fatty forms (Fig. 3).
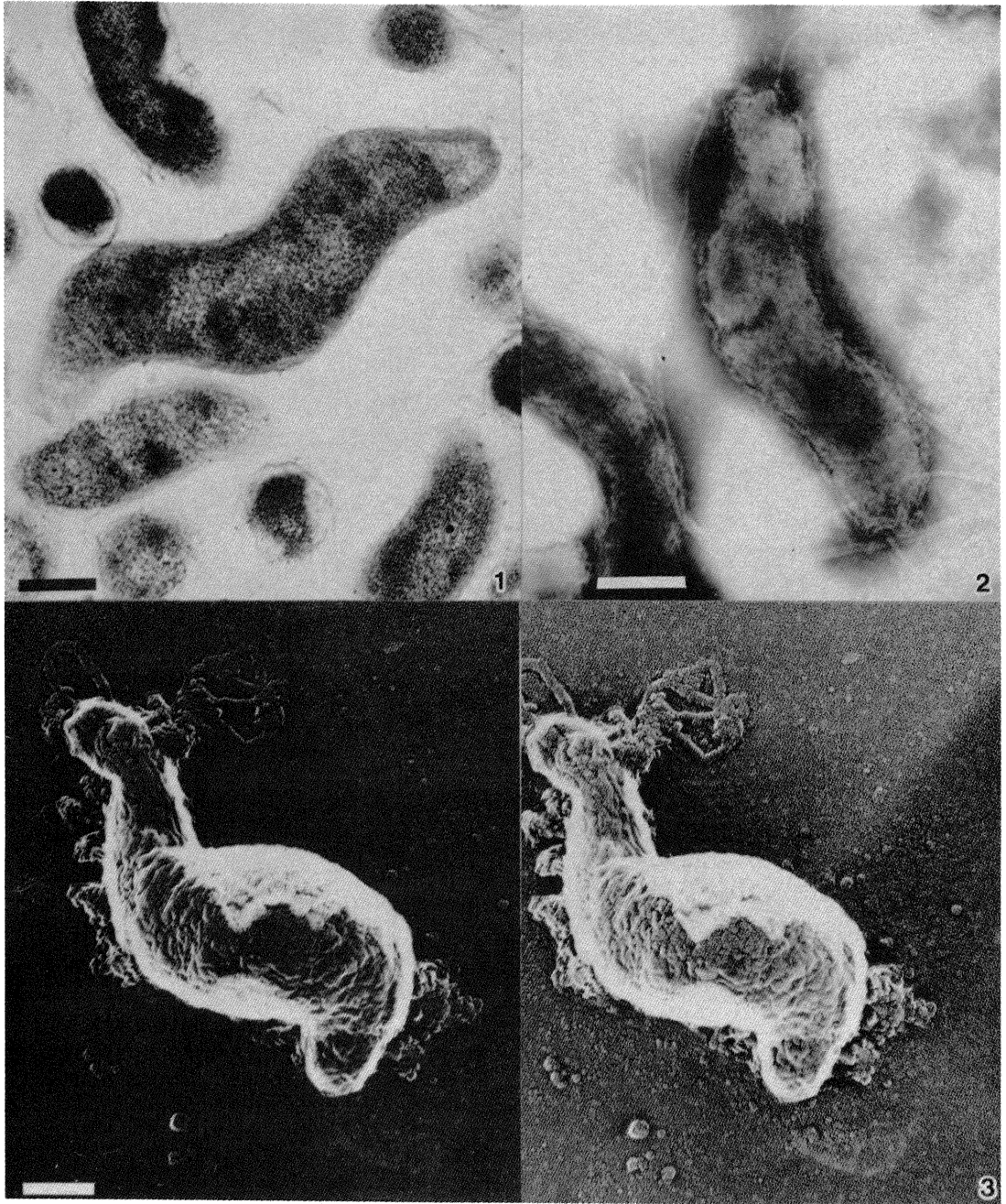
Figs. 1-3. Transmision electron micrographs of *Campylobacter jejuni*. Figs. 1 and 2 are thin sections and negative staining, respectively. The rough surface of the bacteria and the pit of the flagellar insertion (arrow) can be observed in both electron-micrographs. Fig. 3 is a stereo pair of an abnormally "fatty" bacillum, showing its polar unsheathed flagellum. Bar = 0.25 μm.
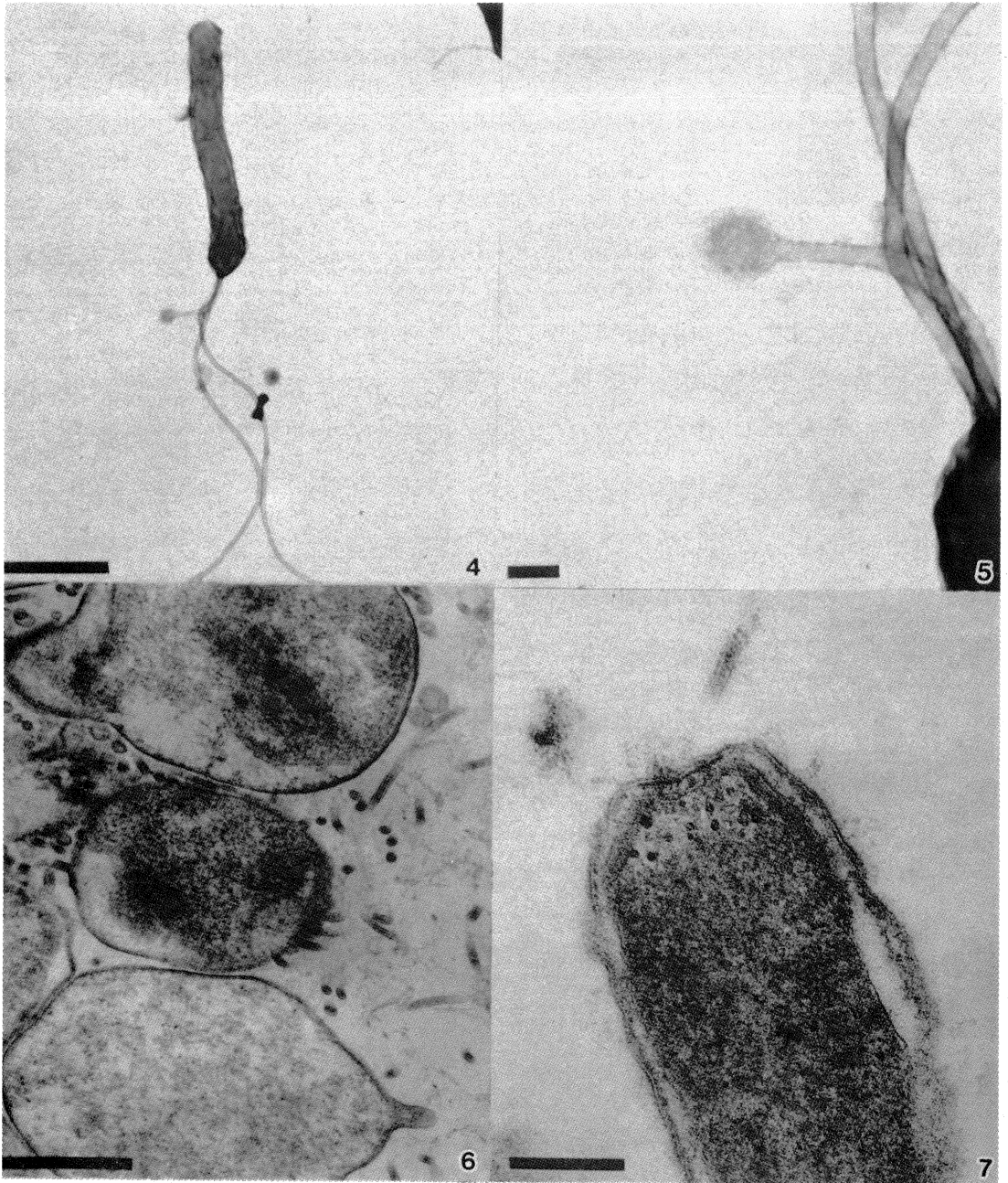
Figs. 4-7. Transmision electron micrographs of *Helicobacter pylori*. Fig. 4 Negative stain of a bacillum, notice typical light curved shape when this agent is grown *in vitro*. Fig. 5 shows the flagellated end of this bacterium, with the terminal bulb in one flagellum. Figs. 6 and 7 are thin sections; the former is a section of a colony, the central bacillum has four sheathed flagella. Fig. 7 corresponds to a thin section of a bacterium and shows their smooth surface and the complex structures associated with flagella. Electron dense grains (small arrows), "polar membrane" (big arrow). Bar = 0.25 μm.
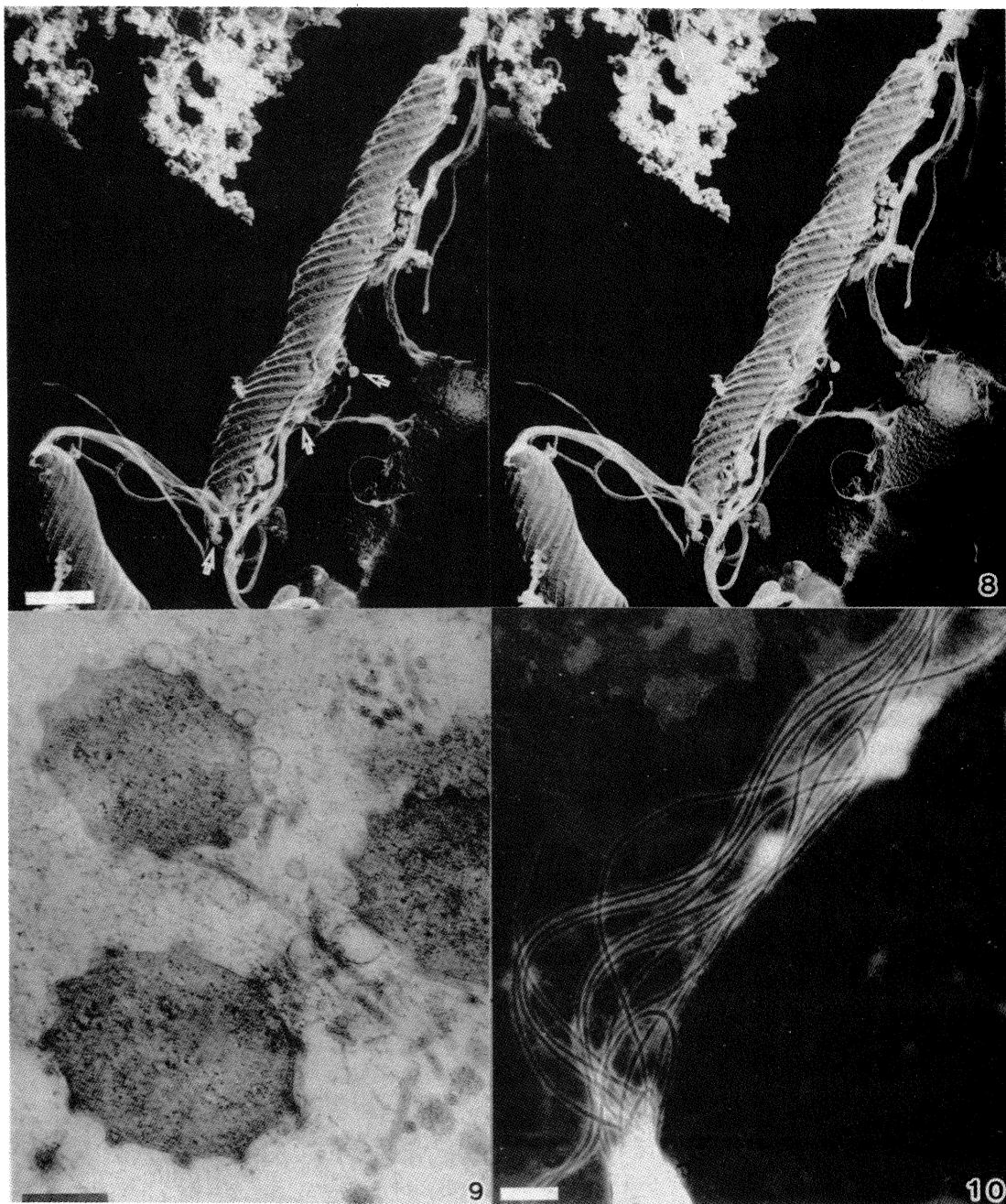
Figs. 8 - 10. Transmission electron micrographs of *H. muridarum.* Fig. 8. Stereo pair showing the screw appearance of this agent, conferred by 10 to 12 periplasmic fibers. (Bar = 0.5 μm). Fig. 9. Trasverse section of bacilli. Some flagella appear in cross section (asterisk), outer membrane (small arrow), cell-wall (big arrow). Bar = 0.25 μm. Fig. 10 corresponds to negative staining of the polar tuft of sheathed flagella of this agent. Bar = 0.5 μm.

H. pylori is a curved bacteria with rounded ends; nevertheless, when it was cultured *in vitro* the predominant forms were more straight than observed *in vivo* (Fig. 4). This agent presents 3 - 6 lophotrichous sheathed flagella (Figs. 4-7) with a terminal bulbous structure (Fig. 5). However, the bulb did not always appear in fixed bacteria; also, the flagellar sheath showed lateral vacuolizations with bulbous apppeareance. This finding suggest that vacuolizations and maybe even the terminal bulb could be artifacts. The structure of the bacterial end associated with its flagella is complex, including a "polar membrane", and electron dense granules (Fig. 6). Fig. 7 shows a section of a colony; the bacteria appear transverse by section and the sheathed flagella are evident in longitudinal and trasverse sections.

H. muridarum, the spiral-shaped bacteria from mice, did not grow *in vitro*, in spite of the large populations observed in the small-intestine content of mice. They were found attached to the microvilli and into the mucus layer, mainly at the ileon level. This agent has an uncommon bacterial appearance due to about 10-12 periplasmic fibers tightly coiled around its body, delimiting grooves; this structure gives it a screw appearance (Fig. 8). The periplasmic fibers are evident in transverse sections. They are localized between the cell wall and the outer membrane; which also covered the flagella constituting the sheath (Fig. 9). This agent presents a bipolar tuft of *ca.* 12 sheathed flagella (Figs. 8 and 10). In some cases a flagellar terminal bulb could be seen, as was described in *H. pylori* (Fig. 8). Dimensions of bacteria and flagella were not calculated, because critical point drying induces volume reduction (Boyde & Boyde 1980, Sinikka & Saukko 1982, Beckett *et al.* 1984).

Ultrastructural analysis of curved bacteria from the gastrointestinal tract of mammals, mainly those agents that still are non-cultivable, is important to determine their relationship with the gastric environment, in order to clear their pathological role.

The taxonomy and systematics of the bacteria are subject to frequent revision which the non-specialist finds difficult to follow. These changes show that the use of cladistic techniques would be of great value in bacteriology. However, the clear rules of cladistic analysis have yet to become widespread in the field, in contrast with vertebrate and invertebrate systematics, in which cladism has become successful after initial resistance (Brusca and Brusca 1990, Monge-Nágera 1994). One special case of cladistic analysis is using the cladogram of a host group to produce a phylogenetic hypothesis for the parasites which have coevolved with it (Hennig 1979, Brooks 1981). Fig. 11 shows a cladogram of some gastric bacteria resulting from their association with hosts of known phylogenetic affinites. The widespread occurrence of *Helicobacter* in unrelated mammals (Eissenberg 1981) suggests that this genus evolved near the Cretaceous-Paleocene boundary 65 million years ago, a suggestion which can be tested by molecular clock analysis. Although this cladogram is in agreement with the phylogenetic inferences of Bronsdon *et al.* (1991), it must be stressed that it represents only a hypotesis to base further work. This type of cladogram is not stronger than its basic assumption, namely that symbiont speciation parallels that of the host group. A perfect coevolution pattern is unlikely to exist in nature because some parasites can become extinct, or can colonize other species horizontally. Also researchers are likely to (1) overlook some parasites, (2) missidentify parasites and hosts, and (3) produce unnatural colonizations by keeping several species in their laboratories.

Thus, the purpose of this study is to present an evolutionary pattern that fits current knowledge but which is likely to change when more information is obtained. A first valuable step would be obtaining new morphological information at least as detailed as that presented here, and its publication in form suitable to elaborate a cladistic matrix (see Henning 1979). Cladism offers a valuable tool for bacterial systematics and deserves more attention.
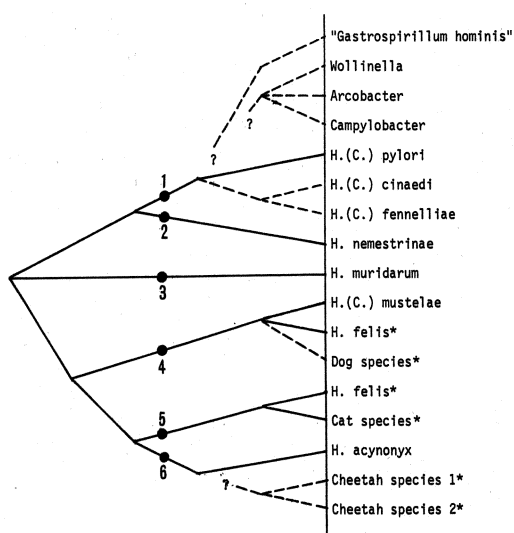
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Fig. 11. A cladistic hypothesis for the gastric bacteria of some mammals, based on mammal cladograms (Eisenberg 1981, Novacek 1992) and using the principles described by Hennig (1979) and Brooks (1981). Discontinuous lines relate those groups for which information is particularly scarce; those species transferred from *Campylobacter* to *Helicobacter* are indicated by "(C.)". Key to numbers: 1 *Homo*, 2 *Macaca* (both Primates), 3 *Mus* (Rodentia), 4 *Mustela* and *Canis*, 5 *Felis*, 6 *Acynonyx* (4-6 Carnivora). Asterisk: *Helicobacter felis* has been reported from domestic cats *(Felis domesticus)* and dogs *(Canis familiaris)*, which also host other unnamed bacteriae described by Lee *et al.* (1988) and Lockard and Boler (1970), respectively. Unnamed species from the cheetah were described by Eaton *et al.* (1991).

RESUMEN

Se describe ultraestructuralmente *Campylobacter jejuni, Helicobacter pylori* y *H. muridarum* (una bacteria curva obtenida de ileon murino) empleando tinción negativa, cortes ultrafinos y réplicas metálicas. Los tres organismos son curvos y presentan flagelos; el primero tiene flagelos monotrichos desnudos, los otros presentan flagelos lobotrichos envainados. En *H. muridarum* hay 10-12 fibras periplásmicas. Se presenta un cladograma de algunas bacterias gástricas, basado en la historia evolutiva de sus hospederos mamíferos. Esta hipótesis sugiere que (1) el género *Helicobacter* evolucionó hace unos 65 millones de años y que (2) parásitos de primates como *H. pylori* y *H. nemestrinae* son parientes cercanos; mientras que *H. mustelae, H. felis* y *H.*

acynoyx, parásitos de carnívoros, también constituyen un grupo natural.

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