An ultrastructural study of *Helicobacter mustelae* and evidence of a specific association with gastric mucosa

**JANI O’ROURKE, A. LEE and J. G. FOX**

*School of Microbiology and Immunology, University of New South Wales, Sydney, NSW, Australia 2033 and *Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA, USA 02139*

**Summary.** The ultrastructure of *Helicobacter mustelae*, a natural inhabitant of the ferret stomach, has been studied and compared with the human gastroduodenal pathogen *H. pylori*. *H. mustelae* is a short, slightly curved rod, 2 μm × 0.5 μm, with four or more sheathed flagella. The most common flagellar configuration is a single flagellum at one terminus, bipolar arrangement at the other end and a lateral flagellum. Dense inclusion bodies were observed below the flagellar insertion sites. It is suggested that this configuration is a specialised adaptation to motility in a viscous environment. On examination of the ferret gastric mucosa, similarities to *H. pylori* were observed such as adherence to gastric tissue and the formation of adhesion pedestals. However, unlike *H. pylori*, significant numbers of bacteria were intracellular. Furthermore, a much greater proportion of *H. mustelae* were attached to the mucosa with few bacteria lying free in the mucus, as is seen with *H. pylori*. It is concluded that the ferret is an important model for the study of adherence of bacteria to gastric mucosa and their possible role in peptic ulceration.

**Introduction**

The isolation of a *Campylobacter*-like organism from the human stomach in 1983 focused attention on what was thought to be a barren, hostile environment—the gastric mucosa. Taxonomic studies on this bacterium have shown that it has different characteristics to *Campylobacter* spp., resulting in the creation of a new genus, *Helicobacter*; the human isolate is now named *H. pylori*. Re-examination of the early literature has revealed the existence of bacteria in the stomachs of many animal species and man. With techniques for culture of *H. pylori*, gastric organisms have been isolated from non-human primates, ferrets, dogs and cats. The isolates from ferrets and cats are morphologically distinct from *H. pylori* and these observations have resulted in the creation of two new species, *H. mustelae* and *H. felis*.

*H. mustelae* is a natural inhabitant of the ferret stomach. Like *H. pylori*, it has a limited host range and appeared, in preliminary studies, to adhere to the gastric epithelium. Significantly, ferrets are prone to peptic ulceration and both gastric and duodenal ulcers have been reported. *H. mustelae* colonises the gastric mucosa in a similar way to *H. pylori*, but the bacterium has a very different morphology; although the spiral morphology has been proposed as a specific adaptation to survival in gastric mucus, *H. mustelae* must have acquired some other survival trait.

The purpose of this study was to make a detailed examination of the ultrastructure of *H. mustelae* both *in vitro* and *in vivo* and to gain further insights as to its association with gastric mucosal surfaces.

**Materials and methods**

**Bacterial strains**

*H. mustelae* isolates ATCC 43773 and NCTC 12032 were used for ultrastructure analysis. All cultures were grown on Blood Agar Base No. 2 (Oxoid), supplemented with horse blood, 5% v/v, incubated under micro-aerophilic conditions for 48 h at 37°C as previously described. Bacteria were harvested from plates and either stained with sodium phosphotungstate 2% or transferred to plus ruthenium red (Sigma) Noble agar 2%.

**Animals**

Ten adult female ferrets were obtained from Marshall Farms, North Rose, NY and housed as previously described. Biopsy specimens were obtained from the stomachs of the animals and placed in Karnovsky’s fixative plus ruthenium red (Sigma) 0.5% and subsequently embedded in Noble agar 2%.

**Electronmicroscopy**

The bacterial preparations from cultures and animals were post-fixed in osmium tetroxide and uranyl
Acetate and embedded as described previously. Negative stains and thin sections (70–80 nm) of the bacteria and gastric tissue were examined with an Hitachi H 7000 transmission electronmicroscope. Ultra-thin sections were cut with a Reichart Jung Ultramicrotome (Ultracut E) with the section thickness set at 20 nm. Sections were collected on to a 400 mesh grid, stained overnight with uranyl acetate and then lead citrate for 5 min. Before examination with an Hitachi H 7000 electronmicroscope, grids were carbon coated on both sides for stability. Freeze-dried and freeze-etched samples were prepared as described previously and examined with a Phillips 300 transmission electronmicroscope.

Results

In-vitro preparations

*H. mustelae* from cultures was found to be a short, slightly curved rod, 2 μm × 0.5 μm, with four or more polar and lateral sheathed flagella as reported previously. Examination of a large number of negatively stained preparations revealed that the most common...
flagellar configuration was a single polar flagellum at
one terminus and bipolar flagella at the other terminus
together with a lateral flagellum (fig. 1A). Higher
magnifications revealed that the surface was covered
with a regular array of circular structures, 8 nm in
diameter. In preparations in which the outer mem-
brane appeared distorted, this array was still visible;
however, it did not appear on the flagella (fig. 1B). The
insertion points of the flagella, visible in some prepar-
ations, measured 80 nm x 100 nm (fig. 1B). In only one
of the negatively stained preparations were paddle-like
structures seen at the end of the flagella. Coccoid
bacterial forms were observed in which the flagella
were located at one end (fig. 1C).

Sections of the bacterial cells showed the ultra-
structure typical of gram-negative organisms, i.e., an
outer membrane and a plasma membrane separated
by a periplasmic space with a total thickness of 30 nm
(fig. 2A). The flagella were sheathed with an external
diameter of 32-40 nm and an internal core diameter of
14-17 nm (fig. 2B). Ultra-thin sections showed that the
outer membrane of the cell wall was continuous with
the flagellar sheath (fig. 2A).

A unique structural feature was found in the H.
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Fig. 3. Thin sections of *H. mustelae* cultures showing (A) glycocalyx external to the outer membrane (bar, 0.5 μm); (B) and (C) paddle-like structures located very close to the cell membrane (bars, 0.1 μm and 0.25 μm, respectively).

**mustelae** preparations. An electron dense area or inclusion body, 80 nm x 100 nm, was observed (fig. 2C) adjacent to most of the flagellar insertion points and in some instances directly beneath them. Occasionally, flagella were seen penetrating the peptidoglycan layer and connecting with this electron-dense area (fig. 2D). This feature was found only in terminal, subterminal or lateral areas corresponding to the typical flagellar arrangement and was located in the cytoplasm below the plasma membrane wall.

Concave structures at lateral (fig. 2E) and terminal regions (fig. 2F) of the cell were seen in freeze fractures of *H. mustelae*. They were 30–42 nm in diameter, often with raised centres. Glycocalyx, 25 nm x 50 nm thick, external to the outer membrane was also evident (fig. 3A). In many instances paddles, oval (80 nm x 100 nm) and circular (110–120 nm in diameter) in shape, were observed (fig. 3B). An outer membrane was visible on these structures; however, it was impossible to determine the location of the paddles due to the procedures prior to electron microscopy. Often the paddles, or a thickening of the flagella, were observed within nanometers of the outer wall of the cell (figs. 3B and C).

**In-vivo preparations**

Examination of semi-thin sections (0.5 μm) of ferret gastric tissue showed bacteria localised within the gastric pits with little evidence of bacteria on the external surface or in the overlying mucus layer. Electron microscopy revealed a very close association of the bacteria with the epithelial cells (fig. 4). Bacteria were found alongside microvilli (fig. 5A), aligning themselves along the surface of the epithelium (fig. 5B), perpendicular to the surface of the epithelial cells and, in some instances, penetrating these cells (fig. 5C). Extensive loss of microvilli was seen and in such denuded areas, occasional adhesion pedestals were visible (fig. 5D). Some organisms were undergoing endocytosis (fig. 5E) and localised within membrane-bound inclusions inside the epithelial cells (fig. 5F).

Bacterial cells were also seen next to intercellular junctions; however, they did not appear to show a particular disposition for this site as has been reported for *H. pylori*. A fibrous-like matrix or glycocalyx was apparent between the epithelial and bacterial cells. This formed a very dense matrix when the two surfaces were very close together (fig. 5D), or showed more
Fig. 4. Thin section of a ferret antral gastric pit showing the location of the bacteria. Note a bacterium apparently moving towards the epithelium surface in a "cartwheel-like" fashion (■■■¿), close association of the bacteria with the epithelial surface including the formation of adhesion pedestals (⇒) and endocytosed bacteria (*) (bar, 1.0 μm).

fibrous strands, especially when the bacterial cells were aligning with microvilli (fig. 5A). On one occasion a bacterial cell with flagella in a "cartwheel"-like format (fig. 4) was observed in a gastric pit. The electron-dense areas near the flagellar insertion points, as described above were also visible on the bacteria in vivo.

Discussion

The mucus-associated epithelial surfaces of man and animals have been shown to contain large numbers of bacteria, many of which have a spiral or helical morphology which is thought to be advantageous in a viscous environment.17,18 The recent isolation of three different bacteria from this area1,9,16 has generated a new genus, Helicobacter.19,20 Their association with gastric disease resulted in extensive studies into the possible mechanisms of pathogenicity and the factors which may allow these organisms to colonise such a harsh environment. With the exception of H. mustelae, members of the Helicobacter genus and the proposed "Gastrospirillum hominis"21 (presently maintained only in vivo22) share characteristics in common with other mucosa-associated organisms, namely, a spiral or helical morphology and motility by means of polar or bipolar flagella.7,10,22 In contrast, H. mustelae is a small, curved rod with polar and lateral flagella.9

Although variations do occur, H. mustelae appears to have a uniform configuration of polar or sub-terminal and lateral flagella. This configuration is rare amongst bacterial species23 and we propose that this rare flagellar configuration compensates for their rod-shaped morphology and allows passage through a viscous environment, such as mucus. Indeed, examination of motile cultures under phase contrast microscopy reveals an unusual spinning type motility.
Fig. 5. Higher magnification of sections of ferret gastric tissue showing: (A) glycocalyx between bacteria and microvilli (bar, 0.25 μm); (B) bacterium aligning on the surface of an epithelial cell (bar, 0.5 μm); (C) bacteria penetrating into epithelial cells (bar, 0.5 μm); (D) formation of an adhesion pedestal (bar, 0.1 μm); (E) a bacterium undergoing endocytosis (bar, 0.25 μm); (F) bacteria located in membrane bound inclusions (bar, 0.25 μm).

This notion is supported by the studies of Greenberg and Canole-Parola in which they examined the motility of spiral-shaped and rod-shaped bacteria; they demonstrated that the spiral morphology of Spirillum spp. and spirochaetes allowed them greater mobility in viscous environments. We have confirmed such observations with Campylobacter jejuni and H. pylori. Greenberg and Canole-Parola selected an organism (strain PFR-3) that was able to move through agar. This bacterium was shown to have the same rare flagellar configuration of H. mustelae, i.e., lateral and polar flagella. Strain PFR-3 attained a maximum speed in excess of the spirochaetes and Spirillum spp. but less than that of E. coli which has peritrichous flagella. However, this bacterium had a minimum immobilising viscosity (MIV), i.e., a velocity that stops the translational movement of cells, far in excess of all the organisms studied, including E. coli.

The basic structure of flagella is well documented. Briefly, they consist of a filament and hook, connected to a basal body consisting of a series of disks located in the outer membrane and peptido-glycan layers (the L and P rings) and within and below the plasma membrane (the S and M rings). Electron-dense areas or “blebs” have been observed in Aquaspirillum spp. and Spirillum spp. in the cytoplasmic side of the plasma membrane and it has been suggested that these are associated with the S and M rings of the flagellar complex. Another feature commonly observed in bacteria with polar flagella, including H. pylori, is the so-called polar membrane. The precise function of this membrane is not known,
though it has been suggested that it may form part of the basal apparatus of flagella or it may be involved in energy production for movement or cell-wall synthesis. This membrane was not seen in *H. mustelae*. The very large electron-dense areas or inclusion bodies, consistently seen in *H. mustelae*, may perform similar functions. This feature has not been observed in *H. pylori* or *H. felis*. The size and nature of this structure, as seen in *H. mustelae*, could reflect a heavy duty flagellar anchor necessary for the type of rotation exhibited by this organism.

The dimensions of the flagella of *H. mustelae*, their insertion points and the surface array correlate with results reported for *H. pylori* and *H. felis*. The regular array of circular structures (8 nm in diameter) associated with the outer membrane of the cell and present when the outer membrane was distorted or showing "blebbing", could be an S layer, i.e., a regular array of glycoproteins external to the outer membrane surface.

Freeze fracture preparations revealed the existence of concave structures at sites associated with flagellar insertions (figs. 2E and 2F) and these resemble the so-called "Coulton-Murray" studs seen in *Aquaspirillum* spp. Similar structures have also been found associated with the flagella of *H. felis*. In both instances the diameter of these structures corresponds with the diameter of the flagella, which supports the concept of Coulton and Murray that these "studs" are associated with the basal organelle of the flagella. It has recently been reported that similar structures now seen in *E. coli* were absent in mutants which lacked genes responsible for flagellar function.

The paddles reported on the flagella of *H. pylori* and *H. mustelae* were rarely seen in our negatively stained preparations; however, in the thin sections of bacterial cultures, these structures were observed more frequently (fig. 3). The exact location of the paddles with respect to the ends of the flagella could not be determined due to the nature of this preparation. Two differently shaped paddles were observed, circular and oval, but neither approach the size of the large paddles reported in *H. pylori* by Steer. In addition, we commonly found similar structures or thickening of flagella quite close to the cell wall and associated with the broken end of a flagellum. The paddles may not be restricted to the terminal end of a flagellum, but may result from physical changes to the flagella if they are broken or fractured or they may be an artefact of electronmicroscopy. Indeed, the regularity with which we noticed such paddle-like structures in sections of *H. mustelae* and the variation in the size of the paddles reported in the literature would support this latter premise. The inclusion of ruthenium red in the initial fixation confirmed the presence of a glycochalyx external to the outer membrane, as has been previously reported.

A detailed examination of the association of *H. mustelae* with ferret gastric tissue at an ultrastructure level has not yet been reported. We stated previously that *H. mustelae* adheres and partly penetrates epithelial cells with microvilli surrounding the bacteria. These observations can now be expanded revealing similarities and differences between the association of *H. mustelae* with ferret gastric tissue and *H. pylori* with human gastric tissue. In the present study, *H. mustelae* was observed aligned along microvilli connected by fibrous strands of glycochalyx (fig. 4). In some instances, the association between the bacterium and the epithelium surface was more direct, occasionally resulting in the formation of pedestals (fig. 4). It has been postulated that pedestals are an extrusion of the cytoplasm resulting from damage to the plasma membrane by toxin produced by the bacteria. However, whereas *H. pylori* is known to possess cytotoxic activity, no *H. mustelae* cytoxin has been detected.

*H. pylori* has been found only rarely intracellularly and then in association with ulcers. The majority of *H. pylori* are found in the overlying mucus with 3-19% of bacteria in biopsies estimated to be adherent to epithelial cells. The proportion of adherent cells is the most striking difference observed between *H. pylori* and *H. mustelae* in tissue specimens. *H. mustelae* is almost exclusively found in association with the epithelial surface, with very few bacteria in the overlying mucus. Furthermore, in ferret tissue, the bacteria are observed in membrane-bound inclusions. Evans et al. have shown that *H. pylori* can be internalised in vitro and they have proposed that receptor-mediated endocytosis occurs. Endocytosis is involved in the pathogenicity of many bacteria but its role in the pathogenesis of infection with *Helicobacter* spp. needs further study.

Demonstration of what appears to be specific adhesion and endocytosis of *H. mustelae* in the gastric mucosa is important, since it will provide a convenient animal model to assess the role of adhesion in pathogenesis. Significantly, our studies with *H. felis* and "*G. hominis*" have shown that these bacteria do not adhere to gastric surfaces yet they are capable of inducing gastritis, which suggests that adhesion may not be essential for gastric colonisation or induction of gastric inflammation. However, *H. pylori* and *H. mustelae* are the only gastric bacteria shown to adhere to the surface of gastric mucosa and are the only bacteria shown to be associated with peptic ulceration; adhesion may be important in ulceration.

In other studies we have confirmed the presence of adhesins in *H. pylori* and *H. mustelae* but not in *H. felis*. Various isolates of *H. pylori* and *H. mustelae* agglutinated red blood cells from man and animals but no such activity was observed in the four isolates of *H. felis* tested.

The more we understand the association of *Helicobacter* spp. with the gastric surface, the more easily we will be able to devise appropriate prophylactic and therapeutic strategies for the prevention of *H. pylori*-induced gastroduodenal disease.
References


