The relationship between *Helicobacter pylori* motility, morphology and phase of growth: implications for gastric colonization and pathology

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To explore the relationship between *Helicobacter pylori* motility, morphology and phase of growth, bacteria were isolated from antral biopsies of patients with duodenal ulcer or non-ulcer dyspepsia, and grown in liquid medium in batch and continuous culture systems. Motilities and morphologies of *H. pylori* in different phases of growth were examined with a Hobson BackTracker and by transmission electron microscopy. Morphologies of bacteria grown *in vitro* were also compared with those of bacteria in antral biopsies from patients with non-autoimmune gastritis. *H. pylori* had poor motility in lag phase, became highly motile in mid-exponential phase and lost motility in the decline phase of growth. Motilities of bacteria in the same phase of growth from patients with duodenal ulcer or non-ulcer dyspepsia were not significantly different. In the mid/late-exponential phase of growth bacteria had helical morphologies and multiple polar flagella, typical of *H. pylori* in the gastric mucus layer. In the decline phase of growth bacteria shed flagella, and had precocoidal or coccoidal morphologies. These findings support the view that helical and coccoidal *H. pylori* are in different phases of growth with different roles in gastric colonization, indicate that bacterial motility per se is unlikely to be a determinant of *H. pylori* pathology, and suggest that *H. pylori* in the antral mucus layer is in a state of continuous (exponential phase) growth.

Keywords: *Helicobacter pylori*, bacterial motility, bacterial morphology, growth phase

INTRODUCTION

Helicobacter pylori is a Gram-negative mucophilic bacterium that colonizes gastric mucosa and damages epithelial cells by association and cytotoxin release (Hessey *et al.*, 1990). As a consequence it is the principal cause of non-autoimmune gastritis and peptic ulcer, and an aetiological factor in gastric carcinoma (Baron, 1993; O'Connor *et al.*, 1996; Nomura *et al.*, 1994).

Bacterial motility is generally held to be a requirement for *H. pylori* colonization of the stomach (Eaton *et al.*, 1989, 1992). Consistent with this view, the bacteria *in vivo* typically possess helical morphologies and multiple polar flagella (Hazell *et al.*, 1986). There have, however,

been reports of apparently non-motile *H. pylori* with U-shaped, doughnut-shaped (precocoidal; Benaissa *et al.*, 1996) and coccoidal (Buck *et al.*, 1986; Chan *et al.*, 1994; Janas *et al.*, 1995; Megraud, 1989; Moshkowitz *et al.*, 1994; Noach *et al.*, 1994) morphologies. *H. pylori* with these atypical structures is seen occasionally *in vivo* (Caselli *et al.*, 1993; Chan *et al.*, 1994; Janas *et al.*, 1995; Noach *et al.*, 1994), but most frequently *in vitro* in old cultures (Andersen *et al.*, 1997; Catrenich & Makin, 1991; Cellini *et al.*, 1994; Jones & Curry, 1990; Kusters *et al.*, 1997; Mai *et al.*, 1989; Moshkowitz *et al.*, 1994; Nilius *et al.*, 1993). It remains uncertain whether coccoidal forms are degenerative (Buck *et al.*, 1986; Catrenich & Makin, 1991; Cellini *et al.*, 1994; Kusters *et al.*, 1997; Nilius *et al.*, 1993), or adaptations to marginal or hostile environments (Janas *et al.*, 1995), with a capacity for transmission and regrowth to the bacillary form (Andersen *et al.*, 1997; Benaissa *et al.*,

Abbreviations: CLV, curvilinear velocity; PL, path length.

1996; Bode *et al.*, 1991; Jones & Curry, 1990; Mai *et al.*, 1989).

In this paper we have examined how *H. pylori* motility is affected by bacterial morphology and phase of growth, with the aim of further clarifying the interrelationship between bacillary and coccoidal forms of the bacterium, and the possible role of motility in gastric colonization and pathology.

METHODS

Bacteria. Antral biopsies were obtained at endoscopy from 13 patients with duodenal ulcer and 14 patients with non-ulcer dyspepsia. *H. pylori* was isolated by plating the biopsies on Columbia agar (BBL) with 7% horse blood (TSCL) made selective by adding 20 mg nalidixic acid l⁻¹, 2 mg amphotericin B l⁻¹ and 3 mg vancomycin l⁻¹. The plates were then incubated at 37 °C for 72 h in a microaerophilic atmosphere generated in a GasPak jar (Oxoid) using a CampyPaks (BBL). Bacterial colonies were finally removed from the plates and stored in 0.5 ml 15% (v/v) sterile glycerol broth at -80 °C until required.

Batch culture. Bacteria were thawed at room temperature, plated out on 7% (v/v) defibrinated horse blood agar, which included *H. pylori* selective supplement (Oxoid), and incubated in a microaerophilic atmosphere (see above) at 37 °C for 48 h. Colonies from these plates were suspended in sterile saline to a turbidity equivalent to that of McFarland's No. 4 standard (10⁸ bacteria ml⁻¹). From this suspension, 100 µl was transferred to 2.9 ml brain heart infusion broth (BBL) supplemented with 10% (v/v) newborn calf serum (Sigma) and *H. pylori* selective supplement. The broth was then incubated with agitation in a 50 ml capacity loose-capped container (Bibby Sterilin) in a microaerophilic atmosphere (10% CO₂, 18% O₂ and 72% N₂) at 37 °C for at least 72 h. Aliquots (totalling 200 µl) were withdrawn periodically for assessment of bacterial motility, and viable count by the method of Miles and Misra as modified by Miles *et al.* (1938). Gram stain, oxidase and catalase tests were used to confirm absence of contamination.

Continuous culture. Bacteria were grown under conditions comparable to those reported by Hudson & Newell (1989). The chemostat apparatus used was a series 00II modular fermenter (LH Fermenters), fitted with 1 l capacity glass culture vessel and autoclavable plate and fittings of nylon and silicon rubber. The top plate had ports for redox and pH electrodes, a thermocouple, and gas, growth medium and sampling lines.

Growth medium (broth pH 7; made *H. pylori*-selective as described above) was introduced into the culture vessel by continuous pumping through anti-growback glass tubing, and the volume of fluid was maintained at 600 ml by an overflow tube. The growth medium was maintained at 37 °C by external heating, stirred at 150–200 r.p.m. and sparged with a 5% O₂, 10% CO₂ and 85% N₂ gas mixture.

The culture vessel was inoculated with 30 ml *H. pylori* (in exponential phase of growth) in selective broth at a concentration of 10⁸ bacteria ml⁻¹. When bacteria had attained exponential growth, fresh medium was pumped into the culture vessel at a dilution rate of 0.05 culture volumes h⁻¹ ($D = 0.12 \text{ h}^{-1}$) and growth was monitored for 14 d thereafter by on-line measurements of OD₅₅₀ with a Perkin Elmer spectro-

photometer against a blank of fresh medium. Aliquots (5 ml) were removed at 6 hourly intervals for assessment of bacterial motility.

Motility measurements. Aliquots (10 µl) of the culture broth (prediluted when necessary with fresh medium to a concentration not exceeding 10⁷ bacteria ml⁻¹) were added to saline (90 µl), and the resulting bacterial suspension (pH 7) was drawn by capillary action into an optical microslide (Camlab) of 0.1 mm path length. The microslide was sealed at one end with vinyl plastic putty (Oxford; Labware), transferred to the warm stage (37 °C) of a phase-contrast microscope at × 40 magnification, and allowed to equilibrate (5 min) before observations were commenced with a Hobson BacTracker. To ascertain the contribution of Brownian movement to bacterial motility, *H. pylori* was killed by exposure to 10% (v/v) formalin for 10 min at room temperature, before examination with the Hobson BacTracker.

The Hobson BacTracker, a new 'blob and track' image-processing technology developed by Hobson Tracking Systems, Sheffield, UK, permits bacterial motility to be quantitatively measured in real time. In this system the microscopic image of the bacteria is recorded by video camera, and displayed on the tracker screen. Movements of up to 120 bacteria may be simultaneously and continuously monitored, with results reported in either histogram or trail draw formats. The Hobson system has the potential to examine 10 parameters of bacterial motility. Two representative parameters were used to assess the motilities of *H. pylori* in this study: curvilinear velocity (CLV; the distance in µm travelled along the path of the bacterium in each second between two stops) and path length (PL; the distance in µm travelled by the bacterium between two stops). Data collected by the BacTracker were analysed by the Mann-Whitney U-test with an SPSS (Social Sciences Version 6) statistics package. A detailed description of the Hobson BacTracker and its operation may be found in the manufacturer's literature.

Transmission electron microscopy

Bacteria in culture. *H. pylori* in lag, exponential and decline phases of growth was prepared for negative contrast transmission electron microscopy as described by Haschemyer & Meyers (1972). Bacteria were transferred to a 400-mesh carbon-coated copper grid by floating the grid, coated side down, on a drop of *H. pylori* culture fluid for about 2 min. Excess liquid was removed by touching the grid with a filter paper, and bacteria were then negatively stained by floating the grid on a drop of 2% (w/v) phosphotungstic acid solution (pH 6.5) for 1 min. Excess liquid was again removed by touching the grid with a filter paper, and the bacterial preparation was examined with a Philips EM 400 transmission electron microscope.

Bacteria in antral biopsies. Antral biopsies from patients with *H. pylori*-associated non-autoimmune gastritis and functional dyspepsia were fixed for 2 h in 4% (w/v) glutaraldehyde. The tissue was then post-fixed in osmium tetroxide, stained with uranyl acetate, dehydrated through an ethyl alcohol sequence until the alcohol content reached 100% and embedded in Taab 812 resin. Sections (0.5 µm) were initially cut for explorative light microscopy. Ultra-thin sections (approx. 100 nm) were then cut with a diamond knife from relevant blocks, collected on copper grids and stained with Reynolds lead stain, before examination with a Philips EM 400 transmission electron microscope.

RESULTS

Growth of *H. pylori* in batch culture

Preliminary experiments (data not included) conducted with residual biopsies revealed that they provide very low inocula, so that broth cultures could take between 6 and 9 d to attain exponential phase of growth. As a result, suspensions from agar plates containing 10^7 – 10^8 bacteria ml^{-1} were used to inoculate batch cultures in the present study. Growth curves were highly reproducible with these consistent and heavy inocula, with bacteria entering the exponential phase of growth within 24 h and stationary phase 1 d later. The mean results for four different isolates are given in Fig. 1(a). Our findings are compatible with those reported by Xia *et al.* (1993).

Fig. 1. Influence of growth phase on motility of *H. pylori* in batch (a) and continuous (b) culture systems. The curves represent the combined data from four (batch culture) and three (continuous culture) isolates. Data are from bacteria in lag phase (A), exponential phase (B), stationary phase (C) and decline phase (D) of growth. Also shown are bacteria in the exponential phase of growth before fresh medium was allowed to flow into the culture vessel (E), and under continuous culture conditions (F) at a dilution rate of 0.05 culture volumes h^{-1} . Bacterial motility (○) is expressed as CLV ($\mu\text{m s}^{-1}$), and bacterial density (●) in c.f.u. ml^{-1} , or as OD₅₅₀.

Fig. 2. CLV and PL of *H. pylori* in batch culture in different phases of growth: (1) about 10 h after inoculation (lag phase); (2) about 15 h after bacteria entered exponential phase (mid/late-exponential phase); (3) about 20 h after bacteria entered stationary phase (late-decline phase). Bacteria in mid/late-exponential phase of growth had significantly ($P < 0.001$) greater CLV and PL than bacteria in lag or decline phases of growth. Motilities of *H. pylori* isolated from patients with duodenal ulcer (⊗) and non-ulcer dyspepsia (○) were not significantly different. *, Brownian movement.

Growth of *H. pylori* in continuous culture

Experiments were initially conducted to establish a dilution rate that would maintain the bacteria in a state of continuous growth. Three different isolates of *H. pylori* were then examined in the continuous culture system for a period of 14 d. The mean results are given in Fig. 1(b). After inoculation into the chemostat vessel *H. pylori* remained in the lag phase of growth for a mean of 14 h. Thereafter, the optical density of the culture increased exponentially, as bacteria entered exponential phase of growth, to attain a maximum (OD₅₅₀ 0.48) within 35 h. The optical density decreased transitorily (to OD₅₅₀ 0.44) when fresh medium was initially allowed to flow into the culture vessel, then recovered, and remained at a high level (OD₅₅₀ 0.48 $\pm$ 0.05) for the duration of the experiment. The pH of the growth medium remained unchanged throughout the experiment. The dilution rate used in our growth experiments (0.05 culture volume h^{-1}) compared favourably with dilution rates (0.05–0.2 culture volume h^{-1}) reported by Hudson & Newell (1989) for optimal chemostat growth.

Fig. 3. Electron micrographs of negatively stained preparations of *H. pylori* in lag and exponential phases of growth, illustrating non-dividing bacteria with helical forms and multiple polar flagella, and (inset; top) a bacterium in division with clearly visible division septum and bunches of flagellar filaments at each pole. Bars, 500 nm.

of *H. pylori* in plasma-supplemented brucella broth (pH 7) at 37 °C.

Motilities of *H. pylori* in different phases of growth

Two parameters were used to quantify the (translational) motions of the bacteria: CLV and PL.

The change in mean *H. pylori* motility throughout the growth cycle in batch culture is shown in Fig. 1(a). The curve demonstrates that CLV is closely linked with bacterial growth in lag and exponential phases of growth, then decreases sharply shortly after bacteria enter the stationary phase of growth.

Translational motions of *H. pylori* from a larger number of isolates were examined at selected points on batch

growth curves, to ascertain whether motilities of bacteria from patients with duodenal ulcer and non-ulcer dyspepsia were significantly different. Results are summarized in Fig. 2. All the bacteria were essentially non-motile in the early-lag phase of growth (mean CLV was 7.0 , SD $5.7 \mu\text{m s}^{-1}$; mean PL was $3.8 \pm 3.1 \mu\text{m}$), became highly motile in late-exponential and early-stationary phases of growth (mean CLV was $28.7 \pm 5.6 \mu\text{m s}^{-1}$; mean PL was $9.9 \pm 1.8 \mu\text{m}$), and lost motility in the late-decline phase of growth (mean CLV was $5.0 \pm 1.0 \mu\text{m s}^{-1}$; mean PL was $3.1 \pm 0.3 \mu\text{m}$).

H. pylori was essentially non-motile in the initial lag phase of growth (mean CLV was $4.5 \pm 1 \mu\text{m s}^{-1}$ and mean PL was $1.4 \pm 0.4 \mu\text{m}$ 12 h after inoculation) in the continuous culture system (Fig. 1b). Motility then increased exponentially as the bacteria entered

Fig. 4. Electron micrographs of negatively stained preparations of *H. pylori* in the decline phase of growth, illustrating early (top left) and late (top right) stages in the genesis of coccoidal forms (bottom) of the bacterium. Precoccal forms (top left; top right) typically carry flagellar filaments on a single pole, which are lost from coccoidal forms. Bars, 500 nm.

the exponential phase of growth (mean CLV was $32 \pm 4.1 \mu\text{m s}^{-1}$ and mean PL was $14 \pm 4.3 \mu\text{m}$ 36 h after inoculation), and remained at a high level throughout the duration of the experiment. The highest motility attained by *H. pylori* in continuous culture was comparable to that attained by the bacterium in batch culture when in late-exponential and early-stationary phases of growth.

Morphologies of *H. pylori* in different phases of growth

Morphologies of *H. pylori* in lag and exponential phases of growth are shown in Fig. 3, and in the decline phase of growth in Fig. 4. Bacteria in lag and exponential phases of growth were morphologically indistinguishable. The populations were composed predominantly of

non-dividing bacilli (about 85% in lag phase, decreasing to about 60% in early-exponential phase, as judged from a small number of photomicrographs), with slight or pronounced helical morphologies and multiple flagella at a single pole, and dividing forms. The latter were distinguishable from morphologically similar U-shaped (precoccal) bacteria in the decline phase by the presence of a division septum and flagellar filaments at each pole. In the early-decline phase of growth a high proportion of bacteria remained flagellate and had precoccal forms, whereas in the late-decline phase almost all bacteria were fully coccoidal, and usually devoid of flagella. The precoccal and coccoidal forms of *H. pylori* did not seem to be degenerative: both the bacterial cells and flagella, for instance, showed no obvious signs of the disintegration that occurs on exposure to bismuth and antibiotics (Armstrong *et al.*, 1987; Nilius *et al.*, 1993).

Fig. 5. Electron micrographs of the luminal surface of the gastric antrum in patients with non-autoimmune gastritis. *H. pylori* with helical morphology is located in the mucus layer close to the epithelial surface. Bars, 166 nm.

Morphologies of *H. pylori* in antral biopsies

Morphologies of *H. pylori* in antral mucosa under the transmission electron microscope are shown in Fig. 5. Non-dividing bacteria with helical morphologies predominated, and were located most frequently within the mucus layer close to the epithelial surface.

DISCUSSION

In this study we explored the relationship between *H. pylori* motility, morphology and phase of growth in batch and continuous culture systems, with the aim of further clarifying how bacterial motility and the

different morphological forms of *H. pylori* influence gastric colonization and pathology.

All the *H. pylori* isolates investigated were highly motile when in exponential and stationary phases of growth, consistent with motility being a colonization factor for the bacterium. Motilities of *H. pylori* from patients with duodenal ulcer or non-ulcer dyspepsia were not found to be significantly different, however, when bacteria were in the same phase of growth, nor could the *H. pylori* isolates investigated, irrespective of source, be divided into two populations with significantly different motilities when in the same phase of growth. These latter findings are consistent with those from an earlier study (Worku *et al.*, 1999), in which we showed that the

motilities of *H. pylori* (in exponential phase) from patients with duodenal ulcer or non-ulcer dyspepsia were not significantly different in viscous media, at viscosities equivalent to those within the gastric mucus layer. Collectively these are potentially important observations, since they argue that an inherent difference in motility between strains is unlikely to be the determinant of *H. pylori* pathology or, for instance, the greater density of epithelial colonization in the antrum of patients with duodenal ulcer, when compared to those with non-ulcer dyspepsia (Khulusi *et al.*, 1995).

H. pylori was found to exhibit highest motility when in late-exponential and early-stationary phases of growth. This observation mirrored our experience with other mucophiles (*Campylobacter jejuni*, *Escherichia coli* and *Pseudomonas aeruginosa*; unpublished observations), and accords with earlier work by other investigators (Kodaka *et al.*, 1982; Macnab, 1996). The onset of *H. pylori* motility coincided with the appearance in the cultures of large numbers of non-dividing bacilli having helical morphologies and multiple polar flagella. Subsequent loss of motility after the bacteria entered the decline phase of growth coincided with the loss of this distinctive morphology [presumably from autohydrolysis of carbohydrate and peptide bonds in the peptidoglycan network (Baker & Park, 1975; Thwaites & Mendelson, 1991; Van Heijenoort, 1996)], and the appearance of flagellate precocoidal, then aflagellate coccoidal forms.

Our data also confirm that the age of a batch culture can have a dramatic effect on flagellation, and support the view of Kodaka *et al.* (1982) and Josenhans *et al.* (1995a) that optimal flagellar development occurs when bacteria are in the exponential phase of growth. Loss of flagella from *H. pylori* in the late-decline phase of growth may be a further manifestation of the stress-induced autohydrolysis that releases surface proteins from the bacterial cell (Phadnis *et al.*, 1996).

We are uncertain of the reasons for the poor motility of *H. pylori* in late-lag and early-exponential phases of growth, particularly as non-dividing flagellated bacteria in lag and exponential growth phases were morphologically indistinguishable. However, (i) the strong relationship that exists between bacterial motility and flagellin expression (Josenhans *et al.*, 1995a) when *H. pylori* is in the exponential or stationary phase of growth (development of high bacterial motility in mid/late-exponential phase, for instance, coincides with a 10–20-fold increase in expression of FlaA and FlaB subunits), and (ii) the dependence of full *H. pylori* motility on the presence of both flagellin subunits (Josenhans *et al.*, 1995b) suggest that the cause is functional, and due to incomplete production of one or both flagellins. Such a deficiency, moreover, would not necessarily alter flagellar morphology (Josenhans *et al.*, 1995b).

Implications for gastric colonization

The interrelationship between bacterial motility, morphology and phase of growth revealed by our *in vitro*

experiments suggests that *H. pylori in vivo* with helical forms are likely to be highly motile and, therefore, suited to colonization of the gastric mucus layer. In contrast, the poorly motile precocoidal or coccoidal form of *H. pylori* ought to be quickly cleared from the mucus environment. If these latter morphological forms are viable, then this characteristic should better fit them for a role in transmission of the infection.

The present study also provides a useful insight into the likely state of a typical population of *H. pylori* within the antral mucus layer. Most of the *H. pylori* colonizing the gastric antrum are to be found within the mucus environment (Terada *et al.*, 1993; Thomsen *et al.*, 1990) and [as we and other investigators (Buck *et al.*, 1986; Hazell *et al.*, 1986; Megraud, 1989) have shown] invariably possess helical morphologies. This observation, in conjunction with results from our *in vitro* studies of bacterial motility and morphology, and the dependence of gastric colonization on bacterial motility (Eaton *et al.*, 1989, 1992), lead us to conclude that the typical *H. pylori* population within the antral mucus layer is in a state of continuous (exponential phase) growth.

To maintain a bacterial population in continuous growth the nutrient supply must at all times exceed that required by the bacteria (Duguid *et al.*, 1978). *H. pylori* in the human stomach should attain this theoretical condition because the bacteria inhabit the gastric mucous barrier, a microenvironment whose physiological properties (Sidebotham & Baron, 1994) will cause it to function in the manner of a chemostat. The nutrient supply to *H. pylori* within the mucous barrier is plasma, which transudes from damaged microcapillaries within the mucosa (Sidebotham *et al.*, 1995). Although the supply of plasma is finite, it should be possible to keep it in excess of requirement because effective clearance mechanisms constantly limit the size of the bacterial population. These mechanisms include the secretion of abnormally viscous mucus (Curt & Pringle, 1969; Markesich *et al.*, 1995), which entraps and removes bacteria from the epithelial surface, and mucus erosion and the 'washout' effect of acid secretion, which displace bacteria into the gastric lumen. The greater susceptibility of *H. pylori* cells with inherently poor motility to clearance from the mucous barrier by these mechanisms will also ensure that bacteria in the exponential phase of growth are preferentially retained within the antral mucus layer.

ACKNOWLEDGEMENTS

We wish to thank Abbott Laboratories and the University of London for their gifts of equipment used in this work. We are indebted to Suresh Ladva for technical expertise in transmission electron microscopy.

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Received 18 February 1999; revised 28 May 1999; accepted 1 June 1999.