

# Morphological transformation of *Helicobacter pylori* during prolonged incubation: Association with decreased acid resistance

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## Abstract

The survival of clinical isolates of *H pylori* at two cultural ages (two and four days) at pH 2, in the presence of different buffers, with and without urea, was investigated.

It was found that the morphological changes which occur with longer incubation of *H pylori* have an inverse correlation with its resistance to an acidic environment. The finding that the addition of urea almost reversed this phenomenon and prolonged survival of the cultures emphasises the role of urea in the survival of *H pylori* in acidic environments.

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*Helicobacter pylori* is one of the few organisms that can survive in the acidic environment of the stomach, despite being sensitive in vitro to such acidic conditions. It has been suggested that production of ammonia by the organism's high urease activity may create an alkaline microenvironment, which promotes its survival in acidic conditions. Marshall *et al* have shown that the presence of physiological concentrations of urea in vitro enhances the survival of this organism at low pH.<sup>1</sup> We have noted that the survival of *H pylori* in acidic buffers decreases as increasingly older cultures are examined, and that this phenomenon is paralleled by changes in bacterial cell morphology.

## Methods

*H pylori* suspensions of two and four day cultures were exposed to high acidity (pH 2) in the presence and absence of urea. Organism survival was investigated as a function of time and type of buffer used.

Clinical isolates from antral biopsy specimens were maintained at -70°C in tryptic soy broth, supplemented with 15% glycerine. Prior to each experiment, a fresh aliquot was thawed to 20°C and inoculated on to Columbia agar, containing 1% yeast extract and 7-8% blood (CYB). Cultures of the same origin were incubated for two or four days in a high humidity microaerophilic environment (Campy pak, BBL). Before use, cultures were tested for colonial and cellular

morphology, and the production of catalase, oxidase, and urease.

Bacterial suspensions of one isolate (20-10-4) were added to prewarmed buffers at pH 2 to produce a final bacterial concentration of 10<sup>7</sup> cfu/ml in one ml volume. The cell density of the original 0.9% saline suspension was adjusted to conform to McFarland standard number 3. Viable counts (cfu/ml) were then performed from each culture. Parallel experiments were conducted with or without urea (5.0 mM/l), in four buffers: Dulbecco phosphate buffer (DPB); citrate phosphate buffer (CPB); NaCl-HCl (HCS); and KCl-HCl (HCK).

Following two, five, 15 and 30 minutes' incubation in a 37°C water bath, 10-fold dilutions were prepared from each solution in prewarmed saline using an octoped pipette (Titertec) and 96 well microtitre plates. Ten microlitres were transferred from each well and incubated on CYB plates for seven days as described above. Assays were performed in triplicate and the data from each set were combined for the determination of the mean count. Statistical evaluation was performed using Student's *t* test.

A similar set of experiments was performed with an additional unrelated isolate (10-10-1).

## Results

Morphologically, the two day cultures showed a cellular morphology typical for *H pylori* (short bacilli, comma, and "S" shaped forms) (fig 1A). Four day cultures were more pleomorphic, and characterised by the appearance of elongated, hooked, ring-shaped and "U" forms (fig 1B).

The survival in an acidic environment of young (two day) cultures of *H pylori* exceeded that of older (four day) cultures (fig 2). In almost all the buffers there was no survival of the old cultures after 15 minutes of exposure to acid, whereas there was considerable survival of the young cultures, even after 30 minutes (*p* < 0.001). There was no significant difference in the survival of cultures in the various buffers. The survival of all cultures at pH 2 was enhanced by the presence of urea, although a difference of at least one log unit in survival was noticed between the two age groups.

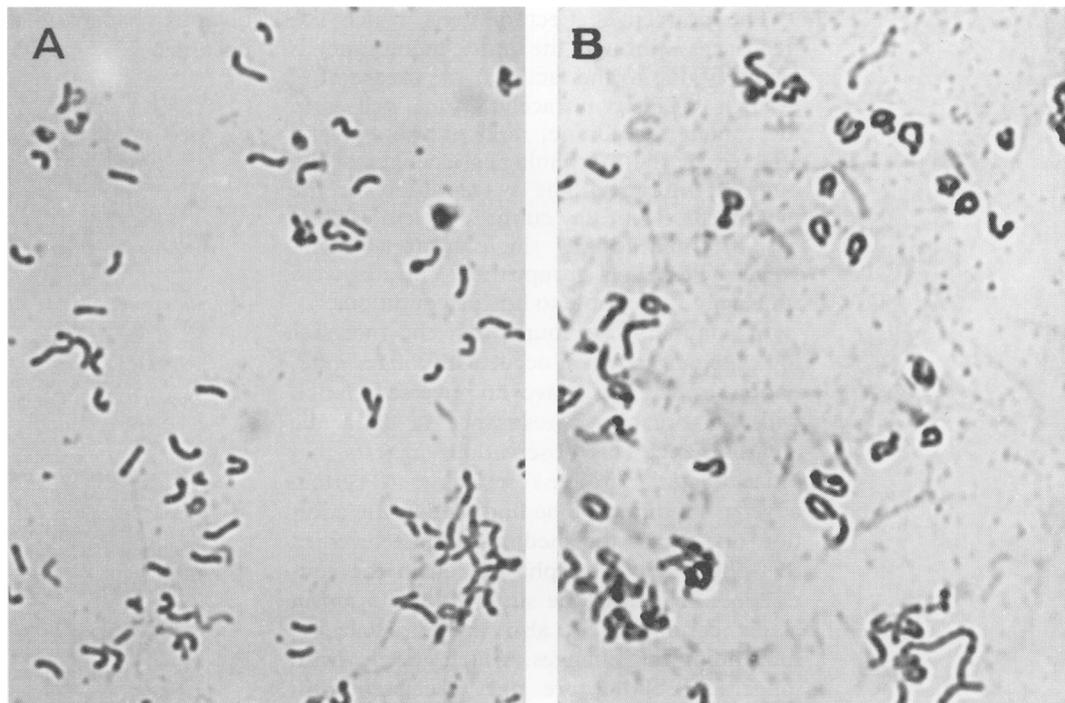
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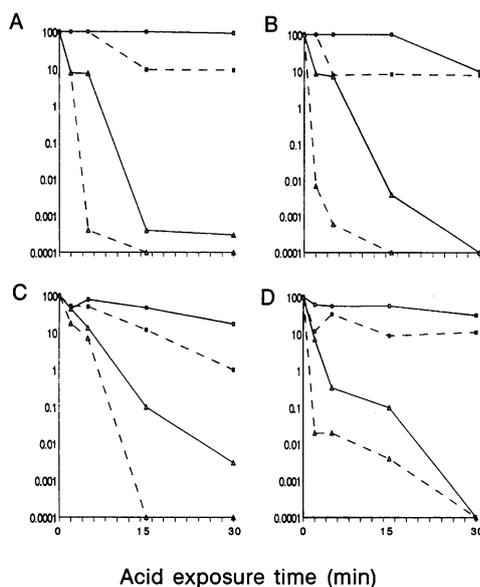
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**Figure 1** Morphological changes in *H pylori* of different cultural ages. (A) Two day cultures showing typical forms of *H pylori*. (B) Four day cultures showing pleomorphic appearance of the bacteria including ring-shaped and "U" forms.



**Figure 2** Survival rate of *H pylori* at pH 2 in different buffers: (A) Dulbecco phosphate buffer (DPB); (B) NaCl-HCl (HCS); (C) citrate-phosphate buffer (CPB); (D) KCl-HCl (HCK). Solid lines represent two day cultures and dotted lines represent four day cultures. Cultures performed with urea are shown as squares and those without urea as triangles. Mean cfu/ml at start was  $5 \times 10^7$  in all culture suspensions in panels A and B;  $4 \times 10^7$  in two day culture suspensions, and  $4 \times 10^6$  in four day culture suspensions in panels C and D.



Identical bacterial morphologies and acid resistance patterns at various conditions were observed in the set of experiments performed with the second bacterial isolate (10-10-1).

### Discussion

Despite the findings of some investigators that *H pylori* can switch from spiral to coccoid forms as seen on electron microscopy,<sup>2-5</sup> very few attempts have been made to study the effect of these changes on bacterial behaviour and resistance at various conditions. One of the most important questions concerning these morphological changes is whether they represent non-culturable but

potentially viable forms of *H pylori* capable of transmission or relapse of infection, or whether they are non-viable degenerative forms which pose no infection risk. Atypical morphological forms of some bacteria reflect a temporary adaptation to hostile environments or the use of antimicrobial agents.<sup>6</sup> It may be expected, therefore, that these atypical morphological forms of *H pylori* will be more resistant to acidic conditions than the normal bacillary forms. The age of a bacterial culture is also known to be a factor in its survival. In hostile environments young, actively multiplying bacteria are less resistant to bactericidal conditions than organisms which are beyond the logarithmic stage of growth.<sup>7</sup>

In the present study we found that young cultures of *H pylori* are more resistant to acid than older cultures. This behaviour was irrespective of the buffer used, but variations in bactericidal intensity and velocity were occasionally noted. The fact that this behaviour was observed in parallel to the morphological changes suggests a common mechanism. Both phenomena are probably a consequence of events taking place at the bacterial wall. The plasma membrane of micro-organisms is relatively impermeable to  $H^+$  and  $OH^-$  ions.<sup>8</sup> However, these ions have free access to the outer surface of the membrane, and may affect enzymes and proteins which are localised there. High concentrations of  $H^+$  have a bactericidal effect as a consequence of direct hydrolysis of enzymes and transport proteins.<sup>7</sup> A microbial cell wall already modified by previous insults may more readily permit  $H^+$  access to its targets. Bacterial cells in the four day cultures were probably more susceptible to such events as the rigid cell wall with its murein layer is the main factor in determining the morphology of the cell.<sup>8</sup>

The protective effect of urea, which has free access through the outer membrane, is probably due to the fact that the urease of *H pylori* is localised intracellularly as well as in the outer membrane, and is not greatly affected by the morphological changes of the outer membrane.<sup>9</sup> The susceptibility of the pleomorphic four day cultures to acid can not be attributed to any single bacterial form. Nevertheless, as a group, these cultures were unusually susceptible to hostile conditions.<sup>7</sup>

In summary, we found that the morphological changes which occur with longer incubation of *H pylori* have an inverse relation with the organism's resistance to an acidic environment. This observation suggests that older bacteria are less resistant to certain adverse conditions. The finding that the addition of urea to the medium almost reversed this phenomenon emphasises again the central role of urea in the survival of *H pylori* in acidic conditions. It also suggests that the morphological changes which occur with longer incubation are not associated with

obvious changes in the urease activity of the bacteria.

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## Bacteraemia caused by *Campylobacter* spp

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### Abstract

The genus *Campylobacter* has become increasingly recognised as the cause of various infections. *Campylobacter jejuni* and *C coli* cause acute gastroenteritis in man all over the world. *C jejuni* enteritis can lead to bacteraemia, but its actual incidence remains unknown.

Seven cases of bacteraemia caused by *C jejuni* or *C coli* are reported, from the blood of seven patients: five immune deficient adults; a newborn baby; and a patient who had had abdominal surgery. Patients who develop diarrhoea as a result of *Campylobacter* infection are at risk of bacteraemia thereafter.

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*Campylobacter jejuni* enteritis may lead to bacteraemia, but its actual incidence remains unknown.<sup>1</sup> A few cases of prolonged *C jejuni* bacteraemia have been noted in adult immune deficient patients.<sup>2</sup>

We have recently isolated *C jejuni* and *C coli* from the blood of seven patients between April 1991 and October 1992.

### Case reports

#### CASE 1

A 2 day old baby with jaundice caused by an isoimmunisation with the isoantibody anti-A had diarrhoea with mucus and blood. The clinical picture was of septicaemia, but examination yielded normal results. *C coli* was recovered from stool and blood cultures after 48 hours of incubation. Erythromycin (1000 mg orally a day) was given, and the patient's symptoms resolved.

#### CASE 2

A 41 year old man with a long history of alcohol related disease, including jaundice and other features of chronic liver disease, was admitted with diarrhoea and fever. Two of three blood cultures grew *C jejuni* after 72 hours of incubation. Fecal culture was also positive for this organism. Erythromycin, to which the bacterium was sensitive, was given and the patient was afebrile after 24 hours. Thereafter, he had only mild diarrhoea which finished on the fourth day.

#### CASE 3

A 33 year old man was admitted to hospital with abdominal pain, fever, and increased

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