

The coccoid forms of *Helicobacter pylori*. Criteria for their viability

G. BODE, F. MAUCH AND P. MALFERTHEINER

Department of Internal Medicine I, University of Ulm, Robert-Koch-Str. 8,
D-89070 Ulm, Germany

(Accepted 9 June 1993)

SUMMARY

The fact that *Helicobacter pylori* can revert to a coccoid form has stimulated speculation about its role in transmission and as a possible cause of reinfection in duodenal ulcer disease. Bismuth subcitrate (32 µg/ml), bismuth subsalicylate (64 µg/ml), amoxicillin (0.05 µg/ml) and erythromycin (4 µg/ml) inhibited the growth of *H. pylori* and stimulated the formation of basically respiring but non-culturable coccoid structures. The presence of polyphosphates as energy and phosphorus source permits a certain level of endogenous metabolism to preserve RNA and DNA, as well as structural components like cell wall, cell membrane and cytoplasm for at least 3 months. However, the applied standard laboratory methods were insufficient for regrowth of *H. pylori* out of the coccoid form.

INTRODUCTION

Possible sources and the mode of transmission of *Helicobacter pylori* are still not known. Foodborne or waterborne transmission is not unusual for an enteric pathogen and *in vitro* experiments have shown that *H. pylori* is able to survive in milk for several days [1] and in water for several months [2, 3]. However, *H. pylori* has not been isolated from the environment by culture techniques. A recent investigation suggests that *H. pylori* infection seems to be a waterborne disease in Peruvian children [4].

The conditions leading to the water contamination are not known but suggest the presence of *H. pylori* organisms in stool. It is difficult to culture *H. pylori* from human stool samples [5], but recently, Fox and colleagues [6, 7], were able to isolate *H. mustelae* from the faeces of infected ferrets.

In leaving its normal habitat in the stomach, *H. pylori* is exposed to physical and chemical stress. The response to the altered environment leads to a change in morphology, metabolism and growth behaviour: the viable but non-culturable stage [8]. This is a stage common to microorganisms that neither sporulate nor encyst but round up and lower their metabolic activity [9, 10].

One characteristic feature of duodenal ulcer disease is frequent relapse of the disease, often within several weeks. It is reasonable to suppose that not all *H. pylori* organisms are completely eradicated and may have survived in a coccoid form under the chemical stress of the therapy. After the apparently successful eradication, recurrence of infection occurs, usually by the same strain of *H. pylori*

as shown by restriction endonuclease RNA analysis or as have been recently shown by numerical analysis of 1D SDS-PAGE protein patterns [11].

In this study we demonstrate that the coccoid form of *H. pylori* shows evidence for basal respiration.

MATERIAL AND METHODS

Isolation and cultivation

Nine different clinical isolates from *H. pylori* positive patients were obtained from gastric tissue collected during endoscopy. Homogenized tissue was plated on Wilkins-Chalgren blood agar plates, supplemented using the method of Skirrow [12]. Cultures were incubated at 37 °C for 2–3 days under microaerophilic conditions. For subculturing, strains were transferred into liquid media containing Brucella broth (Difco Laboratories, Detroit, Michigan, USA) and 6% fetal calf serum and incubated for 2 days under microaerophilic conditions (5% O₂; 5% CO₂; 2% H₂; 88% N₂) on a rotary shaker.

Induction of coccoid forms

An inoculum of about 5×10^8 cells derived from the exponential growth phase, was exposed to 50 ml of culture medium containing the following final concentrations of the applied test substances: bismuth subcitrate (32 µg/ml), bismuth subsalicylate (64 µg/ml), amoxicillin (0.05 µg/ml) and erythromycin (4 µg/ml). Control experiments were done in parallel without the bactericidal substances. After 72 h bacterial cells were isolated and analysed immediately and after a 3 month storage in physiological saline under 4 °C.

Regrowth experiments were performed after 72 h exposure experiments and after 3 months storage, using either Wilkins-Chalgren blood agar plates or Brucella broth.

Conventional electron microscopy

Isolated bacteria were analysed using negative staining (2% (w/v) phosphotungstic acid, pH 7.4) according to Haschemeyer and Myers [13]. In addition, samples were fixed for 2 h in 2.5% (v/v) glutaraldehyde (cacodylate buffer 0.1 M, pH 7.4). After washing in buffer, samples were fixed in buffered 2% (w/v) OsO₄ solution for further 2 h. Samples were washed, dehydrated and block stained with uranyl acetate. After embedding in Epon 812, sections were additionally contrasted with lead citrate. Sections were analysed with a Philips EM 301 transmission electron microscope.

Element-specific electron microscopy

For electron spectroscopic imaging (ESI) and electron energy-loss spectroscopy (EELS), ultrathin sections (15–25 nm) were collected on uncoated 700 mesh grids from samples which had been fixed in cacodylate buffered 2.5% (v/v) glutaraldehyde and examined without further staining in a CEM 902 spectroscopic electron microscope (Carl Zeiss, Oberkochen, Germany) equipped with an image analysis system from Kontron (Munich, Germany).

The EELS spectra of poly P aggregates were recorded with an integrated prism-mirror spectrometer. A photomultiplier system (PMT) recorded the electron intensities. The analogue signals of the detector were digitized by a digital multimeter (Hameg Instruments) and fed into a computer in order to plot relative

intensities against corresponding energy losses. To reduce beam damage, areas for spectra recording were selected with a reduced beam current. Elements were identified analysing the specific energy losses of electrons which are scattered by the atoms of the investigated structures. ESI shows the local distribution and variations in concentration of a selected element using the specific energy loss of the electrons [14].

Detection of DNA synthesis in cocoid forms

In this study we used the uptake and incorporation of 5-bromode-oxyuridine (BrdU), a thymidine analogue, into newly synthesized DNA. Three months old cocoid forms of *H. pylori* were placed in liquid culture medium containing 10 μM BrdU (Sigma, Deisenhofen, Germany) for 18 h at 37 °C. After centrifugation, cocoid forms were fixed for 30 min in Karnovsky solution, rinsed in PBS, dehydrated and embedded in Epon 812. Incorporated BrdU into DNA was localized using a specific monoclonal anti-BrdU-antibody in combination with a streptavidin-biotin colloidal gold complex (Amersham International; Braunschweig, Germany). The immuno-staining was performed on sections of cocoid forms after removing the resin with a mixture of sodium methylate and benzol.

Control experiments were done without the addition of BrdU to the medium or without anti-BrdU-antibody.

RESULTS

It was possible to induce cocoid forms in all nine strains of *H. pylori* with the applied concentrations of the tested substances. A remarkable result is that all substances generated cocoid forms with a range of sizes after 72 h exposure.

The control experiments without the bactericidal substances consisted of differing amounts of cocoid forms (4–10%) after 72 h. After 3 months almost all spiral-shaped bacteria transformed into the cocoidal form. Regrowth of *H. pylori* failed after the 72 h incubation experiments with the test substances and after 3 months of storage.

Morphology and ultrastructure

The negative staining preparation in Fig. 1 (*b, c*) clearly shows that cocoids are endowed with a complete set of flagella providing them with probably almost the same motility as in the spiral form (Fig. 1 *a*). The cocoid forms varied in size from 0.8–1.0 μm . The exposure of *H. pylori* to amoxicillin produced a mini-form of cocoids with a mean diameter of 0.3 μm (Fig. 1 *c*).

Ultrastructural analysis of 3-month-old cocoids show completely intact ultrastructure, like cell wall, cell membrane and cytoplasm (arrowed in Fig. 2 *a*). However, the number of such intact cocoids had decreased remarkably. Even flagella are sometimes visible (Fig. 2 *a*). In addition, dark electron-dense aggregates were found within cytoplasm. The analysis of the electron dense aggregates by elemental specific electron microscopy (EELS) identified phosphorus as main components. Recently, we were able to identify such structures as polyphosphate granules [15]. Electron spectroscopic imaging with the specific electron energy loss of $E = 132$ eV shows the distribution of phosphorus within a sectioned cocoid form (Fig. 2 *b*). A heavy signal was found which corresponds to the electron dense structures shown in Fig. 2 *a*.

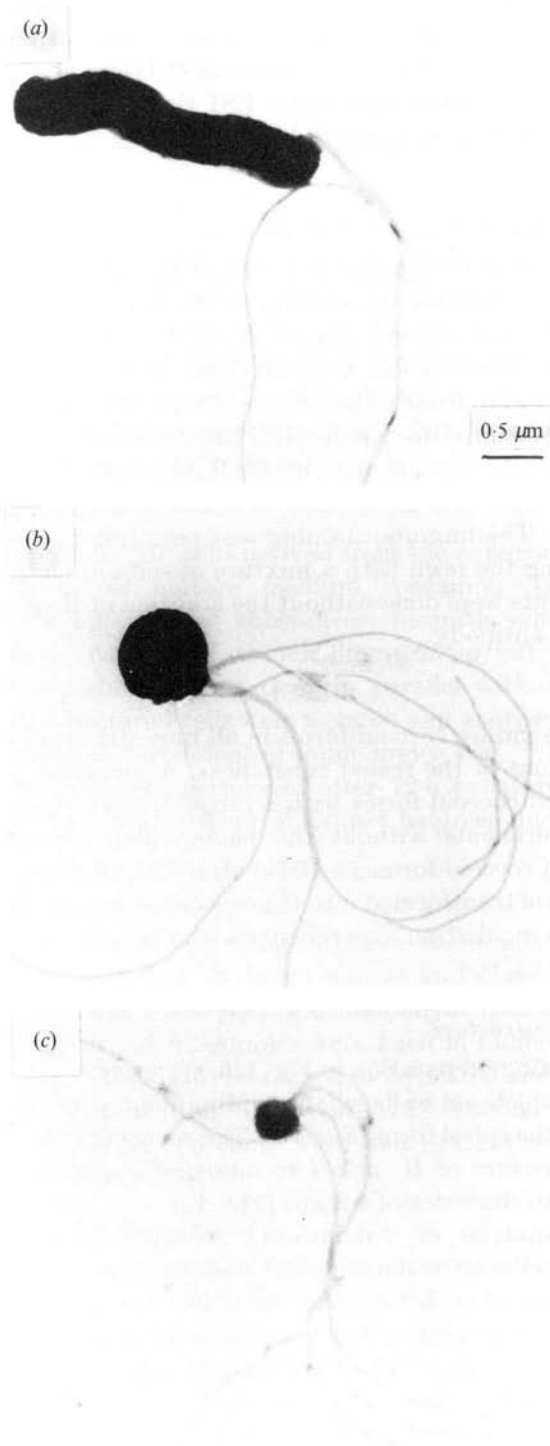


Fig. 1. Morphological appearance of cultured *H. pylori* (a) and induced coccoid form of the same strain (b, c). Negative staining, $\times 22000$.

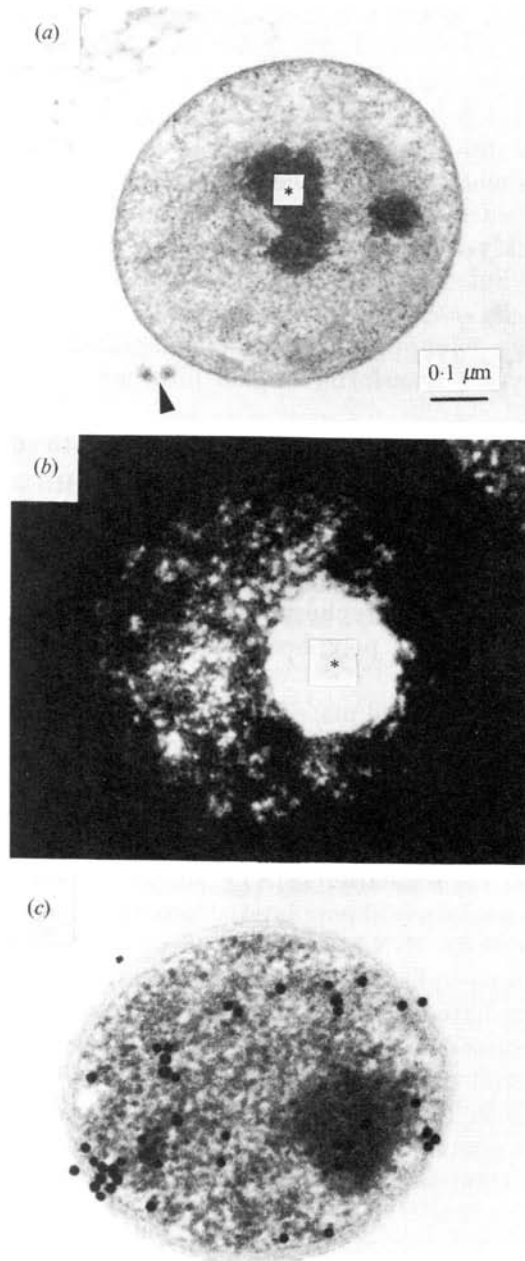


Fig. 2. Electron microscopy of sectioned cocoid forms, $\times 65000$. (a) Intact ultrastructure; (b) electron spectroscopic imaging (ESI) using the specific energy loss of phosphorus ($E = 132$ eV) for demonstrating polyphosphate; (c) immunohistochemical demonstration of BrdU incorporation into newly synthesized DNA segments.

Fig. 2c shows, that coccoids are able to synthesize DNA. This is shown by the incorporation of BrdU, a pyrimidine analogue of thymidine, into newly synthesized DNA. Colloidal gold particles are found within the cytoplasm of a sectioned 3-month-old cocoid form.

Control experiments showed non-specific background staining of single gold particles.

DISCUSSION

Since the first description of coccoid *H. pylori*, results have been controversial and only scanty information is available concerning their viability [2, 16–20].

Coccoid forms are generally regarded sceptically, however it is agreed that they are a possible potent vehicle for the transmission of disease [16, 19–22]. The existence of viable but non-culturable forms is known from other enteric pathogens as *Salmonella enteritidis*, *Escherichia coli*, *Vibrio cholerae*, *Campylobacter coli*, and *Campylobacter jejuni* [23–26]. It is demonstrated however, that after an animal passage, they are transformed again into culturable and virulent rods [26–27].

Our results show, that *H. pylori* is able to transform into coccoid forms during the exposure to antimicrobial agents.

Moreover, after 3 months the coccoid form of *H. pylori* is obviously able to maintain a certain metabolism which allow the preservation of important cellular structures like cytoplasm, cell membrane, flagella and DNA.

The demonstration of large polyphosphate aggregates in the coccoid form of *H. pylori* supports the idea that polyphosphate is essential in this maintenance metabolism.

Specialized reserves of storage material may exert a decisive influence on the survival patterns of microorganisms under unfavourable conditions. By supplying energy and phosphorus, polyphosphates may permit *H. pylori* to maintain its viability in the absence of nutrients. The availability of such storage material by *H. pylori* seems to retard the utilization of cellular constituents such as DNA and protein and to permit the regulation of ATP and nucleotides in the cell [28, 29].

The ability of a distinct coccoid population to synthesize small amounts of DNA is remarkable. Normally, DNA synthesis decreases rapidly upon adverse conditions. Thus, polyphosphates constitute an accessible reverse for the synthesis of metabolic intermediates which may facilitate the initiation of a possible regrowth from the coccoid stage.

If under antibacterial treatment, *H. pylori* transforms into a coccoid stage, it should be considered in future, that the *in vitro* susceptibility of *H. pylori* to antimicrobials is not equivalent to the *in vivo* eradication rate. Despite good *in vitro* activity, many compounds reach only subinhibitory concentrations within the mucus and crypts where *H. pylori* colonizes. Factors which may be responsible for insufficient drug concentrations are decreased activity at an acid pH concentration gradient within gastric mucus, inadequate tissue concentration, and inappropriate formulation [30].

It is reasonable to suppose that not all *H. pylori* organisms are completely eliminated and may escape the techniques usually applied for their detection. In biopsies, coccoid forms may be too small to be recognized by light microscopy, and standard laboratory methods are insufficient for regrowth of the coccoid form. After apparently successful eradication, recurrence of infection occurs usually by the same strain of *H. pylori* within few weeks as has been shown by restriction endonuclease DNA analysis and fingerprinting of DNA [11, 31]. The ultimate goal

of successful therapy must be long-term eradication of both the vegetative rod form and the cocoid survival form.

In conclusion, our results demonstrate, that *H. pylori* is able to convert under physical or chemical stress into a basally respiring but non-culturable cocoid state. It is remarkable that substances which are used in peptic ulcer treatment are able to induce coccoids *in vitro*. Thus, the cocoid form is suspected as being responsible for relapses in duodenal ulcer disease.

ACKNOWLEDGEMENT

We thank Mrs Christine Attenberger for the skilful assistance in preparing this manuscript.

REFERENCES

1. Karim QN, Maxwell RH. Survival of *Campylobacter pylori* in artificially contaminated milk. *J Clin Pathol* 1989; **42**: 778.
2. Mai UEH, Shahamat M, Colwell RR. Survival of *Helicobacter pylori* in the aquatic environment. In: Menge H, Gregor M, Tytgat GNJT, Marshall BJ, McNulty CAM, eds. *Helicobacter pylori* 1990. Berlin: Springer, 1991: 91–4.
3. West AP, Miller MR, Tompkins DS. Survival of *Helicobacter pylori* in water and saline. *J Clin Pathol* 1990; **43**: 609.
4. Klein PD, Graham DY, Gailow A, Opekun AR, O'Brian Smith E. Water source as risk factor for *Helicobacter pylori* infection in Peruvian children. *Lancet* 1991; **i**: 1503–6.
5. Thomas JE, Gibson GR, Darboe MK, Dale A, Weaver LT. Isolation of *Helicobacter pylori* from human faeces. *Lancet* 1992; **340**: 1194–5.
6. Fox JG, Paster BJ, Dewhirst FE, et al. *Helicobacter mustelae* isolation from feces of ferrets: evidence to support fecal–oral transmission of a gastric *Helicobacter*. *Infect Immun* 1992; **60**: 606–11.
7. Fox JG, Blanco MC, Yan L, et al. Role of gastric pH in isolation of *Helicobacter mustelae* from the feces of ferrets. *Gastroenterology* 1993; **104**: 86–92.
8. Kjellberg S, Hermansson M, Marden P. The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. *Rev Microbiol* 1987; **41**: 25–49.
9. Novitsky JA, Morita RJ. Possible strategy for the survival of marine bacteria under starvation conditions. *Mar Biol* 1978; **48**: 289–95.
10. Byrd JJ, Xu H-S, Colwell RR. Viable but nonculturable bacteria in drinking water. *Appl Environ Microbiol* 1991; **57**: 875–8.
11. Costas M, Morgan DD, Owen RJ, Morgan DR. Differentiation of strains of *Helicobacter* by numerical analysis of 1D SDS–PAGE protein patterns: evidence for posttreatment recrudescence. *Epidemiol Infect* 1991; **107**: 607–17.
12. Skirrow MB. *Campylobacter enteritis*: a 'new' disease. *BMJ* 1977; **2**: 9–11.
13. Haschemeyer RH, Myers RJ. Negative staining. In: Hayat MA, ed. *Principles and techniques of electron microscopy*, vol. 2. New York: Van Nostrand Reinhold Company, 1972: 99–147.
14. Bauer R. Electron spectroscopic imaging: an advanced technique for imaging and analysis in transmission electron microscopy. *Meth Microbiol* 1988; **20**: 113–46.
15. Bode G, Mauch F, Ditschuneit H, Malfertheiner P. Identification and characterization of polyphosphates in *Helicobacter pylori*. In press.
16. Jones DM, Curry A. The genesis of coccal forms of *Helicobacter pylori*. In: Malfertheiner P, Ditschuneit H, eds. *Helicobacter pylori*, gastritis and peptic ulcer. Berlin: Springer, 1990: 29–37.
17. Bode G, Malfertheiner P, Ditschuneit H. Cocoid forms of *Helicobacter pylori* are viable. *Ital J Gastroenterol* 1991; **23** (Suppl 2): 35–36.
18. Drumm B, Pérez-Pérez GI, Blaser MJ, Sherman PM. Intrafamilial clustering of *Helicobacter pylori* infection. *New Engl J Med* 1990; **322**: 359–63.

19. Lee A, Fox JG, Otto G, Hegedus-Dick E, Krakowka S. Transmission of *Helicobacter* spp. A challenge to the dogma of faecal-oral spread. *Epidemiol Infect* 1991; **107**: 99–109.
20. Koster T, Vandenbroucke JP. *Helicobacter pylori*, musings from the epidemiologic armchair. *Epidemiol Infect* 1992; **109**: 81–5.
21. Bode G, Malferttheiner P, Ströhle A, Mauch F, Nilius M, Ditschuneit H. Polymorphism in *Helicobacter pylori* – a key function for recurrent infection? *Med Klin* 1992; **87**: 179–84.
22. Ng LK, Sherburne R, Taylor D, Stiles ME. Morphological forms and viability of *Campylobacter* species studied by electron microscopy. *J Bacteriol* 1985; **164**: 338–43.
23. Roszak DB, Grimes DJ, Colwell RR. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Can J Microbiol* 1984; **30**: 334–8.
24. Rollins DM, Colwell RR. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl Environ Microbiol* 1986; **52**: 531–8.
25. Perez-Rosas N, Hazen TC. *In situ* survival of *Vibrio cholerae* and *Escherichia coli* in a tropical rain forest watershed. *Appl Environ Microbiol* 1989; **55**: 495–9.
26. Saha SK, Saha S, Sanyal SC. Recovery of injured *Campylobacter jejuni* cells after animal passage. *Appl Environ Microbiol* 1991; **57**: 3388–9.
27. McFeters GA, Singh A. Effects of aquatic environmental stress on enteric bacterial pathogens. *J Appl Bacteriol* 1991; **70**: 115–20.
28. Kulaev IS, Vagabov VM. Polyphosphate metabolism in micro-organisms. *Adv Microbiol Physiol* 1983; **24**: 83–169.
29. Morita RY. Bioavailability of energy and its relationship to growth and starvation survival in nature. *Can J Microbiol* 1988; **34**: 436–41.
30. Glupczynski Y, Burette A. Drug therapy for *Helicobacter pylori* infection: problems and pitfalls. *Am J Gastroenterol* 1990; **85**: 1545–51.
31. Owen RJ, Bell GD, Desai M, et al. Biotype and molecular fingerprints of metronidazole-resistant strains of *Helicobacter pylori* from antral gastric mucosa. *J Med Microbiol* 1993; **38**: 6–12.