

Ultrastructural observation of *Helicobacter pylori* in glucose-supplemented culture media

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Helicobacter pylori in the human gut can be divided morphologically into spiral and coccoid forms. The spiral form is known to change into the coccoid form in culture *in vitro*. The ultrastructural changes and culturability of *H. pylori* were studied in medium supplemented with different concentrations of glucose. *H. pylori* ATCC 43504^T was cultured in liquid medium containing 10% heat-inactivated horse serum supplemented with glucose (at 0, 10, 100, 300 and 500 mM) for 7 days. Bacterial ultrastructure and culturability were examined daily. With extended time in culture, the spiral forms had transformed into coccoid forms in all media. The coccoid forms could be further divided into two types, A and B, by electron microscopy. The type A coccoid form had an irregular surface with few flagella and indistinct cytoplasmic membrane. The type B coccoid form had a better-maintained integral membrane structure and was the dominant form in 300 mM glucose-supplemented medium. The highest culturability was obtained using 300 mM glucose-supplemented medium. Based on observations of ultrastructural changes in relation to the culturability data, the coccoid forms could be categorized into three stages: dying, viable but non-culturable and proliferating organisms. The optimal glucose concentration for *H. pylori* culture in this liquid medium culture experiment was approximately 300 mM.

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INTRODUCTION

Helicobacter pylori is a microaerophilic bacterium that gives rise to benign and malignant gastro-duodenal diseases (Buck *et al.*, 1986; Asaka *et al.*, 1994). It occurs in spiral and coccoid forms in the human gut (Chan *et al.*, 1994; Hua & Ho, 1996). In liquid culture, it exists in the spiral form and transforms into the coccoid form under certain conditions (Catrenich & Makin, 1991). There is controversy over the existence of the coccoid form. Reports on its ultrastructure and its viability in culture are vague; one report suggested that it might be the morphological manifestation of bacterial cell death (Kusters *et al.*, 1997), and another suggested that it represents a viable form that failed to proliferate in culture (Benaissa *et al.*, 1996). In a previous study by one of the authors (Sato, 2000), it was suggested that coccoid forms might be divided into two types, one with a rough surface and irregular intracytoplasmic density and the other with a smooth spherical surface and well-developed flagella tightly encircling the cell body. From the complete genome sequence of *H. pylori*, it was apparent that glucose is both the only energy source for ATP production and also the major carbon source for this organism (Tomb *et al.*, 1997). Although glucose-supplemen-

ted liquid media have been widely used for the culture of *H. pylori*, there had been no report on the relationship between glucose concentration in the culture medium and changes in ultrastructure or the ability to proliferate in culture, i.e. the culturability. The effects of glucose supplementation on the ultrastructure of *H. pylori* and its ability to proliferate in culture are reported in this paper using a liquid culture method.

METHODS

Bacterial culture. *H. pylori* ATCC 43504^T was cultured in Brucella broth (Difco) with 10% heat-inactivated horse serum (referred to as CLM) and in the same medium supplemented with 10, 100, 300 or 500 mM glucose (referred to as 10 mM-LM etc.) under microaerophilic conditions (10% CO₂, 5% O₂, 85% N₂) with agitation at 37 °C for 7 days. The reason for addition of 300 or 500 mM glucose is as follows. In preliminary experiments, we examined morphological changes and culturability in 10 mM-LM and 100 mM-LM in comparison with those in CLM. By the third day of culture, the spiral form made up less than 1% of the total population in CLM, 1% in 10 mM-LM and 5% in 100 mM-LM. The culturability was 5×10^7 , 8×10^7 and 2×10^9 c.f.u. ml⁻¹, respectively. This suggested that glucose supplementation aided morphological preservation and culturability. Therefore, morphological preservation and culturability were tested using medium supplemented with different concentrations of glucose.

Abbreviation: CLM, control liquid medium.

For counting of *H. pylori* cells, the bacteria were serially diluted (1 : 10) in 0.01 M PBS (pH 7.2) and plated onto Columbia agar medium (BBL). The plates were then incubated at 37 °C for 7 days under microaerobic conditions. The number of colonies was counted on day 7 to determine the rate of bacterial proliferation. This procedure was repeated five times as mentioned above.

Preparation for electron microscopy. On each day of culture, organisms in liquid medium were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 min and post-fixed in 2% osmium tetroxide for 30 min at 4 °C. After dehydration in a graded series of ethanol, the specimens were divided in half, half for SEM and half for TEM. For SEM, dehydrated specimens were soaked in isoamyl acetate, critical-point-dried (Hitachi HCP-2) and coated with platinum (Hitachi E-1030). The samples were observed with a scanning electron microscope (Hitachi S-4500). For conventional TEM, acetone was substituted for ethanol during the process and samples were then embedded in Epok 812 (Oken). Ultrathin sections were stained with both uranyl acetate and lead citrate and finally observed with an electron microscope (Hitachi H-800). For immunoelectron microscopy, bacteria were fixed in a mixture of 0.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M HEPES/NaOH (pH 6.8) for 15 min and then refixed in 2% paraformaldehyde in the same buffer for 2 weeks at 4 °C. After fixation, specimens were dehydrated in 80% ethanol and finally embedded in Lowicryl K4M (Polysciences). Ultrathin sections were made and immuno-stained as reported previously (Saito *et al.*, 1998). Briefly, after ultrathin sections were immersed in a mixture of 0.5% normal goat serum and 0.05 M BSA in 0.1 M Tris/HCl (pH 6.8) for a 3 min pre-incubation, they were incubated in appropriately diluted anti-*H. pylori* antibody (Dakopatts) for 2 h and then rinsed in 10 nm gold particle-labelled goat antiserum specific for rabbit IgG (GAR G10; Amersham) for 1 h. This antibody was used to ascertain that bacteria in the culture were *H. pylori*. Sections were observed after staining with uranyl acetate. As a control, diluted normal rabbit serum was used in place of the first antibody.

Assessment of bacterial structure. The surface structure of cultured bacteria was observed by SEM. The percentages of spiral and coccoid forms on each day were determined by randomly observing the external appearance of more than 1000 organisms at each experimental time-point. The structural assessment was performed five times on each day of culture.

RESULTS

Ultrastructural changes

SEM showed that spiral forms became less frequent with increasing time in culture in all media (Fig. 1; curves a–c). In all culture samples, these rate-of-decrease curves were similar, and spiral forms were sparse and difficult to observe in visual fields on the final day. The percentage of spiral forms on each day in 10 mM-LM or 100 mM-LM was considerably lower level than that in 300 mM-LM, as described in Methods. Although the morphological characteristics of the spiral forms were strikingly similar in each glucose-supplement medium, the coccoid forms could be divided into two types using SEM and TEM (Fig. 2). The length and width of the spiral forms were approximately 2 and 0.5 µm. Some granular structures were observed in the cytoplasm, with numerous ribosomes, by TEM (Fig. 2a). Coccoid forms were observed as spheres of a similar size, approximately 0.8 µm in diameter, and such forms could be divided ultrastructurally

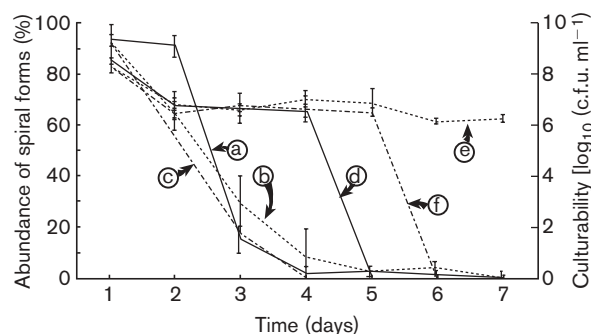


Fig. 1. Morphological changes (curves a–c; percentage of spiral forms) and culturability [curves d–f; $\log_{10}(\text{c.f.u. ml}^{-1})$] during culture of *H. pylori* in CLM (a, d), 300 mM-LM (b, e) or 500 mM-LM (c, f).

into two types, A and B, under more detailed observation. Type A showed an irregular, rough or bumpy surface with few flagella and an indistinct cytoplasmic membrane that obscured much of the intracytoplasmic structure, and these bacteria also tended to adhere each other. Type A was more abundant than type B in the majority of liquid media, with the exception of 300 mM-LM (Fig. 2b). Cells of type B, which showed a smoother surface, had tightly encircled flagella, a comparatively clear membrane structure and a better-maintained intracytoplasmic structure. Type B was observed more often in 300 mM-LM (Fig. 2c). Both coccoid forms were positive for anti-*H. pylori* antibody in the cytoplasm and flagella (Fig. 3). In the controls, no immunostaining was observed. Both types A and B were found to be mixed in each of the media examined, and they became more difficult to differentiate from each other by electron microscopy with increasing time in culture. In 500 mM-LM, types A and B could be distinguished precisely because both spiral and coccoid forms showed irregularly bumpy surfaces. The proportion of type B in 300 mM-LM was higher than in other media (Fig. 4).

Culturability in each medium

Culturability in 300 mM-LM was maintained until the final day of culture. Although culturability was maintained better in 10 mM-LM, 100 mM-LM and 500 mM-LM than in CLM at the beginning of the culture, colonies were scarcely observed on days 6 and 7 (virtually 0 c.f.u. ml⁻¹). On days 3 and 4, culturability was maintained in spite of the reduction of spiral forms (Fig. 1; curves d–f). On the final day in 300 mM-LM, culturability was notably maintained, though spiral forms were difficult to observe in the medium (Fig. 1; curves b and e). During culture, the pH in each medium was maintained between 6.8 and 7.0.

DISCUSSION

In this study, glucose supplementation failed to affect significantly the morphological change of *H. pylori* from

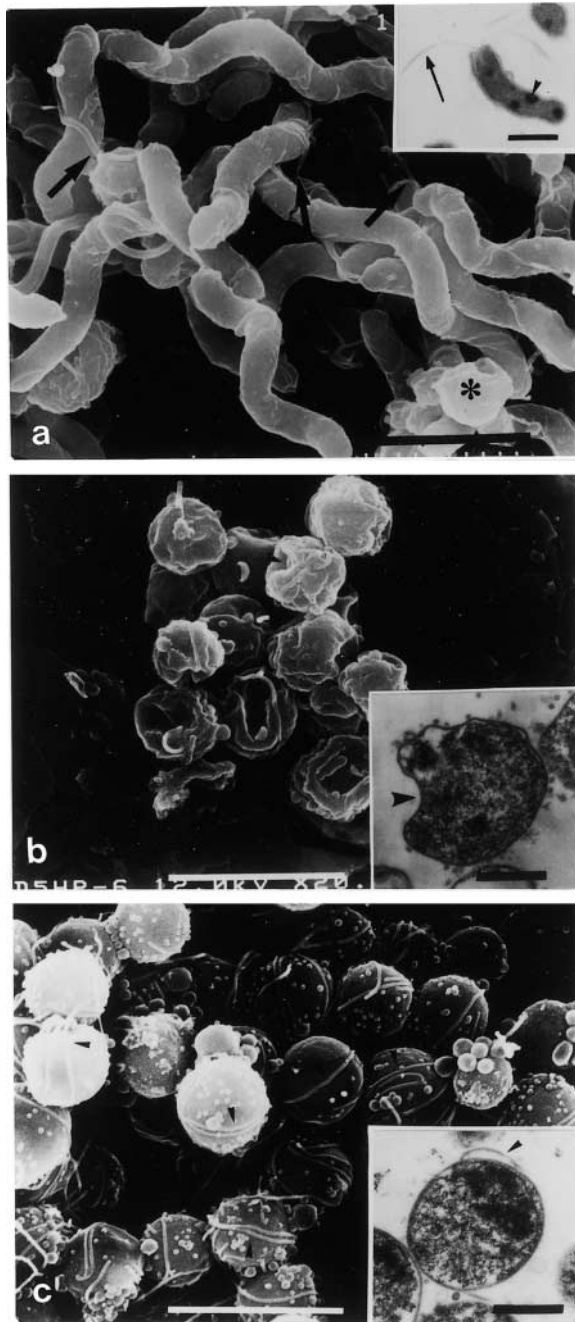


Fig. 2. Ultrastructure of spiral and types A and B coccoid forms of *H. pylori*. (a) SEM of spiral forms on day 1. Flagella are seen on one side of the cells (arrows). A spherical organism is visible (asterisk). Bar, 2 μ m. Inset: TEM of a spiral form. A flagellum (arrow) and an intracytoplasmic granule (arrowhead) are visible. Bar, 0.5 μ m. (b) SEM of type A coccoid forms on day 3 in CLM. Cells are spherical with an irregular surface and possess few flagella. They have a tendency to adhere to each other. Bar, 2 μ m. Inset: TEM of type A coccoid form. The surface is sunken (arrowhead). Membrane and intracytoplasmic structures are not clearly visible. Bar, 0.5 μ m. (c) SEM of type B coccoid forms on day 3 in 300 mM-LM. The smooth surface is tightly encircled by flagella (arrowheads). Bar, 2 μ m. Inset: TEM of type B coccoid form. The membrane structure is well preserved and a flagellum is also visible (arrowhead). Bar, 0.5 μ m.

spiral to coccoid forms, but instead altered the culturability. The coccoid forms could be classified into two types, A and B, by SEM and TEM.

Glucose effectively preserved culturability. Genomic analysis of *H. pylori* has confirmed that glucose is the main energy source of this organism and that it is used to produce ATP. It is suggested that ATP is produced via the glycolytic pathway (Tomb *et al.*, 1997), the pentose phosphate pathway (Mendz *et al.*, 1993) and the Entner–Doudoroff pathway (Chalk *et al.*, 1994). This sugar can also supply membranous components of the organism such as phospholipids and fatty acids of the cell membrane, peptidoglycan and lipopolysaccharide, including lipids A and O (Geis *et al.*, 1990). This study indicated that culturability was maintained better in each glucose-supplemented medium than in CLM without glucose supplementation. The highest proportion of type B coccoid forms was observed in 300 mM-LM, which also showed the best culturability. This type of *H. pylori* has a well-preserved membranous structure compared with the type A coccoid form. The added glucose could be used for cell membrane synthesis, as the type B coccoid form was abundant in 300 mM-LM. This also suggests that mucopolysaccharides in human gastric mucus are important for bacterial proliferation, and *H. pylori* has an enzyme capable of synthesis of bacterial cell membrane that is able to catalyse the production of mucopolysaccharides from glucose (Bode *et al.*, 1988; Slomiany & Slomiany, 1992).

Culturability was worse in 500 mM-LM than in 300 mM-LM. In a preliminary study, using sucrose to alter osmotic pressure, we observed that the surface structure of *H. pylori* became irregular or bumpy in hypertonic liquids. 500 mM-LM might cause dehydration of *H. pylori* and might be too hypertonic for colonies to form. A suitable concentration of glucose might be required for membrane production and cell proliferation. Takeuchi *et al.* (1998) indicated, using a *cdrA*-disrupted *H. pylori* mutant, that *cdrA* was involved in maintaining the rod shape. An intermediate form between the spiral and coccoid forms could not be identified conclusively during these culture experiments. It is supposed that the spiral form is transformed immediately and rapidly into the coccoid form (Saito *et al.*, 2003). Glucose is not thought to be related to the rapid *cdrA*-related deformation, because the transformation from spiral to coccoid forms was not affected by supplementation of glucose in the medium.

Coccoid forms were divided into two types, A and B, by electron microscopy. The function of the two coccoid forms of *H. pylori* is unclear. One possibility is that this form represents a degenerating organism (Hua & Ho, 1996). Kusters *et al.* (1997) indicated that the coccoid *H. pylori* was the morphological manifestation of bacterial cell death, observing the transformation process by electron microscopy. On the other hand, another suggestion is that this form is viable but non-culturable (Eaton *et al.*, 1995). Benaissa *et al.* (1996) asserted that coccoid *H. pylori* was devoid of degenerative change. Many *in vitro* culture studies have reported that coccoid formation was associated with an

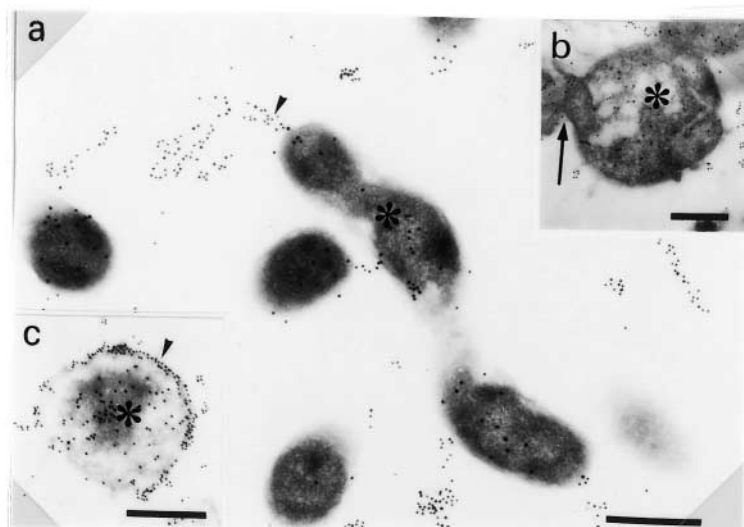


Fig. 3. Immunoelectron micrographs of spiral (a) and type A (b) and B (c) coccoid forms of *H. pylori* stained with anti-*H. pylori* antibody. Flagella (arrowheads) and cytoplasm (asterisks) are positive. In (b), the cell is adhering to an adjacent cell (arrow). Bars, 0.5 μm .

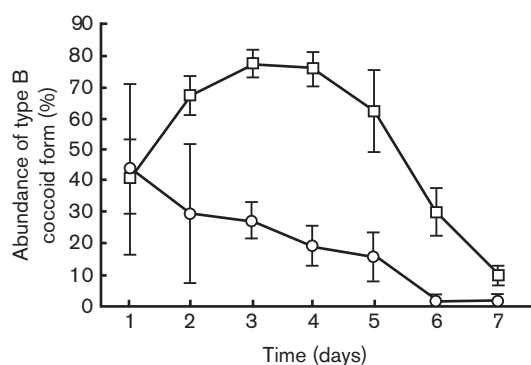


Fig. 4. Type B coccoid forms as a proportion of all spherical organisms in CLM (○) and 300 mM-LM (□).

unsuitable change in the environment, such as nutritional deficiency (Mizoguchi *et al.*, 1998), drug supplementation (Bode *et al.*, 1993), pH change (Cellini *et al.*, 1994), abnormal temperature (Shahamat *et al.*, 1993) or prolonged culture (Sato, 2000). The ATP level of the coccoid forms was also reported to be only 0.1% of that of spiral forms (Sorberg *et al.*, 1996). In spite of these reports, the coccoid form is not necessarily considered to be a completely dead organism. In our study, culturability was well preserved even on days 3 and 4, when coccoid forms were in a majority in all media. This is highly suggestive of some coccoid forms possessing a certain degree of culturability. Both the number and culturability of the type B coccoid form were preserved in media supplemented with glucose. Type B coccoid forms possess comparatively clear membrane structure, and some may play a role in forming colonies on the agar medium after being supplied with ATP that originated from the glucose. In CLM, culturability was lost by day 5, though type B coccoid forms existed at a similar frequency to the previous day. These results mean that some type B coccoid forms are unable to form colonies. It is difficult to explain why almost all type B

coccoid forms should be completely dead on day 5 and should be so different from the previous day, when they were capable of colony formation. A lack of colony formation from cells grown in one medium (CLM in the present circumstances) does not necessarily indicate that bacteria are completely dead. Type B bacteria in 300 mM-LM could produce many colonies on the same cultural agar. Therefore, it is considered that some of the type B coccoid forms may be in a viable but non-culturable stage (the so-called 'VNC' form).

Recently, micro-organisms without colony-forming ability in pure culture were also reported to make colonies in a simulated natural environment (Kaeberlein *et al.*, 2002), and this suggests that a VNC form similar to type B coccoid *H. pylori* may exist in the natural environment. On the other hand, culturability was preserved on day 6 or 7 in 300 mM-LM, despite the rapid reduction in type B coccoid forms. This suggests that even type A coccoid forms possess limited culturability. Proliferative capacity was lost completely within 24 h in liquid media, with the exception of 300 mM-LM. This phenomenon implies that the organisms are switched rapidly from a colony-forming to a non-colony-forming stage through a predetermined pathway. It is suggested that not only spiral forms, but also some type A and B coccoid forms, are viable at the beginning of culture, since both coccoid forms were recognized to possess DNA (Saito *et al.*, 2003). After a lapse of several days in culture, both coccoid forms were severely disrupted morphologically and were confirmed to be dead. They finally degraded in culture medium.

Thus, our study demonstrates that coccoid forms can be divided ultrastructurally into two types, A and B, that consist of three discrete stages, dying, VNC and proliferating stages. An optimal liquid medium supplemented with approximately 300 mM glucose is the most suitable both for increasing the colony-forming ability and for preservation of *H. pylori* membrane structure under pure culture conditions.

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