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NOTES

Changes in *Helicobacter pylori* Ultrastructure and Antigens during Conversion from the Bacillary to the Coccoid Form

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In vitro, *Helicobacter pylori* converts from a bacillary to a full coccoid form via an intermediate U-shaped form. Organisms with a full coccoid form keep a double membrane system, a polar membrane, and invagination structures. Western blots (immunoblots) of sera from colonized patients show that some high-molecular-mass antigenic fractions are expressed only in coccoids. Conversely, fractions of 30 and 94 kDa were more intensively detected in the bacillary forms. These results suggest that (i) coccoid conversion is not a degenerative transformation and (ii) antigens specific to the coccoid forms are expressed in vivo.

Helicobacter pylori is now accepted as being the main etiological agent of human antral gastritis and is an essential factor in peptic ulcer disease (5) and gastric cancer (1). Since its discovery and isolation (18, 27), worldwide investigations have been conducted in an attempt to determine the mode of transmission of *H. pylori*, to explain the chronicity of infection, and to elucidate the virulence of this pathogenic organism.

The fact that *H. pylori* can convert to coccoid forms in prolonged culture has been extensively reported and has stimulated some hypotheses about the possible significance of this conversion. Referring to *Campylobacter jejuni*, some investigators suggested that the coccoid forms are degenerative and pose no infectious risk (6, 20, 21). Others supposed that they may represent a dormant stage, but although some studies have suggested that the coccoid forms of *H. pylori* could be potentially viable (4, 7, 16, 25), standard laboratory methods were found insufficient for regrowth of *H. pylori* from the coccoid forms. The persistance for months of these forms in water has been reported (17, 28). In certain experimental models of *H. pylori* infection, coccoid forms have been found in the feces of infected animals (8). They also have been observed on the gastric mucosa of infected humans (9). As a consequence of these findings, the coccoid forms of *H. pylori* have been suspected to play a role in the transmission of the bacteria and to be partly responsible for recrudescence or relapses of infection after antimicrobial treatments (3, 4, 7, 16, 25).

Finally, the significance of the coccoid forms of *H. pylori* is still controversial and the questions of whether these forms are viable, present in vivo, and infective or degenerative are still open. In the present study, we induced conversion from bacillary to coccoid forms and we studied ultrastructural and antigenic evolutions during this conversion. We showed that coccoid forms retain functional structures. They exhibit antigenic components absent in the bacillary forms and recognized by sera of infected patients.

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Strain and culture conditions. The *H. pylori* strain used (H88D) was isolated in Boucicaut Hospital (Paris, France) from a gastric biopsy of a patient suffering from a gastric ulcer. Cells were grown on Colombia blood agar (BioMérieux, Marcy l'Etoile, France) at 37°C under microaerophilic conditions (8 to 10% [vol/vol] CO_2 and 5 to 7% [vol/vol] O_2).

At day 0, the cells of a 2-day culture of *H. pylori* H88D were harvested from one plate, suspended in phosphate-buffered saline (PBS, pH 7.4), and adjusted to a turbidity of 1 MacFarland unit. After Gram staining, it was microscopically observed that this suspension contained only bacillary forms. The suspension was then used to inoculate 10 plates of Columbia blood agar (0.1 ml per plate). The plates were incubated at 37°C under microaerophilic conditions. The cells of one plate were harvested daily and suspended in 1 ml of PBS (pH 7.4). Enumeration of CFU was performed by standard serial dilution and plate count procedures. Total cell (bacillary and/or coccoid forms) counts were also assessed by turbidimetry at 540 nm. To each suspension, Gram-stained smears were used to assess the relative percentages of coccoid and bacillary forms; these measurements were made blindly by 10 different persons, and the averages of their determinations were considered as the final results. Furthermore, the suspensions of day 2, 3, 4, and 7 were used for electron microscopic observations.

Preparation for electron microscopy. Bacteria were harvested from plates, fixed in 2.5% glutaraldehyde in PBS for 90 min, and then washed three times in the same buffer. Fragments were postfixed (30 min) in osmium tetroxide. After postfixation the material was dehydrated (acetone) and embeded in epoxy medium. Thin sections (50 nm) were cut with a diamond knive on a Reichert ultramicrotome, Ultracut S. The grids were contrasted with 2% uranyl acetate and lead citrate and examined on a JEOL 100 Cx electron microscope.

Western blotting. The evolution of the antigenic profiles during coccoid conversion of *H. pylori* H88D was studied by Western blotting (immunoblotting) using sera from patients known to be colonized by *H. pylori* for a least 6 months. These sera included the serum from the patient infected by strain H88D (homologous serum).

Total antigens of strain H88D were obtained by sonication (Branson Sonifier 450; duty cycle, 50%; output control, 5; time, 5 min) of bacterial cells harvested at different stages of coccoid conversion and suspended in PBS (pH 7.4). After sonication the unlysed bacteria were eliminated by centrifugation (3,000 $\times$ *g*, 15 min), and the supernatants were saved, adjusted to 1 mg of protein per ml, and frozen at -80°C until use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmeli (15) with a 4% stacking gel and a 10% separating gel. Prior to electrophoresis, the protein solutions were heated at 100°C for 5 min in a sample buffer containing 5% (wt/vol) SDS and 0.42% (wt/vol) 2-mercaptoethanol. Electrophoresis was conducted during 1 h under a constant voltage (15 V/cm) using the Bio-Rad minigel system. Proteins were blotted onto a prewetted nitrocellulose membrane (Bio-Rad) by using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) under a constant current of 200 mA for 1 h. The blots were incubated for 1 h with human sera diluted at 1/500; they were then rinsed and incubated in goat serum anti-human immunoglobulin A (IgA)-IgG-IgM conjugated with alkaline phosphatase (Dakopatts, Copenhagen, Denmark). After a final wash the nitrocellulose filters were developed with 5-bromo-4-chloro-3-indolylphosphate (BCIP) as a substrate and nitroblue tetrazolium as a chromogenic indicator. The reactions were stopped after 20 min by washing the filters extensively with distilled water.

Evolution of ultrastructure of *H. pylori* from a bacillary to a full coccoid form via a U-shaped form. As assessed by turbidimetry (Fig. 1A), the bacterial mass increased exponentially from day 1 to day 4; the stationary phase began at day 4, and the bacterial mass remained in a steady state until day 8. The proportion of coccoid forms increased from 0 to 100% from day 2 to day 7 (Fig. 1B), while the CFU increased exponentially from day 1 to day 3 and decreased dramatically from day 5, to become undetectable at day 7 when coccoid forms reached 100% (Fig. 1B and C). This suggests that coccoid forms are unable to divide under the culture conditions used. However, between day 3 and day 5 the fraction of coccoid forms on the plates increased from 20 to 70% with no change in the CFU count.

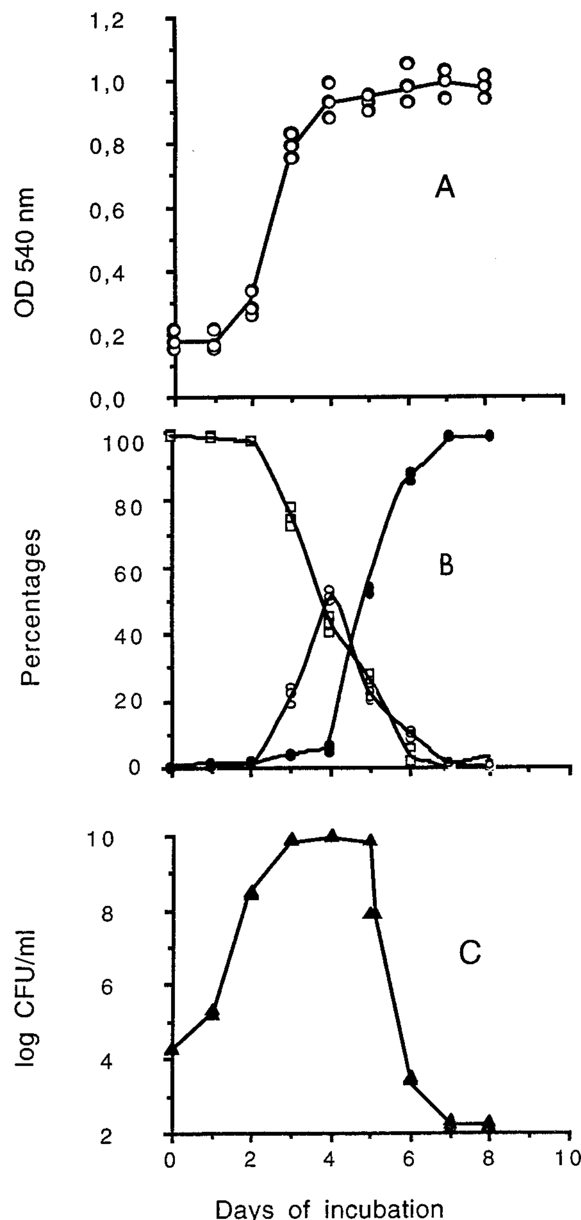

FIG. 1. Growth curves for *H. pylori*. (A) Growth curve was established by turbidimetry. (B) The percentages of bacillary (□), U-shaped (○), and full coccoid forms (●) were determined by electron microscopy (C) CFU were determined by viable count.

Electron micrographs at day 2 showed that coccoid conversion is initiated by ingrowth of the periplasmic space on one side of the bacteria, with an accumulation of a dense material (Fig. 2A and 2B, arrows). This could be a consequence of the increased volume and/or pressure of the periplasmic space. The periplasmic material could be secreted from the cell, or it could result from the lysis of periplasmic components such as peptidoglycan. The nature of this material is under investigation in our laboratory. These forms were mainly observed between the second and the fourth day of incubation (Fig. 1B). At day 4, the relative number of bacillary forms decreased and U-shaped forms became predominant; clearly, the parental bacillary forms were still visible in the coccoid cells (Fig. 2C). They were surrounded only by the inner membrane and bent under torsion apparently created by the globular form of the outer membrane system. The periplasmic material became less dense in the U-shaped forms than in the bacillary forms and exhibited an apparent displacement of the outer membrane. The conversion from U-shaped forms to coccoid forms (called here full coccoid) strongly suggest a dynamic process. Figure 2D shows an increase of the protoplasmic cylinder. At day 7, only globular full coccoid forms were observed (Fig. 2E). These forms keep their double membrane system, but the periplasmic dense material seen in the U-shaped forms is no longer present. Because they appear in the culture while the U-shaped forms disappear, it is likely that the full coccoid forms derive from the U-shaped forms. To confirm this transition suggested by some electronic microscopy observations, percentages of the different forms during conversion were established. The results strongly suggested a transition of bacillary to full coccoid forms via U-shaped forms (Fig. 1B).

On the other hand, electron micrographs showed that there was no difference in morphology between coccoid forms from day 7 and day 30 (data not shown). Both are not viable under our conditions.

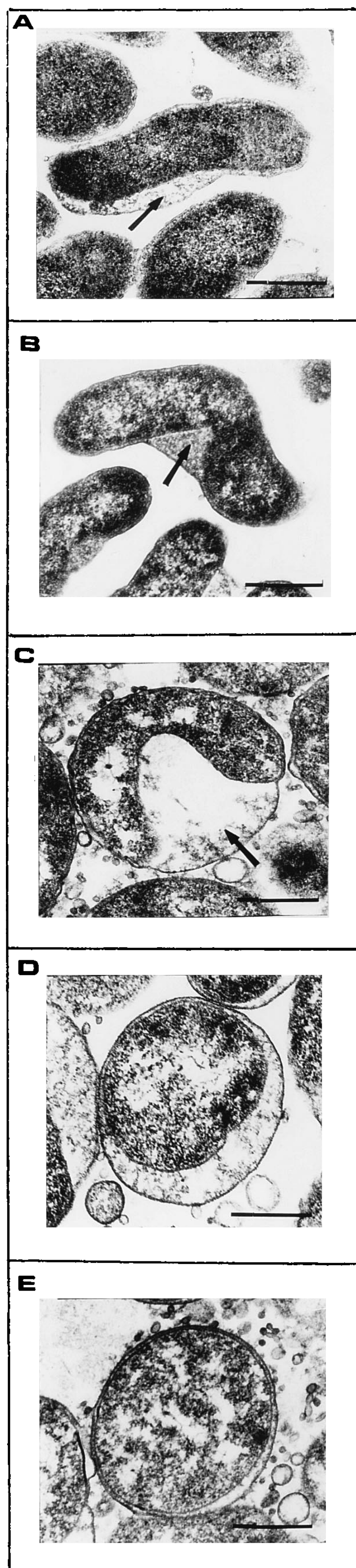

FIG. 2. Ultrastructure of coccoid conversion from the bacillary to full coccoid form. Ultrathin sections of 2-day culture of *H. pylori* (A), showing initiation of conversion from the bacillary to coccoid form with formation of dense periplasmic material (arrow) (bar, 400 nm); at day 3 (B), showing accumulation of dense material in the periplasmic space (arrow) (bar, 400 nm); at day 4 (C), showing the inwardly curved bacillary form which constitutes the intermediate step between the bacillary and coccoid forms (bar, 400 nm); at day 5 (D), showing a change in the protoplasmic cylinder (bar, 400 nm); and at day 7 (E), showing a full coccoid form (bar, 400 nm).

The ultrastructure of full coccoids was studied at day 7. It has been shown that 3-month-old coccoids of *H. pylori* are still able to maintain a certain metabolism, which requires the preservation of cellular structures such as the cytoplasma, cell membrane, flagella, and DNA (4). We add to this finding that polar membranes are also maintained in several intact coccoids (Fig. 3A and B, arrows). Polar membranes have been described for a number of genera including *Spirillum* (22), *Campylobacter* (13, 19), *Ectothiorhodospira*, and *Rhodospirillum* (14) spp. The association of this structure with the basal complex of the flagella remains indefinite. It has been suggested that this specialized structure has evolved as the site of energy generation for motility or cell metabolism (24). In about 1% of the coccoid cells, we also observed invagination membranes (not previously found in *H. pylori*) (Fig. 3C), which originate from and are attached to the plasma membrane and are arranged in a complex pattern. Similar invagination membranes have been described for *Ecthiorhodospira mobilis* and other spirilloid or vibroid gram-negative organisms (2, 23), which divide by binary fission and whose motility necessitates a polar tuft of flagella. In *E. mobilis*, sulfide (normally toxic to the cell) might be oxidized on the extracytoplasmic side of the invaginated membrane. The extracytoplasmic surface of the invaginated membrane of some potentially viable coccoid forms of *H. pylori* might serve as a site for the oxidation of toxic materials.

All of these findings consolidate the potential viability of *H. pylori* coccoids.

Evolution of antigens of *H. pylori* during coccoid conversion. The evolution of the antigenic profiles during coccoid conversion of *H. pylori* H88D was studied by Western blotting using different sera from seropositive patients. These sera were used to reveal the total antigens of the strain cultured for 2 days (0% coccoids), 4 days (50% coccoids), 5 days (80% coccoids), and 7 days (>99% coccoids). Figure 4 shows the results obtained with the serum from the patient from whom the strain was isolated. The same results were obtained using six other sera randomly chosen among those from seropositive patients (data not shown). A high-molecular-mass antigenic fraction (>94 kDa, arrow), not detected in bacilli, was detected more and more intensively from day 2 to day 7 during the coccoid conversion, while the prominent band at 94 kDa appeared to decrease during the same time. On the other hand, an antigenic fraction of 30 kDa (arrow) was more intensively detected in the bacillary forms. The rest of the profiles are identical between bacillary and coccoid forms. This highlights the significance of these antigens. Several antigenic proteins have been found with molecular masses between 29 and 128 kDa (10, 12, 26). These proteins include CagA (120 to 128 kDa) and two antigens with unknown functions (116 and 110 kDa). In the 30-kDa area, a urease subunit (29 to 31 kDa), an adhesin (30 kDa), and a porin (30 kDa) have been identified. Whether or not the antigens specific for coccoid and bacillary forms are among these proteins remains to be established. Another suggestion would be that a 30-kDa antigen seen in the bacillary

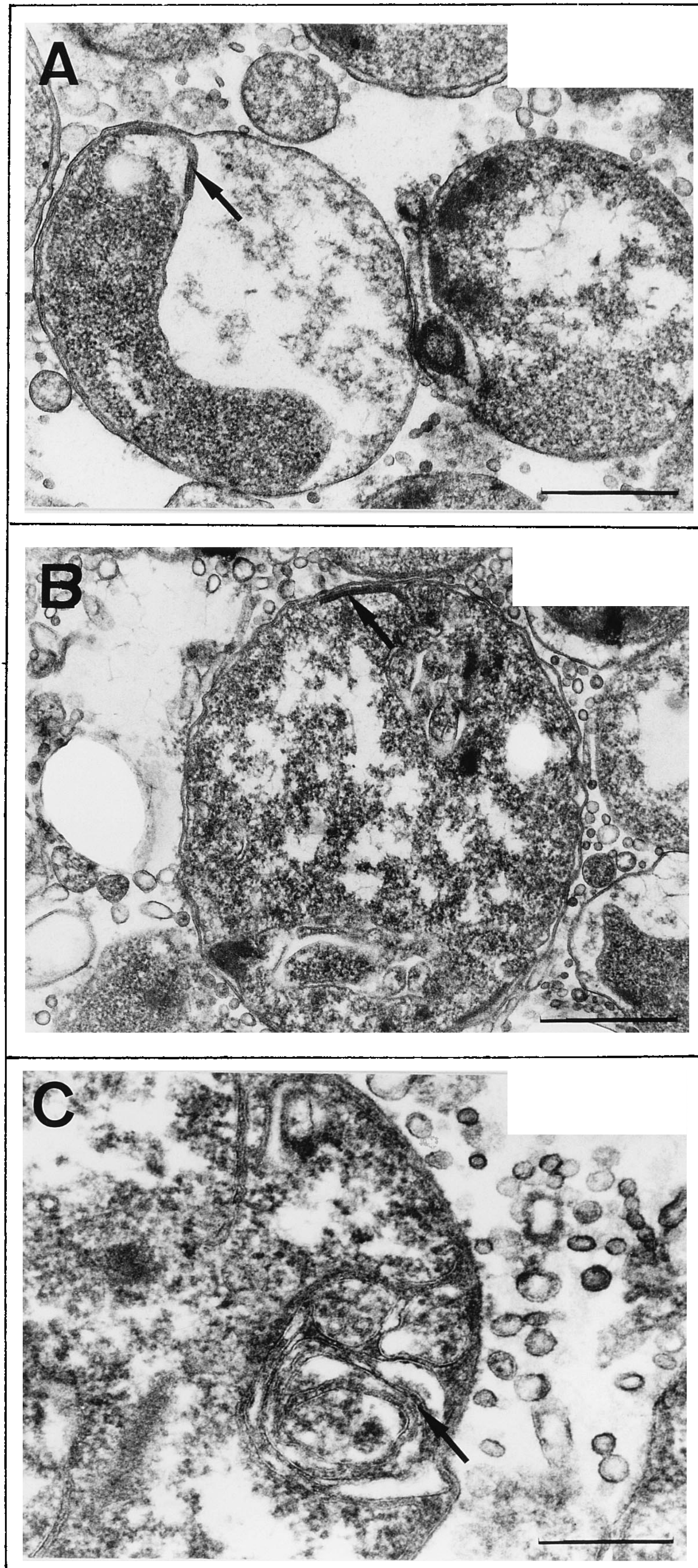

FIG. 3. Ultrastructure of U-shaped (A) and full coccoid (B) forms. Ultrathin sections at 4 and 7 days, respectively, of culture of *H. pylori* showing polar membranes (arrow) (bar, 400 nm). (C) Ultrathin section at 7 days of culture, showing invaginated membranes (arrow) (bar, 800 nm).

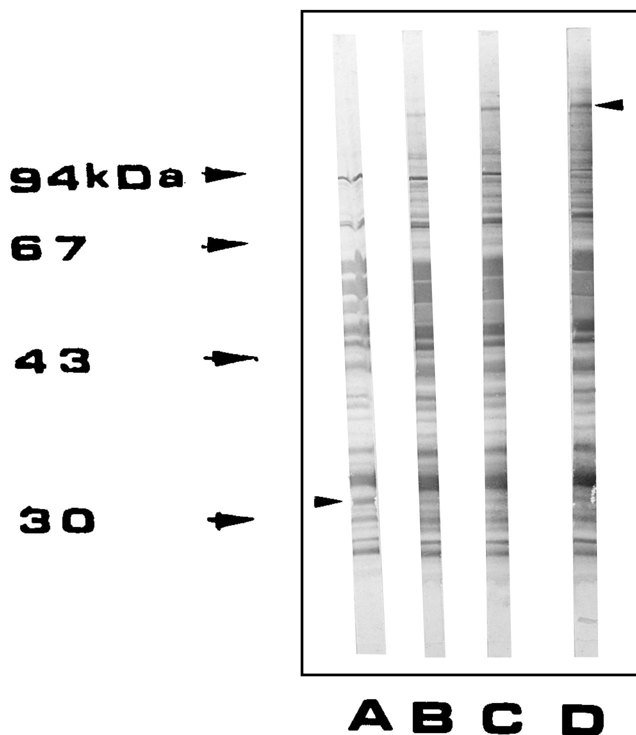

FIG. 4. Evolution of antigenic profiles during coccoid conversion of *H. pylori* H88D. Western blot analysis as described in the text, using homologous serum from the patient from whom *H. pylori* H88D was isolated. This serum was used to reveal the antigens of the strain cultured for 2 days (0% coccoids) (lane A), 4 days (50% coccoids) (lane B), 5 days (80% coccoids) (lane C), and 7 days (>99% coccoids) (lane D).

forms may polymerize during the coccoid conversion and could appear as a trimeric 90-kDa antigen in the coccoid forms (11). However, because electrophoretic separation was performed under denaturating conditions, this is an unlikely hypothesis. These particular antigens are being characterized in our laboratory.

Concluding remarks. This work demonstrates that (i) coccoid forms of *H. pylori* retain cellular structures compatible with viability and may represent one of the stages of a putative *H. pylori* biological cycle and (ii) some of the antigens which appear to be differentially expressed between bacillary and coccoid forms in vitro are expressed in vivo. Whether the coccoid forms are viable in vitro and/or in vivo, whether they represent a temporary adaptation to a particular environment, and whether they are involved in the transmission of the bacterium remain open questions.

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