

# ***Helicobacter pylori*: A Fickle Germ**

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**Abstract:** The morphologic changes from bacillary to coccoid forms of *Helicobacter pylori* were studied. These form changes were analyzed by bacterial growth in Brucella broth plus 2% fetal calf serum. The coccoid forms were observed at five days of incubation and a rapid decrease of CFU/ml was recorded. At two weeks of microaerophilic incubation, all coccoid forms observed were not culturable *in vitro*. The coccoid morphology was observed earlier when the culture of *H. pylori* was incubated in aerobic conditions and with subinhibitory concentrations of omeprazole and roxithromycin. To evaluate the possibility of resistance of coccal forms, before plating, the cultures were heated to 80°C for 10 min and sonicated. In the absence of these treatments the cultures did not show growth *in vitro*. The proteic patterns of the same strains of two different morphologies were studied revealing significant differences.

**Key words:** *Helicobacter pylori*, Bacterial morphology, Bacillary and coccoid forms

A strong association between *Helicobacter pylori* and chronic active inflammation of the gastric antrum and duodenal ulceration has been recognized (4, 14, 20–22). Several therapeutic regimens have been proposed to clear *H. pylori* infections and to obtain a long-term eradication of the microorganism (6, 9, 15, 17).

A possible cause of recrudescence of infections should be due to an incomplete clearing of the germ. *H. pylori*, as well as vibrio-like bacteria, shows two different morphological forms, spiral shape and spherical cells, with the coccal form not culturable *in vitro* (but potentially viable) (2, 10, 11, 13, 16). This morphological conversion may be the expression of transitory adaptation to adverse cultural conditions (3, 13). Particular interest is focused on the viability of the coccoid forms and this aspect is very important to understand the wide diffusion of recrudescence or relapse of *H. pylori* infections (9, 17). The coccal forms should be the transitory resistant cells that in an appropriate environment revert to bacillary or degenerative forms. A large number of studies were made to evaluate the viability of coccal forms in related germs, like *Campylobacter jejuni* (5, 11, 19). These studies demonstrate the presence of degenerative coccoid forms, but in a small portion of these forms the structural integrity was conserved and therefore

probably viable. This last aspect, which can also be suspected for *H. pylori*, can be a probable hypothesis to elucidate the bacterial transmission.

In this work the conditions leading to morphologic changes from bacillary to coccoid forms of *H. pylori* *in vitro* were studied. The time of reversion was calculated, CFU/ml and pH were reported. These parameters were also studied in unsuitable conditions for *H. pylori*. Broth cultures were set up in aerobic environment and in the presence of subinhibiting concentrations of the drugs (omeprazole and roxithromycin). The choice of these drugs is related to their employment in clinical therapy (6).

From the results obtained by Amano and Shibata (1), supposing for *H. pylori* a similar cell wall variation related to morphological changes, and to hypothesize a role of resistance of spherical cells, before plating, the cultures were heated at 80°C for 10 min and sonicated. Moreover, SDS-PAGE was used to differentiate the proteic pattern of the same strains with different morphologies.

## **Materials and Methods**

**Bacterial strains and growth conditions.** *H. pylori* BM46 was the clinical isolate used for this work. Strain, obtained from patient who underwent endoscopy with gastroduodenal disease, was identified by standard methods (7, 18). *H. pylori* NCTC 11639 was used as a standard control strain.

**Abbreviation:** FCS, fetal calf serum.

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All described tests were made in duplicate.

Microorganisms, stored at -70°C by the method of Drumm and Sherman (8), were defrosted to room temperature and rapidly plated in chocolate agar plus 1% of IsoVitaleX (Becton Dickinson & Co., Cockeysville, Md., U.S.A.) at 37°C for three days in a microaerophilic atmosphere (CampyPak Jar-Unipath, Ltd., Basingstoke, London, England).

Strains were inoculated onto Brucella broth (Biolife Italiana, Milano, Italy) supplemented with 2% fetal calf serum (FCS) (Seromed, Biochrom, K.G., Leonorenstr, Berlin, Germany) and incubated in microaerophilic environment at 37°C in a rotary water bath (K.W., Monteriggioni, Siena, Italy). CFU/ml, pH and bacterial morphology were detected at the time of spreading and every day for 14 days.

*Factors inducing the morphological conversion.* At 24 hr of incubation, when the bacillary forms were registered, a sample of broth culture was drawn, divided in aliquots of 3 ml and incubated in rotary water bath (K.W.) in aerobic conditions at 37°C. CFU/ml, pH and bacterial morphology were detected at 1,2,3,4,5,6,24 and 48 hr. The bacterial features indicated as bacillary form (B), U-shaped form (U), coccoid form (C), B/U, U/C, were calculated reading the prevalent form per field on the slide as follows: for each broth studied three slides were read and for each slide four fields were counted. A particular morphology was considered prevalent when the mean of all fields was greater than 90%. When the percentage of the two different forms was between 30-50% both morphologies were registered.

Two samples of 5 ml were also recovered by starting inoculum and added with omeprazole (Astra, Hassle, Sweden) and roxithromycin (Roussel Uclaff, Paris, France) at concentration of 1/4 of respective MIC values and incubated in a controlled environment. CFU/ml, pH and bacterial morphology were detected at 24 hr and 48 hr.

When coccal forms were obtained from each test, two samples of 1 ml were also drawn. Before plating, one of the samples was heated at 80°C for 10 min and the other was sonicated at 100 W for 1 min with a Labsonic 1510 sonicator (Braun, Germany).

*SDS-PAGE of whole cell proteins.* *H. pylori* BM46 and the reference *H. pylori* NCTC 11639 were grown, in duplicate, on Brucella broth plus 2% FCS in microaerophilic environment at 37°C for two and seven days. The sample at two days of incubation showed a bacillary morphology, while after seven

days of incubation the coccoid aspect was the prevalent morphology.

Samples obtained were centrifuged, washed twice in 10 mM potassium phosphate buffer and pellets were suspended in extraction buffer consisting of 80 mM Tris-HCl (pH 6.8), 1.5% dithiothreitol (wt/vol), 2% sodium dodecyl sulfate (wt/vol), 12.5% glycerol (wt/vol), and 0.01% bromophenol blue (wt/vol). The homogenate was heated for 5 min in a boiling water bath. Insoluble debris was removed by centrifugation at 10,000×g for 20 min at 5°C. Proteins extracted were electrophoresed on discontinuous SDS-polyacrylamide slab gels according to Laemmli (12). The SDS concentration was 0.1% (wt/vol), the stacking and the separating gels contained 5% and 12.5% acrylamide, respectively. Gels were stained with 0.2% of Coomassie blue.

Gels were read with a GS 300 densitometer (HSI, San Francisco) connected to an Apple computer (GS 370).

## Results

Figure 1 shows the growth of *H. pylori* on Brucella broth plus 2% FCS. A reduction in CFU/ml initiated after four days of incubation and this decline agreed with the morphologic conversion.

The morphologic conversion of *H. pylori* is shown in Fig. 2. Coccal forms and U-shaped forms were observed at four days of incubation (Fig. 2, b and c). This last morphologic aspect should represent an intermediate step in the genesis of coccoid morphology.

When the CFU/ml declined and the coccoid morphology was prevalent, a rise of pH (7.5-8) of the bacterial culture was observed (Fig. 1). Unfavorable conditions for growth of *H. pylori* produced the change from bacillary to coccoid form

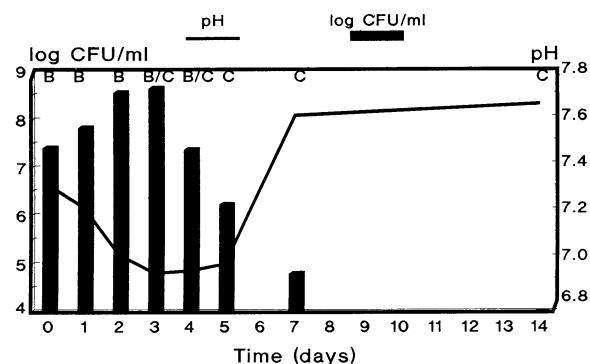


Fig. 1. Expression of growth, pH variation and morphological conversion (B=bacillary forms, C=coccoid forms) of *H. pylori* in broth medium.

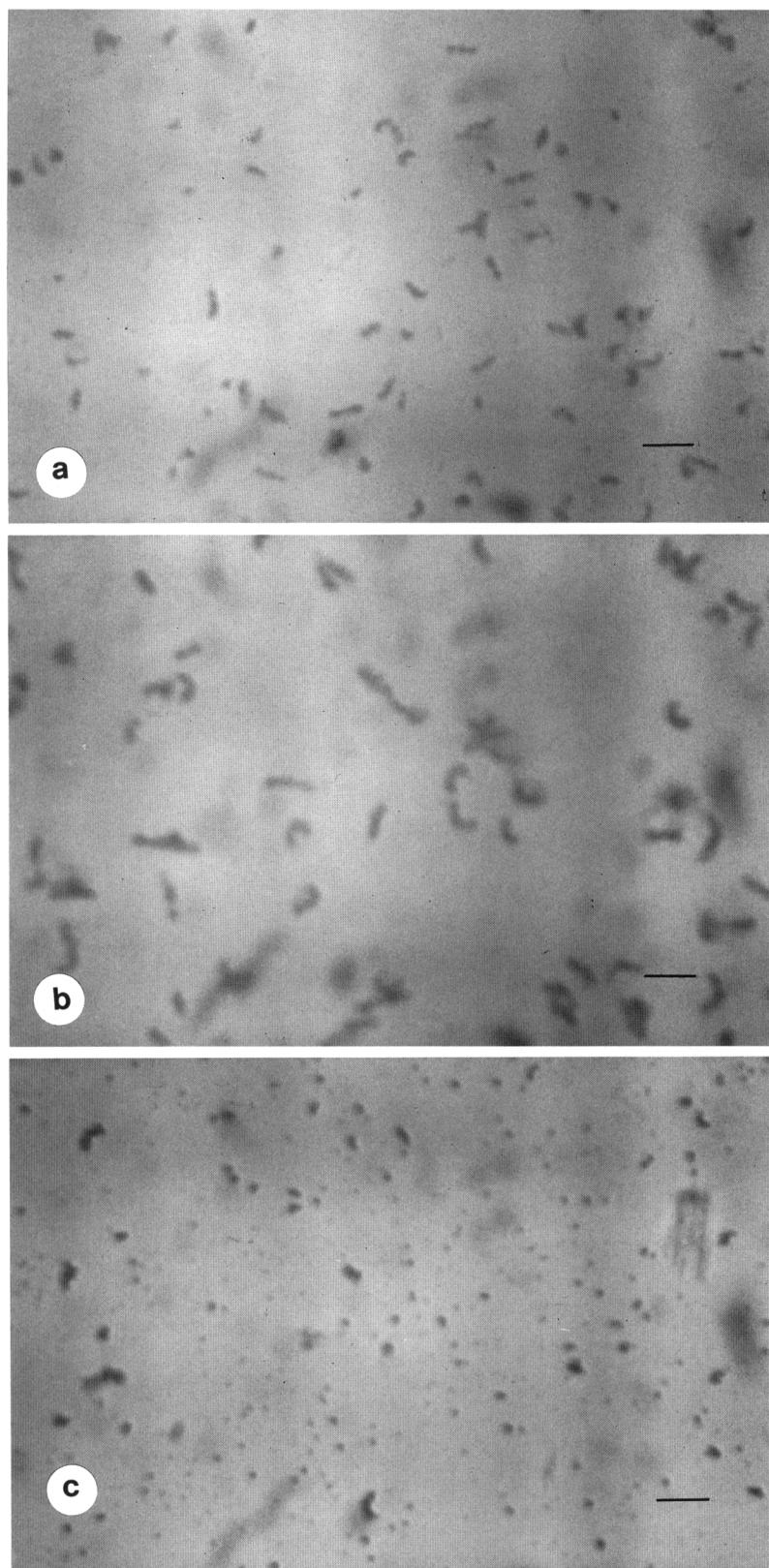


Fig. 2. Aspects of morphologic conversion of *H. pylori*. a) bacillary forms, b) U-shaped forms, c) coccoid forms. Crystal violet stain (1,000 $\times$ ). Bar=10  $\mu$ m.

in a shorter time.

Figure 3 shows the rapid reduction of CFU/ml at 6 hr of aerobic incubation and the prevalent presence of coccoid forms after 3 hr. pH values were increased ( $\text{pH}=8$ ) at the end of the tests. The presence of omeprazole and roxithromycin at values of 1/4 of MIC ( $4 \mu\text{g}/\text{ml}$  and  $0.0015 \mu\text{g}/\text{ml}$  for omeprazole and roxithromycin, respectively, against *H. pylori* BM46 and *H. pylori* NCTC 11639) induces the morphologic change in shorter time. A rapid decline of CFU/ml was observed in the presence of both drugs and the prevalence of coccoid forms was recorded at 24 hr for omeprazole and at 48 hr for roxithromycin (Fig. 4). The pH increased to 7.7 and 7.8 at 48 hr of incubation (in the presence

of omeprazole and roxithromycin, respectively).

All plated broth cultures exhibiting the coccoid form that were treated at  $80^\circ\text{C}$  for 10 min and sonicated, did not grow on plates after three days of incubation in microaerophilic environment.

Figure 5 shows protein profiles of *H. pylori* strains pelleted at two different morphologies. The protein patterns of bacillary and coccoid *H. pylori* were different, having correlation coefficients of 85% between coccoid and bacillary *H. pylori* BM46 and of 71% between *H. pylori* NCTC 11639 at two different morphologies. The respective scans of the patterns, at the left in figure, show more clearly these differences.

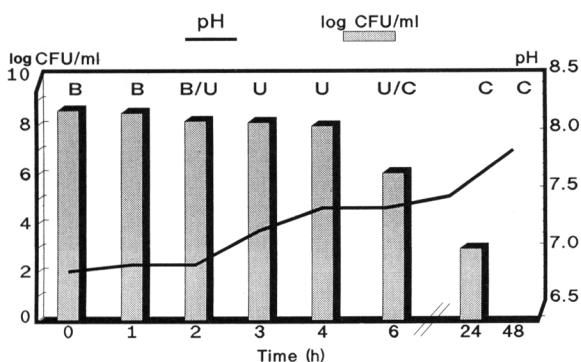


Fig. 3. Expression of growth, pH variation and morphological conversion (B=bacillary forms, U=U-shaped forms, C=coccoid forms) of *H. pylori* in broth medium in aerobic environment.

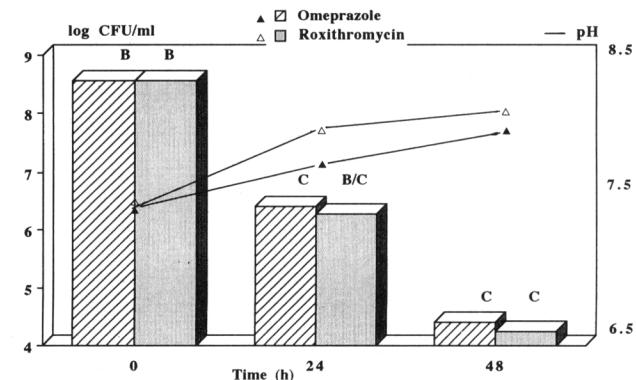


Fig. 4. Expression of growth, pH variation and morphological conversion (B=bacillary forms, C=coccoid forms) of *H. pylori* in broth medium in presence of subMIC of omeprazole and roxithromycin.

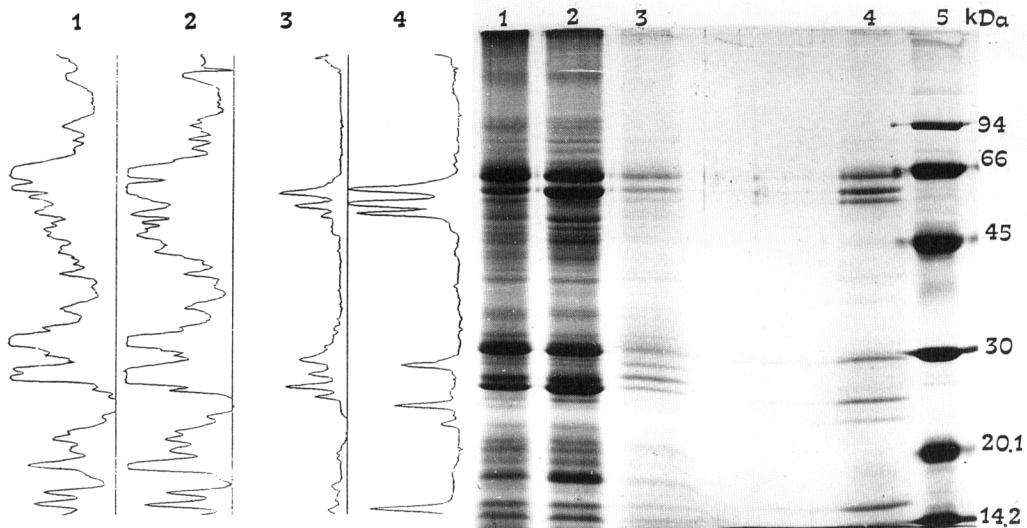


Fig. 5. SDS-PAGE of whole cell proteins of *Helicobacter pylori*. Lane 1, bacillary form of *H. pylori* BM46; lane 2, coccoid form of *H. pylori* BM46; lane 3, bacillary form of *H. pylori* NCTC 11639; lane 4, coccoid form of *H. pylori* NCTC 11639; lane 5, standard and their kDa values, from top to bottom: phosphorylase b (94), bovine serum albumin (66), ovoalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20.1) and  $\alpha$ -lactalbumin (14.2). The scans of the patterns were adjusted to match at the edge.

## Discussion

The conditions inducing the morphologic changes of *H. pylori* were studied in this work. The coccoid forms were obtained with a prolonged incubation of the broth cultures induced by a reduction of nutrients. This form was obtained more readily in "stressed conditions." In fact, coccoid *H. pylori* was produced in the presence of drugs at subMIC and an aerobic atmosphere in a shorter time.

This rapid morphologic modification can represent an adaptation of the germ to survive in unsuitable environments. The significant differences in proteic patterns registered for bacillary and coccoid *H. pylori* show a real change in the germ. These changes produce a bacterial morphology not culturable *in vitro* even when treated at high temperatures.

This "dormant" coccoid form should represent a natural phenomenon or an alternative system of survival. This aspect is important to hypothesize a clinical significance of the coccoid form in relapsing infections when therapy is suspended. In fact, the difficulty in obtaining a long-term eradication of the strain would lead to the supposition that is an incomplete clearing of the germ.

The experimental evidence produced from this work, where the coccoid form of *H. pylori* is not culturable *in vitro* with subMIC of roxithromycin and omeprazole, would lead us to presume an analogous mechanism *in vivo*. Drugs used in the therapeutic protocols may not be sufficient to eradicate the germ and thereby may induce the genesis of coccoid forms. Since the coccoid form is not culturable, the germ is camouflaged, but not eradicated.

Continued studies are required to clarify the clinical significance of the coccoid aspect of *H. pylori*. In particular, the study of the possible viability and reversion of these forms should explain the large diffusion of reinfection of *H. pylori*.

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## References

- 1) Amano, K., and Shibata, Y. 1992. Structural studies of peptidoglycans in *Campylobacter* species. *Microbiol. Immunol.* **36:** 961-967.
- 2) Baker, D.A., and Park, R.W.A. 1975. Changes in morphology and cell wall structure that occur during growth of *Vibrio* sp. NCTC 4716 in batch culture. *J. Gen. Microbiol.* **86:** 12-28.
- 3) Baker, R.M., Singleton, F.L., and Hood, M.A. 1983. Effects of nutrient deprivation on *Vibrio cholerae*. *Appl. Environ. Microbiol.* **46:** 930-940.
- 4) Blaser, J.M. 1992. *Helicobacter pylori*: its role in disease. *Clin. Infect. Dis.* **15:** 386-393.
- 5) Buck, G.E., Parshall, K.A., and Davis, C.P. 1983. Electron microscopy of the coccoid form of *Campylobacter jejuni*. *J. Clin. Microbiol.* **18:** 420-421.
- 6) Cellini, L., Marzio, L., Di Girolamo, A., Allocati, N., Grossi, L., and Dainelli, B. 1991. Enhanced clearing of *Helicobacter pylori* after omeprazole plus roxithromycin treatment. *FEMS Microbiol. Lett.* **84:** 255-258.
- 7) Cellini, L., Allocati, N., Piccolomini, R., Di Campli, E., and Dainelli, B. 1992. New plate medium for growth and detection of urease activity of *Helicobacter pylori*. *J. Clin. Microbiol.* **30:** 1351-1353.
- 8) Drumm, B., and Sherman, P. 1989. Long-term storage of *Campylobacter pylori*. *J. Clin. Microbiol.* **27:** 1655-1656.
- 9) Graham, D.Y., Lew, G.M., Malaty, H.M., Evans, D.G., Evans, D.J., Jr., Klein, P.D., Alpert, L.C., and Genta, R.M. 1992. Factors influencing the eradication of *Helicobacter pylori* with triple therapy. *Gastroenterology* **102:** 493-496.
- 10) Jones, D.M., and Curry, A. 1990. The genesis of coccoid forms of *Helicobacter pylori*, p.29-37. *In* Malfertheiner, P., and Ditschuneit, H. (eds), *Helicobacter pylori, gastritis and peptic ulcer*, Springer Verlag, Berlin, Heidelberg.
- 11) Jones, D.M., Sutcliffe, E.M., and Curry, A. 1991. Recovery of viable but non-culturable *Campylobacter jejuni*. *J. Gen. Microbiol.* **137:** 2477-2482.
- 12) Leammlie, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
- 13) Lai-King, N.G., Sherburne, R., Taylor, D.E., and Stiles, M.E. 1985. Morphological forms and viability of *Campylobacter* species studied by electron microscopy. *J. Bacteriol.* **164:** 338-343.
- 14) Lee, A., Fox, J., and Hazell, S. 1993. Pathogenicity of *Helicobacter pylori*: a perspective. *Infect. Immun.* **61:** 1601-1610.
- 15) Logan, R.P.H., Gummet, P.A., Misiewicz, J.J., Karim, Q.N., Walker, M.M., and Baron, J.H. 1991. One week-eradication regimen for *Helicobacter pylori*. *Lancet* **338:** 1249-1252.
- 16) Mai, U., Geis, G., Leying, H., Ruhl, G., and Opferkuch, W. 1989. Dimorphism of *Campylobacter pylori*, p. 29-33. *In* Megraud, F., and Lamouliatte, H. (eds), *Gastroduodenal pathology and Campylobacter pylori*, Elsevier Science Publishers, Amsterdam.
- 17) Marshall, B.J., Goodwin, C.S., Warren, J.R., Murray,

- R., Blinkow, E.D., Blackbourn, S.J., Phillips, M., Waters, T.E., and Sanderson, C.R. 1988. Prospective double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pylori*. *Lancet* **i**: 1437-1442.
- 18) Megraud, F., Bonnet, F., Garnier, M., and Lamouliatte, H. 1985. Characterization of "Campylobacter pyloridis" by culture, enzymatic profile, and protein content. *J. Clin. Microbiol.* **22**: 1007-1010.
- 19) Moran, A.P., Upton, M.E. 1986. A comparative study of the rod and coccoid forms of *Campylobacter jejuni*. ATCC 29428. *J. Appl. Bacteriol.* **60**: 103-110.
- 20) Moss, S., and Calam, J. 1992. *Helicobacter pylori* and peptic ulcers: the present position. *Gut* **33**: 289-292.
- 21) Ormand, J.E., Talley, N.J., Shorter, R.G., Conley, C.R., Carpenter, H.A., Fich, A., Wilson, W.R., and Phillips, S.F. 1991. Prevalence of *Helicobacter pylori* in specific forms of gastritis. *Dig. Dis. Sci.* **36**: 142-145.
- 22) Peterson, W.L. 1991. *Helicobacter pylori* and peptic ulcer disease. *N. Engl. J. Med.* **324**: 1043-1048.