

Cocoid Form of *Helicobacter pylori* as a Morphological Manifestation of Cell Adaptation to the Environment^{∇†}

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After characterization of preferred conditions for *Helicobacter pylori* survival in the sessile state, it was observed that the bacterium transforms from spiral to cocoid under mild circumstances, whereas under extreme ones it is unable to undergo shape modification. This strongly supports the view that transformation into the cocoid form is an active, biologically led process, switched on by the bacterium as a protection mechanism.

All living organisms are equipped with mechanisms that allow extended survival under adverse environments. For a number of them, this response involves, besides metabolic adaptations, changes in cell morphology (24). Similarly, the gastrointestinal pathogen *Helicobacter pylori* is known to mainly present a spiral shape in the natural habitat within the human host, but it converts into a cocoid shape when exposed to detrimental environmental circumstances (2). In this case, however, the pleiomorphic nature of the bacterium has been the subject of intensive debate over the last 10 years, with part of the scientific community still maintaining that the cocoid shape represents a degraded, nonviable form of the cell (8, 13, 19, 25). There are several factors contributing to this situation. (i) When *H. pylori* transformation into the cocoid form occurs, the cells enter a nonculturable state and are unable to be revived when placed under optimum growth conditions. (ii) Reversion trials (i.e., transformation from the cocoid form to the spiral form) have not been successful so far. (iii) There appears to be little metabolic activity and modification of physiology of the bacterium during conversion. (iv) Transformation to the cocoid form always appears to occur in what are thought to be the most adverse environments when the cells have no chance of survival. On the other hand, several reports have argued that cocoid cells might constitute a survival strategy in adverse environmental conditions (4, 10, 11, 22). The main argument for this is the existence of a state named viable but nonculturable (18, 27, 29). Viable but nonculturable bacteria also tend to possess little activity, which provides an alternative explanation for some of the phenomena observed for *H. pylori*.

Culturability and membrane integrity of water-exposed *H. pylori* adhered to abiotic surfaces. Six strains of *H. pylori* were used in this study, four from culture collections (26695, J99,

NCTC 11637, and 60190) and two clinical isolates from the collection of the National Institute of Health in Lisbon, Portugal (968 and 1152). Cells from 2-day-old cultures were harvested from Columbia agar plates, suspended in 30 ml of autoclaved distilled water, and vortexed. The necessary quantity of this inoculum to obtain a final concentration of approximately 10⁷ CFU/ml (optical density of ~0.020) was then transferred to a bioreactor with 300 ml of distilled water. After 5 min, 10 ml of the suspension were dispensed into each well of a six-well tissue culture plate (Orange Scientific, Braine-l'Alleud, Belgium) containing coupons of different materials. Coupon preparation has been already described elsewhere (3).

One of the problems encountered in earlier studies was to actually recover culturable *H. pylori* at statistically meaningful levels from abiotic surfaces using standard methods (3, 5). It has been found that *H. pylori* is particularly sensitive to sonication and that a 5-s burst at 25% amplitude (GEX 400 ultrasonic processor; Sigma) optimized recovery as opposed to the 1-min cycles more often used in the laboratory to detach and recover microorganisms from heterotrophic biofilms. An obvious concern of having such a short sonication time was that not all the cells were removed from the surfaces. We have therefore analyzed coupons exposed to *H. pylori* for different times after sonication for 5 s by scanning electron microscopy and confirmed that more than 99% of the cells were removed for all materials. The ease by which *H. pylori* is detached from the surface is perhaps due to the apparent lack of extracellular polymer production of the bacterium under these conditions.

After optimization of the detachment procedures, we were able to study the culturability of adhered *H. pylori* over time (Fig. 1A). All strains demonstrated similar behavior, showing that copper and galvanized iron surfaces are deleterious for the survival of the bacterium. A Kruskal-Wallis analysis showed that the results for the different materials were statistically significant. For all strains, the number of culturable cells on the surface increased up to 10⁴ to 10⁶ CFU/cm² in the first 2 h due to the initial adhesion process. Even though it has been previously shown that the total number of cells adhered continues to rise until 48 h (3, 5), culturable cell numbers started to decrease after only 2 hours (for polyvinyl chloride [PVC] and

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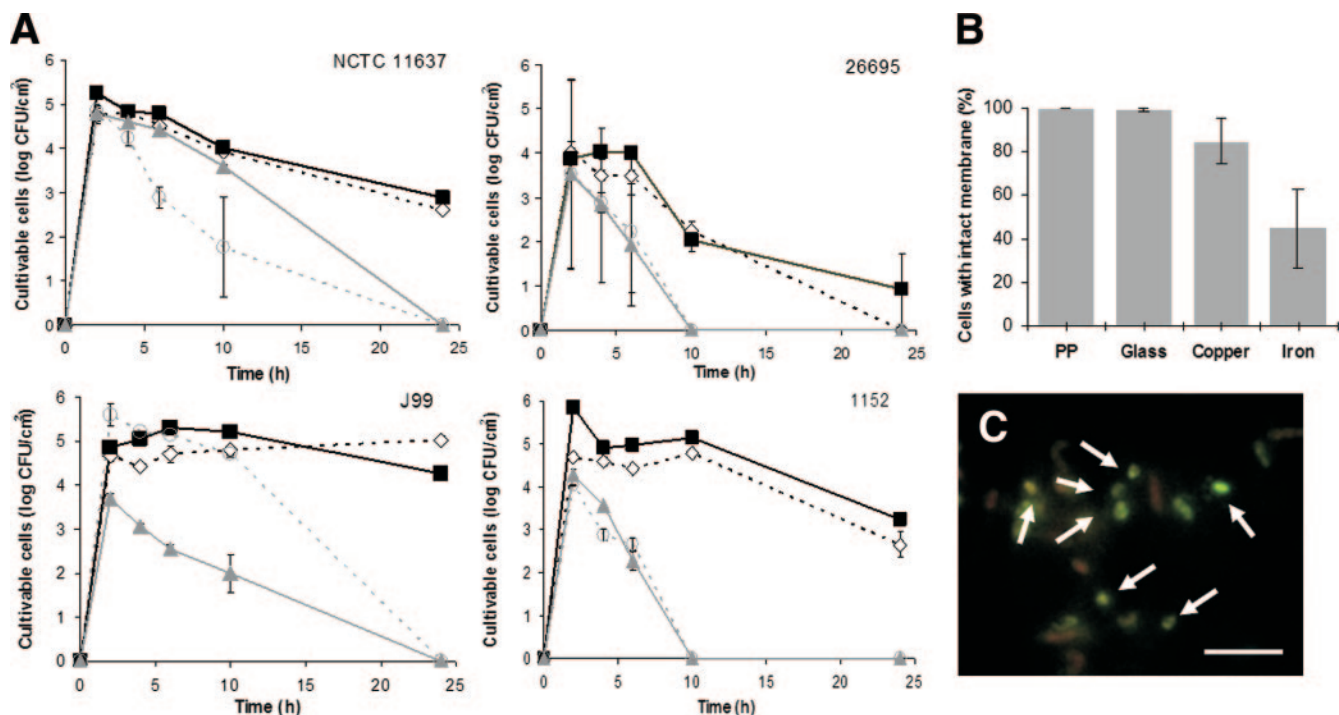


FIG. 1. Detrimental effect of copper and galvanized iron on *H. pylori* survival. (A) Culturability of four strains of water-exposed *H. pylori* over time. Bacteria adhering to glass (■), PVC (◇), copper (▲), and galvanized iron (○) were studied. (B) SYTO9/propidium iodide results obtained for strain 26695 after 24 h of exposure to different materials. Due to the strong autofluorescence of PVC in the red channel, the material was replaced in this experiment with polypropylene (PP), which was shown to induce similar behavior in *H. pylori* (3). The remaining graphs, both for culturability and membrane integrity, can be found in the supplemental material. (C) SYTO9/PI staining showing that coccoid cells appear to retain intact membranes for longer (arrows point to green cocci). The scale bar corresponds to 5 μm .

glass and strain J99, the numbers stabilized). This effect was partly expected, as the culturability time for *H. pylori* in water at this temperature is quite low (1, 4). The decline was much steeper for the metallic materials (copper and galvanized iron) than for glass and PVC. After 24 h, no culturable cells could be recovered from the metallic surfaces for any of the strains tested, which contrasted with the values of 10^1 to 10^4 CFU/cm² obtained for glass and of 0 to 10^5 CFU/cm² for PVC. In previous work, we have shown that the total numbers of *H. pylori* adhered to different materials were of the same order of magnitude (3).

To confirm the results obtained by cultivation methods, we have also assessed the membrane integrity of *H. pylori* on different surfaces using the SYTO9/propidium iodide double staining procedure where intact cells can take up SYTO9 and their DNA is stained with the green fluorochrome while the larger red fluorescent propidium iodide molecule is excluded but can cross compromised cell membranes to stain the DNA; hence, green labeled cells are considered alive and red cells are considered dead. Again, copper and galvanized iron appeared to induce more damage in the cell wall than other materials (Fig. 1B), which is in agreement with a preliminary, nonquantitative assessment that we had already performed (3). More importantly, we were also able to observe that coccoid cells would consistently take longer to stain completely or partially red than the spiral ones (Fig. 1C).

Copper toxicity to planktonic *H. pylori*. Another parameter analyzed during the experiments was the culturability of *H.*

pylori in the planktonic state after 24 h for strains 26695 and 1152 (Fig. 2A). In accordance with the results obtained in Fig. 1, no *H. pylori* could be recovered for the wells where copper and galvanized iron coupons were inserted. Leaching of both iron and copper into the liquid phase is well documented in the literature and is even the cause for some human health concerns when it happens in pipes of the drinking water distribution system (DWDS) (16, 21). Atomic absorption spectroscopy analysis by acetylene flame (Varian Spectra AA-250 Plus) proved that copper leached into the water, causing *H. pylori* cells to enter more quickly into a nonculturable state (Fig. 2B).

Transition metals (such as copper) are usually toxic in excess, but a number of them are also essential trace elements. The levels/concentrations at which copper is toxic certainly depend on the species under study. For instance, the recognition of a copper export system and a copper resistance determinant in *H. pylori* could suggest a higher tolerance of the bacterium for this metal (7, 15, 28). On the other hand, during the characterization of nutritional requirements for *H. pylori*, Testerman et al. determined that copper supplementation of a defined medium was clearly not required (26). In the present study, the results obtained point to an antimicrobial activity of copper at concentrations lower than 1 mg/liter on *H. pylori*. Overall, this behavior is very similar to that of *Campylobacter jejuni* (14), even though the methods used by both studies were slightly different. The potential to control microbial populations on solid supports due to the inhibitory properties of copper has also been well documented for a number of other

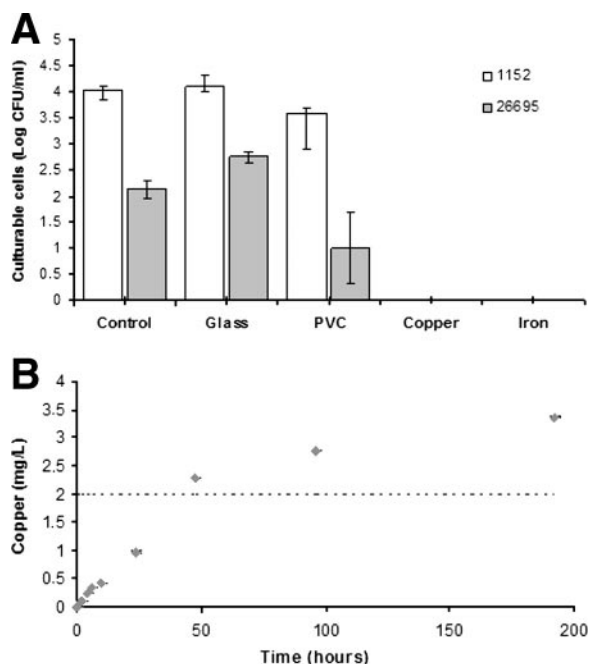


FIG. 2. Culturability of water-exposed *H. pylori* in the planktonic phase and negative effect of copper ions. (A) The culturability of two *H. pylori* strains after 24 h confirms that the deleterious effect of the metals for adhered cells is also observed for cells in the planktonic phase. (B) Using atomic absorption spectroscopy, we confirmed that copper ions were present in suspension at relatively high concentrations. As a reference, the dashed horizontal line represents the internationally accepted maximum value allowed for copper in drinking water, 2.0 mg/liter, and was intersected in our experiments between 24 and 48 h. For the control experiment, no coupons were inserted in the wells.

microorganisms, including *Escherichia coli* and *Salmonella enterica* (14, 20). Interestingly, copper-based compounds are being developed as an alternative to the antibiotics currently used to treat *H. pylori* infection (17).

Relationship between morphology and culturability of *H. pylori*. Based on this evidence and on the fact that coccoid cells form under more adverse circumstances, one would expect that the coccoid form would be more predominant on copper sur-

faces than on the other surfaces. However, and when looking at Fig. 3A, the opposite can be observed: after 192 h of exposure, the coccoid form predominates on glass and PVC but not on copper. To simplify the analysis, a plot of the area below the surface of Fig. 1A versus the percentage of coccoid cells for each strain and surface can be found in Fig. 3B. A clear discrimination between metallic and nonmetallic materials was achieved, with larger areas (and consequently longer culturability times) and percentages of coccoid cells corresponding to nonmetallic materials. This observation gave the first hint that the coccoid shape could in fact be a manifestation of cell adaptation to the environment. It is also interesting to state that discrimination between different materials was evident only after at least 24 h. Up until then, morphological values for cells on different materials were similar (data not shown), which implies that differentiation tends to start after loss of culturability.

Adhesion to copper surfaces and morphology of *H. pylori* when suspended in F-12 medium. Extending the previous results, the time that the cells were exposed to copper and PVC was increased to up to 2 months. The percentage of adhered coccoid cells tended to stabilize with time, but the total number of adhered cells started to differentiate on both materials: on copper, the number of cells continued to increase and the cells even managed to agglomerate sparsely into three-dimensional structures (Fig. 4A), while on PVC, the number of cells started decreasing and cells were nearly absent after this time period. Aggregation and adhesion ability are sometimes referred as ways to assess cell viability (12), but as copper was shown to be a biocidal agent, this study also suggests that adhesion does not necessarily imply viability of a cell and can be governed by purely physical processes.

To further pursue the indications provided by the adhesion of water-exposed *H. pylori* to copper, we devised another experiment with the following rationale: if the water-exposed pathogen was unable to transform on copper due to the deleterious effect of the metal, then suspending *H. pylori* in rich nutrient medium might hopefully provide sufficient protection in order to allow conversion. For that, a suspension of *H. pylori* in F-12 medium was placed in contact with a copper surface. Surprisingly, total conversion of all the cells to the coccoid

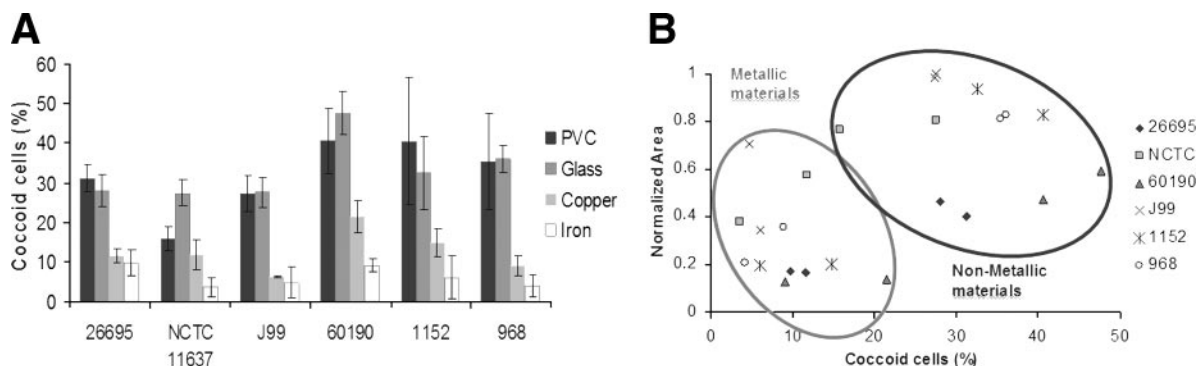


FIG. 3. Correlation between coccoid shape and increased culturability of *H. pylori*. (A) Percentage of coccoid cells on the surfaces of different materials after 192 h as assessed by scanning electron microscopy. Because of the formation of ferrous deposits on the surface of galvanized iron, data obtained for this material used acridine orange staining and epifluorescence microscopy and refer to 4-h exposure only. (B) Plot of the surface area of Fig. 1A versus the percentage of coccoid cells.

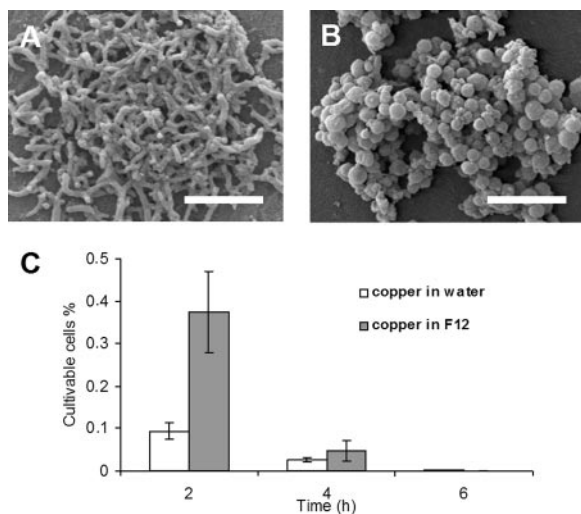


FIG. 4. High-nutrient conditions elicit a response from *H. pylori* in terms of morphology. (A) Spiral cells adhered to copper after 2 months in water; (B) coccoid cells after 48 h in F-12 broth. The scale bars correspond to 5 μm . (C) F-12 broth provides a protective environment for *H. pylori* when this bacterium is adhered to copper. Strain 60190 is shown here as having a higher percentage of culturability in this rich nutrient medium.

form occurred in only 48 h for some strains, which contrasted with the maintenance of the spiral shape in water up until 2 months (Fig. 4B). To ensure that F-12 medium was indeed helping bacterial physiology, the number of culturable cells adhered to copper was controlled. Because in this case the total number of adhered cells is much lower than the one obtained for *H. pylori* exposed to water, comparison between both situations is expressed as a percentage of culturable count (Fig. 4C). As expected, the use of F-12 medium is allowing statistically significant higher percentages of recovery, which indicates the protective effect of the medium, possibly by the neutralization of reactive copper ions in the liquid phase.

Conclusions. Taking all the results together, this study demonstrates that the coccoid shape is in fact a manifestation of cell adaptation to less than optimum environments as the bacterium moves into a viable but nonculturable state. The immediate conclusion from this is that making inferences about *H. pylori* physiology on the basis of morphology, which has been done regularly for the last years, is certainly a flawed approach at least when cells are found in the environment. It was shown here that spiral cells of *H. pylori* can be divided into two categories: one form is the culturable, growing and more infectious state of the bacterium that exists while under optimum conditions for replication, and the other form is certainly "less fit" than coccoid counterparts. Furthermore, coccoid forms have also been classified into three types which the authors claim to represent different transformation processes and consist of the dying bacteria, the living ones with culturability, and the viable but nonculturable ones (22). A more profound understanding of each of these morphological manifestations in terms of molecular biology is now needed to fully understand the mechanisms involved and gain novel knowledge in the life cycle of the bacterium.

A more rational search for the bacterium in DWDS can now

also be accomplished. For instance, the substratum where the pathogen adheres in higher quantities and for longer periods of time is copper. Consequently, it should be more likely to find *H. pylori* by PCR in copper pipes of DWDS than on any other type of material. However, and because copper is deleterious to the bacterium's survival, the best chance for recovery using standard plating procedures is on polymeric surfaces. Besides copper and iron plumbing, areas of the DWDS with high shear stresses (5) and effective chlorination (6) are unlikely environmental reservoirs for *H. pylori*. In fact, the existence of these factors in most DWDSs might have contributed to the decreasing prevalence of *H. pylori* in developed countries. Nevertheless, biofilms are prolific in microenvironments, and the possibility of areas where the bacterium survives cannot be excluded. Future work on screening different types of surface-related microenvironments will allow confirmation of hypotheses developed on the data already acquired on pure culture studies (3, 5) and in developing new hypotheses.

Finally, this study also brings new insights to the *H. pylori* transmission debate. The majority support of the direct person-to-person transmission resided on the fact that nonculturable coccoid cells would be dead. Coupling the results obtained in this study with ones that have indicated that coccoid forms might be able to infect mice (9, 23) suggests that alternative routes of infection are possible. The decreasing prevalence of *H. pylori* infection found in the developed countries has been repeatedly attributed to changes in host lifestyles, but it may be time to consider the ability of the pathogen to adapt to different ecosystems (such as the introduction and dissemination in DWDSs) and whether relatively simple decisions, such as the choice of plumbing materials in buildings, are a major event to allow or prevent transmission of this important global pathogen.

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