Coccoid form of *Helicobacter pylori* as a morphological manifestation of cell adaptation to the environment

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

Keywords: *Helicobacter pylori*; morphology; copper; viable but non-culturable (VBNC); biofilms

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 converts into a coccoid 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 ten years, with part of the scientific community still maintaining that coccoid morphology represents a
degraded, nonviable form of the cell (8, 13, 19, 25). There are several factors contributing to this situation: 1) When *H. pylori* transformation into coccoid morphology occurs, the cells enter a nonculturable state and are unable to be revived when placed under optimum growth conditions; 2) Reversion trials (i.e. transformation from coccoid to spiral) have not been successful so far; 3) There appears to be little metabolic activity and modification of physiology of the bacterium during conversion and; 4) Transformation to coccoid form always appears to occur in what are thought to be the most adverse environments, where cells have no chance of survival. On the other hand, several reports have argued that coccoid 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 denominated viable but nonculturable (VBNC) (18, 27, 29). VBNC 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 (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 approx. $10^7$ CFU/ml (O.D. ~ 0.020) was then transferred to a bioreactor with 300 ml of distilled water. After 5 min, 10 ml of the suspension were dispensed in to wells of a 6-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 5s burst at 25% amplitude (GEX 400 Ultrasonic Processor; Sigma) optimized the 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 by having such a low sonication time was that not all the cells were removed from the surfaces. We have therefore analysed coupons exposed to *H. pylori* for different times after sonication for 5s by scanning electron microscopy (SEM) 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 time of adhered *H. pylori* (Fig.1A). All strains demonstrated similar behaviour, showing that copper and galvanized iron surfaces are deleterious for the survival of the bacterium. A Kruskall-Wallis analysis showed that the results were statistically significant between different materials. For all strains the number of culturable cells on the surface increased up to $10^4-10^6$ CFU/cm$^2$ in the first 2 hours 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 hours (3, 5), culturable cell numbers started to decrease after only two hours (in the case of PVC and glass for 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 hours, no culturable cells could be recovered from the metallic surfaces for any of the strains tested, which contrasted with the values of $10^1-10^4$ CFU/cm$^2$ obtained for glass, and of $0-10^5$ CFU/cm$^2$ for PVC. In previous work, we have shown that the total number of *H. pylori* adhered to different materials was in the same order of magnitude (3).

To confirm the results obtained by cultivation methods, we have also assessed membrane integrity of *H. pylori* on different surfaces using the SYTO9/propidium iodide double staining procedure with time, 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 labelled cells are considered alive, red
cells dead (Fig. 1B). Again, copper and galvanized iron appeared to induce more damage in the cell wall than other materials which is in agreement with a preliminary, non-quantitative 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 analysed during the experiments was the culturability of *H. pylori* in the planktonic state after 24h 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 drinking water distribution system (DWDS) pipes (16, 21). Atomic absorption spectroscopy analysis by acetylene flame (Varian Spectra AA-250 Plus) proved that copper leaching occurred into the water, causing *H. pylori* cells to enter more quickly into a non-culturable state (Fig. 2B). As a reference, the dashed horizontal line represents the internationally accepted maximum value allowed for copper in drinking water, which is 2.0 mg/L and was intersected in our experiments between 24 and 48 hours.

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 depends on the species under study. For instance, the recognition of a copper export system and a copper resistance determinant in *H. pylori* could have suggested 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, results obtained point to an antimicrobial activity of copper at concentrations lower than 1 mg/L on *H. pylori*. Overall, this behaviour 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 microorganisms, including *E. 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 on the more adverse circumstances, one would expect that the coccoid morphology would be more predominant on copper surfaces than on the others. However, and when looking at Fig. 3A, the opposite can be observed: after 192h exposure, coccoid morphology 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 on Fig. 3B. A clear discrimination between metallic and non-metallic materials was achieved, with larger areas (and consequently larger culturability times) and percentage of coccoid cells corresponding to non-metallic materials. This observation gave the first hint that coccoid morphology could be in fact a manifestation of cell adaptation to the environment. It is also interesting to state that discrimination between different materials was only evident after at least 24 hours. Up until then, morphological values between different materials were similar (data not shown), which implies that differentiation tends to start after loss of culturability.

**Adhesion and morphology of *H. pylori* to copper surfaces when suspended in F-12**

As an extension for the previous results, the time at which cells were exposed to copper and PVC was increased for 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 they continued to increase and even managed to agglomerate sparsely into 3D structures (Fig. 4A), while on PVC they started decreasing and cells were nearly absent after this time period. Aggregation and
adhesion ability is sometimes referred as a way 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 cell morphology to the coccoid form occurred in only 48 hours for some strains, which contrasted with the maintenance of spiral morphology in water up until 2 months (Fig. 4B). To ensure that F-12 was indeed helping the 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 the percentage of culturable counts (Fig. 4C). As expected, F-12 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**

Taken altogether, this study demonstrates that coccoid morphology 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 inferring 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: where one is the culturable, growing and more infectious state of the bacterium and exists while under optimum conditions for replication, and the other 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 non-culturable 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 for the bacterium’s survival, the best chance for a recovery using standard plating procedures should be 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 DWDS 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 the screening of 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 ones.

Finally, this study also brings new insights for the *H. pylori* transmission debate. The majority support of the direct person-to-person transmission resided on the fact that non-culturable coccoid cells would be dead. Coupling the results obtained in this study with ones that have indicated that coccoid forms might be able 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 DWDS) and whether relatively simple decisions such as choice of plumbing materials in the built environment are a major event to allow or prevent transmission of this important global pathogen.
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References


Figure 1 Detrimental effect of copper and galvanized iron on *H. pylori* survival. (A) Culturability of four strains of water-exposed *H. pylori* with time when adhered to glass (■), PVC (●), copper (▲) and galvanized iron (○). (B) SYTO9/PI results obtained for strain 26695 after 24 hours exposure to different materials. Due to strong autofluorescence of PVC in the red channel the material was replaced in this experiment with polypropylene (PP), which was shown to induce similar behaviour in *H. pylori* (3). The remaining graphs, both for culturability and membrane integrity, can be found as supplemental material. (C) SYTO9/PI staining showing that coccoid cells appear to retain intact membranes for longer (arrows point to green cocci). Scale bar corresponds to 5 µm.
Figure 2 Culturability of water-exposed *H. pylori* in the planktonic phase and negative effect of copper ions. (A) The culturability of 2 *H. pylori* strains after 24 hours 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. For the control experiment no coupons were inserted in the wells.
Figure 3 Correlation between coccoid morphology and increased culturability of *H. pylori*. (A) Percentage of coccoid cells on the surface of different materials after 192 hours as assessed by SEM. Because of the formation of ferrous deposits on the surface of galvanized iron, data obtained for this material used the acridine orange staining under EF microscopy and refers to 48 hours exposure only. (B) Plot of the surface area of Fig. 1 versus the percentage of coccoid cells.
Figure 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) and coccoid cells after 48h in F-12 broth. Scale bars correspond to 5 µm. (C) F12 provides a protective environment for *H. pylori* when this bacterium is adhered to copper. Strain 60190 is shown here as having higher percentage of culturability in this rich-nutrient medium.