Maintenance of spiral morphology and formation of biofilms on copper surfaces by water-exposed *Helicobacter pylori*

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Abstract There is still a lack of consensus on the way *Helicobacter pylori* is transmitted, but biofilms in drinking water are suspected to create a safe haven for the subsistence of the bacterium and hence promote a waterborne route of infection. Cultivability in water is low when compared to other waterborne pathogens, a situation that is attributed to the conversion of the highly infectious spiral form to the generally regarded as inactive coccoid form of the bacterium. In this study, the adhesion ability to abiotic surfaces (such as copper and PVC), morphology and cultivability of water-exposed *H. pylori* was assessed. In copper, the bacterium was able to retain spiral morphology and form 3D structures for over two months whereas in PVC conversion to the coccoid form occurred in approximately 1 week. Even though the logical interpretation for these results would be that copper favours the bacterium survival, standard plating experiments have shown precisely the opposite: cultivability decreases faster for the cells exposed to copper. Besides demonstrating the deleterious action that copper surfaces have on *H. pylori*, this study also indicates that at least for this case, *H. pylori* coccoid morphology is in fact a manifestation of cell adaptation to the environment.

Keywords: Biofilms; drinking water; *Helicobacter pylori*; morphological aspects; viability.

Introduction

Infection with *Helicobacter pylori* may cause the development of gastritis, gastric and duodenal ulcers, mucosa-associated lymphoid tissue lymphoma, loss of gastric glands (atrophy) and finally adenocarcinoma, a disease with very high morbidity and mortality (Moran *et al.* 2002). Nearly since the discovery of this microorganism by recent Nobel prize winners Marshall and Warren (Marshall and Warren 1984), the scientific community has put in their best efforts to determine how is this pathogen able to infect 50% of the human population.

As humans are currently the only known reservoir for *H. pylori*, transmission of the infection appears to be fecal-oral, gastric-oral or oral-oral (Sherman 2004). Direct person-to-person transmission has however failed to provide enough evidence to account for all infected individuals. Therefore, external environmental reservoirs such as water and associated biofilms have been suggested to harbour the bacterium. To support this view, there are a growing amount of data reporting the identification of the bacterium in water systems using molecular techniques (e. g. Park *et al.* 2001, Watson *et al.* 2004). Nevertheless, a waterborne route of infection for *H. pylori* has been repeatedly dismissed on the basis that the bacterium loses its viability status very rapidly by quickly transforming itself into a non-viable coccoid shaped cell once exposed to water.

The morphological aspects of *H. pylori* have been the subject of intensive debate over the last ten years. Because conversion from spiral to the coccoid shape is induced with exposure to detrimental environmental circumstances (Andersen and Wadstrom 2001), the wider view up until a few years ago was that the latter represented a degrading, nonviable form of the bacterium (Sorberg *et al.* 1996, Kusters *et al.* 1997, Enroth *et al.* 1999). However, several recent reports have now argued that

they might constitute a survival strategy in adverse environmental conditions (e. g. Saito *et al.* 2003, Azevedo *et al.* 2004, Cellini *et al.* 2004, Citterio *et al.* 2004). This issue is of extreme importance because the characterization of biofilm formation by water-exposed *H. pylori* has indicated that up to one week there was a very significant difference between the morphology of the cells adhering to copper and the cells adhering to other materials (Azevedo *et al.* 2006a). However, the study provides no information on cultivability of adhered cells (due to the lack of an appropriate method at the time), and even though spiral morphology was kept for longer in copper, viability stains (Syto9/PI) suggested that the coccoid form constitutes a survival strategy in adverse environmental conditions.

In the present work, different strains of *H. pylori* were suspended in water and tested for their adhesion ability to abiotic surfaces (namely copper, PVC, cast iron and glass) while their cultivability was monitored with time. The main purpose was to establish whether copper plumbing might had or not a protective role for *H. pylori* in drinking water distribution systems (DWDS).

Methods

Culture maintenance

The four strains of *H. pylori* (three culture collection strains and one clinical isolate) were maintained on Columbia Agar (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defribinated horse blood (Biomérieux, Marcy l'Etoile, France). Plates were incubated at 37 °C in a CO_2 incubator (HERAcell® 150; Thermo Electron Corporation, Waltham MA, USA) set to 10% CO_2 and 5% O_2 and streaked onto fresh plates every 2 or 3 days.

Test surfaces preparation

Coupons measuring 2 by 2 cm were prepared at the Centro de Engenharia Biológica from copper, polyvinyl chloride (PVC), galvanized iron (all from Neves e Neves, Trofa, Portugal) and glass (slides 75×25mm, Moreira Costa e Santos, Porto, Portugal). Copper coupons were polished with an alumina suspension (Struers, Copenhagen, Denmark). All materials were immersed in a solution of 5% (v/v) commercial detergent (Top Neils, Tengelmann Portugal, Sintra, Portugal) and pre-warmed distilled water for 30 min while gently mixed. To remove any remaining detergent, coupons were rinsed five times in ultrapure water and air dried. They were subsequently immersed in 90% (v/v) ethanol for 30 min. After being rinsed with ultra-pure water, air dried and wrapped in foil, galvanized iron, copper and glass coupons were autoclaved for 15 min at 121 °C, whereas PVC was heated for 20 min at 70 °C. Coupons were finally placed in wells of a 6-well tissue culture plate (Orange Scientific, Braine-l'Alleud, Belgium).

Suspension preparation and inoculation

Cells from 2 day-old cultures were harvested from Columbia Agar plates, suspended in 30 ml of autoclaved distilled water and vortexed for 30s. 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 sterile bioreactor containing 300 ml of distilled water. The bioreactor was maintained at room temperature (21±2°C) and continuously stirred using a magnetic bar. After 10 min, 10mL of the suspension were dispensed in to each of the wells containing the coupons. At various times of exposure, one coupon of each material was removed from the well and rinsed three times in distilled water. Two types of experiments were performed using this method. The first one assessed adhesion of NCTC 11637 to copper and PVC surfaces after two months exposure by scanning electron microscopy (SEM), whereas the second assessed adhesion, together with cultivability for all strains and materials at different time points up to 24 hours.

Scanning electron microscopy

Coupons with 2 months of exposure were immersed for 15 minutes in solutions with increasing concentrations of ethanol up to 100% (v/v), and placed in a sealed desiccator. The samples were

mounted on aluminium stubs with carbon tape, sputter coated with gold and observed with a Leica Cambridge S-360 scanning electron microscope (SEM) (Leo, Cambridge, UK). The SEM was equipped with an X-ray analyzer, which allowed the identification and quantification of certain atomic elements (such as oxygen) on the surface of the coupons.

Cultivable cell counts of planktonic and adhered bacteria

Cultivable cells of planktonic *H. pylori* were analyzed temporally by plating 100 μL of the appropriate dilution on to Columbia Blood Agar (CBA) plates. For the determination of cultivable adhered bacteria, the coupon was placed in wells of a new 6-well tissue culture plate and immersed in 10 mL of distilled water while under refrigeration. Sonication was undertaken for 5s bursts and 25% amplitude (GEX 400 Ultrasonic Processor; Sigma). Then, 100 μL of the distilled water containing detached cells (or appropriate dilutions) were dispensed on CBA. Plates were incubated microaerophilically at 37 ºC for 6 days to determine the numbers of cultivable *H. pylori*.

Confirmative procedures and analysis of data

Besides checking for typical colony morphology (i.e., round, translucent to yellowish, convex, 0.2-2 mm) (Andersen and Wadstrom 2001), hybridization with a specific peptide nucleic acid (PNA) probe to 16S rRNA (Azevedo *et al.* 2003) was performed to confirm the identity of *H. pylori* isolated in the Petri dishes and adhered to the surfaces.

Results and Discussion

When studying the kinetics of adhesion of *H. pylori* NCTC 11637 to copper for up to two months, it was noticed that the bacterium would predominantly form three-dimensional aggregates of approx. 100-500 cells. These structures appear sparsely after one week of contact, but by the end of two months, the oxidized area of the copper surface becomes completely colonized. One interesting aspect is that they only appeared in the brighter areas of the copper coupon (Figure 1). These brighter areas were identified by the X-ray analyzer as the non-oxidized areas of the coupon. These biofilm-like aggregates were not visible on other types of material (such as PVC) and are totally composed by spiral shaped *H. pylori*, the morphology associated with the pathogenicity of the bacterium. In fact, the numbers of cells on PVC coupons after 2 months was lower than after 1 week [results from 1 week as obtained in (Azevedo *et al.* 2006a)], denoting that detachment occurred during this time period. Aggreggation and adhesion ability is usually referred as a way to assess cell viability (Davey and O'toole 2000), and it was therefore suspected that copper surfaces were able to provide protection for *H. pylori* in DWDS. Nevertheless, cultivability data were still lacking and it would provide the necessary information to solve this conundrum.

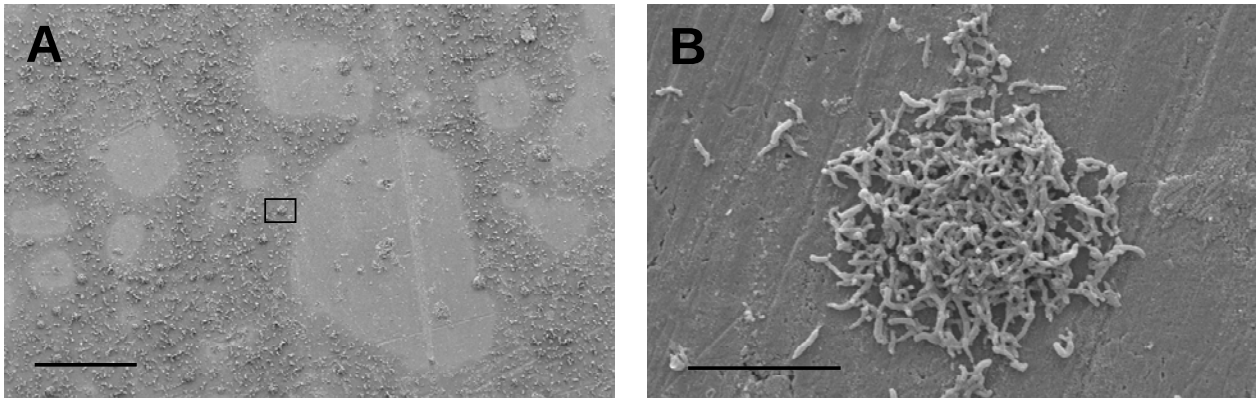

Figure 1 SEM images of the adhesion of *H. pylori* to a copper surface after 2 months of exposure to water. Contrary to what was observed with other type of materials, the copper surface was heavily colonized after two months. Interestingly, this colonization was irregular, as most aggregates were to be found in the more oxidized areas of the coupon. Scale bar, 200 μm (a). In-set of the Figure 1a

showing the formation of aggregates by *H. pylori* in detail. As it can be seen, nearly all cells retain the spiral shape morphology, associated with the infectious state of the bacterium. There is also no evidence of the production of extracellular polymeric substances. Scale bar, 10 μm (b).

Because in an earlier work (Azevedo *et al.* 2006a), we were unable to recover the minimum number of colonies necessary to compare the cultivability of *H. pylori* adhered to different materials, several sonication times were tested to optimize the procedure. Sonication cycles of 1 min have been commonly used in our laboratory to maximize the number of cells from heterotrophic biofilms formed in drinking water systems. Tests have showed that after sonication for 5s, more than 99% of *H. pylori* cells are detached, maybe due to the apparent lack of extracellular polymer production of the bacterium under these conditions. This percentage of removal was obtained for different materials and different adhesion times tested. Furthermore, *H. pylori* proved to be particularly sensitive to sonication procedures, as a nearly 2 log increase in cultivable cells was obtained between sonication times of 5s and 1min.

After the optimization of the detachment procedures, cultivation experiments have shown that both copper and galvanized iron supports were deleterious for the bacterium survival (Figure 2). For all strains the number of cultivable 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 (Azevedo *et al.* 2006a, Azevedo *et al.* 2006b), cultivable 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 survival time (as assessed by cultivation methods) for *H. pylori* in water at this temperature is quite low (Adams *et al.* 2003, Azevedo *et al.* 2004). The decline was much steeper for the metallic materials (copper and galvanized iron) than for glass and PVC. After 24 hours, no cultivable 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.

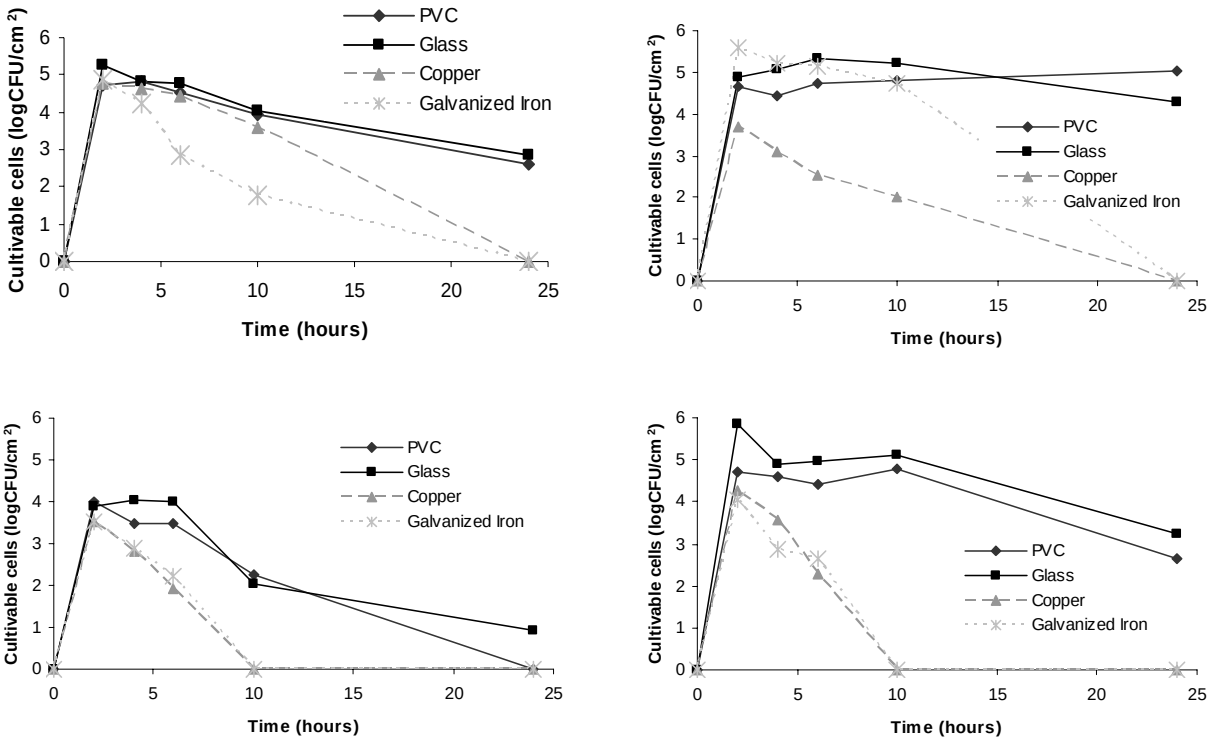

Figure 2 Cultivability of adhered *H. pylori* in different materials over time for 4 of the strains tested, NCTC 11637 (top left), J99 (top right), 26695 (bottom left) and clinical isolate 1152 (bottom

right). In a previous work, we have shown that the total number of *H. pylori* adhered to different materials was in the same order of magnitude (Azevedo *et al.* 2006a).

Another parameter analysed during the experiment was the cultivability of *H. pylori* in the planktonic state after 24h (Figure 3). In accordance with the results obtained in Figure 2, for the wells where copper and galvanized iron coupons were inserted, no *H. pylori* could be recovered. Leaching of both iron and copper from DWDS pipes is well documented in the literature, and is even the cause for some human health issues (Sadiq *et al.* 1997, Georgopoulos *et al.* 2001). PVC caused a slight decrease of the numbers of bacteria in the water, whereas the insertion of a glass coupon appeared to have no effect on the cultivable counts.

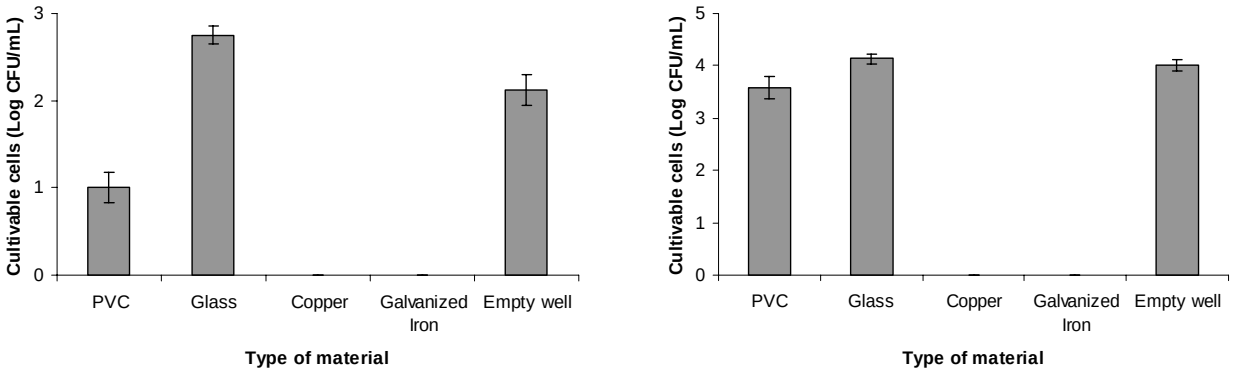

Figure 3 Cultivability of planktonic *H. pylori* after 24 hours for the strain 26695 (left) and clinical isolate 1152 (right) when exposed to coupons of different materials. In the empty well, no coupon was inserted.

H. pylori is one of the few bacteria that has been shown to possess a copper transporter system (Ge *et al.* 1995), which could suggest a higher tolerance of the bacterium for this heavy metal. However, in this study copper and iron proved to be harmful for *H. pylori*. Copper has also been indicated as an effective material to control bacterial growth in several situations (Domek *et al.* 1984, Rogers *et al.* 1994, Wilks *et al.* 2005).
Besides copper and iron plumbing, areas of the DWDS with high shear stresses (Azevedo *et al.* 2006b) and effective chlorination (Baker *et al.* 2002), are unlikely environmental reservoirs for *H. pylori*. In fact, the existence of these factors in most DWDS might have contributed to the decreasing the prevalence of *H. pylori* in the developed countries. Nevertheless, biofilms are prosperous in microenvironments and the possibility of areas where the bacterium subsists cannot be excluded.

Conclusions

Copper and galvanized iron proved to have a very effective biocidal effect against *H. pylori*. The spiral shape maintenance of *H. pylori* in copper can therefore be interpreted as a fast, biocidal effect of the metal upon the pathogen, killing the cell before it has time to undergo the shape modification. For PVC coupons, the transformation into the coccoid morphology is in fact a manifestation of cell adaptation to the environment. This viability indication for different materials might be valuable for future studies attempting to recover *H. pylori* from DWDS.

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