

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

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

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

14 **Methods**

15 **Culture maintenance**

16 The four strains of *H. pylori* (three culture collection strains and one clinical isolate) were
17 maintained on Columbia Agar (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated
18 horse blood (Biomérieux, Marcy l'Etoile, France). Plates were incubated at 37 °C in a CO₂
19 incubator (HERAcell® 150; Thermo Electron Corporation, Waltham MA, USA) set to 10% CO₂
20 and 5% O₂ and streaked onto fresh plates every 2 or 3 days.

21 **Test surfaces preparation**

22 Coupons measuring 2 by 2 cm were prepared at the Centro de Engenharia Biológica from copper,
23 polyvinyl chloride (PVC), galvanized iron (all from Neves e Neves, Trofa, Portugal) and glass
24 (slides 75×25mm, Moreira Costa e Santos, Porto, Portugal). Copper coupons were polished with an
25 alumina suspension (Struers, Copenhagen, Denmark). All materials were immersed in a solution of
26 5% (v/v) commercial detergent (Top Neils, Tengelman Portugal, Sintra, Portugal) and pre-warmed
27 distilled water for 30 min while gently mixed. To remove any remaining detergent, coupons were
28 rinsed five times in ultrapure water and air dried. They were subsequently immersed in 90% (v/v)
29 ethanol for 30 min. After being rinsed with ultra-pure water, air dried and wrapped in foil,
30 galvanized iron, copper and glass coupons were autoclaved for 15 min at 121 °C, whereas PVC was
31 heated for 20 min at 70 °C. Coupons were finally placed in wells of a 6-well tissue culture plate
32 (Orange Scientific, Braine-l'Alleud, Belgium).

33 **Suspension preparation and inoculation**

34 Cells from 2 day-old cultures were harvested from Columbia Agar plates, suspended in 30 ml of
35 autoclaved distilled water and vortexed for 30s. The necessary quantity of this inoculum to obtain a
36 final concentration of approx. 10⁷ CFU/ml (O.D. ~ 0.020) was then transferred to a sterile
37 bioreactor containing 300 ml of distilled water. The bioreactor was maintained at room temperature
38 (21±2°C) and continuously stirred using a magnetic bar. After 10 min, 10mL of the suspension
39 were dispensed in to each of the wells containing the coupons. At various times of exposure, one
40 coupon of each material was removed from the well and rinsed three times in distilled water. Two
41 types of experiments were performed using this method. The first one assessed adhesion of NCTC
42 11637 to copper and PVC surfaces after two months exposure by scanning electron microscopy
43 (SEM), whereas the second assessed adhesion, together with cultivability for all strains and
44 materials at different time points up to 24 hours.

45 **Scanning electron microscopy**

46 Coupons with 2 months of exposure were immersed for 15 minutes in solutions with increasing
47 concentrations of ethanol up to 100% (v/v), and placed in a sealed desiccator. The samples were
48

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

6 **Cultivable cell counts of planktonic and adhered bacteria**

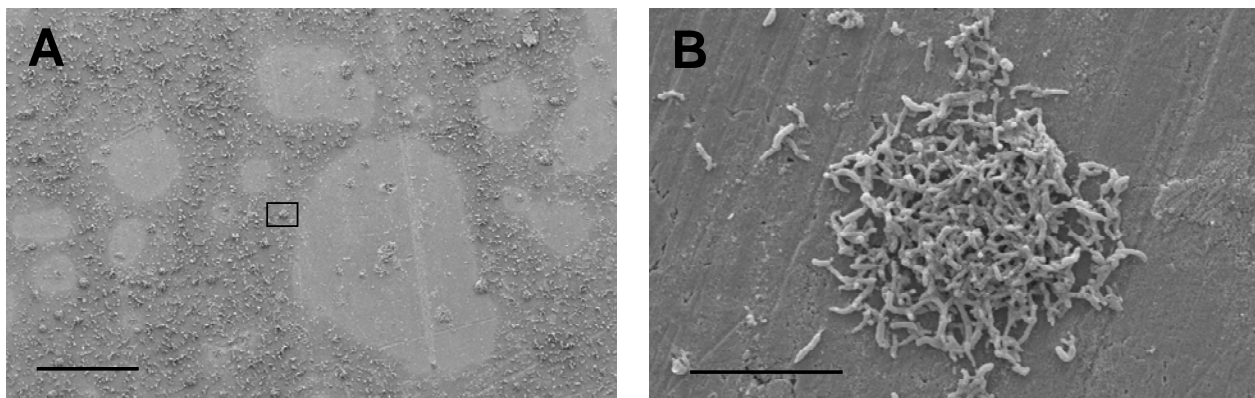
7 Cultivable cells of planktonic *H. pylori* were analyzed temporally by plating 100 μ L of the
8 appropriate dilution on to Columbia Blood Agar (CBA) plates. For the determination of cultivable
9 adhered bacteria, the coupon was placed in wells of a new 6-well tissue culture plate and immersed
10 in 10 mL of distilled water while under refrigeration. Sonication was undertaken for 5s bursts and
11 25% amplitude (GEX 400 Ultrasonic Processor; Sigma). Then, 100 μ L of the distilled water
12 containing detached cells (or appropriate dilutions) were dispensed on CBA. Plates were incubated
13 microaerophilically at 37 °C for 6 days to determine the numbers of cultivable *H. pylori*.

15 **Confirmative procedures and analysis of data**

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

21 **Results and Discussion**

22 When studying the kinetics of adhesion of *H. pylori* NCTC 11637 to copper for up to two months, it
23 was noticed that the bacterium would predominantly form three-dimensional aggregates of approx.
24 100-500 cells. These structures appear sparsely after one week of contact, but by the end of two
25 months, the oxidized area of the copper surface becomes completely colonized. One interesting
26 aspect is that they only appeared in the brighter areas of the copper coupon (Figure 1). These
27 brighter areas were identified by the X-ray analyzer as the non-oxidized areas of the coupon. These
28 biofilm-like aggregates were not visible on other types of material (such as PVC) and are totally
29 composed by spiral shaped *H. pylori*, the morphology associated with the pathogenicity of the
30 bacterium. In fact, the numbers of cells on PVC coupons after 2 months was lower than after 1
31 week [results from 1 week as obtained in (Azevedo *et al.* 2006a)], denoting that detachment
32 occurred during this time period. Aggregation and adhesion ability is usually referred as a way to
33 assess cell viability (Davey and O'toole 2000), and it was therefore suspected that copper surfaces
34 were able to provide protection for *H. pylori* in DWDS. Nevertheless, cultivability data were still
35 lacking and it would provide the necessary information to solve this conundrum.

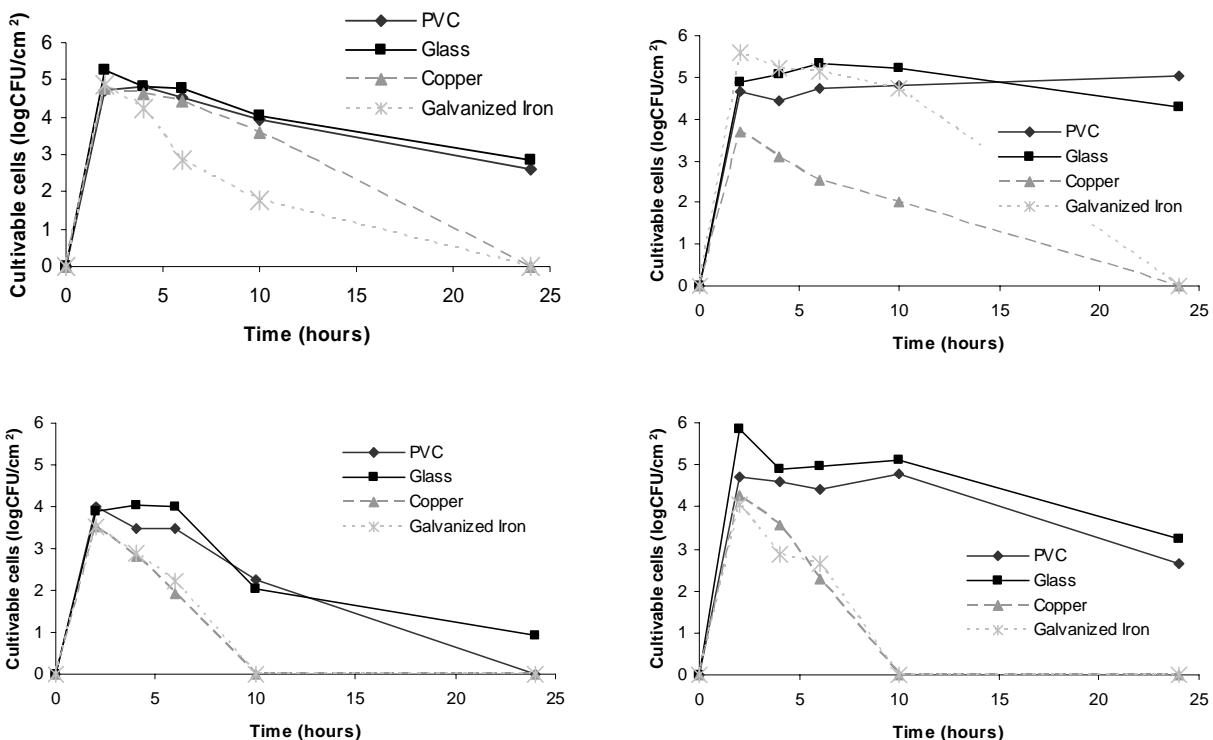


37 **Figure 1** SEM images of the adhesion of *H. pylori* to a copper surface after 2 months of exposure to
38 water. Contrary to what was observed with other type of materials, the copper surface was heavily
39 colonized after two months. Interestingly, this colonization was irregular, as most aggregates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

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

4
 5 Because in an earlier work (Azevedo *et al.* 2006a), we were unable to recover the minimum number
 6 of colonies necessary to compare the cultivability of *H. pylori* adhered to different materials, several
 7 sonication times were tested to optimize the procedure. Sonication cycles of 1 min have been
 8 commonly used in our laboratory to maximize the number of cells from heterotrophic biofilms
 9 formed in drinking water systems. Tests have showed that after sonication for 5s, more than 99% of
 10 *H. pylori* cells are detached, maybe due to the apparent lack of extracellular polymer production of
 11 the bacterium under these conditions. This percentage of removal was obtained for different
 12 materials and different adhesion times tested. Furthermore, *H. pylori* proved to be particularly
 13 sensitive to sonication procedures, as a nearly 2 log increase in cultivable cells was obtained
 14 between sonication times of 5s and 1min.

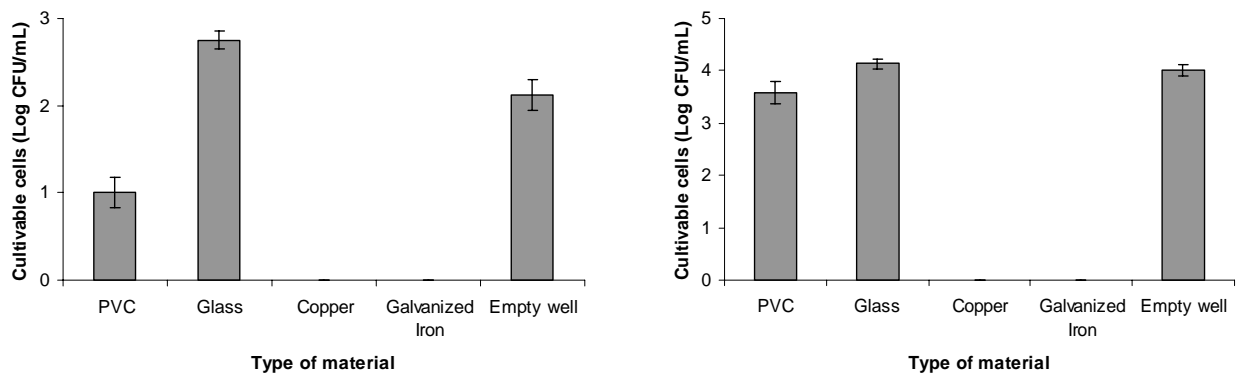
15 After the optimization of the detachment procedures, cultivation experiments have shown that both
 16 copper and galvanized iron supports were deleterious for the bacterium survival (Figure 2). For all
 17 strains the number of cultivable cells on the surface increased up to 10^4 - 10^6 CFU/cm² in the first 2
 18 hours due to the initial adhesion process. Even though it has been previously shown that the total
 19 number of cells adhered continues to rise until 48 hours (Azevedo *et al.* 2006a, Azevedo *et al.*
 20 2006b), cultivable cell numbers started to decrease after only two hours (in the case of PVC and
 21 glass for strain J99 the numbers stabilized). This effect was partly expected, as the survival time (as
 22 assessed by cultivation methods) for *H. pylori* in water at this temperature is quite low (Adams *et al.*
 23 2003, Azevedo *et al.* 2004). The decline was much steeper for the metallic materials (copper and
 24 galvanized iron) than for glass and PVC. After 24 hours, no cultivable cells could be recovered
 25 from the metallic surfaces for any of the strains tested, which contrasted with the values of 10^1 - 10^4
 26 CFU/cm² obtained for glass, and of 0 - 10^5 CFU/cm² for PVC.



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

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

3
4 Another parameter analysed during the experiment was the cultivability of *H. pylori* in the
5 planktonic state after 24h (Figure 3). In accordance with the results obtained in Figure 2, for the
6 wells where copper and galvanized iron coupons were inserted, no *H. pylori* could be recovered.
7 Leaching of both iron and copper from DWDS pipes is well documented in the literature, and is
8 even the cause for some human health issues (Sadiq *et al.* 1997, Georgopoulos *et al.* 2001). PVC
9 caused a slight decrease of the numbers of bacteria in the water, whereas the insertion of a glass
10 coupon appeared to have no effect on the cultivable counts.
11



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

15
16 *H. pylori* is one of the few bacteria that has been shown to possess a copper transporter system (Ge
17 *et al.* 1995), which could suggest a higher tolerance of the bacterium for this heavy metal. However,
18 in this study copper and iron proved to be harmful for *H. pylori*. Copper has also been indicated as
19 an effective material to control bacterial growth in several situations (Domek *et al.* 1984, Rogers *et al.*
20 *et al.* 1994, Wilks *et al.* 2005).

21 Besides copper and iron plumbing, areas of the DWDS with high shear stresses (Azevedo *et al.*
22 2006b) and effective chlorination (Baker *et al.* 2002), are unlikely environmental reservoirs for *H.*
23 *pylori*. In fact, the existence of these factors in most DWDS might have contributed to the
24 decreasing the prevalence of *H. pylori* in the developed countries. Nevertheless, biofilms are
25 prosperous in microenvironments and the possibility of areas where the bacterium subsists cannot
26 be excluded.

27 28 **Conclusions**

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

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1 presented does not represent the opinion of the Community and the Community is not responsible
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