

Water-induced modulation of *Helicobacter pylori* virulence properties

Nuno M Guimarães^{1,2,3/+}, Nuno F Azevedo^{2,3}, Maria J Vieira², Ceu Figueiredo^{1,4}

¹Institute of Molecular Pathology and Immunology ²Department of Chemical Engineering, Faculty of Engineering

³Medical Faculty, University of Porto, Porto, Portugal ⁴Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal

*While the influence of water in *Helicobacter pylori* culturability and membrane integrity has been extensively studied, there are little data concerning the effect of this environment on virulence properties. Therefore, we studied the culturability of water-exposed *H. pylori* and determined whether there was any relation with the bacterium's ability to adhere, produce functional components of pathogenicity and induce inflammation and alterations in apoptosis in an experimental model of human gastric epithelial cells. *H. pylori* partially retained the ability to adhere to epithelial cells even after complete loss of culturability. However, the microorganism is no longer effective in eliciting in vitro host cell inflammation and apoptosis, possibly due to the non-functionality of the *cag* type IV secretion system. These *H. pylori*-induced host cell responses, which are lost along with culturability, are known to increase epithelial cell turnover and, consequently, could have a deleterious effect on the initial *H. pylori* colonisation process. The fact that adhesion is maintained by *H. pylori* to the detriment of other factors involved in later infection stages appears to point to a modulation of the physiology of the pathogen after water exposure and might provide the microorganism with the necessary means to, at least transiently, colonise the human stomach.*

Key words: *Helicobacter pylori* - virulence properties - water - culturability - infection

Helicobacter pylori is an important human pathogen that causes chronic gastritis and is associated with the development of more severe diseases, such as peptic ulcer disease and gastric cancer (Blaser & Atherton 2004). Since the isolation of *H. pylori*, numerous studies have been published addressing the prevalence and epidemiology of infection (Brown 2000, Kikuchi & Dore 2005, Queiroz & Luzzza 2006), its relationship with disease, the identification and characterisation of virulence factors and their role in pathogenesis (Prinz et al. 2003, Blaser & Atherton 2004, Figueiredo et al. 2005). However, it is still unclear to the scientific community how *H. pylori* is transmitted (Azevedo et al. 2009).

The most widely accepted routes of transmission are the oral-oral, faecal-oral and gastric-oral routes. Nevertheless, an increasing number of works report the identification of *H. pylori* in external environmental reservoirs, such as food, domestic animals and, most significantly, water (Dore et al. 2001, Park et al. 2001, Fujimura et al. 2002, Watson et al. 2004, Safaei et al. 2011). In fact, several epidemiological studies have concluded that the drinking water source, or drinking water-related conditions, was a risk factor for *H. pylori* acquisition (Karita et al. 2003, Krumbiegel et al. 2004, Fujimura et al. 2008). Molecular methods, such as fluorescence *in situ* hybridisation and polymerase chain reaction, have been used to

detect the presence of *H. pylori* DNA in water and water-associated biofilms from wells, rivers and water distribution networks (Flanigan & Rodgers 2003, Fujimura et al. 2004, Bragança et al. 2005, Khan et al. 2012). However, the demonstration that *H. pylori* can be detected in water does not imply that the microorganism can then colonise the human host. In fact, while it has been shown that water-exposed *H. pylori* total cell counts did not decrease for a period of two years at 4°C (Shahamat et al. 1993), the complete loss of culturability of the microorganism takes less than 10 h at temperatures over 20°C (Adams et al. 2003, Azevedo et al. 2004). This transition to the non-culturable state is typically accompanied by a morphological transition of the bacteria from spiral to coccoid form (Andersen & Rasmussen 2009). Depending on the authors, the latter state has been considered a manifestation of cell death (Kusters et al. 1997) or a cellular adaptation to less than optimal environments (Azevedo et al. 2007a). In the determination of the physiological state of these non-culturable bacteria, which are still able to retain their structure for a much longer period, lies the key to our understanding of the exact role of water in *H. pylori* transmission. More specifically, it is important to address the effect of water exposure on several *H. pylori* mechanisms that are, under favourable conditions, able to induce a response in host cells. At the moment, apart from a few studies that concluded that the water-induced coccoid form of *H. pylori* can colonise the gastric mucosa and cause gastritis in mice (Cellini et al. 1994, She et al. 2003), there is still a lack of information regarding the capacity of water-exposed bacteria to induce a response in host cells.

In this study, we assessed the culturability of water-exposed *H. pylori* and determined whether this bacterium retains the capacity to adhere and elicit host cell

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+ Corresponding author: nguimaraes@fe.up.pt

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responses, such as inflammation and apoptosis, using an experimental model of human gastric epithelial cells. Because these host cell responses may be related to components of bacterial pathogenicity, we also evaluated the capacity of water-exposed *H. pylori* to assemble a functional *cag* type IV secretion system (T4SS).

MATERIALS AND METHODS

Bacterial strains and growth conditions - The experiments were performed with *H. pylori* 26695, obtained from the American Type Culture Collection. The bacteria were grown in tryptic soy agar (TSA) supplemented with 5% sheep blood (BioMérieux, France) and incubated at 37°C under a microaerophilic atmosphere for 48 h.

Water-exposed *H. pylori* - After 48 h of culture growth, *H. pylori* was harvested from TSA plates and suspended in 5 mL of autoclaved tap water in a 10⁹ bacteria/mL concentration. The suspensions were kept at 25°C under aerophilic conditions. The bacteria were then exposed to water for 2 h, 6 h, 24 h and 48 h. *H. pylori* inocula that were not exposed to water were used as controls.

Culturability - The number of culturable bacteria at the different time points was determined by plating serial dilutions of the suspensions on TSA plates containing 5% sheep blood. The culturability was analysed by comparing the number of colony-forming units of each time point.

Cell line maintenance and bacterial co-cultures - The AGS cells, derived from a human gastric carcinoma, were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% foetal bovine serum (FBS) and 1% Pen-Strep (Invitrogen) at 37°C and kept under a 5% CO₂ humidified atmosphere. All co-culture experiments of *H. pylori* with the AGS cells were performed at a multiplicity of infection of 100. The co-cultures were maintained at 37°C under a 5% CO₂ humidified atmosphere.

Adhesion assay - An *H. pylori* suspension corresponding to the different times of water exposure was added to the AGS cells and the plate was gently agitated for 30 min at 37°C. The cultures were fixed with 1% paraformaldehyde and blocked with 1% phosphate buffered saline (PBS)-bovine serum albumin (BSA) for 30 min. Bacterial adhesion was determined by ELISA as previously described (McGuckin et al. 2007) using a rabbit polyclonal anti-*H. pylori* (Cell Marque) and an anti-rabbit IgG-horseradish peroxidase (HRP) as a secondary antibody. The binding was visualised after incubation with tetramethylbenzidine and 1 M HCl. The absorbance was read at 450 nm. The controls for the *H. pylori* binding to the wells experiment comprised wells with no AGS cells, to which bacteria were added and allowed to adhere to the plastic before fixation. The negative controls contained neither AGS cells nor *H. pylori*. The bacterial adhesion was expressed as a percentage of the adhesion to AGS cells of *H. pylori* that were not exposed to water.

Interleukin (IL)-8 production - The AGS cells were grown in six-well plates for 48 h in RPMI supplemented with 10% FBS at 37°C and 5% CO₂. Bacterial suspensions corresponding to each water exposure time period

were added to the cells and incubated for 24 h at 37°C. The IL-8 levels were detected in co-culture supernatants by ELISA using the Quantikine Human CXCL8/IL8 kit (R&D Systems, USA).

Apoptosis assay - The AGS cells were grown in six-well plates for 48 h in RPMI supplemented with 10% FBS at 37°C and 5% CO₂. A volume of bacterial suspension corresponding to each water exposure time period was added to the cells and incubated for 24 h at 37°C. Apoptotic cell death was determined by the terminal uridine deoxynucleotide nick end-labelling assay (In Situ Cell Death Detection Kit, Roche Diagnostics). Apoptotic cells were detected using a Leica DM IRE2 fluorescence microscope.

Western blot (WB) analysis - The co-cultures and AGS uninfected control cells were lysed in cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% NP-40, 3 mM sodium vanadate, 20 mM NaF, 1 mM PMSF, 10 µg/mL aprotinin and 10 µg/mL leupeptin) and the lysates were separated by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto Hybond nitrocellulose membranes (Amersham), which were then blocked with 4% BSA or 5% non-fat milk in PBS with 0.5% Tween-20. The membranes were incubated with a mouse monoclonal antibody against tyrosine phosphorylated residues (α -PY-99, Santa Cruz Biotechnology) and, after stripping, re-probed with a mouse monoclonal anti-*cagA* antibody (Santa Cruz Biotechnology). Goat anti-rabbit (Santa Cruz Biotechnology) or rabbit anti-mouse HRP-conjugated secondary antibodies (Amersham) were used, followed by ECL detection (Amersham). As a loading control, the membranes were also incubated with a mouse monoclonal anti- α -tubulin antibody (Sigma).

Protein lysates of the *H. pylori* suspensions of each timepoint of water exposure were used as parallel controls for the amount of bacterial proteins present. Twenty micrograms of proteins of each sample was separated by 6% SDS-PAGE and transferred onto Hybond nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in PBS with 0.5% Tween-20 and incubated overnight with mouse monoclonal anti-*cagA* or with rabbit polyclonal anti-urease B (Santa Cruz Biotechnology) antibodies.

Statistical analyses - The data were analysed with Student's *t* test using Statview for Windows software v.5.0 (SAS Institute Inc, USA) and were expressed as the mean values of, unless otherwise stated, three independent experiments $\pm$ standard deviations. Differences in data values were considered significant at $p < 0.05$.

RESULTS

***H. pylori* culturability after water exposure** - The culturability of *H. pylori* was evaluated after 0 h, 2 h, 6 h, 24 h and 48 h of water exposure. Based on previous studies (Adams et al. 2003, Azevedo et al. 2004), we anticipated that the longest timepoints would be sufficient to turn the bacterium into the non-culturable state. The results obtained confirmed our expectations, as the

culturability of *H. pylori* progressively decreased and, after 24 h of water exposure, *H. pylori* was no longer culturable (Fig. 1). The subsequent studies were performed at all time points as well and we were able to observe the modulation of the virulence properties of *H. pylori* as the bacteria transitioned from the culturable to non-culturable state.

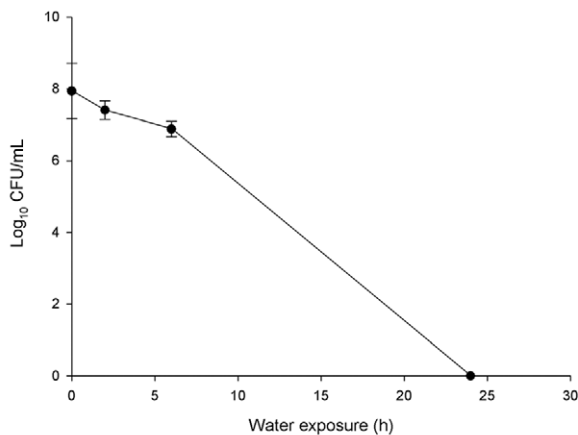


Fig. 1: effect of water exposure on *Helicobacter pylori* culturability. After water exposure, bacteria suspension was plated on tryptic soy agar plates and incubated for seven days at 37°C in microaerophilic conditions. The colony-forming units (CFUs) formed were counted to assess the culturability. Each experiment was performed in triplicate.

Influence of water exposure on the adhesion of *H. pylori* to host cells - To assess whether the adherence ability of *H. pylori* to gastric epithelial cells is altered by the contact of the bacterium with water, we performed an adhesion assay in an ELISA format using the human gastric epithelial AGS cell line. Whereas exposure of *H. pylori* to water for only 2 h led to a statistically significant decrease in its ability to adhere to AGS cells ($p < 0.05$), the adhesion levels remained constant for bacteria that were exposed to water for longer time periods (Fig. 2). Compared to non-exposed *H. pylori*, the decrease in adhesion of water-exposed bacteria was approximately 40%. Nevertheless, the observation that water-exposed *H. pylori* are still capable of adhering to cells suggests that in these conditions, the bacterium may still exert effects on host gastric cells.

Influence of water exposure on *H. pylori* induction of IL-8 secretion by host cells - *H. pylori* leads to increased production by the epithelium of the proinflammatory cytokine IL-8 when in close contact with the gastric mucosa (Shimoyama & Crabtree 1998). Because water-exposed *H. pylori* were able to adhere to epithelial cells, we studied the capability of *H. pylori* to induce inflammation by evaluating the secretion levels of IL-8 from AGS cells infected with *H. pylori* inocula exposed to water for different time periods (Fig. 3). Results show that *H. pylori* with 2 h and 6 h of water exposure re-

tain the ability to induce IL-8 secretion similarly to unexposed bacteria. However, after 24 h of exposure, *H. pylori* are no longer able to induce IL-8 production by AGS cells. Therefore, the case of inflammation induced by the bacterium appears to be more related to the culturability status of *H. pylori* than to the ability of this microorganism to adhere to epithelial cells. In fact, although adhesion to host cells is immediately decreased after contact with water, after short time periods (up to 6 h), adhering *H. pylori* cells are still able to induce pro-inflammatory IL-8 secretion in these cells.

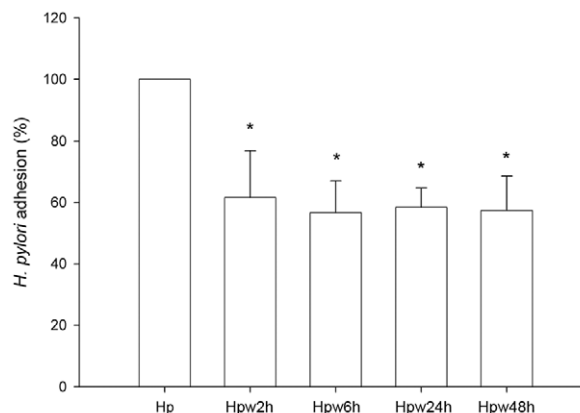


Fig. 2: effect of water exposure on *Helicobacter pylori* adhesion to host epithelial cells. AGS cells were infected with *H. pylori* 26695 inocula that have been exposed to water for 2 h (Hp2h), 6 h (Hp6h), 24 h (Hp24h) and 48 h (Hp48h) at a multiplicity of infection of 100. As control, *H. pylori* 26695 that was not exposed to the water was used (Hp). Cells were washed to remove non-adherent bacteria and adhesion was evaluated by ELISA. Data are expressed as percentage of control. Graphics represent mean $\pm$ standard deviation and are representative of three independent experiments. Asterisks mean significantly different from non-exposed *H. pylori* ($p < 0.05$).

Influence of water exposure on *H. pylori* deregulation of host cell apoptosis - *H. pylori* infection has been shown to modify epithelial cell apoptosis (Moss et al. 2001, Cover et al. 2003). To elucidate whether water-exposed *H. pylori* is able to induce such impairment, AGS cells were infected with bacteria previously exposed to water and cell apoptosis was evaluated. As expected, non-exposed *H. pylori* increased AGS cell apoptosis (Fig. 4). In contrast, water-exposed *H. pylori* induced significantly lower levels of apoptosis than non-exposed bacteria ($p < 0.01$ for all water exposure times). Furthermore, no statistically significant differences were observed between apoptosis in uninfected cells and those infected with water-exposed *H. pylori* ($p > 0.05$), except for cells infected with *H. pylori* exposed to water for 24 h ($p < 0.05$). These experiments indicate that water exposure, although still permitting *H. pylori* to adhere, limits the influence of the bacteria on host cell apoptosis.

Influence of water exposure on the *H. pylori* *cag* T4SS - The T4SS is a molecular syringe that allows the injec-

tion of bacterial effectors into the host cell cytoplasm, altering host cellular processes including the induction of inflammation and deregulation of apoptosis (Segal et al. 1999, Moss et al. 2001, Viala et al. 2004, Cabral et al. 2006). After water exposure, *H. pylori* were still able to adhere to epithelial cells; therefore, our next experiment aimed at elucidating if water-exposed *H. pylori* had a functional T4SS. To assess the functionality of the T4SS, we used a WB to evaluate *cagA* tyrosine phosphorylation in AGS cells after infection with *H. pylori* 26695 inocula exposed to water at four different time periods (Fig. 5A). *cagA* is a T4SS effector injected into the host cell and can be phosphorylated by host protein kinases (Odenbreit et al. 2000, Backert & Selbach 2008). *cagA* phosphorylation only occurs inside the host cell and is an indirect measure of the T4SS functionality. *H. pylori* that was not exposed to water was used as a positive control for this experiment (Fig. 5A). In parallel and to control for the amount of proteins present in bacterial suspensions that were incubated in water, a WB analysis for *H. pylori* *cagA* and urease B was performed (Fig. 5B).

While water-exposed *H. pylori* remained culturable for at least 6 h, *cagA* tyrosine phosphorylation was not observed in any of the co-cultures of water-exposed bacteria. After just 2 h in water, *H. pylori* were no longer able to translocate *cagA* into the host cells. This was not due to the lower *cagA* levels present in bacteria incubated in water (for at least 48 h) because water exposure affected neither the levels of *cagA* nor urease B, which remained similar to those of non-exposed *H. pylori*. These data suggest that water-exposed bacteria are not able to produce a functional *cag* T4SS and, consequently, are not able to translocate *cagA* into the host cells. In combination with our previous experiments, these results sug-

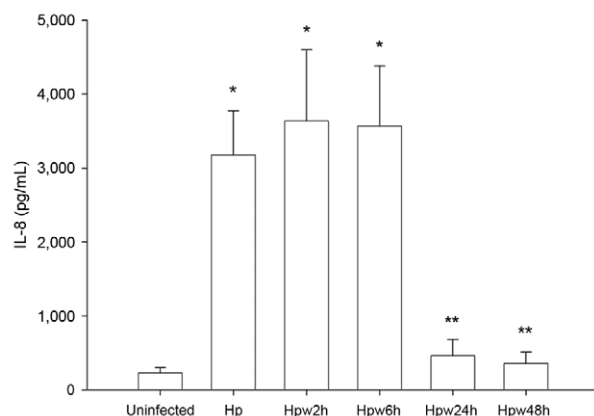


Fig. 3: effect of water exposure on *Helicobacter pylori* induction of interleukin (IL)-8 secretion by host epithelial cells. AGS cells were infected with *H. pylori* 26695 inocula that have been exposed to water for 2 h (Hp2h), 6 h (Hp6h), 24 h (Hp24h) and 48 h (Hp48h) at a multiplicity of infection of 100. As control, *H. pylori* 26695 that was not exposed to the water was used (Hp). IL-8 production was evaluated by ELISA. Graphics represent mean $\pm$ standard deviation and are representative of three independent experiments. *: significantly different from uninfected cells; **: significantly different from non-exposed *H. pylori* ($p < 0.05$).

gest that after being in water for periods longer than 6 h, *H. pylori* is still able to adhere to host cells, but is not effective in eliciting in vitro IL-8, a pro-inflammatory chemokine and apoptosis, possibly due to the non-functionality of the *cag* T4SS.

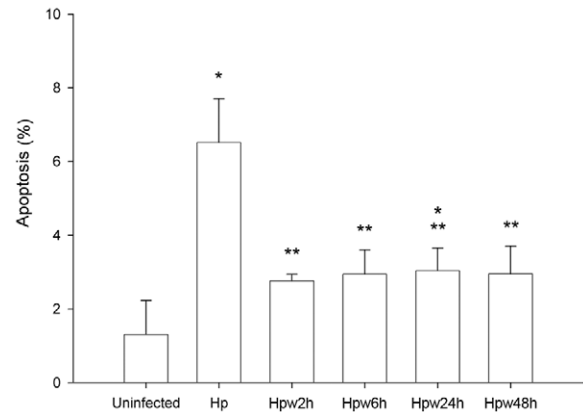


Fig. 4: effect of water exposure of *Helicobacter pylori* on apoptosis of host epithelial cells. AGS cells were infected with *H. pylori* 26695 inocula that have been exposed to water for 2 h (Hp2h), 6 h (Hp6h), 24 h (Hp24h) and 48 h (Hp48h) at a multiplicity of infection of 100. As control, *H. pylori* 26695 that was not exposed to the water was used (Hp). Apoptosis was detected at single cell level using the terminal uridine deoxynucleotide nick end-labelling assay. Graphics represent mean $\pm$ standard deviation and are representative of at least two independent experiments. *: significantly different from uninfected cells; **: significantly different from non-exposed *H. pylori* ($p < 0.05$).

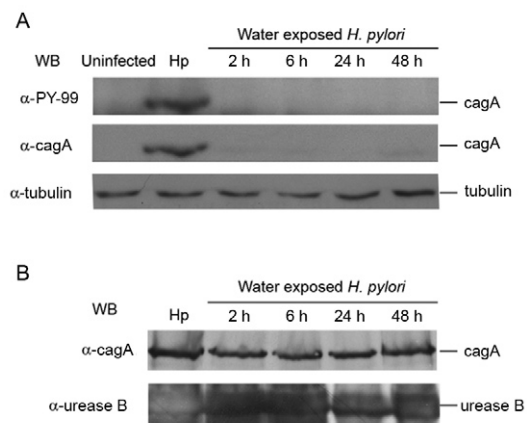


Fig. 5: effect of water-exposure on *Helicobacter pylori* *cag* type IV secretion system formation. A: AGS cells were infected with *H. pylori* 26695 inocula that have been exposed to water for 2 h, 6 h, 24 h and 48 h at a multiplicity of infection of 100. As control, *H. pylori* 26695 that were not exposed to water were used (Hp). *cagA* tyrosine phosphorylation levels were evaluated by western blot using an anti-PY99 antibody against tyrosine phosphorylated motifs and after membrane stripping, *cagA* was detected by re-probing with an anti-*cagA* antibody. Tubulin was used as equal protein loading control for co-cultures; B: protein lysates of *H. pylori* 26695 suspensions of each time-point of water exposure were used as parallel controls of the amount of bacterial *cagA* and urease B proteins present. *H. pylori* 26695 that were not exposed to water (Hp) were also used as control.

DISCUSSION

Epidemiological evidence has pointed to environmental water as a risk factor for *H. pylori* infection among humans (Klein et al. 1991, Goodman et al. 1996, Karita et al. 2003). To elucidate if there are mechanisms that might allow water-exposed *H. pylori* to colonise the human stomach, several properties related to the survival and pathogenicity of *H. pylori* when exposed to water were studied. Our results showed that after being exposed to water for 24 h at 25°C, *H. pylori* was no longer culturable. Studies have reported that when exposed to water, *H. pylori* enter a viable, but non-culturable state as a response to unfavourable environmental conditions (Azevedo et al. 2007a), which means that even though *H. pylori* cannot be recovered by plating techniques, bacterial cells might remain viable.

Adhesion is one of the most important pathogenic determinants of *H. pylori* because attachment to the host cells allows bacterial maintenance and gastric colonisation. Our results showed that water-exposed *H. pylori* has a decreased adhesion capacity compared to *H. pylori* that has not been in contact with water. Nevertheless, water-exposed bacteria still retain a significant adhesion capacity and this capacity does not significantly change with the time of water exposure. Our findings, in combination with the discovery that *H. pylori* would only grow under conditions mimicking the stomach if adhered to the surface of epithelial cells (Tan et al. 2009), could be a means for allowing *H. pylori* to remain in the host long enough for the occurrence of genetic recombination with other *H. pylori* strains that could be present in the same host, resulting in a higher genetic diversification (Azevedo et al. 2007b). This genetic diversification may help *H. pylori* adapt to a new host after transmission (Dorer et al. 2009).

Inflammation of the gastric mucosa is a universal consequence of *H. pylori* interaction with the host (Shimoyama & Crabtree 1998). Although water-exposed *H. pylori* still retained a considerable capacity to adhere to gastric cells, at 24 h of exposure, *H. pylori* was not able to influence IL-8 secretion. This is concurrent with the absence of nuclear factor (NF)- κ B activation and the lack of IL-8 production in epithelial cells observed after the morphologic transition from bacillar into coccoid form, in which the *H. pylori* peptidoglycan structure is modified (Chaput et al. 2006). In our experiments, bacteria exposed to water for short time periods still triggered signalling that led to IL-8 production, which could represent bacteria with an unmodified peptidoglycan. Whether water exposure leads to altered peptidoglycan structure and to which extent these bacterial cell wall modifications allow these bacterial forms to temporarily escape detection by the host immune system remain to be elucidated.

Infection with *H. pylori* leads to increased host epithelial cell turnover with an increase in both apoptosis and proliferation rates (Peek et al. 1997, Moss et al. 2001, Cabral et al. 2006, 2007). Water-exposed bacteria were not able to induce alterations in the apoptotic index of host cells. As gastric epithelial cells have a rapid turnover, the lack of influence of water-exposed *H. pylori* on epithelial cell apoptosis may be an advantage for coloni-

sation and persistence in the host. In addition, the lack of an ability to induce inflammation may also contribute to decreased host cell proliferation (Lynch et al. 1999), therefore slowing cell turnover.

Several lines of evidence have pointed to the importance of the *cag* T4SS in *H. pylori*-mediated host inflammation and apoptosis (Segal et al. 1999, Moss et al. 2001). In co-cultures of water-exposed *H. pylori* with gastric cells, we could not detect *cagA* phosphorylation, suggesting that the *cag* T4SS becomes non-functional. The absence of a functional T4SS may underlie the lack of influence of water-exposed *H. pylori* on host cell IL-8 secretion and apoptosis. It has been shown that activation of NF- κ B leading to IL-8 secretion may not only be influenced by *cagA* (Brandt et al. 2005), but also stimulated by the T4SS itself. Indeed, it has been shown that *H. pylori* use the T4SS to deliver fragments of peptidoglycan that are sensed by the host nucleotide-binding oligomerisation domain 1 receptor, resulting in NF- κ B activation and IL-8 production (Viala et al. 2004). In animal models, *H. pylori* exposed to sterile tap water can colonise mice and induce gastric inflammation (She et al. 2003). However, further studies are needed to determine whether water-exposed *H. pylori* are still able to recover the functionality of the T4SS in vivo.

Our results show that water-exposed *H. pylori* retain adhesion properties while other interactions with the host cells are decreased. This may be beneficial for the bacterium in the sense that it may improve the likelihood of the establishment and persistence of the infection.

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