FEMS Microbiology Ecology, 96, 2020, fiaa182

doi: 10.1093/femsec/fiaa182
Advance Access Publication Date: 8 September 2020
Research Article

RESEARCH ARTICLE

Role of amoebae for survival and recovery of 'non-culturable' *Helicobacter pylori* cells in aquatic environments

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One sentence summary: Free-living amoebae were shown to play a role in the survival and recovery of *H. pylori* in aquatic environments, demonstrating a new mechanism for their distribution and persistence in these habitats.

Editor: Julie Olson
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ABSTRACT

Helicobacter pylori is a fastidious Gram-negative bacterium that infects over half of the world's population, causing chronic gastritis and is a risk factor for stomach cancer. In developing and rural regions where prevalence rate exceeds 60%, persistence and waterborne transmission are often linked to poor sanitation conditions. Here we demonstrate that *H. pylori* not only survives but also replicates within acidified free-living amoebal phagosomes. Bacterial counts of the clinical isolate *H. pylori* G27 increased over 50-fold after three days in co-culture with amoebae. In contrast, a *H. pylori* mutant deficient in a cagPAI gene (*cagE*) showed little growth within amoebae, demonstrating the likely importance of a type IV secretion system in *H. pylori* for amoebal infection. We also demonstrate that *H. pylori* can be packaged by amoebae and released in extracellular vesicles. Furthermore, and for the first time, we successfully demonstrate the ability of two free-living amoebae to revert and recover viable but non-cultivable coccoid (VBNC)-*H. pylori* to a culturable state. Our studies provide evidence to support the hypothesis that amoebae and perhaps other free-living protozoa contribute to the replication and persistence of human-pathogenic *H. pylori* by providing a protected intracellular microenvironment for this pathogen to persist in natural aquatic environments and engineered water systems, thereby *H. pylori* potentially uses amoeba as a carrier and a vector of transmission.

Keywords: *Helicobacter pylori*; free-living amphizoic amoebae; intracellular multiplication; extracellular vesicles; phagosomal pH; bacterial recovery

INTRODUCTION

Recently, the World Health Organization (WHO) published a catalogue of 12 families of antimicrobial resistant bacteria that pose the greatest threat to human health, one of which was *Helicobacter pylori* (Willyard 2017). *Helicobacter pylori* is an antibiotic resistant, gastric carcinogenic pathogen, described in 1984 as the

Received: 14 June 2020; **Accepted:** 2 September 2020

principal cause of peptic ulcer disease (Blaser 1997; Dunn, Cohen and Blaser 1997). This fastidious Gram-negative bacterium colonizes the gastric mucosa of over half of the world's population, yet its microbial ecology outside of the human host is poorly understood (Aziz, Khalifa and Sharaf 2015). Over the last decade or so, however, various studies implicate or support the theory that free-living amoebae (FLA) could be aquatic environmental hosts for *H. pylori* (Winiecka-Krusnell *et al.* 2002; Smith and Ashbolt 2012; Ashbolt 2015; Moreno-Mesonero *et al.* 2016, 2017).

FLA are one of the most ubiquitous organisms found in aquatic environments, being primary predators controlling bacterial populations (Rodriguez-Zaragoza 1994; Samba-Louaka *et al.* 2019). However, they are known natural environmental reservoirs for a range of amoeba-resisting bacterial pathogens (ARB) and a vector of human infection for, water-associated infections (Thomas *et al.* 2010; Thomas and Ashbolt 2011; Dey, Hoffman and Glomski 2012). Interestingly, the intracellular growth of free-living bacteria within FLA has been shown to increase bacterial resistance to antibiotics (Maurin, Bryskier and Raoult 2002) and biocides (King *et al.* 1988) and upregulate virulence (Molmeret *et al.* 2005). In addition to FLA being present in water systems, they have been identified within the gastrointestinal track of humans from the stomach to faeces (Thamprasert, Khunamornpong and Morakote 1993; Bradbury 2014), they are considered as an amphizoic organisms and live as parasites within host tissue (ability to live endozoically) (Page 1967; Jadin 1973; Martinez and Visvesvara 1997).

The significance of FLA in human intestinal tract infection goes back to 1912 when Chatton and Lalung-Bonnaire isolated the FLA *Vahlkampfia* sp. from a human intestine and proposed the idea that the amoeba could be the pathogen and/or an important vector of infection (Chatton and Lalung-Bonnaire 1912). Almost a century later, Steele and colleagues reported the implication of the FLA *Willaertia magna* in the gastric infection of a dog (Steele *et al.* 1997).

Since *H. pylori* and FLA co-occur in human intestinal tract, we considered the possibility that FLA might serve as natural hosts and be vectors for *H. pylori* transmission. We tested this hypothesis by studying the interaction between *H. pylori* and two different amoebae species that are also facultative parasites of humans and animals. The selected FLA are, *W. magna* belonging to the Vahlkampfiidae family and *Vermamoeba vermiformis*, belonging to Vermamoebidae family and one of the most common free-living protists in human environments (de Moura *et al.* 1985; Bradbury 2014; Delafont *et al.* 2018). Moreover, many reports have implicated the survival of *H. pylori* in aquatic environments within FLA, hence this pathogen is considered to be an ARB (Giao *et al.* 2011; Santiago, Moreno and Ferrus 2015; Moreno-Mesonero *et al.* 2016, 2017). However, no direct demonstration of *H. pylori* replication within amoebae hots had been provided.

It has been concluded that during harsh environmental conditions *H. pylori* adopts a dormant stage and remains infectious, which represent a potential public health concern (Nilsson *et al.* 2002), transforming from a spiral shape to the coccoid form that includes viable but non-culturable (VBNC) cells (Sarem and Corti 2016) and subsequently becomes highly tolerant to antibiotics (Bates, Adams and Oliver 2003). However, one of the most important challenges has been determining the mechanism of the coccoid form reactivation (Loke *et al.* 2016). Interestingly, it has been speculated that VBNC enteric bacteria can be recovered by co-culture with eukaryotic cells (Senoh *et al.* 2012). Similar results were obtained with VBNC *Legionella pneumophila* when co-cultured with FLA (Garcia *et al.* 2007). To investigate this further, we analyzed the morphological changes of *H. pylori* in co-culture with FLA using an imaging flow cytometric approach, that we already have developed (Dey *et al.* 2019). In addition, we investigated growth and survival strategies of *H. pylori* in co-culture with FLA by culture, molecular and imaging techniques.

MATERIALS AND METHODS

H. pylori strains and growth conditions

A total of four different *H. pylori* strains were used in this study: *H. pylori* G27; two derivative mutant strains (Δ*cagE* and Δ*cagM*) obtained from Dr Monika Keelan, Faculty of Medicine and Dentistry, University of Alberta; and Green Fluorescent Protein (GFP) expressing *H. pylori* from Dr John Kao, University of Michigan, USA. *Helicobacter pylori* were grown on Brain Heart Infusion (BHI) agar plates (BactoTM, Le Pont de Claix) supplemented with 15 mg/mL of Amphotericin B and Vancomycin to suppress the growth of other bacteria and fungi (Jiang and Doyle 2000), the plates incubated under microaerophilic conditions (anaerobic jars filled with 5% CO_2, 5% H_2 and balance N_2) at 37°C and the bacteria cultivated for 3–4 days to maintain a stationary growth phase for bacterial optimal growth (Worku *et al.* 1999).

Amoebae growth conditions

The amoebae used in this study were *W. magna* (ATCC 50035) isolated from bovine faeces and *Vermamoeba vermiformis* (ATCC 50237) isolated from a hospital cooling tower drain. Amoebae were grown in sterile tissue culture flasks (Thermo Scientific, Edmonton, Canada 130192 or 130193) in SCGYEM (Serum-Casein-Glucose-Yeast-Extract-Medium: ATCC medium 1021) at 25°C in a 5% CO_2 incubator. The trophozoites were maintained in exponential growth phase by sub-culturing every 3–4 days in fresh SCGYEM, and then harvested by tapping the flasks and washing three times with sterile distilled water by centrifugation at 2000 *g* for 10 min to remove carried-over nutrients in the supernatants. Experiments were carried out in sterile 15 mL screw-cap tubes, using 3 mL of medium. FLA were used at a final cell concentration of 10^5/mL determined by a haemocytometer counting (Hausser Scientific, Horsham, USA HSR3110) and distributed in autoclaved lake water (autoclaved for 20 min at 121°C) from the Edmonton area (Alberta, Canada).

Co-culture of *H. pylori* with FLA

Bacterial suspensions (*H. pylori* G27, Δ*cagE* and Δ*cagM*) in sterile distilled water (OD_{550} 1.0, ~10^9/mL; Kavermann *et al.* 2003) were used to inoculate amoeba cultures at a multiplicity of infection (MOI) of 100. Tubes (FALCON, Fischer Scientific, Edmonton, Canada 3033) containing 3 mL of autoclaved lake water were seeded with 10^5/mL trophozoites of the different amoebae, low speed centrifugation at 500 *g* for 5 min was used to initiate physical interaction between bacteria and amoebae and then the co-cultures were incubated under microaerophilic conditions at 25 and 37°C and performed in triplicate ($n = 4$–6). After 2 h incubation, the co-cultures were treated with 100 μg/mL of sterile gentamicin (Sigma-Aldrich, Ontario, Canada G1397) for 1 h in order to kill extracellular *H. pylori* and subsequently quantify intracellular bacteria. After centrifugation (10 min at 2500 *g*) the medium was removed and the amoebae pellet was washed twice in sterile Phosphate Buffered Saline (PBS) medium to eliminate residual gentamicin and the possible remaining extracellular bacteria. The co-cultures were resuspended in sterile lake water, which was unable to support

bacterial outgrowth in the absence of amoebae and to establish conditions that mimic aquatic environments.

The concentrations of the amoebae and *H. pylori* were determined at 24 h intervals from days 0 to 4 (d0–d4). The growth of amoebae was determined by counting resuspended cells per unit volume with a haemocytometer after staining with trypan blue (Gibco, Edmonton, Canada fischer scientific 15 250–061) for amoebae exposed and unexposed to *H. pylori*. Simultaneously, the bacterial concentrations (CFU/mL and genomic copies/mL) with or without FLA from aliquots of the same experimental samples were estimated with culture and qPCR methods after lysing the FLA by passing the sample through a sterile 20-gauge syringe needles (BD, Franklin lakes, USA 304827) 5–6 times.

The total number of *H. pylori* (with or without FLA) was established by performing serial 10-fold dilutions of the co-culture medium containing lysed amoeba and bacteria with sterile distilled water that were subsequently spread in triplicate on BHI agar plates supplemented with Amphotericin B and Vancomycin and incubated at 37°C under microaerophilic conditions.

Cytotoxicity of *H. pylori*

The cytotoxicity of *H. pylori* towards FLA was studied qualitatively by fluorescent microscopy EVOS FL (ThermoFisher Scientific, Edmonton, Canada) using the GFP-*H. pylori*, amoebae cells quantified with a haemocytometer (Hausser Scientific HSR3110) and trypan blue (Gibco 15 250–061) staining as previously described (Dey *et al.* 2009). The influence of bacteria on amoebal monolayer formation in 6-well plates containing 10^5 amoebae/well infected with GFP-*H. pylori* at a MOI of 100 were photographed after 72 h of co-culture.

Transwell assay

To examine whether *H. pylori* can grow extracellularly and damage the host cells by direct contact with amoebae, a transwell plate assay was used and *H. pylori* separated from amoebae (*V. vermiformis* or *W. magna*) by a 0.4 μm membrane. The transwell plates consisted of 6 wells (Corning, New york, USA), each well containing two chambers and separated by a 0.4 μm polycarbonate membrane. FLA (*V. vermiformis* or *W. magna*) were placed in the apical compartment and *H. pylori* G27 strain placed in the basal compartment (amoebae free) in sterile lake water and with the same concentration (FLA/bacteria) as described in co-culture section. After 24 h of incubation, *H. pylori* cells were plated on BHI agar plates supplemented with Amphotericin B and Vancomycin, and incubated at 37°C under microaerophilic conditions for Colony Forming Unit (CFU) determination.

Quantitative PCR (qPCR) analysis

A total of 1 mL of the co-culture samples were taken at different post-infection times and lysed mechanically by pumping several times through a 20-gauge syringe needle (BD 304827) prior to the DNA extraction process. A total of 100 μL from the original volume (1 mL) were taken for DNA extraction using DNeasy blood and tissue extraction kit (Qiagen, Toronto, Canada). Abundance of total *H. pylori* was quantified in triplicate using a qPCR assay, designed and optimized in this study to be specific to the 23S rRNA gene of *H. pylori*, but no other *Helicobacter*, *Arcobacter* or *Campylobacter* species (see Supplementary data SD 1). The limit of detection (LOD) is <10 copies/qPCR rxn with the following sequences:

HpyP3: 5′- 6FAM-AAGATATATGAGAATTGTATCCGCC—NFQMGB - 3′
HpyF3: 5′- GCGGTATGAAGTGAGCATGCA -3′
HpyR3: 5′- GACCATCGCGTAGGAAACCTT -3′

qPCR amplifications were performed using an ABI 7500 Fast cycler (ThermoFisher®, Waltham, USA) in a 20 μL volume containing 1x IDT PrimeTime qPCR Mastermix (ThermoFisher®), 450 nM of each primer, 200 nM of probe and 5 μL of DNA template. The amplification conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

pH measurement

For the intracellular pH measurement, *H. pylori* G27 was used and the infected amoebae (*W. magna*) were incubated with 10 μM of pHrodo Green or Red intracellular pH indicators for 30 min at 37°C according to the manufacturer's protocol (Life technologies, Edmonton, Canada P35373). Standard curves of cytosolic pH were created using pHrodo Green or Red with intracellular pH calibration buffer kit (Life technologies P35379) of pH 4.5. After incubation, the samples treated with pHrodo Red were washed and re-suspended in PBS prior to processing through the Attune NxT acoustic focusing flow cytometer with (blue/red/violet/yellow) lasers (Thermo Fisher Scientific) and the pH Rodo data was collected on the YL2 detector. The fluorescence images of the cells treated with pHrodo Green were osecbtained using an EVOS FL fluorescent cell imaging system (Thermo Fisher Scientific), that capture the green fluorescence channel combined with transmitted light channel. Images were acquired at 100x magnification.

Induction of viable but nonculturable state in *H. pylori* cells

Entry into the VBNC state was induced by incubation of GFP-*H. pylori* suspensions in sterile lake water (5 mL), covered in the dark at 4°C. To assess the culturability of the bacteria, samples were taken at intervals and inoculated on BHI agar plates with amphotericin and vancomycin and incubated at 37°C under microaerophilic conditions. The GFP-*H. pylori* entered into the VBNC state after 5 months incubation and was confirmed by the absence of growth on BHI, in accordance with previous studies which reported similar prolonged incubation times to induce VBNC cells (Asakura *et al.* 2007; Su *et al.* 2015), and demonstrated by absence of any colonies. Apparent VBNC cells and spiral GFP-*H. pylori* were co-cultured with amoebae and compared to controls (VBNC and spiral cells alone), with a MOI of 100 and incubated at 25 °C for up to 72 h. *H. pylori* cells (VBNC and spiral) were plated on BHI agar plates supplemented with Amphotericin B and Vancomycin, and incubated at 37°C under microaerophilic conditions for CFU determination. To aid in resolving 'viable' and damaged cells, 1.5 μL/mL of propidium iodide (Thermo Fisher Scientific P3566) was added to the samples after incubation time (VBNC cells and reverted cells), followed by mixing and 5–10 min incubation in the dark.

Morphological characteristics of assumed VBNC (coccoid) cells and reverted (spiral) cells were analyzed by imaging flow cytometer (ImageStreamx® MarkII, Millipore Sigma, Seattle, USA).

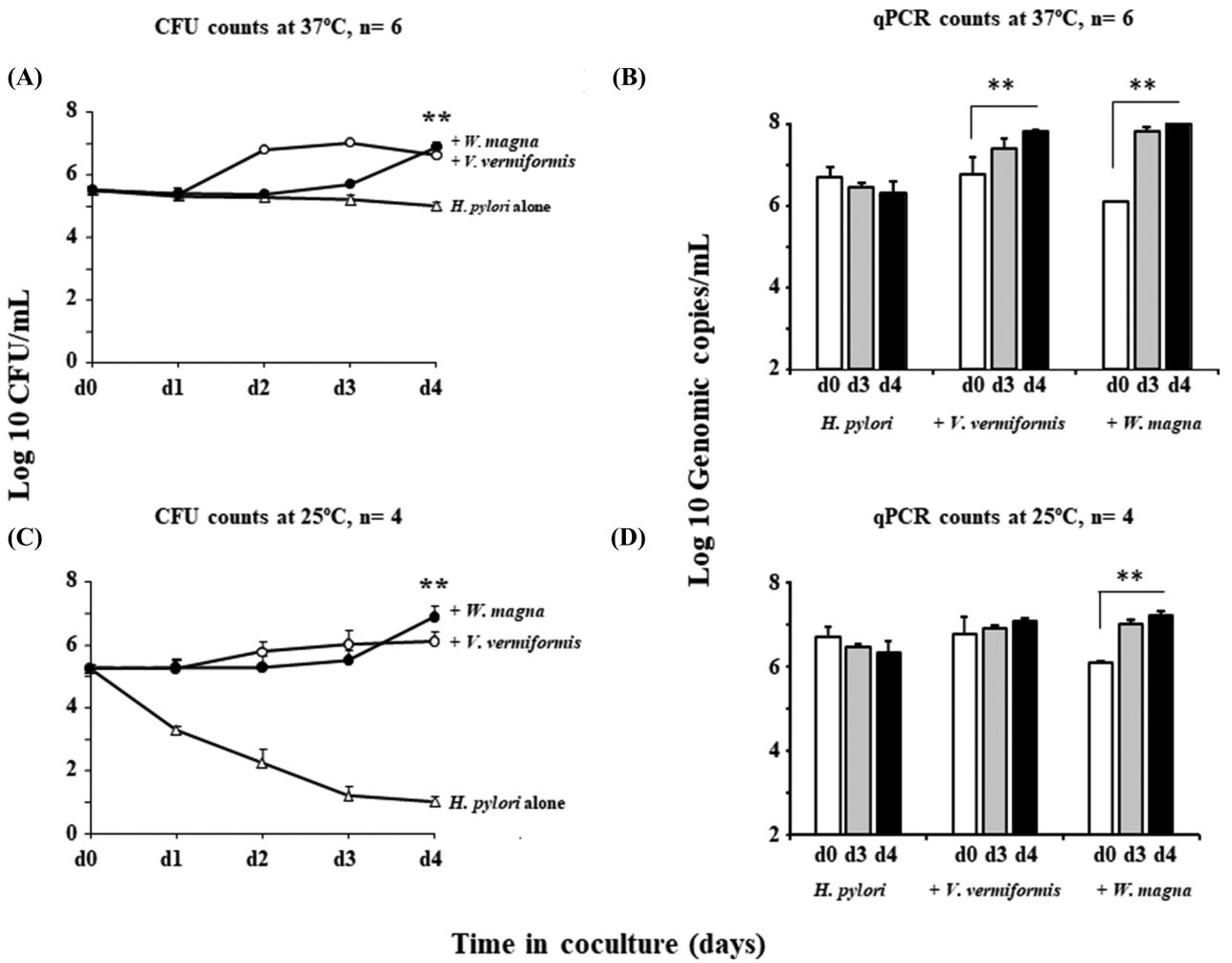

Figure 1. Intracellular growth of *H. pylori* in co-culture with FLA. **(A)** and **(C)**: Growth Kinetics of *H. pylori* G27 with *V. vermiformis* (open circles) and *W. magna* (closed circles) or without amoebae (open triangles) by CFU counts at 37°C and 25°C respectively for up to 4 days. **(B)** and **(D)**: Growth Kinetics of *H. pylori* G27 with or without amoebae by qPCR at 37°C and 25°C respectively for up to 4 days, white bars represent the number of *H. pylori* on day 0 of experiments; grey and black bars represent, respectively, day 3 and day 4 of co-culture. Data are the mean ± SEM, $n = 4$–6 performed in triplicate. Statistical differences by Student's *t*-test comparing the indicated strains to the control in the absence of amoeba after 4 days (**: $P < 0.001$).

Imaging Flow Cytometry (IFC) analysis

Intracellular locations of GFP-*H. pylori* in *W. magna* coculture was performed using ImageStream® cytometry as previously described (Dey *et al.* 2019). Briefly, FLA were infected for 2 h with viable spiral GFP-*H. pylori* at a MOI of 100, washed and re-suspended in PBS prior to processing through the ImageStream®X Mark II (Millipore Sigma). Cells were acquired at 60x magnification. Analysis was performed using the IDEAS software (Amnis, Seattle, USA) and cells were identified on the basis of bright field morphology and size. For the recovering analysis and after 30 min of coinfection with FLA, GFP-*H. pylori* were gated based on small size and low side scatter (to remove any internal control SpeedBeads® from the gate). They were then further gated based on GFP signal, degree of focus and all doublets/ clumps were removed from the analysis. Spiral and coccoid bacteria were then gated based on Aspect Ratio Intensity (Ch01) and Major Axis Intensity (Ch01) to discriminate between the two bacterial cell morphologies (spiral vs VBNC cell forms). The bacteria gating strategy as previously described (Dey *et al.* 2019) and briefly provided in (Supplementary data SD. 2).

MICROSCOPY

Fluorescence microscopy

Co-cultures of GFP-*H. pylori* and FLA in 12-well tissue culture plates (Fisher Scientific 130185) overlaid with microscopy cover slips (Fisher Scientific 12–546-1) were incubated at 37°C with 5% CO_2. After 2 h of infection, the sterile lake water medium was removed and cells fixed with 4% paraformaldehyde for 5 min at room temperature and then washed with PBS three times. Images were taken with an EVOS FL fluorescent cell imaging system (ThermoFisher Scientific).

Scanning electron microscopy (SEM)

The overnight co-culture samples (*H. pylori* G27-*W. magna*) were fixed for 15–60 min with glutaraldehyde (Electron Microscopy Sciences, Hatfield, USA glutaraldehyde 16537–10) and followed by three 5-min rinse steps in 1x Dulbecco's Phosphate Buffered Saline (DPBS). Samples were then dehydrated using increasing concentrations of ethanol (Fisher BioReagents BP82031GAL), 5 min each in 10, 30, 50, 70, 80, 90 and 100% (ethanol in distilled

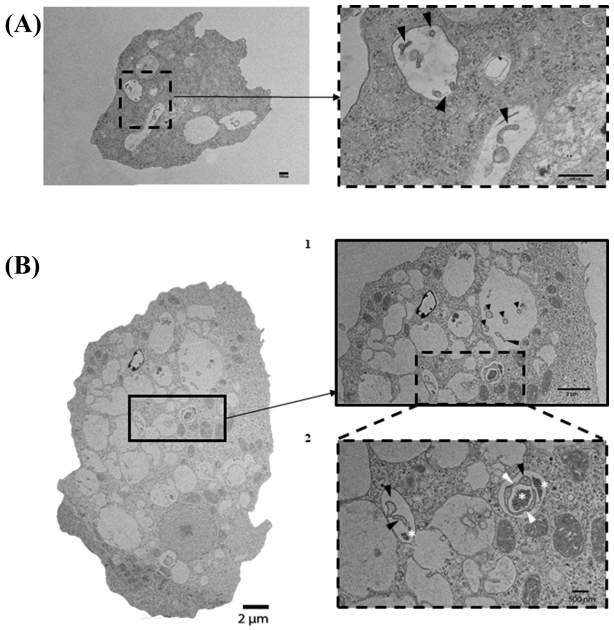

Figure 2. Transmission Electron Microscopy (TEM) of spiral shaped rod morphology of *H. pylori* contained within **(A)** *V. vermiformis* and (B1 and B2) *W. magna* phagosomes after 6 h of co-culture at 37°C (black arrowheads). Note, B2 demonstrates *H. pylori* in the process of septation (white arrowheads) as well as the bacterial remnants (white stars).

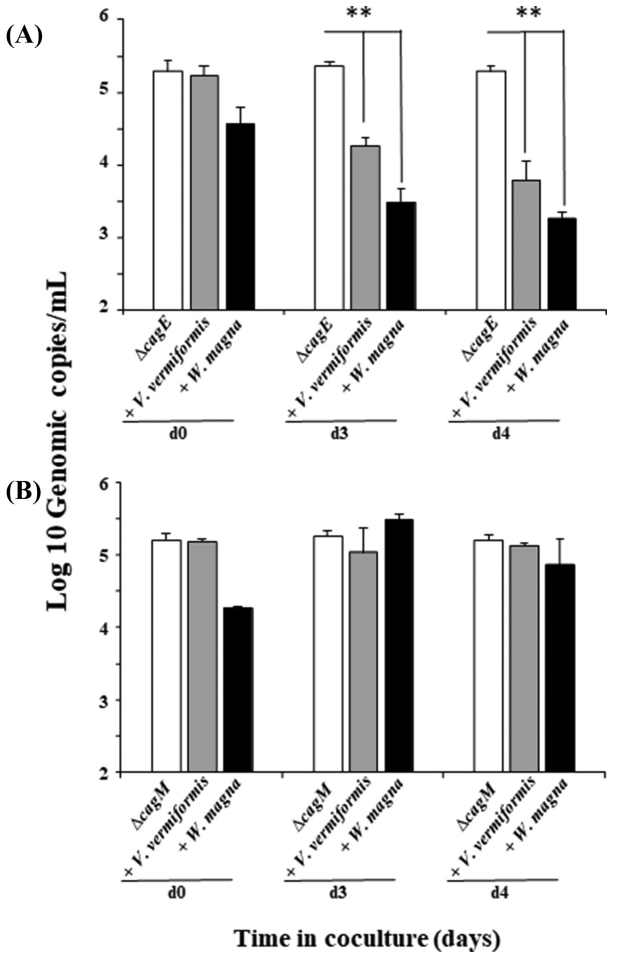

Figure 3. qPCR analysis of *H. pylori* mutant deficient in cagPAI genes growth in co-culture with FLA. **(A)** Growth Kinetics of *H. pylori* mutant Δ*cagE* with *V. vermiformis* (grey bars) and *W. magna* (black bars) or without amoebae (open bars) respectively at 37°C for up to 4 days. **(B)** Growth Kinetics of *H. pylori* mutant Δ*cagM* with *V. vermiformis* (grey bars) and *W. magna* (black bars) or without amoebae (open bars) respectively at 37°C for up to 4 days. Data are the mean ± SEM, $n = 3$ performed in triplicate. Statistical differences by Student's *t*-test comparing the indicated strains to the control in the absence of amoeba after 4 days (**: $P < 0.001$).

water). The samples were further dried for 10 min in hexamethyldisilazane (Sigma-Aldrich H4875). Finally, the membranes were stuck to SEM stubs with adhesive carbon strips and sputter coated with gold using a Leica EM ACE600 sputter coater. The final samples were imaged using a Sigma VP HD Field-emission SEM (Zeiss, Pleasanton, CA) at 10 000x magnification through the University of Alberta Microscopy Facility.

Transmission electron microscopy (TEM)

Axenic cultures of amoebae were infected for 6 h with *H. pylori* G27 at a MOI of 100 on Thermanox cover slips (Thermo Fisher 174985). After decanting the medium, amoebae were fixed at room temperature with 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences 15960). Samples were submitted for processing at the imaging core at University of Alberta, Faculty of Medicine and Dentistry.

Statistical analysis

Data were analyzed with a two-tailed Student's *t*-test (Microsoft Excel 2007), with an alpha value of 0.05, under the assumption that data were normally distributed and had equal variances (see details in figure legends).

RESULTS AND DISCUSSION

Intracellular multiplication of *H. pylori* and their effect on FLA viability

To determine the number of *H. pylori* in amoebae, estimates were made by CFUs and qPCR targeting its 23S rRNA gene. Analysis showed the bacterial numbers of *H. pylori* from both FLA species significantly increased over 50-fold by day 4 at 37C (Fig. 1A and B), suggesting that FLA contribute to the amplification of *H. pylori* and promote their multiplication.

Similar qPCR analysis results were also obtained at 25 °C, but with somewhat slower kinetics. However, as seen in Fig. 1C, viable counts did not increase ($P < 0.001$), and viable *H. pylori* without amoeba hosts also decreased by over 4 $\log_{10}$ after 3 days, suggesting that *H. pylori* cells may entered the VBNC state period after time; This suggested that lower temperature may affect the growth of *H. pylori* and increase the likelihood of VBNC cell formation. The reported optimum temperature range for growth of *H. pylori* is from 30 to 37°C (Jiang and Doyle 1998), which is consistent with our observation that the growth of *H. pylori* alone declined in lake water and viable cells only persisted for 2 days at 25°C. In contrast, at the same temperature but in the presence of amoebae *H. pylori* continue to grow and survived for up to 4 days.

Therefore, it was of interest to further view, by electron microscopy, the formation of numerous replicative phagosomes within *V. vermiformis* and *W. magna* filled with *H. pylori* (Fig. 2A and B).

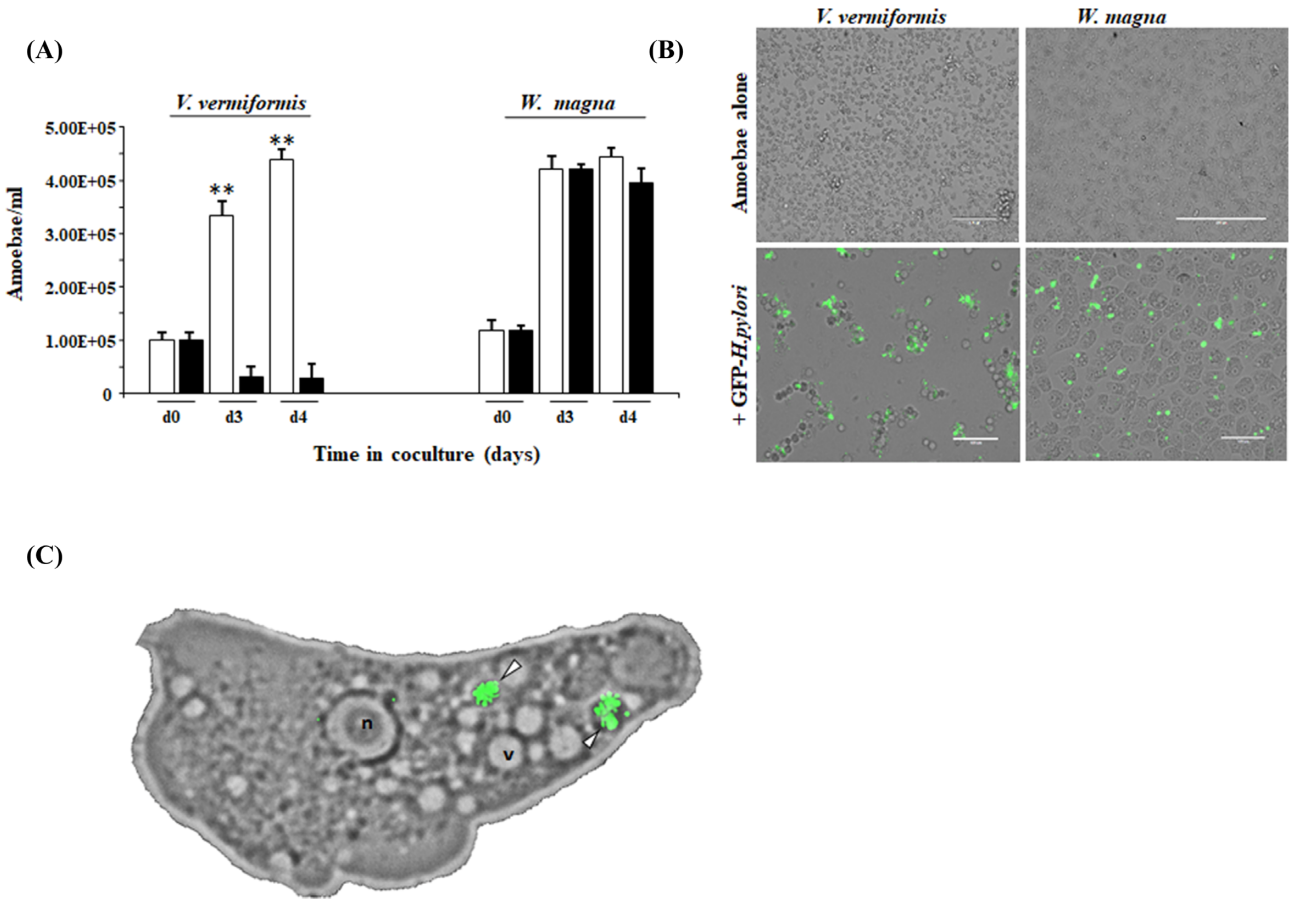

Figure 4. Effect of *H. pylori* on the amoebic viability. **(A)** Effect of *H. pylori* infection on the growth of amoebae: The different amoebae were cultured at 37°C for 4 days either with (black bars) or without (white bars) *H. pylori* at MOI of 100 as described in methods. The data are expressed as the number of amoebae/mL of medium and are the average ± SEM of four independent experiments performed in triplicate. Statistical differences (Student *t*-test) between the growth of amoebae cultured either with or without *H. pylori* are indicated (**$P < 0.001$). **(B)** Effect of *H. pylori* on monolayer formation by amoebae: Representative fluorescent images of the two *amoebae* cultured in 6-well plates either with or without *H. pylori* after 72 h. **(C)** Micrograph (60x) of *W. magna* infected with GFP–*H. pylori* after 6 h of co-culture. The white arrowhead points to replicative phagosomes containing *H. pylori*. nucleus (n), vacuole (v).

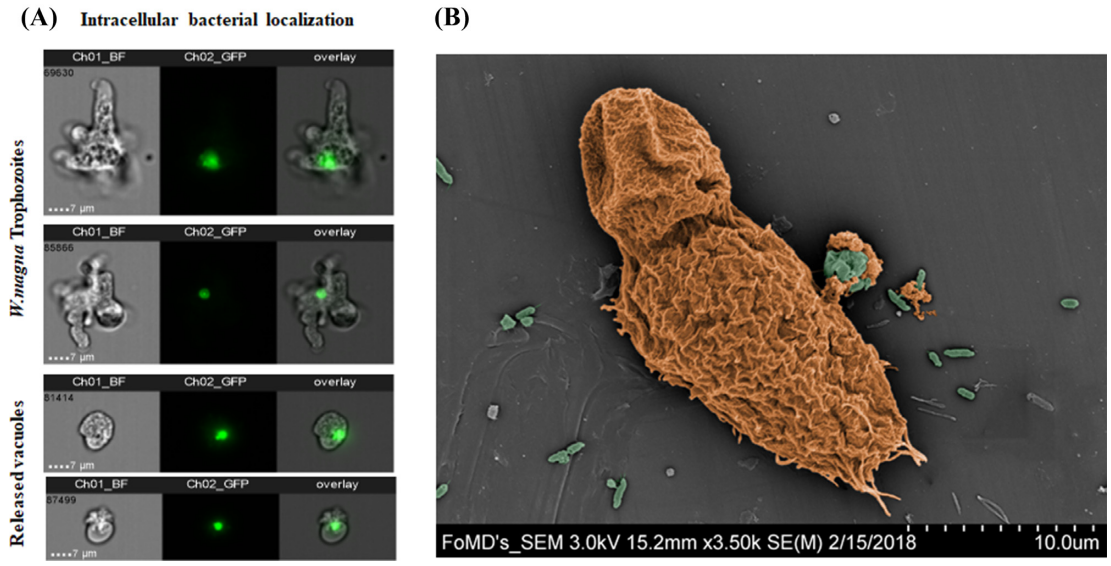

Figure 5. Packaging of *H. pylori* by amoebae. **(A)** Internalized GFP-*H. pylori* in live *W. magna* and located in released vacuoles using ImageStream flow cytometry. **(B)** Colorized scanning electron micrograph of *H. pylori* packaged in excreted vesicles by *W. magna*. *H. pylori* (highlighted in green) was cocultured with *W. magna* (in orange) for 24 h, at an MOI of 1:100.

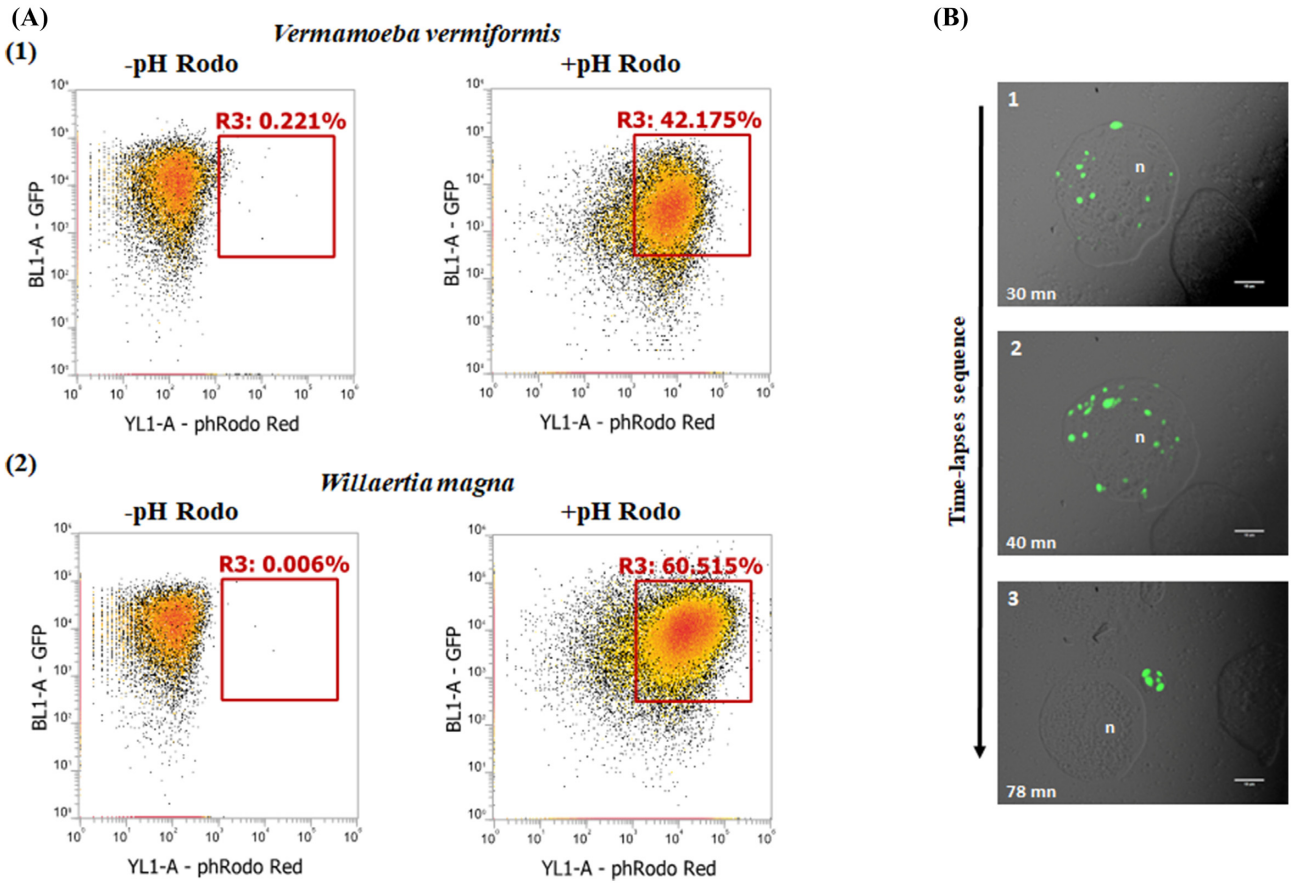

Figure 6. Amoebal intracellular pH analysis. **(A)** Amoebae infected with GFP-*H. pylori* for 2 h at 37°C, analysis of intracellular pH dynamics evaluated by flow cytometry. The fluorescence percentage represents the occurrence of acidic phagosomes with or without treatment with red fluorescent intracellular pH indicator (pHrodo Red); (1) *V. vermiformis* (2) *W. magna*. **(B)** Green fluorescent intracellular pH indicator in *W. magna* infected with non GFP-*H. pylori* and treated with pHrodo Green. Time-lapse fluorescent micrographs representing: Panel (1) and (2) acidic phagosomes formation; and (3) release of phagosome containing *H. pylori* from trophozoite after 78 min; n, nucleus; (100x objective).

Since the cag Pathogenicity Island (cagPAI) that encodes a type IV secretion system (T4SS), is considered to be involved in bacterial internalization and is a major putative virulence factor in *H. pylori* (Tegtmeyer, Wessler and Backert 2011; Boonyanugomol *et al.* 2013; Tegtmeyer *et al.* 2017), we tested whether two different genes belonging to the cagPAI family, *cagE* and *cagM* were required for bacterial multiplication in presence of FLA.

As seen in Fig. 3 and based on qPCR analysis, *H. pylori* mutant deficient in cagPAI gene *cagE* (Δ *cagE*) failed to multiply in amoebae and growth kinetics dropped by over two $\log_{10}$ by day 3 post infection compared to Δ *cagM* mutant (Fig. 3 A and B); suggesting that deficient *cagE* gene cells within FLA are eventually digested but not with deficient *cagM* cells, showing the importance of *cagE* gene in *H. pylori* infection and multiplication within amoebae.

Similar results were also obtained at 25 °C, indicating that the observed phenomenon was not affected by a lower temperature (Supplementary data SD 3).

Interestingly, several studies indicate that a functional crosstalk exists between the two virulence factors, vacuolating toxin VacA and the effector protein CagA (encoded by the cag pathogenicity island together with a T4SS); this relationship could be a strategy to improve the bacterium's fitness within the hostile gastric environment (Gangwer *et al.* 2010; Boquet and Ricci 2012; Djekic and Muller 2016; Ricci 2016). Indeed, our results demonstrates that *CagE*-deficient mutants did exhibit an intracellular proliferation defect. It has been demonstrated that *CagE* and *CagM* are required for CagA translocation through Cag-T4SS and are also needed for the secretion of interleukin-8 (IL-8) by the host gastric epithelial cells (Fischer *et al.* 2001; Shariq *et al.* 2015; Bats *et al.* 2018). Unexpectedly, the *CagM*-deficient gene did not affect the growth of the bacteria; implying that *CagE* gene may be essential for *H. pylori* survival within amoebae and additional studies will be required to understand the genes involved in *H. pylori* entering and replicating within amoebae.

Furthermore, we screened the impact of *H. pylori* infection on the growth of amoebae. Within 3 days the number of viable *V. vermiformis* trophozoites in co-culture with GFP-*H. pylori* had dropped significantly, the growth was reduced by 94% compared to those of uninfected amoebae or those cocultured with *W. magna* (Fig. 4A). The effect of *H. pylori* towards *V. vermiformis* is further illustrated in Fig. 4B, showing that *H. pylori* prevented the formation of a *V. vermiformis* cell monolayer and affected their appearance (encystment) after 3 days of infection in sterile lake water, while *W. magna* was apparently unaffected.

Helicobacter pylori has long been viewed as an extracellular bacterium, attached to gastric epithelial cells and adapting different mechanisms to avoid phagocytosis and evade immune responses (Allen 2000, 2007). Based on our transwell assay results, no bacterial increase was observed in amoeba-free compartments (Supplementary data SD 4), suggesting that *H. pylori* replicates intracellularly within the two amoebae tested. On closer observation using fluorescent microscopy showed *W.*

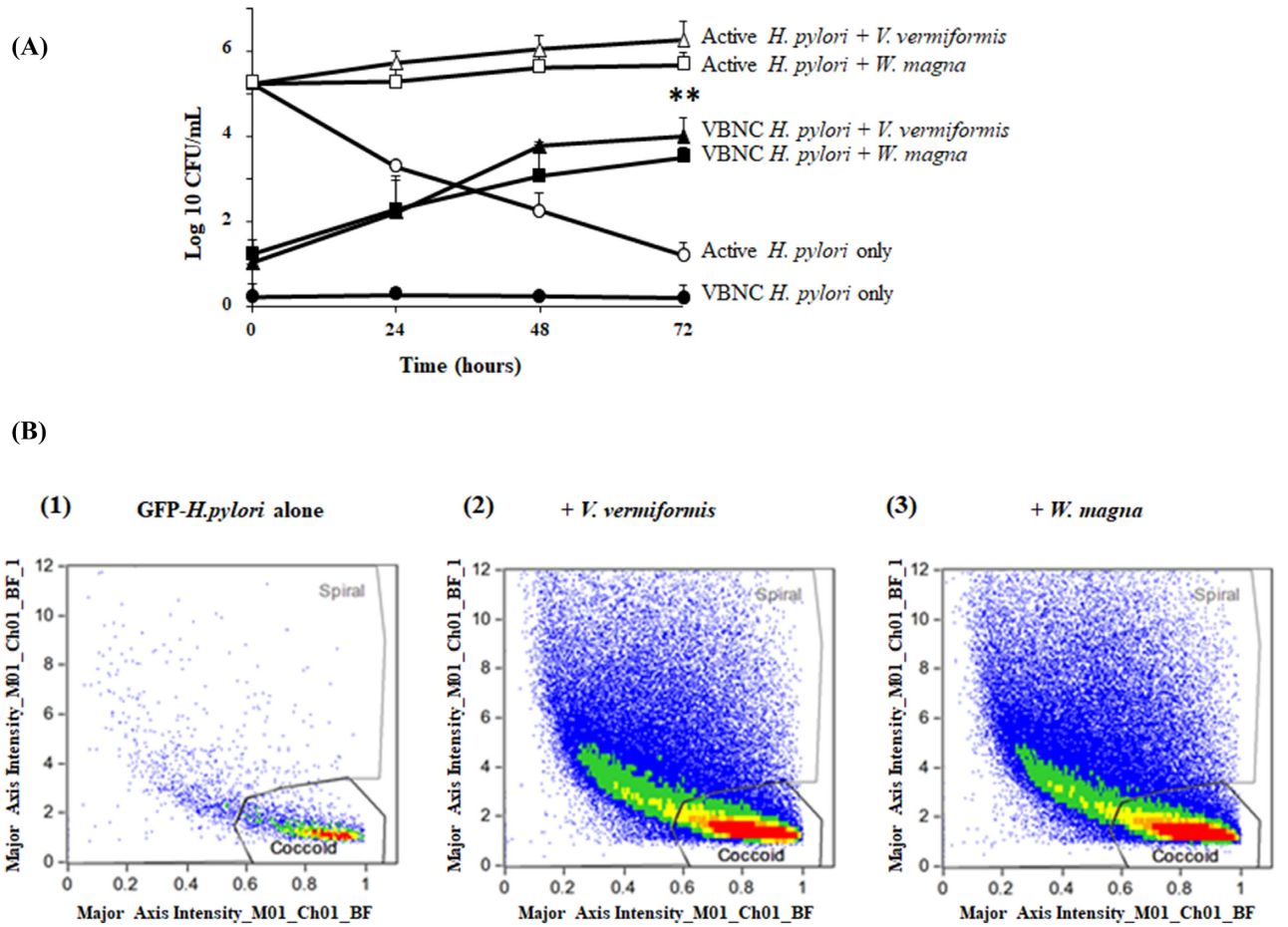

Figure 7. VBNC *H. pylori* recovery analysis. **(A)** Growth kinetics of active or VBNC *H. pylori* with *V. vermiformis* (open and closed triangles) and *W. magna* (open and closed squares) or without amoebae (open and closed circles respectively) by CFU counts at 25°C for up to 72 h. Data are the mean ± SEM, $n = 3$ (biological repeats) performed in triplicate. Statistical differences by Student's *t*-test comparing the indicated strains to the control in the absence of amoeba after 72 h (**$P < 0.001$). **(B)** ImageStream flow cytometry analysis of the VBNC GFP-*H. pylori* reactivation induced by adding amoebae. Percentage of coccoid and spiral shape bacteria after 30 mn incubation: without amoebae (1), with *V. vermiformis* (2) and with *W. magna* (3).

magna phagosomes filled with GFP-*H. pylori* (Fig. 4C). Interestingly, our study demonstrates that intracellular replication of *H. pylori* had a cytotoxic effect on the *V. vermiformis* host by disrupting the culture-flask monolayer and damaging the host cells. However, our results also demonstrate that *H. pylori* is able to replicate and escape from within *W. magna* without killing the host cell.

Release of packaged *H. pylori* by *W. magna*

From the results depicted in Fig. 4 (A and B) showing unaffected *W. magna* cells exposed to *H. pylori* and the successful growth of the bacterium, it is reasonable to hypothesize that *H. pylori* is able to replicate and escape from within *W. magna* without killing the host cell. To this end, we applied an imaging flow cytometry approach to study infected amoebae and localize phagocytized GFP- *H. pylori* cells. Fig. 5A shows live images of GFP-*H. pylori* internalized by *W. magna* and located in amoebal-released vesicles. Using scanning microscopy, we can see *W. magna* in the process of releasing a vesicle filled with bacteria (Fig. 5B). Tracked by fluorescence microscopy, different stages of *H. pylori* contained within *W. magna* phagosomes were observed (Supplementary data SD. 5). Overall these observations demonstrate that *H. pylori* indeed most likely replicates within *W. magna* and exits via released vesicles.

Interestingly, this phenomenon was first observed with *L. pneumophila* as a survival strategy that enable them to avoid digestion and to be packaged in the egested pellets from FLA and ciliates (Rowbotham 1980; Berk *et al.* 1998, 2008; Bouyer *et al.* 2007). More importantly, the released vesicles can serve as natural reservoirs for many opportunistic bacteria pathogens, including respiratory strains when expelled within aerosolized amoebal vesicles (Berk *et al.* 1998; Shaheen and Ashbolt 2018). Hence the growth outcome of *H. pylori* is likely dependent upon the species of host amoebae and its growth environment (Allen 2000; Dey *et al.* 2009).

H. pylori adaptation within acidified phagosomes

Considering that *H. pylori* is a neutralophile known to colonize the acidic human stomach by using a variety of acid-adaptive mechanisms (Wen *et al.* 2003; Scott *et al.* 2007), we next examined and tracked the intracellular pH of amoebae after infection. Previous studies showed that GFP fluorescence decreases significantly under acidic conditions (Campbell and Choy 2001; Roberts *et al.* 2016), hence we used an intracellular pH indicator (green or red at pH 4–5) and flow cytometry to illustrate phagosome acidification.

Figure 6 (A1 and A2) shows the co-culture samples of GFP *H. pylori*-amoebae incubated with pHrodo Red becoming

increasingly fluorescent over time. There was a clear distinction between positive and negative pHrodo, indicating the presence of GFP-*H. pylori* in acidified amoeba phagosomes. In a separate experiment, we tracked the intracellular pH of *W. magna* after infection with a non-GFP *H. pylori* using a green intracellular pH indicator (pHrodo green). As seen in Fig. 6B1 and after 30 min of incubation, the formation of acidified phagosomes followed ingestion by *W. magna*. Our observations suggest that *H. pylori* adapt the acidified phagosomal compartment and resist digestion by free-living amoebae.

Culturability recovering of VBNC *H. pylori* cells

Helicobacter pylori is well known to exist in two morphologies in the culturable and nonculturable states; spiral and coccoid forms (Nilius *et al.* 1993; Benaissa *et al.* 1996; Willen *et al.* 2000; Young, Allaker and Hardie 2001; Saito *et al.* 2003), the coccoid form has also been described for a VBNC form of *H. pylori* (Nilius *et al.* 1993; Vijayakumari *et al.* 1995; Mizoguchi, Fujioka and Nasu 1999). Morphological change to the coccoid form appears to be a survival strategy that allows VBNC cells to persist under harsh environmental conditions (Mizunoe *et al.* 2000; Oliver 2010; Zeng *et al.* 2013; Zhao *et al.* 2013).

As we have previously observed (Fig. 1C), *H. pylori* lost almost all culturability after 3 days at 25°C in the absence of amoeba host but the cells persisted and were detectable by qPCR. This discrepancy between the two methods could be explained by the fact that qPCR is able to detect viable, dead and VBNC cells, while the culture method detects only the living culturable bacteria. Interestingly, it has been previously reported that amoebae are able to reactivate VBNC bacteria into a viable pathogenic state (Steinert *et al.* 1997; Garcia *et al.* 2007; Dey *et al.* 2019). We thus investigated VBNC-*H. pylori* reactivation in co-culture with amoebae using culture and imaging flow cytometry. As shown in Fig. 7A, VBNC cells cocultured with *V. vermiformis* and *W. magna* trophozoites appeared to revert to a culturable state, and that coculture with amoebae led to over 3-$\log_{10}$ increase ($P < 0.001$) of culturable *H. pylori* after 48 h compared to VBNC *H. pylori* alone.

Morphological changes between spiral and VBNC *H. pylori* were detected by using Imaging flow cytometry (Supplementary data SD 6). After 2 h of incubation time, the VBNC *H. pylori*-amoebae co-culture was treated with gentamicin and then cultured for another 30 min, at which time the bacterial population analyzed by IFC. In Fig. 7B–D and based on bacterial morphology and size, the co-cultures with *V. vermiformis* and *W. magna* resulted in a significant increase in spiral-shape GFP-*H. pylori* (50%) when compared with GFP-*H. pylori* alone (26%). Taken together, the recovery from VBNC cells by co-incubation with amoebae was associated with a morphological change from coccoid to spiral-shape cells as described by IFC. Hence we demonstrate for the first time the ability of free-living amoebae to revert VBNC-*H. pylori* cells into a viable spiral form. Thus, FLA may also be a promising tool for the enrichment of environmentally-stressed *H. pylori*, particularly from water samples with low concentrations, as used for other amoeba-resisting bacterial pathogen recovery from waters (Corsaro *et al.* 2010).

Our study demonstrates that *H. pylori* is likely to replicate and survive in FLA under conditions that simulate aquatic environments in which *H. pylori* has been previously detected by PCR (Linke *et al.* 2010; Moreno and Ferrus 2012; El-Sharouny, El-Shazli and Olama 2015). Prospectively, FLA could be a promising cell model for the identification of the genes encoding the proteins involved in the adaptation of helicobacters to withstand digestion within acidic phagosomes. We also report evidence that FLA may act as a biological mediator of activation of dormant, VBNC *H. pylori*. Our findings also highlight and support the hypothesis put forth by Chatton over 100 years ago that amoebae could serve as vectors (Chatton and Lalung-Bonnaire 1912), and in this case sustain water-associated *H. pylori* transmission. Further studies are necessary in order to examine the transmissibility of VBNC and spiral *H. pylori* contained within FLA (trophozoites and/or vesicles), which would help inform future risk assessments of *H. pylori* in water.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

ACKNOWLEDGMENTS

We would like to thank Monika Keelan (University of Alberta) and John Kao (University of Michigan) for supplying the different *Helicobacter* strains used in this study. We are also very grateful for the technical support provided by Sieren Wang, Candis Scott and Homun Yee. Flow cytometry was performed at the University of Alberta, Faculty of Medicine and Dentistry Flow Cytometry Facility, which receives financial support from the Faculty of Medicine and Dentistry and Canadian Foundation for Innovation (CFI) awards to contributing investigators. Aja Rieger is an ISAC Shared Resource Lab Emerging Leader (2017–2021).

FUNDING

These studies were supported by Alberta Innovates (grant number 201300490), Alberta, Canada.

Conflicts of interest. None declared.

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