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TOPIC HIGHLIGHT

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Helicobacter pylori: A chameleon-like approach to life

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Abstract

Helicobacter pylori (*H. pylori*) is widely adaptable for colonization in human stomachs in more than half of the world's population. The microorganism is characterized by an unusual capability of arranging itself in both genotypic and phenotypic ways. Stressing conditions, including antimicrobial agents in sub-inhibitory concentrations, facilitate entering the viable but non-culturable state in which bacterial cells acquire the coccoid form. This morphotype represents an important strategy for bacterial survival in unsuitable conditions and also allows escape from the immune system. *H. pylori* is capable of forming biofilm outside and inside the host. For the bacterial population, the sessile growth mode represents an ideal environment for gene rearrangement, as it allows the acquiring of important tools aimed to improve bacterial "fitness" and species preservation. Biofilm formation in *H. pylori* in the human host also leads to recalcitrance to antibiotic treatment, thus hampering eradication. These lifestyle changes of *H. pylori* allow for a "safe haven" for its survival and persistence according to different ecological niches, and strongly emphasize the need for careful *H. pylori* surveillance to improve management of the infection.

Key words: *Helicobacter pylori*; Viable But Non Culturable state; Route of transmission; Biofilm *in vitro*; Biofilm *in vivo*

Core tip: *Helicobacter pylori* (*H. pylori*) is a Gram negative bacterium that colonizes the human stomach early in the life of the host and tends to persist. The present review is focused on the general phenomenon of the fickleness in *H. pylori* and analyses the significance and role of this "chameleon-like" approach to life in the persistence of this fastidious bacterium outside and inside the host.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram negative bacterium that colonizes the human stomach early in the life of the host and tends to persist. It is estimated that the bacterium is present in the gastric mucosa of half of the world population, but disease only occurs in about 15% of colonized individuals[1].

Today, *H. pylori* is recognized as the most common cause of gastritis, peptic, and duodenal ulcers, and is responsible for an increasing incidence of gastric cancer[2-4]. The natural habitat of the microorganism is the mucus layer of the stomach, but it may also need to survive in other environments to become a life-long infection threat[5]. In fact, a large number of studies support evidence of the microorganism in dental plaque (detected by culture and PCR techniques), in houseflies, in human and animal feces[6-10], and in natural environmental waters[11-17]. Therefore, water supplies contaminated by sewage containing fluids or feces from infected people have been considered as a potential route of *H. pylori*

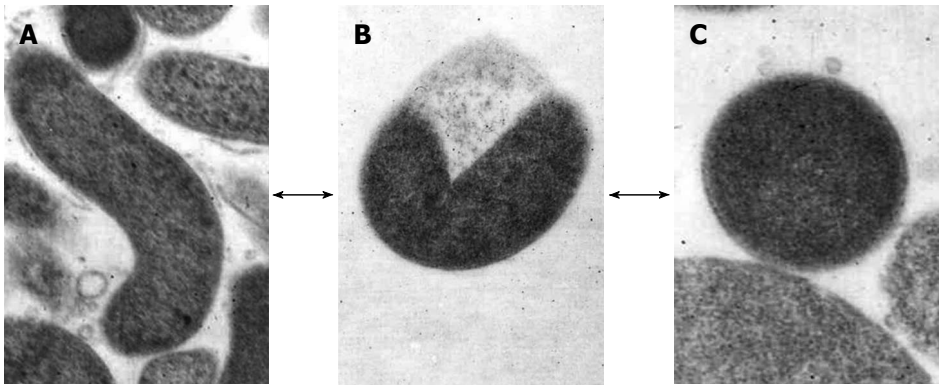

Figure 1 Morphological appearance of *Helicobacter pylori*. A: Rod-shaped; B: U-shaped; C: Coccoid form. Arrows show the hypothetical alternative pathway between the different forms. Transmission electron microscope: original magnification × 20000.

transmission[13,14,18,19].

H. pylori is able to overcome environmental stressed conditions, such as sub-inhibitory concentrations of drugs or non-permissive atmosphere, by entering the viable but nonculturable (VBNC) state, in which the microorganism modifies its morphology from a spiral to coccoid (spherical) form with a loss of cultivability[20,21]. This important strategy of survival is emphasized when bacterial cells organize themselves into microbial communities, establishing a sort of "free multicellularity" forming biofilm[22-26].

Moreover, as a species, *H. pylori* possesses one of the most fluid genomes within the prokaryotic kingdom[27,28], with many investigators asserting that *H. pylori* polymorphisms reflect human phylogeography and historical migrations[29-31], as it is virtually impossible to find two identical DNA patterns in microorganisms isolated from different hosts[28,32,33]. Furthermore, a host individual can harbor more than one isolate, which can derive either from a micro-evolutionary change among strains coming from a unique host microorganism or from a multi-strain infection. This typology of colonization may offer a condition for a more efficacious bacterium-host association during long-term harboring colonization[33,34].

The present review is focused on the general phenomenon of the fickleness in *H. pylori* and analyzes the significance and role of this "chameleon-like" approach to life in the persistence of this fastidious bacterium outside and inside the host.

VBNC STATE: A GENERAL VIEW

Bacteria, when subjected to inauspicious environmental conditions such as an insufficient supply of nutrients, non-permissive temperature, oxygen, or pH conditions, irradiation, or toxic chemicals, can survive by entering the VBNC state[35-37]. In this "survival state" that is well-documented in both Gram negative and Gram positive bacteria (including those of medical interest and widely-recognized in aquatic environments), the bacteria are not detectable by conventional culture techniques, and can undergo changes in morphology[38], cell wall composition[39], gene expression[36], and protein synthesis[40].

This protective condition, first described by the Rita Colwel group[35], represents a viable survival strategy in unsuitable situations that has contributed to the formation of environmental reservoirs of non sporulating bacteria[39].

It has been demonstrated that bacteria in the VBNC state are able to maintain their metabolic activity and pathogenicity, as well as, in some cases, the ability to revert to active re-growth conditions[41-44].

The broad distribution of VBNC cells among bacterial species underlines their significance in the ability to cope with stresses, and also draws attention to their potential risk for human health[37].

VBNC STATE IN *H. PYLORI*

H. pylori, which can be defined "a master of adaptation", is able to overcome stressed conditions, such as sub-inhibitory drug concentrations or non-permissive atmospheres which occur outside and inside the host, by entering the VBNC state[45]. This protective state occurs when the microorganism modifies its elongated, spiral morphology to transform into the coccoid morphotype through a U-shaped intermediate form (Figure 1), which results in it becoming unculturable[20]. Thus, *H. pylori* essentially displays three different cellular types: the spiral cells which grow under optimum conditions for replication and are both virulent and capable of inducing inflammation in experimental models; the viable coccoid forms that are unable to grow on solid media and are characteristically more persistent in the host and environment; and the degenerative spiral and coccal dead forms[21].

The conversion into the VBNC state represents an active process in which the microorganism switches on their adaptive machinery as a protection mechanism. In support of this, a study by Costa *et al*[46] demonstrated that the change in shape in *H. pylori* was related to its more resistant condition, due to a significant modification in the cell wall which resembled those of endospores. In fact, the peptidoglycan of *H. pylori* coccoid cells was similar to those of sporulating *Bacillus sphaericus*.

In another work, Chaput *et al*[47] demonstrated the ability of *H. pylori* coccoid cells to escape detection by the immune system because of a significant modification of the cell wall peptidoglycan which had no IL-8 stimulatory activity in gastric epithelial cells. Thus, *H. pylori* in the VBNC state may be able to escape or modulate the host response and thereby persist in the human stomach.

The capability of viable *H. pylori* coccoid cells to be persistent outside the host has been demonstrated in many works. In a study by Shahamat *et al*[48], it was demonstrated that VBNC *H. pylori* cells could be present for up to 1 year in fresh water. In another work, the authors[49] verified the entrance of *H. pylori* into a VBNC state as the cells aged in a natural freshwater environment by using viability assays, also confirming that coccoid viable cells continued to transcribe several genes, including those responsible for its virulence.

Regarding this last aspect, Wang *et al*[50] obtained a coccoid *H. pylori* population by exposure to sub-inhibitory concentration of antibiotics, with the target fragment of the *cagA* gene of these cells being amplified and cloned into a plasmid, and then transformed in *Escherichia coli*. By sequence analysis, the authors demonstrated that coccoid *H. pylori* contained a completed *cagA* gene that displayed a homology with the reported original sequence of vegetative forms of *H. pylori* (99.7%), thus supporting speculation about the pathogenicity of these cells.

The contamination of drinking water by human feces has been suggested as one of the possible routes of *H. pylori* transmission, and it has been demonstrated that the microorganism is present in the VBNC state in this unsuitable environment[51], meaning that their role in fecal-oral transmission *via* contaminated water sources cannot be disregarded.

In our study[19], we demonstrated, by Nested-PCR, the presence of *H. pylori* DNA in seawater both free and bound to zooplanktonic organisms, such as copepod and cladocerans. Considering that the intensive activity of enzymes produced by prokaryotic and eukaryotic cells in seawater favors the instability and degradation of nucleic acids[52], we assumed that the detected nucleic acids were part of viable resistant coccal cells able to survive in marine environments. Indeed, *H. pylori* was isolated by culture from marine zooplankton, supporting speculation about the potential role of zooplankton in *H. pylori* survival and transmission[15]. This isolated microorganism, named *H. pylori* MDC1, harbored a genotype coding for the most important virulence markers and, in particular, contained cagPAI, which could both exert a role in adapting to the marine environment and also be acquired by different species. In this regard, a *cagA* like gene of *H. pylori* was found to be present in environmental isolates of *Aeromonas* spp. from different water samples in India[53].

All of these considerations strongly underline that the morphological fickleness of *H. pylori* is in response to external stimuli entering the VBNC state, and representing, during the lifespan of the microorganism, a powerful response to improve bacterial "fitness" and species preservation.

MICROBIAL BIOFILM: A GENERAL VIEW

Bacterial biofilms may be considered an ancestral selective event used by prokaryotes to adapt to the environment. In this way, microorganisms are organized in communities that settle and proliferate on biotic and abiotic surfaces embedded in a highly hydrated self-produced matrix constituted of extracellular polymeric substances[54-57].

Bacteria aggregated in biofilm represent a complex dynamic system that could be considered the best program of survival in unsuitable conditions[57]. Many bacterial species match their lifecycle to the human host and environment, and thus change their regulatory processes to adapt to this new niche[54].

It is widely recognized that an ever increasing number of infections arise from biofilm-producing microorganisms that are extremely difficult to eradicate. Infections caused by sessile bacteria are characterized both by a strong tolerance to antimicrobial/biocidal agents and by an extraordinary resistance to phagocytosis, which allows them to evade the hosts' defenses[58,59]. These processes are thought to be the major contributors to the etiology and the persistence of infectious diseases.

Biofilm growing bacteria represent a major cause of exacerbating chronic infections with persistent inflammation and damage of tissue[60].

Many signals and gene products are involved in biofilm development under a cyclic and dynamic process depending on different bacteria and surfaces[61,62]. Into these microbial communities, bacteria may convey their presence to one another by producing, detecting, and responding to small diffusible signal molecules referred to as autoinducers, which carry out the Quorum-Sensing[63].

Moreover, bacteria organized in a biofilm can find a protected environment to facilitate horizontal gene transfer, thus providing a bacterial population with newly-modified genomes[64].

The biofilm represents an ideal environment for gene rearrangement and also for the horizontal bacteriophage and plasmid transfer that contributes significantly to strain variability and adaptability[65].

BIOFILM FORMATION IN *H. PYLORI*

It is well known that *H. pylori* is capable of forming biofilm both outside and inside the human host, which likely provides greater protection under stressful conditions.

The first evidence of biofilm formation in *H. pylori* was provided by Stark *et al*[66] in 1999, which characterized the water-insoluble biofilm accumulated at the air/liquid interface of a continuous culture of *H. pylori* NCTC 11637 by gas chromatography and mass spec-

trometry. Fucose, glucose, galactose, glycero-manno-heptose, N-acetylglucosamine, and N-acetylmuramic acid were identified, suggesting their role in enhancing resistance to host defense factors, antibiotics, and micro-environmental pH homeostasis, which facilitates the growth and survival of *H. pylori in vivo*.

When studied on abiotic surfaces, *H. pylori* forms a structured biofilm with an extracellular polymeric substance (EPS) matrix in which are mixed exogenous DNA fragments (eDNA)[67]. This extracellular DNA (eDNA), detected in the 2 d-old EPS biofilm matrix of *H. pylori* strains, showed some remarkable differences when compared by RAPD-PCR analysis to the intracellular DNA (iDNA). The different profiles of eDNA and iDNA indicated that lysed cells were not the primary source of eDNA release, which suggested a role in the active dynamic flow of information, such as recombination processes (*via* transformation), and contributing to the wide genomic variability of this microorganism that has been defined as a "quasi-species". Moreover, promotion of genetic transfer was studied by our group[27] between two clinical *H. pylori* strains when grown in the biofilm mode. Two co-cultured *H. pylori* clinical strains were analyzed for their cooperative/competitive behavior and selected clones, coming from their mixed mature biofilm, were compared through DNA fingerprinting and main virulence factors analysis.

Biofilms developed by mixed *H. pylori* strains were well-structured, with a higher amount of EPS matrix and viable cells than those detected by the parental strains. Finally, genetic analysis by both RAPD-PCR and *cagA* (EPIYA motifs)/*vacA* virulence genes of 45 clones showed a high number of recombinant clones together with the generation of more virulent strains. Thus, these recombinant clones might provide an advantage to the bacterial population by promoting the development of a more adhesive and stable biofilm. These data demonstrated that the biofilms developed by multiple *H. pylori* strains were more complex and structured than the ones associated with single strains. Such conditions might promote the genetic exchange favored by the protected environment and explain the development, in a single host, of more virulent and difficult to eradicate strains.

In an *in vitro* study, Cole *et al*[68] demonstrated the negative effect of mucin on *H. pylori* biofilm formation, suggesting that in the mucus-rich stomach, *H. pylori* planktonic growth is favored over biofilm formation. Moreover, these authors found, in the *H. pylori luxS* mutant, that biofilm formation was affected by overproduction of LuxS, as was observed in a *Streptococcus mutans luxS* mutant by Merritt *et al*[69]. In this study, Cole indicated the relative importance of the Quorum-Sensing gene, *luxS*, and also the *cagE* type Ⅳ secretion gene to the production of biofilms by *H. pylori*.

However, biofilm production and its characterization are strongly influenced by the different methods and media used for biofilm culture[70]. In a recent study by Bessa *et al*[24], the authors reported on the important influence of culture media on *H. pylori* growth, both in its free-living and biofilm growth modes. In particular, they suggested that the adherence and ability of *H. pylori* to form biofilm were not accomplished by the same mechanism in different media.

Finally, they demonstrated that sub-Minimal Inhibitory Concentration (MIC) values of amoxicillin and clarithromycin could increase biofilm biomass. The sub-MIC drug influence on *H. pylori* biofilm-forming capability may have clinical consequences, as during any antibiotic treatment focused to a particular infection, *H. pylori* bacteria can be exposed to sub-MICs of antibiotics, which constitute a condition that can stimulate the switching from planktonic to sessile cells forming biofilm, and consequently lead to recalcitrance to antibiotic treatment, and thus hampering eradication.

Similar results were obtained by Yonezawa *et al*[71] that displayed the increasing of biofilm biomass after various concentration of clarithromycin treatment. They also demonstrated that biofilm-forming capability in *H. pylori* affects the generation of clarithromycin resistance with the presence of a point mutation at positions 2142 and 2143 in the domain V loop of the *23 r*RNA gene more frequently detected in sessile cells than their planktonic counterparts.

These conclusions strongly underline that biofilm formation can affect the generation of antibiotic resistance mutations in *H. pylori*.

The first evidence of an *ex vivo H. pylori* biofilm was raised by the Carron group[25,26]. They showed, *via* Scanning Electron Microscopy (SEM) analysis, the presence of dense, mature *H. pylori* biofilm detectable in urease *H. pylori* positive biopsy specimens that were absent in urease-negative controls. Of the patients who tested urease positive for *H. pylori*, the average percentage of total surface area covered by biofilms was 97.3%. Those testing negative had average surface area coverage of only 1.64%. This study demonstrated that, compared with controls, urease-positive specimens have significant biofilm formation, whereas urease-negative specimens have little to none. This was reflected in the significantly-increased biofilm surface density in urease positive specimens compared with urease-negative controls.

The dynamic behavior of *H. pylori* in the colonization of human gastric mucosa was investigated in patients previously treated for *H. pylori* infection by us[23]. In our study, biopsy samples were taken and analyzed for *H. pylori* detection by cultural, molecular, and ultra-structural methods. Viable *H. pylori* cells were isolated in 33% of performed cultures, whereas the expression of the *glmM* constitutive gene and the Quorum-Sensing related l*uxS* gene were detected in 90% of the analyzed biopsies. In these positive cases, the analysis of *glmM* and *luxS* sequences confirmed *H. pylori* identity. The SEM analysis of biopsies coming from patients harboring culturable bacteria revealed a prevalent "S-shape" *H. pylori* morphotype co-existent with coccoid aggregated bacteria embedded in abundant matrix; samples coming from *H.*

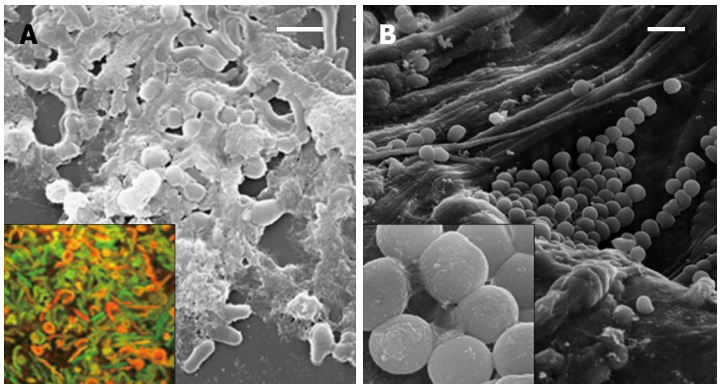

Figure 2 Biofilm of *Helicobacter pylori*. A: Scanning electron micrograph of mature biofilm on a polystyrene surface with rod shaped and coccoid cells embedded in an abundant matrix. Insert: Confocal laser scanning microscopy image of mature *in vitro* biofilm with viable (green) and dead (red) cells, live/dead staining; B: Clusters of coccoid *Helicobacter pylori* cells in the gastric mucosa also embedded in a matrix (insert). Bars represent 5 μm.

pylori positive patients showed clustered coccoid bacteria arranged in a microbial biofilm only through molecular method (Figure 2).

The undoubted clinical significance of coccoid *H. pylori* cells in epithelial gastric cells[72], also described in cases of adenocarcinoma[73], alone or grouped in clusters, underlines the need for planning of more efficacious testing protocols, such as RT-PCR methodology, to avoid underestimating *H. pylori* colonization by identifying camouflaged and protected clustered bacteria, and taking into account this serious microbial problem in medicine in the recommendation of therapeutic regimens.

CONCLUSION

H. pylori, more than other microorganisms, displays an amazing adaptive ability when confronted with stress conditions.

The viable coccoid morphotypes able to retain virulence factors and the aggregative behavior among *H. pylori* cells growing as a biofilm suggest a long-term survival of these bacterial communities outside and inside the host, enabling bacterial transmission with important clinical repercussions. In particular, these new living conditions, consisting of new self-organized populations, guarantee persistence, genetic variability, and antimicrobial resistance, as well as prolonging protection. For successful therapy, it may be essential not only to eliminate the bacillary forms but also to rapidly suppress and/or destroy the coccoid forms that are clustered in biofilm as well[74,75].

A recent study suggested a new effective treatment for the demolition of *H. pylori* biofilm which includes in the therapeutic regimen N-acetylcysteine (NAC), a mucolytic agent used in medical practice for the treatment of patients with chronic respiratory diseases[76]. In a clinical trial[77], the authors obtained a significantly higher percentage of *H. pylori* eradication (65% *vs* 20%) in patients with at least 4 treatment eradication failures by using NAC pretreatment prior to a culture guided antibiotic regimen. N-acetylcysteine may act by disrupting the biofilm agent and favoring the planktonic growth mode of *H. pylori*, thus overcoming the tolerance phenomenon described for bacterial biofilms[78].

Novel therapeutic regimens including plant extracts[79] or substances capable of inhibiting or destabilizing the formation of *H. pylori* biofilm should be explored to improve management of the infection.

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