A proposed role for diffusible signal factors in the biofilm formation and morphological transformation of Helicobacter pylori

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Abstract
Due to the increasing resistance of Helicobacter pylori *to antibiotics, there is a growing need for new strategies for the effective eradication of this pathogen. The inhibition of quorum-sensing activity in most microorganisms leads to a decrease in virulence. A different reaction is observed in* H. pylori*, as interfering with the production of autoinducer-2 initiates biofilm formation and increases the survival of these bacteria. Therefore, it is believed that there is an alternative way to control the physiological changes of* H. pylori *exposed to environmental stress. In this article, we present the compounds probably involved in the modulation of* H. pylori *virulence. Diffusible signal factors (DSFs) are fatty acid signal molecules involved in communication between microbes. DSFs are likely to stimulate* H. pylori *transition into a sedentary state that correlates with bacterial transformation into a more resistant coccoid form and initiates biofilm formation. Biofilm is a structure that plays a crucial role in protecting against adverse environmental factors (low pH, oxidative stress, action of immune system) and limiting the effective concentration of antimicrobial substances. This article has suggested and characterized the existence of an alternative DSF-mediated cell-cell signaling of* H. pylori*, which controls autoaggregative behaviors, biofilm formation, and the transition of microorganisms into the coccoid form.*
Keywords: *Coccoid form, DSF, fatty acids,* Helicobacter pylori*, morphological transformation*

INTRODUCTION

Helicobacter pylori is a gram-negative, microaerophilic, spiral-shaped rod (1). This bacterium causes infections in humans with different incidences in the world, but only in about 20% of infected people the signs of infection are observed (2). The *H. pylori* infection manifests itself in gastrointestinal disorders, such as chronic gastritis, gastric or duodenal ulcers, gastric cancer, and mucosa-associated lymphoid tissue lymphoma (MALT type) (3). The development of these ailments depends on many factors, including the infectious dose of the pathogen, age and condition of the infected immune system, status of the microbial flora, and environmental pressures (4,5).

Colonization of the stomach environment is very difficult because it has a low pH value of about 2. The lowest pH is in the lumen of the stomach, but it becomes near neutral in the mucosa. *H. pylori* enters the stomach through the mouth and rapidly colonizes the mucosa of both the gastric body and antrum (6). Because the colonization step is crucial for *H. pylori*, this bacterium exhibits a number of adaptive features that facilitate the smooth operation of this process. These factors include flagellar motility, bacterial adhesins, spiral shape, proteases, and mucinases. In addition, a large amount of urease enzymes leads to ammonia-dependent alkalinity of the local environment (7).

H. pylori morphological forms

All living organisms have the ability to react to environmental stimuli. These adaptations include metabolic and often morphological changes. *H. pylori* can be found in three morphological forms. Spiral forms are live, culturable, and have strong colonization capacity; coccoid forms are viable but nonculturable (VBNC) and represent persistent forms of this bacterium; and degenerated forms are notable for a disintegrated cytoplasmic membrane and are most likely a manifestation of bacterial death (8). The ability to create coccoid forms is a common phenomenon among gram-negative rods (9). It is believed that the transition of *H. pylori* from the spiral to

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the coccoid form has a protective function against environmentally disadvantageous factors such as increases in oxygen concentration (aerobiosis), changes in pH (acidic or alkaline), elevated temperature, reduced nutrient content, prolonged *in vitro* incubation, and exposure to biocides (9-11). Similarly, gram-negative rods of *Salmonella* spp. and *Campylobacter jejuni* have been shown to alter their morphology from bacillary to spherical upon exposure to adverse conditions, including low temperature, nutrient deficiency, and exposure to biocides (12,13).

Coccoid forms of *H. pylori* possess increased capacity to aggregate into monomicrobial bacterial clusters surrounded by a thick matrix of exopolysaccharide (14). Biofilm is a bacterial structure composed of microorganisms embedded within an extracellular matrix consisting of proteins, polysaccharides, and nucleic acids (15). This structure plays a very important role in protecting against elevated oxygen levels, osmotic stress, pH changes, and immune responses. It also protects against antibiotic substances by limiting their diffusion and reducing the effective concentration of biocides that act on microbes (16). Cellini et al. (14) demonstrated in biopsy samples of patients from whom they isolated *H. pylori* that the dominant phenotype was the spiral form, which coexisted with aggregated coccoid subpopulations. In contrast, in some patients only spherical forms embedded within a large amount of extracellular matrix were found. The *in vitro* analysis of the *H. pylori* biofilm revealed that the spiral shape was the dominant phenotype in the bacterial biofilm on the first day (66.49±14.76%). On the second day, the morphotype changed into coccoid (59.26±5.77%). It was estimated that most bacteria were alive. The presence of single dead microorganisms, located in the centers of the microcolonies, was also identified (17). Bacteria in the center of microcolonies are exposed to the highest concentrations of toxic metabolites with limited availability of nutrients (18). Such conditions might promote the disintegration of the cell membrane and the release of nucleic acids into the extracellular environment. Nucleic acids induce intraspecific variation in the process of horizontal gene transfer, and this enhances the formation of heterologous *H. pylori* subpopulations and the emergence of bacteria with mosaic-like genetic material (19).

Bessa et al. (20) demonstrated that subinhibitory concentrations (sub-MICs) of antibiotics, such as amoxicillin and clarithromycin, increase the biofilm biomass of *H. pylori*. These results correlate with the findings from other studies in which the *H. pylori* biofilm was treated with MIC of clarithromycin. A 4-fold and 16-fold increase in biofilm biomass was observed in 2-day and 3-day culture, respectively (21). For the three antibiotics most commonly used for eradication of this pathogen, i.e. metronidazole, clarithromycin, and amoxicillin, Faghri et al. (10) demonstrated inducible effects of sub-MIC biocide concentrations on the *H. pylori* morphological transition from the spiral to the coccoid form. Compared to the antibiotic-free control, where in 6-day culture 50% of *H. pylori* were seen in coccoid form, the highest degree of induction was obtained for the sub-MIC concentrations of amoxicillin in which 99.9% of the bacteria were in the coccoid form. Sub-MICs of metronidazole also increased the transition of *H. pylori* into the coccoid form (60%). For clarithromycin, the level was comparable to the control. That experiment also tested the effectiveness of these antibiotics against *H. pylori* preincubated with sub-MICs of biocides. An inverse correlation has been demonstrated between the antibiotic bactericidal activity and the number of coccoid forms because amoxicillin did not reduce the viability of *H. pylori* spherical forms preincubated with this biocide. These results indicate the importance of combating the bacterial biofilm, which can limit the diffusion of active bactericidal substances and may promote the conversion of *H. pylori* to more resistant coccoid forms (9,10,22,23).

The transition of spiral *H. pylori* forms, exposed to unfavourable environmental conditions, to more resistant coccoid forms is relatively well documented (9,11,22). Still, many controversies have arisen regarding the possibility of reversing bacteria from the VBNC state to the culturable state through a process called resuscitation (24). There have been reports of the possibility of converting *H. pylori* from the coccoid to the spiral form by stimulation and nutritional supplementation, while others deny the possibility of getting revertants of this bacterium (25-27). In mouse model studies, it has been shown that the transition into the spiral form is likely to occur *in vivo* because the colonization of animals with the coccoid *H. pylori* forms allowed researchers to isolate spiral morphotype from infected mice (28). Hence, it is probable that under strictly defined physicochemical and biological conditions coccoid forms may return to the spiral phenotype. The experiment conducted by Ayrapetyan et al. (29) demonstrated that it is possible to induce the resuscitation of VBNC forms of *Vibrio vulnificus* by incubating these bacteria with supernatant derived from

culturable, rejuvenated *V. vulnificus* or by exogenous autoinducer (AI)-2 supplementation. These results suggest that AI-2 molecules might be sufficient and/or necessary for the transformation of microorganisms from the VBNC form into the culturable state.

The role of AI-2 in H. pylori communication

H. pylori possess the ability to express a homologous gene for *luxS,* involved in the production of N-acyl homoserine lactones (AHLs) associated with the quorum-sensing process, and AI-2 is a primary example of these molecules (30,31). AHLs are compounds whose synthesis is related to the entrance of bacteria into idiophase, increased production of secondary metabolites and virulence factors, sporulation, and biofilm formation. Inhibition of quorum sensing might be associated with a decrease of pathogenic bacteria virulence, but there are no scientific reports for therapies aimed at AI-2-dependent inhibition of *H. pylori* communication. This is probably related to the different, compared to most bacteria, use of AI-2 as a chemorepulsive signal that leads to the escape of *H. pylori* from the source of high concentrations of AI-2 molecules. The reduction of AI-2 synthesis by *H. pylori* correlates with a decrease in bacterial motility and the promotion of biofilm formation (30-32).

The experiment conducted by Rader et al. (32), using microscopic observation and motility tests on soft agar, showed the decreased motility of *H. pylori* mutants deprived of the *luxS* gene. The lack of *luxS* gene expression resulted in the reduced expression of flagellar genes *flaA*, *flaE*, *flhA*, and *fliI*. The altered phenotype and transcription of these genes was restored by the exogenous addition of AI-2 or 4,5- dihydroxy-2,3-pentandione (DPD, an AI-2 precursor) to the culture medium or by complementing the *luxS* gene. The experiment conducted by Cole et al. (33) showed that *H. pylori luxS* mutants have the ability to grow in a similar manner as wild-type strains, i.e. there were no defects in adhesion, formation of microcolonies or spatial biofilm structure, or in physiological transition of bacteria from the spiral to the coccoid form. The biofilm formation capacity was two- to three-fold better than in wild type strains with a functional *luxS* gene. The study investigating the effect of sub-MIC concentrations of antibiotics revealed the reduced expression of *H. pylori luxS* gene compared to the control bacteria not treated with antibiotic (20). These results suggest that there is an alternative way to control physiological changes in *H. pylori* exposed to environmental stress and that this system is associated with biofilm formation and conversion of bacteria to the coccoid form.

Communication of microorganisms via diffusible signal factors

Diffusible *signal* factors (DSFs) are a newly discovered class of fatty acid derivatives that are involved in quorum-sensing activity (34). Many species of gram-negative bacteria have the capacity for inter- and intraspecies communication via fatty acids, and this is mediated by the cluster of *rpfA-G* genes (regulation of pathogenicity factor) (35). In gram-negative bacteria, these systems have been described in γ-Proteobacteria (*Xanthomonas campestris*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Xylella fastidiosa*) and β-Proteobacteria (*Burkholderia cenocepacia*) (36). Moreover, the fatty acid communication system has also been described in gram-positive *Streptococcus mutans*, *S. mitis*, *S. oralis*, and *S. sanguinis* (37). The DSF-dependent autoinductive activity involves a classic two-component system in which DSF molecules, upon reaching a critical concentration, induce sensor kinase RpfC, which in turn phosphorylates the RpfG regulator. RpfG causes transduction of the signal and expression of the relevant genes (35,38,39). Communication via DSFs is involved in many processes that are crucial to the physiology and pathogenicity of bacteria, such as the production of extracellular enzymes (proteases, endoglucanases, chitinases), microcolony and biofilm formation, autoaggregative behavior, cell motility, resistance to toxins and biocides, and oxidative stress activation (40). DSFs accumulation occurs in the early stationary phase, and RpfF, which is similar to enoyl-CoA hydratase, is responsible for their production (39). RpfF through thioesterase activity also determines the formation of saturated free fatty acids. These, in turn, can compete with DSFs for RpfC receptors and decrease their autoinductive activity (35).

Morphological changes due to quorum sensing are well characterized in *Candida albicans* (41,42). Tyrosol is an aromatic alcohol that is a product of tyrosine catabolism and promotes the transition of *C. albicans* from yeasts to hyphal forms (41). Farnesol (a structural DSF analogue), on the other hand, exhibits antagonistic activity against tyrosol (41,42). The results of the study conducted by Alem et al. (42) indicate the ability of tyrosol to induce *C. albicans* transition to the hyphal morphotype only in the early stages of biofilm formation. Farnesol, however, was shown to play a key role in the late stages of biofilm for-

mation and to disperse biofilm structure, what is accompanied by the colonization of new ecological niches. The experiment conducted by Ryan et al. (43) also demonstrated that DSFs can actively participate in morphological changes of nonfermentative gram-negative rods. *S. maltophilia* forms filamentous biofilms, while *P. aeruginosa* creates flat biofilms of low heterogeneity. A change was noted in the bacterial biofilm architecture and its transformation from the flat to filamentous form during coincubation of *S. maltophilia* with *P. aeruginosa* or exogenous supplementation of *P. aeruginosa* culture with 10 or 50 μM DSFs. In addition, for *P. aeruginosa* an increase in proteins associated with a response to environmental stress and resistance to cationic antimicrobial peptides was also observed.

The ability to synthesize and communicate via DSFs is also likely in *H. pylori*. The *in vitro* studies conducted by Yamashita et al. (44) demonstrated the presence of autoinhibitory substances secreted by *H. pylori*, and the identification of these substances revealed the presence of fatty acids such as cis-7-tetradecenic acid (TDA) and lauric acid. Antimicrobial tests for various aerobic and anaerobic microorganisms have shown a poor inhibitory effect of these compounds. A different reaction was observed for *H. pylori* strains for which the MIC was 8–16 μg/mL. BacLight Live/Dead staining after 48 h incubation revealed that most of these bacteria were alive. These authors have thus suggested the ability of *H. pylori* to produce self-inhibiting substances with bacteriostatic activity. It appears, however, that the effect of the apparent inhibition of the bacteria was due to the transition of the microorganisms into the VBNC state, which in the case of *H. pylori* is connected with spiral-to-coccoid transformation (9,10,14,22,33). Cis-2-tetradecenic acid (C_{14}:Δ^2), which is produced by *X. fastidiosa*, is responsible for biofilm formation and autoaggregative behavior associated with the increased synthesis of hemagglutinin-like HxfA/B proteins. This compound is a positional isomer of TDA (C_{14}:Δ^7) produced by *H. pylori* (44).

There is currently no evidence for the ability of TDA to be able to activate gene expression, and it is assumed that only cis-2-unsaturated acids (C_x:$\underline{\Delta^2}$) has such activity (34,44). The studies conducted by Ionescu et al. (38) showed that in *X. fastidiosa* cis-2-tetradecenic acid (C_{14}:$\underline{\Delta^2}$, XfDSF) and cis-2-hexadecenic acid (C_{16}:$\underline{\Delta^2}$, XfDSF2) are the strongest gene expression activators. *X. fastidiosa* and *X. campestris* were used to show the structural and isomeric effect of DSF molecules on the activation of DSF-dependent processes (the production of extracellular enzymes). Induction of exoenzymes production has been demonstrated for both cis-9-hexadecenic acid (C_{16}:$\underline{\Delta^9}$) and trans-2-dodecenoic acid (trans-C_{12}:$\underline{\Delta^2}$). Contrary to the reports on the exclusive autoinductive activity of cis-2-unsaturated fatty acids, these results suggest the inductive activity of other structural DSF isomers. Thus, despite the presence of an unsaturated bond in a position other than the second carbon, the potential biological activity of TDA (C_{14}:$\underline{\Delta^7}$) produced by *H. pylori* cannot be excluded.

The possibility of initiating the formation of coccoid *H. pylori* forms by fatty acids was indirectly confirmed by numerous *in vitro* studies demonstrating the strong activity of free fatty acids against *H. pylori,* despite the lack of efficacy of such therapies in *in vivo* studies. These observations suggest that in response to the fatty acids *H. pylori* can transform into the VBNC form in which we observe the apparent lack of viability and conversion to the coccoid morphotype. Among the fatty acids, the most potent activity against *H. pylori* is attributed to lauric acid (C_{12}:Δ^0), myristoleic acid (C_{14}:Δ^9), and linolenic acid (C_{18}:$\Delta^{9,12,15}$) (45). Yamashita et al. (44) showed that the fatty acids with autoinhibitory activity produced by *H. pylori* are lauric acid and TDA (C_{14}:Δ^7; which is myristoleic acid structural analogue). For this reason, these compounds would most likely have a strong ability to induce *H. pylori* transition from the culturable spiral form to the non-culturable coccoid form. The ability to initiate morphological changes in *H. pylori* has also been demonstrated for other fatty acids. Correia et al. (46) investigated the influence of polyunsaturated docosahexaenoic acid (DHA) on the growth of *H. pylori* by observing bacterial viability using CFU/mL measurement and by analyzing the morphology of this pathogen. The exposure of *H. pylori* 26695 and SS1 to 100 μM DHA resulted in the conversion from the spiral to the coccoid form and the initiation of the VBNC state.

It was reported that various species of *Streptococcus* spp. (*S. mutans*, *S. mitis*, *S. oralis*, *S. sanguinis*) have the ability to communicate via DSFs, i.e. trans-2-decenoic acid (SDSF, trans-C_{10}:Δ^2) (37). Khosravi et al. (47) determined the effect of probiotic bacteria on *H. pylori* viability and found that the coincubation of *H. pylori* with *Lactobacillus fermentum* for 7 days did not significantly reduce the viability of this pathogen. Different results were obtained during coincubation of *H. pylori* with *S. mitis*. Co-cultiva-

tion of these two microorganisms resulted in a significant decrease in *H. pylori* proliferation after the first day of incubation, with complete inhibition after 48 h. It was found that a decrease in the number of microorganisms was due to the conversion of *H. pylori* from the spiral to the coccoid form. The same effect was obtained when the *H. pylori* culture was supplemented with *S. mitis* supernatant from 2-day or 4-day culture of *S. mitis*, but not from 1-day culture. The authors of the article pointed out that the tenovin-6 analogue - a p53 protein activator and tumor suppressor - was the potential substance involved in *H. pylori* transformation and growth inhibition. It appears, however, that the compound involved in the morphological transformation of *H. pylori* might be another substance because of the increased risk of gastric cancer mediated by coccoid *H. pylori* forms (9,14). It is also suggested that *H. pylori* can form dental plaques and that the oral cavity might serve as a reservoir for this bacteria (48,49). The oral cavity is largely colonized by *Streptococcus* spp., which may be a source of SDSF-type DSFs, thus explaining the presence of *H. pylori* in the dental plaque in both spiral and coccoid forms embedded within the polysaccharide matrix (37,47).

The holistic model of H. pylori infection

The considerations presented in this paper were used to develop a hypothetical, holistic model of human infection with *H. pylori* taking into account the dual quorum sensing system, including AI-2 and DSF compounds, and their function in modulating the physiology of this pathogen.

The infection with *H. pylori* occurs by ingestion of contaminated water/food or contact with a person in whom the microorganisms are present in the oral cavity. Spiral and coccoid forms of *H. pylori* enter the human stomach. Coccoid forms, which are exposed to stimulatory/stress-inducing factors that occur in this environment and are induced by AI-2 secreted by the gastric microflora, are converted from the spherical to the spiral form (9-11,13,14,22,29,48,49). The possible lack of AI-2 stimulation causes *H. pylori* to remain in the coccoid form with the decreased ability to adhere to and penetrate the mucosa, potentially contributing to the removal of this bacterium from the human body (6). Spiral forms actively seek the gastric mucosa to protect against the low pH of the stomach environment. This is supported by chemotaxis away from AI-2 and protons and towards nutrients (amino acids and metal ions), urea, and HCO_3-. In addition, the spiral shape enables effective penetration of mucin (6,22,31). After bacterial proliferation in the gastric mucosa, microorganisms enter the stationary phase accompanied by a decrease in AI-2 concentration and an increase in DSFs autoinductive activity. This stage is promoted by another conversion of bacteria to the coccoid form (10,17,31,34,46). The spherical forms display enhanced survival capabilities as shown by the increased potential for autoaggregation, biofilm formation, and biocide resistance. Furthermore, the coccoid forms are also responsible for creating a carcinogenic environment in the stomach (14,22,31,33). The late form of *H. pylori* biofilm is associated with low nutrient levels and elevated concentrations of AI-2 (17,31,32). The DSF-dependent autoinductive activity is reduced due to the generation of free saturated fatty acids by the RpfF synthase. Saturated fatty acids, competing for the RpfC receptors, reduce the activation of processes conditioned by *rpf* genes, i.e. autoaggregative behavior and biofilm formation (35,38,39,45,46). Reduction of DSFs activity and an increase in AI-2 concentration results in the transformation of some coccoid *H. pylori* forms to the spiral morphotype, followed by chemorepulsion from the AI-2 source and migration in search of new niches in the gastric environment (6,17,30-32,44).

CONCLUSION

The considerations set forth in this article were used to develop a holistic model of *H. pylori* infection that includes a morphological transformation cycle and a double quorum-sensing communication system using AI-2 and DSFs. It seems that the more accurate identification and characterization of coccoid forms of this pathogen should be an important focus of future research into methods of effective *H. pylori* eradication. This form is probably associated with a more aggressive process of carcinogenesis in the gastric environment and increased resistance of bacteria to stress factors. *In vivo*, some coccoid forms of *H. pylori* probably have the potential to revert to the spiral morphotype, which promotes the spreading of this microorganisms. The *in vitro* and *in vivo* conformation of DSF involvement in the modulation of biofilm formation and conversion of *H. pylori* to the coccoid form will enable to create new strategies for effective eradication of this pathogen.

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