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Overview on chemotaxis and acid resistance in *Helicobacter pylori*

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INTRODUCTION

Sensing chemicals in the environment and responding to changes in their concentrations is a fundamental property of a living cell. It is particularly important for unicellular organisms that constantly interact with the environment. Many bacterial pathogens colonize the hosts assisted by the flagella, but little is known about how these microbes use the ability to swim when they are inside of animal hosts. Motility is often driven by flagella, which is a complex extracellular structure that requires energy for operation. Motile flagellated bacteria swim toward chemical attractants and away from repellents by a mechanism known as chemotaxis. Chemotaxis is a response to microenvironmental changes and is controlled by probably the best-studied signal transduction system (Liu et al., 1997; Manson et al., 1998). Taxis responses allow motile microorganisms to rapidly move toward a microenvironment optimal for their growth and survival. The mechanism of flagellar motility and its control via chemotaxis have been studied in great detail in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (Bren and Eisenbach, 2000; Stock and Levit, 2000; Stock et al., 2000). Enteric motile bacteria can measure concentrations of chemicals outside the cell using transmembrane receptors that transmit information into the cell interior. They normally express the *mcp* and *che* genes in order to regulate chemotaxis behavior. The methyl accepting chemotaxis proteins (MCPs) interact with specific ligands, while the Che proteins relay the appropriate signals from the MCPs to the flagellar motor. When attractants such as amino acids, sugars and dipeptides are present, bacteria sense the concentration gradient of attractants and swim towards them.

Enteric pathogens must survive at the acid pH of the stomach in order to gain entrance into the stomach or intestine to cause diseases. Pathogenic microorganisms that prefer to grow at neutral pH exhibit widely varying abilities to survive at extreme pH values. The enteric pathogens can protect themselves from acid in several basic ways. They can prevent protons from entering the cell, pump protons out of the cell, or once internal pH reaches a dangerous acid zone, they could protect or repair damage to macromolecules. Also, they could synthesize key enzymes that can function when internal pH falls. Our laboratory is currently investigating the basic response of acid tolerance in *Helicobacter pylori* that protect the cell further down to pH 6 and the chemotactic properties of this microorganism.

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The microorganism

Helicobacter pylori is a neutralophilic, Gram-negative, ureolytic and motile bacterium unique in its ability to colonize the normal human stomach. It is microaerophilic, spiral-shaped bacillus, 3-5 μm long and about 0.5 μm in diameter, with six flagella at one pole (Dunn et al., 1997). Its genome has been sequenced and it has about 1500 open reading frames coding for a variety of proteins, and about two-thirds of known function by homology with other proteins (Tomb et al., 1997). The spiral body of this microorganism, with its bundle of unipolar flagella, is well adapted for penetrating the gastric mucous layer and for swimming rapidly in a viscous environment (Warren and Marshall, 1983; Hazel et al., 1986; Jung et al., 1997; Nakamura et al., 1998). It is also well established that *H. pylori* colonizes the mucous layer of gastric epithelium and is a causative agent for peptic ulcer disease, gastric adenocarcinoma, and gastric lymphoma (Warren and Marshall, 1983; Parsonnet et al., 1994; Dunn et al., 1997). The bacterium resides mostly within the gastric mucus layer, but a few microorganisms (20%) are associated with the gastric epithelia, and the former population is present to replenish the latter (Kirschner and Blaser, 1995; Yoshiyama and Nakazawa, 2000). *H. pylori* has adapted remarkably well to variations of pH in their environment. Many neutralophilic organisms, such as *Yersinia enterocolitica*, *Vibrio cholera, Escherichia coli* or S*almonella typhimurium*, are adapted to transit the acidity of the gastric juice (Young et al., 1996; Merrell and Camilli, 1998; Chevelli et al., 1996). They do not colonize the stomach but instead have developed acute acid resistance mechanisms. Only one organism, *H. pylori*, has adapted itself to allow not only survival in, but also habitation of the human stomach.

Several factors are thought to be involved in the colonization by *H. pylori* of the gastric mucosa, including urease activity and motility using flagella (Mobley et al., 1995; Eaton et al., 1996).

Acid Resistance in H. pylori

H. pylori is also unique in having a large amount of urease in the cytoplasm at neutral pH but is greatest in the outer portion at acidic pH in the wild-type strain (Mobley et al., 1995; Hong et al., 2003). Urease is synthesized constitutively by the microorganism accounting for about 10-15% of the total protein synthesized by the bacterium (Bauerfeind et al., 1997). The enzyme is a two-subunit Ni^{+2} containing protein, and it does not have a signal peptide at the N-terminal suggesting a cytoplasmic localization. However, a portion of the bacterial population releases the urease by cellular autolysis allowing the anchorage of the enzyme on the surface of intact cells (Phadnis et al., 1996). The main role of urease is thought to be the neutralization of acidic microenvironments by producing NH_3 and CO_2. Thereby, urease is an obvious candidate for the acid resistance mechanism of *H. pylori*. The urease activity of intact cells increased nearly exponentially when the external pH decreases. This activation is not due to enhanced gene expression at low external pH values. In cell extracts the pH optimum of urease activity is dependent on the buffer system and is about pH 5 in sodium citrate buffer. Since this is the cytoplasmic pH of the cells at pH 1 to 2, Stingl et al. (2002) propose that the cytoplasmic pH is a key factor in the *in vivo* activation of the urease at low external pH values.

With the activity of carbonic anhydrase also encoded in the genome of *H. pylori*, the CO_2 generated will be able to increase the buffering capacity of the cytoplasm to resist internal alkalinization due to the generation of NH_3. The NH_3 produced can act as a buffer leaving the bacterial cytoplasm and entering the bacterial periplasm, by the formation of NH_4^+. It was previously believed that urease located on the cell surface created a neutral microenvironment that was conducive to bacterial survival, however it has been shown that intracellular urease actually plays a key role in promoting acid resistance (Scott et al., 2002). This role results from the availability of urease.

There are seven genes within the urease operon, *ureABIEFGH*, *ureA* and *ureB* are the structural subunits of the urease, whereas *ureE, F, G* and *H* are thought to be accessory genes necessary for formation of an active urease by insertion of Ni^{+2} into the UreA/UreB protein complex. Their removal results in loss of urease activity. *ureI* is not essential for the activity of urease (Sachs et al., 2003).

The dilemma about how intracellular urease gains access to its extracellular substrate was recently solved by the identification of UreI as an inner membrane proton-gated urea-specific channel (Weeks et al., 2000). This work demonstrated that the UreI pore opens, as the pH of the medium drops below pH 5.5 and cytoplasmatic urease is able to gain access to the urea. As the enzyme activity neutralizes the local environment, the pore closes and the urea transport stops, thus providing a regulated level of urease activity. Urea in the gastric juice is able to access the intrabacterial urease if periplasmatic pH falls below 6 owing to pH-gating of the urea channel, UreI. As a result of this, NH_3 is formed and neutralizes the bacterial periplasm to a pH 6.2. Urease shifts from the cytoplasm to the outside of the cell as a result of an extracellular decrease in pH. This difference is independent of the presence of urea, but it is UreI-dependent, suggesting an additional role of UreI in urease-dependent acid resistance (Hong et al., 2003).

Site-directed mutagenesis and chimeric analysis have identified several amino acids involved in maintaining the closed state of channel UreI at neutral pH (Sachs et al., 2003). Also it was concluded that the first periplasmatic loop of UreI is implicated in channel opening/closing at acidic and neutral pH, and the second periplasmic loop and the C terminus require protonatable amino acid residues to allow the function of the channel (Weeks and Sachs, 2001).

The UreI of *H. pylori* is homolog to UreI of *H. hepaticus* and *S. salivarius* (Beckwith et al., 2001; Chen et al., 1998). The UreI products from the three bacteria have considerable homology in their predicted membrane domains, and some in the cytoplasmic domain, and little in the periplasmic domain. Although all are urea channels, those of *S. salivarius* are equally active at neutral and acidic pH, whereas those of *H. pylori* and *H. hepaticus* are acid activated.

In addition to this acid resistance mechanism it has been shown by 2-D isoelectric focusing non-equilibrium pH gel electrophoresis that *H. pylori* has a urease-independent acid stress response system (Toledo et al., 2001; Toledo et al. 2002). This mechanism shows that about 49 proteins change their levels of expression due to the acidic pH, operate at mild acidic pH and they are under Fur control (Valenzuela and Toledo, personal communication). In addition to these mechanisms, other acid-inducible systems have been described: the acid tolerance response (Toledo et al., 2001, personal communication; Toledo et al., 2002; Karita and Blaser, 1998), the acid-induced expression of LPS (McGowan et al., 1998), and the inducible hsp70 stress protein (Huesca et al., 1998). Also, by using the whole genome approaches, proteomic and promoter analysis, it is known that *H. pylori* varies its gene expression when the bacteria is grown under acidic conditions (Bijlsma and Lie-A-Ling, 2000; Jungblunt et al., 2000; Ang et al., 2001; Allan et al., 2001; Dong et al., 2001; Toledo et al., 2002; Merrell et al., 2003; McGowan et al., 2003). However, there is a general lack of agreement between these studies emphasizing the complexity of *H. pylori*'s response to acid and the difficulty in comparing single time point experiments for the assessment of global transcription or global translation.

Chemotaxis in H. pylori

H. pylori flagella contain two flagellin molecules, FlaA the major species, and FlaB, which is expressed in minor amounts (Josenhans et al., 1995; O´Toole et al., 2000). Flagellar motion appears to be essential for infection of animal models, since deletion mutants of the flagellar system are also unable to colonize (Foynes

et al., 1999). Both flagellin molecules are necessary for full motility on soft agar plates and for full colonization of gnotobiotic piglets, whereas an aflagellated strain colonizes the stomach less frequently (Eaton et al., 1996). *H. pylori* apparently expresses six Che cytoplasmatic signal transduction proteins that regulate the swimming of the bacteria and are the products of the following ORFs: HP0019 (*cheV*), HP0393 (*cheV*), HP0616 (*cheV*), HP0391 (*cheW*), HP1067 (*cheY*), and HP0392 (*cheA*) (Tomb et al., 1997; Foynes et al., 2000; Pittman et al., 2001). In addition to these components, there is a family of transmembrane proteins, the methyl-accepting chemotaxis proteins or MCPs, with putative receptor functions, known as HP0082, HP0099, HP0103; these are orthologs to *tlpC*, *tlpA* and *tplB* from *Bacillus subtilis*, respectively (Tomb et al., 1997).

In the stomachs of infected patients, the bacteria reside mainly in the mucous layer (Yoshida et al., 1993; Kirschner and Blaser, 1995). Because the gel layer has a rapid turnover (Messier and Leblond, 1960), the bacterium proliferating in the mucous layer should have the ability to move toward the epithelial cell surface, against the mucous flow toward the duodenum. We hypothesize that chemotaxis in *H. pylori* must be crucial for bacteria colonization and persistent infection.

Recently, it has been demonstrated that *H. pylori* has the ability to sense and move towards urea, sodium bicarbonate, sodium ions (Mizote et al., 1997) and also to aspartate and serine (Toledo and Rivas, 1999, personal communication; Cerda et al., 2003). Urea is synthesized in the liver, circulated by the blood stream, and secreted into the gastric juice through a capillary network beneath the gastric epithelial surface (Niethercut et al., 1993). Thus, a concentration gradient of urea is formed in the gastric mucus layer, which should be sensed by *H. pylori*. Bicarbonate is also secreted into the gastric mucosa by chloride-bicarbonate exchangers localized in parietal cells and Na^+-H^+ exchangers distributed in the mucous neck, chief, and mucous surface cells, respectively (Stuart-Tilley et al., 1994). In addition, *H. pylori* might be able to swim fast at low pH because the flagellar motor is powered by a proton motive force (Yoshiyama et al., 1999). Thus, orally uptaken *H. pylori* can promptly evade the acidic periphery of the mucous layer and move towards the epithelial surface by chemoattraction of substances such as urea and bicarbonate, which diffuse out from the gastric epithelial surface.

The chemotactic response to urea could be crucial not only for acid resistance, but also for colonization in the hostile environment. *H. pylori* in the mucus layer may sense urea and move toward the epithelial cell surface, which must be important for persistent infection of this microorganism. Our results about urea chemotaxis with four different strains indicated that *H. pylori* is not attracted by urea even if the assay is done at pH 7 or mild acid pH (Cerda and Toledo, personal communication; Cerda et al., 2003). Nevertheless, these results are in disagreement with the observation of Mizote et al. (1997) that reported chemotaxis to urea in *H. pylori* CPY3401. This different response could be attributed to the presence of the surface urease in *H. pylori* (Phadnis et al., 1996), that may hydrolyze the urea rapidly before becoming a signal to chemotactic system or, each *H. pylori* clinical isolate differs genetically from most other independent isolates based on DNA fingerprint and sequence analysis (Akopyanz et al., 1992; Achtman et al., 1999). Superimposed on this great general diversity, several sub-populations of *H. pylori* have been identified. Those are relatively distinct genetically, being each of them specific to a different geographic region or human ethnic group (Achtman et al., 1999; Jeong et al., 2000; Kersulyte et al., 2000).

Physiological significance of chemotaxis in H. pylori

Nitric oxide (NO), the NO synthase product, is known to play an important role in host defense against a variety of microbes (Doi et al., 1993; Fang, 1997; Nathan, 1997) although NO itself does not show sufficient

antimicrobial activity (Yoshida et al., 1993; Kaplan et al., 1996). Peroxynitrite ($ONOO^-$), a metabolite of NO, is considered to be responsible for the antimicrobial effect. NO and superoxide (O_2^-) react forming $ONOO^-$, a strong oxidant and nitrating agent (Beckman et al., 1990; Ischiropoulos, 1998). In recent years, increased expression of inducible NO synthase (iNOS) has been confirmed in *H. pylori*-infected gastric tissues of patients and experimental animals (Tatemichi et al., 1998; Fu et al., 1999; Goto et al., 1999). Furthermore, it has recently been reported that not only phagocytic inflammatory cells but also *H. pylori* itself produce O_2^- (Nagata et al., 1998), which indicates that $ONOO^-$ may be formed into and around the bacteria *in vivo*. Consequently, $ONOO^-$ may function as a major bactericidal effector for *H. pylori* in the stomach. Recently, Kuwahara et al. (2000) reported that CO_2 formed by bacterial urease inhibits the reactivity of $ONOO^-$ with the bacterial components and accelerates its decomposition outside the bacterial cells. Also, formation of nitrotyrosine in *H. pylori* was suppressed by the addition of urea or sodium bicarbonate. In this context, it is quite reasonable to think that *H. pylori* has evolved with a system capable of detoxifying $ONOO^-$, and hence steady and sustained colonization in the infected stomach is facilitated.

Bicarbonate is also secreted into the gastric mucosa (Stuart-Tilley et al., 1994) and chemotactic response to sodium bicarbonate (Cerda and Toledo, personal communication; Cerda et al., 2003; Mizote et al, 1997) may contribute to the persistence of *H. pylori* in the gastric epithelia and in the gastric mucus layer. Gastric mucosa is markedly adverse to bacterial colonization, as the physical and chemical barriers encountered (mucus, enzymes, and acid) inhibit colonization by common bacteria.

Arginine has a guanidine chemical group that is close to the chemical structure of urea. Cerda demonstrated that *H. pylori* also shows chemotaxis to arginine (Cerda and Toledo., personal communication; Cerda et al., 2003). Amino acid metabolism is essential for *H. pylori* growth (Marais et al., 1999). This microorganism does not synthesize L-arginine (Tomb et al., 1997; Doig et al., 1999) and therefore it must obtain that amino acid from extracellular sources. In this way chemotaxis to arginine could play a role allowing the bacteria to find the arginine source.

It has been propose that *H. pylori* arginase inhibits nitric oxide production by activated macrophages at physiological concentrations of L-arginine, the common substrate for NO synthase and arginase. On the other hand, inactivation of the gene *rocF*, encoding constitutively expressed arginase in *H. pylori*, restored high-output NO production by macrophages, resulting in marked NO-dependent killing of *H. pylori* (Gobert et al., 2001). This observation indicates that the bacterial arginase has evolved as a survival mechanism that may contribute to the ability of *H. pylori* to successfully colonize the human stomach. Also, arginine could protect the microorganism from toxic effects of gastric acidity by raising the pH of its microenvironment. Being a substrate of arginase, a highly active enzyme of the urea cycle (Mendz and Hazell, 1996), arginine would be converted to urea, which in turn is the substrate of urease, an enzyme that has been accepted as an environmental modulator. In addition, the high-frequency usage of arginine, and also lysine, in *H. pylori* proteins (Tomb et al., 1997) may be one adaptation that favors survival in acidic environments.

Arginine is not used as a nitrogen source. It has been postulated that ammonium can be obtained by deamination of asparagine, aspartate and glutamine (Mendz and Hazell, 1995). It has also been proposed that it could be the major source of carbon, converting it into α-ketoglutarate, which can be routed throughout the majority of central metabolism.

The scavenging of arginine, bicarbonate and other substances like urea is an important factor for *H. pylori* colonization and persistence in the gastric mucus layer and motility plays a key role. In this way chemotaxis may play a fundamental role in these processes.

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