

# Factors involved in the pathogenesis of *Helicobacter pylori* infection

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Olivares D, Gisbert JP. Factors involved in the pathogenesis of *Helicobacter pylori* infection. *Rev Esp Enferm Dig* 2006; 98: 374-386.

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## ABBREVIATIONS

*vacA*: vacuolizing cytotoxin A; VEGF: vascular endothelial growth factor; *cagA*: cytotoxin-associated gene A; MAP: mitogen-activated protein kinases; IL: interleukin; LPS: lipopolysaccharide; MHC II: major histocompatibility complex II; COX: cyclooxygenase; ROS: reactive oxygen species; SOD: superoxide dismutase; iNOS: inducible nitric oxide synthase; NO: nitric oxide.

## INTRODUCTION

*Helicobacter pylori* is a bacterium that chronically colonizes the gastric epithelium and infects approximately half of the human population worldwide. This pathogen is responsible for chronic gastritis and a high percentage of peptic ulcers, and its presence has been correlated to gastric cancer development (1-4).

Several factors have been associated with this germ's aggressiveness and hence implicated in epithelial damage, including vacuolizing cytotoxin (VacA), cytotoxin-associated gene A (*cagA*), surface lipopolysaccharide (LPS), bacterial urease, flagella, surface adhesins, oxidiz-

ing radicals, and cytokines produced by leukocytes in response to infection.

On the other hand, there is increasing evidence that *H. pylori* species are genetically diverse, that such diversity is associated with different aggressiveness degrees on the mucosa, and hence with gastric mucosal inflammation to different extents and a variety of clinical prognoses for infected patients (5). *H. pylori* genome includes more than 1,000 preserved genes and strain-specific genes. This bacterium may acquire or lose exogenous DNA, and thus follows an ongoing microevolution model allowing high genetic variability, which may result in strains adapted to multiple adverse environments. Such variability also results from a high recombination rate during colonization of one host by non-related *H. pylori* species, in addition to a high frequency of mutation (6-11).

The objective of this paper is to review the characteristics, and both the *in vivo* and *in vitro* effects, of major bacterial factors related to *H. pylori* virulence on human epithelial cells, as well as the effects deriving from *H. pylori*'s intrinsic genetic variability.

## ADHESINS

The process of *H. pylori* infection acquisition may be divided in two stages: a) adhesion to the epithelial cell layer; and b) induction of proinflammatory cytokines (12-14). Adhesion is mediated by *H. pylori* adhesins; these include BabA, which binds the host's Lewis<sup>b</sup> epitope, a molecule that has been suggested to play a crucial role in the development of gastric adenocarcinoma, peptic ulcer, and chronic gastritis (15-21). The polymorphic *babA* gene has two alleles (*babA1* and *babA2*), with the latter coding for protein BabA. They only differ in a number of 10-base-pair repeats containing a transcription start codon (19). The presence of *babA2* has been seen to correlate to that of other pathogenic genes (*cagA* and the *s1* and *m1* alleles of *vacA*) (22).

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Recibido: 01-03-05.  
Aceptado: 22-06-05.

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Another protein involved in adhesion is NAP (neutrophil-activating protein), coded for by the *napA* gene and isolated from the plasma membrane protein fraction of *H. pylori*. Besides its function as a bacterial ferritin for iron capture, it plays different roles when secreted or presented on the bacterial surface, including affinity for ceramides present in cell plasma membranes. The NAP protein also has a high affinity for sulphated ceramides, the SO<sub>3</sub>-Lewis<sup>a</sup> blood antigen group, and to a lesser extent the Lewis<sup>x</sup> and SO<sub>3</sub>-Lewis<sup>x</sup> groups, which demonstrates NAP function as an adhesin (23).

*H. pylori* also has a hemagglutinin coded for by the *hpaA* gene, which binds N-acetylneuraminyl α (2,3)-lactose and sialic acid remnants, since the reversibility of *H. pylori*-to-gastric cell binding has been demonstrated using sialyllactose or the glycoprotein fetuin (24-26).

In summary, it may be concluded that the presence of adhesins allows the involved strain a greater infective capability on the mucosa.

### VACUOLIZING CYTOTOXIN (VacA)

All *H. pylori* strains have a *vacA* gene coding for a 87 kDa cytotoxin that induces epithelial cell vacuolization (27-32), but only 50 to 65% of *H. pylori* strains do produce the cytotoxic protein. Infection by toxin-producing *H. pylori* strains has been seen to be more frequent in patients with peptic ulcer (30,33-35) and gastric cancer (35-38) when compared to patients with only gastritis.

Two polymorphic regions have been documented within *vacA* (39). One is on the second half of the signal sequence (s1a/s1b/s1c or s2), the other on the central region (m1 or m2). Sequence s1, but not sequence s2, is closely linked to the cytotoxin's *in vitro* activity, peptic ulcer, and the presence of gene *cagA* (33,39). In contrast, *cagA* is not associated to cytotoxicity in epithelial cells, as has been seen in a study following *cagA* gene mutation (40). Therefore, the presence of *cagA* is not essential for cytotoxicity induction.

In contrast to s1 strains, s2 strains only express the VacA protein in small amounts, and only exhibit mild cell vacuolization (38). In this regard, it has been postulated that the clinical progression of *H. pylori* infection is dependent upon the *vacA* genotype. This hypothesis was demonstrated in a study with patient-derived strains, which were s1-m1 or s1-m2 in most cases, and the s1 strain was shown to be present in all patients with peptic ulcer analyzed, and in most individuals diagnosed with gastritis, whereas all s2 strains derived from patients with gastritis exclusively. On the other hand the s2-m1 combination was found in no patient in this study (38). It may be concluded that the s1 allele is linked to peptic ulcer development, and hence to greater virulence. However, despite this association between s1/m1 *vacA* genotype and presence of peptic ulcer, together with an increased infiltration by neutrophils and lymphocytes *in vivo*,

greater in ulcer patients *versus* gastritis patients, this association is not so obvious in Asian countries, where both conditions exhibit a similar incidence regarding the s1/m1 genotype (41,42).

The mechanism through which VacA induces vacuolization is only partially understood. This protein is thought to be hexameric, and its assembly is favored by an acidic pH, resulting in an anion-selective channel across the cell's lipid bilayer (43-46). It is then translocated to the cytosol, where it interferes with the vesicular transit of lysosomes, and may again set up an ion channel across endosomal membranes, which is partly responsible for the vacuolization process (47,48).

The process by which VacA induces apoptosis (genetically "programmed" cell death) is not so clear. Fas/CD95 receptor activation (49) has been suggested, which would lead to apoptosis through the activation of caspases 3 and 8. A study has observed that the microinjection of cytoxin-coding DNA results in cytochrome c release and apoptosis. VacA has been recently seen to interact with mitochondria, thus enhancing apoptosis (50). However, factors other than VacA must be involved in apoptosis, since VacA immunodepletion *in vitro* does not result in a complete abolition of apoptosis (43).

On the other hand, the vascular endothelial growth factor (VEGF), a well-characterized angiogenic factor, may induce mucosal recovery by stimulating vascularization and nutrient supply (51). VEGF overexpression has been seen in human gastric carcinomas (52-54), as has overexpression of factors similar to the endothelial growth factor and cyclooxygenase-2 (COX-2), a prostaglandin-producing enzyme, both *in vitro* (55,56) and *in vivo* (57-59); the latter may in turn stimulate VEGF production (29). A study has shown that *H. pylori* strains expressing the VacA protein (also called Tox<sup>+</sup>) overexpress VEGF production through MAP kinase activation, whereas Tox<sup>-</sup> strains do not (51). Therefore, the presence of VacA may induce the expression of VEGF and lead to the development of tumorigenic processes.

### THE *cagA* GENE

The cytotoxin-associated gene A (*cagA*) codes for a highly immunoreactive, high-molecular-weight (120-140 kDa) protein present in approximately 60% of *H. pylori* strains (28). *In vitro* studies have shown that *H. pylori* capability to induce chemokines in gastric epithelial cell lines is variable, and this only occurs when a CagA phenotype is present (60-62). *In vivo*, infection by CagA+ strains induces a greater immune response and more severe gastritis (63-65). Several studies have shown that infection by *cagA*<sup>+</sup> strains is associated with a higher frequency of peptic ulcer (65,66), gastric atrophy (63-65) and gastric cancer (67-69) except in Asian subjects. As is the case with *vacA*, no association exists between *cagA* genotype and clinical status in Asian subjects: both

asymptomatic individuals and patients diagnosed with duodenal ulcer or gastric cancer express CagA and VacA with the same frequency (70,71).

The *cagA* gene is a part of a pathogenicity island (cag-PAI) around 40 kDa in size, which includes 31 genes whose products play a role in chemokine stimulation and MAP kinase (a class of substrate-phosphorylating proteins involved in multiple cell signaling pathways) activation, with the subsequent induction of proinflammatory factors. The capability for interleukin-8 (IL-8) production by epithelial cells has been shown to remain unaffected when *cagA*-deleted mutant cells (i. e., strains lacking this gene) are used. In contrast, the deletion of other cag-PAI genes does eventually suppress it (20,21,72,73).

Since *H. pylori* may easily lose or acquire exogenous DNA, cag-PAI may have been acquired from other organism by horizontal transfer, as its G+C contents differs from that in the remaining genome (74-77). Several studies have revealed that cag-PAI may present as a single uninterrupted unit, separated in two regions by the insertion sequence IS605 or a chromosome fragment, or partially deleted (28,76,78,79).

The capability of *H. pylori* strains for IL-8 induction correlates with the presence of cag-PAI. While cag-PAI prevails in patients with dyspepsia or duodenal ulcer, no significant differences exist between both conditions regarding its presence (78,80).

Some cag-PAI products show homology to the type-IV translocation system in *Agrobacterium tumefaciens* and *Bordetella pertussis*, which operate to transfer bacterial factors into the host cell (76,81,82). Using this system, *H. pylori* may directly "inject" a number of factors into epithelial cells, including protein CagA, which has been seen to be phosphorylated (83) and may bind the cell's SHP-2 phosphatase (37), involved in cell growth and motility; its dysregulation may therefore result in cell proliferation changes and gastric cancer development.

Structural analyses have shown that CagA varies in size among the various *H. pylori* strains (84-87). Such variation derives from the presence of a number of repeats in an amino acid sequence at the carboxylic end, which may influence the pathogenicity of *cagA*<sup>+</sup> strains as a result of varied phosphorylation sites, and hence protein activity in terms of the effective binding of SHP-2 (51).

Another gene within cag-PAI is *cagE*, which is a part of the type-IV secretion system (81). It is required for IL-8 induction, and shows homology with genes *ptfF* and *virB4* of *B. pertussis* and *A. tumefaciens*, respectively (82). Finally, a positive correlation between the presence of *H. pylori cagE*<sup>+</sup> strains and duodenal ulcer has been found.

## LIPOPOLYSACCHARIDE

*H. pylori*'s lipopolysaccharide (LPS) has been involved in the bacteria-host interaction. LPS is made up of

three parts: hydrophobic lipid A, hydrophilic O-antigenic polysaccharide region, and polysaccharide core, which connects the former two (88). Lipid A is the component responsible for LPS immune and endotoxic properties. *H. pylori* LPS has a low toxicity when compared for instance to that *Salmonella*'s or *Escherichia coli*'s (89). This low biological activity may contribute to prolonged infection and chronic mucosal inflammation (90).

LPS antigenic structure is similar to that of antigens in the host's Lewis<sup>x</sup> and Lewis<sup>y</sup> blood groups (91), which may explain the presence of *H. pylori*-induced auto-antibodies, which in turn may contribute to the development of atrophic gastritis. In addition, the H<sup>+</sup>/K<sup>+</sup> pump has been seen to have Lewis<sup>y</sup> epitopes, and may be a target for the immune system in chronic gastritis (92). *H. pylori* antigens similar to Lewis<sup>x</sup> have also been seen to be more commonly expressed in *cagA*<sup>+</sup> strains versus *cagA*<sup>-</sup> strains, which would stimulate a stronger autoimmune response in bacteria carrying the *cagA* gene (93).

On the other hand, selected regions have been identified within *H. pylori* genome with a high number of repeats for a one or two nucleotides; some of these repeats lie within ORF (*Open Reading Frame*) sequences that potentially correspond to genes (77). Nucleotide unpairing upon the displacement of a DNA strand on the other strand is a phenomenon that results in a "gained" or "lost" unit in the reading schedule during transcription, which may in turn result in a missed start codon or mutated proteins, this being a factor increasing *H. pylori* genetic variability—this process has been designated *slipped-strand mispairing* (94)—. Similar repeat sequences have been found in other organisms such as *Haemophilus influenzae* (95,96). In the "phase variable gene" class belong a number of genes coding for enzymes involved in LPS biosynthesis, membrane proteins, or other proteins (e.g., *cagA*); these genes may produce variants unlike the genic product in one same bacterial population.

## FLAGELLA AND MOBILITY

*H. pylori* mobility is facilitated by flagella, complex extracellular structures that require energy for operation. *H. pylori* has 5 to 7 flagella on one of its poles. Human immunoglobulins are usually directed against *H. pylori* flagellar proteins (97). A peculiar characteristic of these bacterial flagella is that they are covered in a lipoprotein sheath protecting them against gastric acid (98).

*H. pylori* strains with mutated flagella are less virulent than wild-type strains, which suggests that flagella are crucial for the pathogenetic process. Every flagellum is made up of two flagellins, FlaA and FlaB (99), an unusual characteristic not shared by the rest of flagellar proteins, which are homopolymeric in nature. FlaB is located at the flagellum's base, whereas FlaA—which is more abundant—is on the outside. Removal of both flagellins (FlaA/FlaB<sup>-</sup>) results in nonmobile bacteria (100) that

nonetheless keep a wild-type-like adherence capacity (14), and that can only efficiently infect in early infection stages.

## UREASE

*H. pylori* contains urease, which is the enzyme more abundantly produced by these bacteria. It is a significant survival factor for *H. pylori*, since ammonium ( $\text{NH}_4^+$ ) is produced from urea in the stomach.  $\text{NH}_4^+$  neutralizes HCl and allows *H. pylori* to colonize the gastrointestinal tract.  $\text{NH}_4^+$  concentration in the stomach of infected patients is significantly higher than in non-infected subjects (101). Similarly,  $\text{NH}_4^+$  levels are higher in infected patients than in these same patients following *H. pylori* eradication (102).  $\text{NH}_4^+$  has a number of toxic effects within the bowel, including DNA synthesis disorders, increased risk for viral infection, and carcinogenesis (103). Furthermore, a decrease in oxygen use by gastric cells has been reported in cultures (104). *In vitro*, the high levels of  $\text{NH}_4^+$  generated by *H. pylori* have a significant effect on reduced cell viability, an effect unseen in the absence of urea and in strains with non-functional urease. These effects have been seen to be reversible when urease is added in the medium, and the number of viable cells diminishes anew (103).

Urease is essential for colonization, as has been demonstrated in experiments using *H. pylori* strains with non-functional urease (105). These strains are unable to colonize under hypochlorhydria conditions (106), which demonstrates that urease is essential for survival regardless of its role in gastric acid neutralization.

On the other hand, major histocompatibility complex II (MHC II) molecules regulate immune response through antigen presentation to CD4 T cells. The binding of *H. pylori* to MHC II through urease has been examined, with a resulting increase in gastric cell apoptosis (107). Apoptosis induction is dependent upon MHC II expression, and may become eventually blocked when anti-MHC II antibodies are used; cells deficient in MHC II expression also show no *H. pylori*-induced apoptosis (107).

## *H. PYLORI*-INDUCED FREE RADICALS

Oxygen radicals (superoxide and hydrogen peroxide ions) derived from neutrophils activated by *H. pylori* infection damage the gastric mucosa (108-113). A positive association between reactive oxygen species (ROS) production and *H. pylori*-related infection and histologic changes (108). Cell protection against ROS is induced by the activation of ROS-sequestering enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase.

Some authors, using an epithelial cell line, found that exposure to ROS in the absence of *H. pylori* reduced cell survival to 84% (114). On the other hand, exposure of these cells to ROS following incubation with *H. pylori* reduced survival to 73 and 39% for *cagA*<sup>+</sup> and *cagA*<sup>-</sup> strains, respectively. SOD activity has also been estimated, and was found to be higher in cells incubated with *cagA*<sup>+</sup> strains versus *cagA*<sup>-</sup> strains. Similarly, higher catalase and glutathione peroxidase levels have been described in *cagA*<sup>+</sup> strains (114). This increased activity of enzymes suppressing agents that may potentially damage DNA following exposure to *cagA*<sup>+</sup> strains is likely a source of higher viability for cells after exposure to ROS (73%), when compared to cells exposed to *cagA*<sup>-</sup> strains (39%) (114).

On the other hand, chloramine ( $\text{NH}_2\text{Cl}$ ) is a toxic oxidizing agent produced in the gastric mucosa as a consequence of *H. pylori* invasion. In neutrophils, the enzyme myeloperoxidase catalyzes chloride oxidation by  $\text{H}_2\text{O}_2$  (mainly resulting from the aerobic metabolism of neutrophils activated by the presence of pathogenic organisms) to hypochlorous acid. This reacts with  $\text{NH}_4^+$  as derived from *H. pylori* metabolism, and produces chloramine (115), a very toxic substance because of its lipophilicity and low molecular weight, which may easily cross the cell's plasma membrane.

## INDUCTION OF ENZYMES AND CYTOKINES BY *H. PYLORI*

COX enzymes catalyze the conversion of arachidonic acid to prostanoids such as prostaglandin  $\text{E}_2$ , which protect the gastric mucosa from apoptosis by increasing cell proliferation (116,117). Two isoenzymes exist: COX-1 and COX-2; the former is constituent and the latter inducible in case of injury, and mediates inflammation among other processes (116-119). *H. pylori*-related gastritis has been shown to induce COX-2 expression according to type of bacterial strain (58,59,120-127), which may in part account for differential pathogenicity (127,128). Infection by *H. pylori cagA*<sup>+</sup> strains has been seen to overexpress COX-2 in patients with gastric cancer (127). In addition, some studies have demonstrated that organism eradication is associated with a decreased gastric COX-2 expression (59,125,129).

Another molecule, nitric oxide (NO), has been documented to contribute to gastric mucosal protection by increasing blood flow and inhibiting leukocyte adhesion to the endothelium (130). There is no inducible NO synthase (iNOS) in the normal gastric mucosa, but its expression increases in patients with *H. pylori*-related gastritis (59). Both iNOS and COX-2 are induced by cytokines, including IL-1 $\beta$ , tumor necrosis factor  $\alpha$ , interferon  $\gamma$ , phorbol esters, and growth factors, as well as bacterial lipopolysaccharides (131-133).

Epithelial gastric cells substantially contribute to the cytokine-induced proinflammatory response to *H. pylori* infection, both through active production and the capture of cytokines derived from the lamina propria and intraepithelial leukocytes. Epithelial IL-1 $\beta$ , IL-6, IL-8, and tumor necrosis factor  $\alpha$  levels have been shown to be significantly higher in infected patients *versus* healthy subjects (134). There is also interferon  $\gamma$ , but not IL-4, overexpression in infected patients, which suggests a Th1 lymphocyte-mediated response (134-137).

Finally, increased interferon  $\gamma$  levels may contribute to gastric inflammation not only through phagocyte and neutrophil activation, but also the induction of MHC II overexpression in epithelial cells, with an ensuing increase in *H. pylori* adhesion (138).

## CONCLUSIONS

*H. pylori* is a highly efficient pathogenic organism in terms of colonization, with the potential to cause a number of conditions ranging from chronic gastritis to gastric cancer. A major part of its colonizing efficiency results from genetic dynamism given the small size of the organism's genome, which confers a higher feasibility for recombination processes, and hence may give rise to a high amount of recombinant gene products with a high potential for adaptation. In addition, mutation frequency is high given the lack of a number of DNA-repairing enzymes, which together with the presence of "phase variable genes" allows genetic variability, thus facilitating bacterial host adaptation. *H. pylori*'s adaptive process is mainly mediated by LPS, which allows it to evade the immune system. Other virulence factors such as *cagA*, *vacA*, or *babA* are also subject to genetic variability, which extends the range of potentially virulent strains, and difficulties both strain characterization and the establishment of an accurate clinical prognosis in patients infected by one of these strains. Nevertheless, a more detailed understanding of *H. pylori*-related pathogenic factors will likely be of help in establishing which strains are more virulent, and will allow a selection of patients who most likely will benefit from eradicating therapy.

## ACKNOWLEDGMENTS

This review has been partly funded by a grant from Instituto de Salud Carlos III (03/02).

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