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Minireview

Molecular and cellular activities of *Helicobacter pylori* pathogenic factors

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Abstract Stomach infection with pathogenic strains of *Helicobacter pylori* causes in some patients severe gastroduodenal diseases. These bacteria produce various virulence factors and, here, we review the recent acquisition on the biochemical mode of action of three major factors. We discuss the role of urease both as buffer of the stomach pH and as source of ammonia. The vacuolating toxin alters the endocytic pathway of non-polarized cells, inducing the release of acid hydrolases, the depression of extracellular ligand degradation and of antigen processing and, in the presence of ammonia, swelling of late-prelysosomal compartments. In polarized epithelial monolayers, vacuolating toxin induces an increase of the paracellular permeability, independent of vacuolation. The neutrophil activating protein induces the production of oxygen radicals in human neutrophils and could contribute to the damage of the stomach mucosa. The activities of these factors are discussed in terms of the need of the bacterium of increasing the supply of nutrients from the stomach lumen and from the mucosa.

Key words: Urease; Stomach pH; Ammonia; Vacuolation; VacA; *Helicobacter pylori*; HPNAP; Cytotoxin associated gene A (CagA)

1. Introduction

The relevance of *Helicobacter pylori* for the pathogenesis of gastritis and gastroduodenal ulcers has been proposed about 15 years ago [1,2] and the presence of this bacterium is now established as the main risk factor in the development of stomach carcinomas and lymphomas [3]. *H. pylori* infection interests billions of people in the world, with peaks of 90% of the population infected in countries with poor sanitary conditions and a low socio-economical level. The circular genome of *H. pylori* encodes about 1500 genes, depending on the strain [4,5]. Several aspects of *H. pylori* infection and of the host reactions have been uncovered and current research is beginning to clarify the molecular mechanisms of action of *H. pylori* virulence factors [6,7].

H. pylori is a spiral-shaped Gram-negative bacterium endowed with a very powerful urease activity and with polar flagella [8]. These features allow the bacterium to survive in the stomach lumen. Urease activity buffers the pH at the cell surface, allowing bacterium survival until it enters the mucus layer, a protective barrier against the high proton concentration (Fig. 1). *H. pylori* resides within the mucus and on the apical surface of epithelial cells, as depicted in Fig. 1, where it attaches firmly via adhesin molecules and via modifications of cell membrane proteins and of cytoskeletal proteins [8–10]. This ecological niche 'chosen' by *H. pylori* requires special features to survive, but offers the advantage of little competition from other bacterial species.

Although *H. pylori* infections last for decades, only a minority of infected patients (15–25%) develop severe gastroduodenal diseases. Several factors contribute to this outcome (diet, genetic factors, acid hypersecretion, stress etc.), but the most important one is the virulence factor produced by a given strain [7]. Major *H. pylori* factors are listed in Table 1 and available evidence indicates that pathogenic strains contain a cluster of genes forming a pathogenicity island [11] in addition to genes encoding a vacuolating toxin (VacA), a neutrophil activating protein (HPNAP) and the urease.

2. VacA

The culture supernatants of about half of the *H. pylori* isolates induce large cytoplasmic vacuoles in eukaryotic cells [12], which eventually fill up the entire cytosol, leading to cell sufferance. A protein toxin of 95 kDa, termed therefore VacA, is responsible for such an activity [13]. Secondary structure prediction programs indicate the presence of four distinct domains within the VacA protein. A N-terminal signal sequence necessary for export into the periplasm precedes a domain of 37 kDa (p37), predicted to contain α-helices and amphipatic β-pleated segments, some of which are amphipatic. This domain begins with a 32 residues long hydrophobic segment and ends with a protease-sensitive hydrophilic double repeat, frequently cleaved in the VacA toxin released in the medium [14]. The following domain corresponds to a protein of 58 kDa (p58), predicted to contain a first region with both α an β secondary structures and a second region containing mainly β elements, suggesting the existence of two sub-domains, possibly involved in different aspects of cell intoxication. The second sub-domain exhibits a considerable genetic diversity, whereas the first sub-domain is strongly conserved [15]. The C-terminal part is similar to a bacterial domain capable of translocating the rest of the molecule across the outer membrane, which was first characterized in *Neisseria gonorrhoeae*.

After translocation, part of the 95 kDa protein is released by selective proteolysis from the outer bacterial membrane and it can be additionally cleaved within the repeat connecting the p37 and p58 domains, which remain associated via non-covalent forces [14]. The released toxin has a strong tendency

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Abbreviations: CagA, cytotoxin-associated gene A; HPNAP, *Helicobacter pylori* neutrophil activating protein; VacA, vacuolating toxin

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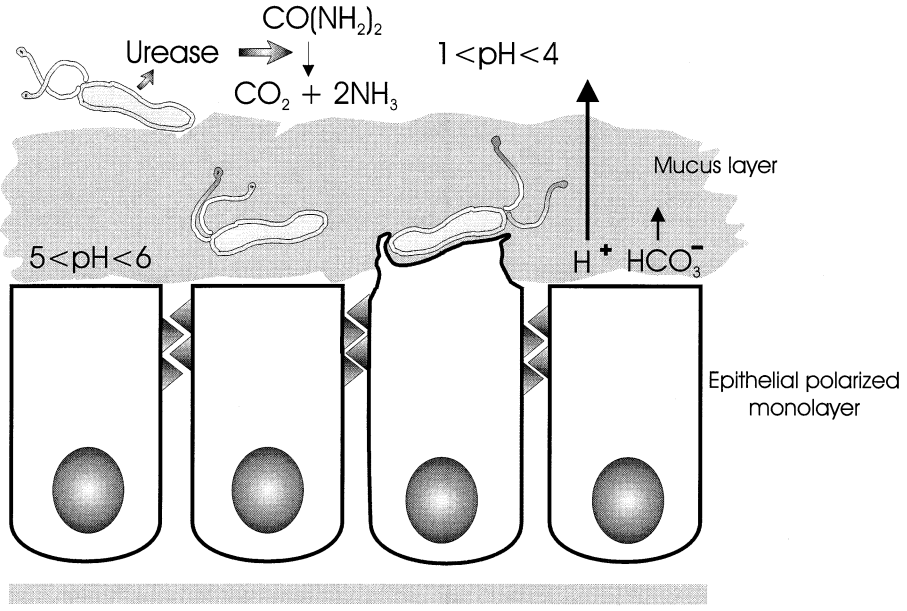

Fig. 1. Localization of *H. pylori* in the host. The bacterium is acquired via the oral route and survives the low pH of the stomach lumen because of the protective action of the urease, which buffers the pH around the bacterial surface. The bacterium, propelled by powerful flagella and helped by the helicoidal shape, penetrates into the mucus layer covering the superficial stomach cells. This layer is poorly permeable to bicarbonate anions, while it is very permeable to protons in the direction of the stomach lumen, but not vice versa [59,60]. As a consequence, the pH above the cells is much less acidic than in the lumen. *H. pylori* adheres strongly to the apical cell surface via adhesins and VacA (Table 1) and induces a rearrangement of the underlying cytosol.

to form flower-shaped oligomers, composed of six or seven monomers [16,17].

Oligomeric VacA is poorly active, but is strongly activated by transient exposure to pH values <5.5 [18]. A low pH induces monomerization with exposure of hydrophobic segments is likely to be involved in VacA penetration into the lipid bilayer [17,19]. Little is known on the structural association of the VacA molecules still bound to the outer membrane of *H. pylori*. Surface-retained toxin may be as important as the released oligomer, if not the predominant form in vivo, as suggested by the recent finding that the bacterium-associated toxin is constitutively very active and does not require pre-treatment at a low pH [20].

3. Cell vacuolation

In the presence of ammonium ions, VacA causes the rapid appearance of small translucid vacuoles in the perinuclear area of sparse cells in culture. Vacuoles contain membrane protein markers of late endosomes (LEs) and of lysosomes (LY) [21,22] and are accessible to extracellular fluid phase markers as well as to BSA-gold pre-loaded inside LYs ([21,23,24], our unpublished results). Vacuoles are acidic because their limiting membrane contains the vacuolar ATPase proton pump (V-ATPase), whose activity is essential for vacuole formation and enlargement [25,26]. They also contain the small GTPase rab7 and an active rab7 is required for vacuole biogenesis [27]. Electron microscopy reveals that vacuoles are largely devoid of the array of multivescicular bodies, characteristic of LEs and LYs ([12,23,24 28] and our unpublished results).

Hence, it appears that VacA induces a reorganization of LE and LY compartments, with extensive membrane fusion and compartment swelling. Already in the early phases of such alterations, before the development of large vacuoles, the following functional defects are evident, (i) a marked decrease of the cell proteolytic activity within the endocytic pathway and (ii) an extensive alteration of protein trafficking from of the *trans* network (TGN) to LE, as judged by the mistargeting of acid hydrolases destined to LYs, which are released in the extracellular medium. A partial pH neutralization of the lumen of LEs and LYs induced by VacA can account, or at least contribute at early intoxication stages, for these effects [29]. Their consequences are likely to be directly relevant for

Table 1
Major virulence factors

Factor	Function	Distribution
Urease	Buffers stomach acid	All strains
Flagella	Motility	All strains
NAP	Neutrophil activation	All strains
BabA	Adhesin for Le^b	Prevalent on type I strains
VacA	Cytotoxicity	Most strains
PAI	31 Genes encoding type IV secretion system	Type I strains
CagA	Immunodominant antigen (part of PAI)	Type I strains
PicB	Equivalent to CagE	Type I strains

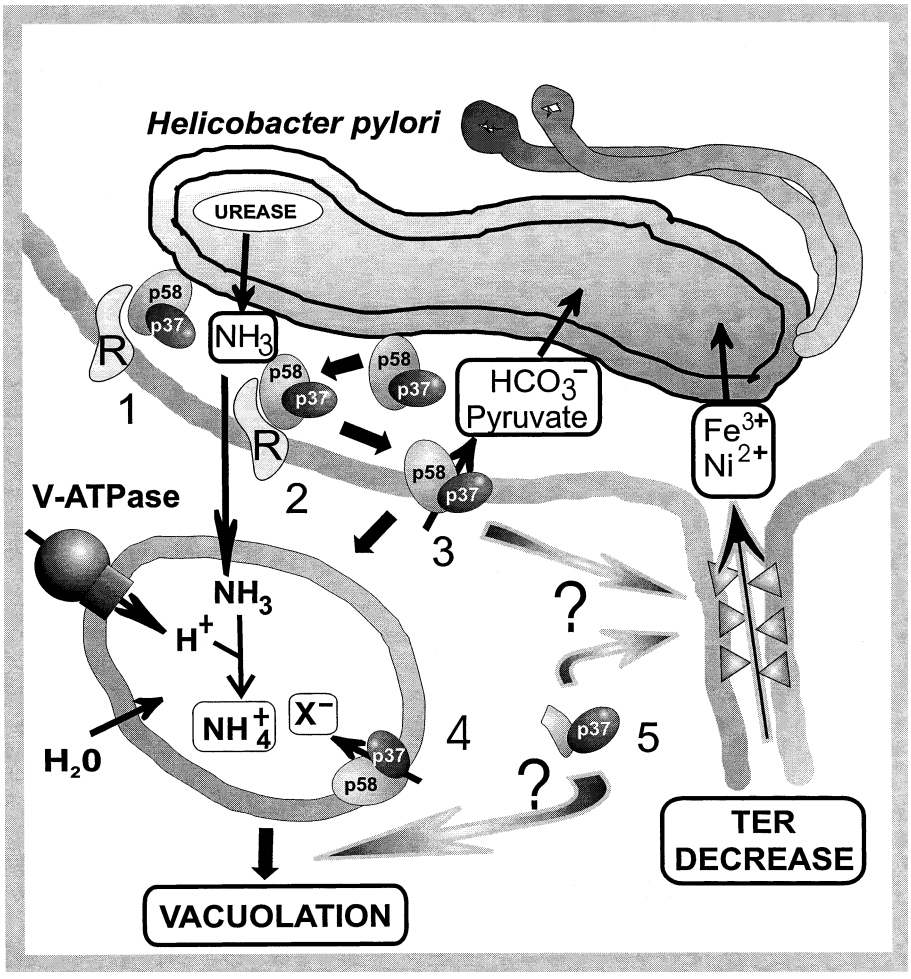

Fig. 2. Hypothetical model of cell intoxication by VacA. The toxin is proposed to bind to the apical portion of epithelial cells via its carboxy-terminal domain (p58) to a receptor (R), whose nature is still unidentified. Active monomeric toxin, but not oligomeric inactive complexes, insert into the plasma membrane via hydrophobic protein-lipid interactions. The insertion step, which requires and involves the amino-terminal domain (p37), results in the formation of anion-selective channels of a low conductance. These toxin channels are likely to result from re-assembling of single toxin molecules into a new oligomeric structure (not pictured here). Endocytosis and transport to endosomes of the toxin, a step which again requires p37, is proposed to increase the anionic permeability of these compartments, which in turn would enhance the vacuolar ATPase proton pumping activity. In the presence of weak bases and in particular of the ammonia generated by the *H. pylori* urease, the endosomal accumulation of osmotically active acidotropic ions (NH_4^+) is predicted to increase. This leads to water influx and vesicle swelling, an essential step in vacuole formation. Changes in the paracellular route of permeability of polarized epithelial cell monolayers might result from a still unknown secondary mechanism, triggered by plasma membrane-associated channels. Alternatively, cytosol-delivered active portions of VacA, formed by p37 plus an amino-terminal region of p58, would act on cell-cell junctions, to modify the *trans*-epithelial electrical resistance. Such a putative cytosolic activity of the toxin could be of a catalytic nature and may play a role in vacuole formation as well.

infection and disease pathogenesis. In fact, protein degradation is an essential function of the cell life, which allows the removal of non-functional cell membrane proteins and of extracellular ligands and re-utilization of amino acids. Moreover, the processing of protein antigens is a selected hydrolysis taking place mainly inside the antigen processing compartment, which is a specialized form of LE/LY compartment, capable of fusion with the plasma membrane [30]. VacA was recently shown to inhibit the degradation of tetanus toxoid in the antigen processing compartment [31]. Consequently, the stimulation of T-cell clones specific for epitopes generated in the antigen processing compartment was strongly inhibited by VacA, while that of T-cell clones specific for epitopes generated in the early compartment was unaffected [31]. Such an inhibition of antigen processing and presentation by VacA may be part of a strategy of survival of *H. pylori* since a depression of antigen processing within the mucosa would significantly contribute to the long lasting infection that *H. pylori* establishes in the human stomach. A second consequence of the VacA-induced alteration of the LE/LY compartments is the release of lysosomal acid hydrolases which are made in the ER as pre-pro-enzymes and are tagged in the Golgi to be recognized by tag-specific receptors, which drive them from the TGN to LE and LY. Within these latter compartments, the pre- and pro- segments are removed with activation of their hydrolase activity [32]. If pre-pro-acid hydrolases are released on the acidic apical domain of the stomach epithelial cells intoxicated by VacA, they can be converted into the active form and thus degrade the protective mucus layer depicted in Fig. 1. This degradation activity would cooperate with *H. pylori* mucin degradating enzymes [33] in decreasing the thickness of this layer (Fig. 1) with the possible aim of increasing the supply of nutrients from the stomach lumen to the bacterium.

4. VacA induces an increase in the permeability of polarized epithelial monolayers

H. pylori adheres strongly to the apical surface of the stomach epithelial cells. Polarized epithelial monolayers maintain a *trans*-epithelial electrical resistance (TER) which measures their degree of sealing [34,35]. Low pH-activated VacA, added apically, causes the rapid drop of TER from the initial value to 1000–1500 Ohm/cm^2, a low resistance state which is maintained for days [36]. Parallely, the paracellular route of permeability to small organic molecules and to ions such as Fe^{3+} and Ni^{2+}, which are essential for *H. pylori* growth, is increased. On the basis of these results, we suggest that the major role of VacA is that of increasing the supply of essential nutrients to the bacterium. The supply from the stomach lumen might increase indirectly via partial degradation of the mucus layer, while the supply of nutrients from the mucosa would result directly via loosening of the epithelial layer tightness.

The increase of epithelial conductivity induced by the toxin occurs in the absence of cell vacuolation, is not inhibited by V-ATPase inhibitors and does not require weak bases, suggesting that epithelial permeabilization induced by VacA is distinct from vacuolization. The biochemical activity of VacA is not known and it is also unknown whether it is a membrane acting toxin or an A/B toxin acting intracellularly. VacA transfected in HeLa cells induces vacuole formation [37] and this activity is associated to the N-terminal domain [38,39]. In addition, recombinant VacA p58 domain permeabilizes potassium-filled liposomes [40] and native VacA forms anion-selective and voltage-dependent channels in planar lipid bilayers only at a low pH or after low pH pre-activation [41]. These results may indicate that VacA is similar to diphtheria toxin and related to three domain toxins with respect to the cell entry process [42], but there is a major difference. In fact, VacA cell intoxication is clearly not inhibited by ammonium ions, which prevent the membrane translocation of diphtheria toxin from the endosomal lumen into the cytosol. This opens the possibility that VacA can enter the cytosol from the plasma membrane. If this were the case, VacA should form ion channels on the plasma membrane of cells in culture after low pH activation. Such a membrane permeabilization might be taken as an indication that VacA is an A/B toxin and that it reflects a possible translocation step [42]. However, it might also indicate that VacA acts via formation of an anion channel, a significant variation with respect to other known cytosolic acting toxins. As depicted in Fig. 2, formation of anion-selective pores by VacA on the cell surface would lead to an efflux of Cl^- and HCO_3^- from the cell cytosol into the extracellular medium, thus causing a decrease in the intracellular pH and contributing to cell intoxication. Moreover, such an increased anionic permeability of the membrane is predicted to favor, after endocytosis and targetting to the membrane of LEs of VacA channels, the turn-over of the electrogenic V-ATPase, thus increasing vacuolation by generation of an osmotically driven water uptake in the endosomal vesicle [41].

5. HPNAP

H. pylori gastritis is characterized by infiltration of the infected stomach mucosa by phagocytic inflammatory cells [1,2,43–45] and there is a good correlation between the degree of mucosal damage and neutrophil infiltration [1,44–46]. A protein capable of promoting neutrophil adhesion to endothelial cells was identified in bacterial extracts [47]. The purified protein is a 150 kDa oligomer composed of identical 15 kDa subunits, with sequence similarities to bacterioferritins and DNA binding proteins [48,49]. It was termed HPNAP because of its capability of inducing neutrophils to produce reactive oxygen radicals. Recombinant HPNAP stimulates the production of reactive oxygen radicals via a cascade of intracellular activation events, including an increase of the cytosolic calcium ion concentration and phosphorylation of proteins leading to assembly of functional NADPH oxidase on the neutrophil plasma membrane (Satin et al., in preparation). HPNAP as neutrophil activator is however less powerful than PMA or fMLP, which is in keeping with the suggestion that *H. pylori* induces a moderate inflammatory reaction leading to alteration of the epithelial tight junctions and basal membranes such as to promote the release of nutrients from the mucosa to support the growth of *H. pylori* [8]. Such an activity of HPNAP will add to the nutrient supply promoting activity of VacA, discussed above. Alternatively, this may be an unnecessary side activity of HPNAP, secondary to its main biological function, which remains to be determined.

6. Urease

Urease is an essential virulence factor which allows *H. pylori* to survive the very acidic conditions of the stomach lumen before the bacterium swims into the mucus layer [50–52].

The biosynthesis of urease is governed by a seven gene cluster which includes the genes encoding the UreA (26.5 kDa) and UreB (60.3 kDa) subunits of the urease and five accessory proteins which are responsible for Ni^{2+} uptake and insertion into the active site of the apo-enzyme [53]. Urease is a dodecameric protein composed of six UreA and six UreB subunits arranged as a double ring of 13 nm diameter and the residues involved in the coordination of the two active site Ni^{2+} ions are strictly conserved between *H. pylori* and *Klebsiella aerogenes* ureases [54].

Urease is synthesized without a leader sequence and accumulates in the cytosol together with the heat shock protein HspB, but these proteins are released upon bacterial autolysis and adsorb efficiently on the extracellular surface of viable bacteria [55–57].

The urease may contribute to toxicity via the production of ammonia, which is toxic to cells by itself and after reaction with neutrophil metabolites, though the extent of this effect in vivo remains to be assessed. The picture is more defined in in vitro systems. In the presence of ammonium chloride, VacA-induced vacuolization proceeds faster and vacuoles are larger and swollen with respect to the vacuoles generated by the toxin alone and to those induced by ammonia [23,28], which suggests a substantial cooperation of urease and VacA in damaging cells.

7. Cytotoxin associated gene A (CagA)

The *cagA* gene encodes a 128 kDa protein, an immunodominant surface antigen always present in *H. pylori* strains associated to the more severe form of diseases [7,11]. Although the function of CagA is not presently known, the *cagA* gene is part of a large pathogenicity island which has

been acquired by horizontal transfer of DNA [11]. The origin of this pathogenicity island is not known but a difference in the GC content from the rest of the *H. pylori* genome indicates that it has been acquired from another species or even genus [58]. It includes at least 40 genes which are involved in different pathogenic processes including the induction of inflammatory mediators in gastric epithelial cells, induction of pedestal formation and modification of intracellular signaling. Similarities between some of these genes and genes of a known function in other bacteria indicate that the pathogenicity island encodes a complex secretion system involved in the release of macromolecules from the bacteria [11].

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