# Polyamine- and NADPH-dependent generation of ROS during *Helicobacter pylori* infection: A blessing in disguise

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## Abstract

*Helicobacter pylori* is a Gram-negative bacterium that specifically colonizes the gastric ecological niche. During the infectious process, which results in diseases ranging from chronic gastritis to gastric cancer, the host response is characterized by the activation of the innate immunity of gastric epithelial cells and macrophages. These cells thus produce effector molecules such as reactive oxygen species (ROS) to counteract the infection. The generation of ROS in response to *H. pylori* involves two canonical pathways: 1) the NADPH-dependent reduction of molecular oxygen to generate $O_2^{\bullet-}$, which can dismute to generate ROS; and 2) the back-conversion of the polyamine spermine into spermidine through the enzyme spermine oxidase, leading to $H_2O_2$ production. Although these products have the potential to affect the survival of bacteria, *H. pylori* has acquired numerous strategies to counteract their deleterious effects. Nonetheless, ROS-mediated oxidative DNA damage and mutations may participate in the adaptation of *H. pylori* to its ecological niche. Lastly, ROS have been shown to play a major role in the development of the inflammation and carcinogenesis. It is the purpose of this review to summarize the literature about the production of ROS during *H. pylori* infection and their role in this infectious gastric disease.

## Graphical Abstract

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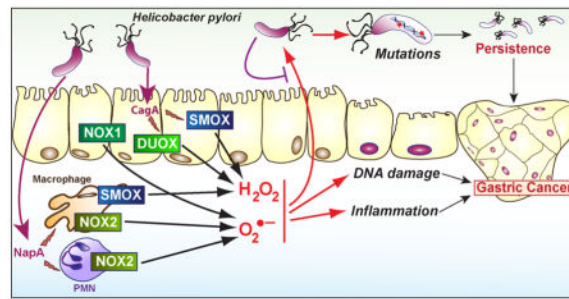
## Keywords

*Helicobacter pylori*; Reactive oxygen species; Polyamines; NADPH oxidase; Gastric cancer

## 1. Infections with *Helicobacter pylori*

### 1.1. Epidemiologic aspects

*Helicobacter pylori* is a Gram-negative microaerophilic bacterium that colonizes the human stomach and it is estimated that half of world's human population is infected. Although *H. pylori* potentially confers protection against diseases, notably in childhood [1], long-term infection has been associated with the development of chronic active gastritis. Moreover, approximately 10% of *H. pylori*-infected patients develop peptic ulcer disease, less than 0.1% develop mucosa-associated lymphoid tissue lymphoma, and 1 to 3% develop gastric adenocarcinoma [2, 3], the third leading cause of death by cancer worldwide corresponding to 10% of total cancer-related mortality. Therefore, *H. pylori* is considered as the most common etiologic agent of infection-related cancers and has been classified as a class I carcinogen.

Eradication of *H. pylori* by antibiotic-based therapy has been proposed to decrease the incidence of gastric malignant transformation [4]. However, the extraordinary genomic plasticity of *H. pylori* has led to considerable antibiotic resistance and the efficacy of the treatments has declined in recent years [5]. Thus, treatment failure is common and high rates of infection recurrence have been reported, principally in developing countries [6]. Lastly, the impact of *H. pylori* eradication on development of gastric cancer appears to be of little value once the development of precancerous lesions is observed [7].

This information highlights that *H. pylori* infection is a serious concern for human health in a world with increasing movement of populations and that non-antibiotic treatment therapy should be considered to limit the risk of development of gastric cancer. This could be achieved by a better understanding of the crosstalk of *H. pylori* and the host.

### 1.2. Determinism of pathogenicity

*H. pylori* strains express three major virulence factors. First, the persistence of the bacterium is principally due to the activity of the bacterial urease that neutralizes gastric acidity by generating ammonium from urea. Second, the cytotoxin-associated gene A (CagA) is a bacterial factor that belongs to the *cag* pathogenicity island (cagPAI) and is directly injected

into human epithelial cells through a type 4 secretion system (T4SS). CagA is then sequentially phosphorylated on tyrosine residues of Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs by the host kinases SRC proto-oncogene (SRC) and ABL proto-oncogene 1 (ABL1) [8]. Four distinct EPIYA motifs (A-D) have been identified and most *H. pylori* strains exhibit EPIYA motif repeats on the C-terminal regions of CagA. The Western isolates exhibit the EPIYA-A/B/(1–3)C motifs, whereas the EPIYA-D motif replaces the C motif in East Asian strains [9]; in Amerindian strains, the last motif is a chimera of the D and C motifs [10]. It has been demonstrated that SRC phosphorylates EPIYA-C and EPIYA-D, whereas ABL phosphorylates all four motifs [8]. Epidemiological studies have emphasized that gastric cancer in East Asian countries is most often associated with the EPIYA-ABD motif [11] and more severe disease in Western countries is associated with multiple EPIYA-C motifs [12]. When CagA is phosphorylated in gastric epithelial cells, it activates the tyrosine-protein phosphatase non-receptor type 11 (also known as SHP2) [13], which then activates extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent cytoskeletal rearrangements, increased motility, loss of cell polarity, resistance to apoptosis, and chromosomal instability, which have been linked to malignancy [14–16]. The phosphorylation of CagA has been also shown to stimulate the activation of the transcription factor nuclear factor-kappa B (NF-κB) [17–19]. It should be also underlined that CagA also possesses a CRPIA (conserved repeat responsible for phosphorylation-independent activity) motif, which is responsible for the phosphorylation-independent signaling of CagA that induces the phosphoinositide 3-kinase (PI3K) signaling pathway [18]. Consistent with these data, large epidemiologic studies have associated the strains harboring the gene *cagA* with higher rates of gastric cancer [20]. Third, the vacuolating cytotoxin A (VacA) contributes to *H. pylori* pathogenesis by regulating inflammatory process [21] and by reducing cell death by autophagy, thus favoring gastric colonization and oxidative damage [22]. Although the contribution of VacA to precancerous gastric intestinal metaplasia has not been directly demonstrated using animal models, epidemiological studies have emphasized that the signal region s1 and the middle region m1 of the *vacA* gene belong to strains that are associated with increased risk for the development of peptic ulcers and/or gastric cancer, compared to s2 or m2 strains [23].

However, the sole expression of virulence factors is not sufficient to explain *H. pylori* pathogenesis, mainly because only 1–3% of *H. pylori*-infected patients develop gastric cancer. Several studies have shown that an environmental component, including iron deficiency [24] or high-salt diets [25], may modulate *H. pylori*-induced inflammation and related carcinogenesis. In addition, the clinical outcome of *H. pylori* infection-induced gastric carcinogenesis is determined by the progression along the histologic cascade from non-atrophic gastritis to adenocarcinoma [26]. In this context, the level of gastritis and the regulation of proteins with potential effects on cell transformation are fundamental features that determine *H. pylori*-related diseases. Among the effectors that can affect cellular function or disrupt signaling, reactive oxygen species (ROS) have been unequivocally recognized as the yin and yang of the innate immune response towards pathogens. Identification of the source of ROS in *H. pylori*-infected mucosa, determination of the mechanism by which *H. pylori* survives the oxidative challenge imposed by the host, and comprehension of the deleterious effect of endogenous ROS are critical topics for a better understanding of *H. pylori* pathogenesis.

## 2. Polyamine-dependent ROS synthesis during *H. pylori* infection

Polyamines are critical homeostatic regulators also involved in the modulation of pathogenesis of numerous diseases, including in the gastrointestinal tract. The inducible synthesis of the biogenic polyamines during *H. pylori* infection necessitates three steps (Fig. 1): First, the amino acid L-arginine is transported into the cells through the activity of the L-arginine transporter solute carrier family 7 (cationic amino acid transporter, y+ system), member 2 (SLC7A2). Second, L-arginine is catabolized by the enzyme arginase into L-ornithine. Third, L-ornithine is converted by ornithine decarboxylase (ODC) into the first polyamine putrescine, which is then catabolized to spermidine and spermine by the action of spermidine synthase and spermine synthase, respectively. Importantly, the back-conversion of spermine to spermidine by spermine oxidase (SMOX) generates $H_2O_2$ (Fig. 1), which plays a critical role in *H. pylori* pathogenesis.

### 2.1. L-arginine bioavailability

The expression of the gene *Slc7a2* and the transporter SLC7A2 is induced in the murine macrophage cell line RAW 264.7 and in murine peritoneal macrophages infected with *H. pylori in vitro* and in lamina propria mononuclear cells of mice and humans with *H. pylori* gastritis [27]; this increase in SLC7A2 protein expression enhances L-arginine uptake in the cells [27]. It has been shown that *Slc7a2*-deficient macrophages produce less polyamines than wild-type (WT) cells when stimulated with M-CSF [28]. Although this has never been reported in the context of *H. pylori* infection, it provides a strong rationale for the contention that polyamine synthesis depends on SLC7A2 activity.

Interestingly, it has been reported that spermine generated endogenously during *H. pylori* infection or added exogenously inhibits L-arginine uptake by macrophages [27]. Blocking *Odc* mRNA expression or ODC activity favors L-arginine entry in macrophages, but does not regulates *Slc7a2* mRNA or SLC7A2 protein levels [27], suggesting that spermine is an inhibitor of SLC7A2 activity and may be considered as a negative feedback regulator of polyamine synthesis in immune cells.

Our lab has reported that *H. pylori*-infected *Slc7a2*$^{-/-}$ mice exhibit more colonization and less gastritis than WT animals. These events were accompanied by a defect in recruitment of macrophages in the infected gastric tissues and by an altered activation of dendritic cells and Th1 cells [29]. These data demonstrate that SLC7A2 plays a major function in the activation of the innate immune response during *H. pylori* infection; but the involvement of polyamines and/or SMOX-derived $H_2O_2$ in this mechanism remains unknown. Work is ongoing in our laboratory to address the contributions of myeloid versus epithelial SLC7A2 in mouse models of *H. pylori* infection. In that context, the potential role of modulation of L-arginine availability in the generation of ROS derived from polyamines that are downstream products of L-arginine metabolism is a theme that will be investigated.

### 2.2. Arginase activity

Two isoforms of arginase exist in mammals: the cytosolic arginase 1 (ARG1), and arginase 2 (ARG2) that is sublocalized to the mitochondria. ARG1, but not ARG2, is a marker of

alternative (M2) macrophages and ARG2 is not induced by IFN-$\gamma$, the classical inducer of M1 phenotype, but can be express in M1 macrophages. Thus, it has been shown that cultured murine macrophages infected with *H. pylori* and gastric macrophages of infected mice display an M1/Mreg phenotype [30], associated with the expression of *Arg2* mRNA expression and ARG2 protein synthesis through an NF-$\kappa$B-dependent pathway [31–33]. However, the ARG1 isoform is not directly induced by *H. pylori* [31]. Arginase activity in infected RAW 264.7 macrophages has been principally detected in the mitochondrial fraction and not in the cytoplasm [32], further demonstrating that the ARG2 isoform is induced in response to *H. pylori*. In addition, it has been described using flow cytometry and immunostaining that ARG2, but not ARG1, localized to gastric macrophages isolated from C57BL/6 mice infected with *H. pylori* [33].

In mice infected with *H. pylori*, the genetic deletion of the gene *Arg2* results in a decrease of putrescine synthesis, demonstrating that ARG2 regulates polyamine production during the infection [34]. Two studies have reported that *H. pylori*-infected *Arg2$^{-/-}$* mice show more gastritis, characterized by enhanced M1 macrophage and Th1/Th17 responses, and less colonization [33, 34] than WT animals, suggesting that macrophage ARG2 decreases the inflammatory process during the infection. Therefore, the induction of ARG2 by *H. pylori* could represent a strategy by which this pathogen dampens and escapes the host immune response. Importantly, ARG2 is also a natural competitor for inducible nitric oxide synthase (NOS2), the enzyme that generates nitric oxide (NO) from L-arginine. NO is a free radical produced by *H. pylori*-infected macrophages that exhibits anti-microbial properties and can regulate inflammation and carcinogenesis [35]. In *H. pylori*-infected macrophages, blocking ARG2 activity results in increased NO production [32], highlighting that the induction of the arginase metabolic pathway by the bacterium is one way of controlling NO production. However, we have recently reported that *Arg2$^{-/-}$* mice and *Arg2$^{-/-}$*; *Nos2$^{-/-}$* mice have similar levels of colonization and gastritis when infected with *H. pylori* [34], indicating that the role of ARG2 in controlling immune response is NOS2-independent. However, more evidence is needed to ascertain that the effect of ARG2 on *H. pylori* pathogenesis occurs through a polyamine-dependent pathway, in particular in human populations.

## 2.3. Induction of ODC

Studies have reported an increase of *ODC* mRNA expression in the gastric tissues of *H. pylori*-infected patients [27, 36], independently of the *cagA* status of the bacteria [36]. In one study, increased ODC activity was mainly observed in the areas of gastric atrophy in *H. pylori*-infected patients [37]. The concentration of total polyamines, but mainly spermidine, is enhanced by more than two times in the stomach of *H. pylori*-infected patients compared to *H. pylori*-negative subjects [38]. In contrast, Elitsur et al. reported no significant change in ODC activity in the mucosa of children infected with *H. pylori* compared to uninfected [39]; the low level of colonization and the absence of gastric atrophy, in which ODC was observed in adults [37], could be responsible for the lack of ODC induction. Interestingly, Chaturvedi *et al.* further demonstrated that *ODC* mRNA expression is increased patients with *H. pylori* gastritis, but not in those with *H. pylori*-negative gastritis [27]; in the same way, Patchett *et al.* reported that the presence of *H. pylori*, and not the severity of gastric inflammation is associated with increased ODC activity in humans [40]. These observations indicate that the

presence of the bacteria is required for ODC induction, and that this is not a global response to inflammation. Supporting this, *ODC* mRNA expression [36], ODC activity in the corpus and antrum of the stomach [41, 42], and polyamine concentrations [38, 43] are decreased after successful eradication of *H. pylori*. In the same way, a concomitant decrease of *H. pylori* colonization, gastric ODC activity, and gastric polyamine concentration has been observed in patients treated with the probiotic *Lactobacillus brevis* strain CD2 [38].

Although it has been described that ODC can be induced in vitro in the gastric epithelial cell lines AGS and BGC-823 and in the immortalized epithelial cell line GES-1 through a phospho-CagA-dependent mechanism and a signal involving ERK1/2 and V-Myc avian myelocytomatosis viral oncogene homolog (MYC) [44], in vivo and in vitro evidence suggest that ODC induction is predominant in myeloid cells in response to *H. pylori*. Hence, ODC has been immunolocalized in gastric macrophages in both humans and mice infected with *H. pylori* [27]. Moreover, *Odc* mRNA expression and ODC activity are induced in the murine macrophage cell line RAW 264.7 stimulated with *H. pylori* SS1 [31]. This induction first requires the activation of the transcription factor activator protein 1 (AP-1), which corresponds to a phospho-FBJ murine osteosarcoma viral oncogene homolog (FOS)/Jun proto-oncogene (JUN) complex, through an ERK1/2-dependent pathway; then AP-1 initiates the transcription of the gene encoding the proto-oncogene MYC [45, 46]. Finally, this transcription factor binds the *Odc* promoter region and stimulates the expression of the gene [45]. Notably, chemical inhibition of ERK1/2 phosphorylation led to a decrease of ODC protein expression in gastric macrophages from *H. pylori*-infected mice [45], supporting the likelihood that this mechanism is not specific to macrophage cell lines. However, the bacterial factors involved in the AP-1/MYC signaling pathway in macrophages remain unknown.

## 2.4. Generation of ROS by SMOX

The homeostasis of polyamine content within a non-toxic range is a considerable challenge for cells, notably during a pathophysiological process in which the genes encoding enzymes involved in polyamine metabolism and catabolism are induced. In this regard, the back conversion of spermine to spermidine and putrescine is a critical step in the regulation of polyamine levels that can prevent the cytotoxic effects of spermine accumulation. Polyamine metabolism can involve several biochemical pathways that may have direct significance in *H. pylori* pathogenesis, ROS generation, and risk for carcinogenesis. First; the spermidine/ spermine N(1)-acetyltransferase 1 (SAT1, also known as SSAT) catalyzes the acetylation of spermine into N(1)-acetylspermine and spermidine into N(1)-acetylspermidine; then, the oxidation of N(1)-acetylspermine and N(1)-acetylspermidine through the enzyme polyamine oxidase (PAOX) leads to the formation of spermidine and putrescine, respectively. However, this pathway is poorly induced in macrophages stimulated in vitro with *H. pylori* and in mice infected for 4 months with the strain SS1 [47]. In contrast, the flavoenzyme SMOX, which directly catalyzes the oxidation of spermine to spermidine and yields the generation of 3-aminopropanal and $H_2O_2$ [48], is overexpressed in macrophages [47] and in gastric epithelial cells [49] infected in vitro with *H. pylori* as well as in the gastric epithelium and monocytes of infected humans, mice, and gerbils [50].

Two *cagA*-deficient strains of *H. pylori* and a *cagE* mutant each induced less *Smox* mRNA expression in conditionally immortalized murine gastric epithelial cells and less *SMOX* mRNA in human epithelial cells than the WT strains [50], suggesting that CagA and the T4SS are involved in SMOX expression. These *in vitro* observations were confirmed by the analysis of gastric tissues from patients with *H. pylori* infection: individuals infected with *cagA*-positive *H. pylori* showed a significant increase of *SMOX* mRNA expression, and SMOX protein level in the gastric epithelium compared to patients harboring *cagA*-negative strains [50]. Similar data were obtained in mice infected with *cagA*-positive *H. pylori* versus a *cagE* mutant that cannot translocate CagA, and in gerbils infected with *cagA*-positive versus *cagA*-negative strains [50, 51].

## 3. NADPH oxidases

The mammalian NADPH oxidases (NOX) are enzymes that transport electrons across the plasma membrane. The electron acceptor is oxygen and therefore the product of the reaction is $O_2^{\bullet-}$. Seven homologs have been identified, referred as five NOX enzymes (NOX1 to NOX5) and two dual oxidase (DUOX) enzymes. Expression of NOX1, NOX2, and DUOX2 have been reported during *H. pylori* infection (Fig. 2).

### 3.1. NOX1

The human and mouse *NOX1* gene is located on the X chromosome and is mainly expressed in the human colon [52]. Although NOX1 expression and function have been evidenced in the gastric mucosa of the guinea pig [53], it has been first suggested that this isoform is not expressed in the human stomach [54]. However, Tominaga et al. have reported that the gene encoding NOX1 is induced in tissues from *H. pylori*-infected patients with gastric adenocarcinoma [55]. These authors also showed by immunofluorescence and confocal microscopy that the proteins NOX1 and the partners NOX organizer 1, NOX activator 1, and p22$^{phox}$ are sublocalized in the Golgi apparatus in gastric cancer cells; and that their expression levels are increased in gastric cancers compared to the surrounding tissue, and compared to areas of chronic atrophic gastritis or adenomas in patients without carcinoma [55]. This suggests that NOX1 could represent a marker of neoplastic transformation. In addition, non-transformed antral-derived primary epithelial cells and the gastric epithelial cell line AGS infected with *H. pylori* 26695 express NOX1 through a signal transduction process involving Ras-related C3 botulinum toxin substrate 1 (RAC1), which is also a protein partner for NOX1, and produce ROS rapidly and transiently [56]. Similarly, NOX1 induction and ROS production has been observed in primary guinea pig gastric mucosal cells infected with *H. pylori* lipopolysaccharide (LPS) [57] through a PI3K/RAC1-dependent pathway [58]. Therefore, RAC1 seems to play an important role in regulating NOX1 expression and activity in *H. pylori*-infected cells. It is exciting to note that the *H. pylori* protein CagL, which is a part of the T4SS, activates the host enzyme SRC [59], and that SRC is also a strong activator of RAC1.

Interestingly, the endonuclease apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1, also known as APE1), a multifunctional enzyme that plays a central role in the cellular response to oxidative stress, including DNA repair and redox regulation of transcriptional

factors, is induced by endogenous ROS in *H. pylori*-infected AGS cells [56]. APEX1 inhibits RAC1 activation and consequently NOX1-dependent ROS production, and blocking APEX1 leads to sustained NOX1 activation and increased $O_2^-$ generation [56]. This could represent a mechanism by which cells limit NOX1-dependent DNA damage and/or a strategy developed by the bacterium to promote its own survival by inhibiting ROS production. In addition, as an another striking example of host-pathogen interactions, it has been established that *H. pylori* superoxide dismutase and catalase decompose NOX1-mediated $O_2^{\bullet-}$ and $H_2O_2$, respectively, thus protecting gastric epithelial cells from ROS-induced cytotoxicity [60]. The authors proposed that the bacterium may use this mechanism to limit the elimination of the transformed cells, thus favoring tumor progression [60].

### 3.2. NOX2

The enzyme NOX2, also known as gp91$^{phox}$ or phagocyte NOX, is principally expressed in neutrophils and macrophages. *H. pylori*, *H. pylori* lysate, or *H. pylori* LPS can each induce a rapid oxidative burst in human primary blood-derived polymorphonuclear neutrophils (PMN) [61–63]. However, it has been reported that *H. pylori* does not efficiently induce the recruitment of the domains p47$^{phox}$ and p67$^{phox}$ to the phagosome, which normally leads to the release of large amounts of $O_2^{\bullet-}$ extracellularly [63]. This unique ability to prevent NOX2 assembly at the phagosome allows *H. pylori* to evade the oxidative killing and may result in neutrophil-derived oxidative damage to surrounding cells, mainly from the epithelium.

The gene *napA* of *H. pylori* [64] encodes the 17 kDa virulence factor called neutrophil-activating protein A (NapA) that forms hexagonal rings [65]. It has been shown that NapA is a chemoattractant for leukocytes and induces NOX2 in human neutrophils through a SRC/PI3K signaling pathway [66]. However, no alteration in gastric colonization has been observed in animals infected for 3 weeks with the *napA* mutant compared to the parental strain [67]. Chronic infection of mice or the use of animal models of *H. pylori*-mediated gastric dysplasia, e.g., transgenic FVB/N insulin-gastrin (INS-GAS) mice, or gerbils, would be useful to determine the role of NapA and the associated induction of NOX2 in carcinogenesis.

Similarly, the stimulation of blood-derived monocytes or differentiated THP-1 cells with *H. pylori* or its LPS yields a rapid and sustained production of $O_2^{\bullet-}$ [68]. More particularly, it has been reported that *H. pylori* cecropin-like peptide Hp(2–20) is a chemoattractant for monocytes through the N-formyl peptide receptors 1 and 2, and that monocytes stimulated with Hp(2–20) release ROS, which inhibit the function of antineoplastic lymphocytes [69].

### 3.3. DUOX2

DUOX1 and DUOX2 are NOX isoforms that generate $H_2O_2$ in a $Ca^{2+}$- and NADPH-dependent manner [70]. DUOX(1–2) must interact with the activator proteins DUOXA(1–2) that organize DUOX for surface expression and activity. The mechanism by which DUOX isoforms produce $H_2O_2$ and not $O_2^{\bullet-}$ remains unknown, but it has been reported that the non mature DUOX2 releases $O_2^{\bullet-}$ whereas the glycosylated form of DUOX2 generates $H_2O_2$ [71].

A microarray analysis performed on the stomach of rhesus macaques (2–3 years-old) has shown that *DUOX2* is one of the 119 genes induced in *H. pylori*-infected animals [72]. Moreover, the expression of *DUOX2* is significantly decreased in animals infected with a cagPAI mutant strain although the level of colonization by the WT and the mutant is similar [72]. The authors also found that other factors with antimicrobial properties, e.g., defensins and the protein DMBT1 (for 'deleted in malignant brain tumors 1'), have been induced in the infected monkeys and therefore proposed that the antimicrobial response activated by the cagPAI may affect the resident gastric microbiota to favor *H. pylori* colonization [72]. In this context, the study of DUOX2 deserves further investigation, even if it has been reported that *H. pylori*-infected patients, mainly those carrying a *cagA*/*vacA*-positive strains, show less DUOX2 levels than uninfected individuals [73].

## 4. Resistance of *H. pylori* to oxidative stress

The goal of the production of ROS by the host cells is to limit the development of pathogens. However, bacteria, including *H. pylori*, have elaborated strategies to counteract the deleterious effect of ROS by different mechanisms (Fig. 3). Superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\bullet-}$ to $H_2O_2$, which is subsequently dismutated into $H_2O$ through two electron reactions by catalase. *H. pylori* also express enzymes with reducing activity on peroxides.

### 4.1. SOD

*H. pylori* expresses a single SOD encoded by the gene *sodB* [74, 75], which contrast to *Escherichia coli* that possesses three SODs [76]. *H. pylori* SOD consist of two identical subunits of 24 kDa and is an iron SOD [75]. *H. pylori sodB* mutants do not exhibit SOD activity and are highly susceptible to $O_2$ and $O_2^{\bullet-}$ [77, 78] and, more surprisingly, to $H_2O_2$ [77]. Consequently, accumulation of ROS in an *sodB*-deficient strain led to an increase of mutagenesis frequency compared to WT strain [77]. Moreover, it has been reported that the enzyme SodB is essential for *H. pylori* to colonize mice [77], demonstrating that $O_2^{\bullet-}$ and/or $H_2O_2$ are effectively released in vivo and target the bacteria in the gastric mucosa. It has been also shown that the *H. pylori* strains that are associated with gastric cancer exhibit more SOD activity than those carried by patients with gastritis or gastric and duodenal ulcers, further evidencing that SodB has a major function in the virulence of *H. pylori* [79].

### 4.2. Catalase

The gene *katA* of *H. pylori* encodes a catalase that does not require NADPH binding for its activity [80]. Catalase possesses a high isoelectric point and resists a very high concentration of $H_2O_2$ [80, 81], but is inhibited by organic hydroperoxides [82]. The KatA-associated protein (KapA), which is encoded by the *kapA* gene located downstream of *katA*, is involved in the resistance of *H. pylori* to $H_2O_2$ [81]. Through a mechanism commonly described as "hitchhiking", KatA binds to KapA and the heterologous protein complex is translocated to the periplasm by the twin-arginine translocation system [83, 84].

*H. pylori katA*⁻ strains are viable in vitro [85], demonstrating that $H_2O_2$ endogenously generated by the bacterium itself is not a significant problem for *H. pylori*. However,

catalase is essential for resistance to ROS because high concentrations of $H_2O_2$ kill *katA*-and *kapA*-deficient strains [81, 83]. In addition, strains lacking catalase activity and exposed to sublethal doses of ROS show more DNA oxidative damage than the parental *H. pylori* [78]. Accordingly, *katA⁻* strains cannot survive in neutrophils or macrophages after phagocytosis [86] and, as with the *sodB* mutant, the *katA* and *kapA* mutants fail to successfully colonize the stomach of mice when compared to the matched WT *H. pylori* [83]. This demonstrates that *H. pylori* catalase activity is implicated in the persistence of *H. pylori* within its ecological niche.

### 4.3. Peroxiredoxins

Bacterial peroxiredoxin (Prx) enzymes reduce $H_2O_2$, organic hydroperoxides, and peroxinitrite that is formed by the reaction of $O_2^{\bullet-}$ and NO. *H. pylori* contains three Prx forms: the thiol-specific peroxidase (Tpx), the alkyl hydroperoxide reductase (AhpC), and the bacterioferritin comigratory protein (BCP).

A two-dimensional gel analysis has evidenced that Tpx is an abundant protein in *H. pylori* [87]. The increased sensitivity of the *tpx* mutant to $O_2^{\bullet-}$, $H_2O_2$, and organic hydroperoxide [88] confirms an in vivo role for Tpx as an important antioxidant protein in *H. pylori*. Hence, the *H. pylori* SS1 *tpx* strain was recovered from the stomach of only 5% of the inoculated mice at 3-weeks post-infection, whereas the WT strain colonized 78% of animals in the same experiment [89].

AhpC is the most abundant antioxidant protein in *H. pylori*. Oligomers of AhpC exhibit a peroxide-reductase activity, whereas high-molecular-weight complexes are molecular chaperones for prevention of protein misfolding under oxidative stress [90]. A mutant of *H. pylori* lacking the gene *ahpC* was more sensitive to oxidative stress conditions induced by $H_2O_2$ and also contained significantly higher amounts of 8-OHdG associated with its DNA, compared to WT bacteria [78, 82]. It has also been shown that AhpC is involved in the resistance to $O_2^{\bullet-}$ [91], but the molecular mechanism has not been demonstrated and it may correspond to the resistance to $H_2O_2$ generated by the activity of SOD. Interestingly, the *ahpC* mutant contains three times more hydroperoxides than WT [82]; since hydroperoxides inhibit KatA, the *ahpC* strain exhibits a significant decrease of catalase activity [82], demonstrating a cooperation between the different systems of resistance to ROS to further improve the survival of the bacterium against hostile oxidative conditions. Finally, it has been described that the *ahpC* mutant does not colonize mice [89].

Comtois *et al.* reported that the role of BCP in resistance to oxidative stress is minimal [88]. This could be attributed to the fact that BCP is less abundant than AhpC in *H. pylori* [91]. Wang et al. further demonstrated that BCP preferentially reduces linoleic acid hydroperoxide, and not $H_2O_2$ [91]. The deletion of *bcp* in *H. pylori* is associated with a modest reduction of gastric colonization compared to WT [91]. The specificity of BCP for organic hydroperoxide substrates probably justifies why the *bcp* deletion has a less dramatic effect on gastric colonization than the mutation of *tpx* or *ahpC* genes.

*H. pylori* lacks the glutathione system, which is essential for cellular thiol:disulfide balance and survival under oxidative stress in many Gram-negative bacteria [92]. Consequently, this

bacterium uses the thioredoxin (Trx):Trx reductase (TrxR) system as an electron donor [91]. Two Trx exist in *H. pylori*: TrxA (also called Trx1) and TrxC (Trx2). While TrxA is more involved in preventing nitrogen-dependent damage than TrxC [88], both reductases have been shown to play a major function in the resistance against oxygen stress from $O_2^{\bullet-}$ and $H_2O_2$ [88, 93]. However, if TrxA forms a reductase system for AhpC [94] and BCP [91] and is a chaperone for *H. pylori* arginase RocF [95], the mechanism by which TrxC protects bacteria remains unknown. Nonetheless, the deletion of *trxA* yields a diminution of stomach colonization by *H. pylori* [88, 93], while the *trxC* mutant does not colonize the animals [93], demonstrating that both Trx enzymes, but mainly TrxC, are essential for *H. pylori* colonization and persistence. In regard to this data, the study of TrxC deserves further investigation.

### 4.4. NapA

As described above, NapA is an essential bacterial factor that induces ROS production by PMNs. In addition to this inducing effect, NapA also displays a role in the bacterium itself. Thus, the *napA* mutant is more sensitive to oxygen [96] and oxidative stress [67, 96] and therefore exhibits more bacterial DNA damage than the WT strain [67]. It has been proposed that NapA could bind and protect bacterial DNA from oxidative damages [97]. Remarkably, NapA is mainly induced when the main proteins involved in ROS detoxification, e.g., SodB, AhpC, Tpx, are absent in *H. pylori* [98], demonstrating an elaborated transcriptional plasticity of the genes encoding antioxidant effectors.

In a co-infection model, the *napA⁻* strain exhibited reduced ability to survive in the mouse stomach when competing with the WT parental strain [67]. This suggests that NapA is also involved in the combat of *H. pylori* against oxidative stress by an unidentified mechanism.

### 4.5. NADPH quinone reductase

The NADPH quinone reductase MdaB is a flavoprotein that catalyzes two-electron transfer from NADPH to ubiquinones and menaquinones. MdaB is involved in the resistance of *H. pylori* against $O_2$, $H_2O_2$, and the $O_2^{\bullet-}$ donor paraquat [98, 99], but the molecular mechanism remains unidentified. It could be proposed that MdaB may act as an $O_2^{\bullet-}$ scavenger as its human homolog NAD(P)H:quinone oxidoreductase 1 [100]. Moreover, the *mdaB* mutant colonized mice less efficiently than the WT [99].

## 5. Genomic plasticity in response to ROS

The oxidative environment that surrounds *H. pylori* can result in DNA alterations in the pathogen. Exposure of *H. pylori* to methyl viologen, an $O_2^{\bullet-}$ donor, significantly increases point mutations, intergenomic recombination, and rearrangements between direct DNA repeats [101]. These mutations are transient and limited to the duration of stress, and do not lead to a global hypermutator phenotype. Intriguingly, in silico analyses have demonstrated that DNA repeats are especially concentrated in the *H. pylori cag* pathogenicity island [101]. Intriguingly, a study performed in rhesus macaques has shown that a mutation burst is observed in the *H. pylori* genome during the acute infection phase that is over 10 times faster

than the mutation rate during chronic infection [102], demonstrating that the innate immune response may play a critical role in *H. pylori* genomic changes.

Nevertheless, compared to other pathogenic bacteria, the arsenal of *H. pylori* to counteract DNA oxidative damages is limited: it lacks translesion DNA synthesis polymerases, the DNA methyl-directed mismatch repair system (MutSHL), the SOS response, and the stress-induced sigma factors RpoS and RpoH [103, 104]. O'Rourke et al. have demonstrated that an *H. pylori* mutant lacking the endonuclease III Nph, is more sensitive to $H_2O_2$, exhibits more mutation frequencies when exposed to $H_2O_2$ or to activated macrophages [105]. NpH is a DNA glycosylase that removes oxidized nucleobases and repairs oxidized pyrimidines [106], which are recognized to block DNA replication and transcription and are thus more cytotoxic. This highlights that the ROS produced by the host are effectively able to provoke lethal DNA adducts in the *H. pylori* genome and that the presence of NpH can favor *H. pylori* survival. A *mutS* homolog has also been identified in the *H. pylori* genome. *H. pylori* $mutS^-$ is more sensitive to $H_2O_2$ and paraquat and shows increased mutation (mainly G:C to T:A) rates under oxidative stress conditions [107], demonstrating that MutS is involved in DNA repair in response to ROS. The authors have also demonstrated that MutS has high specific affinity to dsDNA containing oxidized purines (such as 8-oxoguanosine), which are mutated bases that are mostly mutagenic [107].

Of importance, a 1–2-log order decreased colonization of the *mutS* mutant compared to the parental strain has been demonstrated at day 90 post-infection in mice [107]. In contrast, the *nph*-deficient strain completely fails to colonize mice after 60 days [105]. These data emphasize that DNA repair in *H. pylori* seems to have higher specificity for cytotoxic base lesions than mutagenic base lesions, thus contributing to the high genetic variability that is observed among *H. pylori* strains [102].

To conclude, ROS-induced high mutation rate, associated with a reduced proofreading capacity of its DNA polymerase I [108], increases the chance of *H. pylori* acquiring mutations. This can then confer an adaptive advantage in the gastric environment and in response to the selective pressure imposed by host immunity.

## 6. Endogenous effect of ROS on the host

### 6.1. Cell signaling and inflammation

Inflammation is an important hallmark of *H. pylori* infection that represents a universal response to the infection, and production of ROS has been strongly implicated in the risk for neoplastic progression of *H. pylori*-induced disease. There are numerous potential targets of ROS within the cell, and their reaction with thiol residue-containing proteins, such as phosphatase or thioredoxin, can therefore regulate cell signaling [109].

Hence, the stimulation of AGS cells by *H. pylori* results in a rapid phosphorylation of V-Akt murine thymoma viral oncogene homolog 1 (AKT1) independently of CagA, leading to the induction of autophagy through p53 downregulation [110]. In this study, the authors also demonstrate that the treatment of the cells with the antioxidant N-acetylcysteine inhibits AKT1 activation [110], demonstrating the role of ROS in this molecular process. In the same

way, Ngo *et al.* demonstrated that the oxidative burst induced by *H. pylori* in human gastric epithelial cells is responsible for the PI3K/AKT1-dependent expression of the transcription factor Snail family zinc finger 1 (SNAI1, also known as SNAIL) [111], which is essential for epithelial-mesenchymal transition and therefore in gastric cancer progression and metastasis. Additionally, it has been shown that the transcription factor signal transducer and activator of transcription 3 (STAT3), which plays a major function in inflammation and angiogenesis, is also induced in gastric epithelial cells via upregulated autocrine IL-6 signaling, partially mediated by endogenous ROS [112]. Moreover, α-lipoic acid, a naturally occurring thiol compound that exhibits antioxidant properties, inhibits NOX-derived ROS production in AGS cells and concomitantly dampens NF-κB and AP-1 activation and expression of the oncogenes β-catenin (CTNNB1) and MYC [113]. Lastly, it has been described that the production of vascular endothelial growth factor by *H. pylori*-infected gastric epithelial cells is dependent on the stabilization of the transcription factor hypoxia-inducible factor 1-alpha (HIF-1α) through ROS synthesis [114]. These data indicate that ROS signaling in gastric epithelial cells may contribute to cell transformation and gastric cancer development.

ROS production may also regulate the signaling events in myeloid cells. Thus, *H. pylori* induces IL-1β secretion through the inflammasome in murine bone marrow-derived macrophages and human peripheral blood mononuclear cells; this is inhibited by the use of the anti-oxidant molecule ebselen, suggesting that *H. pylori*-induced ROS are involved in the activation of the immune response of myeloid cells [115]. This effect was observed in the early events post-infection [115], suggesting that the oxidative burst, rather than the effect of SMOX is involved. These results are also consistent with *in vitro* data showing that the partial scavenging of ROS by N-acetylcysteine led to a decrease of *H. pylori*-induced inflammasome formation and production of IL-1β and IL-18 by the human monocytic cell line THP-1 [116].

Tsugawa et al have shown that VacA signals in human gastric epithelial cells through the receptor LDL receptor related protein 1 (LRP1) to decrease glutathione (GSH) levels and therefore increase ROS accumulation, leading to CagA degradation by autophagy [110]. Of note, CagA accumulates in CD44-positive gastric cancer stem-like cells because they retain a high level of GSH and resist ROS [110]. This study further highlights the important role of ROS in *H. pylori* carcinogenesis, not only by inducing ROS producing enzyme, but by altering GSH content.

Chronic granulomatous disease (CGD) mice are animals with a targeted disruption of the NOX2/gp91$^{phox}$ subunit. At 12 and 30-weeks post-infection *with H. pylori*, CGD mice showed no differences in colonization, but more glandular atrophy, proliferation of gastric epithelial cells, and neutrophil infiltration [117]. But overall, there were no significant changes in gastritis score between WT and CGD mice [117]. In contrast, a second study reported that *gp91*$^{phox-/-}$ mice exhibit more inflammation and increased mononuculear cell infiltration in the mucosa during infection with *H. pylori* [118].

### 6.2. Apoptosis and DNA damage

Apoptosis and formation of DNA damage are critical for *H. pylori*-induced carcinogenesis. Thus, the implication of ROS in the regulation of these both events has been extensively studied.

An increase of 8-hydroxy-2-deoxyguanosine (8-OHdG), a reliable biomarker of DNA oxidative damage, has been observed in *cagA*-positive *H. pylori*-infected patients compared to those uninfected or infected with *cagA*-negative strains [50, 119–121]; further, a correlation between the production of ROS and the level of 8-OHdG [121] and between 8-OHdG and the severity of the gastritis [120] has been evidenced. In addition, increased 8-OHdG staining has been associated with more advanced histopathologic lesions of atrophic gastritis and intestinal metaplasia in subjects from a high cancer risk region of Colombia [51, 122].

The gastric epithelial cell lines AGS or Kato III infected with *H. pylori* undergo apoptosis through a mechanism involving mitochondrial depolarization caused by $H_2O_2$ [49, 123–125], and this can be blocked by catalase or by antioxidants like vitamin E or N-acetylcyteine [49, 124, 125]. Further, Hayakawa et al. have also described that the rapid oxidative burst of *H. pylori*-infected gastric epithelial cells in vitro activates the apoptosis signal-regulating kinase 1 (ASK1) that ultimately triggers c-Jun N-terminal kinase (JNK) phosphorylation and cell apoptosis [125]. Remarkably. Ding et al. have reported that generation of ROS by both the bacteria themselves and the infected gastric epithelial cells mediate apoptosis [123].

It has been shown that SMOX is associated with apoptosis in macrophages [47] and in gastric epithelial cells [49], because the use of the SMOX inhibitor MDL 72527 or gene silencing by *Smox* siRNA in the mouse cells and *SMOX* siRNA in human cells reduces $H_2O_2$ synthesis, mitochondrial membrane depolarization, cytochrome c release, and caspase-3 activation. Moreover, SMOX expression in isolated epithelial cells from the gastric tissues of *H. pylori*-infected gerbils correlates with 8-oxoguanosine formation, as detected by flow cytometry [50]. Of note, there is a subpopulation of SMOX-expressing cells with oxidative DNA damage that were resistant to apoptosis [50], which may contribute to the development of gastric cancer. Further, the treatment with either the ODC inhibitor α-difluoromethylornithine or MDL 72527, reduces the abundance of the subpopulation of 8-oxoguanosine+ cells that were resistant to apoptosis, and were associated with gastric dysplasia and carcinoma in *H. pylori*-infected gerbils [51]. Furthermore, reduced levels of SMOX, DNA damage, and DNA damage[high], apoptosis^{low} cells have been observed in *H. pylori*-stimulated gastric epithelial cells derived from mice lacking the gene encoding the epidermal growth factor receptor (EGFR) [126]. Similar results were obtained in cells isolated from infected mice with disruption of EGFR signaling (*Egfr[wa5]* mice) when compared to WT mice [126]. Together, these data suggest that EGFR is involved in *H. pylori*-mediated SMOX expression. Additionally, the kinase Erb-B2 receptor tyrosine kinase 2 (ERBB2) was shown to be critical for the cellular events associated with EGFR signaling in gastric epithelial cells; specifically, heterodimerization of pEGFR and ERBB2 was shown to lead to phosphorylation of ERBB2 and apoptosis resistance [126]. These data strongly support the contention that SMOX-derived ROS are key metabolites that link *H. pylori*

infection to gastric cancer. Further evidence should arise from studies in *Smox*-deficient mice, which are ongoing in our laboratory.

### 6.3. Regulation of CagA signaling

CagA is phosphorylated in gastric epithelial cells by SRC [8]. pCagA then activates SHP2 by binding to SH2 domains, causing conformation change and activation without phosphorylation of C-terminal tyrosine residues [13]. SHP2 activation leads to signaling through ERK1/2 to stimulate cell proliferation, differentiation, and survival [14–16].

It has been shown that ROS induce SRC activation [127] and that CagA-independent generation of ROS contributes to *H. pylori*-induced SHP2 activation [128]. Specifically, the treatment of the human gastric epithelial cell line, MKN45, and the human monocytic cell line, U937, by the anti-oxidant N-acetylcyteine decreases *H. pylori*-induced SHP2 phosphorylation [128]. In contrast, it has been proposed that ROS, and mainly $H_2O_2$, reversibly inactivates protein tyrosine phosphatases, such as SHP2, by causing the oxidation of cysteine residues and formation of disulfide bonds [129, 130].

The contribution of ROS in the regulation of CagA phosphorylation and signaling in *H. pylori*-infected gastric epithelial cells deserves further investigation. The purported paradoxical effects of ROS could represent another example of the bacterium manipulating host cells to affect signaling events that benefit the bacterium.

## 7. Conclusions

The parasite-like lifestyle of *H. pylori* has been shaped by hundreds of thousands of years of coevolution with the human host. Thus, since the host expresses multiple ROS-producing enzymes that are redundant in gastric somatic and immune cells, *H. pylori* has elaborated strategies to counteract oxidative damage and increase its own survival. However, it is interesting to note that this bacterium possesses a limited arsenal to repair DNA damage; the bacterium does not tend to respond to ROS-induced mutagenic mutations, thus contributing to the high genetic variability that is observed among *H. pylori* strains. Therefore, we can raise the question of whether *H. pylori* favors its own genetic variation and adaptability to its niche by inducing ROS production by the host.

The current knowledge about the resistance of *H. pylori* to the effectors of the innate immune response has evidenced one intriguing feature: the bacterium fights the nitrosative stress and also inhibits NO production by the host [35]; however, it appears to only inhibit ROS-dependent oxidative damage without attenuating the expression/activity of SMOX or NOX. This could be explained by the fact that the most reactive nitrogen species result from the reaction of NO and ROS (e.g. peroxinitrite or dinitrogen trioxide), and that *H. pylori* may conserve energy by inhibiting just NO production, and not both NO and ROS. Therefore, it is conceivable that ROS are beneficial for the bacterium by favoring genomic variation required for long-term persistence and are deleterious for the host since their production is not inhibited by *H. pylori*.

As experimental systems have continued to evolve, in addition to standard animal models (mouse, gerbils, and monkeys) and in vitro models, we now have access to various three and two dimensional gastric organoid systems from rodents and humans in which to recapitulate the microenvironment of the bacterium and the host, which will be very powerful. We are hopeful that these models and increased studies of human and bacterial interactions will counteract some of the inherent limitations of animal models, to better ascertain the role of polyamines and ROS in *H. pylori* infection.

Recent analysis of the phylogeographic origins of both the human hosts and the infecting *H. pylori* strains have highlighted that the risk of developing neoplastic lesions is mainly associated with a lack of coevolution between the host and the bacterium [131]. In this context, a future challenge will be to include human ancestry and *H. pylori* phylogeographic origin in the analysis of the expression and the role of ROS-producing systems.

## Acknowledgments

## Abbreviations

| | |
|---|---|
| **8-OHdG** | 8-hydroxy-2-deoxyguanosine |
| **ABL1** | ABL proto-oncogene 1 |
| **AhpC** | alkyl hydroperoxide reductase |
| **AKT1** | V-Akt murine thymoma viral oncogene homolog 1 |
| **APEX1** | endonuclease apurinic/apyrimidinic endodeoxyribonuclease 1 |
| **ARG1** | arginase 1 |
| **ARG2** | arginase 2 |
| **ASK1** | apoptosis signal-regulating kinase |
| **BCP** | bacterioferritin comigratory protein |
| **CagA** | cytotoxin-associated gene A |
| **cagPAI** | *cag* pathogenicity island |
| **CGD** | chronic granulomatous disease |
| **DUOX** | dual oxidase |
| **EGFR** | epidermal growth factor receptor |
| **EPIYA** | Glu-Pro-Ile-Tyr-Ala |
| **ERBB2** | Erb-B2 receptor tyrosine kinase 2 |

| | |
|---|---|
| **ERK1/2** | extracellular signal-regulated kinase 1/2 |
| **FOS** | FBJ murine osteosarcoma viral oncogene homolog |
| **GSH** | glutathione |
| **HIF-1α** | hypoxia-inducible factor 1-alpha |
| **JNK** | c-Jun N-terminal kinase |
| **JUN** | Jun proto-oncogene |
| **LPS** | lipopolysaccharide |
| **LRP1** | receptor LDL receptor related protein 1 |
| **MYC** | V-Myc avian myelocytomatosis viral oncogene homolog |
| **NapA** | neutrophil-activating protein A |
| **NF-κB** | nuclear factor-kappa B |
| **NO** | nitric oxide |
| **NOS2** | inducible nitric oxide synthase 2 |
| **NOX** | NADPH oxidase |
| **ODC** | ornithine decarboxylase |
| **PAOX** | polyamine oxidase |
| **PI3K** | phosphoinositide 3-kinase |
| **PMN** | polymorphonuclear neutrophil |
| **Prx** | peroxiredoxin |
| **RAC1** | Ras-related C3 botulinum toxin substrate 1 |
| **ROS** | reactive oxygen species |
| **SAT1** | spermidine/spermine N(1)-acetyltransferase 1 |
| **SLC7A2** | solute carrier family 7 (cationic amino acid transporter, y+ system), member 2 |
| **SMOX** | spermine oxidase |
| **SNAI1** | snail family zinc finger 1 |
| **SOD** | superoxide dismutase |
| **SRC** | SRC proto-oncogene |
| **STAT3** | signal transducer and activator of transcription 3 |

| **T4SS** | type 4 secretion system |
|---|---|
| **Tpx** | thiol-specific peroxidase |
| **Trx** | thioredoxin |
| **TrxR** | thioredoxin reductase |
| **VacA** | vacuolating cytotoxin A |
| **WT** | wild-type |

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**Highlights**

- ROS are locally produced in the gastric mucosa during *H. pylori* infection

- Spermine oxidase and NADPH oxidases are the main sources of ROS in the stomach

- *H. pylori* has multiple strategies to resist oxidative damage

- ROS-induced mutagenic changes in *H. pylori* leads to genetic variation and adaptation

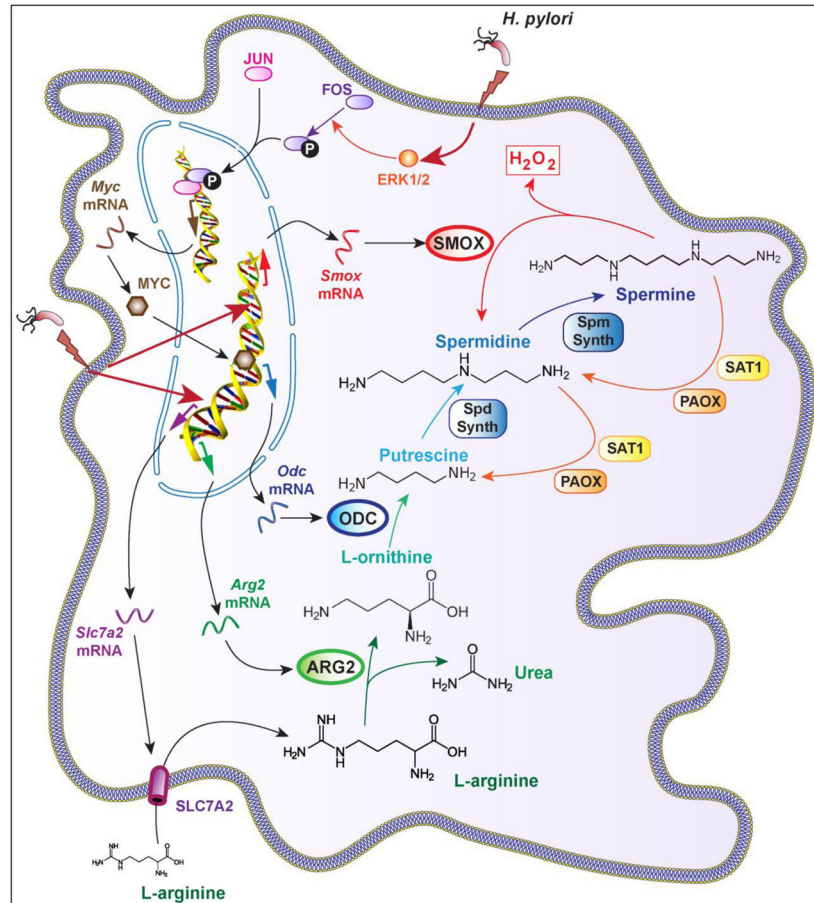- Oxidative stress contributes to *H. pylori*-induced inflammation and carcinogenesis

**Fig. 1.**

*H. pylori*-infected macrophages produces $H_2O_2$ from L-arginine. *H. pylori* induces SLC7A2 expression allowing the uptake of L-arginine in macrophages. The induction of ARG2, ODC, which requires a MYC-dependent pathway, and SMOX leads to the release of $H_2O_2$.
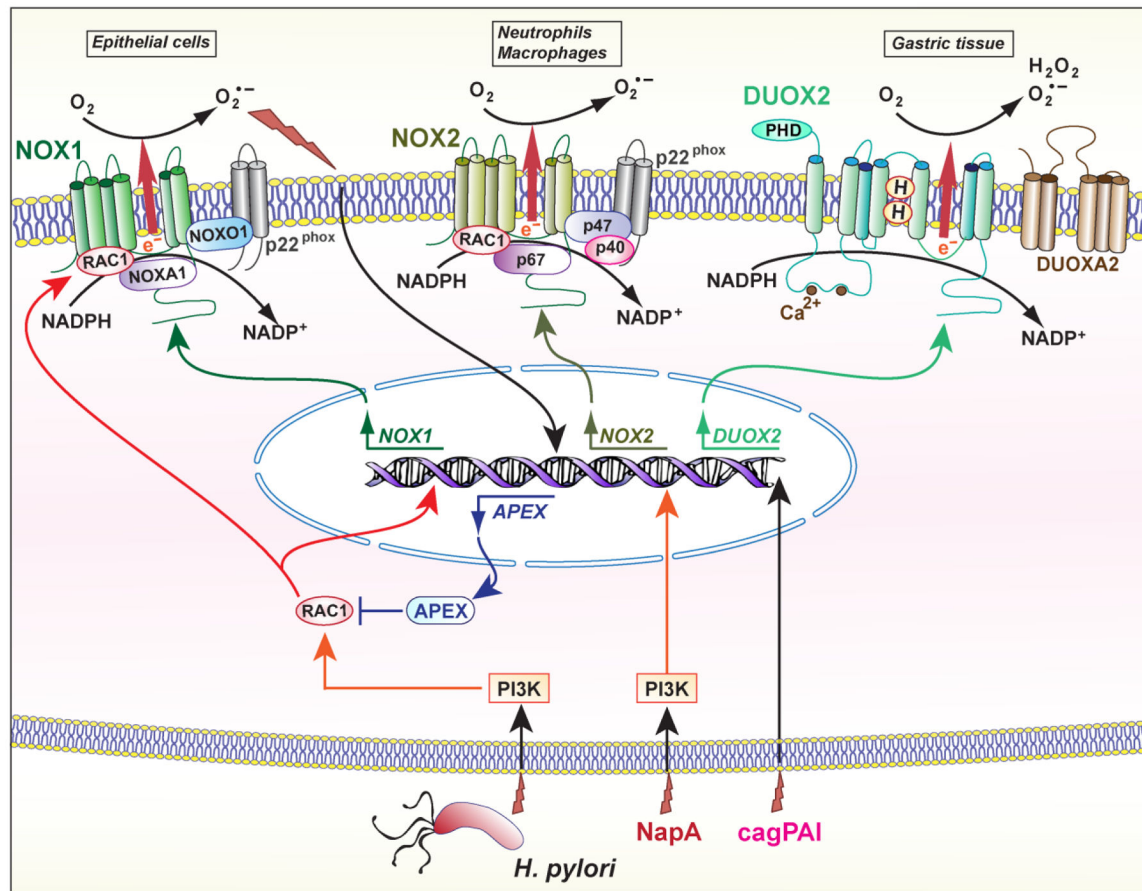
**Fig. 2.**
Regulation of NOX by *H. pylori*. NOX1 is induced in gastric epithelial cells through a PI3K/ RAC1 signaling pathway; in these cells, the production of $O_2^{\bullet-}$ stimulate the expression of APEX that blocks RAC1. In myeloid cells, the expression of NOX2 requires a NapA/PI3K signals. DUOX2 has been shown to be induced in the gastric tissue of infected animals, but the subcellular localization of this enzyme remains unknown. PHD, peroxidase homology domain.
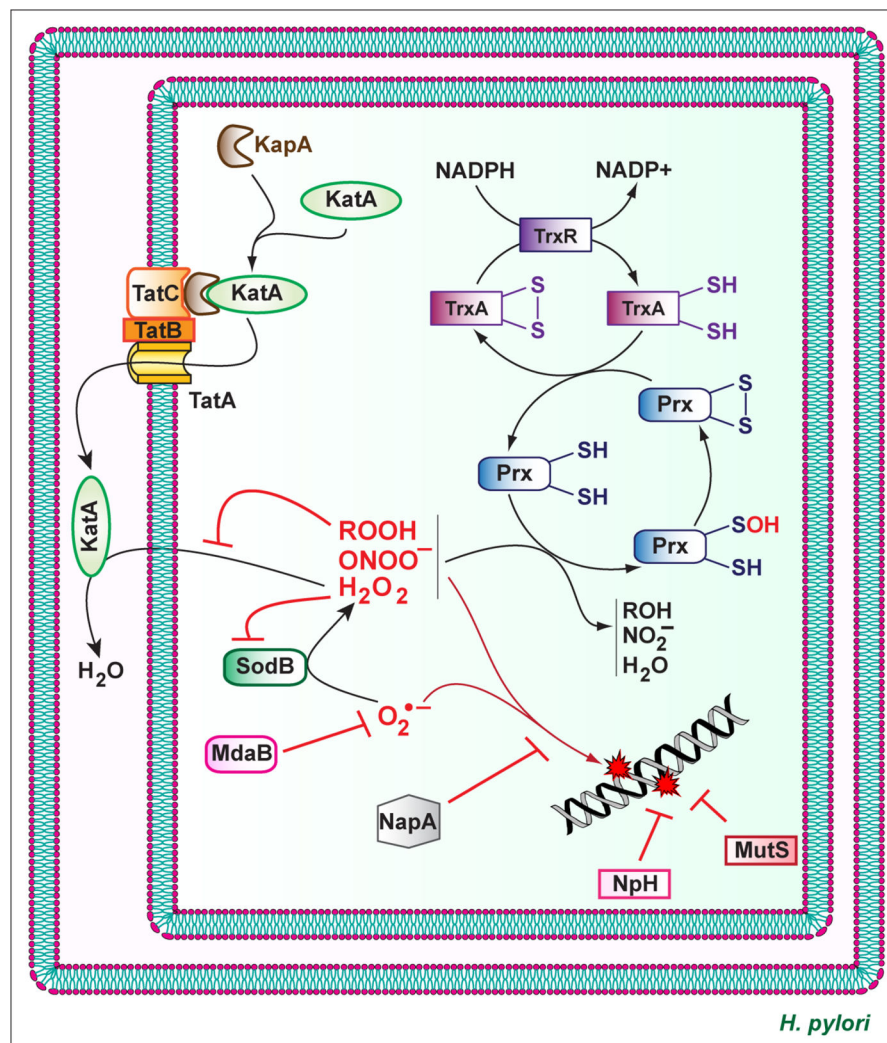
**Fig. 3.**
Biochemical pathways used by *H. pylori* to counteract ROS. The enzyme KatA is transported to the periplasm by the action of KapA and the Tat system. $O_2^{\bullet-}$ is converted into $H_2O$ by SodB and KatA. Hydroperoxides, peroxynitrites, and $H_2O_2$ are reduced by the Prx/TrxA system. NapA protects *H. pylori* from DNA oxidation by an unknown mechanism. NpH and MutS are involved in oxidative DNA repair.