ANTIOXIDANTS & REDOX SIGNALING
Volume 17, Number 9, 2012

DOI: 10.1089/ars.2011.4304

Helicobacter pylori Has an Unprecedented Nitric Oxide Detoxifying System

Marta C. Justino,[1,2] Chantal Ecobichon,[2,3] André F. Fernandes,[1] Ivo G. Boneca,[2,3] and Lígia M. Saraiva[1]

Abstract

Aims: The ability of pathogens to cope with the damaging effects of nitric oxide (NO), present in certain host niches and produced by phagocytes that support innate immunity, relies on multiple strategies that include the action of detoxifying enzymes. As for many other pathogens, these systems remained unknown for *Helicobacter pylori*. This work aimed at identifying and functionally characterizing an *H. pylori* system involved in NO protection. ***Results:*** In the present work, the *hp0013* gene of *H. pylori* is shown to be related to NO resistance, as its inactivation increases the susceptibility of *H. pylori* to nitrosative stress, and significantly decreases the NADPH-dependent NO reduction activity of *H. pylori* cells. The recombinant HP0013 protein is able to complement an NO reductase-deficient *Escherichia coli* strain and exhibits significant NO reductase activity. Mutation of *hp0013* renders *H. pylori* more vulnerable to nitric oxide synthase-dependent macrophage killing, and decreases the ability of the pathogen to colonize mice stomachs. ***Innovation:*** Phylogenetic studies reveal that HP0013, which shares no significant amino acid sequence similarity to the other so far known microbial NO detoxifiers, belongs to a novel family of proteins with a widespread distribution in the microbial world. ***Conclusion:*** *H. pylori* HP0013 represents an unprecedented enzymatic NO detoxifying system for the *in vivo* microbial protection against nitrosative stress. *Antioxid. Redox Signal.* 17, 1190–1200.

Introduction

Helicobacter pylori is a Gram-negative pathogen that chronically colonizes the human gastric mucosa, and is a major cause of several gastroduodenal pathologies (33). In gastric epithelial cells, neutrophils, and macrophages, *H. pylori* causes up-regulation of inducible nitric oxide synthase (iNOS) expression as judged by the higher systemic and intraluminal levels of stable NO metabolites (nitrite and nitrate) measured in *H. pylori* infected patients (13, 17, 25). In addition, the host niche exposes *H. pylori* to a second source of NO from the acid-induced chemical decomposition of dietary and salivary nitrites present in the gastric lumen.

NO and derived reactive nitrogen species (RNS) are crucial to innate immunity for the control and clearance of pathogens (11), as RNS damage several biological targets, including DNA, lipids, protein metal centers, and amino acid residues of proteins, therefore causing inactivation of key metabolic functions. Hence, to sustain the long-term colonization, *H. pylori* needs to resist and subvert the effects of the nitrosative stress.

Regarding RNS defense mechanisms, *H. pylori* counteracts host NO production by expressing arginase RocF that competes with iNOS for the same substrate, L-arginine (15). *H. pylori* has also been proposed to induce, within macrophages, an alternative pathway of L-arginine metabolization, through

Innovation

On infection, the innate immune system produces nitric oxide (NO), a reactive molecule that helps eradicate pathogens. To date, no system devoted to removing NO has been reported for *Helicobacter pylori*, a pathogen with a high human health burden. This work provides evidence for the first NO detoxifying enzyme present in *H. pylori*, namely HP0013. An extensive functional characterization of the gene/protein demonstrates that the enzyme catalyzes the reduction of NO and provides the pathogen with an added defense against inducible nitric oxide synthase-mediated host immune attack. Moreover, it is shown that HP0013 constitutes a novel family of NO detoxifying microbial enzymes that is widely spread in prokaryotes.

[1]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal.
[2]Group Biology and Genetics of the Bacterial Cell Wall, Institut Pasteur, Paris, France.
[3]INSERM, Groupe AVENIR, Paris, France.

up-regulation of host arginase II and ornithine decarboxylase that altogether deplete arginine and cause inhibition of iNOS translation *via* the production of polyamine spermine (8, 14). Regarding RNS removal, so far, only alkyl hydroperoxidase AphC was shown to reduce peroxynitrite (5), a highly reactive species formed on reaction of NO with superoxide. Moreover, analysis of the genome of *H. pylori* does not allow identification of homologs of the canonical bacterial NO detoxification systems, such as the respiratory membrane-bound NO reductases (qNOR), flavohemoglobins (HMP) flavodiiron proteins (FDP), nitrite reductase (NrfA), and cytochrome c′-type proteins (35). To fill this gap, we screened a library of *H. pylori* mutants for NO resistance phenotypes, and found that the *hp0013* gene product contributes to the survival of *H. pylori* under NO stress.

Results

HP0013 increases resistance of H. pylori *to RNS*

A mini-*Tn*3-Km transposon mutant library of *H. pylori* 26695 (21) was screened for resistance to the NO donor S-nitrosoglutathione (GSNO) using phenotypic disk-diffusion assays. Mutation of the *hp0013* gene was shown to yield a strain with higher susceptibility to GSNO (Fig. 1A). *H. pylori hp0013* codes for a hypothetical protein that forms a putative operon with the upstream gene *hp0012* which encodes the DNA primase DnaG, and the downstream gene *hp0014* that encodes a hypothetical protein. However, the *hp0014* mutant was equally susceptible to nitrosative stress as the wild-type strain (Fig. 1A).

To analyze the role of *hp0013*, a nonpolar mutant strain was generated in *H. pylori* 26695 by gene replacement with a kanamycin resistance cassette. The growth behavior of both transposon and nonpolar *hp0013* mutants was examined in cultures treated with 200 μM GSNO. Although in the absence of stress the two mutants behaved as the parental strain (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/ars), GSNO treatment significantly reduced the mutants viability in comparison to the wild-type strain (Fig. 1B),

To further substantiate the results, the nonpolar *hp0013* gene deletion was introduced into another *H. pylori* strain, namely *H. pylori* B128, a strain that has the ability to colonize mice and Mongolian gerbils (20). Similar growth assays were performed, and the results revealed that deletion of *hp0013* also impairs the resistance of *H. pylori* B128 to GSNO (Fig. 1C), even though these strains appeared less sensitive than their counterpart *H. pylori* 26695 derived strains. We further observed that expression of HP0013 from the inducible plasmid pILL2157-HP0013 was sufficient to rescue the phenotype of the *H. pylori* B128 *hp0013* mutant (Fig. 1C).

To confirm the role of HP0013, *H. pylori* 26695 and B128 nonpolar *hp0013* mutant strains were exposed to other sources of nitrosative stress. As seen in Figure 2A, B, *H. pylori* 26695 was less resistant than the B128 wild-type strain to stress induced by dipropylenetriamine (DPTA)-NONOate, an NO donor that releases two molecules of NO with a half-life of 3 h at 37°C. This result was similar to that seen when the strains were tested against GSNO-induced stress. Nevertheless, in both backgrounds, the deletion of *hp0013* caused significantly higher NO-dependent killing, with an up to 10-fold decrease of survival relative to the correspondent wild-type strain, an effect that could be reversed with the expression in *trans* of the HP0013 protein (Fig. 2B).

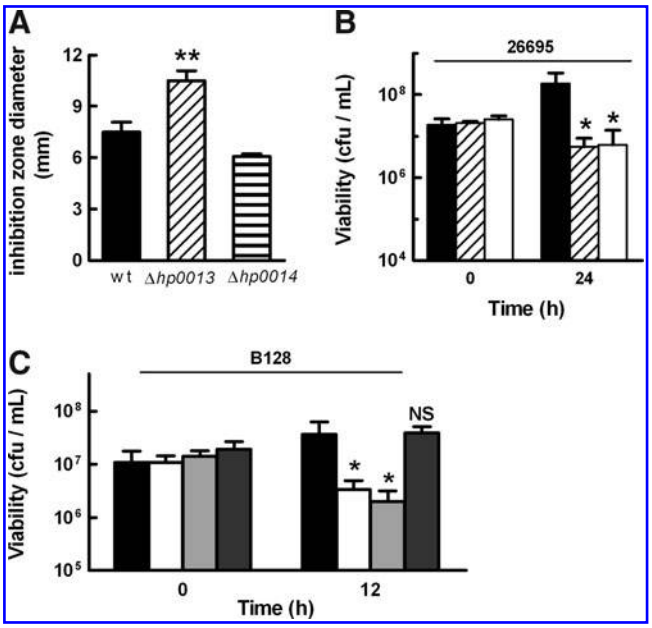

FIG. 1. Deletion of *hp0013* increases susceptibility of *Helicobacter pylori* to S-nitrosoglutathione, nitric oxide donor (GSNO). (A) Comparison of the sensitivity to GSNO of *H. pylori* 26695 wild-type cells (*black*), Δ*hp0013*::mini*Tn*3 (*dashed*) and Δ*hp0014*::mini*Tn*3 (*horizontal stripes*) by disk diffusion assays, performed with 6-mm discs containing 15 μl of 10 m*M* GSNO. Values are means ± standard deviation SD ($n = 4$). $^{**}p < 0.01$ (analysis of variance [ANOVA] and Bonferroni test). **(B)** Cell viability of GSNO-treated cultures of *H. pylori* 26695 wild-type (*black*), 26695 Δ*hp0013*::mini*Tn*3 (*stripes*) and nonpolar 26695 Δ*hp0013*ΩK2 (*white*). **(C)** Cell viability of GSNO-treated cultures of *H. pylori* B128 wild-type (*black*), B128 Δ*hp0013*ΩK2 (*white*), B128 Δ*hp0013*ΩK2 carrying the empty vector pILL2157 (*light gray*), and B128 Δ*hp0013*ΩK2 with pILL2157 expressing HP0013 (*dark gray*). In **B** and **C**, *H. pylori* cultures were inoculated at OD_{600} of 0.05 and treated with 200 μM GSNO. The number of viable cells were determined for, at least, four independent cultures and are expressed as means ± SD. $^{*}p < 0.05$, NS, nonsignificant ($p > 0.05$) (ANOVA and Bonferroni test).

In contrast, on exposure to 200 μM peroxynitrite, wild-type and mutant strains suffered, independently of the time, a small decrease of viability (~30%) (Fig. 2C), indicating that HP0013 is not involved in the detoxification of the NO congener, peroxynitrite.

Altogether, these results revealed that HP0013 protects the *H. pylori* species against NO stress.

HP0013 confers NO detoxification ability to H. pylori

Given the phenotypes of the *hp0013* mutants, we tested whether HP0013 was involved in the reduction of S-nitrosothiol adducts or in NO detoxification.

The first hypothesis was disproved as *H. pylori* 26695 and *hp0013* mutant cell lysates exhibited similar levels of GSNO reduction activity (Fig. 3A). On the contrary, the rates of anaerobic NO consumption, measured with an NO-electrode, were ~50% lower in cell extracts of the 26695 *hp0013* mutant strain when compared with the wild-type (Fig. 3B).

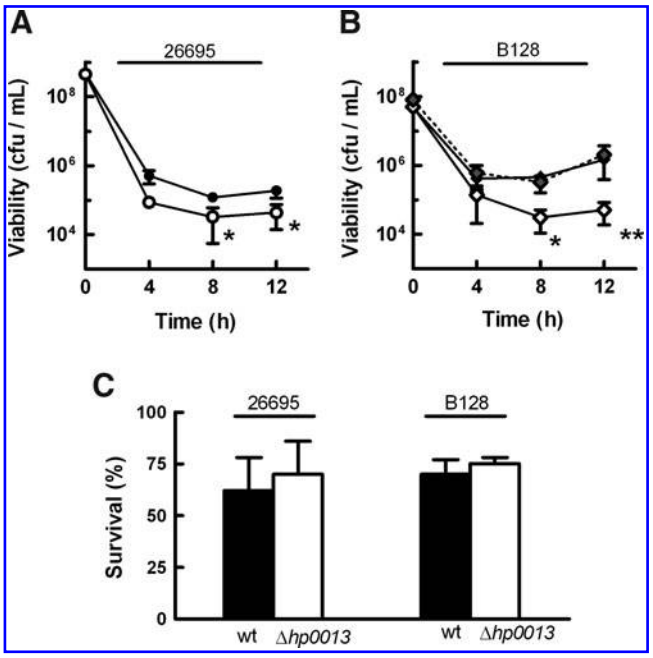

FIG. 2. *H. pylori* HP0013 confers resistance to nitric oxide (NO). (A) Cell viability of *H. pylori* 26695 wild-type (*filled circle*) and nonpolar *hp0013* mutant (*open circle*), on treatment with 150 μM dipropylenetriamine-NONOate, NO donor (DPTA-NONOate). Values are means of four independent cultures ± SD. *$p < 0.05$ (ANOVA). **(B)** Cell viability of *H. pylori* B128 wild type (*black diamond*), the derived *hp0013* mutant carrying pILL2157 (*open diamond*), and the *hp0013* mutant carrying pILL2157 expressing HP0013 (*gray diamond*, *dotted line*), after exposure to 150 μM DPTA-NONOate. Values are means of at least four independent cultures ± SD. *$p < 0.05$; **$p < 0.01$ (ANOVA and Bonferroni test). **(C)** Survival of *H. pylori* 26995 and B128 wild-type strains (*filled bar*) and their *hp0013* derived mutants (*open bar*) after 6 h exposure to 200 μM peroxynitrite. Values are means ± SD ($n = 4$).

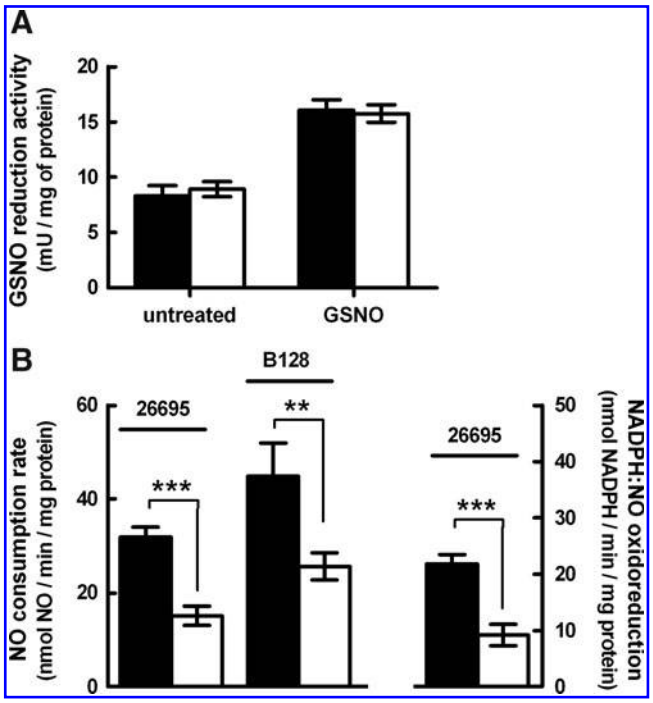

FIG. 3. HP0013 contributes to the NO consumption activity of *H. pylori*. (A) GSNO reduction activity measured in cell lysates of *H. pylori* 26695 (*black*) and derived *hp0013* mutant (*white*) prepared from untreated cultures and cultures exposed for 1 h to 200 μM GSNO. Values are means ± SD ($n = 4$). One unit corresponds to 1 μmol of GSNO and 1 μmol of NADPH consumed per minute. **(B)** NO reduction activity of *H. pylori* cell lysates, determined amperometrically measuring the consumption of NO (*left axis*), and spectrophotometrically monitoring NADPH oxidation coupled to NO reduction (*right axis*). *Black bars* represent the wild-type strain, and *white bars* represent the corresponding *hp0013* mutant. Values are means ± SD ($n \geq 4$). **$p < 0.01$, ***$p < 0.001$ (ANOVA performed for each wild-type/mutant pair).

The rate of NAD(P)H oxidation coupled to NO reduction was also assayed in spectrophotometric assays. The cell lysates of *H. pylori* 26695 wild-type and *hp0013* mutant showed rates of NADPH:NO oxidoreduction that were similar to the rates of NO consumption measured amperometrically (Fig. 3B, left), while no activity was detected when using NADH as an electron donor.

We next investigated whether *hp0013* deletion also affected the NO reduction ability of *H. pylori* B128 cells. *H. pylori* B128 wild-type cell lysates exhibited higher levels of NO consumption than *H. pylori* 26695. Moreover, the B128 strain mutated in *hp0013* had significantly lower activity (Fig. 3B).

We also observed that lysates prepared from *H. pylori* 26695 cultures pre-exposed for 1 h, to 200 μM GSNO presented NO consumption rates equivalent to untreated cells (Supplementary Fig. S2). In accordance, the *hp0013* gene expression was found to be essentially unchanged by the presence of nitrosative stress (data not shown).

Hence, we concluded that HP0013 is involved in the NO detoxification of *H. pylori*, as the activity of both strains was found to be lowered by ~50% in the absence of the *hp0013* gene.

HP0013 protein exhibits NO reduction activity and rescues the NO-deficient phenotype of the Escherichia coli *NO reductase mutant strain*

The HP0013 protein was recombinantly produced, isolated, and its enzymatic NO activity was assayed. The purified protein had an NO reduction activity of 0.10 ± 0.03 nmol NO.(s.nmol protein)$^{-1}$ and a rate of coupled NADPH:NO oxidoreduction of 0.07 ± 0.03 s^{-1}, consistent with a stoichiometry of two molecules of NO reduced to N_2O, per molecule of NADPH oxidized, similar to what is generally described for other NO reductases (16). The NO turnover of the recombinant HP0013 protein is lower than that of qNORs and FDPs (10–50 s^{-1}), but within the same range of the NO reductase activity of flavohemoglobins (HMP) (31). Nevertheless, we hypothesize that such a low turnover rate may be due to the instability exhibited by the protein. To further confirm the role of the HP0013 protein as an NO reductase, we tested whether the expression of HP0013 rescued the anaerobic NO-phenotype of *Escherichia coli* cells deficient in the FDP-type NO reductase. As shown in Figure 4, the *E. coli fdp* mutant cells carrying the plasmid expressing *H. pylori* HP0013 were more resistant to NO stress than those carrying the empty vector.

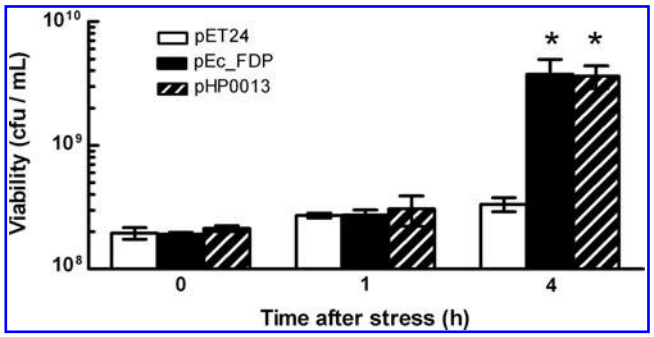

FIG. 4. *H. pylori* HP0013 complements an *E. coli* strain lacking the flavodiiron protein (FDP)-NO reductase. Viability of anaerobic cultures of *E. coli* LMS2710 (FDP-type NO reductase mutant strain) harboring an empty vector (pET24, *white*), pME2337 that expresses the *E. coli* FDP-NO reductase (pEc_FDP, *black*) and pET24 expressing HP0013 (pHP0013, *black striped*), after treatment with 30 μM spermine-NONOate. Values are expressed as means of four independent cultures ± SD. $^*p < 0.05$ (ANOVA and Bonferroni test).

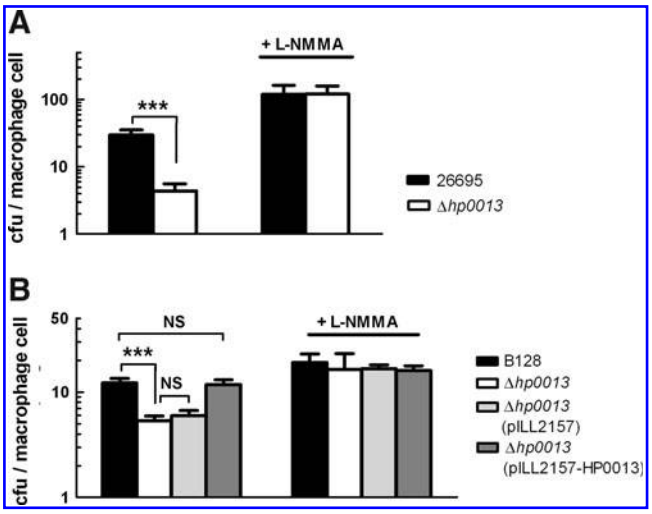

FIG. 5. Survival of *H. pylori* wild-type and *hp0013* mutant strains with macrophages. (A) *H. pylori* 26695 wild-type (*black bars*) and nonpolar Δ*hp0013* mutant (*white bars*). Values are means ± standard error of the mean (SEM; $n \geq 10$). $^{***}p < 0.001$ (ANOVA). **(B)** *H. pylori* B128 wild-type (*black*), B128 Δ*hp0013* mutant (*white*), B128 Δ*hp0013* carrying the empty vector pILL2157 (*light gray*), and B128 Δ*hp0013* with pILL2157 expressing HP0013 (*dark gray*). Values are means ± SEM ($n \geq 6$). $^{***}p < 0.001$, NS, non significant ($p > 0.05$) (ANOVA and Bonferroni test). Macrophages RAW264.7 cells (5×10^5) were seeded in 24-well plates and infected at an MOI of 100, and the bacterial survival was determined after 24 h. When indicated, the inducible nitric oxide synthase (iNOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) (0.8 mM) was added to the infection medium and for infections with bacteria carrying plasmids, the medium was supplemented with IPTG (1 mM).

Moreover, the expression of HP0013 complemented the NO-phenotype to an extent similar to that achieved on expression of the *E. coli*'s own FDP protein (Fig. 4) from the same vector. These results showed that the NO reductase activity of HP0013 was able to protect the *E. coli* cells to an extent similar to the one conferred by the *E. coli* FDP.

HP0013 contributes to the survival of H. pylori *in macrophages*

Since NO production is a crucial factor of macrophage-dependent eradication of *H. pylori* (8, 15), we evaluated the killing of *H. pylori* strains (26695, B128) and their isogenic *hp0013* mutants by RAW264.7 macrophages. After 24 h of incubation with RAW264.7 cells, the survival of the two *hp0013* mutant strains was ~50% lower than that of the correspondent parental strains (Fig. 5A, B), that is, the absence of the *hp0013* led to a significant increase of RAW264.7 mediated killing of *H. pylori*. Moreover, expression of the HP0013 protein from an inducible plasmid in the B128 *hp0013* mutant strain restored the wild-type phenotype (Fig. 5B), in an expression level-dependent way (Supplementary Fig. S3).

H. pylori is known to influence macrophage NO production, for instance, by depleting the substrate of iNOS, L-arginine, *via* the action of arginase (RocF). Hence, we performed the macrophage-infection assays in a serum-free medium supplemented with L-arginine to ensure that the RAW264.7 cells were fully able to produce NO. Among infections with *H. pylori* 26695, B128, and *H. pylori* 26695 *rocF* deficient strain, the levels of nitrite accumulated in the macrophages' supernatants were found to be similar (31.2 ± 7.0, 22.9 ± 4.7 and 31.6 ± 6.0 μM nitrite, respectively). Hence, under the conditions used, *H. pylori* arginase had indeed a negligible influence on iNOS activity.

To confirm that the lower viability of *hp0013* mutants when infecting macrophages is attributable to their susceptibility to nitrosative stress, assays were repeated in the presence of the iNOS inhibitor N^G-monomethyl-L-arginine (L-NMMA). In the absence of NO produced by the RAW264.7 macrophages, as judged by the lack of nitrite accumulated in the supernatants, the survival of wild-type *H. pylori* 26695 and B128 increased (Fig. 5A, B), confirming that NO contributes to the killing of *H. pylori*. Moreover, in these conditions the survival *of H. pylori* wild-type and *hp0013* mutant strains was no longer different (Fig. 5A, B). These results corroborate the relevance of HP0013 to the *H. pylori* resistance against NO-dependent macrophage-mediated killing.

HP0013 contributes to the fitness of H. pylori *to colonize iNOS-proficient mice*

To further examine the HP0013-dependent NO detoxification activity *in vivo*, we determined the ability of *H. pylori* B128 strain and Δ*hp0013* derived mutant to colonize the stomach of C57BL/6J mice. First, an equal number of mice were orogastrically inoculated with the wild-type and mutant strains. The levels of gastric colonization were evaluated at the peak of infection (15 days) that is characterized mainly by the infiltration of innate immune cells, and at a later time of infection (45 days), when *H. pylori*'s numbers have been drastically reduced by the innate and adaptive immune response (but not eradicated) and an equilibrium between *H. pylori* and the host has been reached. At these times, mice were sacrificed, and their stomachs were removed to determine the bacterial load. After 15 days, the mean colonization levels of B128 Δ*hp0013* were comparable to the wild-type B128, but after 45 days, while wild-type *H. pylori* B128 colonization had reached a steady state, the mutant had been eliminated in seven out of eight mice

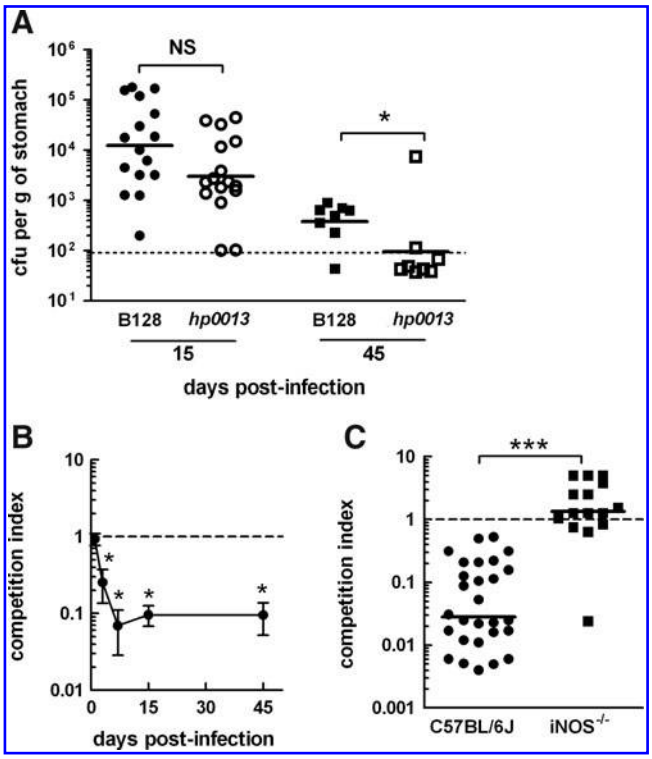

FIG. 6. HP0013 confers fitness to colonization of iNOS competent mice. (A) Single-strain colonization of C57BL6/J mice by *H. pylori* B128 or *hp0013* mutant. Each data point corresponds to a single mouse (16 mice after 15 days infection, and 8 mice after 45 days infection); *horizontal bars* are the geometric mean, and the *dashed line* indicates the detection limit of cfu/g of stomach. *$p < 0.05$ and NS, nonsignificant (Mann–Whitney test). **(B)** Competition index of the *hp0013* mutant during co-colonization of C57BL6/J mice by a mixture of B128 wild-type and Δ*hp0013* (1:1). The colonization level of each strain was analyzed after 1, 3, 7, 15, and 45 days (8 mice per time point, except 15 days, where 31 mice were used). Values are calculated as the ratio of the stomach load of the mutant to the wild-type, divided by the ratio of the initial inocula. A minimum competition index was calculated for mice in which the *hp0013* mutant could not be detected, considering the value of colonization as half of the detection limit. Values are expressed as means ± SEM. *Dotted line* indicates the level of equal efficiency of colonization.*$p < 0.01$, (Mann–Whitney test). **(C)** Competition index of the *hp0013* mutant after 15 days co-colonization by a mixture of B128 wild-type and Δ*hp0013* (1:1) of mice of the wild-type (C57BL6/J, *circles*, 31 mice) and iNOS-deficient (iNOS$^{-/-}$, *squares*, 16 mice). Each data point corresponds to a single mouse, *horizontal bar* represents the geometric mean, and *dotted lines* indicate the level of equal efficiency of colonization. ***$p < 0.001$ (Mann–Whitney test). All mice were orally inoculated with 2×10^8 bacteria, and the stomach was bacterial load assayed after the indicated days of infection.

(Fig. 6A). Hence, the mutant strain exhibited a lower capacity to sustain a chronic infection of mouse stomachs.

In a second set of experiments, we assessed the colonization efficiency of the *H. pylori* B128 Δ*hp0013* mutant during competition with the wild-type strain for the same niche. We inoculated mice with equal proportions of both strains and performed a kinetic analysis of the levels of each strain after 1–45 days. For each mouse, the competition index of the *hp0013* mutant *versus* the wild-type was calculated. The results showed a very clear and rapid displacement of the *hp0013* mutant (Fig. 6B), as the competition index started to drastically decline immediately after the first day of infection, and the strain was almost completely eliminated after 7 days. In fact, after 15 days of co-infection, the competition index values determined for 31 C57BL/6J mice were all below 1, with a geometric mean of 0.028, demonstrating that the inactivation of *hp0013* renders *H. pylori* less proficient to colonize the stomach of mice (Fig. 6C). In contrast, when co-infection was performed in iNOS-deficient C57BL/6J mice that lack the capacity to produce NO as an antimicrobial defense, both *H. pylori* strains showed similar levels of colonization. The geometric mean value of the competition index was determined to be 1.2 (Fig. 6C), which reflects an equal fitness of both *H. pylori* strains to colonize when no NO is produced by the host immune response. This shows that HP0013 contributes to *H. pylori*'s virulence through the role in NO metabolization.

H. pylori *HP0013 protein is widely spread in the microbial world*

The NCBI protein database BLAST search of *H. pylori* 26695 HP0013 retrieved, using as selection criterion an E-value below 10^{-10} and a span of the query sequence above 60%, ~200 proteins with sequence identity and similarity ranging from 16% to 99% and 26% to 99%, respectively. This analysis revealed that HP0013 is encoded in all 21, so far available, *H. pylori* completed genomes. Moreover, the occurrence of HP0013 homologs is restricted to neither the *Campylobacterales* family nor the ε-Proteobacteria class, as they exist in more than 120 distinct species widely distributed through eight bacterial phyla (with a higher predominance in Proteobacteria, Fusobacteria, Firmicutes, and Aquificae) (Fig. 7A). A unifying characteristic of these organisms is their low affinity for oxygen, as they are mainly anaerobes or microaerobes. Around 40%, spread over distant phylogenies, colonize as commensal or pathogenic flora the oro-gastrointestinal tract of humans or animals. These include members of *Helicobacter*, *Campylobacter*, *Clostridium*, *Fusobacterium*, *Brachyspira*, and *Desulfovibrio* genus, as well as of *Francisella philomiragia* and *Wolinella succinogenes*.

To the best of our knowledge, no HP0013-like protein has been characterized so far, and the majority of these proteins are annotated as hypothetical or putative argininosuccinate synthetase, as TrmU-like tRNA methyltransferases or ThiI-like proteins involved in thiamine biosynthesis. The annotations are based on sequence similarity confined to the *N*-terminal region carrying an adenine nucleotide binding motif and a potential Rossman fold motif. However, it was reported that the annotation of ThiI-like proteins is, in most cases, incorrect, as it is based on the presence of these domains, which were shown not to be required for the function of ThiI proteins (2).

The alignment of the amino acid sequences of all retrieved HP0013 homologs revealed a stronger conservation within the first 250 amino acid residues (referring to *H. pylori* 26695 HP0013), and several highly conserved sequence regions were identified (Fig. 7C and Supplementary Fig. S4).

The first motif (LxSGGLD(S/A)) shares similarity to the P-loop adenosine nucleotide binding motif, which interacts with the phosphate group (36). According to the HP0013 predicted

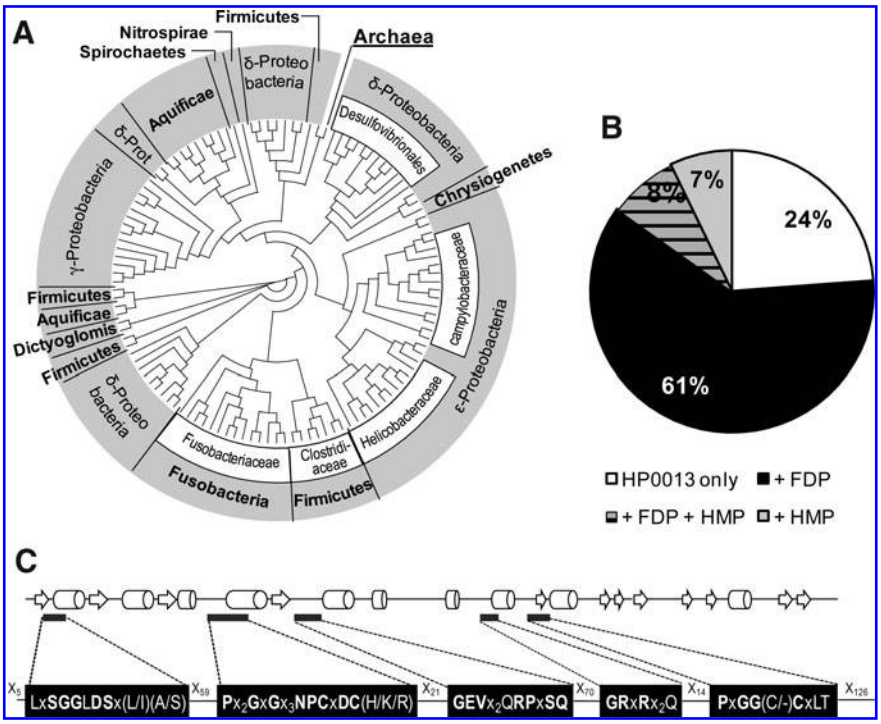

FIG. 7. Distribution of HP0013 homologs in microbes. (A) Unrooted dendrogram of HP0013 homologs considering the alignment of 120 sequences (Supplementary Fig. S4). The outer sphere *shaded boxes* indicate different bacterial Phyla (*bold*) or Class. The inner *white boxes* specify the Order or Family of clusters of sequences. δ-Proteobacteria class is represented as δ-Prot. **(B)** Occurrence of microbial canonical NO detoxification systems—flavohemoglobins (HMP) and flavodiiron proteins (FDP) in organisms containing HP0013-like proteins. Microorganisms with no HMP or FDP (*white*), only FDP (*black*), only HMP (*gray*), and with both HMP and FDP (*striped*). **(C)** Highly conserved regions present in all analyzed sequences are shown in *black boxes*. *Uppercase letters* denote amino acids residues conserved in more than 95% of the sequences, and letters in *bold* highlight strictly conserved ones. The position of the conserved regions in the *H. pylori* 26695 HP0013 is schematized below the graphical representation of the PSIPRED predicted secondary structure. *Arrows* depict β-sheets, cylinders represent α-helices, and lines depict coils. *Small dashes* denote conserved regions in-scale.

secondary structure, this motif is within a Rossman fold structural motif (Fig. 7C), spanning between the first β-sheet and α-helix structures, a configuration that is typical of NAD(P)/FAD binding motifs (24). Given that the purified recombinant HP0013 was not associated to an FAD group, we propose that this region represents the binding site for a NADPH molecule, which acts as the electron donor for HP0013's catalytic activity.

In the second highly conserved sequence segment ($Px_2GxGx_3CxDC(H/K/R)$), in many organisms including all *Helicobacter* species, the second cysteine residue is followed by a histidine residue. The CXXCH motif is typical of *c*-type heme containing proteins, which are periplasmic localized (26); however, the predicted cytoplasmic nature of HP0013 suggests that it does not bind a *c*-type heme.

A previous *in silico* study aimed at identifying potential disulfide reductases encoded in *Campylobacterales* genomes reported that the HP0013 protein of *H. pylori* contains an alternative motif (CXXT) characteristic of the disulfide reductases (23). Our current analysis revealed that this motif is located within one of the conserved regions (Fig. 7C), and that the cysteine residue (numbered 221 in HP0013) is strictly conserved while the threonine residue is highly conserved. This motif is present in the primary sequences of a wide range of diversely functional proteins including those that perform reduction of disulfide bonds and peroxynitrite (23, 34). However, cells of *H. pylori* 26695 and of the *hp0013* mutant showed significant differences neither in the levels of oxidized glutathione (GSSG) reduction activity (144.1 ± 8.9 and 157.9 ± 19.6 mU/mg of protein, respectively [$n = 3$]) nor in the rates of peroxynitrite reduction, as judged by the degree of inhibition of peroxynitrite-mediated oxidation of dihydrorhodamine (respectively, 21.1% ± 0.5% and 23.8% ± 1.9% [$n = 3$]).

As previously mentioned, *H. pylori* lacks any of the known NO detoxifying systems, namely HMP and FDP that, in general, are proposed to act as NO detoxifiers under aerobic and anaerobic conditions, respectively (1, 35). Analysis of the organisms which contain HP0013 homologs showed that ~20% also lack both HMP and FDP systems (Fig. 7B). The co-occurrence of HP0013-like proteins and FDPs exits in ~70% of the microbes, while the co-presence with HMPs is lower (15%). Hence, one may speculate a possible association of HP0013 to FDPs and that, similar to FDPs, HP0013 acts predominantly as oxygen-independent NO reductase in organisms growing under low oxygen tension.

Discussion

In this work, we show that *H. pylori* cells metabolize NO at rates that are within the range of values which were previously reported (32). However, while in this earlier study, NO was assumed to be chemically scavenged, we now reveal

that NO consumption is indeed associated to an NADPH-dependent enzymatic activity present in *H. pylori* cells.

When compared with *H. pylori* 26695, *H. pylori* B128 had higher NO consumption rates and, in agreement, increased resistance toward NO. This seems consistent with a different tolerance of several *H. pylori* strains to nitrosative stress, as *H. pylori* 26695 and B128 exhibited higher resistance to peroxynitrite when compared with the NCTC 11637 and ATCC 43504 strains (27, 37). GSNO did not induce any morphological changes in *H. pylori* 26695 and B128 (data not shown), a result that contrasts with previous studies with *H. pylori* SD14 (9). Although at this stage it is not possible to exclude that these differences are due to the variation of the growth conditions utilized, they may result from the high genetic variability associated to *H. pylori* (10).

Independently of the *H. pylori* background, all *hp0013* mutant strains had increased susceptibility to NO, and the NO reduction activity of *H. pylori* cells was found to be significantly lowered on deletion of the *hp0013* gene. Moreover, the purified HP0013 protein showed *in vitro* activity as NADPH:NO oxidoreductase, and its expression in *trans* complemented an *E. coli* strain lacking the FDP-type NO reductase. We further verified that the purified HP0013 protein specifically used NADPH and not NADH as a reducing agent for its catalytic activity. Accordingly, *H. pylori* cells displayed no consumption of NADH for NO reduction. The preferential usage of NADPH over NADH by *H. pylori* cells is in line with an earlier work which suggested that *H. pylori* lacks NADH generating enzymes (12).

HP0013 seems to be dedicated to NO removal, as the deletion of the gene did not influence the peroxynitrite and S-nitrosothiol reduction activities of *H. pylori* cells. As mentioned in the introduction, *H. pylori* has been reported to utilize several strategies to survive the damage inflicted by nitrosative stress. However, HP0013 is the first NO detoxifying enzyme, as no genes encoding canonical bacterial NO detoxifiers are found in all the so far available *H. pylori* genomes.

We further addressed the relevance of HP0013-mediated NO protection in the evasion of host immune response. *In vivo* studies revealed that deletion of *hp0013* in *H. pylori* significantly reduced the survival of the strains on incubation with macrophages, a phenotype that was shown to be associated to the NO production by macrophages.

Murine colonization assays further demonstrated that the *H. pylori* B128 *hp0013* mutant was also less efficient at sustaining a chronic colonization of mice stomachs. Moreover, in competition assays, in which mice were co-infected with the *hp0013* mutant and the wild-type *H. pylori* B128 strains (1:1), the mutant was much less infectious than the wild-type mice, being eliminated after 7 days of infection. These results contrast somehow with the data from independent infection assays, where the mutant was still found colonizing mice after 15 days of infection. One possible explanation may come from the fact that *H. pylori* seems to exploit the inflammatory and immune response to its own benefit. Intensifying inflammation creates damages to the epithelial cell barrier and, therefore, conditions to acquire essential nutrients from the submucosa. However, the damage also leads to the infiltration of immune cells (particularly of neutrophils and macrophages that produce NO). Thus, *H. pylori* has developed a myriad of mechanisms to cope with the immune response, including NO generation by producing an arginase to scavenge the substrate of the iNOS. Hence, it might be postulated that, during the first stages of the infection, *H. pylori* B128 *hp0013* may still deal with a moderate inflammatory response, as the mutant is not completely devoid of NO reductase activity. However, once the adaptive immune response is engaged recruiting an even higher number of neutrophils and macrophages, the threshold of NO is reached, leading to HP0013 eradication. In the competition assays, while the *H. pylori* B128 wild-type keeps its NO defenses and can sustain a higher inflammatory response, the HP0013 mutant not only has to deal with NO but also has to compete for the access of nutrients with the fitter wild-type strain. Thus, when both strains are co-infecting, the threshold of NO production required to kill a weakened mutant strain is encountered much earlier. In support of this hypothesis, we could attribute the phenotype of the mutant to its lowered tolerance for NO stress, as during competition for a niche in the stomach of iNOS-deficient mice, *H. pylori* B128 wild-type and *hp0013* were equally proficient in colonizing the mouse stomach. Hence, HP0013 was shown to be an important factor for *H. pylori* to resist macrophages and infect the mouse stomach, constituting an added advantage against NO-dependent immune defenses.

Given that neither the NO consumption activity of *H. pylori* cells nor the expression of the *hp0013* gene were induced by nitrosative stress, HP0013 seems to be a constitutive NO detoxifying protein that may result from the need of *H. pylori* to survive in an *in vivo* niche where it is constantly exposed to NO stress.

Finally, phylogenetic analysis revealed that HP0013 homologs have a widespread distribution in microbes (Fig. 6A). The presence of highly conserved residues and motifs in all sequences analyzed further suggests that HP0013-like proteins constitute a family that share a common function in NO detoxification. Although the current knowledge is insufficient to understand the relevance of these conserved regions, their presence in a protein that is able to detoxify NO suggests an unprecedented link between these motifs and NO metabolism. Therefore, we propose to rename HP0013 to NADPH-dependent NO reductase of *Helicobacter pylori* (NorH).

In conclusion, this work has disclosed a distinct system devoted to the *in vivo* protection of *H. pylori* and other prokaryotes, including several pathogens, against NO associated stress.

Materials and Methods

Reagents, strains, plasmids, and growth conditions

GSNO (31), spermine-NONOate, DPTA-NONOate, peroxynitrite (Cayman Chemical), and saturated NO (Gasin) solution (29) were used as NO donors.

All bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. Inactivation of *hp0013* in *H. pylori* 26695 and B128 was done by gene replacement with the nonpolar *aphA-3* kanamycin cassette as described in Supplementary Materials and Methods.

H. pylori was routinely cultivated microaerobically, at 37°C, on blood agar (BA) plates and in brain heart infusion (BHI) liquid medium with 10% (v/v) decomplemented fetal calf serum (Gibco-Invitrogen), supplemented with an antibiotic-antifungic mix (7) and when required with kanamycin (20 μg/ml), chloramphenicol (5 μg/ml). Liquid *H. pylori* cultures, inoculated from 24 h plates at OD_{600} $\sim$0.05, were incubated under microaerobic conditions.

Table 1. Bacterial Strains, Oligonucleotides, and Plasmids Used in This Work

Strain	*Description*	*Source*
Escherichia coli		
MC1061	F$^-$, *araD139*, Δ(*ara-leu*)7696, *galE*15, *galK*16, Δ(*lacX*74), *rpsl*, *hsdR2*, *mcrA*, *mcrB1*	Lab stock
XL2-Blue	F$^-$ *pro*AB *lac*IqZΔM15 *Tn*10 (Tet^r) *end*A1, *sup*E44, *thi*-1, *recA*1, *gyrA*96, *relA*1, *lac*	Lab stock
Bl21(DE3)Gold	F$^-$, *ompT*, *hsdS*($r_B^-m_B^-$) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte	Stratagene
LMS2710	K-12 ATCC23716 Δ*norV*ΩCm^R	(22)
Helicobacter pylori		
26695	Parental strain	(38)
B128	Parental strain	(20)
Δ*hp0013*::mini*Tn*3	26695 Δ*hp0013*::mini*Tn*3-Km, Km^R	(21)
Δ*hp0014*::mini*Tn*3	26695 Δ*hp0014*::mini*Tn*3-Km, Km^R	(21)
26695 Δ*hp0013*	26695 Δ*hp0013*ΩK2, nonpolar Km^R mutant	This work
B128 Δ*hp0013*	B128 Δ*hp0013*ΩK2, nonpolar Km^R mutant	This work
26695 Δ*rocF*	26695 Δ*hp1399*Ω*cat*, nonpolar Cm^R mutant	(7)

Oligonucleotide	*Description*	*Sequence (5′→3′)*
u5	deletion of *hp0013*	TACAAAGCGAATACAAGCCC
u3	deletion of *hp0013*	TTATTCCTCCTAGTTAGTCATCATAT GGCGACTAATTCTCC
d3	deletion of *hp0013*	TACCTGGAGGGAATAATGAAAGGG AGTTTTTGAGGGTTTTAGG
d5	deletion of *hp0013*	CCATAAACAAACCTGTCAATGG
hp13NdeI	expression of HP0013	TTAGTCGCATATGAAAAAGA
hp13XhoI	expression of poly(His)HP0013	CCCTCGAGAGCGAACAAATA
hp13EcoRI	expression of HP0013, without tag	AAAGAATTCCCCTAAAACCCTC

Plasmid	*Description*	*Source*
pUC18-K2	pUC18 carrying the nonpolar kanamycin *aphA-3* cassette between *Kpn*I and *Bam*HI sites	(30)
pILL2150	*E. coli* - *H. pylori* shuttle vector, for controlled low expression of genes in *H. pylori*	(4)
pILL2157	*E. coli* - *H. pylori* shuttle vector for controlled high expression of genes in *H. pylori*	(4)
pILL2150-HP0013	pILL2150 with *hp0013* cloned between *Nde*I and *Sac*I	This work
pILL2157-HP0013	pILL2157 with *hp0013* cloned between *Nde*I and *Sac*I	This work
pET23a-HP0013	pET23a with *hp0013* cloned between *Nde*I and *Xho*I, expression of HP0013 bearing a poly(His) tail at the C-terminus	This work
pET24b	*E. coli* expression vector	Novagene
pET24b-HP0013	pET24b with *hp0013* cloned between *Nde*I and *Eco*RI, expression of HP0013	This work
pME2337	pET24b-*norV*, expression of *E. coli* FDP NO reductase	(16)

FDP, flavodiiron proteins; NO, nitric oxide.

E. coli strains were grown on Luria Bertani medium (LB) and, when required with 30 μg/ml chloramphenicol, 100 μg/ml ampicillin and 30 μg/ml kanamycin.

The susceptibility of *H. pylori* 26695 and derived mutants to GSNO was analyzed by disk diffusion assays using cell suspensions prepared in BHI ($OD_{600}\sim2$), and spread on HP selective plates (BioGerm Laboratórios), which were incubated at 37°C for 48 h. The disks received 15 μl of GSNO 10 m*M*. Each strain was analyzed in two independent experiments, performed with duplicate plates.

For tests with NO donors, liquid cultures were treated, at OD_{600} of 0.05–0.1, with 200 μ*M* GSNO and peroxynitrite, and at $OD_{600}\sim0.5$ with 150 μ*M* DPTA-NONOate. The number of viable cells was monitored at intervals of ~4 h.

E. coli LMS2710 cells (22) were transformed with pET24b, pME2337, and pET24b-HP0013, obtained by *Nde*I/*Eco*RI cloning of the *hp0013* gene that was amplified with hp13NdeI and hp13EcoRI oligonucleotides. Cells were grown anaerobically in minimal salts medium, and at an $OD_{600}\sim0.3$, 30 μ*M* spermine-NONOate was added and colony forming unit (cfu) was analyzed after 1, 2, and 4 h.

For complementation in *H. pylori*, the *hp0013* gene was extracted *Nde*I/*Sac*I from pET24b-HP0013 and cloned into pILL2157 and pILL2150, rendering pILL2157-HP0013 and pILL2150-HP0013. Each vector was introduced by tri-parental conjugation into *H. pylori* B128 *hp0013*, using *E. coli* GC7(pRK2013) as mobilizer (19). *H. pylori* strains were grown and exposed to NO donors as just described, but isopropyl-

β-D-thiogalactoside (IPTG) 1 m*M* was added to the media to induce protein expression.

Production of recombinant HP0013 protein: enzymatic assays in cells and protein samples—phylogenetic analysis

Gene *hp0013* was polymerase chain reaction amplified with primers hp13NdeI and hp13XhoI, cloned *Nde*I/*Xho*I into pET23a (Novagen) to generate a protein with a C-terminal poly(His) tail fusion, and introduced in *E. coli* BL21(DE3)-Gold. Cells were grown in LB plus 0.4 m*M* $FeSO_4$. At $OD_{600} \sim 1$, IPTG (1 m*M*) was added, and cultures were grown for 6 h, at 20°C. After cell disruption and centrifugation, the soluble fraction was loaded into a Ni^{2+}-loaded Chelating Sepharose Fast Flow (GE Healthcare) equilibrated with Tris-HCl 20 m*M* pH 7.5 and 500 m*M* NaCl, and the protein was eluted with 500 m*M* imidazole, as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel.

H. pylori cell lysates from at least three independent cultures were prepared by incubation for 15 min with 0.1 mg/ml lysozyme and 0.02% sodium deoxycholate and used in enzymatic assays. The GSNO reductase activity was determined in cell lysates of *H. pylori* wt and Δ*hp0013* mutant after the combined consumption of NADPH and GSNO at 340 nm (ε_{340} [NADPH + GSNO] = 7.04 mM^{-1} cm^{-1}), considering a reaction stoichiometry of one mol of NADPH oxidized per 1 mol of GSNO reduced. Reactions contained 20 m*M* Tris-HCl pH 7.5, 0.2 m*M* NADPH, and cell lysates, and were initiated with 0.4 m*M* GSNO. The NADPH consumption of cell lysates (measured in blank reactions that did not receive GSNO) was subtracted.

NO reduction assays were performed anaerobically, in phosphate-buffered saline with 20 m*M* glucose, catalase 130 U/ml and glucose oxidase 17 U/ml (Sigma), 0.2 m*M* NADPH, and 4–6 μ*M* NO and were monitored both amperometrically and spectrophotometrically. The NO consumption was monitored amperometrically using an NO electrode (ISO-NOP) connected to an APOLLO-4000 Free Radical Analyzer (WPI). The NADPH (/NADH) coupled oxidation was monitored spectrophotometrically at 340 nm (ε(NAD(P)H) = 6.22 mM^{-1} cm^{-1}), subtracting the NAD(P)H consumption of each lysate in the absence of NO. The specific activity of HP0013 was assayed using 0.5 nmol of purified protein in each reaction. Two batches of purified protein were analyzed in triplicate.

Oxidized glutathione (GSSG) and peroxynitrite reductase activities are described in Supplementary Materials and Methods. Activities (25°C) are defined as unit (μmol substrate consumed/min) per milligram of total protein.

The search for homologs of *H. pylori* 26695 HP0013 was performed with protein-protein BLAST algorithm at NCBI-BLAST against the nonredundant protein sequences database. The alignment of the retrieved sequences and generation of the unrooted dendrogram (see Supplementary Materials and Methods) were performed using Clustal X 2 (28). Prediction of the secondary structure of HP0013 was done with PSIPRED v3.0 (6), and signal peptide prediction with SignalP 3.0 Server (3).

Macrophage and mouse colonization experiments

For the macrophage killing assays, murine macrophages RAW264.7 (ATCC Tib71) were seeded (5×10^5 cells per well), in 24-well plates and after 24 h, the medium was changed to Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 m*M* sodium pyruvate and 0.4 m*M* L-arginine. Bacterial suspensions were prepared in DMEM, using 24 h grown BA plates, and were added to the macrophages at a multiplicity of infection of 100. After 24 h of infection, bacterial survival was determined by serial diluting on BA plates the content of each well (further details in Supplementary Materials and Methods). The iNOS inhibitor L-NMMA from Sigma, 0.8 m*M* was used, and the nitrite concentration was determined in supernatants by the Griess method (18). For complementation studies, media was supplemented with IPTG (1 m*M*).

Five-week-old female wild-type C57BL/6J mice (Charles River, France) and iNOS-deficient C57BL/6J(iNOS$^{-/-}$) mice (Jackson Laboratories) were infected with wild-type *H. pylori* B128, B128 Δ*hp0013*, and co-infected with a 1:1 mixture of both strains by oral route with feeding needles (2×10^8 bacteria per mouse). After the indicated time, mice were sacrificed, their stomachs removed, homogenized, and plated to determine the bacterial load as described in Supplementary Materials and Methods. Stomach homogenates from co-infected mice were plated in duplicate, one plate to determine the total bacterial load (wt + mutant), and another with 20 μg/ml kanamycin to measure the survival of the *hp0013* mutant.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software). According to the data, statistical comparisons were assessed using one-way analysis of variance, followed by a Bonferroni multiple comparison test (for more than two conditions), or the Mann–Whitney *t* test (murine colonization data), with the significance threshold at $p < 0.05$ (95% confidence level).

Acknowledgments

This work was funded by Fundação para a Ciência e Tecnologia (FCT) through projects PTDC/SAU-MII/098086/2008 and PEst-OE/EQB/LA0004/2011. Marta C. Justino is a recipient of the FCT fellowship SFRH/BPD/43172/2008.

Author Disclosure Statement

No competing financial interests exist.

References

1. Angelo M, Hausladen A, Singel DJ, and Stamler JS. Interactions of NO with hemoglobin: from microbes to man. *Methods Enzymol* 436: 131–168, 2008.
2. Bender RA. The danger of annotation by analogy: most "*thiI*" genes play no role in thiamine biosynthesis. *J Bacteriol* 193: 4574–4575, 2011.
3. Bendtsen JD, Nielsen H, von Heijne G, and Brunak S. Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340: 783–795, 2004.
4. Boneca IG, Ecobichon C, Chaput C, Mathieu A, Guadagnini S, Prevost MC, Colland F, Labigne A, and de Reuse H. Development of inducible systems to engineer conditional mutants of essential genes of *Helicobacter pylori*. *Appl Environ Microbiol* 74: 2095–2102, 2008.
5. Bryk R, Griffin P, and Nathan C. Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* 407: 211–215, 2000.

6. Bryson K, McGuffin LJ, Marsden RL, Ward JJ, Sodhi JS, and Jones DT. Protein structure prediction servers at University College London. *Nucleic Acids Res* 33: W36–W38, 2005.
7. Bury-Mone S, Thiberge JM, Contreras M, Maitournam A, Labigne A, and De Reuse H. Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori*. *Mol Microbiol* 53: 623–638, 2004.
8. Bussiere FI, Chaturvedi R, Cheng Y, Gobert AP, Asim M, Blumberg DR, Xu H, Kim PY, Hacker A, Casero RA Jr., and Wilson KT. Spermine causes loss of innate immune response to *Helicobacter pylori* by inhibition of inducible nitric-oxide synthase translation. *J Biol Chem* 280: 2409–2412, 2005.
9. Cole SP, Kharitonov VF, and Guiney DG. Effect of nitric oxide on *Helicobacter pylori* morphology. *J Infect Dis* 180: 1713–1717, 1999.
10. de Reuse H and Bereswill S. Ten years after the first *Helicobacter pylori* genome: comparative and functional genomics provide new insights in the variability and adaptability of a persistent pathogen. *FEMS Immunol Med Microbiol* 50: 165–176, 2007.
11. Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* 2: 820–832, 2004.
12. Finel M. Does NADH play a central role in energy metabolism in *Helicobacter pylori*? *Trends Biochem Sci* 23: 412–413, 1998.
13. Fu S, Ramanujam KS, Wong A, Fantry GT, Drachenberg CB, James SP, Meltzer SJ, and Wilson KT. Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. *Gastroenterology* 116: 1319–1329, 1999.
14. Gobert AP, Cheng Y, Wang JY, Boucher JL, Iyer RK, Cederbaum SD, Casero RA Jr., Newton JC, and Wilson KT. *Helicobacter pylori* induces macrophage apoptosis by activation of arginase II. *J Immunol* 168: 4692–4700, 2002.
15. Gobert AP, McGee DJ, Akhtar M, Mendz GL, Newton JC, Cheng Y, Mobley HL, and Wilson KT. *Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc Natl Acad Sci U S A* 98: 13844–13849, 2001.
16. Gomes CM, Vicente JB, Wasserfallen A, and Teixeira M. Spectroscopic studies and characterization of a novel electron-transfer chain from *Escherichia coli* involving a flavorubredoxin and its flavoprotein reductase partner. *Biochemistry* 39: 16230–16237, 2000.
17. Goto T, Haruma K, Kitadai Y, Ito M, Yoshihara M, Sumii K, Hayakawa N, and Kajiyama G. Enhanced expression of inducible nitric oxide synthase and nitrotyrosine in gastric mucosa of gastric cancer patients. *Clin Cancer Res* 5: 1411–1415, 1999.
18. Granger DL, Taintor RR, Boockvar KS, and Hibbs JB Jr. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. *Methods Enzymol* 268: 142–151, 1996.
19. Heuermann D and Haas R. A stable shuttle vector system for efficient genetic complementation of *Helicobacter pylori* strains by transformation and conjugation. *Mol Gen Genet* 257: 519–528, 1998.
20. Israel DA, Salama N, Arnold CN, Moss SF, Ando T, Wirth HP, Tham KT, Camorlinga M, Blaser MJ, Falkow S, and Peek RM Jr. *Helicobacter pylori* strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. *J Clin Invest* 107: 611–620, 2001.
21. Jenks PJ, Chevalier C, Ecobichon C, and Labigne A. Identification of nonessential *Helicobacter pylori* genes using random mutagenesis and loop amplification. *Res Microbiol* 152: 725–734, 2001.
22. Justino MC, Vicente JB, Teixeira M, and Saraiva LM. New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. *J Biol Chem* 280: 2636–2643, 2005.
23. Kaakoush NO, Sterzenbach T, Miller WG, Suerbaum S, and Mendz GL. Identification of disulfide reductases in *Campylobacterales*: a bioinformatics investigation. *Antonie Van Leeuwenhoek* 92: 429–441, 2007.
24. Kleiger G and Eisenberg D. GXXXG and GXXXA motifs stabilize FAD and NAD(P)-binding Rossmann folds through C(alpha)-H... O hydrogen bonds and van der waals interactions. *J Mol Biol* 323: 69–76, 2002.
25. Kodama K, Sumii K, Kawano M, Kido T, Nojima K, Sumii M, Haruma K, Yoshihara M, and Chayama K. *Helicobacter pylori* infection increases serum nitrate and nitrite more prominently than serum pepsinogens. *Helicobacter* 7: 9–13, 2002.
26. Kranz RG, Richard-Fogal C, Taylor JS, and Frawley ER. Cytochrome *c* biogenesis: mechanisms for covalent modifications and trafficking of heme and for heme-iron redox control. *Microbiol Mol Biol Rev* 73: 510–528, 2009.
27. Kuwahara H, Miyamoto Y, Akaike T, Kubota T, Sawa T, Okamoto S, and Maeda H. *Helicobacter pylori* urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. *Infect Immun* 68: 4378–4383, 2000.
28. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, and Higgins DG. Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947–2948, 2007.
29. Lim MD, Lorkovic IM, and Ford PC. The preparation of anaerobic nitric oxide solutions for the study of heme model systems in aqueous and nonaqueous media: some consequences of NO x impurities. *Methods Enzymol* 396: 3–17, 2005.
30. Menard R, Sansonetti PJ, and Parsot C. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J Bacteriol* 175: 5899–5906, 1993.
31. Nobre LS, Goncalves VL, and Saraiva LM. Flavohemoglobin of *Staphylococcus aureus*. *Methods Enzymol* 436: 203–216, 2008.
32. Park AM, Nagata K, Sato EF, Tamura T, Shimono K, and Inoue M. Mechanism of strong resistance of *Helicobacter pylori* respiration to nitric oxide. *Arch Biochem Biophys* 411: 129–135, 2003.
33. Peek RM Jr., Fiske C, and Wilson KT. Role of innate immunity in *Helicobacter pylori*-induced gastric malignancy. *Physiol Rev* 90: 831–858, 2010.
34. Rhee SG, Chae HZ, and Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med* 38: 1543–1552, 2005.
35. Saraiva LM, Vicente JB, and Teixeira M. The role of the flavodiiron proteins in microbial nitric oxide detoxification. *Adv Microb Physiol* 49: 77–129, 2004.
36. Saraste M, Sibbald PR, and Wittinghofer A. The P-loop—a common motif in ATP- and GTP-binding proteins. *Trends Biochem Sci* 15: 430–434, 1990.
37. Tecder-Unal M, Can F, Demirbilek M, Karabay G, Tufan H, and Arslan H. The bactericidal and morphological effects of peroxynitrite on *Helicobacter pylori*. *Helicobacter* 13: 42–48, 2008.

38. Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weidman JM, Fujii C, Bowman C, Watthey L, Wallin E, Hayes WS, Borodovsky M, Karp PD, Smith HO, Fraser CM, and Venter JC. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 388: 539–547, 1997.

Address correspondence to:
Dr. Lígia M. Saraiva
Instituto de Tecnologia Química e Biológica
Universidade Nova de Lisboa
Av. da República (EAN)
Oeiras 2780-157
Portugal

E-mail: lst@itqb.unl.pt

Dr. Ivo G. Boneca
Group Biology and Genetics of the Bacterial Cell Wall
Institut Pasteur
26 rue du Dr. Roux
Paris 7015
France

E-mail: bonecai@pasteur.fr

Date of first submission to ARS Central, September 26, 2011; date of final revised submission, January 09, 2012; date of acceptance, January 10, 2012.

Abbreviations Used

ANOVA = analysis of variance
BA = blood agar
BHI = brain heart infusion
DHR = dihydrorhodamine 123
DMEM = Dulbecco's modified Eagle's medium
DPTA-NONOate = dipropylenetriamine-NONOate, NO donor
FDP = flavodiiron protein
GSNO = S-nitrosoglutathione, NO donor
GSSG = oxidized glutathione
HMP = flavohemoglobin
HP = *Helicobacter pylori*
iNOS = inducible nitric oxide synthase
IPTG = isopropyl-β-D-thiogalactoside
LB = Luria Bertani
L-NMMA = N^G-monomethyl-L-arginine, iNOS inhibitor
NO = nitric oxide
PCR = polymerase chain reaction
qNOR = respiratory membrane bound nitric oxide reductase
RNS = reactive nitrogen species
SD = standard deviation
SEM = standard error of the mean