

Helicobacter pylori arginase inhibits nitric oxide production by eukaryotic cells: A strategy for bacterial survival

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The antimicrobial effect of nitric oxide (NO) is an essential part of innate immunity. The vigorous host response to the human gastric pathogen *Helicobacter pylori* fails to eradicate the organism, despite up-regulation of inducible NO synthase (iNOS) in the gastric mucosa. Here we report that wild-type strains of *H. pylori* inhibit NO production by activated macrophages at physiologic concentrations of L-arginine, the common substrate for iNOS and arginase. Inactivation of the gene *rocF*, encoding constitutively expressed arginase in *H. pylori*, restored high-output NO production by macrophages. By using HPLC analysis, we show that L-arginine is effectively consumed in the culture medium by wild-type but not arginase-deficient *H. pylori*. The substantially higher levels of NO generated by macrophages cocultured with *rocF*-deficient *H. pylori* resulted in efficient killing of the bacteria, whereas wild-type *H. pylori* exhibited no loss of survival under these conditions. Killing of the arginase-deficient *H. pylori* was NO-dependent, because peritoneal macrophages from iNOS^{-/-} mice failed to affect the survival of the *rocF* mutant. Thus, bacterial arginase allows *H. pylori* to evade the immune response by down-regulating eukaryotic NO production.

Helicobacter pylori is a Gram-negative microaerophilic bacterium, which selectively colonizes the human stomach. Current prevalence of *H. pylori* is ≈40% of the population in the U.S. (1) and substantially higher in underdeveloped regions. *H. pylori* causes chronic gastritis, peptic ulcers, and gastric carcinoma and lymphoma, leading to its classification as a Class I carcinogen (2). Despite inciting substantial acute and chronic immune and inflammatory responses, *H. pylori* infection generally persists for the life of the host. Understanding how the bacterium evades the host response remains a critical issue in managing the public health burden of this infection.

Nitric oxide (NO) is a central component of innate immunity and an effective antimicrobial agent (3). This activity is especially marked for intracellular pathogens such as *Mycobacterium tuberculosis* (4) and *Leishmania major* (5), which are killed by an NO-dependent mechanism. Reactive nitrogen intermediates can also effectively kill extracellular parasites (6, 7) and bacteria such as *Escherichia coli* (8). Chemical sources of NO and peroxynitrite have a direct toxic effect on *H. pylori* (9, 10). However, the effect of cell-derived NO on *H. pylori* has not been investigated. The survival of *H. pylori*, despite marked induction of inducible NO synthase (iNOS) in macrophages (11) and gastric tissues (12), suggests that the bacterium has developed mechanisms to avoid NO-dependent killing.

Arginases are a primordial enzyme family, which are highly conserved across kingdoms (13). Mammalian arginases compete with NO synthases for the common substrate L-arginine (14), hydrolyzing the amino acid to urea and L-ornithine. Therefore, arginases can regulate cellular NO production (15, 16) and counteract the biological effects of NO (7, 17). *H. pylori* pos-

sesses the gene *rocF*, which encodes an arginase (18). Our previous work showed that *H. pylori* added to macrophages under culture conditions of high L-arginine availability resulted in abundant levels of NO production (11). Here we report the effect of *H. pylori* arginase on macrophage NO production under conditions of physiologic L-arginine concentration. Although wild-type (WT) *H. pylori* and strains deficient in *rocF* stimulate similar levels of iNOS mRNA expression, significantly greater levels of NO are released by macrophages stimulated with *rocF*-deficient strains. This finding was attributable to L-arginine consumption by the WT but not by the arginase-deficient bacteria. Loss of arginase activity results in marked NO-dependent killing of *H. pylori*, indicating that the bacterial arginase has evolved as a survival mechanism that may contribute to the ability of *H. pylori* to successfully colonize the human stomach for the life of the host. To our knowledge, our data constitute the first description of the modulation of eukaryotic NO production by a prokaryotic arginase.

Materials and Methods

Bacteria. *H. pylori* strains SS1 (19) and 26695 (20) were used. In these strains, the *rocF* gene was disrupted by insertion of an *aphA3* cassette, yielding mutants (*rocF::aphA3*) completely devoid of arginase activity (18). The *aphA3* cassette used to disrupt *rocF* is unlikely to exert any polar effect on other genes, because the upstream gene (*hp1398*) is in the opposite orientation to that of *rocF*, and the 5' end of the downstream gene (*hp1400*) is more than 500 bp beyond the 3' end of *rocF*. Additionally, *rocF* is followed by a predicted near-consensus rho-independent transcriptional terminator (CUUUUCAAAACCN₁₁GGUUGAA-AAAG), followed by an AU-rich region (18). Both WT and *rocF* mutants were passaged on *Brucella* agar plates containing 10% sheep blood or cultured in Mueller–Hinton broth with 3% FBS (Life Technologies, Grand Island, NY). For the current experiments, the passage number from the frozen stock was always identical for the WT and mutant strains. *H. pylori* strains were harvested from growth medium at exponential phase, washed twice, suspended in PBS, and used in the experiments. All cultures of bacteria were grown under microaerobic conditions.

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Abbreviations: iNOS, inducible NO synthase; WT, wild-type strains of *H. pylori*; moi, multiplicity of infection.

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Bacterial concentration was determined by spectrophotometry, using OD₆₀₀ as described (11).

To develop additional preparations of *H. pylori*, the following manipulations were performed. French press lysates of *H. pylori* were obtained by passage through a French pressure cell at 20,000 psi (1 psi = 6.89 kPa; ref. 11). Water extracts were obtained by vortexing 10⁹ bacteria in 1 ml of water for 5 min (21). Lysates and water extracts were subjected to centrifugation at 12,000 × *g* for 10 min, and supernatants were used for the assays. Bacteria were also boiled for 30 min in PBS.

For the *H. pylori* bactericidal studies, *H. pylori* colony-forming units were determined before and after cocultures by serial dilution and culture on blood agar plates.

Cells. The murine macrophage cell line RAW 264.7 was maintained in DMEM (Life Technologies) and supplemented with 10% FBS/100 units/ml penicillin/100 μg/ml streptomycin/1 mM sodium pyruvate/10 mM Hepes in a humidified 5% CO₂ atmosphere. For the experiments, RAW 264.7 cells were cocultured with intact or lysed *H. pylori*, with or without filter supports (0.2 μm pore size; Transwell, Nunc), at a multiplicity of infection (moi) of 10 to 100. To control L-arginine concentrations in the cocultures, serum-free DMEM, devoid of L-arginine and phenol red (Life Technologies) and supplemented with 1 mM sodium pyruvate/10 mM Hepes/4 g/l BSA (Sigma), was used. Macrophages (10⁶ cells per ml) were plated in 24-well plates, and medium was changed after 4 h. The iNOS-specific inhibitor, N-iminoethyl-L-lysine (100 μM; Alexis Biochemicals, San Diego), and various concentrations of L-arginine (Sigma) were then added with the bacteria. In some experiments, macrophages were first activated with 100 units/ml of IFN-γ for 24 h before addition of *H. pylori* for 6 h.

Peritoneal macrophages were isolated from C57BL/6 WT and iNOS^{-/-} mice (The Jackson Laboratory) as described (22) and cocultured with *H. pylori* in DMEM-BSA.

Measurement of NO Concentration. The concentration of the oxidized product of NO, NO₂⁻, was assessed by the Griess reaction, as described elsewhere (11). Concentration of total reactive nitrogen compounds (23) was measured by using an NO analyzer, NOA 280 (Sievers, Boulder, CO), a high-sensitivity detector based on a gas-phase chemiluminescent reaction between NO and ozone as described (24).

Measurement of L-Arginine Concentration. *H. pylori* SS1 WT and *rocF::aphA3* (10⁸/ml) were cultured for 24 h in the medium used for the coculture experiments, containing 0.4 mM L-arginine. Amino acid analysis was performed by HPLC on a Beckman Coulter 7300 Amino Acid Analyzer, using anion-exchange chromatography. This system utilizes four lithium chloride elution buffers of increasing pH and ionic strength, postcolumn reaction with ninhydrin, and dual-wavelength detection at 570 and 440 nm. Data capture and integration of peaks were performed by using Beckman Coulter System Gold.

Northern Blot Analysis. RAW 264.7 macrophages (10⁶/ml) were seeded in 75-cm² flasks and incubated with *H. pylori* SS1 WT or *rocF::aphA3* (10⁷/ml). After 6 h, medium was removed and cells were washed twice with PBS. Extraction of total RNA and Northern blot analysis were performed as described (11). For iNOS mRNA detection, a ³²P random primer-labeled full-length (3.9 kb) cDNA probe for murine iNOS (obtained from Q. W. Xie and C. Nathan, Cornell University Medical College, New York) was used. The concentration of mRNA was standardized by hybridization with a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; CLONTECH).

Results

Analysis of NO Generation by RAW 264.7 Macrophages in Response to WT and Arginase-Deficient *H. pylori*. Because *H. pylori* possesses the gene *rocF*, which encodes a bacterial arginase, we used isogenic *rocF* mutant strains to investigate the role of *H. pylori*-derived arginase on NO production by host cells. At an L-arginine concentration of 0.4 mM, *H. pylori* SS1 WT inhibited production of NO₂⁻ by RAW 264.7 macrophages (Fig. 1A). In contrast, arginase-deficient *H. pylori* stimulated concentration-dependent increases in NO₂⁻ levels. Differences in stimulated macrophage NO₂⁻ generation between the WT and *rocF::aphA3* strains occurred at L-arginine concentrations of 0.8 mM or lower, but not at higher levels (Fig. 1B), indicating that the competitive inhibition between bacterial arginase and macrophage iNOS can be overwhelmed by addition of excess substrate. Very similar results were obtained by using WT and *rocF::aphA3* mutant strains of *H. pylori* 26695 (not shown).

By using an NO analyzer (NOA 280), we verified that total NO metabolites were decreased by 3.1 ± 0.1-fold (*P* < 0.05, *n* = 6) with *H. pylori* SS1 WT compared with the arginase-deficient strain, in studies conducted at 0.4 mM L-arginine and an moi of 100.

Macrophages alone did not produce detectable levels of NO₂⁻, and the iNOS-selective inhibitor, N-iminoethyl-L-lysine, completely inhibited macrophage NO₂⁻ production in response to both mutant and WT *H. pylori* strains (not shown). The differences in NO₂⁻ generation by macrophages exposed to WT or *rocF::aphA3* are not attributable to variation in macrophage survival, as determined by cell counts using Trypan blue exclusion or by analysis of macrophage apoptosis using histone-DNA fragmentation ELISA.

Utilization of L-Arginine by WT and Arginase-Deficient Strains of *H. pylori*. HPLC analysis was used to assess L-arginine concentration in cell culture medium containing *H. pylori*. After 24 h, L-arginine concentration was dramatically decreased in the culture of WT *H. pylori*, but not in the culture of the *rocF* mutant strain (Fig. 1C). These data support the hypothesis that *H. pylori* arginase effectively decreases L-arginine in the medium, leading to the decrease of macrophage NO production by means of loss of substrate availability for iNOS.

Induction of iNOS mRNA Expression in Macrophages by *H. pylori* WT and *rocF::aphA3*. iNOS Northern blot analysis was performed to exclude a difference in macrophage activation by the WT vs. the *rocF::aphA3* mutant of *H. pylori*. Stimulation of macrophages by *H. pylori* SS1 WT and *rocF::aphA3* resulted in comparable increases in iNOS mRNA levels, compared with undetectable basal iNOS levels in unstimulated macrophages (Fig. 1D). Because we have demonstrated that iNOS mRNA levels correlate directly with iNOS enzymatic activity in response to *H. pylori* stimulation (11), these data indicate that bacterial arginase regulates macrophage NO production independently of effects on iNOS expression.

Effect of *H. pylori* Arginase on NO₂⁻ Production by IFN-γ-Activated Macrophages. To establish whether macrophage NO inhibition by bacterial arginase can occur regardless of the macrophage stimulation process, we pretreated RAW 264.7 cells with IFN-γ, followed by the addition of *H. pylori*. In a concentration-dependent manner, *H. pylori* SS1 WT inhibited macrophage NO₂⁻ production by the preactivated cells (Fig. 2). In contrast, the arginase-deficient *H. pylori rocF::aphA3* did not affect NO₂⁻ levels. These data strongly suggest that bacterial arginase acts to inhibit NO release by preformed iNOS.

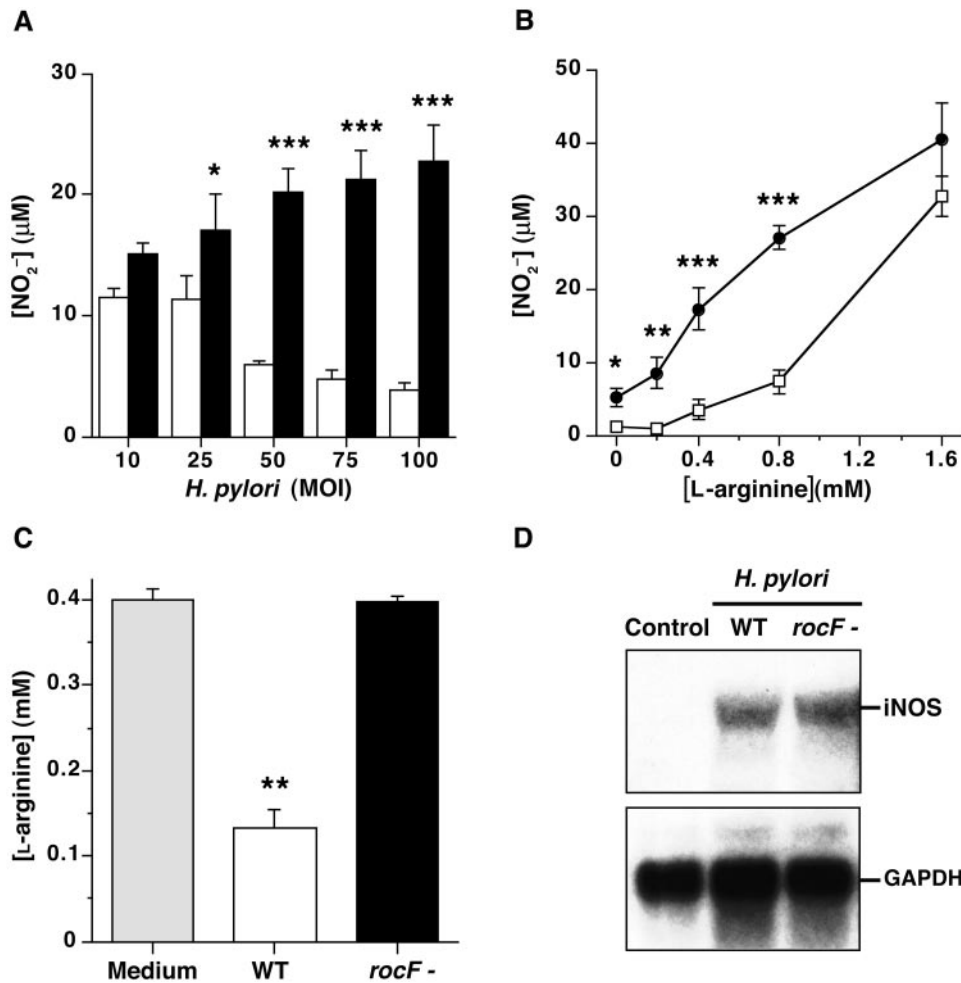


Fig. 1. *H. pylori* arginase inhibits NO₂⁻ generation by RAW 264.7 cells. (A) *H. pylori* SS1 WT (open bars) or *rocF::aphA3* (solid bars) was cocultured with macrophages in DMEM-BSA containing 0.4 mM L-arginine at an moi between 10 and 100. Production of NO₂⁻ was determined in supernatants after 24 h by the Griess reaction. Values are expressed as mean ± SEM of four experiments performed in duplicate. (B) *H. pylori* SS1 WT (□) or the *rocF::aphA3* (●) strains were added to RAW 264.7 macrophages at an moi of 100 and varying L-arginine concentrations. [NO₂⁻] was measured after 24-h incubation. Data represent mean ± SEM of three experiments performed in duplicate. For A and B, *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 for *H. pylori* WT vs. *rocF::aphA3* mutant strain by unpaired *t* test. (C) *H. pylori* SS1 WT (open bar) or *rocF::aphA3* (*rocF*⁻; solid bar) was incubated in the presence of 0.4 mM L-arginine. After 24 h, [L-arginine] was assessed by HPLC analysis. Data represent mean ± SEM of two separate experiments performed in triplicate. **, *P* < 0.01 compared with medium alone and *rocF::aphA3* strain by Student–Newman–Keuls multiple analysis comparison. (D) Northern blot analysis of RNA isolated from RAW 264.7 macrophages incubated with *H. pylori* SS1 WT and *rocF::aphA3* mutant (*rocF*⁻) at an moi of 100. After 6 h, total RNA from macrophages was harvested, and 10 µg was loaded per lane. Blots were hybridized sequentially with murine iNOS and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes.

Live *H. pylori*-Containing Arginase Is Required to Inhibit Macrophage NO₂⁻ Generation. To further assess the ability of *H. pylori* to prevent NO production, we stimulated macrophages with different *H. pylori* preparations. Addition of heat-killed bacteria, water extracts, or French press lysates of *H. pylori* resulted in loss of the inhibitory effect on NO production by the WT *H. pylori* (Table 1). Prevention of NO₂⁻ release occurred with intact *H. pylori* or with bacteria separated from the host cells by a filter support. These data suggest that live bacteria, but not contact with the macrophages, are required for functional inhibition of iNOS. Thus, viable bacteria efficiently consume L-arginine available in the extracellular environment, resulting in effective inhibition of NO production.

Regulation of Host Cell NO Production by Bacterial Arginase Is an *H. pylori* Survival Mechanism. The ability of NO to kill microbes makes it an important part of primordial host defense. Because we found that only live *H. pylori* can modulate L-arginine substrate availability for iNOS, we reasoned that bacterial

arginase contributes to the viability of *H. pylori*. We tested the effect of *H. pylori* arginase on NO-mediated killing of *H. pylori* by macrophages. After coculture with RAW 264.7 macrophages for 24 h, *H. pylori* SS1 WT did not have any loss of survival (Fig. 3A), whereas the arginase-deficient mutant strain had more than a 5-log-order decrease in colony-forming units (Fig. 3B). To demonstrate that killing of *H. pylori* was NO-dependent, we exposed the *rocF::aphA3* strain to resident peritoneal macrophages obtained from WT or iNOS^{-/-} mice. Although there was more than a 450-fold decrease in survival with the WT macrophages (Fig. 3C), there was no killing of the arginase-deficient *H. pylori* after coculture with the iNOS^{-/-} macrophages.

Discussion

iNOS-derived NO is a central effector molecule in the innate immune response to pathogens. A major finding of our current study is that extracellular *H. pylori* can be killed by NO released from activated macrophages. We show that *H. pylori* arginase competes with host cell iNOS for the common substrate L-

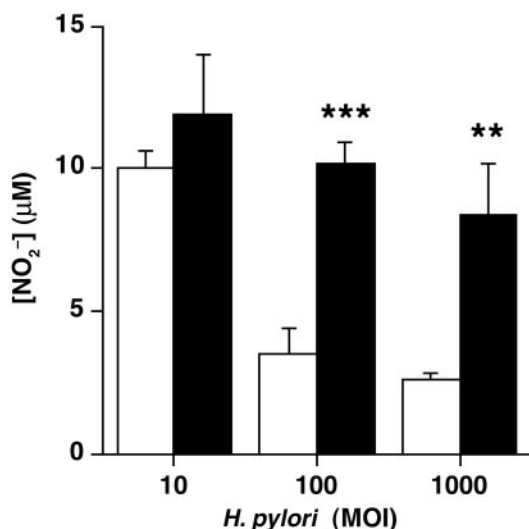


Fig. 2. *H. pylori* arginase inhibits NO₂⁻ production by preactivated macrophages. RAW 264.7 macrophages were stimulated with IFN-γ (100 units/ml) for 24 h. Cells were washed and *H. pylori* SS1 WT (open bars) or *rocF::aphA3* (solid bars) was added in medium containing 0.4 mM L-arginine at an moi of 10, 100, or 1,000. [NO₂⁻] was assessed in the supernatant after 6 h. Values are expressed as mean ± SEM of six experiments performed in duplicate. **, *P* < 0.01; ***, *P* < 0.001, for *H. pylori* SS1 *rocF::aphA3* compared with WT by unpaired *t* test.

arginine. Thus, WT *H. pylori* prevents NO production by host cells. Arginase-deficient *H. pylori* cannot limit macrophage NO production and are efficiently killed by macrophage-derived NO. These observations are independent of any effect on iNOS transcriptional regulation. We suggest that our results represent the description of a unique pathway of immune escape by extracellular-infecting bacteria. The protozoan, *Leishmania*, possesses arginase activity; however, this is an obligate intracellular parasite and the biological importance of the arginase is unrelated to modulation of NO synthesis (25). Mammalian arginases have the ability to modulate NO production in host-pathogen interactions as well, but our data now indicate that a prokaryotic arginase can have the same effect.

The inhibition of macrophage NO production by WT *H. pylori* occurred at physiologic concentrations of L-arginine (7) and at mois that mimic conditions documented *in vivo* (26), supporting the likelihood that the data we have observed have direct

Table 1. Effect of different preparations of *H. pylori* SS1 and SS1 *rocF::aphA3* on NO₂⁻ generation by RAW 264.7 macrophages

Preparation	[NO ₂ ⁻], µM		Fold increase	<i>P</i>
	SS1	SS1 <i>rocF::aphA3</i>		
Bacteria, untreated	2.5 ± 1.1	17.3 ± 1.8	6.8	<0.0001
Bacteria in Transwell	5.9 ± 1.9*	19.1 ± 1.5	3.2	0.0002
Boiled bacteria	26.3 ± 4.6	30 ± 4.8	1.1	NS
Water extract	19.6 ± 1.2	17 ± 2.1	1.1	NS
French press lysate	22.3 ± 3.3	21.3 ± 3.8	0.9	NS

RAW 264.7 macrophages were cocultured with intact *H. pylori* SS1 WT or *rocF::aphA3* or with the preparations of bacteria listed. *H. pylori* were also separated from macrophages by a filter support. Experiments were performed with an moi of 100, at 0.4 mM L-arginine in the medium. [NO₂⁻] in culture supernatants was measured after 24 h. Results are the mean ± SEM of three separate experiments performed in duplicate. Statistical comparison of values for SS1 WT vs. SS1 *rocF::aphA3* were performed by the unpaired *t* test.

*No significant difference vs. untreated bacteria by unpaired *t* test. NS, not significant.

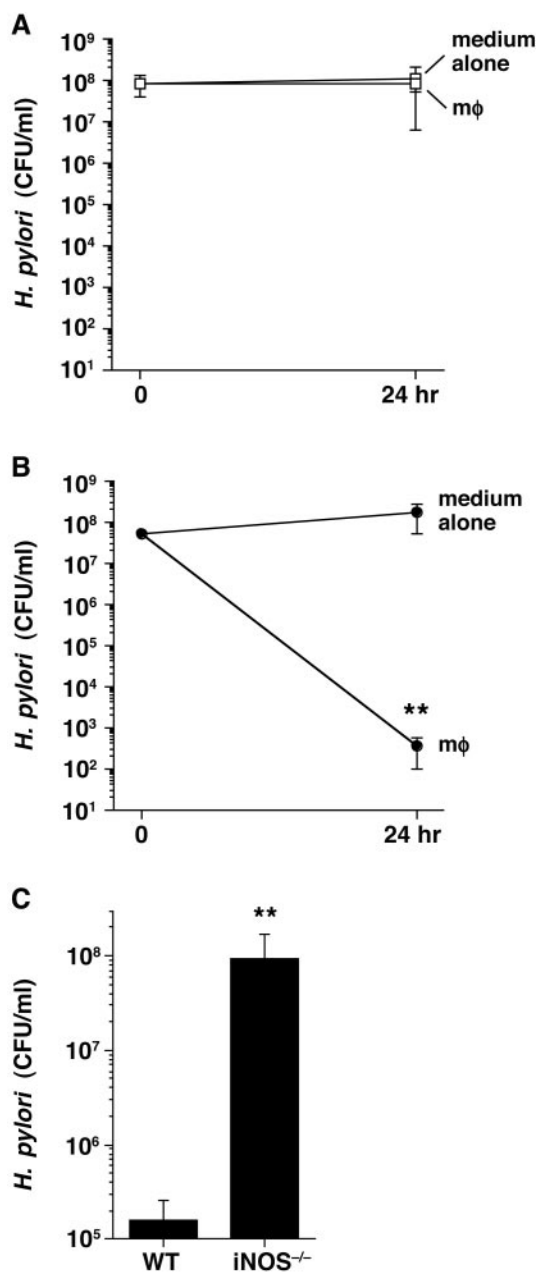


Fig. 3. *H. pylori* escapes the effect of macrophage-derived NO by its arginase activity. *H. pylori* WT (A) or *rocF::aphA3* (B) strains were cultured alone or above filter supports in the presence of macrophages (mφ) at an moi of 100 with 0.4 mM L-arginine in the medium. Colony-forming unit and NO₂⁻ concentrations were determined before and after 24 h of coculture. Results from four independent experiments performed in quadruplicate are represented by mean values ± SEM. **, *P* < 0.01 compared with medium alone by Mann-Whitney *U* test. NO₂⁻ levels were 5.8 ± 0.7 µM for SS1WT and 20.3 ± 1.2 µM for SS1 *rocF::aphA3*. (C) *H. pylori* SS1 *rocF::aphA3* were added above filter supports to peritoneal macrophages from WT or iNOS^{-/-} mice at an moi of 100 with 0.4 mM L-arginine in the medium. **, *P* < 0.01 by Mann-Whitney *U* test. NO₂⁻ levels were 16.3 ± 2.5 µM and 2.1 ± 0.1 µM for WT and iNOS^{-/-} peritoneal macrophages, respectively.

relevance *in vivo*. In fact, the SS1 *rocF* mutant strain used in the current study has attenuated ability to colonize mice (18). This finding raises the question of the role of iNOS-derived NO in *H. pylori* infection *in vivo*. Because we have previously shown that iNOS expression is up-regulated in human *H. pylori* gastritis (12), we have infected iNOS^{-/-} mice and WT C57BL/6 mice with WT

SS1. We found no significant difference in colonization levels or histologic injury scores, despite consistent expression of iNOS in the WT mouse stomach (K.T.W., unpublished observations). Based on the *in vitro* data presented here, we hypothesize that the lack of difference between iNOS-deficient and WT mice in *H. pylori* infection could be related to the ability of *H. pylori* arginase to modulate mucosal NO synthesis by iNOS in WT mice. With this unique insight, experiments have been initiated in our lab to compare the *rocF::aphA3* to WT SS1 strains in iNOS^{-/-} vs. WT mice, which are expected to determine the relative importance of host iNOS induction and *H. pylori* arginase *in vivo*. *H. pylori* are located in close proximity to the gastric epithelium in the mucous layer of the stomach, which results in iNOS expression in the epithelium and lamina propria (12). Thus, it is likely that the *H. pylori* arginase could directly modulate the iNOS-derived NO levels because of this close interaction between bacteria and the iNOS-expressing cells. There is also nonenzymatically formed NO in the gastric juice caused by acidification of salivary nitrate and nitrite, but this is unlikely to play any significant role in the *H. pylori* survival as a result of the location of the bacterium in the mucous layer.

Amino acid metabolism is essential for *H. pylori* growth (27). L-Arginine is not synthesized by *H. pylori* and therefore the bacterium must obtain this amino acid from extracellular sources (28). In fact, L-arginine is one of the amino acids completely consumed by *H. pylori* in broth culture (29, 30). By direct analysis of L-arginine utilization, we found that *H. pylori* consumed ≈70% of the L-arginine available in the cell culture medium over 24 h. The incomplete utilization of the L-arginine is most likely the result of the reduced metabolism of *H. pylori* in the minimal medium of serum-free DMEM. Nonetheless, our data indicate that L-arginine consumption by WT *H. pylori* strains explains the inhibition of host NO production. The lack of significant NO production by macrophage iNOS, despite the residual level of 0.1 mM L-arginine in the medium, may be caused by factors such as altered L-arginine uptake by the macrophages or metabolism of L-arginine by other biochemical pathways. The loss of the inhibitory effect on NO production when WT *H. pylori* are heat-killed is consistent with denaturation of arginase. Lack of effects on NO production by water extracts or French press lysates is consistent with the loss of active L-arginine transport (31) for subsequent metabolism by arginase or with the loss of

bacterial arginase after centrifugation of lysates, because arginase is associated with the cell envelope (32). Our direct analysis of L-arginine levels in media exposed to *H. pylori* indicates that viable bacteria efficiently consume L-arginine available in the extracellular environment, providing a means to decrease macrophage NO production.

Bacteria have developed various mechanisms to escape the effect of the immune system. For example, the intracellular pathogen, *Burkholderia cepacia*, which causes lung infection in cystic fibrosis patients, seems to enhance its own survival after macrophage apoptosis by inhibiting host NO production, but the mechanism has not been established (33). In response to NO, *E. coli* defends itself by up-regulating expression of *soxR*, a gene essential for the resistance to oxidative stress (34). *H. pylori* have similarly developed strategies for defense against oxidative stress, such as constitutively expressing superoxide dismutase and catalase, which detoxify superoxide anion and hydrogen peroxide, respectively (35), and alkylhydroperoxide reductase (36), which detoxifies peroxyxynitrite. Thus, *H. pylori* arginase is not the only potential strategy for enhancement of bacterial survival, but it has several unusual characteristics: Rather than defending against a toxic radical, it prevents NO production, and it does not require *de novo* gene transcription. Contrary to inducible arginases from other bacteria (37, 38) and yeast (39), *H. pylori* arginase is constitutively expressed (29). Its activity is present in all strains tested to date (18, 32), suggesting that arginase has evolved in *H. pylori* as an important enzyme.

Inhibition of host NO production by *H. pylori* not only protects the bacterium, but may also prevent mucosal injury. NO and peroxyxynitrite at high levels can kill eukaryotic cells and cause DNA damage (40, 41) and lipid peroxidation (42). Limiting host NO production by consuming L-arginine may be an evolutionary advantage in the successful adaptation of *H. pylori* to its ecologic niche of the gastric mucosa. A chronic infection for the life of the host would benefit from limitation of host mucosal destruction, which has been likened to a form of parasitism (43) in the case of *H. pylori*.

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