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Maintenance of Type IV Secretion Function During *Helicobacter pylori* Infection in Mice

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**ABSTRACT** The *Helicobacter pylori* type IV secretion system (T4SS) encoded on the cag pathogenicity island (cagPAI) secretes the CagA oncoprotein and other effectors into the gastric epithelium. During murine infection, T4SS function is lost in an immune-dependent manner, typically as a result of in-frame recombination in the middle repeat region of cagY, though single nucleotide polymorphisms (SNPs) in cagY or in other essential genes may also occur. Loss of T4SS function also occurs in gerbils, nonhuman primates, and humans, suggesting that it is biologically relevant and not simply an artifact of the murine model. Here, we sought to identify physiologically relevant conditions under which T4SS function is maintained in the murine model. We found that loss of *H. pylori* T4SS function in mice was blunted by systemic *Salmonella* coinfection and completely eliminated by dietary iron restriction. Both have epidemiologic parallels in humans, since *H. pylori* strains from individuals in developing countries, where iron deficiency and systemic infections are common, are also more often cagPAI than strains from developed countries. These results have implications for our fundamental understanding of the cagPAI and also provide experimental tools that permit the study of T4SS function in the murine model.

**IMPORTANCE** The type IV secretion system (T4SS) is the major *Helicobacter pylori* virulence factor, though its function is lost during murine infection. Loss of function also occurs in gerbils and in humans, suggesting that it is biologically relevant, but the conditions under which T4SS regulation occurs are unknown. Here, we found that systemic coinfection with *Salmonella* and iron deprivation each promote retention of T4SS function. These results improve our understanding of the cag pathogenicity island (cagPAI) and provide experimental tools that permit the study of T4SS function in the murine model.

**KEYWORDS** *Helicobacter pylori*, *Salmonella*, type IV secretion system, cagY, pathogenicity island

Infection with *Helicobacter pylori* causes chronic gastric inflammation that sometimes progresses to peptic ulcer disease or gastric adenocarcinoma, which is the third most common cause of cancer mortality worldwide (1). The virulence locus most strongly associated with disease is the type IV secretion system (T4SS) encoded on the cytotoxin-associated gene pathogenicity island (cagPAI). The *H. pylori* T4SS is essential for injection of several known bacterial effectors into host cells, including the CagA...
oncoprotein (2, 3), chromosomal DNA (4), peptidoglycan (5), and ADP-heptose, an intermediate metabolite of lipopolysaccharide biosynthesis (6–8). T4SS-dependent translocation of effectors activates the NF-κB inflammatory pathway and induces interleukin 8 (IL-8), a chemokine that recruits neutrophils to the site of infection and promotes chronic inflammation (9, 10).

Experimental *H. pylori* infection in mouse models usually results in loss of T4SS function, typically measured by the capacity of the recovered strains to induce IL-8 or translocate CagA (11). We recently found that this is most often due to recombination in *cagY*, an ortholog of *virB10* that encodes an essential protein found in all known T4SSs (12), though changes in other essential *cag* PAI genes also occur commonly (13). Recombination in *cagY* occurs in what has been called the middle repeat region (MRR)—a segment of *cagY* that has an extraordinary number of direct repeats (14)—leaving the open reading frame intact and the protein expressed but altering T4SS function (12). The DNA repeats encode a series of amino acid motifs, typically consisting of 30 to 40 residues (15), one or more of which is lost (or sometimes gained) by recombination events. This yields a potentially large number of variant *cagY* alleles, some functional and some not, though to date, it has not been possible to distinguish them based simply on sequence. Loss of T4SS function is driven by the host immune response (16) and is dependent on CD4+ T cells and interferon gamma (IFN-γ). The precise mechanism by which alterations in the MRR motif structure regulate T4SS function is unclear. However, the MRR is expressed on the bacterial surface, and motifs that confer function also enable the bacterial cell to bind β1 integrin (17). Since β1 integrin was previously shown to be essential for T4SS function (18, 19), these results suggested that alteration in CagY binding to β1 integrin might mediate changes in T4SS function. However, this conclusion remains speculative in view of recent studies showing that carcinoembryonic antigen-related cell adhesion molecule (CEACAM) receptors but not integrins are essential for CagA translocation (20).

CagY-mediated loss of T4SS function has been observed not only in mice but also in rhesus macaques (12) and in gerbils (21), though some gerbil-adapted strains have retained function (22). Therefore, it seems likely that loss of T4SS by changes in CagY is not simply an artifact observed in animal models but rather reflects an aspect of *H. pylori* biology that is also critical for chronic human infection. This is also supported by the fact that all known *cagY* sequences contain an MRR and that isogenic *H. pylori* strains with *cagY*-dependent differences in T4SS function have been recovered from chronically infected humans (16). Since the T4SS enhances gastritis and reduces bacterial load (23, 24), loss of T4SS function from the bacterial perspective may serve to reduce inflammation so as to maintain adequate colonization and transmission to a new host. Yet, most *cagPAI*+ *H. pylori* strains recovered from humans (25) and from naturally infected rhesus monkeys (26) have a functional T4SS, and so this is apparently the homeostatic state. But there must be circumstances in natural human infection during which loss of T4SS function is selected. One possibility is that concurrent systemic infection with another pathogen may induce inflammatory cytokines, which suppress *H. pylori* bacterial load in a non-antigen-specific manner, and selects for strains that can overcome this immune pressure by loss of T4SS function. Since the *cagPAI* is important for iron acquisition (27, 28), another possibility is that PAI function is maintained under iron-limiting conditions but not when iron is replete. Here, we address these hypotheses in the *H. pylori* mouse model by examining the effects of *Salmonella* coinfection and iron deprivation on PAI function. The results demonstrate that T4SS function is actually maintained by systemic coinfection with *Salmonella* and also by iron deprivation, both of which are relevant to natural human infection, particularly in developing countries where *H. pylori* is most common.

**RESULTS**

**Characterization of the *H. pylori-Salmonella* coinfection model.** The *Salmonella enterica* serovar Typhimurium challenge model with live-attenuated strain BRD509 was
previously described (29). Intravenous inoculation is followed rapidly by high bacterial burden in the spleen and expansion of IFN-γ CD4+ T cells, which peak 1 to 2 weeks later and largely dissipate by 6 to 8 weeks. C57BL/6 mice were orally gavaged with *H. pylori* PMSS1, injected intravenously with *Salmonella* Typhimurium (St) 1 week later, and sacrificed (Sac) 3, 5, or 8 weeks after *H. pylori* infection. (B) There was robust colonization of the spleen with *Salmonella*, which decreased over the course of infection. *Salmonella* was also present initially in the stomach at much lower quantities but was undetectable by 8 weeks. (C) Serum IFN-γ levels were high 3 weeks after *Salmonella* infection and declined rapidly. Data represent the means ± SEMs from 4 to 8 mice at each time point.

FIG 1 Characterization of the *H. pylori*-Salmonella coinfection model. (A) Schematic time frame of the *H. pylori*-Salmonella coinfection model. Mice were orally gavaged with *H. pylori* PMSS1 (Hp), infected intravenously with *Salmonella* Typhimurium (St) 1 week later, and sacrificed (Sac) 3, 5, or 8 weeks after *H. pylori* infection. (B) There was robust colonization of the spleen with *Salmonella*, which decreased over the course of the 8-week experiment (Fig. 1B). *Salmonella* was also recovered from gastric tissue, though in much smaller numbers. Serum IFN-γ detected by enzyme-linked immunosorbent assay (ELISA) was markedly elevated 3 weeks after *Salmonella* challenge in mice infected with *H. pylori* and declined rapidly (Fig. 1C). Eight weeks p.i., when *H. pylori* colonization and T4SS were characterized, IFN-γ levels were low, and mice had largely recovered from infection with *Salmonella*, which was no longer detectable in the stomach.

Salmonella coinfection decreases *H. pylori* colonization and enhances local and systemic inflammation. *H. pylori* colonization was assessed 8 weeks p.i. (7 weeks after *Salmonella* challenge), when cagY recombination and loss of T4SS function become apparent. Mice challenged with wild-type (WT) *H. pylori* harbored $10^5$ CFU/g of gastric tissue, which was approximately 10-fold lower in mice that also received S. Typhimurium (Fig. 2A). Colonization with *H. pylori* SS1cagY or *H. pylori* ΔcagE, which have a nonfunctional T4SS, was greater than with WT *H. pylori* but showed a similar decrease when coinfected with *Salmonella* (Fig. 2B). Gastric inflammation was induced by *H. pylori* infection and was somewhat increased in mice coinfected with S. Typhimurium (Fig. 3A). Since IFN-γ is induced by *Salmonella* challenge (Fig. 1) and is important for control of *H. pylori* (16), we
also examined IFN-γ levels in serum and gastric tissue 8 weeks p.i. The results showed that *Salmonella* coinfection increased IFN-γ expression in gastric tissue (Fig. 3B) and in serum (Fig. 3C) compared to that with *H. pylori* alone, though only the latter was statistically significant. Together, these results show that coinfection with *Salmonella* induces a systemic and local inflammatory response that is associated with reduced *H. pylori* colonization, independent of T4SS function.

**Gastric overexpression of IFN-γ is sufficient to decrease *H. pylori* colonization.**

To further characterize the role of IFN-γ in controlling *H. pylori* colonization, we challenged heterozygous mice overexpressing mouse IFN-γ under the control of the stomach-specific H+/K+ ATPase β promoter (tgIFN-γ). Gastric IFN-γ transcript levels were increased by *H. pylori* infection and were markedly greater in tgIFN-γ than in WT mice (Fig. 4A). Gastric inflammation was also increased in tgIFN-γ compared to that in WT mice, both uninfected and 4 weeks p.i. (Fig. 4B), and was accompanied by decreased *H. pylori* bacterial load at 4 and at 8 weeks p.i., when most animals had cleared the infection (Fig. 4C). Functional T cells are required to control *H. pylori* infection (16), and they are also the major source of IFN-γ. To determine if IFN-γ is sufficient to reduce *H. pylori* colonization in the absence of T cells, we compared infection in TCR-/- mice and TCR+/-mice bearing the IFN-γ transgene. As in mice with functional T cells (Fig. 4A), IFN-γ expression was increased in T cell receptor-deficient (TCR-/-) mice expressing the IFN-γ transgene (see Fig. S1A in the supplemental material), while *H. pylori* colonization was decreased (Fig. S1B). Similar to previous results (16), *cagY* recombination

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**FIG 2** *Salmonella* coinfection decreases *H. pylori* colonization. (A) Colonization of WT *H. pylori* in the stomach was decreased 8 weeks p.i. in *Salmonella*-coinfected animals (+St) compared to that in animals with *H. pylori* infection alone (−St). (B) Colonization with *H. pylori* SS1cagY (PMSS1 with cagY from SS1) and *H. pylori* ΔcagE, which have defective T4SSs, was also decreased when mice were coinfected with *Salmonella*. Each data point represents one mouse. Bars indicate means ± SEMs. *, P < 0.05; ***, P < 0.005.

**FIG 3** *Salmonella* coinfection enhances local and systemic inflammation. Gastric inflammation (mean percent fields containing neutrophils, mononuclear cells, or metaplasia) (A) and IFN-γ (B) were induced by *H. pylori* infection alone and increased slightly in coinfections with *Salmonella*. (C) Infection with *Salmonella* markedly increased serum IFN-γ compared to that with *H. pylori* alone or in uninfected mice. Data are from mice 8 weeks after *H. pylori* infection or at an equivalent age for uninfected mice. Each data point represents one mouse. Bars indicate means ± SEMs. ***, P < 0.01; ***, P < 0.005.
was eliminated in TCR⁻/⁻ mice but increased somewhat by overexpression of IFN-γ, even in the absence of functional T cells (Fig. S1C). These results suggest that reduction in H. pylori colonization in the Salmonella coinfection model may be explained, at least in part, by increases in IFN-γ-mediated inflammation.

Salmonella coinfection promotes retention of H. pylori T4SS function. We previously found that increased gastritis in IL-10⁻/⁻ mice is associated with decreased H. pylori colonization (16), similar to our findings here in tgIFN-γ mice. However, some IL-10⁻/⁻ mice were colonized at levels similar to those of WT mice, and H. pylori recovered from them typically recombined cagY, suggesting that loss of T4SS function permits increased colonization in the face of a robust immune response. Consistent with this observation, we also found that overexpression of IFN-γ caused increased cagY recombination (Fig. 4D), even in the absence of functional T cells (Fig. S1C). Since Salmonella coinfection enhances the systemic and local inflammatory responses and decreases H. pylori colonization, we hypothesized that it too might select for cagY-mediated loss of T4SS function and enable H. pylori to partially escape increased immune pressure from IFN-γ and other inflammatory cytokines. However, we observed the opposite result. cagY recombination was reduced (Fig. 5A) and T4SS function was increased (Fig. 5B) in H. pylori colonies recovered from coinfected mice compared to those in mice infected with H. pylori alone. H. pylori colonization was inversely correlated with T4SS function in coinfected mice (Fig. 5C) but not in mice infected with H. pylori alone, where colonization was overall greater and less variable (Fig. 5D).

H. pylori T4SS function is retained in iron-deficient mice. In vitro experiments suggest that CagA translocation via the T4SS serves to increase iron acquisition from host cells (28). These results are consistent with in vivo studies demonstrating that deletion of cagA impairs H. pylori colonization in iron-deficient, but not iron-replete,
Moreover, *H. pylori* strains recovered from iron-deficient gerbils, or grown in vitro under iron-decient conditions, demonstrate greater numbers of T4SS pili and show enhanced T4SS function (30). Since *Salmonella* induces anemia (31) and inflammation-mediated iron sequestration (32), we hypothesized that *Salmonella* coinfection might select for a functional T4SS by competing with *H. pylori* for iron. To first test the effects of anemia on colonization and T4SS function, we gavaged *H. pylori* into EPO2/2 mice bearing a homozygous disruption in the 5' untranslated region of the erythropoietin gene (Epo-Tagh), which reduces whole-body erythropoietin expression (33). EPO2/2 mice are severely anemic, with a hematocrit level approximately half that of WT mice (see Fig. S2A), though they may not be iron deficient. Hematocrit was further reduced by *H. pylori* infection in both WT and EPO2/2 mice, but *H. pylori* colonization and T4SS function were unaffected (Fig. S2B and C).

Anemia can occur with or without iron deficiency. To test specifically for the role of iron deficiency on *H. pylori* colonization and T4SS function, we compared *H. pylori* infections in mice fed for 5 weeks with an iron-deficient diet (2 to 6 ppm iron) to infections in mice fed a standard diet (200 ppm iron). *H. pylori* infection was followed by a decrease in food intake, which was restored in mice fed an iron-replete diet but not in those fed an iron-deficient diet (see Fig. S3A). The iron-deficient mice also weighed slightly less than control mice at the end of the experiment (Fig. S3B). Serum iron levels were highly variable in mice fed an iron-deficient diet, though anemia was severe and uniform (Fig. S3C and D). Similar to anemia in EPO−/− mice, iron deficiency anemia did not affect *H. pylori* colonization (Fig. 6A). However, the effect on T4SS function was dramatic, with no *cagY* recombination and no loss of IL-8 induction in iron-deficient mice 8 weeks p.i. (Fig. 6B and C). Gastric IFN-γ levels were also increased in *H. pylori*-infected iron-deficient mice, likely as a result of the increased T4SS activity (Fig. 6D).

![FIG 5](http://mbio.asm.org/) *Salmonella* coinfection enhances *H. pylori* T4SS function. (A) Fewer *H. pylori* colonies (3 to 6 per mouse) from coinfect ed mice showed recombination of *cagY* by RFLP analysis (Fisher's exact test, *P* < 0.05). (B) T4SS function (IL-8 induction) was greater for *H. pylori* isolated from *Salmonella*-coinfected mice than from mice infected with *H. pylori* alone. Each data point represents a sweep of *H. pylori* colonies isolated from one mouse. In *Salmonella*-coinfected mice, the colonization level of *H. pylori* correlated inversely with the capacity to induce IL-8 in AGS cells (C), while this was not the case with *H. pylori* alone (D). Bars indicate means ± SEMs. *P* < 0.05.
Role of iron deficiency in *Salmonella* coinfection. Since iron deficiency maintains T4SS function in mice, we hypothesized that retention of T4SS function during *Salmonella* coinfection may be a result of iron starvation. To determine if *Salmonella* coinfection induces iron deficiency, we analyzed gastric expression of lipocalin-2, hepcidin, and fur, which are known to be regulated by iron and therefore serve as a functional readout of iron deficiency. *Salmonella* induces lipocalin-2 in the gut, which binds siderophores from *Enterobacteriaceae* and enables *Salmonella* to overcome colonization resistance (32). Although *Salmonella* coinfection increased gastric lipocalin-2 expression somewhat more than *H. pylori* alone (Fig. 7A), it seems unlikely that this is relevant for iron sequestration from *H. pylori* in the stomach, since *H. pylori* is not known to produce siderophores. Hepcidin is also upregulated in *Salmonella* infection, causing iron retention in macrophages (34). We confirmed previous observations (35) that *H. pylori* decreases hepcidin expression in mice, but this was unaffected by *Salmonella* coinfection (Fig. 7A). Finally, we investigated expression of the *H. pylori* ferric uptake regulator, *fur*, which is activated by iron restriction (36). Since we could not readily detect low levels of *H. pylori* gene expression from infected gastric tissue, we examined *fur* expression in *H. pylori* cocultured with AGS cells to mimic interactions with the gastric epithelia. Low-passage-number *H. pylori* isolated from iron-deficient mice showed greater expression of *fur* than *H. pylori* from iron-replete mice, which validated that the assay served as a bacterial readout of iron deprivation (Fig. 7B). However, *fur* expression in *H. pylori* was unaffected by coinfection with *Salmonella* (Fig. 7C). Together, these results suggest that neither host nor bacterial expression
support the hypothesis that Salmonella coinfection in mice limits iron availability for H. pylori.

**DISCUSSION**

After the initial demonstration in mice (11), loss of T4SS function has been demonstrated in gerbils (21), rhesus macaques (12), and humans (16, 37), suggesting that modulation of T4SS function is an important feature of the biology of H. pylori. The mechanism is typically in-frame recombination in the middle repeat region of cagY, which encodes an essential T4SS protein, though single nucleotide polymorphisms (SNPs) in cagY or in other genes that are essential for T4SS function may also occur (13). While we know how T4SS function is lost during experimental infection—or even gained (12)—we do not know why. The T4SS is typically functional during chronic infection in humans (38, 39) and in rhesus monkeys (26), unless essential genes are absent or present as pseudogenes. Yet, the extraordinary repetitive genetic structure of cagY and the demonstration that recombination in these repeat regions can alter T4SS function strongly suggest that there must be conditions under which T4SS-dependent inflammation is advantageous for the bacterium and others under which it is...
not. We know from studies with genetically modified mice that cagY recombination does not occur in the absence of CD4 T cells expressing IFN-γ, and so loss of T4SS function is immune sensitive (16). But these are not physiological experiments. Here, we sought to use the murine model to identify conditions relevant to human infection under which H. pylori modulates T4SS function.

Just as loss of T4SS function and cagY recombination do not occur in immunodeficient mice, they occur more commonly when the immune response is increased in IL-10 knockout mice, which have more severe gastritis and lower bacterial load when challenged with H. pylori (16). Since deletion of the cagPAI reduces inflammation and increases colonization (23, 24), loss of T4SS function may serve to maintain colonization in the face of increased inflammation, such as might occur during a severe, transient systemic infection. The notion that one infection can alter the outcome of another has been repeatedly demonstrated and is thought to occur via enhancement of nonspecific innate immunity (40–43). To test this hypothesis, we coinfect mice with H. pylori and Salmonella, predicting that, like in IL-10 knockout mice, we would find increased inflammation, lower bacterial load, and reduced T4SS function compared to that in mice infected with H. pylori alone. As expected, Salmonella coinfection decreased H. pylori colonization and was accompanied by increased gastritis as well as local and systemic levels of IFN-γ. The importance of IFN-γ was supported by the observation that its overexpression was itself sufficient to increase gastritis and reduce H. pylori colonization (Fig. 4). Similar to that in IL-10 knockout mouse (16), H. pylori colonization in coinfect mice was inversely correlated with T4SS function, which we initially interpreted as consistent with immune escape leading to increased bacterial load.

However, in contrast to our prediction, T4SS function was increased in Salmonella-coinfect mice, and cagY recombination was reduced (Fig. 5). This suggested the possibility that Salmonella coinfection selected strains with a functional T4SS, which would be expected to be associated with more inflammation and therefore a lower bacterial load. Decreased H. pylori colonization during coinfection might then be a result of increased inflammation, not just from Salmonella infection but also from retention of T4SS function. Since the T4SS enables H. pylori to acquire iron from the host (28) and systemic infection causes iron sequestration (44), we asked whether iron deficiency might cause retention of T4SS function and explain why it is enhanced in Salmonella coinfection. While anemia per se had no effect, anemia induced by dietary iron restriction completely eliminated cagY recombination and loss of T4SS function (Fig. 6).

Together, these studies have identified two physiologically relevant variables that affect T4SS function in the murine model, Salmonella coinfection and especially dietary iron restriction, which completely eliminated loss of T4SS function. Both have epidemiologic parallels in humans, since H. pylori strains from individuals in developing countries, where iron deficiency and systemic infections are common, are also more often cagPAI− than strains from developed countries. However, there are important caveats to our findings. For example, while the results of Salmonella coinfection prompted the iron restriction studies and both promote retention of T4SS function, it appears that they are not mechanistically linked, though we did not directly measure gastric iron levels. We currently do not have an explanation for why H. pylori in coinfect mice more often retains T4SS function. While iron deficiency maintains T4SS function and increases gastric IFN-γ, it does not affect colonization, which might be expected to decrease. It seems likely that the effects of Salmonella coinfection, and perhaps iron restriction, are pleomorphic and will require additional studies to understand their impact on T4SS function. One approach to separate the effects of Salmonella infection from the inflammation it causes might be to pharmacologically induce innate immunity, for example, lipopolysaccharide (LPS) administration. Nonetheless, here we have identified two physiological variable factors relevant to human infection that affect T4SS function. The results have implications for our fundamental understanding
MATERIALS AND METHODS

Ethics statement. Experiments were carried out at the University of California, Davis, under protocols approved by the U.C. Davis Institutional Animal Care and Use Committee, which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with NIH guidelines, the Animal Welfare Act, and U. S. federal law.

Bacterial culture. H. pylori PMSS1 was cultured on brucella agar (BBL/Becton, Dickinson, Sparks, MD) supplemented with 5% heat-inactivated newborn calf serum (NCS; Invitrogen, Carlsbad, CA), ABPN antibiotics (ampicillin B, 20 μg/ml; bacitracin, 200 μg/ml; polymixin B, 3.3 μg/ml; nalidixic acid, 10.7 μg/ml; vancomycin, 100 μg/ml), and selective antibiotics (kanamycin, 25 μg/ml), or chloramphenicol, 5 μg/ml, where appropriate) (all antibiotics from Sigma). Prior to experimental mouse challenge, H. pylori was cultured overnight in brucella broth supplemented with 5% NCS and TVPA antibiotics (trimethoprim, 5 mg/liter; vancomycin, 10 mg/liter; polymixin B, 2.5 IU/liter; amphotericin B, 2.5 mg/liter). Cultures were incubated at 37°C under microaerophilic conditions at 5% CO2 generated by an Anoxomat (Advanced Instruments, Norwood, MA). Construction of H. pylori PMSS1 ΔcagE and PMSS1 ΔcagY replaced with cagY from either PMSS1 (H. pylori PMSS1 cagY) or S11 (H. pylori S11 cagY) was previously described (16, 23). Salmonella enterica serovar Typhimurium BRD509 (strain SL1344ΔaroA mutant) was grown overnight in LB broth without shaking at 37°C prior to experimental challenge and enumerated by culture on MacConkey agar plates.

Animals. Female C57BL/6J WT and TCR β/β−/− mice were purchased from the Jackson Laboratory (Sacramento, CA). A male mouse with an extra copy of the mouse IFN-γ gene under the control of the H/K ATPase β promoter (H/K-IFN-γ line 944 mice) was provided by Andrzej Dlugosz (45). These tgIFN-γ mice were bred with WT and TCR−/− mice to obtain heterozygous H/K-IFN-γ mice and H/K-IFN-γ−/− TCR−/− mice as well as littermate controls without the H/K-IFN-γ gene. Heterozygous erythropoietin knockout mice obtained from Nicolas Voituron (46) were bred in-house to obtain EPO−/− mice and littermate controls. Mice were housed in microisolator cages and provided with irradiated food and autoclaved water ad libitum. Iron deficiency was induced by providing mice with food containing traces of iron in the range of 2 to 6 ppm (TD 10210; Envigo Teklad Diets, Madison, WI) starting from 3 weeks of age. Control mice were given an equivalent food but with 200 ppm iron (TD 150282, Envigo Teklad Diets). Food intake for each cage of 4 mice was monitored, and iron-deficient and -replete mice were weighed before and after H. pylori infection.

H. pylori and Salmonella challenge. At 8 to 9 weeks of age, mice were challenged with 1 × 105 CFU of H. pylori suspended in 0.25 ml of brucella broth administered by oral gavage with a ball-end feeding needle. For coinfection experiments, mice were infected with Salmonella 1 week after H. pylori challenge by intravenous (i.v.) injection in the lateral tail vein with 5 × 107 CFU of bacteria diluted in 0.2 ml phosphate-buffered saline (PBS). Bacterial concentrations were estimated by optical density at 600 nm and confirmed by plating serial dilutions. At the endpoint, mice were euthanized with an overdose of pentobarbital sodium injection (50 mg/ml intraperitoneally [i.p.]). Blood was collected in microcapillary tubes for hematocrit measurement and into serum separator tubes (BD Microtainer). The forestomach was minced in microisolator cages and provided with irradiated food and autoclaved water ad libitum. Iron deficiency was induced by providing mice with food containing traces of iron in the range of 2 to 6 ppm (TD 10210; Envigo Teklad Diets, Madison, WI) starting from 3 weeks of age. Control mice were given an equivalent food but with 200 ppm iron (TD 150282, Envigo Teklad Diets). Food intake for each cage of 4 mice was monitored, and iron-deficient and -replete mice were weighed before and after H. pylori infection.

Histology. A quarter of the stomach was fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin. The percentage of fields containing neutrophil infiltration (polymorphonuclear leukocytes), gastritis (mononuclear cells), and metaplasia was identified by a veterinary pathologist blinded to experimental condition, using a scoring system previously validated in mice (47). The results for the three histological criteria were averaged and defined as percent gastric inflammation.

Gene expression and iron analysis. Serum IFN-γ levels were analyzed with the mouse IFN-γ uncoated ELISA kit according to the manufacturer’s protocol (Invitrogen). Murine gene expression of IFN-γ, hepcidin, and lipocalin-2 was analyzed from gastric tissue homogenized in TRIzol reagent (Ambion). Bacterial expression of fur was analyzed from sweeps isolated from gastric tissue and cocultured for 20 h with AGS cells as described below. RNA was purified by phenol-chloroform phase separation as described by the manufacturer or by the Direct-zol RNA miniprep kit (Zymogen). RNA was transcribed to cDNA by Superscript III (Qiagen) and added to quantitative PCR (qPCR) mixtures with TB green premix Ex Taq (Takara) using primers shown in Table S1 in the supplemental material. Gene expression was normalized to murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or bacterial 16S rRNA expression. Amplification was performed using a QuantStudio 6 Flex real-time PCR system (Applied Biosystems). Total serum iron was analyzed on a chemistry analyzer by the Comparative Pathology Laboratory at UC Davis.

IL-8 ELISA. IL-8 was measured as described previously (48). Briefly, human AGS gastric adenocarcinoma cells (ATCC, Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 5% CO2 and 37°C.
were seeded in six-well plates at a density of approximately 5 × 10^5 cells/cm² with 1.8 ml antibiotic-free RPMI medium-10% fetal bovine serum, incubated overnight, and then cocultured with H. pylori sweeps from an individual mouse diluted in 200 μl brucella broth at a multiplicity of infection (MOI) of 100:1. Supernatants were harvested after 20 to 22 h of culture (37°C, 5% CO2) and diluted 1:8 prior to IL-8 assay by ELISA (Invitrogen) performed according to the manufacturer’s protocol. WT H. pylori PMSS1 and the isogenic cagY deletion mutant were included on every plate as positive and negative controls, respectively. IL-8 expression was normalized to that for WT PMSS1.

**cagY PCR restriction fragment length polymorphism.** cagY genotyping was performed on isolated single colonies by PCR-restriction fragment length polymorphism (RFLP) essentially as previously described (12). cagY was amplified with Herculase II fusion DNA polymerase (Agilent Technologies), digested with Ddel and BfuCI or Sau3AI restriction enzymes (New England BioLabs, Ipswich, MA), visualized by agarose gel electrophoresis, and compared to that for WT H. pylori PMSS1. For convenience, cagY recombination was defined as a change in PCR-RFLP pattern compared to that for the WT, though we did not formally measure recombination and changes could occur by other mechanisms.

**Statistics.** All statistical analyses were performed using GraphPad Prism 7.00 (GraphPad Software, San Diego, CA). Multiple groups were compared using analysis of variance (ANOVA) with Tukey’s or Bonferroni’s post hoc tests. Differences in H. pylori colonization (CFU/g) and IL-8 expression between two groups were analyzed using the Mann-Whitney test. Proportions of samples with changed cagY were compared between groups using the Fisher’s exact test. Correlation between IL-8 expression and H. pylori CFU was analyzed by linear regression. Data are reported as means ± standard errors of the means (SEMs), and a P value of <0.05 was considered statistically significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available only online.

**FIG S1** PDF file, 0.1 MB

**FIG S2** PDF file, 0.1 MB

**FIG S3** PDF file, 0.1 MB

**TABLE S1** DOCX file, 0.1 MB

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**REFERENCES**

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68:394–424. https://doi.org/10.3322/caac.21492.

2. Odenbreit S, PulS J, Sedimaier B, Gerland E, Fischer W, Haas R. 2000. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. Science 287:1497–1500. https://doi.org/10.1126/science.287.5477.1497.

3. Ohnishi N, Yuasa H, Tanaka S, Sawa H, Miura M, Matsui A, Higashi H, MusashI M, Iwabuchi K, Suzuki M, Yamada G, Azuma T, Hatakeyama M. 2008. Transgenic expression of Helicobacter pylori CagA induces gastrointestinal and hematopoietic neoplasms in mouse. Proc Natl Acad Sci U S A 105:1003–1008. https://doi.org/10.1073/pnas.0711183105.

4. Varga MG, Shaffer CL, Sierra JC, Suarez G, Piazuelo MB, Whitaker ME, Romero-Gallo J, Krishna US, Delgado A, Gomez MA, Good JA, Almqvist F, Backert S, Haas R, Gerhard M, Naumann M. 2017. The Helicobacter pylori type IV secretion system encoded by the cag pathogenicity island: architecture, function, and signaling. Curr Top Microbiol Immunol 413:187–216. https://doi.org/10.1007/978-3-319-75241-9_8.

5. Zhou P, She Y, Dong N, Li P, He H, Borio A, Wu Q, Lu S, Ding C, Cao Y, Xu Y, Gao W, Dong M, Ding J, Wang DC, Zamyatina A, Shao F. 2018. Alpha-kini-nase 1 is a cytosolic innate immune receptor for bacterial ADP-htepose. Nature 561:122–126. https://doi.org/10.1038/s41586-018-0433-3.

6. Stein SC, Faber E, Bats SH, Munillo T, Speidel Y, Coombs N, JansenB N, Josenhans C. 2017. Helicobacter pylori modulates host cell responses by CagT4SS-dependent translocation of an intermediate metabolite of LPS inner core heptose biosynthesis. PloS Pathog 13:e1006514. https://doi.org/10.1371/journal.ppat.1006514.

7. Cariaga TA, Suarez G, Peek RM, Jr, Cover TL, Solnick JV. 2013. Functional architecture, function, and signaling of the Helicobacter pylori type IV secretion system encoded by the cag pathogenicity island: architecture, function, and signaling. Curr Top Microbiol Immunol 413:187–216. https://doi.org/10.1007/978-3-319-75241-9_8.

9. Backert S, Haas R, Gerhard M, Naumann M. 2017. The Helicobacter pylori type IV secretion system encoded by the cag pathogenicity island: architecture, function, and signaling. Curr Top Microbiol Immunol 413:187–220. https://doi.org/10.1007/978-3-319-75241-9_8.

10. Backert S, Tegtmeier N, 2017. Cell Microbiol 20(3):296–307. https://doi.org/10.1111/cmi.12967.

11. Pfannkuch L, Hurwitz R, Traulsen J, Signulla J, Poeschke M, Matzner L, Kosma P, Schmid M, Meyer TF. 2019. ADP heptose, a novel pathogen-associated molecular pattern identified in Helicobacter pylori. FASEB J 33:9087–9099. https://doi.org/10.1096/fj.201802555R.

12. Zhou P, She Y, Dong N, Li P, He H, Borio A, Wu Q, Lu S, Ding C, Cao Y, Xu Y, Gao W, Dong M, Ding J, Wang DC, Zamyatina A, Shao F. 2018. Alpha-kine-nase 1 is a cytosolic innate immune receptor for bacterial ADP-heptose. Nature 561:122–126. https://doi.org/10.1038/s41586-018-0433-3.

13. Stein SC, Faber E, Bats SH, Munillo T, Speidel Y, Coombs N, Josenhans C. 2017. Helicobacter pylori modulates host cell responses by CagT4SS-dependent translocation of an intermediate metabolite of LPS inner core heptose biosynthesis. PloS Pathog 13:e1006514. https://doi.org/10.1371/journal.ppat.1006514.
48. Israel DA, Salama N, Arnold CN, Moss SF, Ando T, Wirth HP, Tham KT, Camorlinga M, Blaser MJ, Falkow S, Peek RM, Jr. 2001. Helicobacter pylori strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. J Clin Invest 107:611–620. https://doi.org/10.1172/JCI11450.

49. Thomson MJ, Pritchard DM, Boxall SA, Abuderman AA, Williams JM, Varro A, Crabtree JE. 2012. Gastric Helicobacter infection induces iron deficiency in the INS-GAS mouse. PLoS One 7:e50194. https://doi.org/10.1371/journal.pone.0050194.

50. De la Cruz MA, Ares MA, von Bargen K, Panunzi LG, Martinez-Cruz J, Valdez-Salazar HA, Jimenez-Galicia C, Torres J. 2017. Gene expression profiling of transcription factors of Helicobacter pylori under different environmental conditions. Front Microbiol 8:615. https://doi.org/10.3389/fmicb.2017.00615.