# Vitamin B₆ Is Required for Full Motility and Virulence in *Helicobacter pylori*

Alexandra Grubman,[a]* Alexandra Phillips,[a] Marie Thibonnier,[b]* Maria Kaparakis-Liaskos,[a]* Chad Johnson,[c] Jean-Michel Thiberge,[d]* Fiona J. Radcliff,[e] Chantal Ecobichon,[d]* Agnès Labigne,[d] Hilde de Reuse,[b] George L. Mendz,[f] and Richard L. Ferrero[a,d]*

Department of Microbiology, Monash University, Melbourne, Australia[a]; Institut Pasteur, Unité de Pathogenèse de Helicobacter, Paris, France[b]; Monash Micro Imaging, Monash University, Melbourne, Australia[c]; Institut Pasteur, Unité de Pathogénie Bactérienne des Muqueuses, Paris, France[d]; Department of Molecular Medicine and Pathology, University of Auckland, Auckland, New Zealand[e]; and School of Medicine, the University of Notre Dame, Sydney, Australia[f]

* Present address: Alexandra Grubman, Maria Kaparakis-Liaskos, and Richard L. Ferrero, Centre for Innate Immunity and Infectious Diseases, Monash Institute of Medical Research, Monash Medical Centre, 3168 Victoria, Australia; Marie Thibonnier, Institut Pasteur, Unité de Génétique des Génomes Bactériens, Paris, France; Jean-Michel Thiberge, Institut Pasteur, PF8 Génotypage des Pathogènes et Santé Publique, Paris, France; Chantal Ecobichon, Institut Pasteur, G5 Biologie et Génétique de la Paroi Bactérienne, Paris, France.

**ABSTRACT** Despite recent advances in our understanding of how *Helicobacter pylori* causes disease, the factors that allow this pathogen to persist in the stomach have not yet been fully characterized. To identify new virulence factors in *H. pylori*, we generated low-infectivity variants of a mouse-colonizing *H. pylori* strain using the classical technique of *in vitro* attenuation. The resulting variants and their highly infectious progenitor bacteria were then analyzed by global gene expression profiling. The gene expression levels of five open reading frames (ORFs) were significantly reduced in low-infectivity variants, with the most significant changes observed for ORFs HP1583 and HP1582. These ORFs were annotated as encoding homologs of the *Escherichia coli* vitamin B₆ biosynthesis enzymes PdxA and PdxJ. Functional complementation studies with *E. coli* confirmed *H. pylori* PdxA and PdxJ to be *bona fide* homologs of vitamin B₆ biosynthesis enzymes. Importantly, *H. pylori* PdxA was required for optimal growth *in vitro* and was shown to be essential for chronic colonization in mice. In addition to having a well-known metabolic role, vitamin B₆ is necessary for the synthesis of glycosylated flagella and for flagellum-based motility in *H. pylori*. Thus, for the first time, we identify vitamin B₆ biosynthesis enzymes as novel virulence factors in bacteria. Interestingly, *pdxA* and *pdxJ* orthologs are present in a number of human pathogens, but not in mammalian cells. We therefore propose that PdxA/J enzymes may represent ideal candidates for therapeutic targets against bacterial pathogens.

**IMPORTANCE** Approximately half of the world's population is infected with *H. pylori*, yet how *H. pylori* bacteria establish chronic infections in human hosts remains elusive. From gene array studies, we identified two genes as representing potentially novel colonization factors for *H. pylori*. These genes encoded enzymes involved in the synthesis of vitamin B₆, an important molecule for many metabolic reactions in living organisms. Little is currently known regarding vitamin B₆ biosynthesis in human pathogens. We showed that mutant *H. pylori* bacteria lacking an enzyme involved in *de novo* vitamin B₆ biosynthesis, PdxA, were unable to synthesize motility appendages (flagella) and were unable to establish chronic colonization in mice. Thus, this work identifies vitamin B₆ biosynthesis enzymes as novel virulence factors for bacterial pathogens. Interestingly, a number of human pathogens, but not their mammalian hosts, possess these genes, which suggests that Pdx enzymes may represent ideal candidates for new therapeutic targets.

Address correspondence to Richard L. Ferrero, Richard.Ferrero@monash.edu.

There are currently only a limited number of antibiotics suitable for the routine treatment of *Helicobacter pylori* infection; moreover, the efficacies of these compounds have been severely hindered by the rise in resistance among *H. pylori* isolates (1, 2). It is therefore necessary to develop novel therapeutic and prophylactic strategies for the management of *H. pylori*-related disease. In an effort to develop such strategies, we need to greatly enhance our understanding of the molecular basis of *H. pylori* adaptation to the specialized niche of the gastric mucosa (3). *In silico* analyses have been fruitful in this regard; however, they are not always accurate (4). Thus, several groups have combined genome information and

functional data derived from the use of molecular techniques to shed new insights into different facets of *H. pylori* virulence (5–7). Although these techniques are very powerful, they rely, among other things, on the availability of *H. pylori* strains that can be readily transformed *in vitro*. These strains are usually laboratory-adapted isolates that are not able to colonize small rodents. To overcome this problem, workers have used suckling mice, which support the growth of highly transformable laboratory-adapted *H. pylori* strains that do not colonize adult mice (5). Suckling mice, however, have underdeveloped immune systems and do not possess the normal flora of adult animals, which may bias results

**TABLE 1** Mouse colonization experiments with low- and high-infectivity *H. pylori* SS1 isolates

| *In vitro* passage no. of isolate[a] | Inoculum/mouse (log$_{10}$ CFU) | No. of mice infected (total)[b] | Mean ± SD log$_{10}$ CFU recovered/g[c] |
|---|---|---|---|
| 58 | 6.3 | 9 (10)[d] | —[e] |
| 8 | 5.0 | 9 (9) | — |
| | | | |
| 74 | 6.7 | 5 (5)[d] | — |
| 9 | 6.7 | 5 (5) | — |
| | | | |
| 109 | 4.6 | 3 (10)[f] | 3.74 ± 0.50[g] |
| 15 | 4.6 | 10 (10) | 6.08 ± 0.52 |
| | | | |
| 179 | 5.5 | 0 (10)[g] | <2.0[h] |
| 24 | 5.5 | 9 (9) | 6.46 ± 0.8 |

[a] For each experiment, mice were separated into 2 groups, and each group was inoculated with either a low- or high-infectivity *H. pylori* SS1 isolate.
[b] *H. pylori* infection was detected by culture and urease activity at 4 or 6 weeks postchallenge.
[c] Bacterial load determinations were performed by quantitative culture. Mean values were calculated from infected mice only (assay sensitivity, 3.0 log CFU/g).
[d] $P > 0.99$.
[e] —, not determined.
[f] $P = 0.0031$.
[g] $P \leq 0.0001$.
[h] $P \leq 0.0003$.

toward the identification of factors that are important in the establishment of infection over those required for chronic colonization (5).

Historically, *in vitro* attenuation has been used as a strategy to study how microorganisms cause disease (8, 9). This strategy has the advantage of being applicable to any microorganism that can be propagated *in vitro*. Moreover, when combined with modern molecular techniques, such as whole-genome sequencing (10), it has been possible to identify novel virulence factors in otherwise well-characterized bacterial pathogens. The current study describes the use of a dual strategy of *in vitro* attenuation and gene expression profiling for the identification of novel virulence factors in *H. pylori*. Using this approach, we identified PdxA, an enzyme involved in *de novo* vitamin B$_6$ biosynthesis, as being an important factor required for the chronic colonization of mice by *H. pylori*. Although it is widely accepted that vitamin B$_6$ is a cofactor in a wide array of cellular metabolic reactions, this study is the first to describe a link between this metabolite and bacterial pathogenesis. Specifically, we demonstrated that vitamin B$_6$ is important for flagellar structure, glycosylation, and motility in a human pathogen. Given that mammalian hosts are unable to perform *de novo* vitamin B$_6$ biosynthesis, we propose that this work opens new avenues for the development of innovative therapeutic targets against bacterial pathogens.

## RESULTS

**Generation of low-infectivity isolates of *H. pylori* SS1 by prolonged *in vitro* passage.** To identify novel colonization factors of *H. pylori*, we applied the technique of *in vitro* attenuation to the well-characterized mouse-colonizing SS1 strain (11). We observed a progressive loss in the ability of *H. pylori* SS1 variants to infect mice, with significant reductions apparent both in the proportion of mice infected and in gastric bacterial levels after 109 passages *in vitro* (Table 1) ($P = 0.0031$ and $P \leq 0.0001$, respectively). By 179 subcultures, *H. pylori* SS1 isolates were no longer

able to colonize mice. Only minor differences in the DNA profiles were detected between low- and high-infectivity isolates (see Fig. S1A and B in the supplemental material). Furthermore, no differences were observed between isolates with regard to either their abilities to perform urease-mediated protection against acid, essential for *H. pylori* colonization of the stomach (12), or their *in vitro* growth properties (Fig. S1C and D).

**Downregulation of gene expression in low-infectivity *H. pylori* SS1.** To determine molecular changes correlating with decreased colonization in high-passage-number isolates, we performed global gene expression profiling of high- and low-infectivity isolates. The expression levels of five coding sequences were found to be significantly downregulated in low-infectivity isolates compared to those of high-infectivity bacteria (Fig. 1A). These genes encode proteins involved in vitamin B$_6$ biosynthesis (*pdxJ*, *pdxA*), iron metabolism (*frpB1*), restriction modification (*hsdR*), and autotransporter (*vacA* paralog) functions. The expression levels of these genes were confirmed by quantitative reverse transcription-PCR (qRT-PCR) to be significantly reduced in low-infectivity *H. pylori* SS1 isolates (Fig. 1B). The reduction in the expression levels of these genes correlated with a decrease in infectivity, first observed in *H. pylori* variants having undergone at least 109 *in vitro* subcultures, suggesting that the genes may encode factors that contribute to host colonization. The greatest variations in significance analysis of macroarray (SAM) scores were observed for *H. pylori pdxJ* and *pdxA*, annotated as encoding homologs of the vitamin B$_6$ biosynthesis proteins PdxJ and PdxA in *Escherichia coli*. As the role of vitamin B$_6$ has not been extensively studied in a bacterial pathogen, we chose to focus on the functions of the proteins encoded by these genes, including their roles in host colonization by *H. pylori*.

**PdxJ and PdxA are involved in *de novo* vitamin B$_6$ biosynthesis in *H. pylori*.** To determine whether *H. pylori pdxJ* and *pdxA* encode *bona fide* homologs of vitamin B$_6$ biosynthesis enzymes, we constructed isogenic *H. pylori pdxA* mutants (13) in several laboratory-adapted (251, N6, G27) and mouse-colonizing (SS1 and X47-2AL) *H. pylori* strains. These mutants could be recovered only when the growth medium was supplemented with pyridoxal-5′-phosphate (PLP), the product of PdxJ/PdxA activities. In contrast, despite numerous attempts and approaches, we were unable to disrupt *pdxJ*, suggesting that this mutation may be lethal for *H. pylori* bacteria (data not shown). It is possible that PdxJ may have another vital function(s) besides the synthesis of vitamin B$_6$, as has been reported for certain other *H. pylori* proteins (14). Alternatively, it is possible that disruption of *pdxJ* may have downstream effects on *pdxA* expression. In *H. pylori*, the *pdxJ* and *pdxA* genes are clustered together on the genome, with the stop codon of *pdxJ* preceding the start codon of *pdxA* by just 1 nucleotide (Fig. S2A and B). From qRT-PCR analyses, it appears that *pdxA* is transcribed as part of a longer *pdxJ-pdxA* cotranscript (Fig. S2C).

We next investigated the growth properties of the *H. pylori pdxA* mutants *in vitro*. In the absence of PLP supplementation, *H. pylori pdxA* mutants exhibited slower growth in liquid medium than the parent strain (Fig. 2A and B). This defect could, however, be entirely compensated for by the addition of PLP to the media or by complementation using insertion in *cis* of an intact copy of *pdxA* into the *H. pylori* genome (Fig. 2A and B). These data suggest a role for *H. pylori* PdxA in *de novo* vitamin B$_6$ biosynthesis. To demonstrate definitively that *pdxA* and *pdxJ* encode homologs of vitamin B$_6$ biosynthesis enzymes in *H. pylori*, we used a cross-

**A**

| TIGR no. | Symbol | Putative function | Score | Fold change |
|----------|--------|-------------------|-------|-------------|
| HP0846 | *hsdR* | Type I restriction enzyme R protein | -3.8 | 0.08 |
| HP0876 | *frpB1* | Iron-regulated outer membrane protein | -1.5 | 0.34 |
| HP0922 | *vacA* | Paralogue of vacuolating cytotoxin A (VacA) | -4 | 0.04 |
| HP1582 | *pdxJ* | Pyridoxal phosphate (Vitamin B6) biosynthetic protein J | -11.2 | 0.19 |
| HP1583 | *pdxA* | Pyridoxal phosphate (Vitamin B6) biosynthetic protein A | -6.8 | 0.21 |
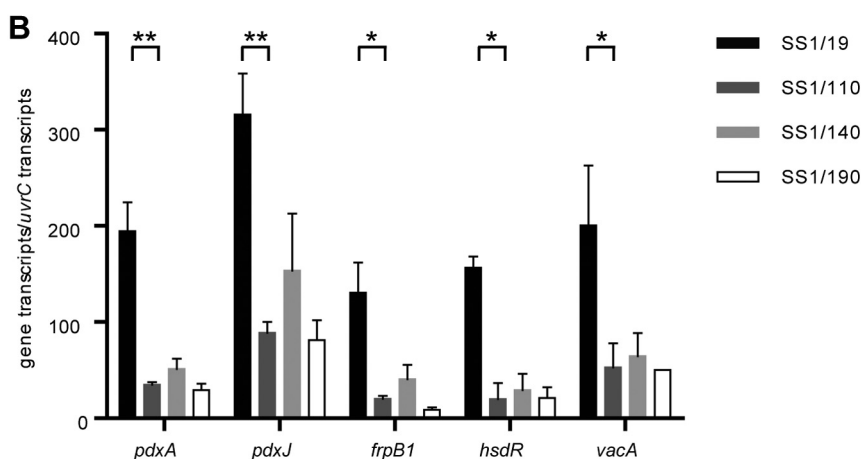


FIG 1 Downregulation of gene expression in low-infectivity *H. pylori* SS1 isolates. (A) Radioactively labeled cDNA samples from paired low- and high-infectivity *H. pylori* SS1 isolates were hybridized to macroarrays. TIGR numbers refer to the coding sequence numbers as identified by the J. Craig Venter Institute. Score values represent the relative levels of significance assigned by SAM analysis (34). Fold changes represent the fold changes between low- and high-infectivity isolates (four experiments). (B) qRT-PCR analyses of *pdxA*, *pdxJ*, *frpB1*, *hsdR*, and *vacA* paralog mRNA expression in liquid cultures of *H. pylori* SS1 isolates having undergone different numbers of passages. Gene expression was normalized to the housekeeping gene, *uvrC* (35). Error bars indicate standard errors of the means (SEM) (three experiments). *, $P < 0.05$; **, $P < 0.01$.

complementation strategy with *E. coli* K-12 Δ*pdxA* and Δ*pdxJ* mutants (CGSC8631 and CGSC10027, respectively [15]). For this, we transformed the *E. coli* mutants with IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible expression plasmids harboring intact copies of either *H. pylori pdxA* or *pdxJ*. The untransformed *E. coli* mutants were unable to grow on minimal medium without PLP added (Fig. 2C). In contrast, *E. coli pdxA* and *pdxJ* mutant bacteria that had been transformed with either *H. pylori pdxA*- or *pdxJ*-expressing plasmids, respectively, were able to grow normally on minimal medium containing IPTG without PLP added. Thus, the ability of *H. pylori pdxA* and *pdxJ* to cross-complement a PLP deficiency in *E. coli* provides functional evidence that these genes encode vitamin B$_6$ biosynthesis proteins in *H. pylori*.

**PdxA is essential for gastric colonization of C57BL/6 mice by *H. pylori*.** To determine whether vitamin B$_6$ biosynthesis is important for bacterial colonization, we performed mouse infection studies with *H. pylori pdxA* mutants generated on both the SS1 and X47-2AL genetic backgrounds. In parallel, colonization experiments were performed with mutants in the other putative colonization genes (Fig. 1A). Colonization efficiency was assessed by at least two methods: quantitative culture, serology, and/or RT-PCR (Fig. 3A).

Significantly lower bacterial loads were observed in mice challenged with an *H. pylori vacA* paralog mutant ($P < 0.01$), whereas mutations in either *frpB1* or *hsdR* had no significant effects on *in vivo* colonization by *H. pylori* (Fig. S3). Interestingly, the most dramatic effects on *H. pylori* colonization were observed for *H. pylori pdxA* mutants. Indeed, *H. pylori* bacteria were not cultured from the gastric biopsy specimens of mice inoculated with *pdxA* mutants in either *H. pylori* strain at both 7 and 30 days postinfection (Fig. 3B and C). Similarly, no IgG was detectable in the sera of mice infected with *H. pylori pdxA* mutant bacteria at 30 days postinfection in either of the strains tested (Fig. 3E). Interestingly, however, low levels of colonization, as indicated by IgM titers, were observed at day 7 postinfection in mice challenged with *H. pylori pdxA* mutants on the X47-2AL genetic background, whereas no evidence of colonization could be found for *H. pylori* SS1 *pdxA* mutants (Fig. 3A and D). In both cases, however, the *H. pylori pdxA* mutant bacteria were cleared by 30 days.

Complementation of *H. pylori pdxA* mutants with a wild-type copy of *pdxA* restored infectivity, as demonstrated by a significant increase in both the numbers of mice colonized and the serum IgG levels at day 30 postinfection (Fig. 3B, C, and E) ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). Although the restoration of in-
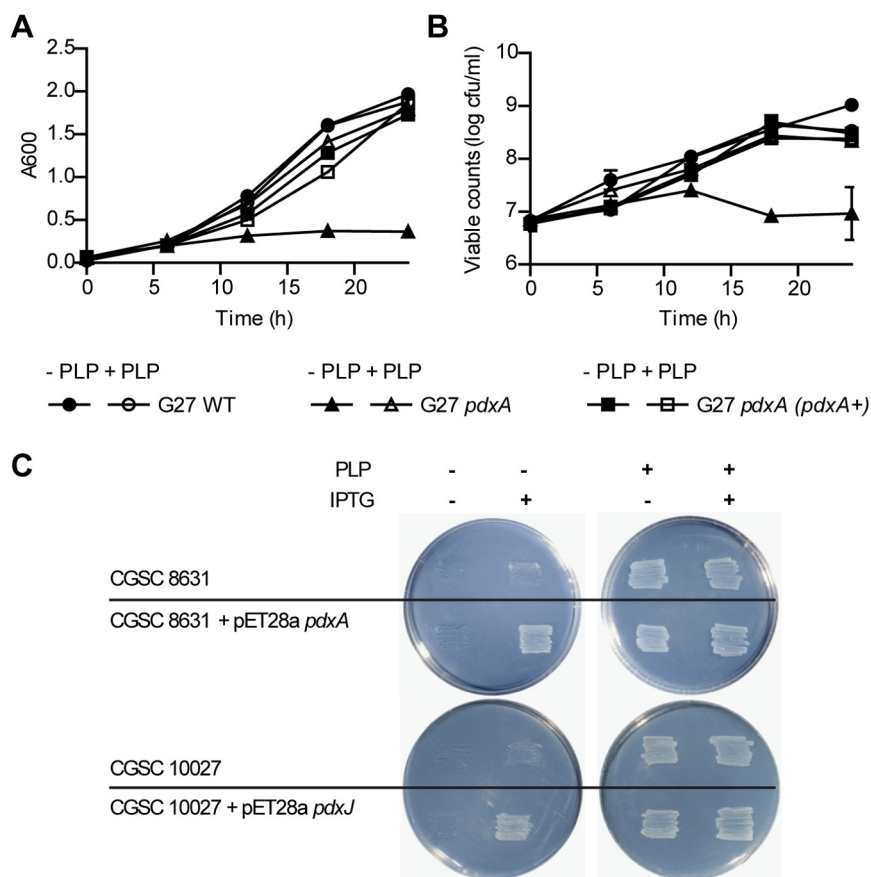
FIG 2 *In vitro* growth of *H. pylori pdxA* mutants and functional characterization of PdxJ/A. (A and B) *In vitro* growth properties of *H. pylori* G27 wild type (WT), *pdxA*, and *pdxA* (*pdxA+*) strains were determined in liquid media supplemented or not supplemented with PLP. At each time point, $A_{600}$ readings (A) and duplicate viable cell count determinations (B) were performed (three experiments). Data are presented as the log of the mean viable cell count (for each time point) $\pm$ SEM. (C) Growth of *E. coli* CGSC8631 ($\Delta pdxA$) and CGSC10027 ($\Delta pdxJ$) that had been transformed, or not, with pET28a *pdxA* and pET28a *pdxJ*, respectively, in the presence or absence of PLP and IPTG.

fectivity for the complemented mutants was incomplete, this can most likely be attributed to the reduced expression levels of *pdxJ* observed in *H. pylori pdxA* mutant bacteria (Fig. S4A). Cumulatively, these data identify the essential role of PdxA in *H. pylori* colonization of the mouse gastric mucosa.

**Vitamin B$_6$ contributes to motility and flagellar glycosylation in *H. pylori*.** We next wished to investigate the reason(s) for the severe impairment in colonization efficiency of the *H. pylori pdxA* mutants. Vitamin B$_6$, or its active form, PLP, is classically thought to be a cofactor for many metabolic enzymes and has recently been identified as a cofactor of *H. pylori* PseC, an aminotransferase involved in flagellar glycosylation (16). Glycosylation of flagella is necessary for the assembly of functional flagellar filaments, motility, and infectivity in *H. pylori* (17). Therefore, we hypothesized that a defect in flagellar structure or motility may account for the impaired virulence *in vivo* of the *H. pylori pdxA* mutants. To test this hypothesis, we examined *H. pylori* motility in soft agar plates and by live-cell imaging. For each of the *H. pylori* strains tested (G27, X47-2AL, and SS1), parental bacteria were motile, whereas *H. pylori pdxA* mutants were nonmotile (Fig. 4A) ($P <$ 0.01). Addition of exogenous PLP to the motility media, however, resulted in a diffuse growth pattern for *H. pylori pdxA*

mutants, indicative of motile bacteria (Fig. 4A). Consistent with these findings, in the absence of PLP supplementation, parental *H. pylori* bacteria exhibited an average velocity of 27.92 $\pm$ 1.437 $\mu$m/s (see Movie S1 in the supplemental material), whereas *H. pylori pdxA* mutants moved at an average velocity of only 2.865 $\pm$ 0.5626 $\mu$m/s (Fig. 4B; Movie S2) ($P <$ 0.0001). Supplementation of broth cultures with PLP restored the motility of *H. pylori pdxA* mutants to an average velocity of 28.92 $\pm$ 3.160 $\mu$m/s (Fig. 4B; Movie S3) ($P <$ 0.0001).

To determine whether the defect in motility of *H. pylori pdxA* mutants was a result of altered flagellar structure, we examined the flagella from *H. pylori* G27 parental and *pdxA* mutant bacteria. Parental *H. pylori* bacteria and complemented *pdxA* mutants displayed polar, sheathed flagella, whereas *H. pylori pdxA* mutants appeared to express no flagella (Fig. 5A to C), indicating a potential impairment in flagellar assembly. To elucidate further the role of vitamin B$_6$ in the production of flagella, we performed Western blot analyses of whole-cell lysates and sheared flagellar preparations using a monoclonal antibody directed against the major *H. pylori* flagellin, FlaA. Whole-cell lysates prepared from *H. pylori* X47-2AL *pdxA* mutants contained amounts of FlaA protein equivalent to those in lysates prepared from parental and comple-
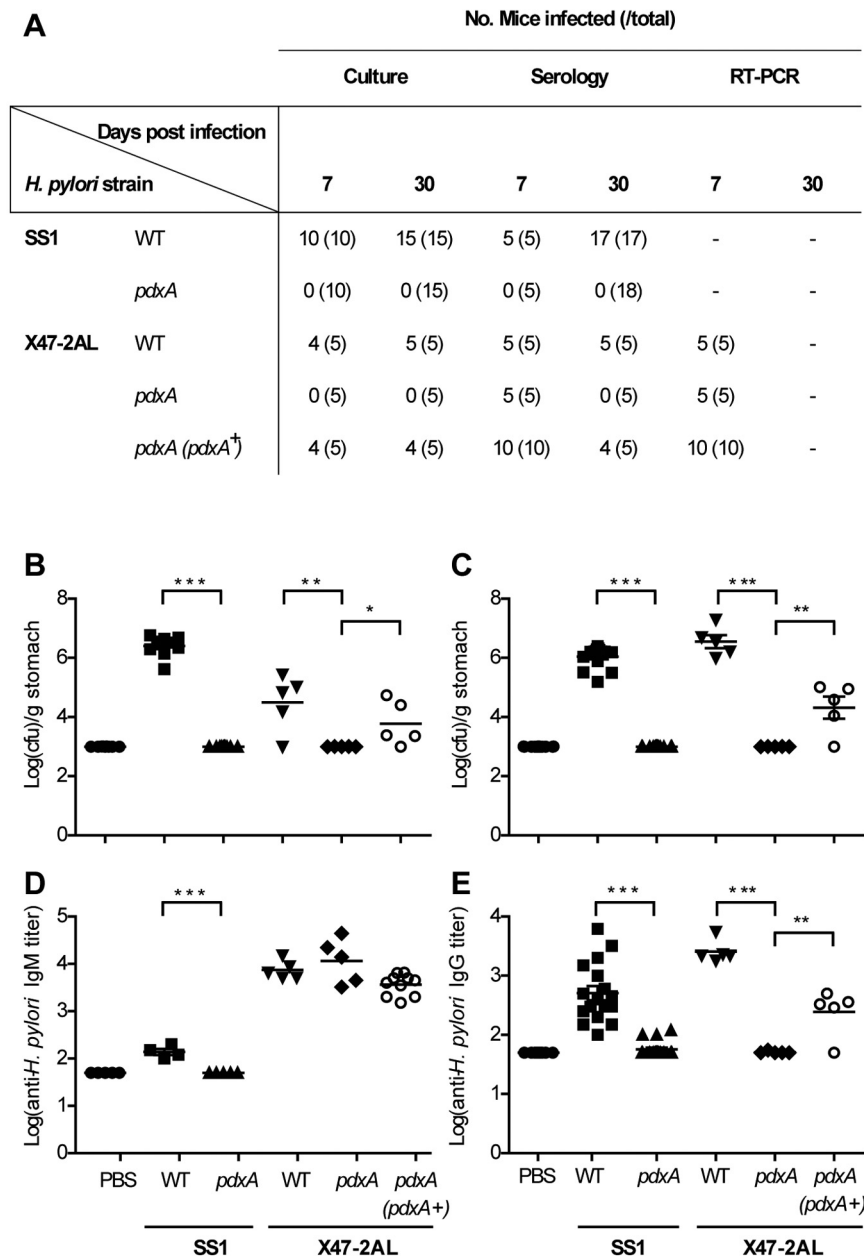
FIG 3 PdxA is required for colonization of mice by *H. pylori*. C57BL/6 mice (five per group) were inoculated with 10$^7$ CFU of *H. pylori* by intragastric gavage. (A) Colonization efficiency was assessed by culture, serology, and/or RT-PCR. −, not determined. (B and C) Quantitative culture assays were performed at 7 (B) and 30 (C) days postinfection. Each point represents the mean of two estimations for a single mouse. Data are pooled from three separate experiments for *H. pylori* SS1 and two separate experiments for *H. pylori* X47-2AL. Two independent *H. pylori* SS1 *pdxA* clones and two independent *H. pylori* X47-2AL *pdxA* (*pdxA*$^+$) clones were used. Data are presented as log units (CFU/gram stomach). Horizontal bars represent the geometric means of viable cell count determinations for each *H. pylori* strain. The limit of detection for the assay is 3 log CFU/gram. (D and E) Specific IgM (D) and IgG (E) class antibodies in the sera of *H. pylori*-infected mice were detected by ELISA at 7 (D) and 30 (E) days postinfection. Data are presented as logs of titers, corresponding to the reciprocal of the serum dilution giving an $A_{595}$ value that was 5-fold above that obtained for background controls. Horizontal bars represent the geometric means for each *H. pylori* strain. The limit of detection for the assay is a log titer of 1.7. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.0001$, as determined by the Mann-Whitney U test.

mented *H. pylori* bacteria (Fig. 5D). Importantly, however, the sheared flagellar preparations from *H. pylori pdxA* bacteria contained much less FlaA than those prepared from parental bacteria (Fig. 5D). Consistent with previous work (18), exported *H. pylori* FlaA proteins in the flagellar preparations from parental and complemented *H. pylori* bacteria were glycosylated, whereas FlaA proteins in the cytoplasm were nonglycosylated (Fig. 5E). Signifi-

cantly, only the exported FlaA in the flagellar preparations from parental *H. pylori* bacteria, but not those from the *pdxA* mutant organisms, were glycosylated (Fig. 5E). The data suggest that the inability of *H. pylori pdxA* mutants to glycosylate and export functional flagellar filaments was not due to a defect in cellular FlaA production but rather to the absence of a posttranslational modification that requires the PLP-dependent enzyme PseC. Taken
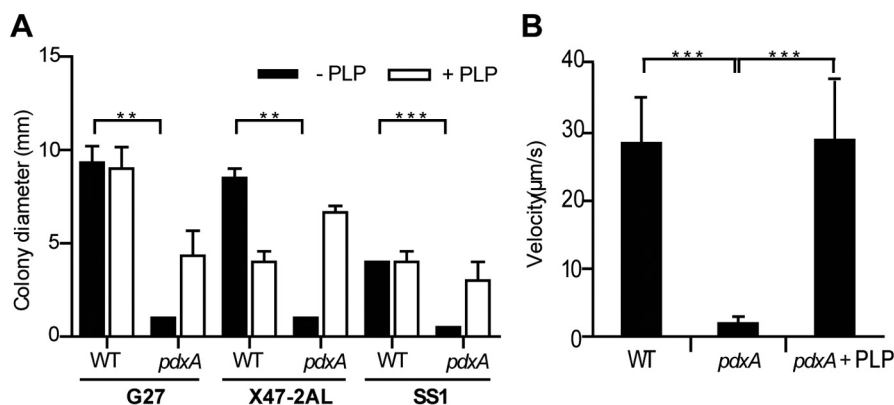
**FIG 4** PdxA is required for motility in *H. pylori*. (A) The bacterial motility of wild-type and *pdxA* mutant strains on different *H. pylori* genetic backgrounds was assayed using 0.4% (wt/vol) semisolid agar plates with (white bars) or without (black bars) the addition of PLP. Bars represent the mean determinations of triplicate measurements of colony diameter ± SEM (three experiments). **, $P < 0.01$, and ***, $P < 0.0001$, as determined by Student's *t* test. (B) Bacterial motility in 6-h broth cultures was determined by visualization on a Leica AF6000 LX live-cell imager and analyzed using MetaMorph software. Data are presented as means ± SEM derived from over 400 positional data points for each strain (three experiments).

together, the data indicate that vitamin B$_6$ biosynthesis is important for flagellar biosynthesis and motility, which are vital for *H. pylori* colonization of the gastric mucosa.

## DISCUSSION

This study reports the identification of novel virulence factors in a pathogenic bacterium using the classical approach of *in vitro* attenuation in combination with global gene expression profiling. Although some of the factors (i.e., HsdR and FrpB1) were not essential for colonization, we suggest that the cumulative effects of lowered gene expression levels for these and other factors, including PdxA and the VacA paralog, are likely to have contributed to the difference in colonization levels between the low- and high-passage-number isolates of *H. pylori* SS1. Most importantly, how-
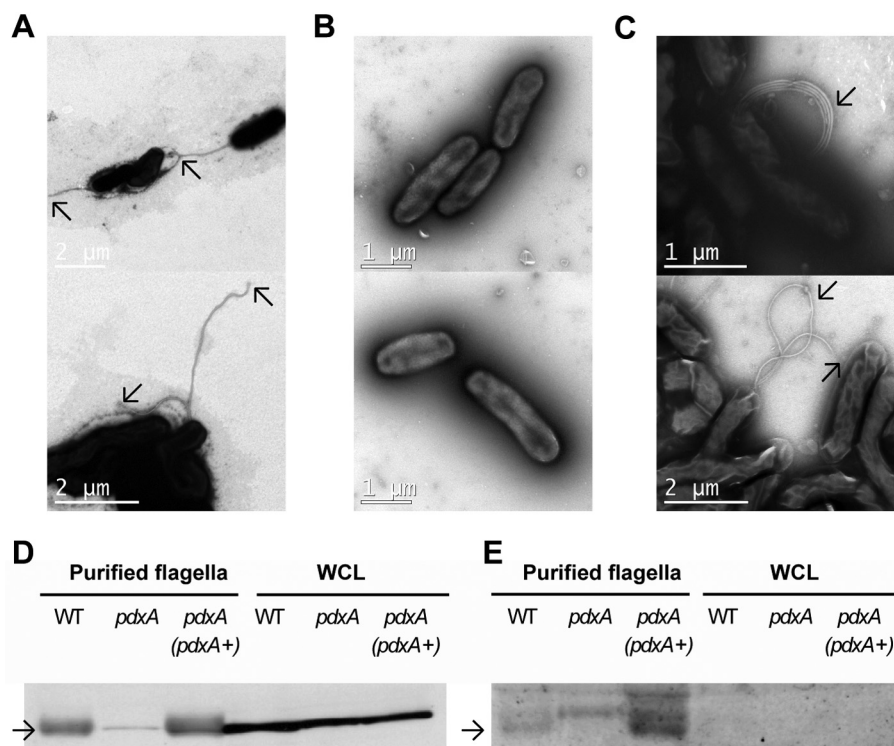


**FIG 5** PdxA is required for functional flagellar assembly and flagellar glycosylation in *H. pylori*. (A to C) TEM micrographs of negatively stained *H. pylori* G27 wild-type (A), G27 *pdxA* (B), and G27 *pdxA* (*pdxA*⁺) strains (C). Arrows indicate the ends of the flagella in each strain. (D) Western blot of whole-cell lysates (WCL) and sheared flagellar preparations, detected with monoclonal anti-*H. pylori* 54-kDa flagellin antibodies. (E) Detection of glycosylated proteins in WCL and sheared flagellar preparations of *H. pylori*, oxidized with periodate and visualized with Pro-Q Emerald 488 reagent. Arrows indicate the bands corresponding to FlaA.

ever, this approach has allowed us to demonstrate, for the first time, the importance of *de novo* vitamin B$_6$ biosynthesis for host colonization by a human pathogen. Vitamin B$_6$ is an enzyme cofactor in a plethora of cellular reactions, including transamination, decarboxylation, and racemization (reviewed in reference 19). PdxA and PdxJ catalyze two sequential reactions in the DXP-dependent *de novo* vitamin B$_6$ biosynthesis pathway (20); the byproduct of these reactions is pyridoxine-5′-phosphate (PNP) (21). In turn, PNP is oxidized to form PLP, the bioactive vitamer of vitamin B$_6$ (22). The DXP-dependent pathway is found predominantly in a small subset of gammaproteobacteria (23–25) but has been extensively studied only in *E. coli* (20–23). In *H. pylori*, only the genes *pdxJ* and *pdxA* are annotated as belonging to the *pdx* family and are clustered together on the *H. pylori* genome (Fig. S2A). Interestingly, the genetic organization of *pdxJ* and *pdxA* appears to be conserved for all *Helicobacter* and *Campylobacter* spp., as well as for several other related organisms (Fig. S2A). Conversely, these genes are spatially separated in all other sequenced bacterial genomes containing the *pdxJ* and *pdxA* genes, including *E. coli*. Thus, there appears to have been divergent evolution of bacterial vitamin B$_6$ biosynthesis genes. We suggest that this further highlights the differences between the physiology of the model bacterium *E. coli* and those of other bacteria and emphasizes the need to study the physiology of pathogenic bacterial species.

From the data presented here, it appears that *H. pylori pdxJ* and *pdxA* are transcriptionally coregulated. This finding is consistent with reports that both *pdxA* and *pdxJ* are repressed in the stationary phase of growth (26) but that both genes are induced by low pH (pH 4.5) (27). Furthermore, these findings are in agreement with a recent study describing the primary transcriptome of *H. pylori*, where *pdxJ* and *pdxA* were found to be transcribed as an operon (28). This further validates PdxJ and PdxA as colonization factors for *H. pylori*, as low pH is encountered by *H. pylori* in its preferred ecological niche (27). No differences in the upstream DNA regions of *pdxJ* and *pdxA* were identified between low- and high-infectivity variants of *H. pylori* SS1 (Fig. S2B), suggesting that transcriptional repression by a proximal *cis*-acting sequence is unlikely to be responsible for altered gene expression in the low-infectivity variants. Recently, a new layer of complexity in *H. pylori* gene expression and regulation, which is encoded by antisense and/or *trans*-acting regulatory short RNAs, was uncovered (28) and may be the mechanism of regulation of *pdxJ* and *pdxA* expression. The reason for the reduced levels of gene expression in the low-infectivity variants requires further investigation.

In this study, we identified *H. pylori* PdxA (HP1583) as being essential for *in vivo* colonization of mice. We therefore reasoned that the colonization defect of *H. pylori pdxA* mutants may be the result of the loss of a PLP-dependent function involved in colonization. One such function is the glycosylation of flagella, which is necessary for motility and colonization in *H. pylori* (16–18). *H. pylori* glycosylates its flagellins with a pseudaminic acid (Pse) moiety, synthesized via a PLP-dependent reaction mediated by PseC (16). We therefore hypothesized that the absence of PLP in *H. pylori pdxA* mutants may result in defective flagellar synthesis. Indeed, *H. pylori pdxA* mutants were nonmotile and expressed no flagella. Furthermore, these mutants accumulated nonglycosylated flagellins of lower molecular weights within the cytoplasm, indicative of an impairment in flagellar glycosylation (Fig. 5A to E). Hence impairment of PseC activity, due to a lack of available PLP, is likely

to be one factor contributing to the colonization defect of *H. pylori pdxA* mutants.

It is widely accepted that vitamin B$_6$ plays an important cellular role as an enzyme cofactor. To our knowledge, however, this study is the first to describe a link between this vitamin and bacterial pathogenesis. This finding is consistent with emerging evidence supporting the importance of vitamin B$_6$ in cellular processes involved in the biosynthesis of surface-exposed structures that are likely to contribute to adhesion and colonization in some pathogenic bacteria (16, 29, 30). Indeed, PLP-dependent enzymes are involved in the synthesis of surface-exposed glycosyl moieties, such as flagella and lipopolysaccharide (LPS), in *Salmonella enterica* serovar Typhimurium (29), *Campylobacter jejuni* (16), *Vibrio cholerae* (30), and *E. coli* O157:H7 (30); LPS is necessary for efficient bovine colonization by O157:H7 (30). It is worth noting that in addition to the bacteria named above, the PdxJ/A-dependent pathway of vitamin B$_6$ biosynthesis is present in several other human pathogens, including *Neisseria* spp. and *Yersinia pestis*, but is absent in mammals. Thus, this work identifies PdxJ and PdxA as novel potential therapeutic targets for the treatment of infections by microbial pathogens. Indeed, various PLP-dependent enzymes have already been identified as drug targets for the treatment of a diverse range of diseases (20–22). We suggest that the disruption of *de novo* PLP biosynthesis *per se*, via the inhibition of PdxJ/A, offers the added advantage of selectively inhibiting microbial growth without nonspecifically affecting vital host cell functions. Thus, this study opens new avenues for the treatment of microbial infections.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Bacterial strains used in this study are listed in Table S1 in the supplemental material. *H. pylori* isolates were routinely subcultured as previously described (31). For *in vitro* growth studies, *H. pylori* bacteria were grown for up to 24 h in 10 ml brain heart infusion (BHI; Oxoid) broth supplemented or not supplemented with 10 mg/liter PLP (Sigma). Viable cell counts and optical density ($A_{600}$) determinations were performed at the selected time points. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar (containing 1.5% [wt/vol] agar) supplemented with kanamycin (20 mg/liter), ampicillin (100 mg/liter), or chloramphenicol (25 mg/liter), as appropriate.

**Infection of mice with *H. pylori*.** Animal handling and experimentation were performed in accordance with national and institutional guidelines (French Laws 87 to 848 and Monash University AEC no. BAM/M2005/44, respectively). *H. pylori* suspensions for mouse inoculation were prepared directly in broth (31). Six- to eight-week-old female specific-pathogen-free mice were each intragastrically administered a single 100-μl aliquot of the inoculating suspension (10$^7$ CFU/mouse) using polyethylene catheters (31).

**Assessment of *H. pylori* infection in mice.** The presence of *H. pylori* infection in mice was determined by quantitative culture, detection of anti-*H. pylori* antibodies in sera, and/or RT-PCR on RNA isolated from gastric biopsy specimens, as previously described (31).

**Molecular biology techniques.** Genomic and plasmid DNA were isolated using the Masterpure DNA purification (Epicentre) and PureLink plasmid midi kits (Invitrogen), respectively. Restriction endonuclease digestions, ligations, and other enzymatic reactions were conducted by standard procedures as described by Sambrook et al. (32).

**Macroarray analyses.** Macroarrays (Eurogentec, SA, Belgium), consisting of the complete set of genes from the *H. pylori* 26695 strain, were hybridized to [α-$^{33}$P]dCTP-labeled cDNA which had been reverse transcribed from RNA isolated from paired liquid cultures of high (17 to 23 *in vitro* passages)- or low (180 to 192 passages)-infectivity *H. pylori* SS1 variants (33). The intensities of hybridizing spots from four independent

biological replicates were determined using the XdotsReader program COSE (33). Significant differences in gene expression were determined using the program significance analysis of macroarrrays (SAM) (34).

**RNA isolation.** Mouse gastric biopsy specimens, stored in RNA later (Ambion), were placed in TRIzol reagent (Invitrogen) and homogenized on ice using a PRO-200 homogenizer (PRO Scientific, CT). RNA was purified from gastric biopsy specimens or harvested *H. pylori* bacteria ($10^7$ CFU) using TRIzol reagent and Phase Lock gel tubes (Eppendorf) and then DNase treated using RQ1 DNase (Promega). RNA was subsequently purified using the RNeasy minikit (Qiagen).

**qRT-PCR.** RNA (200 ng to 2 $\mu$g) was reverse transcribed using random hexamers and SuperScript III (Invitrogen). Primers (Table S2) were designed based on the *H. pylori* 26695 genomic DNA sequence (GenBank accession no. AE000511) using the Primer Express primer design software (Applied Biosystems). qRT-PCR was performed as previously described (35). cDNA concentrations of the target genes for each sample were normalized to expression of the housekeeping gene, *uvrC* (26).

**Construction of *H. pylori* mutants by allelic exchange.** PCR fragments, amplified using the primers listed in Table S2 in the supplemental material, were purified and then ligated into pILL570 or pGEM-T Easy (Promega). Constructs were amplified by inverse PCR (Failsafe PCR System; Epicentre) using primers SL7 and SL8, SL3 and SL4, or SL12 and SL13, respectively. The digested PCR products were ligated with a BamHI/KpnI-digested nonpolar kanamycin resistance cassette (*aphA3*) (13). The construct used to inactivate the *vacA* paralog gene was generated by PCR with the 3T primer pool (3T-PCR), using the oligonucleotides H206, H207, H208, and H209 (Table S2). Isogenic *H. pylori* mutants were created by either electroporation (36) or natural transformation (37) and selected on horse blood agar (HBA) plates containing kanamycin (20 mg/liter). PLP was added to media used to culture *H. pylori pdxA* mutant strains. Kanamycin-resistant transformants in each strain of *H. pylori* were verified by sequencing and PCR analyses (primer H17 or H50) (Table S2).

**Complementation of *H. pylori pdxA* mutants.** A 150-bp fragment containing the *H. pylori* 26695 *ureA* promoter sequence was amplified by PCR from the pUGO plasmid (M. Kaparakis-Liaskos, unpublished data) using primers AG14F and AG14R (Table S2). *H. pylori pdxA* was amplified from *H. pylori* 26695 chromosomal DNA using primers AG15F and AG5R. The partially complementary PCR products (1 ng) were used as templates in splicing by overlap extension (SOE) PCR, consisting of five cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min without the addition of primers, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min in the presence of 20 pmol of each of the primers AG14F and AG5R. The resultant PCR products were cloned into the BamHI/EcoRI sites of the linearized pRdxA vector (38). Complemented *H. pylori pdxA* mutants were selected on HBA plates supplemented with metronidazole (8 mg/liter) in the absence of PLP. These mutants were verified by PCR sequencing using the primers AG1F, AG1R, AG5F, and AG5R.

**Detection of antibodies by ELISA.** *H. pylori* antigen-specific antibodies in mouse serum samples were detected by enzyme-linked immunosorbent assay (ELISA) (39). Bound *H. pylori*-specific immunoglobulins were detected with biotinylated goat anti-mouse antibodies (IgM $\mu$-chain [Millipore] or IgG [Jackson ImmunoResearch Laboratories, Inc.]) and streptavidin-peroxidase conjugate (Chemicon).

**Cross-complementation in *E. coli*.** *pdxA* and *pdxJ* were amplified from *H. pylori* 26695 genomic DNA using the primers AG5F and AG5R and AG6F and AG6R, respectively, and ligated into the BamHI/EcoRI-linearized pET28a vector (Novagen). These plasmids were transformed by electroporation (40) into *E. coli* CGSC8631 (*pdxA*) and CGSC10027 (*pdxJ*) and selected on Glu B1 56/2 minimal medium (41), supplemented or not supplemented with PLP and IPTG (1 mM).

**Motility testing.** Bacterial motility was determined using semisolid agar plates (17). The velocities of individual bacteria were determined by differential interference contrast (DIC) imaging using an Integrated Leica

AF6000 LX live-cell workstation (Leica Microsystems). Aliquots (10 $\mu$l) of *H. pylori* ($5 \times 10^7$ CFU/ml), which had been harvested from 6-h liquid broth cultures supplemented or not with PLP, were prepared in 0.4% methylcellulose in a FluoroDish (World Precision Instruments Inc.). Image analysis was performed using MetaMorph software (Molecular Devices). For each experiment, positional data for 20 bacteria over a minimum of 20 frames each were used to determine average velocity.

**Electron microscopy.** Bacteria were applied to Formvar-carbon-coated copper grids (400 mesh) and negatively stained for 5 to 10 s with 1% phosphotungstic acid (pH 6.8). Grids were examined using a Hitachi H-7500 transmission electron microscope.

**Flagellin preparation.** Flagella were isolated according to a modified method of Reeves et al. (42). Briefly, bacteria were harvested from 500-ml liquid cultures, resuspended in phosphate-buffered saline (PBS), and sheared for 1 min with an MSE homogenizer (MSE Ltd.). Cell debris was removed by centrifugation, and flagella were concentrated from the supernatants by ultracentrifugation using a TH-641 rotor in the Sorvall RC-90 ultracentrifuge (285,000 $\times$ *g* for 1 h at 10°C). Pellets containing flagella were resuspended in 100 $\mu$l Milli-Q $H_2O$. Protein concentrations were determined by the Bradford (Bio-Rad) or Qubit (Invitrogen) assay.

**SDS-PAGE and Western blotting.** Samples were separated in 10 to 12.5% (vol/vol) acrylamide gels and transferred to nitrocellulose. Flagellin was detected using mouse monoclonal antibodies to the *H. pylori* 54-kDa flagellin antigen (Bioscientific), followed by biotinylated secondary goat anti-mouse IgG antibodies (Chemicon) and streptavidin-horseradish peroxidase (HRP) (Chemicon). Antigen-antibody complexes were detected using ECL detection reagent (GE Healthcare). Glycosylated proteins were detected using the Pro-Q Emerald 488 glycoprotein gel and blot stain kit (Invitrogen).

**Statistics.** Differences in colonization levels between low- and high-infectivity *H. pylori* SS1 variants were determined using contingency tables (Fisher's exact test). Differences in the bacterial loads or antibody responses between animal groups were determined by the Mann-Whitney U test (two-sided). Differences in expression levels determined by qRT-PCR were analyzed using the Student *t* test. Differences were considered significant when *P* was ≤0.05.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00158-10/-/DCSupplemental.

Supplemental Text and Methods, DOC file, 0.025 MB.
Table S1, DOC file, 0.034 MB.
Table S2, DOC file, 0.062 MB.
Figure S1, TIF file, 0.696 MB.
Figure S2, TIF file, 0.284 MB.
Figure S3, TIF file, 0.163 MB.
Figure S4, TIF file, 1.007 MB.
Movie S1, MOV file, 0.548 MB.
Movie S2, MOV file, 0.271 MB.
Movie S3, MOV file, 0.644 MB.

## REFERENCES

1. **Katelaris, P. H., D. Adamthwaite, P. Midolo, N. D. Yeomans, G. Davidson, and J. Lambert.** 2000. Randomized trial of omeprazole and metronidazole with amoxycillin or clarithromycin for *Helicobacter pylori*

eradication, in a region of high primary metronidazole resistance: the HERO study. Aliment. Pharmacol. Ther. **14:**751–758.

2. **Mollison, L. C., N. Stingemore, R. A. Wake, D. J. Cullen, and D. B. McGechie.** 2000. Antibiotic resistance in *Helicobacter pylori*. Med. J. Aust. **173:**521–523.

3. **Ferrero, R. L., and A. Labigne.** 2001. *Helicobacter pylori* vaccine development in the post-genomic era: can *in silico* translate to *in vivo*. Scand. J. Immunol. **53:**443–448.

4. **Marais, A., G. L. Mendz, S. L. Hazell, and F. Megraud.** 1999. Metabolism and genetics of *Helicobacter pylori*: the genome era. Microbiol. Mol. Biol. Rev. **63:**642–674.

5. **Guo, B. P., and J. J. Mekalanos.** 2002. Rapid genetic analysis of *Helicobacter pylori* gastric mucosal colonization in suckling mice. Proc. Natl. Acad. Sci. U. S. A. **99:**8354–8359.

6. **Kavermann, H., B. P. Burns, K. Angermuller, S. Odenbreit, W. Fischer, K. Melchers, and R. Haas.** 2003. Identification and characterization of *Helicobacter pylori* genes essential for gastric colonization. J. Exp. Med. **197:**813–822.

7. **Salama, N. R., B. Shepherd, and S. Falkow.** 2004. Global transposon mutagenesis and essential gene analysis of *Helicobacter pylori*. J. Bacteriol. **186:**7926–7935.

8. **Steenken, J. W.** 1935. Lysis of tubercle bacilli *in vitro*. Proc. Soc. Exp. Biol. Med. **33:**253–255.

9. **Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small.** 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science **284:**1520–1523.

10. **Brosch, R., S. V. Gordon, C. Buchrieser, A. S. Pym, T. Garnier, and S. T. Cole.** 2000. Comparative genomics uncovers large tandem chromosomal duplications in *Mycobacterium bovis* BCG Pasteur. Yeast **17:**111–123.

11. **Lee, A., J. O'Rourke, De Ungria, M. C., B. Robertson, G. Daskalopoulos, and M. F. Dixon.** 1997. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology **112:**1386–1397.

12. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59:**2470–2475.

13. **Skouloubris, S., J. M. Thiberge, A. Labigne, and H. De Reuse.** 1998. The *Helicobacter pylori* UreI protein is not involved in urease activity but is essential for bacterial survival *in vivo*. Infect. Immun. **66:**4517–4521.

14. **Cover, T. L., and S. R. Blanke.** 2005. *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. Nat. Rev. Microbiol. **3:**320–332.

15. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2:**2006.0008.

16. **Schoenhofen, I. C., D. J. McNally, E. Vinogradov, D. Whitfield, N. M. Young, S. Dick, W. W. Wakarchuk, J. R. Brisson, and S. M. Logan.** 2006. Functional characterization of dehydratase/aminotransferase pairs from *Helicobacter* and *Campylobacter*: enzymes distinguishing the pseudaminic acid and bacillosamine biosynthetic pathways. J. Biol. Chem. **281:**723–732.

17. **Schirm, M., E. C. Soo, A. J. Aubry, J. Austin, P. Thibault, and S. M. Logan.** 2003. Structural, genetic and functional characterization of the flagellin glycosylation process in *Helicobacter pylori*. Mol. Microbiol. **48:**1579–1592.

18. **Josenhans, C., L. Vossebein, S. Friedrich, and S. Suerbaum.** 2002. The neuA/flmD gene cluster of *Helicobacter pylori* is involved in flagellar biosynthesis and flagellin glycosylation. FEMS Microbiol. Lett. **210:**165–172.

19. **Fitzpatrick, T. B., N. Amrhein, B. Kappes, P. Macheroux, I. Tews, and T. Raschle.** 2007. Two independent routes of *de novo* vitamin B6 biosynthesis: not that different after all. Biochem. J. **407:**1–13.

20. **Banks, J., and D. E. Cane.** 2004. Biosynthesis of vitamin B6: direct identification of the product of the PdxA-catalyzed oxidation of 4-hydroxy-l-threonine-4-phosphate using electrospray ionization mass spectrometry. Bioorg. Med. Chem. Lett. **14:**1633–1636.

21. **Laber, B., W. Maurer, S. Scharf, K. Stepusin, and F. S. Schmidt.** 1999. Vitamin B6 biosynthesis: formation of pyridoxine 5′-phosphate from 4-(phosphohydroxy)-L-threonine and 1-deoxy-D-xylulose-5-phosphate by PdxA and PdxJ protein. FEBS Lett. **449:**45–48.

22. **Zhao, G., and M. E. Winkler.** 1995. Kinetic limitation and cellular amount of pyridoxine (pyridoxamine) 5′-phosphate oxidase of *Escherichia coli* K-12. J. Bacteriol. **177:**883–891.

23. **Ehrenshaft, M., P. Bilski, M. Y. Li, C. F. Chignell, and M. E. Daub.** 1999.

A highly conserved sequence is a novel gene involved in *de novo* vitamin B6 biosynthesis. Proc. Natl. Acad. Sci. U. S. A. **96:**9374–9378.

24. **Mittenhuber, G.** 2001. Phylogenetic analyses and comparative genomics of vitamin B6 (pyridoxine) and pyridoxal phosphate biosynthesis pathways. J. Mol. Microbiol. Biotechnol. **3:**1–20.

25. **Tanaka, T., Y. Tateno, and T. Gojobori.** 2005. Evolution of vitamin B6 (pyridoxine) metabolism by gain and loss of genes. Mol. Biol. Evol. **22:**243–250.

26. **Thompson, L. J., D. S. Merrell, B. A. Neilan, H. Mitchell, A. Lee, and S. Falkow.** 2003. Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. Infect. Immun. **71:**2643–2655.

27. **Wen, Y., E. A. Marcus, U. Matrubutham, M. A. Gleeson, D. R. Scott, and G. Sachs.** 2003. Acid-adaptive genes of *Helicobacter pylori*. Infect. Immun. **71:**5921–5939.

28. **Sharma, C. M., S. Hoffmann, F. Darfeuille, J. Reignier, S. Findeiss, A. Sittka, S. Chabas, K. Reiche, J. Hackermuller, R. Reinhardt, P. F. Stadler, and J. Vogel.** 2010. The primary transcriptome of the major human pathogen *Helicobacter pylori*. Nature **464:**250–255.

29. **Noland, B. W., J. M. Newman, J. Hendle, J. Badger, J. A. Christopher, J. Tresser, M. D. Buchanan, T. A. Wright, M. E. Rutter, W. E. Sanderson, H. J. Muller-Dieckmann, K. S. Gajiwala, and S. G. Buchanan.** 2002. Structural studies of *Salmonella typhimurium* ArnB (PmrH) aminotransferase: a 4-amino-4-deoxy-L-arabinose lipopolysaccharide-modifying enzyme. Structure **10:**1569–1580.

30. **Sheng, H., J. Y. Lim, M. K. Watkins, S. A. Minnich, and C. J. Hovde.** 2008. Characterization of an *Escherichia coli* O157:H7 O-antigen deletion mutant and effect of the deletion on bacterial persistence in the mouse intestine and colonization at the bovine terminal rectal mucosa. Appl. Environ. Microbiol. **74:**5015–5022.

31. **Ferrero, R. L., J. M. Thiberge, M. Huerre, and A. Labigne.** 1998. Immune responses of specific-pathogen-free mice to chronic *Helicobacter pylori* (strain SS1) infection. Infect. Immun. **66:**1349–1355.

32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

33. **Philpott, D. J., D. Belaid, P. Troubadour, J. M. Thiberge, J. Tankovic, A. Labigne, and R. L. Ferrero.** 2002. Reduced activation of inflammatory responses in host cells by mouse-adapted *Helicobacter pylori* isolates. Cell. Microbiol. **4:**285–296.

34. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U. S. A. **98:**5116–5121.

35. **Grubman, A., M. Kaparakis, J. Viala, C. Allison, L. Badea, A. Karrar, I. G. Boneca, L. Le Bourhis, S. Reeve, I. A. Smith, E. L. Hartland, D. J. Philpott, and R. L. Ferrero.** 2010. The innate immune molecule, NOD1, regulates direct killing of *Helicobacter pylori* by antimicrobial peptides. Cell. Microbiol. **12:**626–639.

36. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J. Bacteriol. **174:**4212–4217.

37. **Wang, Y., K. P. Roos, and D. E. Taylor.** 1993. Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J. Gen. Microbiol. **139:**2485–2493.

38. **Smeets, L. C., J. J. Bijlsma, S. Y. Boomkens, C. M. Vandenbroucke-Grauls, and J. G. Kusters.** 2000. *comH*, a novel gene essential for natural transformation of *Helicobacter pylori*. J. Bacteriol. **182:**3948–3954.

39. **Ferrero, R. L., J. M. Thiberge, I. Kansau, N. Wuscher, M. Huerre, and A. Labigne.** 1995. The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. Proc. Natl. Acad. Sci. U. S. A. **92:**6499–6503.

40. **Morrison, S. L.** 2001. Transformation of *E. coli* by electroporation. Curr. Protoc. Immunol.

41. **Monod, J., G. Cohen-Bazire, and M. Cohn.** 1951. The biosynthesis of beta-galactosidase (lactase) in *Escherichia coli*; the specificity of induction. Biochim. Biophys. Acta **7:**585–599.

42. **Reeves, E. P., T. Ali, P. Leonard, S. Hearty, R. O'Kennedy, F. E. May, B. R. Westley, C. Josenhans, M. Rust, S. Suerbaum, A. Smith, B. Drumm, and M. Clyne.** 2008. *Helicobacter pylori* lipopolysaccharide interacts with TFF1 in a pH-dependent manner. Gastroenterology **135:**2043–2054.