BACTERIAL PATHOGENICITY

The *Helicobacter pylori* flbA flagellar biosynthesis and regulatory gene is required for motility and virulence and modulates urease of *H. pylori* and *Proteus mirabilis*

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*Helicobacter pylori* and *Proteus mirabilis* ureases are nickel-requiring metallo-enzymes that hydrolyse urea to NH₃ and CO₂. In both *H. pylori* and in an *Escherichia coli* model of *H. pylori* urease activity, a high affinity nickel transporter, NixA, is required for optimal urease activity, whereas the urea-dependent UreR positive transcriptional activator governs optimal urease expression in *P. mirabilis*. The *H. pylori* flbA gene is a flagellar biosynthesis and regulatory gene that modulates urease activity in the *E. coli* model of *H. pylori* urease activity. All flbA mutants of eight strains of *H. pylori* were non-motile and five had a strain-dependent alteration in urease activity. The flbA gene decreased urease activity 15-fold when expressed in *E. coli* containing the *H. pylori* urease locus and the nixA gene; this was reversed by disruption of flbA. The flbA gene decreased nixA transcription. flbA also decreased urease activity three-fold in *E. coli* containing the *P. mirabilis* urease locus in a urea- and UreR-dependent fashion. Here the flbA gene repressed the *P. mirabilis* urease promoter. Thus, FlbA decreased urease activity of both *H. pylori* and *P. mirabilis*, but through distinct mechanisms. *H. pylori* wild-type strain SS1 colonised gerbils at a mean of 5.4 × 10⁶ cfu/g of antrum and caused chronic gastritis and lesions in the antrum. In contrast, the flbA mutant did not colonise five of six gerbils and caused no lesions, indicating that motility mediated by flbA was required for colonisation. Because FlbA regulates flagellar biosynthesis and secretion, as well as forming a structural component of the flagellar secretion apparatus, two seemingly unrelated virulence attributes, motility and urease, may be coupled in *H. pylori* and *P. mirabilis* and possibly also in other motile, ureolytic bacteria.

Introduction

*Helicobacter pylori* causes gastritis [1], is strongly associated with the development of peptic ulcers [2] and constitutes a risk factor for gastric adenocarcinoma [3, 4]. The mechanisms behind the development of these diseases are not well understood.

Urease, which catalyses the hydrolysis of urea to CO₂ and NH₄⁺, is central to the pathogenesis of *H. pylori* infection and urease-negative mutants fail to colonise various animal models [5–7]. Because urease is a nickel-requiring enzyme, nickel transporters, such as NixA, are required for full urease activity in both *H. pylori* [8] and in an *Escherichia coli* model of *H. pylori* urease [9, 10]. *H. pylori* urease was thought initially to be constitutively expressed [11, 12], but mounting evidence suggests otherwise. Recently, a number of genes, including a flagellar biosynthesis and regulatory gene, flbA, was demonstrated to modulate urease activity [9]. Furthermore, urease protein levels [13] and activity [14, 15] are elevated under acidic conditions and nickel has been shown to activate *H. pylori* urease expression transcriptionally [16]. Thus, *H. pylori* may modulate urease activity *in vivo* in response to specific environmental cues.
Proteus mirabilis causes urinary tract infections including pyelonephritis and kidney stone formation, particularly in patients with indwelling catheters or structural abnormalities of the urinary tract [17–19]. Like H. pylori, the urease of P. mirabilis is required for virulence [20]. However, in contrast with H. pylori, P. mirabilis urease is activated transcriptionally by UreR [21] in the presence of urea [22]; no UreR homologues exist in the H. pylori genome [23, 24], and H. pylori urease is not urea-inducible [25]. UreR is transcribed from its own promoter and then activates the divergently transcribed ureDABCEFG urease operon [21]. E. coli carrying the P. mirabilis urease gene cluster also has urease activity that is inducible by urea [22] and requires UreR [26], but optimal urease activity does not require addition of a nickel transporter gene, as it does in E. coli containing the H. pylori urease gene cluster. Urea-induced expression of the P. mirabilis urease (ureD) promoter–lacZ transcriptional fusion is likewise dependent on a functionally intact UreR [26]. Clearly, P. mirabilis urease is regulated differently from that of H. pylori.

The flbA gene, which modulates H. pylori urease activity, is a cytoplasmic membrane protein of 80 kDa of the LcrD protein family that is thought to be a structural component of the flagellar secretion apparatus [9, 27, 28]. Although much in-vitro data suggest that FlbA homologues are involved in virulence [29–33], these have not been assessed in vivo for virulence. H. pylori motility is required for colonisation of gnotobiotic piglets [34, 35], mice [36] and gerbils [37]. However, in the gerbil study, undefined and non-isogenic non-motile variants of H. pylori were employed. Thus, no specific H. pylori flagellar biosynthesis gene has been tested for its role in virulence in the gerbil model.

A previously described isogenic flbA mutant of one strain of H. pylori had both loss of motility and elevated urease activity in a qualitative assay [27], and another study indicated that flbA significantly decreased urease activity and protein levels in E. coli containing the H. pylori urease gene cluster and the nixA nickel transporter gene (on pH8080) [9]. New mutants were generated by these studies. For example, urease activity and motility have not been quantified in flbA mutants of H. pylori, nor has the effect of flbA on urease of other motile bacterial species, such as P. mirabilis, been addressed. Furthermore, the in-vivo relevance of flbA homologues has not been demonstrated. The present study examined these matters.

Materials and methods

Bacterial strains, growth conditions, primers and plasmids

H. pylori strains (Table 1) were grown at 37°C on Campylobacter blood agar (CBA) containing defibri-
Table 1. Oligonucleotide primers, plasmids and bacterial strains

<table>
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<tr>
<th>Oligonucleotide primers</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>FlhA-F1</td>
<td>GCCGGATCCCTGGGAAACCGCCCTATAGAT</td>
<td>Forward primer; upstream region of <em>H. pylori</em> flhA</td>
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<tr>
<td>FlhA-R1</td>
<td>GCACAGATCGTGTTAAACCTGCATCTCC</td>
<td>Reverse primer; downstream of translational stop codon of <em>H. pylori</em> flhA</td>
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<tr>
<td>KanDM-F2</td>
<td>GCCGCAAGCTGATGATTTTT</td>
<td>Forward primer; 3’ end of aphA3</td>
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<td>KanDM-R2</td>
<td>CCAATCTACTGGTCTTGCAT</td>
<td>Reverse primer; 5’ end of aphA3</td>
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<tr>
<td>LacZ-R1</td>
<td>TGTGCTGAGAGGCGATTAAG</td>
<td>Reverse primer; 5’ end of lacZ; used for sequence confirmation of pRS415-ureAP and pLX2106-ureAP</td>
</tr>
<tr>
<td>NixA-F2</td>
<td>TTCGCGGGAGAAGGTTCAGCCCAACAAA</td>
<td>5’ <em>H. pylori</em> nix promoter primer</td>
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<tr>
<td>NixA-R2</td>
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<td>3’ <em>H. pylori</em> nix promoter primer</td>
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<td>UreDA-F1</td>
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<td>5’ <em>H. pylori</em> ureA promoter primer</td>
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<td>UreDA-R1</td>
<td>GGGAATACCTTCTTGGTTAGGTTTCTCTC</td>
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<th>Source or reference</th>
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<td>pBluescript II SK and KS (-)</td>
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<td>ColE1 ori; Ap'; cloning vectors</td>
<td>Stratagene New England Biolabs</td>
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<td>pBR322</td>
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<td>New England Biolabs</td>
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<td>pKKS303</td>
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<td>J. Kyle Hendricks</td>
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<td>phP1</td>
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<td>pRS-Kan</td>
<td>pBluescript II SK (-)</td>
<td>1.2-kb EcoRI aphA3 non-polar cassette from pHPl cloned into same site in pBluescript II SK (-)</td>
<td>H. Kleanthous, Acambas</td>
</tr>
<tr>
<td>pRS-flhA</td>
<td>pBluescript II SK (-)</td>
<td>ColE1 ori; Ap'; <em>H. pylori</em> 2.4 kb flhA gene including promoter cloned into the BamHI and ColI sites of pBluescript II SK (-)</td>
<td>This study</td>
</tr>
<tr>
<td>pRS-flhA:aphA3 (flhA3)</td>
<td>pRS-flhA</td>
<td>Ap'; Ks'; flhA disrupted by insertion of blunted 1.2-kb <em>aphA3</em> cassette from pHPl into NheI site (bp 727 within flhA coding sequence) in pRS-flhA</td>
<td>This study</td>
</tr>
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<td>pRS22-flhA</td>
<td>pBR322</td>
<td>PCR-amplified 2.4-kb flhA gene including promoter (primers FlhA-F1 and FlhA-R1) from pRS-flhA using Vent DNA polymerase and cloned into unique SpeI site of pRS22</td>
<td>This study</td>
</tr>
<tr>
<td>pRS22-flhA::aphA3 (flhA3)</td>
<td>pRS22-flhA</td>
<td>flhA disrupted by insertion of the 1.2-kb Smal-HincII <em>aphA3</em> cassette from pHPl into the unique SpeI site of pRS22</td>
<td>This study</td>
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<td>pMD1010</td>
<td>pBR322</td>
<td>ColE1 ori; Ap'; pBR322 encoding the entire <em>P. mirabilis</em> urease gene cluster</td>
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<td>pAR100ureD-lacZ</td>
<td>pBluescript II SK (-)</td>
<td>ColE1 ori; Ap'; encodes P. mirabilis ureR and a ureD-lacZ transcriptional fusion</td>
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<td>pCC038</td>
<td>pKKS303</td>
<td>p15A ori; Cm'; 2.4-kb SpeI-KpnI flhA gene including promoter from pRS22-flhA ligated to same sites in pKKS303</td>
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<td>pRS415-ureAP</td>
<td>pRS415</td>
<td><em>H. pylori</em> ureA promoter primer</td>
<td>This study</td>
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<td>ColE1 ori; Cm'; 11.3 kb; promoterless lacZ/I regulatory gene cluster</td>
<td>Xin Li</td>
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<td>pLX2106-ureAP</td>
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<td>600-bp PCR-amplified fragment (primers UXA-F2 and UXA-R2) containing the ureA promoter from pLX2106-ureAP</td>
<td>Susan Harrington</td>
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<td>pWSK29</td>
<td>pWSK29</td>
<td>2.4-kb BamHI and ColI flhA gene including promoter from pWSK29-flhA cloned into same sites in pLX2106-ureAP; transcriptional fusion</td>
<td>63</td>
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<td>pUEF201</td>
<td>pBluescript II SK (-)</td>
<td>nixA-R2 containing clone from <em>H. pylori</em> strain 43504</td>
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<td>pIP8000</td>
<td>pACYC184</td>
<td>nixA containing clone from strain UMAB41 and nixA cloned from strain 43504</td>
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continued overleaf
effects on adjacent downstream genes. Chromosomal DNA was isolated [41] from kanamycin-resistant transformants and was used to re-transform wild-type strains to remove potential background mutations. Because *H. pylori* urease activity decreases significantly upon in-vitro passage (D. J. McGee and H. L. T. Mobley, unpublished observations), first-passage transformants were used for urease extracts and transformants were passaged a second time for isolation of chromosomal DNA and subsequent PCR-based confirmation of mutants. The following PCR primer pairs were used (Table 1 and Fig. 1): FlhA-F1 and KanDM-R2 (1.2-kb product only in mutants), FlhA-R1 and KanDM-F2 (1.8-kb product only in mutants), FlhA-F1 and FlhA-R1 (2.4-kb product in wild-type, 3.6-kb product in mutants). PCR conditions were: 94°C for 5 min (first cycle only), 30 cycles of 94°C for 60 s, 60°C for 90 s, 72°C for 90 s, followed by extension for 5 min at 72°C. The expected size product (or lack of product) was obtained in all cases (data not shown).

![Fig. 1.](image)

**Table 1.** (continued)

<table>
<thead>
<tr>
<th>Bacterial strains</th>
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<td>American Type Culture Collection</td>
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<td>SE5000</td>
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<td><em>P. mirabilis</em></td>
<td>Wild-type, pyelonephritis strain</td>
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<td>H4320</td>
<td>Wild-type, genome sequenced, non-motile variant</td>
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<tr>
<td>26695</td>
<td>Wild-type, type strain</td>
<td>American Type Culture Collection</td>
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<tr>
<td>ATCC 43504</td>
<td>Wild-type, clinical isolate obtained by biopsy from a patient with suspected gastritis at the University of Maryland Hospital, Baltimore, MD</td>
<td>This study</td>
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<td>368</td>
<td>Doodeinal ulcer isolate, cagT (–)</td>
<td>Richard Peek</td>
</tr>
<tr>
<td>J75</td>
<td>Gastritis isolate, cagT (+)</td>
<td>Richard Peek</td>
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<tr>
<td>J166</td>
<td>Gastritis isolate, cagT (+)</td>
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<td>B194A</td>
<td>Gastritis isolate, cagT(+)</td>
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<tr>
<td>SS1</td>
<td>Wild-type mouse-adapted strain</td>
<td>Adrian Lee</td>
</tr>
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<td>UMAAB41</td>
<td>Wild-type, clinical isolate obtained by biopsy from a patient with suspected gastritis at the University of Maryland Hospital, Baltimore, MD</td>
<td>45</td>
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</tbody>
</table>

![Fig. 1.](image)

**ROLE OF flhA IN VIRULENCE, MOTILITY AND UREASE**
Urease extract preparations, protein determinations and urease activity determinations

For *H. pylori* [42] or for *E. coli* containing the *H. pylori* urease gene cluster [9], extracts were prepared and protein concentration was determined as described. The phenol hypochlorite assay for urease activity was as described previously [9, 42]. For *P. mirabilis* or for *E. coli* containing the *P. mirabilis* urease gene cluster, extracts were prepared and measured for protein concentration and for urease activity by the phenol red urease assay as described previously [25].

β-galactosidase activity determinations

*E. coli* cells were grown to mid-exponential phase with appropriate antibiotics as described above. β-Galactosidase activity was determined by the method of Miller [43].

Inoculation of gerbils, tissue processing and recovery of *H. pylori*

Animal experiments were performed at the University of Maryland, with the approval of the Institute Animal Care and Use Committee. Male Mongolian gerbils (*Meriones unguiculatus*; Charles River) were inoculated twice orally (2 days apart) with 50 ml of F12-broth-grown *H. pylori* strains (SS1 background) suspended in sterile PBS (pH 7.4) to 10⁷ viable cfu/ml. Control animals received PBS. At 4 weeks after infection, animals were anaesthetised (Avertin, 125 mg/kg), exsanguinated by cardiac puncture and euthanased by cervical dislocation. Stomachs were removed, dissected longitudinally along the greater curvature and washed several times in sterile PBS. The antrum was dissected into two halves. One was weighed and then homogenised (Ultra-Turrax T25, IKA Works) in 1 ml of sterile PBS. The other half was fixed in formalin 10% for histology. Antrum homogenates and dilutions of them in PBS were plated for viable counts in triplicate on CBA containing nalidixic acid 10 µg/ml, vancomycin 10 µg/ml, amphotericin B 2 µg/ml, bacitracin 30 µg/ml, polymyxin B 10 U/ml and trimethoprim 10 µg/ml to suppress normal flora.

Histology

Antrum sections were embedded in paraffin, sectioned (5 μm), stained with haematoxylin and eosin and evaluated in a blind fashion.

Statistical analysis of data

Statistical analyses of urease and β-galactosidase activities were calculated by the alternative Welch’s *t* test; statistical analysis of colonisation data was made by the Mann-Whitney *t* test. Instat 2.03 software (GraphPad Software, San Diego, CA, USA) was employed. *p* < 0.05 was considered statistically significant.

Results

Effect of flbA on *H. pylori* motility

To understand the role of *flbA* in *H. pylori* motility, urease activity and virulence, *flbA* mutants were generated in nine strains by two different strategies (Table 1, Fig. 1 and Materials and methods). All *flbA* mutants tested in strain backgrounds SS1, UMAB41, 43504, HPDJM17, J68, J75, B194A and J166 were non-motile on F12-modified soft agar, in contrast to the corresponding wild-type strain. It was confirmed that wild-type strain 26695 was non-motile [44]; this strain could not be distinguished from the *flbA* mutant in the soft agar assay. However, a revertant of wild-type strain 26695, designated as 26695m, was isolated that was motile in the soft agar assay.

Urease activity of *H. pylori* *flbA* mutants

Eleven of 14 *flbA* mutants of strain UMAB41 [45] and two of three *flbA* mutants of strain 43504 had elevated urease activity compared with the corresponding wild-type strain (Fig. 2a and b, respectively; representatives shown). In contrast, *flbA* mutants of strains 26695 (21 of 31 mutants tested) and HPDJM17 (4 of 4 mutants tested), showed reduced urease activity (Fig. 2c and d, respectively; representatives shown), whereas *flbA* mutants of the fresh clinical isolate J75 had no detectable urease activity (2 of 2 mutants tested; Fig. 2e). Other strains of *H. pylori* containing the *flbA* mutation exhibited no effect on urease activity: strain SS1 (3 of 3), fresh clinical isolates J68 (2 of 2), B194A (3 of 3), and J166 (3 of 3). The urease data were consistent regardless of the construct (*flbA1* or *flbA2*) used to obtain the *flbA* mutants. Two-thirds (42 of 65) of the *flbA* mutants and five of nine *H. pylori* strain backgrounds showed changed urease activity, but whether this was an increase or decrease was clearly strain-dependent. Consistent data were obtained for wild-type and mutants of a single strain. The second observation from these experiments was that urease activity among wild-type *H. pylori* strains varied widely, ranging from 7000 units for strain 26695 to 55 000 units for strain HPDJM17 (Fig. 2a–e).

*H. pylori* *flbA* effect on urease activity in *E. coli* containing genes for *H. pylori* urease and NixA nickel transporter

Because *flbA* caused a strain-dependent modulation of urease activity in *H. pylori*, it would be difficult to decipher the reasons for the strain differences without extensive genetic characterisation of them. Therefore, an *E. coli* model of *H. pylori* urease activity was used, in which genetic variability could be minimised. In this model, the *H. pylori* urease gene cluster and the nixA
Fig. 2. Urease activity of wild-type and flbA mutants of H. pylori. Extracts of wild-type and isogenic flbA mutants of blood agar-grown H. pylori were measured for urease activity by the phenol-hypochlorite urease assay. Data are given as urease specific activity (nmol NH₄⁺/min/mg of protein) and SD. Representative flbA mutants are shown, each of which was a separate transformant. #Transformant number. *Except where noted, p < 0.05 compared with the corresponding wild-type strain. (a) Strain UMAB41 and isogenic flbA mutants. Data shown are duplicate or triplicate samples from one experiment representative of three. Each experiment was a separate transformation procedure. (b) Strain 43504 and isogenic flbA mutants. Data shown are duplicate or triplicate samples from one experiment. (c) Strain 26695 and isogenic flbA mutants. Data shown are duplicate or triplicate samples from one experiment representative of five. Each experiment was a separate transformation procedure. (d) Fresh clinical isolate HPDJM17 and isogenic flbA mutants. Data shown are duplicate or triplicate samples from one experiment. (e) Fresh clinical isolate J75 and isogenic flbA mutants. Data shown are duplicate samples of two independent mutants from one experiment representative of two. Each experiment was a separate transformation procedure. p = 0.0001 between wild-type and flbA mutant.
Effect of flbA on the H. pylori urease promoter and on expression of the nixA promoter

The flbA gene may decrease urease activity by decreasing promoter activity of urease or nixA within pH8080, or by increasing turnover of urease subunits. It was shown previously that flbA decreased expression of the urease subunits UreA and UreB, supporting the increased turnover model [9]. To address the other two possibilities, flbA was transformed into E. coli containing nixA promoter- or urease promoter-lacZ transcriptional fusion plasmids (Table 1) and the resultant strains were assayed for β-galactosidase activity. H. pylori flbA had no effect on the H. pylori urease promoter in E. coli strain MC1061 containing pKS415-ureAP (urease promoter-lacZ fusion) and pCC038 (Table 1; low copy plasmid harbouring flbA); the mean β-galactosidase activity was 1275 Miller Units in MC1061 (pKS415-ureAP/pCC038) versus 1529 Miller Units in the vector control strain, MC1061 (pKS415-ureAP/pKHKS303) (p > 0.05). Similarly, no differences were observed when the same constructs were transformed into E. coli strain DH5α. No β-galactosidase activity was observed when the H. pylori urease promoter was omitted from the construct (as plasmid pRS415). In contrast, the flbA gene significantly decreased expression of the nixA promoter in E. coli MC1061 (pLX2106-nixAP) by about three-fold (p < 0.0001) (Fig. 4). No β-galactosidase activity was observed when the H. pylori nixA promoter was omitted from the construct (as plasmid pLX2106).

Effect of flbA on P. mirabilis urease activity in E. coli

Because flbA decreased urease activity of some H. pylori strains, it was of interest to investigate the effect of flbA on other bacterial ureases. The P. mirabilis urease was chosen as a model because urease regulation is well understood in this system [7]. The P. mirabilis urease gene cluster has a positive transcriptional activator of urease gene expression, UreR, which is divergently transcribed from the rest of the ureDABCEFG operon only in the presence of urea. Plasmid pCC038, containing flbA in low copy, was transformed into E. coli DH5α harbouring pMID1010, which encodes the entire P. mirabilis urease gene cluster. Transformants were grown in the presence of 100 mM urea and assayed for urease activity. Urease activity was decreased seven-fold when compared with the vector control-containing strain, DH5α (pKHKS303/pMID1010) (p < 0.001) (Fig. 5a). Only very low basal levels of urease activity were observed for both strains cultured in the absence of urea.

![Fig. 3](image-url) Effect of H. pylori flbA on urease activity in an E. coli model of H. pylori urease. E. coli strain SE5000 containing pH8080 (H. pylori urease generating system) and the constructs listed in the figure were grown overnight in M9 minimal medium containing 1 μM nickel chloride and urease activities of cytosolic extracts were determined by the phenol-hypochlorite urease assay. Data are given as urease specific activity (nmol NH₄⁺/min/mg of protein) and SD and are the average of at least three experiments each conducted in duplicate or triplicate. *p < 0.0001 compared with the vector control strain and with the flbA::aphA3 vector-containing strain.

![Fig. 4](image-url) Effect of flbA on the nixA promoter by β-galactosidase assays in E. coli. E. coli strain MC1061 containing pLX2106-nixAP plus either pBS or two independent, confirmed transformants containing pBS-flbA, were grown to mid-log phase in M9 minimal medium and β-galactosidase activity was determined. Data shown are representative of two experiments each conducted in triplicate. The average β-galactosidase activity in Miller Units and SD are shown. *p < 0.0001 compared with the vector control strain. #Transformant number.
Fig. 5. Effect of flbA on urease of *P. mirabilis*. (a) Effect of flbA on urease activity of *E. coli* (pMID1010). *E. coli* strain DH5α containing pMID1010 (*P. mirabilis* urease generating system) and the constructs listed in the figure were grown to mid-log phase in L broth and the urease promoter was induced by growth with 100 mM urea for 1 h, or left un-induced. Supernatant (cytosolic) extracts from French-pressed lysates were assayed for urease activity by the phenol red assay. Data shown are representative of six experiments each conducted in triplicate. The average urease activity and 2 SD are shown. *p < 0.001 compared with the vector control strain. (b) Effect of flbA on urease activity of *P. mirabilis*. *P. mirabilis* HI4320 or HI4320 containing the constructs listed in the figure were grown and processed as described in Fig. 5a. Data shown are representative of three experiments each conducted in triplicate. Urease activity from vector-free HI4320 (average 35,000 nmol NH₄⁺/min/mg of protein) was set to 100%. The percent decrease from vector-free HI4320 was calculated by the following formula: 100 \[(urease activity of vector-free HI4320) – (urease activity of vector-containing or flbA-containing HI4320)\]/urease activity of vector-free HI4320. The average and 2 SD are shown. *p < 0.001 compared with HI4320 and with HI4320 (pKHKS303). (c) Effect of flbA on the *P. mirabilis* urease promoter. *E. coli* strain DH5α containing the *P. mirabilis* urease promoter (as construct pCR10bareD-lacZ) and the constructs listed were grown as described in Fig. 5a and β-galactosidase activity was determined. Data shown are representative of three experiments each conducted in triplicate. The average β-galactosidase activity in Miller Units and 2 SD are shown. *p < 0.001 compared with the vector control strain.
Effect of flbA on urease activity in *P. mirabilis* HI4320

When grown in the presence of 100 mM urea, *P. mirabilis* HI4320 (pCC038), which contains flbA, produced 20% less urease activity than *P. mirabilis* containing the vector control, pKHKS303 (Fig. 5b). This suggested that the flbA gene product of *H. pylori* repressed the expression of urease produced by *P. mirabilis*. Only very low basal levels of urease activity were observed for both strains when cultured in the absence of urea.

Effect of flbA on P. mirabilis ureD promoter expression in E. coli

To determine whether flbA repressed the *P. mirabilis* urease promoter, plasmid pCC038 containing flbA or the vector control, pKHKS303, was transformed into *E. coli* DH5α harbouring plasmid pAR10bureD-lacZ, which encodes the *P. mirabilis* ureD promoter transcriptionally fused to lacZ*Y* [26]. This plasmid also has the functional ureR gene, which is required for expression of the ureD promoter. β-Galactosidase activity from urea-induced cultures of DH5α (pAR10bureD-lacZ/pCC038) was decreased 4–5-fold (p <0.001), as compared with the vector control strain, DH5α (pAR10bureD-lacZ/pKHKS303) (Fig. 5c). Only nominal basal levels of β-galactosidase activity were detected in the uninduced controls grown in the absence of urea. This suggested that the flbA-mediated decrease of urease activity was dependent on urea and a functional UreR.

Requirement for flbA for colonisation of gerbils by *H. pylori*

Previous work based solely on in-vitro data has speculated that flbA homologues are important in virulence [29–33]. To determine whether flbA was important for virulence *in vivo*, gerbils were inoculated with either wild-type *H. pylori* strain SS1, the isogenic flbA mutant or sterile buffer. Of six animals inoculated with either wild-type SS1, the isogenic flbA mutant was colonised and this one animal had a mean of only 5.5 × 10³ cfu/g of antrum, barely above the detection limit of 9 × 10² cfu/g. Lack of colonisation by the flbA mutant was not due to loss of urease activity, because the flbA mutant of strain SS1 had wild-type urease activity. No *H. pylori* or Helicobacter-like organisms were recovered from animals inoculated with buffer alone. These results indicated that flbA was required for *H. pylori* to colonise gerbils.

Occurrence of chronic gastritis and ulcers in antral tissue from gerbils infected with wild-type *H. pylori* and the flbA mutant

The antrum from gerbils inoculated with wild-type *H. pylori* strain SS1 exhibited micro-ulcer formation (three of six animals) (Fig. 6a), lymphoid follicle formation and lymphocytic infiltration (one of six animals) (Fig. 6b), disruption of the ordered gastric pit and glandular architecture (six of six animals) and small foci of necrosis. In contrast, the antra from gerbils inoculated with the flbA mutant (six of six animals) (Fig. 6c) or sterile buffer (Fig. 6d) exhibited no lesions.

Discussion

*H. pylori* FlbA is a cytoplasmic membrane protein that is required for motility and virulence and acts as an urease-decreasing factor in *E. coli* models containing either the *H. pylori* or the *P. mirabilis* urease gene clusters. Although FlbA affects the urease of both human pathogens, the mechanisms are distinct due to dissimilarities in the regulation of the urease gene clusters (Table 3). For *P. mirabilis*, the urease gene cluster is activated by the transcriptional regulator UreR in the presence of urea and a high affinity nickel transporter is not required for optimal urease activity in the *E. coli* model (Table 3). When provided in trans, *H. pylori* flbA decreased urease activity in *E. coli* (pMID1010) – i.e., with the urease-generating system of *P. mirabilis* – and in wild-type *P. mirabilis* HI4320 in the presence of urea and UreR (Fig. 5a and b) by specifically repressing transcription from the *P. mirabilis* urease promoter (Fig. 5c). In contrast with *P.

Table 2. Role of *H. pylori* flbA in colonisation of gerbils

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>mean cfu/g of antrum</th>
<th>flbA mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.9 × 10⁷</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.8 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&lt;9.0 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.2 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.9 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6.8 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>5.4 × 10⁷(7.3 × 10⁵)</td>
<td>Mean (SD)</td>
</tr>
</tbody>
</table>

Animals were inoculated with either *H. pylori* wild-type strain SS1 or its isogenic flbA mutant and antrum samples were processed as described in Materials and methods. Homogenised antrum samples were plated in triplicate.

¹No *H. pylori* recovered. Limit of detection was 9 × 10² cfu/g of antrum.

²*p = 0.027* between wild-type SS1 and the flbA mutant.
mirabilis urease, the \textit{H. pylori} urease is not regulated by a UreR homologue nor by urea, but requires the \textit{NixA} high affinity nickel transporter for urease activity in the \textit{E. coli} model [9, 10] (summarised in Table 3). In the presence of \textit{flbA}, urease activity was almost abolished in \textit{E. coli} containing the \textit{H. pylori} urease gene cluster (Fig. 3). However, this was not due to repression of the urease promoter as it was for \textit{P. mirabilis}, but rather of the \textit{nixA} promoter (Fig. 4).

Because \textit{NixA} is required for urease activity in the \textit{E. coli} model of \textit{H. pylori} urease, it is believed that the \textit{flbA}-mediated decrease in \textit{H. pylori} urease activity is due to decreased \textit{nixA} expression, whereby less nickel would be delivered to apo-urease. This may render the protein more susceptible to proteolytic degradation and would explain the observation that the urease structural subunits \textit{UreA} and \textit{UreB} are markedly reduced in \textit{E. coli} containing \textit{flbA} and PHP8080 [9].

The contrasting mechanisms of \textit{flbA}-mediated modulation of urease in \textit{H. pylori} and \textit{P. mirabilis} may reflect the differences in the importance of a high affinity nickel transporter (Table 3) and the distinct niches that these two organisms occupy in vivo. \textit{H. pylori}, which has very few regulatory genes [23, 24], may exert regulatory control of gene expression through more subtle mechanisms than observed for organisms with larger genomes and more regulatory genes such as \textit{P. mirabilis}. \textit{H. pylori} is found in a very low nickel environment (0.1–0.5 \textit{µg/g} \textit{L} in serum and presumably in similar amounts in the gastric milieu [46–48]) and thus has evolved the high affinity nickel transporter \textit{NixA} for optimal delivery of nickel to apo-urease. In contrast, \textit{P. mirabilis} resides in the urinary tract, where nickel concentrations are about 10-fold higher (1–3 \textit{µg/L} [47, 49]) and thus a high affinity nickel transporter is unnecessary.

Although both \textit{H. pylori} and \textit{P. mirabilis} ureases were examined in \textit{E. coli} models that were optimised for urease activity, urease activities in both models were significantly lower (10–30-fold for \textit{H. pylori} urease,

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\hline
\textit{H. pylori} & ureABIEFGH & FlbA, pH, NikR & Yes & Serum/gastric milieu: 0.1–0.5 \textit{µg/L} & \textit{NixA} expression repressed \\
\textit{P. mirabilis} & ureRDABCEFG & H-NS (repressor), UreR + urea (activator), FlbA (repressor) & No & Urine: 1–3 \textit{µg/L} & Urease promoter repressed \\
\hline
\end{tabular}
\caption{Differences in urease properties and \textit{flbA}-mediated modulation of urease in \textit{H. pylori} and \textit{P. mirabilis}}
\end{table}
In addition to the evidence for decreased urease activity by \( \text{flbA} \) in the \( E.\ coli \) models described above, evidence was also obtained that \( \text{flbA} \) played a significant role in urease modulation in \( H.\ pylori \) itself. Some \( H.\ pylori \) \( \text{flbA} \) mutants in some strain backgrounds had elevated urease levels, whereas \( \text{flbA} \) mutants of other strain backgrounds had a decrease or loss in urease activity (Fig. 2) [27]. This suggested that urease regulation differs among \( H.\ pylori \) isolates. This observation was complicated by the finding that \( H.\ pylori \) urease activity decreased (50–90%, depending on the strain) by the tenth in-vitro passage in nearly all strains, regardless of whether \( \text{flbA} \) was present or not (D. J. McGee and H. L. T. Mobley, unpublished observations). This problem was minimised by transforming DNA from \( \text{flbA} \) mutants back into the wild-type strain to remove background mutations, and by measuring urease activity from first-passage transformants. Differences in urease activity between wild-type and \( \text{flbA} \) mutants of various \( H.\ pylori \) strains may be explained by the observations of high mutation frequency leading to genetic variability [51–54] of urease, \( \text{flbA} \) or \( \text{nixA} \), or to numerous strain-specific genes [23]. For example, recent studies have found: two different \( cag+ \) strains [55] exhibiting different effects on interleukin-8 production by gastric epithelial cells; strain differences with respect to urease activity in \( \text{nixA} \) mutants [8, 56]; and strain differences in arginase and urease activity (Fig. 2a–e) [25, 42, 57]. Clearly, phenotypic variation of \( H.\ pylori \) strains can be explained by genetic variability leading to genetic variability [51–54] of urease, \( \text{flbA} \) or \( \text{nixA} \) or to numerous strain-specific genes [23].

This study confirmed the original observation of Schmitz et al. [27] that \( \text{flbA} \) is required for \( H.\ pylori \) motility and extended it by using more strains and by employing a transparent soft agar containing F-12, a chemically defined medium that supports the growth of \( H.\ pylori \) [38]. The homologous gene \( \text{flbA} \) is likewise required for motility in \( P.\ mirabilis \) [58]. Because inhibition of \( H.\ pylori \) urease activity by urease inhibitors abolishes motility and chemotaxis through a viscous medium, the proton motive force required for flagellar movement may be generated by the hydrolysis of urea [15, 59–61]. Indeed, urease-negative \( H.\ pylori \) mutants fail to swarm on motility agar [61]. These data, taken together with those of the present study, suggest that \( \text{flbA} \) alters urease activity in both \( H.\ pylori \) and \( P.\ mirabilis \) and provides a crucial link between two virulence attributes in both human pathogens—urease and motility.

The \( \text{flbA} \) gene was required for \( H.\ pylori \) to colonise gerbils. This is the first demonstration of a specific flagellar biosynthesis gene being required for \( H.\ pylori \) colonisation of gerbils. One other study suggested that motility is important for colonisation, but the allflagellate variant was of an undefined mutation, was not isogenic with the wild-type strain, and the mutation could potentially be reversible [37]. Notably, no lesions were observed in the antrum of gerbils inoculated with the \( \text{flbA} \) mutant, whereas lesions of gastritis were common in gerbils infected with wild-type \( H.\ pylori \).

The \( H.\ pylori \) strains used for the gerbil study were \( \text{SS1} \) and the isogenic \( \text{flbA} \) mutant, which have identical urease activities. Thus, the lack of colonisation by the \( \text{flbA} \) mutant was not due to altered urease activity. Attempts to complement the mutant have so far been unsuccessful. Other \( H.\ pylori \) mutants that affect flagellar biosynthesis are likewise severely attenuated in other animal models [34–36], emphasising the important role of motility in enabling \( H.\ pylori \) to penetrate the viscous mucous layer to adhere to the gastric epithelial cell surface and avoid the harsh gastric acidity through urease activity.

In summary, the flagellar biosynthesis and regulatory gene \( \text{flbA} \) was shown to modulate urease of both \( H.\ pylori \) and \( P.\ mirabilis \), but this modulation was by distinct mechanisms. \( \text{flbA} \) was required for motility and for virulence in \( H.\ pylori \).

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**References**


