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

DAVID J. McGEE, CHRISTOPHER COKER*, TRACI L. TESTERMAN, JANETTE M. HARRO*, SUSAN V. GIBSON† and HARRY L. T. MOBLEY*

*Departments of Microbiology & Immunology and †Comparative Medicine, University of South Alabama College of Medicine, Mobile, AL 36688 and *Department of Microbiology & Immunology, University of Maryland School of Medicine, Baltimore, MD 21201, USA*

Helicobacter pylori **and** ***Proteus mirabilis*** **ureases are nickel-requiring metallo-enzymes that hydrolyse urea to NH_3 and CO_2. 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 5.4×10^6 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 CO_2 and NH_4^+, 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.

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Corresponding author: Dr D. J. McGee (e-mail:dmcgee@jaguar1.usouthal.edu).

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 pHP8080) [9]. New matters 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 defibrinated sheep blood 10% v/v in a CO_2 5% incubator with 100% humidity for 2 days. Alternatively, *H. pylori* was grown on F-12 agar or in F-12 broth containing fetal bovine serum 4% [38]. Kanamycin (5–20 μg/ml) was added to the growth medium for selection and maintenance of transformants. *E. coli* and *P. mirabilis* strains (Table 1) were grown on Luria (L) agar and in L broth plus appropriate antibiotics at 37°C. For urease assays with *E. coli* containing the *H. pylori* urease gene cluster on pHP8080, bacteria were grown in M9 minimal medium as described previously [9]. For urease assays with *E. coli* containing the *P. mirabilis* urease gene cluster on pMID1010, bacteria were grown in L broth to mid-log phase, and urea (100 mM) was added to induce expression of the urease promoter in the presence of UreR (1 h). Cultures grown in the absence of urea served as uninduced controls. For β-galactosidase assays with the *H. pylori* urease or *nixA* promoters, *E. coli* strains were grown to mid-log phase in M9 minimal medium. For β-galactosidase assays with the *P. mirabilis* urease promoter, *E. coli* strains were grown in L broth to mid-log phase and urea (100 mM) was added to induce urease promoter expression.

Oligonucleotide primers used for PCR and sequence analyses are listed in Table 1. Plasmids (detailed in Table 1) were constructed by standard molecular biology techniques [39, 40]. Constructs were verified by restriction endonuclease digestions or PCR analyses, or both, and subsequently confirmed by sequence analysis of restriction endonuclease site junctions. Plasmids pBS-*flbA*, pBR322-*flbA* and their corresponding kanamycin disruption constructs are represented diagrammatically in Fig. 1.

Motility assay

Motility was assessed on F-12 soft agar. F-12 powder mix (Life Technologies) was reconstituted to a 2× stock, filter sterilised and mixed with an equal volume of Bacto agar (0.7%). Fetal bovine serum was added to a final concentration of 4%. Strains were inoculated by stabbing the agar and the plates were incubated at 37°C for several days in a CO_2 5% incubator with 100% humidity. Strains were considered motile if they moved away from the initial stab within 2–3 days, whereas non-motile strains remained at the inoculation site.

Construction and confirmation of H. pylori isogenic mutants of flbA

H. pylori strains were electroporated (800 ohms, 2.5 kV, 25 μF; Gene Pulser II, BioRad, Hercules, CA, USA) with insertionally inactivated *flbA* to generate *flbA1* mutants (derived from pBS-*flbA::aphA3*) or *flbA2* mutants (derived from pBR322-*flbA::aphA3*) (Fig. 1 and Table 1). Two different *flbA* mutant constructs were employed to ensure reproducibility of the data. The kanamycin cassette was non-polar, thereby minimising

Table 1. Oligonucleotide primers, plasmids and bacterial strains

Oligonucleotide primers	Sequence (5′–3′)	Remarks
FlhA-F1	GCGCGGATCCGTGGCAAACGCCTTAATGAT (*Bam*HI site underlined)	Forward primer; upstream region of *H. pylori flbA*
FlhA-R1	GCGCATCGATTGGTAAACTTGCATCATTCTCC (*Cla*I site underlined)	Reverse primer; downstream of translational stop codon of *H. pylori flbA*
KanDM-F2	GCGCGAGCTGTATGATTTTT	Forward primer; 3′ end of *aphA3*
KanDM-R2	CCAATTCACTGTTCCTTGCAT	Reverse primer; 5′ end of *aphA3*
LacZ-R1	TGTGCTGCAAGGCGATTAAG	Reverse primer; 5′ end of *lacZ*; used for sequence confirmation of pRS415-*ureAP* and pLX2106-*nixAP*
NixA-F2	TCCCCCGGGGGAGAAGGTTCAGCCCAACAAA (*Sma*I site underlined)	5′ *H. pylori nixA* promoter primer
NixA-R2	CGGGATCCCGCAATTTCACAACACGCCTTT (*Bam*HI site underlined)	3′ *H. pylori nixA* promoter primer
UreDA-F1	GCGAATTCGAAATACTTGCAAATCCTTTTGA (*Eco*R1 site underlined)	5′ *H. pylori ureA* promoter primer
UreDA-R1	GCGGATCCTCTTTTGGGGTGAGTTTCATC (*Bam*HI site underlined)	3′ *H. pylori ureA* promoter primer

Plasmids	Parent	Description	Source or reference
pBluescript II SK and KS (–)		ColE1 *ori*; Ap^r; cloning vectors	Stratagene
pACYC184		p15A *ori*, Cm^r, Tc^r; low copy cloning vector	New England Biolabs
pBR322		ColE1 *ori*; Ap^r ; Tc^r; cloning vector	New England Biolabs
pKHKS303	pBluescript II KS (–)	p15A *ori*; Cm^r; low copy cloning vector with blue-white screen	J. Kyle Hendricks
pHP1	pUC19	ColE1 *ori*; Ap^r; Kn^r; source of *aphA3* non-polar cassette	H. Kleanthous, Acambis
pBS-Kan	pBluescript II SK (–)	1.2-kb *Eco*RI *aphA3* non-polar cassette from pHP1 cloned into same site in pBluescript II SK (–)	This study
pBS-*flbA*	pBluescript II SK (–)	ColE1 *ori*; Ap^r; *H. pylori* 2.4 kb *flbA* gene including promoter cloned into the *Bam*HI and *Cla*I sites of pBluescript II SK (–)	9
pBS-*flbA::aphA3* (*flbA1*)	pBS-*flbA*	Ap^r; Kn^r; *flbA* disrupted by insertion of blunted 1.2-kb *aphA3* cassette from pHP1 into *Nhe*I site (bp 727 within *flbA* coding sequence) in pBS-*flbA*	This study
pBR322-*flbA*	pBR322	PCR-amplified 2.4-kb *flbA* gene including promoter (primers FlhA-F1 and FlhA-R1) from pBS-*flbA* using Vent DNA polymerase and cloned into unique *Ssp*I site of pBR322	This study
pBR322-*flbA::aphA3* (*flbA2*)	pBR322-*flbA*	*flbA* disrupted by insertion of the 1.2-kb *Sma*I-*Hin*cII *aphA3* cassette from pBS-Kan into the unique *Ssp*I site (bp 669 within *flbA* coding sequence) in pBR322-*flbA*	This study
pMID1010	pBR322	ColE1 *ori*, Ap^r; pBR322 encoding the entire *P. mirabilis* urease gene cluster	21
pΔR10b*ureD-lacZ*	pBluescript II SK (–)	ColE1 *ori*; Ap^r; encodes *P. mirabilis ureR* and a *ureD-lacZ* transcriptional fusion	26
pCC038	pKHKS303	p15A *ori*, Cm^r; 2.4-kb *Spe*I-*Kpn*I *flbA* gene including promoter from pBS-*flbA* ligated to same sites in pKHKS303	This study
pRS415	pBR322	ColE1 *ori*; Ap^r; 10.8 kb; promoterless *lacZYA*	62
pRS415-*ureAP*	pRS415	651-bp PCR-amplified fragment (primers UreDA-F1 and UreDA-R1) containing the urease promoter, with pHP8080 as template, and cloned into the *Bam*HI/*Eco*RI sites of pRS415 (transcriptional fusion)	This study
pLX2106	pACYC184 and pRS415	p15A *ori*; Cm^r; 11.3 kb; promoterless *lacZYA* from pRS415 cloned into *Eag*I and *Eco*RV sites in pACYC184; has *Bam*HI and *Sma*I cloning sites upstream of *lacZYA*	Xin Li
pLX2106-*nixAP*	pLX2106	606-bp PCR-amplified fragment (primers NixA-F2 and NixA-R2) containing the *nixA* promoter from pUEF201 (contains *nixA* from *H. pylori* strain 43504) cloned into *Sma*I and *Bam*HI sites of pLX2106 (transcriptional fusion)	Susan Harrington
pWSK29		pSC101 *ori*, Ap^r; low copy cloning vector; 5.4 kb	63
pWSK29-*flbA*	pWSK29	2.4-kb *Bam*HI and *Cla*I *flbA* gene including promoter from pBS-*flbA* cloned into same sites in pWSK29	This study
pUEF201	pBluescript II SK (–)	*nixA*-containing clone from *H. pylori* strain 43504	10
pHP8080	pACYC184	*H. pylori* urease gene cluster cloned from strain UMAB41 and *nixA* cloned from strain 43504	9

continued overleaf

Table 1. (*continued*)

Bacterial strains	Relevant genotype or description	Source or reference
E. coli		
DH5α	*F*$^-$*supE44*, Δ*lacU169* (ϕ80 *lacZ* Δ*M15*), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*	Bethesda Research Laboratories
MC1061	*araD*139, Δ(*ara-leu*)7696, Δ(*lac*)174, *galU*, *galK*, *rpsL*, *thi-1*, *hsdR2* (r_k-m_k+), *mcrB1*	American Type Culture Collection
SE5000	F$^-$, *araD*139, Δ(*argF-lac*)*U169*, *rpsL*, *relA*1, *ptsF*25, *rbsR*, *flbB*5301, *recA*56	64
P. mirabilis		
HI4320	Wild-type, pyelonephritis strain	17
H. pylori		
26695	Wild-type, genome sequenced, non-motile variant	24
ATCC 43504	Wild-type, type strain	American Type Culture Collection
HPDJM17	Wild-type, clinical isolate obtained by biopsy from a patient with suspected gastritis at the University of Maryland Hospital, Baltimore, MD	This study
J68	Duodenal ulcer isolate, *cag*(−)	Richard Peek
J75	Gastritis isolate, *cag*(−)	Richard Peek
J166	Duodenal ulcer isolate, *cag*(+)	Richard Peek
B194A	Gastritis isolate, *cag*(+)	Richard Peek
SS1	Wild-type mouse-adapted strain	Adrian Lee
UMAB41	Wild-type, clinical isolate obtained by biopsy from a patient with suspected gastritis at the University of Maryland Hospital, Baltimore, MD	45

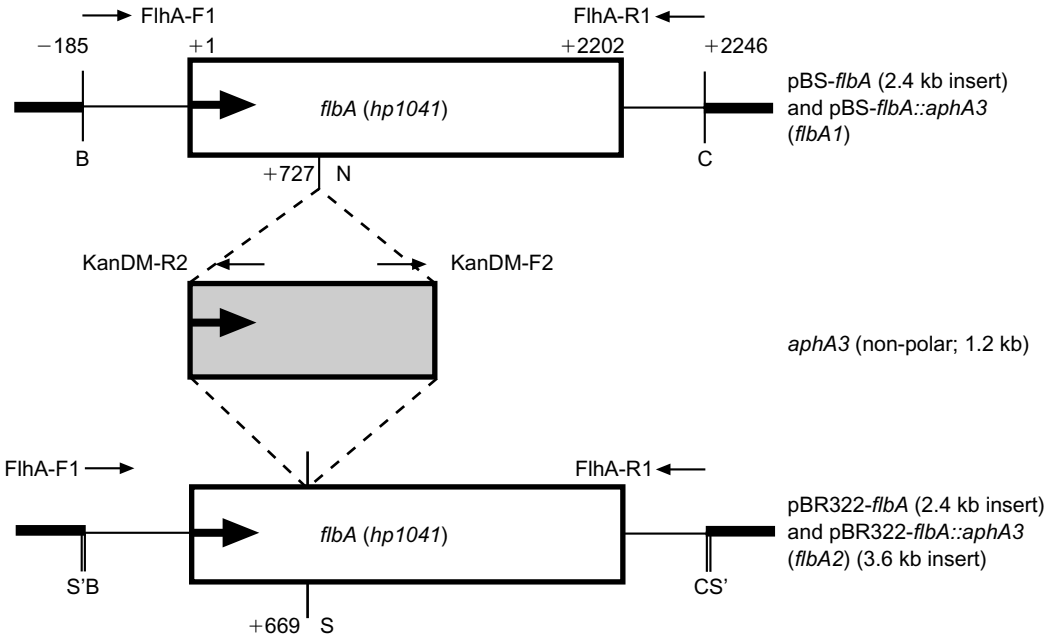

Fig. 1. *flbA* constructs used. Plasmids pBS-*flbA*, pBS-*flbA::aphA3* (for generation of *flbA1* mutants in *H. pylori*), pBR322-*flbA* and pBR322-*flbA::aphA3* (for generation of *flbA2* mutants in *H. pylori*) were constructed as indicated in Table 1. Large arrows, direction of transcription; small arrows, primers used for PCR confirmation of *H. pylori flbA* mutants; B, *Bam*HI site; C, *Cla*I site; N, *Nhe*I site; S, *Ssp*I site; S', *Ssp*I site lost upon cloning; +1 refers to translation start site. The *Nhe*I and *Ssp*I sites within *flbA* were lost upon cloning of the blunted kanamycin resistance cassette.

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 and 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).

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 μl of F12-broth-grown *H. pylori* strains (SS1 background) suspended in sterile PBS (pH 7.4) to 10^9 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*

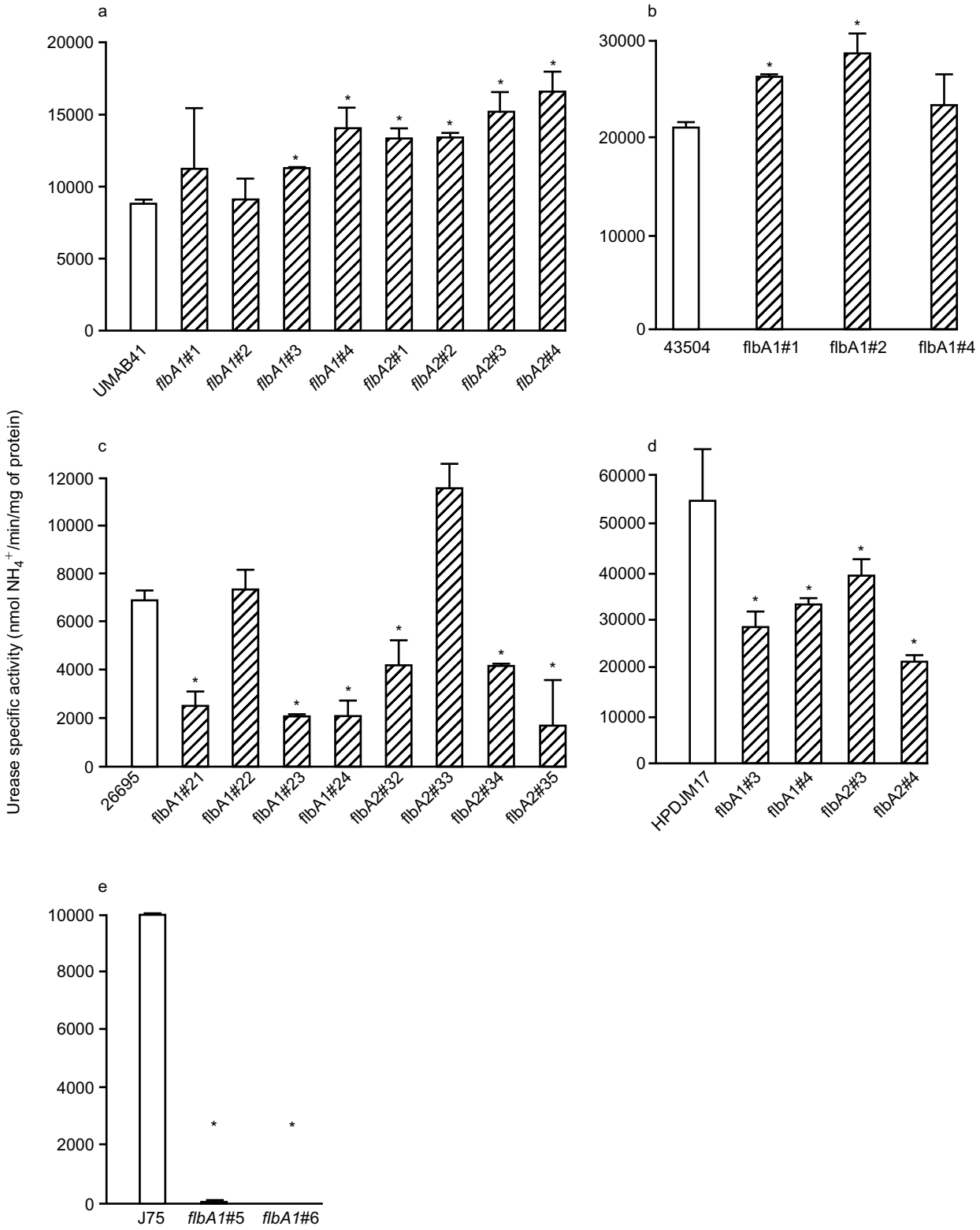

Fig. 2. Urease activity of wild-type and *flbA* mutants of *H. pylori.* Extracts of wild-type and isogenic *flbA* mutants of blood agar-grown *H. pylori* were measured for urease activity by the phenol-hypochlorite urease assay. Data are given as urease specific activity (nmol NH_4^+/min/mg protein) 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.

nickel transporter gene were on plasmid pHP8080 and this permitted urease activity in *E. coli* [9]. The model allowed exploration of genes that affect urease or *nixA* expression without the excessive variability observed with *H. pylori*. To investigate further the role of *flbA* in modulating *H. pylori* urease activity, the *flbA* gene was subcloned and the resultant plasmid (pBS-*flbA*) DNA was transformed into *E. coli* (pHP8080). Urease activity was reduced 15-fold compared with the vector control strain (Fig. 3). Disruption of *flbA* with a kanamycin resistance cassette restored urease activity in *E. coli* (pHP8080/pBS-*flbA::aphA3*) to levels of the vector control strain, *E. coli* (pHP8080/pBS) (Fig. 3). This suggested that *flbA* functioned as a urease-decreasing factor in the *E. coli* model.

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 pHP8080, 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 pRS415-*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 (pRS415-*ureAP*/pCC038) *versus* 1529 Miller Units in the vector control strain, MC1061 (pRS415-*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* urease gene cluster and activates transcription of itself and 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.

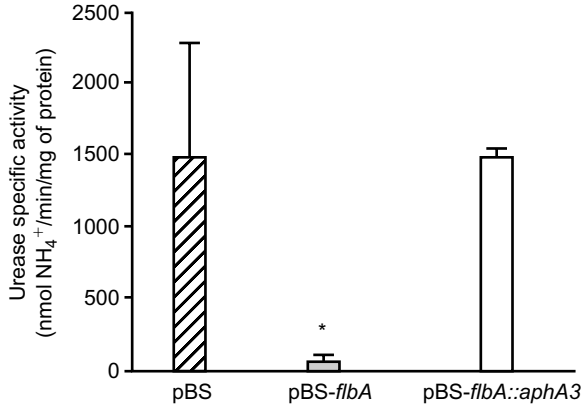

Fig. 3. Effect of *H. pylori flbA* on urease activity in an *E. coli* model of *H. pylori* urease. *E. coli* strain SE5000 containing pHP8080 (*H. pylori* urease generating system) and the constructs listed in the figure were grown overnight in M9 minimal medium containing 1 μM nickel chloride and urease activities of cytosolic extracts were determined by the phenol-hypochlorite urease assay. Data are given as urease specific activity (nmol NH_4^+/min/mg of protein) and SD and are an 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.

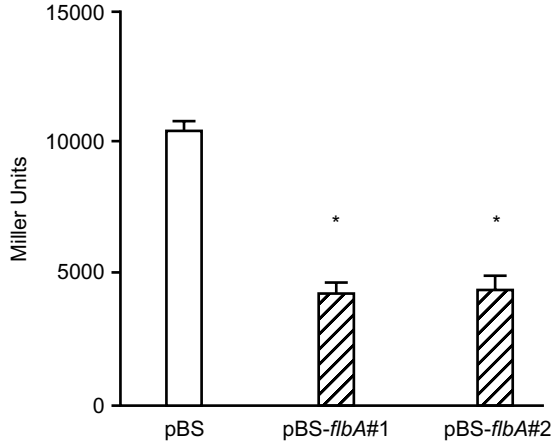

Fig. 4. Effect of *flbA* on the nixA promoter by β-galactosidase assays in *E. coli*. *E. coli* strain MC1061 containing pLX2106-*nixAP* plus either pBS or two independent, confirmed transformants containing pBS-*flbA*, were grown to mid-log phase in M9 minimal medium and β-galactosidase activity was 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.

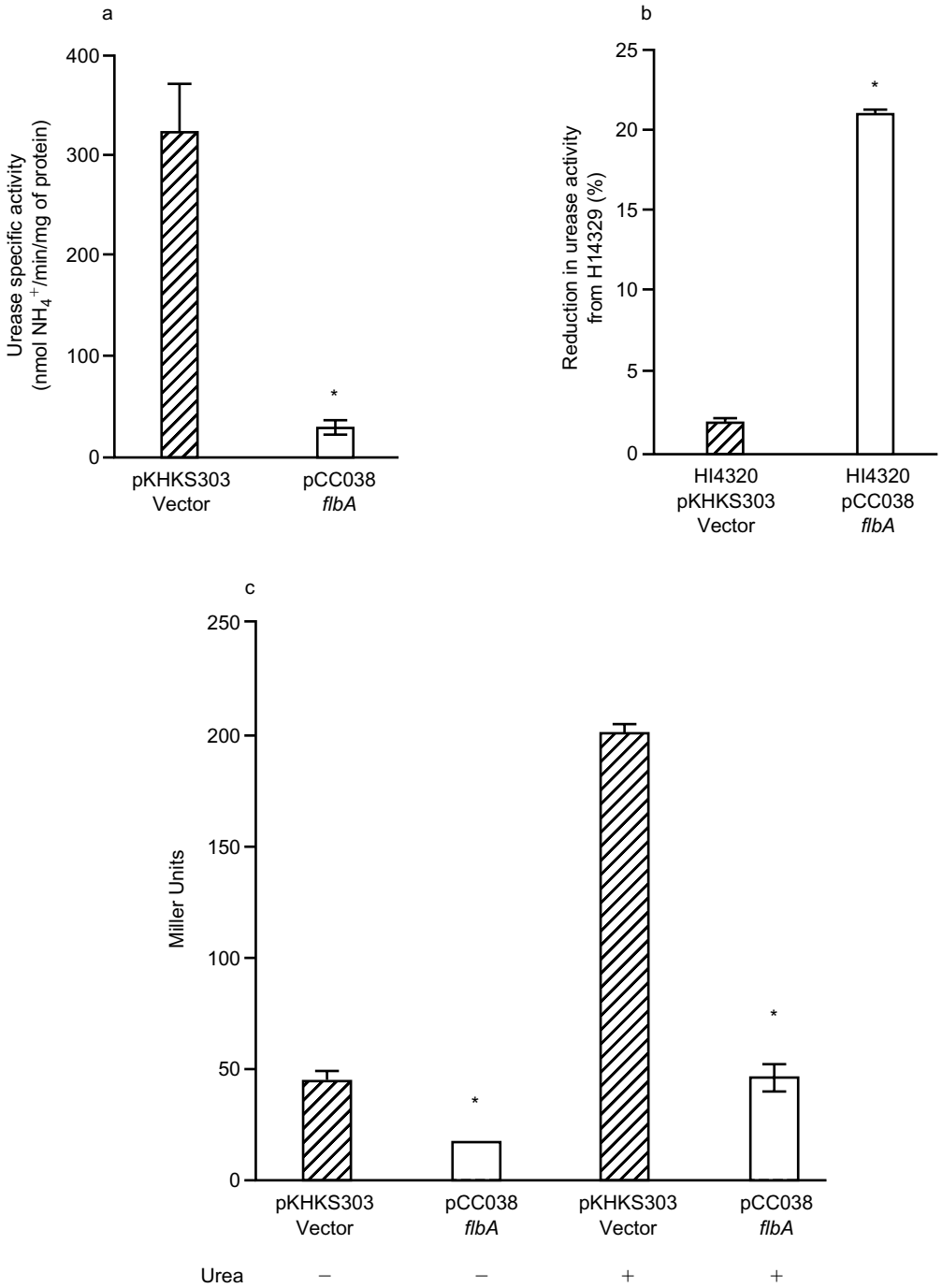

Fig. 5. Effect of *flbA* on urease of *P. mirabilis.* (**a**) Effect of *flbA* on urease activity of *E. coli* (pMID1010). *E. coli* strain DH5α containing pMID1010 (*P. mirabilis* urease generating system) and the constructs listed in the figure were grown to mid-log phase in L broth and the urease 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 ($\sim$average 35 000 nmol NH_4^+/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 pΔR10b*ureD-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* DH5*α* harbouring plasmid pΔR10b*ureD-lacZ*, which encodes the *P. mirabilis ureD* promoter transcriptionally fused to *lacZYA* [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 DH5*α* (pΔR10b*ureD-lacZ*/pCC038) was decreased 4–5-fold ($p < 0.001$), as compared with the vector control strain, DH5*α* (pΔR10b*ureD-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 SS1, five were colonised with a mean of 5.4×10^6cfu/g of antrum (Table 2). In contrast, only one of six animals inoculated with the *flbA* mutant was colonised and this one animal had a mean of only 5.5×10^3cfu/g of antrum, barely above the detection limit of 9×10^2cfu/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

SS1		*flbA* mutant	
Animal no.	mean cfu/g of antrum	Animal no.	mean cfu/g of antrum
1	1.9×10^7	7	$< 9.0 \times 10^{2*}$
2	5.8×10^6	8	$< 9.0 \times 10^{2*}$
3	$< 9.0 \times 10^{2*}$	9	$< 9.0 \times 10^{2*}$
4	1.2×10^6	10	$< 9.0 \times 10^{2*}$
5	1.9×10^3	11	$< 9.0 \times 10^{2*}$
6	6.8×10^6	12	5.5×10^3
Mean (SD)	$5.4 \times 10^6 (7.3 \times 10^6)$	Mean (SD)	$1.6 \times 10^3 (1.9 \times 10^3)^{\dagger}$

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.

*No *H. pylori* recovered. Limit of detection was 9×10^2 cfu/g of antrum.

†$p = 0.027$ between wild-type SS1 and the *flbA* mutant.

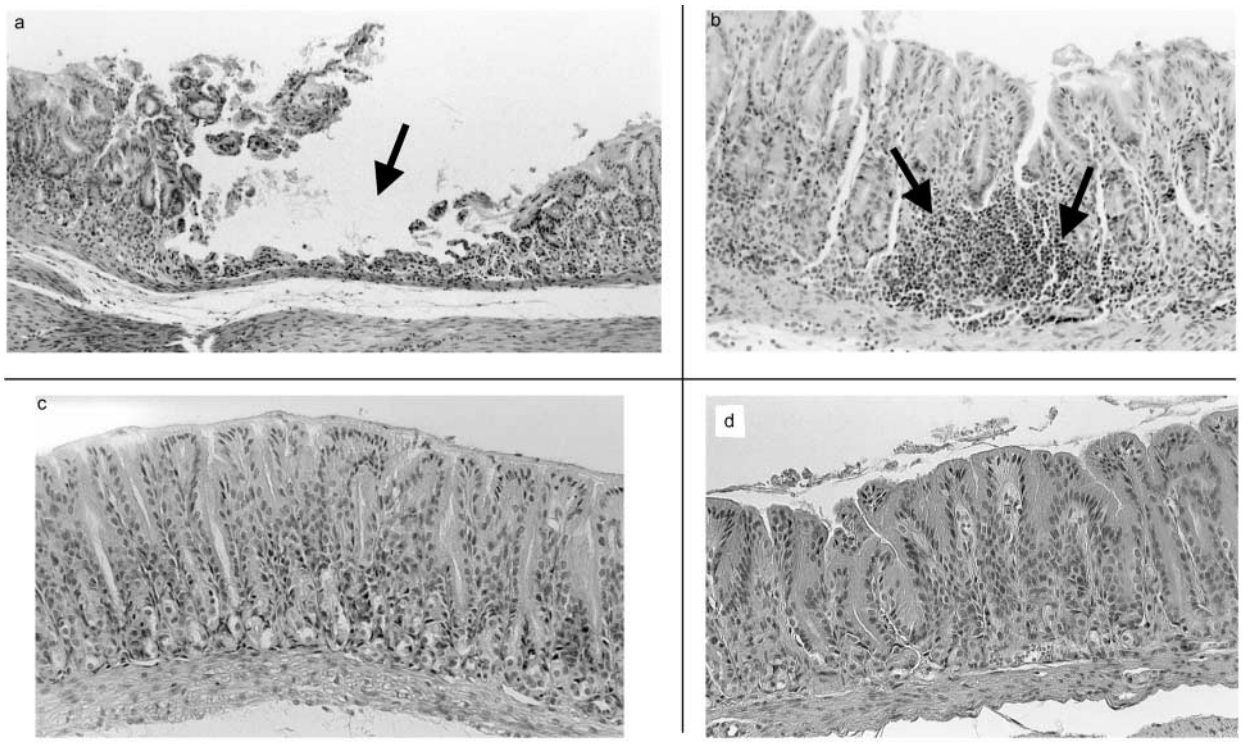

Fig. 6. Effect of *H. pylori flbA* on histopathology in the antrum of gerbils. All sections (5 μm thick) are antrum tissue of the stomach and were stained with haemotoxylin and eosin. Magnification, 180×. (**a**) Antrum from gerbils inoculated with wild-type *H. pylori* strain SS1. Arrow, micro-ulcer formation. (**b**) Antrum from gerbils inoculated with wild-type *H. pylori.* Arrow, lymphoid follicle formation and lymphocytic inflammation. (**c**) Antrum from gerbils inoculated with the *flbA* mutant of *H. pylori.* (**d**) Antrum from gerbils inoculated with sterile buffer.

Table 3. Differences in urease properties and *flbA*-mediated modulation of urease in *H. pylori* and *P. mirabilis*

Organism	Urease gene cluster	Urease regulation	High affinity nickel transporter required for urease activity?	Nickel concentration in in-vivo niche	Mechanism of FlbA-mediated urease modulation
H. pylori	*ureABIEFGH*	FlbA, pH, NikR	Yes	Serum/gastric milieu: 0.1–0.5 μg/L	NixA expression repressed
P. mirabilis	*ureRDABCEFG*	H-NS (repressor), UreR + urea (activator), FlbA (repressor)	No	Urine: 1–3 μg/L	Urease promoter repressed

mirabilis urease, the *H. pylori* urease is not regulated by a UreR homologue nor by urea, but requires the NixA high affinity nickel transporter for urease activity in the *E. coli* model [9, 10] (summarised in Table 3). In the presence of *flbA*, urease activity was almost abolished in *E. coli* containing the *H. pylori* urease gene cluster (Fig. 3). However, this was not due to repression of the urease promoter as it was for *P. mirabilis*, but rather of the *nixA* promoter (Fig. 4). Because NixA is required for urease activity in the *E. coli* model of *H. pylori* urease, it is believed that the *flbA*-mediated decrease in *H. pylori* urease activity is due to decreased *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 UreA and UreB are markedly reduced in *E. coli* containing *flbA* and pHP8080 [9].

The contrasting mechanisms of *flbA*-mediated modulation of urease in *H. pylori* and *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*. *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 *P. mirabilis*. *H. pylori* is found in a very low nickel environment (0.1–0.5 μg/L in serum and presumably in similar amounts in the gastric milieu [46–48]) and thus has evolved the high affinity nickel transporter *nixA* for optimal delivery of nickel to apo-urease. In contrast, *P. mirabilis* resides in the urinary tract, where nickel concentrations are about 10-fold higher (1–3 μg/L [47, 49]) and thus a high affinity nickel transporter is unnecessary.

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

100-fold for *P. mirabilis* urease) than those observed in the native organisms, suggesting that additional loci and compounds are necessary to achieve peak urease activity. In support of this hypothesis, Soriano and Hausinger have shown that bicarbonate and GTP are needed to achieve high urease activities in an *E. coli* model of *Klebsiella* urease [50]. Furthermore, a previous study uncovered a number of genes, including *flbA* and a putative DNA helicase, which influenced urease activity [9].

In addition to the evidence for decreased urease activity by *flbA* in the *E. coli* models described above, evidence was also obtained that *flbA* played a significant role in urease modulation in *H. pylori* itself. Some *H. pylori flbA* mutants in some strain backgrounds had elevated urease levels, whereas *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 *flbA* was present or not (D. J. McGee and H. L. T. Mobley, unpublished observations). This problem was minimised by transforming DNA from *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 *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, *flbA* or *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 *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 make it difficult for investigators to make generalisations. Therefore, it is recommended that researchers use multiple *H. pylori* strains to investigate phenotypes of wild-type strains and their corresponding isogenic mutants because misleading or premature conclusions might be reached with only one strain.

This study confirmed the original observation of Schmitz *et al.* [27] that *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 *flhA* 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 *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 *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 aflagellate 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 *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 SS1 and the isogenic *flbA* mutant, which have identical urease activities. Thus, the lack of colonisation by the *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 *flbA* was shown to modulate urease of both *H. pylori* and *P. mirabilis*, but this modulation was by distinct mechanisms. *flbA* was required for motility and for virulence in *H. pylori*.

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