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Adherence of Isogenic Flagellum-Negative Mutants of *Helicobacter pylori* and *Helicobacter mustelae* to Human and Ferret Gastric Epithelial Cells

MARGUERITE CLYNE,[1,2]* TADHG OCROININ,[1,2] SEBASTIAN SUERBAUM,[3]†
CHRISTINE JOSENHANS,[3]‡ AND BRENDAN DRUMM[1,2]

Department of Paediatrics and The Conway Institute of Molecular and Biomedical Research, University College Dublin, Belfield, Dublin 4,[1] and The Childrens Research Centre, Our Ladys Hospital for Sick Children, Crumlin, Dublin 12,[2] Ireland, and Ruhr-Universitat Bochum, D-4630 Bochum, Germany[3]

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Isogenic flagellum-negative mutants of *Helicobacter pylori* and *Helicobacter mustelae* were screened for their ability to adhere to primary human and ferret gastric epithelial cells, respectively. We also evaluated the adherence of an *H. pylori* strain with a mutation in the *flbA* gene, a homologue of the *flbF*/*lcrD* family of genes known to be involved in the regulation of *H. pylori* flagellar biosynthesis. *H. pylori* and *H. mustelae* mutants deficient in production of FlaA or FlaB and mutants deficient in the production of both FlaA and FlaB showed no reduction in adherence to primary human or ferret gastric epithelial cells compared with the wild-type parental strains. However, adherence of the *H. pylori flbA* mutant to human gastric cells was significantly reduced compared to the adherence of the wild-type strain. These results show that flagella do not play a direct role in promoting adherence of *H. pylori* or *H. mustelae* to gastric epithelial cells. However, genes involved in the regulation of *H. pylori* flagellar biosynthesis may also regulate the production of an adhesin.

Adherence of *Helicobacter pylori* to the gastric mucosa is thought to be an important virulence determinant of the organism. *Helicobacter mustelae* infects ferrets naturally and is the only *Helicobacter* species other than *H. pylori* known to be associated with both gastritis and peptic ulcer disease in its natural host (8, 9). *H. mustelae* has been shown to adhere tightly to the gastric mucosa of ferrets (20). *H. mustelae* infection of the ferret may prove to be a very useful natural animal model of *H. pylori* infection in humans and for the study of adherence of bacteria to the gastric mucosa and their role in peptic ulceration.

Motility and the production of an active urease enzyme are two factors which have been shown to be essential for the colonization of experimental animals by either *H. pylori* or *H. mustelae* (1, 2, 4, 5, 6, 7, 25). Motility is conferred on both organisms by the possession of flagella. *H. pylori* possesses a bundle of unipolar flagella, while *H. mustelae* exhibits both polar and lateral flagella. The flagellar filaments of *H. pylori* and *H. mustelae* are composed of two constituents. FlaA, encoded by *flaA*, is the major component and has a molecular mass of 53 kDa (16). FlaB, encoded by *flaB*, has a molecular mass of 54 kDa and seems to be located mainly at the proximal part of the filament (15). *H. pylori* and *H. mustelae* FlaA are 72% identical (14). The FlaB proteins of *H. pylori* and *H. mustelae* share 81.7% identical amino acids (24). *H. pylori* FlaA has 58% homology with *H. pylori* FlaB, and *H. mustelae* FlaA and FlaB have 56% amino acid similarities (14). The *H. pylori flaA* and *flaB* genes are unlinked on the chromosome, and their products share only limited amino acid sequence similarity. It seems likely that both genes can be regulated differentially by environmental conditions. The flagellar filament is linked to the flagellar basal body by means of the hook which itself is a polymer of the flagellar hook protein FlgE (21). Mutations in FlgE do not prevent the synthesis of either FlaA or FlaB. Each flagellum is enveloped by a sheath that is thought to serve as a protective shield against gastric acidity for the acid-labile flagellar filament (11).

Isogenic *flaA*-, *flaB*-, and *flaA flaB*-negative mutants of both *H. pylori* and *H. mustelae* have been constructed (14, 24). FlaA mutants produce truncated flagella and are only weakly motile, whereas FlaB mutants produce normal flagella but have diminished motility in a soft-agar assay. Mutants lacking both FlaA and FlaB are aflagellate and nonmotile. By using aflagellate *flaA flaB* double mutants it has been shown that motility is essential for the ability of *H. pylori* and *H. mustelae* to colonize the stomachs of gnotobiotic piglets (7) or specific-pathogen-free ferrets (1), respectively. In the piglet model, mutations in a single flagellin gene were sufficient to prevent persistent colonization. Single-gene mutants of *H. mustelae* were still able to colonize ferrets, although only at a low density. Whether flagella per se are involved solely in motility or also in attachment of the organisms to the gastric mucosa is an important question. To answer this question we have screened isogenic *flaA*-, *flaB*-, and *flaA flaB*-negative mutants of *H. pylori* and *H. mustelae* for adherence to primary gastric cells isolated from humans and ferrets respectively. Adherence of all mutants to Kato III cells was assessed for the purpose of comparison. We have also looked at the adherence of an *H. pylori* strain with a mutation in the *flbA* gene, a homologue of the *flbF*/*lcrD* family of genes (23). This mutant strain expresses neither the FlaA nor the FlaB flagellin protein, and expression of the FlgE hook protein is reduced in comparison with that of the wild-type strain. The *flbA* mutants are aflagellate and completely nonmotile.

* Corresponding author. Mailing address: The Childrens Research Centre, Our Ladys Hospital for Sick Children, Crumlin, Dublin 12, Ireland. Phone: 353-1-4096594. Fax: 353-1-4555307. E-mail: MARGUERITE.CLYNE@ucd.ie.

† Present address: Institute of Hygiene and Microbiology, University of Wuerzburg, D-97080 Wuerzburg, Germany.

‡ Present address: Microbial Pathogenesis Unit, Institute of Cellular Pathology, Universite Catholique de Louvain, B-1200 Bruxelles, Belgium.

TABLE 1. Wild-type and isogenic flagellum-negative mutants of *H. pylori* and *H. mustelae* used in this study

Bacterium	FlaA	FlaB	Motility	Flagellation	Reference
H. pylori					
N6 (wild type)	+	+	Motile	Normal	23
N6 *flaA*::Km	−	+	Weakly motile	Short truncated flagella	14, 23
N6 *flaB*::Km	+	−	30–40% reduced motility	Normal	23
N6 *flaA*::cat/*flab*::Km	−	−	Nonmotile	Aflagellate	14
N6 *flbA*::Km	−	−	Nonmotile	Aflagellate	23
H. mustelae					
F1 (wild type)	+	+	Motile	Normal	14
F1 *flaA*::Km	−	+	Weakly motile	Short truncated flagella	14
F1 *flaB*::Km	+	−	30–40% reduced motility	Normal	14
F1 *flaA*::cat/*flaB*::Km	−	−	Nonmotile	Aflagellate	14

H. pylori parental wild-type strain N6 produces both FlaA and FlaB and is fully motile. Four isogenic mutants of *H. pylori* strain N6 were used in this study (Table 1). For *H. mustelae*, the parental wild-type strain F1 and three isogenic mutants were used in this study (Table 1). The isogenic mutant strains of *H. pylori* and *H. mustelae* were constructed by electroporation-mediated allelic exchange (14, 23, 24). *H. pylori* and *H. mustelae* strains were grown on Columbia blood agar (Gibco) plates containing 7% (vol/vol) defibrinated horse blood for 3 days at 37°C in an atmosphere of 10% CO_2 and 5% O_2. Strains with isogenic mutations in *flaA*, *flaB*, and *flbA* were grown on blood agar plates containing kanamycin (20 mg/liter), and double mutants were grown on blood agar plates containing both kanamycin (25 mg/liter) and chloramphenicol (25 mg/liter). Quantitation of bacteria in suspension was accomplished by optical density measurements at 450 nm and by viable counts. Appropriate dilutions of the bacterial suspension were spread on Columbia blood agar plates and after incubation of plates at 37°C under microaerobic conditions for 5 days, CFU per milliliter were enumerated.

Kato III cells are gastric adenocarcinoma cells obtained from the American Type Culture Collection. They were grown in RPMI 1640 medium (BioWhittaker) containing 20 mM HEPES buffer and 12 mM sodium bicarbonate supplemented with 5% (vol/vol) fetal calf serum. For adherence assays cells were scraped from the base of the flask and centrifuged at 200 × *g* for 5 min. Cells were resuspended in RPMI 1640 medium and counted by microscopy using a hemocytometer.

Primary human gastric epithelial cells were isolated from biopsy tissue by digestion with 0.1 mM EDTA and 0.1 mM dithiothreitol followed by digestion with 0.05% (wt/vol) collagenase, as previously described (3). Cells were resuspended in RPMI 1640 tissue culture medium and counted using a hemocytometer. Viability was determined using the trypan blue exclusion assay and ranged from 65 to 85% on a day-to-day basis. Cells were used immediately in the adherence assays.

Ferret gastric tissue was obtained by sacrificing 10- to 12-week-old ferrets and removing the stomach. All ferrets were negative for *H. mustelae* by culture and by urease activity of biopsy specimens taken from the antrum, fundus, and duodenum (three samples from each site). The serosa was separated from the tissue by injecting RPMI 1640 tissue culture medium containing 10% (vol/vol) fetal calf serum underneath the serosa and peeling it away. The remaining tissue was minced using scalpels, and epithelial cells were isolated as described above. Cells were either used immediately in the adherence assay or suspended in a solution of RPMI 1640 medium containing 50% (vol/vol) fetal calf serum and 10% (vol/vol) dimethyl sulfoxide (DMSO), frozen using a Planar Kryo 10 controlled-rate freezer, and stored in liquid nitrogen. Vials of cells were then thawed as required. Viability of cells was assessed using the trypan blue exclusion assay prior to use in the adherence assays. Viability ranged from 60 to 80% on a day-to-day basis.

The adherence assay used in these experiments has been previously described (3). Briefly, gastric cells and bacteria were incubated together at 37°C for 30 min under microaerobic conditions. Cells were stained with whole-cell *H. pylori* or *H. mustelae* antibody raised in rabbits (1/200 dilution); then they were washed and subsequently stained with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma). Adherence was assessed by phase-contrast microscopy, fluoresence microscopy, and flow cytometry.

By using a dot plot display of forward- and right-angle scatter, the flow cytometer (Becton Dickinson) was gated to include single cells and to exclude cell debris, clumps of cells, and nonadherent bacteria. A total of 10,000 gated events were collected, and analysis of the data was performed by using the Lysis II software program from Becton Dickinson. This program produces histograms of each cell sample and calculates the mean channel fluorescence (mean channel number) of the cell population, which is directly related to the surface density of fluorescently labeled *H. pylori* adhering to the cell. Mean channel numbers of cells with adherent bacteria and without bacteria were compared. Results are also expressed as the percent fluorescent cells (i.e., the percent positive events or the percentage of cells with bacteria attached); this value is calculated from the fluoresence frequency distribution histograms and is based on the relative number of cells versus the relative fluoresence intensity. The threshold for positivity was set for each experiment by flow cytometric analysis of cells without adherent bacteria which had been stained with anti-*H. pylori* or anti-*H. mustelae* whole-cell antibody and the fluorescein isothiocyanate-labeled second antibody. Unless otherwise stated, results are expressed as the mean results of at least three separate experiments ± the standard error of the means. Results were analyzed using the Student *t*-test.

H. pylori strain N6 and the mutants N6 *flaA*::Km, N6 *flaB*::Km, and N6 *flaA*::cat/*flaB*::Km all bound to Kato III cells and to primary human gastric cells (Fig. 1). These results were confirmed by phase-contrast microscopy (results not shown). Strain N6 *flaB*::Km consistently bound better than the parental strain, N6, to both Kato III and primary human gastric cells. This difference reached statistical significance ($P < 0.05$) for Kato III cells but not for primary human gastric cells. In contrast, adherence of *H. pylori* strain N6 *flbA*::Km to Kato III and to primary human gastric cells (Fig. 1) was significantly re-

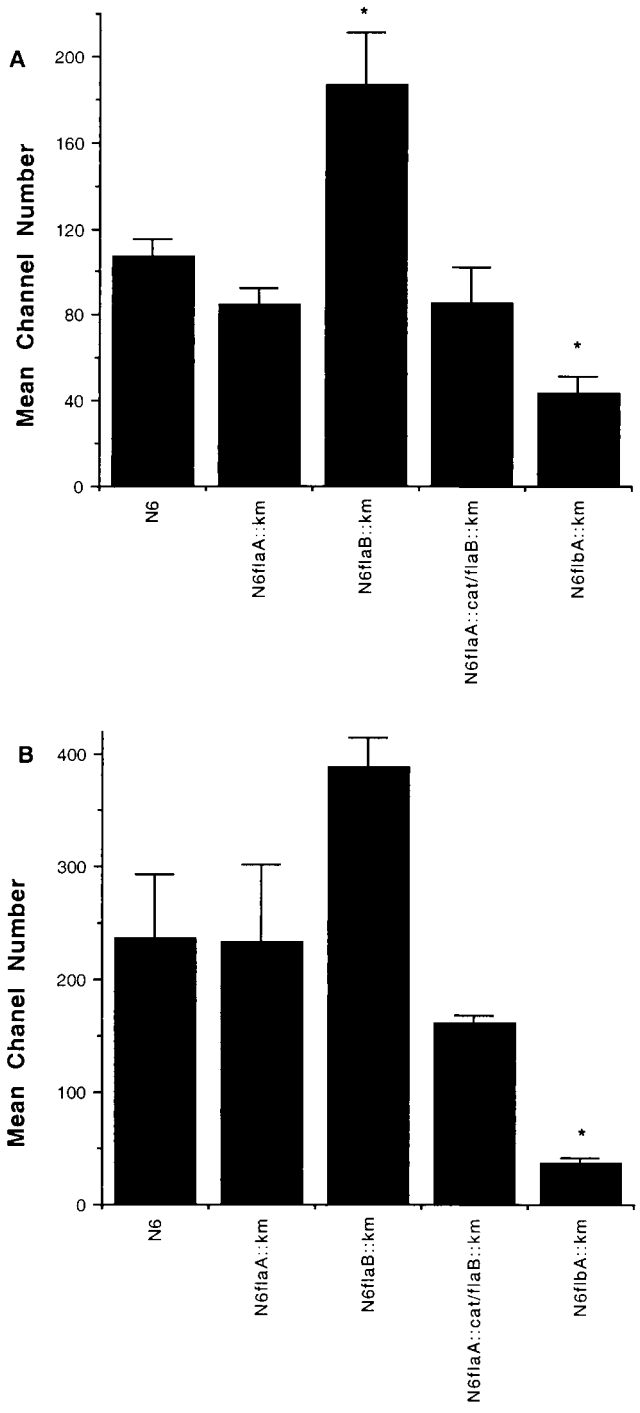

FIG. 1. Binding of *H. pylori* strain N6 and isogenic flagellar negative mutants to Kato III cells (A) and to primary human gastric cells (B). Bacteria and cells were incubated together at a ratio of 200:1, and adherence was assessed by flow cytometry as outlined in the text. The results are means ± standard errors of the means for three experiments. *, statistically significantly different result from that obtained with *H. pylori* strain N6 ($P < 0.05$).

duced compared to that of N6. This result was confirmed by immunofluorescent microscopy (Fig. 2).

H. mustelae strain F1 and the isogenic flagellum-negative mutant strains F1 *flaA*::Km, F1 *flaB*::Km and F1 *flaA*::cat/*flaB*::Km were tested for binding to Kato III cells and to primary ferret gastric epithelial cells at a range of different ratios of bacteria to cells. All strains bound to both cell types. This result was confirmed by phase-contrast microscopy (results not shown). Adherence of the bacteria to Kato III cells revealed that strain F1 *flaA*::Km showed a decrease in adherence at the lower bacterium/cell ratio ($P < 0.05$) (Fig. 3). However, all mutants showed similar levels of adherence to ferret gastric epithelial cells and at the lower bacterium/cell ratio of 50:1 they showed greater adherence than the wild type, but this result was not statistically significantly different ($P > 0.05$) (Fig. 3).

It has previously been speculated in the literature that the flagellar sheath of *H. pylori* plays a role in promoting adherence of the organism to the gastric mucosa (11, 17). Flagellin has also been proposed as an adhesin in the binding of *Campylobacter jejuni* to cultured cells (19). However, the role of flagella in the interactions of *C. jejuni* with nonpolarized and polarized epithelial cells was examined with flagellar mutants. There was no statistical difference in binding of the parental wild type strain and the aflagellate, nonmotile mutants to the cells indicating that flagella are not involved in adherence to

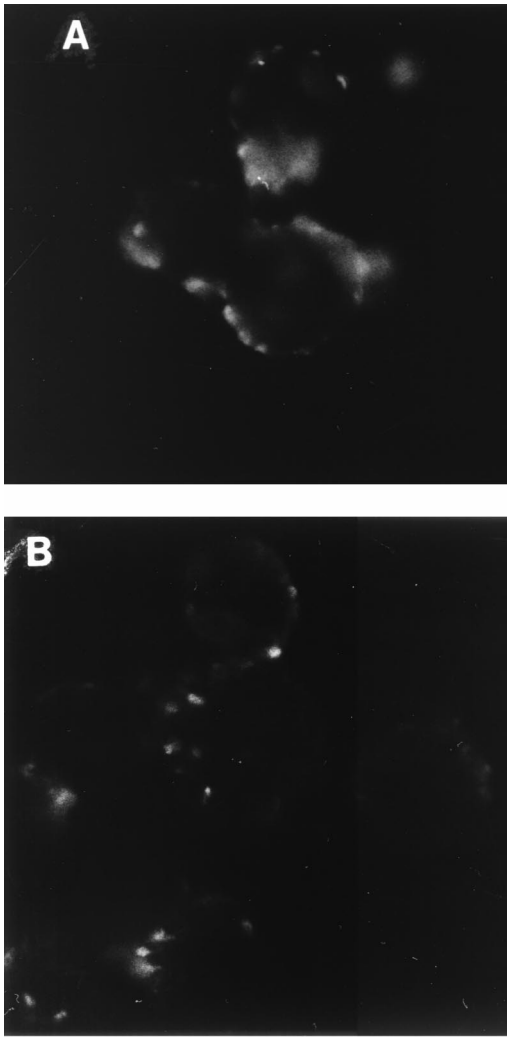

FIG. 2. Adherence of *H. pylori* strain N6 (A) and strain N6 *flbA*::Km (B) to primary human gastric cells isolated from a gastric biopsy specimen. Bacteria and cells were incubated together at a ratio of 200:1, and adherence was assessed by fluorescent microscopy using a 50× water immersion lens.

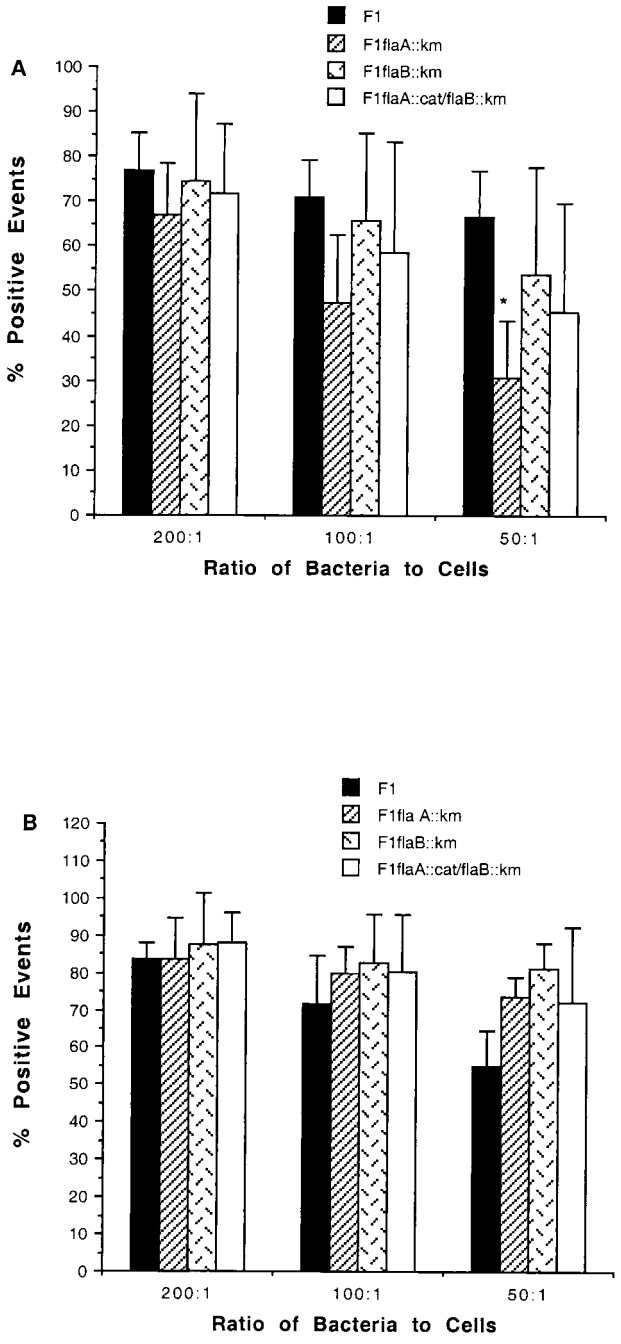

FIG. 3. Adherence of *H. mustelae* strain F1 and isogenic flagellar negative mutants to Kato III cells (A) and to primary ferret gastric cells (B). Different concentrations of bacteria were incubated with the cells, and adherence was assessed by flow cytometry as outlined in the text. The results are means ± standard errors of the means for three experiments. *, statistically significantly different result from that obtained with *H. mustelae* strain F1 ($P < 0.05$).

epithelial cells (12). Likewise, the present study has shown that flagella of *H. pylori* and *H. mustelae* do not play a direct role in promoting adherence of the organisms to gastric cells of their respective natural hosts, i.e., humans and ferrets. Wild-type strains, mutants deficient in the production of single flagellin species, and aflagellate double flagellin mutants were able to adhere to Kato III and to primary gastric cells. The fact that the *flaA flaB* double mutants were able to adhere to gastric cells as well as the wild-type strains did show that the flagellar sheath is unlikely to play a role in mediating adherence of the bacteria to the gastric mucosa.

Surprisingly, strain N6 *flaB*::Km consistently adhered better than the wild-type strain or the other mutants to both Kato III and primary human gastric cells. However, this difference in binding failed to reach statistical significance when we used primary human gastric cells. Strain N6 *flaB*::Km did grow more rapidly than the wild type and other mutant strains on blood agar plates, and although we used the same number of organisms in each experiment, it is possible that this strain was in a slightly different phase of growth. It is unlikely that mutation of *flaB* induces upregulation of an adhesin since the double mutant strain N6 *flaA*::cat/*flaB*::Km did not display increased binding compared to the wild-type parental strain.

Strain N6 *flbA*::Km, which is mutated in the regulatory gene *flbA*, behaved very differently from the other mutants. This mutant showed a marked reduction in adherence to primary gastric cells which was very different from that seen with strain N6 *flaA*::cat/*flaB*::Km, despite the fact that phenotypically the two mutant strains were very similar (i.e., aflagellate and nonmotile). FlbA is a membrane protein involved in the expression of flagellin genes encoded by the *flbA* gene which is a member of the *lcrD*/*flbF*/*invA* gene family (23). These proteins are components of type III secretion systems which are involved in the export of flagellar proteins or the injection of virulence factors into eukaryotic target cells. The FlbA protein itself, being a cytoplasmic membrane protein, is unlikely to function as an adhesin. This suggests that *flbA* is involved in the regulation of genes which play a role in mediating adherence of the organism to gastric cells. It has recently been shown that the *flbA* gene of *H. pylori* can play a role in modulating urease activity in *E. coli*. An *E. coli* strain which expressed both *H. pylori* urease and the NixA nickel transporter was constructed. Almost no urease activity could be detected in this strain when it contained the subcloned *flbA* gene. Thus, flagellar biosynthesis and urease activity may be linked in *H. pylori* (18). A number of other flagellar regulatory genes have been described. One of these genes is *fliQ*, which encodes FliQ, a protein with homology to the FliQ protein found in *Salmonella enterica* serovar Typhimurium and shown to be membrane bound. FliQ is required for flagellation, but *fliQ* does not encode any structural or regulatory component (10). An isogenic *fliQ* mutant of *H. pylori* showed a reduced level of adherence to AGS cells, a gastric cell line, compared with the parental wild-type strain. FliI is an ATPase protein found to be necessary for flagellar assembly in *H. pylori* (13). An *H. pylori fliI* knockout mutant showed reduced expression of OMP4, an outer membrane protein (22). The results of those studies combined with the results of the present study suggest that genes involved in the regulation of *H. pylori* flagellar biosynthesis can also play a role in regulating the production of adhesins and other potential virulence factors in *Helicobacter* organisms.

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