Role of the Flagellar Hook-Length Control Protein FliK and σ28 in cagA Expression in Gastric Cell–Adhered Helicobacter pylori

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Adherence of Helicobacter pylori to the gastric epithelial cell line AGS strongly induces expression of fliK encoding a flagellar hook-length control protein. FliK has a role in triggering dissociation of the alternate sigma factor, σ28, from a nonfunctional σ28-FlgM complex, releasing free, functional σ28. The σ28-RNA polymerase initiates transcription of cagA, the major virulence gene, from a promoter identified in this study. Consequently, significant up-regulation of cagA was observed in AGS-adhered H. pylori. Direct binding of σ28 to the cagA promoter was demonstrated by chromatin immunoprecipitation and the transcription start site was identified by 5′ RACE (rapid amplification of complementary DNA ends). The σ28-dependent cagA promoter was active specifically in AGS-adhered H. pylori, and this motif might be associated with high cagA expression and severity of disease. These results also indicate that H. pylori has evolved to integrate expression of the major virulence gene cagA with the flagellar regulatory circuit, essential for colonization of the human host.

Keywords. Sigma 28; FliK; CagA; host-pathogen interaction.

Helicobacter pylori is a gram-negative, spiral shaped bacterium that colonizes the stomach and may maintain life-long association with the human host without any apparent clinical symptom [1]. However, in a small proportion of infected individuals, H. pylori causes peptic ulcer, gastric cancer, Mucosa associated lymphoid tissue lymphoma, and other gastroduodenal disorders [2, 3]. Associated with these clinical outcomes are the major virulence factors produced by H. pylori, including the vacuolating cytotoxin VacA and the cytotoxin-asso ciated gene CagA [4].

Flagella-dependent motility of H. pylori has been shown to be essential for colonization of the stomach of Mongolian gerbils [5] and enhances pathological outcomes in humans [6]. As in many motile bacteria, regulation of flagellar gene expression and biosynthesis is a complex process in H. pylori [7]. The assembly of the flagellar structure is a well-coordinated process initiated by the assembly of the basal body on the cell membrane. On the basal body, the flagellar hook forming proteins are assembled exterior to the cell forming the hook basal body complex [8, 9]. When the flagellar hook substructure reaches its optimal length, sensed by the “checkpoint control” protein FliK, export of the anti-sigma factor FlgM is triggered, releasing σ28 from a σ28-FlgM complex, which in turn allows the subsequent expression of σ28-dependent genes. This results in a switch of export substrate specificity from rod/hook type to filament type, consequently initiating filament assembly [10–17]. In fliK mutants, the hook to filament transition does not occur, and long hooks of unregulated length, termed polyhooks are formed [18]. Moreover, the lack of substrate-specificity switching in the fliK mutants results in failure to secrete the anti-σ28 factor FlgM, and σ28 remains sequestered by FlgM, consequently class 3 genes are not expressed [12, 19–24]. In H. pylori, it is not clear whether release of σ28 from the σ28-FlgM complex is actually dependent on export of FlgM, but it doubtlessly requires the hook to filament switch [25].

It has been reported that contact of H. pylori with gastric epithelial cells strongly induces expression of...
the flagellar hook-length control protein FliK [26]. We have recently demonstrated that host cell contact also up-regulates expression of the H. pylori major virulence gene cagA [27]. In this report, we demonstrate that FliK is necessary for cagA up-regulation in gastric cell-associated H. pylori and have elucidated the role of FliK and σ28 in cagA up-regulation.

**MATERIALS AND METHODS**

The following were performed according to standard procedures: bacterial strains and culture conditions, construction of H. pylori mutants, construction of H. pylori strain expressing FLAG-tagged FliA (σ28), complementation of ΔfliK and ΔfliA mutants and overexpression of fliK and fliA genes in H. pylori G27, immunoprecipitation and Western blot analysis, interleukin 8 (IL-8) estimation by enzyme-linked immunosorbent assay, adherence assays, RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR), chromatin immunoprecipitation (ChIP) assay, 5′ RACE (rapid amplification of complementary DNA ends), microscopy of H. pylori-infected AGS cells, and statistical analysis. Details are provided in the Supplementary Materials.

**RESULTS**

**Up-regulation of the FliK Homolog HP0906 on Adherence of H. pylori to AGS Cells**

Up-regulation of the HP0906 gene, later annotated as fliK, has been demonstrated in AGS cell-associated H. pylori strain 969a using complementary DNA macroarrays [26, 28]. To examine whether similar up-regulation occurred in the H. pylori strains 26695 and G27 after adherence to AGS cells, RNA was isolated from bacteria at different intervals after adherence and also from unadhered bacteria grown under identical conditions without cell line or isolated from the supernatant of H. pylori–infected AGS monolayer. Expression of fliK was examined in the adhered and unadhered bacteria with quantitative RT-PCR. Significant increase in fliK expression was consistently observed in both strains within 2 hours after adherence of H. pylori to the AGS cells, compared with unadhered controls (Figure 1), and fliK expression was consistently up-regulated up to 8 hours examined (Supplementary Figure 1A).

**Adherence of H. pylori ΔfliK Mutant to AGS Cells and Effect on Cell Morphology**

To examine whether FliK has a role in AGS-adhered H. pylori, 2 ΔfliK mutants of each H. pylori strain were independently constructed and analyzed. Two independent mutants were used to decrease the possibility of erroneous results arising from unidentified spontaneous mutations. The mutants were completely nonmotile in contrast to the highly motile parent strains (Supplementary Figure 1B). As has been reported elsewhere, mutation in fliK did not result in any polar effect (data not shown) [28].

H. pylori wild-type (WT) and ΔfliK strains were added to AGS cells at different multiplicities of infection (MOIs), and the adhered bacteria were enumerated. With increasing MOI, the number of adhered bacteria increased progressively. No difference in the number of AGS-adhered bacteria was observed between H. pylori WT and the ΔfliK mutants at any MOI (Supplementary Figure 1C and 1D).

The effect of adherence of the WT and ΔfliK mutant strains on AGS cell morphology was next examined. When infected by WT strains at low MOI (1, 5, and 10) very few AGS cells exhibited elongation and scattering, known as the hummingbird phenotype (Supplementary Figure 1E). At an MOI of 50, a pronounced hummingbird phenotype was observed in a large majority (about 70%) of AGS cells within 2 hours of adherence of
However, much lower scattering and very little elongation (about 15%) was observed in AGS cells after adherence of the ΔflK mutant strains (Figures 2A and 3A).

To confirm that the observed phenotype was indeed due to mutation in the flK gene and not due to an unidentified secondary mutation, the G27ΔflK mutant was complemented by
Figure 3. Adherence of *Helicobacter pylori* G27 and mutants to AGS cells and cagA expression. A, Morphological changes in AGS cells after *H. pylori* adherence. AGS cells were incubated with *H. pylori* G27 (wild type [WT]), ΔflK, ΔflA, ΔflK/ΔflK, or ΔflA/ΔflA strains for 2 hours, washed to remove unadhered bacteria, and observed on an inverted microscope in phase contrast at ×20 magnification. The elongated cells were counted and expressed as the percentage of total cells in each sample. Bar diagram represents means and standard deviations (SDs) of 3 independent experiments; *P* values (above bars), significance of the differences in number of elongated cells between WT and the mutant strains. B, Interleukin 8 (IL-8) estimation in culture supernatants of AGS
integration of the full-length \( fliK \) gene into the chromosome; the complemented strain, designated G27\( fliK \)/\( fliK \), produced elongation and scattering on adherence to AGS cells, similar to the WT cells (Figure 3A). The strain AM1 that lacks the \( cagA \)-PAI, used as a negative control, did not produce the hummingbird phenotype (Supplementary Figure 1E).

**Reduced Induction of the \( cagA \) Gene After Adherence to AGS Cells in \( H. pylori \) \( fliK \) Mutant Strain**

Induction of the hummingbird phenotype in gastric cells after \( H. pylori \) infection has been attributed to the translocation and subsequent phosphorylation of CagA from the bacteria into host cells [4, 29]. In view of the remarkable difference in AGS morphology after adherence of \( H. pylori \) WT and \( fliK \) mutant strains, expression of the \( cagA \) gene was examined by quantitative RT-PCR in adhered bacteria after infection of AGS cells at different MOIs and compared with the expression in unadhered bacteria. As reported elsewhere [27], about 3.5–5-fold up-regulation of \( cagA \) expression was consistently observed in AGS-adhered \( H. pylori \) WT strains; however, practically no up-regulation was observed in the \( fliK \) strains (Figures 2C, 3C, and Supplementary Figure 1F). In the complemented G27\( fliK \)/\( fliK \) strain, \( cagA \) expression after adherence was similar to that in the WT strain (Figure 3). Thus, host cell contact-dependent up-regulation of \( cagA \) expression observed in the WT \( H. pylori \) [27] did not occur in the \( fliK \) mutants. Consistently, Western blot analysis indicated that the levels of CagA protein increased in AGS-adhered WT bacteria but not in the \( fliK \) mutants (Figures 2D and 3D).

To assess the ability of the WT and the \( fliK \) strains to translocate CagA into AGS cells, we examined CagA phosphorylation in the infected AGS cells using anti-phosphotyrosine and anti-CagA antibodies. Substantial CagA phosphorylation was observed in AGS cells infected with WT strains, only minor amounts were detected in \( fliK \)-infected AGS cells, and the amounts increased to WT levels in G27\( fliK \)/\( fliK \) (Figures 2D and 3D). CagA phosphorylation is known to induce cytokine IL-8 secretion from host cells. Indeed, AGS cells infected with \( H. pylori \) WT bacteria secreted substantial amounts of IL-8, but much lower amounts were secreted by \( H. pylori \) \( fliA \) mutant strains (Figures 2 and 3), strongly suggesting that host cell contact-dependent up-regulation of \( cagA \) is dependent on \( fliA \). To further confirm that the observed phenotype was indeed due to \( fliA \) mutation, the G27\( fliA \) was complemented with the full-length \( fliA \) gene inserted in the chromosome, and complementation restored \( cagA \) expression in the AGS-adhered

**\( fliA \) Expression and \( fliA \) (\( \sigma^{28} \))-Dependent Gene Expression in AGS-Adhered Bacteria**

It has been reported that in \( \Delta fliK \) mutants of several bacterial species, the anti-\( \sigma^{28} \) factor FlgM sequesters \( \sigma^{28} \) within the cell and the concentration of free, functional \( \sigma^{28} \) is lower in the \( \Delta fliK \) mutant than in the WT strain [15, 24]. To examine the status of functional \( \sigma^{28} \) in the \( H. pylori \) \( fliK \) mutants, expression of the \( flaA \) gene known to be dependent on \( \sigma^{28} \) [28] was examined in unadhered and AGS-adhered \( H. pylori \) \( fliK \) strains and compared with that in the isogenic WT strains (Supplementary Figure 2A). Interestingly, \( flaA \) expression increased about 5-fold after adherence of WT \( H. pylori \) to AGS cells, as has been reported elsewhere [30], but no up-regulation was observed in the AGS-adhered \( fliK \) strains (Supplementary Figure 2A). Taken together, these results suggested that the level of functional \( \sigma^{28} \) increased after adherence to AGS cells and that the increase was dependent on \( fliA \).

Because expression of the \( \sigma^{28} \)-dependent \( flaA \) gene was strongly up-regulated after adherence of \( H. pylori \) to AGS cells, we next examined whether the \( fliA \) gene encoding \( \sigma^{28} \) was itself up-regulated in AGS-adhered \( H. pylori \). About 4-fold induction of \( fliA \) was observed in the AGS-adhered \( H. pylori \). However, \( fliA \) induction in the corresponding \( fliK \) strains was not significant (Supplementary Figure 2B).

**\( \sigma^{28} \)-Dependent Up-regulation of \( cagA \) Expression in AGS Cell–Adhered Bacteria**

In view of the fact that \( \sigma^{28} \) activity and \( cagA \) expression were significantly lower in the AGS-adhered \( fliK \) mutant than in the adhered WT strain, we examined whether \( \sigma^{28} \) has a role in the up-regulation of \( cagA \) after adherence to AGS cells. For this purpose, \( fliA \) mutant strains of 26695 and G27 were constructed. As expected, \( flaA \) expression was drastically reduced in the \( fliA \) strains (Supplementary Figure 2A). Adherence of the \( fliA \) strain to AGS cells was similar to that of the WT strain (Supplementary Figure 1). Similar to the \( fliK \) mutant, no increase in \( cagA \) expression was observed in the AGS-adhered \( fliA \) mutant (Figures 2 and 3), strongly suggesting that host cell contact-dependent up-regulation of \( cagA \) is dependent on \( \sigma^{28} \). To further confirm that the observed phenotype was indeed due to \( fliA \) mutation, the G27\( fliA \) was complemented with the full-length \( fliA \) gene inserted in the chromosome, and complementation restored \( cagA \) expression in the AGS-adhered
Figure 4. The $\sigma^{28}$ promoter sequence upstream of cagA. A, Alignment of the nucleotide sequence upstream of the cagA open reading frame (ORF) in different Helicobacter pylori strains. Strains are indicated on the left. A putative $\sigma^{28}$ promoter is highlighted, and the transcription start site (TSS) identified by 5' RACE (rapid amplification of complementary DNA ends) is indicated. Numbers in brackets on the right indicate numbers of mismatches in the region from the $\sigma^{28}$ binding site to the TSS (37 bases), with respect to the sequence in strain 26695. B, Alignment of $\sigma^{28}$-dependent promoters of H. pylori. Consensus $-10$ element and an A/T rich upstream region $(-35)$ are highlighted. C, Nucleotide sequence of the cagA promoter from $-144$ base pairs (bp) to $+180$ bp of the translation start site ATG. The designated $\sigma^{30}$ and putative $\sigma^{28}$ recognition sites are underlined, the RACE primer (cagArace) binding site is highlighted, and the transcription start sites obtained by sequencing the 5' RACE products is indicated by an asterisk. D, Size of transcripts in H. pylori strains 26695 and G27 estimated by 5' RACE in unadhered wild type (WT) (a), AGS-adhered WT (b), unadhered $\Delta$flkK mutant (c), and AGS-adhered $\Delta$flkK mutant (d), unadhered $\Delta$flkA mutant (e), and AGS-adhered $\Delta$flkA mutant (f). Lower panel shows transcripts in the complemented G27 strains, unadhered $\Delta$flkK/flK (g), AGS-adhered $\Delta$flkK/flK (h), unadhered $\Delta$flkA/flkA (i), and AGS-adhered $\Delta$flkA/flkA (j). M, molecular size markers. E, Expected size of transcripts initiating from the $\sigma^{30}$ and $\sigma^{28}$ promoters amplified using the RACE primers (inclusive of 39-nucleotide forward primer).
G27ΔfliA/ΔfliA to WT levels (Figure 3C). Consistent with the lower cagA expression in AGS-adhered ΔfliA mutant, much less elongation and scattering of the AGS cells was observed after adherence of the ΔfliA mutants than with the WT strain and lower amounts of IL-8 were secreted by ΔfliA-infected AGS cells (Figures 2A, 2B, 3A, and 3B).

We also examined cagA expression in strains overexpressing flIK or flIA >100-fold (Supplementary Figure 2C). In these strains, cagA expression remained at WT levels in both unadhered and AGS-adhered bacteria (Supplementary Figure 2D), although expression of the σ28-dependent flaA gene was significantly higher (Supplementary Figure 2A). Furthermore, in the ΔflgM strain lacking the anti-σ28 factor FlgM, and consequently producing high levels of free σ28 [15], cagA expression and translocation of CagA after AGS adherence was found to be similar to what was seen in the WT strain, although a small decrease was observed in unadhered bacteria (Figure 2C and 2D). The IL-8 secretion and morphological changes in ΔflgM-infected AGS cells were similar to those in WT H. pylori-infected AGS cells (Figure 2A and 2B). Morphological observation of ΔflgM-infected AGS cells indicated changes similar to those observed in WT H. pylori-infected AGS cells (Figure 2).

**cagA Transcription Initiation in Adhered and Unadhered H. pylori**

A consensus σ28 binding site consisting of a well-conserved −10 element, and a spacer length of 13–15 nucleotides between the −10 and −35 boxes has been proposed for H. pylori [30, 31]. Nucleotide sequence of the upstream region of the cagA open reading frame (ORF) (http://www.ncbi.nlm.nih.gov/nuccore/?term=helicobacter%20pylori) was examined for the presence of a σ28 recognition site. A putative σ28-RNA polymerase (RNAP) promoter motif was detected between the designated σ80-RNAP promoter [32] and the cagA ORF in 11 H. pylori strains whose genome sequences are available, but not in the cagA promoter of strain J99 (Figure 4A). The σ28–RNAP promoter motif was also detected in the cagA promoter of strain S11 sequenced in this study (GenBank accession number KP137390). Overall, a high degree of conservation of the promoter sequence from σ28–RNAP binding site to the cagA transcription start site was noted in the strains except strain J99 (Figure 4A). The σ28 motif in the cagA promoter had high homology with σ28-dependent H. pylori promoters described previously (Figure 4B).

Next, 5’ RACE PCR was used to determine which promoter was active in unadhered and AGS-adhered H. pylori. A single transcript was detected in unadhered WT strains, the size of which indicated that transcription initiated from immediately downstream of the reported σ80-RNAP promoter. In AGS-adhered WT strains, an additional shorter transcript was detected, whose size indicated that it might have initiated from the downstream putative σ28-RNAP promoter (Figure 4D). The latter transcript was not detected in unadhered and AGS-adhered ΔflIK and ΔflIA strains but could be detected in the AGS-adhered complemented G27ΔflIK/ΔflK and G27ΔflIA/ΔflA strains, suggesting that the shorter transcript originated from a σ28-dependent promoter (Figure 4D). To map the transcription start sites, the PCR products were cloned in plasmid pBS and sequenced. The results obtained indicated that the shorter transcript initiated from a C residue located 22 nucleotides upstream of the cagA translational start codon (Figure 4C). As reported elsewhere, the longer σ80-RNAP–dependent transcript initiated from a G residue located 104 nucleotides upstream of the translational start site [30, 32].

To examine whether σ28 binds directly to the cagA promoter, ChIP assay was performed using the strain H. pylori 26695/ΔflIA-FLAG. Expression of the σ28-dependent flaA gene in this strain was similar to that in the WT strain (data not shown), indicating the FLAG-tagged σ28 functioned normally. After immunoprecipitation with FLAG antibodies, the precipitated DNA from adhered and unadhered bacteria was PCR amplified using primers PChIP. Using immunoprecipitated DNA from adhered bacteria, amplification was observed for the region containing the predicted σ28 binding site. Mock assays performed without FLAG antibody or using strain H. pylori 26695 WT instead of H. pylori 26695/ΔflIA-FLAG indicated no nonspecific binding (Figure 5). Taken together, the ChIP assays indicated that σ28 binds specifically to the cagA promoter in AGS-adhered H. pylori but not in unadhered bacteria.

**σ28-Dependent Expression of cagA and Cell Line Specificity**

It has been reported that H. pylori is capable of adhering to a variety of cell lines of different origins [33, 34]. The expression of cagA gene in H. pylori WT and ΔflIK, ΔfliA, and ΔflgM mutants after adherence to HeLa and HEp-2 cells was comparable to that observed after adherence to the AGS monolayers (Figure 6A). Microscopic observation revealed that adherence of ΔflIK and ΔfliA mutant to HeLa and HEp-2 cell lines resulted in much less cell scattering than adherence of the WT and

![Figure 5](https://academic.oup.com/jid/article-abstract/211/11/1779/858148/fig1)
ΔfigM mutant strains (Figure 6B). These results suggested that the mechanism of host cell contact–dependent up-regulation of cagA might not be cell line specific.

**Role of FliK and FliA in Other H. pylori Strains**

The role of FliK and σ²⁸ in regulating cagA expression in AGS-adhered H. pylori, was examined in 2 more strains, SS1 and J99. Similar to findings in the strains 26695 and G27, fliK, fliA, and flaA expression was significantly up-regulated after adherence of strain SS1 to AGS cells (Figure 7A), and cagA expression was also about 3-fold higher in the AGS-adhered SS1 bacteria than in unadhered bacteria (Figure 7B). To examine whether the up-regulation of cagA was dependent on σ²⁸, ΔfliK and ΔfliA mutants of strain SS1 were constructed. Although adherence of both mutants to AGS cells was similar to the WT strain, practically no up-regulation of cagA was observed in the mutants after adherence, and, unlike the WT strains, the mutants failed to induce the hummingbird phenotype in AGS cells (Figure 7B and 7D). These results suggested that σ²⁸ might be involved in up-regulation of cagA expression in AGS-adhered H. pylori SS1 strain, similar to strains 26695 and G27. In contrast, little up-regulation of fliK, fliA, or flaA was observed in AGS-adhered-strain J99, and cagA expression in this strain was similar between AGS-adhered and unadhered bacteria.

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**Figure 6.** Adherence of Helicobacter pylori strains to different cell lines and cagA expression. A, H. pylori strain 26695 (Hp wild type [WT]), ΔfliK mutant, ΔfliA mutant, and ΔfigM mutant were used to infect the cell lines HeLa and HEp-2 under identical conditions. Two hours after adherence, cagA expression was assayed in the unadhered and adhered bacteria with quantitative reverse-transcription polymerase chain reaction. Expression of cagA in unadhered H. pylori 26695 (Hp WT) was arbitrarily designated as 1. P values (above bars) represent significance of the differences in cagA expression between AGS-adhered and unadhered H. pylori strains. B, Uninfected and infected cell lines were observed on an inverted microscope in phase contrast at ×20 magnification. Abbreviation: NS, not significant (P > .05).
J99 also failed to produce the hummingbird phenotype after adherence to AGS cells (Figure 7D). To clarify the reason for this difference, we sequenced the cagA promoter region of strain SS1 and compared it with that of strain J99. A distinct σ28-RNAP promoter motif was detected in the cagA promoter of strain SS1, with a position similar to that in 26695, G27, and several other strains (Figure 4A). Furthermore, the sequence of the cagA promoter in these strains was highly conserved; however, in strain J99 considerable divergence was noted (Figure 4A). The σ28-RNAP binding site diverged from the consensus. In particular, the spacer region is reduced to 7 base pairs (bp) instead of 15 bp in J99. These results further support the hypothesis that up-regulation of cagA expression after adherence of H. pylori to AGS cells is dependent on σ28, because the up-regulation was observed in strains 26695, G27, and SS1, all of which have a σ28-RNAP binding site in the cagA promoter, but it was not observed in strain J99, that lack σ28-RNAP consensus in the cagA promoter.

DISCUSSION

In many bacterial pathogens, contact with eukaryotic cells is recognized as a signal that triggers expression of specific bacterial genes required for host pathogen interactions leading to

Figure 7. AGS infection by Helicobacter pylori strains SS1 and J99. A, Expression of flk, flaA, and flaA was examined in unadhered and AGS-adhered strain SS1 with quantitative reverse-transcription polymerase chain reaction (qRT-PCR). B, qRT-PCR analysis of cagA expression in unadhered and AGS-adhered SS1 wild-type (WT) and mutant strains. Expression levels are given relative to that in unadhered strain SS1 WT, arbitrarily designated as 1. Error bars represent standard deviation (SDs). C, Expression of cagA, flk, flaA, and flaA was examined in unadhered and AGS-adhered strain J99 with qRT-PCR. Expression levels are given relative to that in unadhered strain J99, arbitrarily designated as 1. Error bars represent SDs; P values, significance of the differences in gene expression between AGS-adhered and unadhered strains. D, Morphological changes in AGS cells after adherence of strain SS1, SS1 Δflk, SS1 ΔflaA, and J99. Elongated cells were counted and expressed as the percentage of total cells in each sample. Bar diagram represents means and SDs of 3 independent experiments; P values, significance of the differences in number of elongated cells between SS1 WT and mutant strains; Abbreviation: NS, not significant (P > .05).
disease. It has been reported that 22 genes were consistently up-regulated on adherence of *H. pylori* strain 69a to AGS cells, and maximal induction (5.2-fold) was observed in expression of the *flk* gene [26]. In strains 26695 and G27, we observed even higher up-regulation of *flk* (Figure 1 and Supplementary Figure 1). Moreover, the *cagA* gene was strongly induced after adherence of *H. pylori* 26695 and G27 to AGS cells (Figure 2C) [27], although no up-regulation of *cagA* was reported in strain 69a [26]. The difference may be due to heterogeneity between the strains. Indeed, marked variation in levels of *cagA* expression among individual isolates has been reported [35].

In this study, it has been demonstrated that FliK is necessary for up-regulation of *cagA* expression on adherence of *H. pylori* to AGS cells. The Δ*flik* mutant strain adhered efficiently to AGS cells but failed to produce the scattering and elongation or hummingbird phenotype (Figures 2A, 3A, and 7B), consistent with the observation that practically no *cagA* up-regulation occurred in the AGS-adhered Δ*flik* mutant strain (Figures 2C, 3C and 7C). We considered possible reasons for the failure to induce *cagA* expression in AGS-adhered *H. pylori* Δ*flik* mutant strain. It is known that FliK is required to sense the optimal length of the flagellar hook, which triggers secretion of the anti-σ²⁸ factor FlgM. In a Δ*flik* mutant, FlgM is not exported and sequesters σ²⁸ within the bacterial cell, preventing expression of σ²⁸-dependent genes [9]. In this context, we examined if the expression of *cagA* was dependent on σ²⁸. Our results clearly indicated that in unadhered *H. pylori*, *cagA* expression occurred at low levels from a σ⁸⁰-dependent promoter, but in AGS-adhered bacteria *cagA* expression is up-regulated and occurs from an additional σ²⁸-dependent promoter located downstream of the σ⁸⁰ promoter (Figures 4 and 5).

It is attractive to hypothesize that on adherence of *H. pylori* to gastric cells, FliK up-regulation might be necessary to sense optimal hook length, switch substrate specificity from hook to filament proteins, trigger secretion of the anti-σ²⁸ factor FlgM, releasing free σ²⁸, which then activates expression from the σ²⁸-dependent promoter located upstream of the *cagA* gene. Consistent with the hypothesis that the concentration of free σ²⁸ increases in AGS-associated *H. pylori*, a strong up-regulation of the known σ²⁸-dependent flagellin gene flaA was observed in the AGS-adhered bacterium as has been reported elsewhere [30].

If increased levels of free functional σ²⁸ were the only cause for increased *cagA* expression in AGS-adhered *H. pylori* WT strains, increasing σ²⁸ levels in unadhered bacteria by overexpression of *flik* or *flaA* or in Δ*flgM* strain (Supplementary Figure 2) should lead to increased *cagA* expression, even in unadhered bacteria. However, *cagA* expression in these strains was comparable to that in the WT strain (Figures 2C and 3C). Taken together, these results suggested that although functional σ²⁸ is essential for up-regulation of *cagA* expression in AGS-adhered *H. pylori*, some other still-unidentified factor may also be involved, which requires further investigation.

Findings of a study on *H. pylori* clinical isolates [35] have suggested a strong correlation between the presence of a DNA motif (AATAAGATA) upstream of the *cagA* ORF, with a high level of *cagA* expression and severity of disease. However, to our knowledge, how this motif influences *cagA* expression has not been addressed previously. In this context, it was interesting to note that the AATAAGATA motif is included within the σ²⁸-RNAP promoter motif identified upstream of the *cagA* in this study. It is likely that strains harboring the AATAAGATA motif use an alternative σ²⁸-RNAP promoter apart from the previously annotated promoter, thereby contributing to the increased level of *cagA* expression and consequent disease severity.

It has been demonstrated that the flagella is essential for the colonization of human gastric mucosa by *H. pylori* [5]. This study demonstrates that the flagellar regulatory system of *H. pylori* is directly required for up-regulation of the major virulence gene *cagA* in gastric cell associated *H. pylori*. Thus, an intriguing feature of the host adaptation strategy of *H. pylori* seems to be the integration of *cagA* expression with the flagellar regulatory system that presumably ensures that 2 essential virulence determinants, motility and CagA, are coordinately expressed.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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