Helicobacter pylori FlhB processing-deficient variants affect flagellar assembly but not flagellar gene expression

Todd G. Smith,1 Lara Pereira2 and Timothy R. Hoover1

1Department of Microbiology, University of Georgia, Athens, GA 30602, USA
2Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA

Regulation of the Helicobacter pylori flagellar gene cascade involves the transcription factors σ54 (RpoN), employed for expression of genes required midway through flagellar assembly, and σ28 (FlaA), required for expression of late genes. Previous studies revealed that mutations in genes encoding components of the flagellar protein export apparatus block expression of the H. pylori RpoN and FliA regulons. FlhB is a membrane-bound component of the export apparatus that possesses a large cytoplasmic domain (FlhBC). The hook length control protein FliK interacts with FlhBC to modulate the substrate specificity of the export apparatus. FlhBC undergoes autocleavage as part of the switch in substrate specificity. Consistent with previous reports, deletion of flhB in H. pylori interfered with expression of RpoN-dependent reporter genes, while deletion of fliK stimulated expression of these reporter genes. In the ΔflhB mutant, disrupting fliK did not restore expression of RpoN-dependent reporter genes, suggesting that the inhibitory effect of the ΔflhB mutation is not due to the inability to export FliK. Amino acid substitutions (N265A and P266G) at the putative autocleavage site of H. pylori FlhB prevented processing of FlhB and export of filament-type substrates. The FlhB variants supported wild-type expression of RpoN- and FliA-dependent reporter genes. In the strain producing FlhBN265A, expression of RpoN- and FliA-dependent reporter genes was inhibited when fliK was disrupted. In contrast, expression of these reporter genes was unaffected or slightly stimulated when fliK was disrupted in the strain producing FlhBP266G. H. pylori HP1575 (FlhX) shares homology with the C-terminal portion of FlhBC (FlhBCC) and can substitute for FlhBCC in flagellar assembly. Disrupting flhX inhibited expression of a flaB reporter gene in the wild-type but not in the ΔfliK mutant or strains producing FlhB variants, suggesting a role for FlhX or FlhBCC in normal expression of the RpoN regulon. Taken together, these data indicate that the mechanism by which the flagellar protein export apparatus exerts control over the H. pylori RpoN regulon is complex and involves more than simply switching substrate specificity of the flagellar protein export apparatus.

INTRODUCTION

Bacterial flagellar biosynthesis involves transcriptional hierarchies that coordinate flagellar gene expression with assembly. Flagellar gene hierarchies are well characterized in a few bacteria, including Salmonella enterica serovar Typhimurium (S. typhimurium) and Caulobacter crescentus (Chilcott & Hughes, 2000; Wu & Newton, 1997). Flagellar gene regulation in γ-Proteobacteria, such as Helicobacter pylori and Campylobacter jejuni, shares some similarities with these established paradigms but also differs significantly. As in S. typhimurium, H. pylori flagellar genes required late in assembly, such as the major flagellin (FlaA) and filament cap protein, are transcribed by σ28 (FlaA)-RNA polymerase holoenzyme (Kim et al., 1999; Leying et al., 1992), and, as in C. crescentus, many H. pylori flagellar genes require σ34 (RpoN) for their transcription (Spohn & Scarlato, 1999; Suerbaum et al., 1993). Transcription of genes needed early in H. pylori flagellar assembly are dependent on σ54 (RpoD), the primary H. pylori sigma factor (Beier et al., 1997; Porwollik et al., 1999; Schmitz et al., 1997). Expression of early flagellar genes generally requires a transcriptional activator, referred to as the master regulator. While a master regulator has yet to be identified in H. pylori, some studies have shown that the quorum-sensing autoinducer synthase LuxS affects motility.
by altering expression of early and late flagellar genes (Loh et al., 2004; Osaki et al., 2006; Rader et al., 2007). The effect of LuxS on flagellar gene expression, however, is strain-specific (Joyce et al., 2000; Lee et al., 2006), and may be due to global changes in gene expression caused by decreased fitness in the luxS mutant rather than loss of quorum sensing (Lee et al., 2006).

The H. pylori RpoN regulon contains genes that encode proteins needed midway through flagellar assembly, including the hook protein FlgE and the minor flagellin FlaB (Niehus et al., 2004; Spohn & Scarlato, 1999). Transcription of the RpoN regulon is controlled by a two-component regulatory system consisting of the sensor kinase FlgS and the response regulator FlgR (Beier & Frank, 2000; Spohn & Scarlato, 1999), which activates transcription with 54-RNA polymerase holoenzyme without binding upstream activation sequences in the DNA (Brahmachary et al., 2004). The cellular cues that initiate FlgS/FlgR signal transduction are unknown. Some RpoN-dependent genes are responsive to exposure to low pH or heat shock response, but these stresses appear to affect expression of the RpoN regulon indirectly (Merrell et al., 2003; Roncarati et al., 2007).

Transcription of the H. pylori Flia regulon is negatively regulated by the anti-sigma factor FlgM (Colland et al., 2001; Josenhans et al., 2002). S. typhimurium FlgM is secreted by the flagellar protein export apparatus upon completion of the hook–basal body complex, resulting in expression of the Flia-dependent flagellar genes (Hughes et al., 1993). In H. pylori, disrupting the flagellar protein export apparatus inhibits expression of Flia-dependent genes (Schmitz et al., 1997), indicating that the H. pylori export apparatus may secrete FlgM. Inactivation of the H. pylori export apparatus also results in decreased levels of some RpoN-dependent gene products (Allan et al., 2000; Schmitz et al., 1997), and reduced transcript levels of RpoN-dependent genes (Niehus et al., 2004). Similarly, disruption of the C. jejuni flagellar protein export apparatus inhibits expression of RpoN-dependent reporter genes (Hendrixson & DiRita, 2003). The mechanism that couples export apparatus function with expression of RpoN-dependent genes in H. pylori or C. jejuni is unknown.

The flagellar protein export apparatus is a type III secretion system (T3SS) that transports flagellar substrates across the cell membrane (Minamino & Macnab, 1999). In S. typhimurium, the export apparatus consists of six membrane proteins (FlhA, FlhB, FlhO, FlhP, FlhQ and FlhR) and three cytoplasmic proteins (FliH, Flii and FliJ) (Macnab, 2003). FlhB possesses a large C-terminal cytoplasmic domain referred to as FlhB C (Kutsukake et al., 1994; Minamino et al., 1994). The export apparatus transports rod-/hook-type substrates until completion of the hook–basal body complex, at which point it switches substrate specificity to filament-type substrates (Minamino et al., 1999a). The switch in substrate specificity involves FlhB and the hook length control protein, FljK. Completion of the mature hook is communicated by FljK to FlhB C, which is believed to cause conformational changes that are required for the switch between export states (Williams et al., 1996). FlhB C undergoes autocleavage at a conserved NPTH motif which is required for the switch in substrate specificity, as substitutions at the cleavage site inhibit processing of FlhB and export of filament-type substrates (Fraser et al., 2003; Minamino & Macnab, 2000). The C-terminal subdomain of FlhB C (FlhB CC) remains tightly associated with the rest of FlhB following processing (Minamino & Macnab, 2000). FlhB homologues in the virulence factor T3SS used by pathogenic bacteria also undergo autocleavage, and like those of FlhB, the C-terminal subdomains of these proteins remain associated with the rest of the protein after autocleavage (Deane et al., 2008; Minamino & Macnab, 2000; Zarivach et al., 2008). FlhB processing has been proposed to serve as a molecular clock to regulate temporal gene expression with the spatial assembly of the flagellum (Ferris et al., 2005).

H. pylori has an ORF (HP1575) that shares homology with FlhB CC (Tomb et al., 1997; Wand et al., 2006). Orthologues of HP1575 occur in several other bacteria and are referred to as FlhX proteins (Pallen et al., 2005; Wand et al., 2006). Wand and co-workers reported that FlhX can functionally substitute for FlhB CC in H. pylori (Wand et al., 2006), suggesting that in the absence of FlhB CC, FlhX can associate with the N-terminal subdomain of FlhB C (FlhB CN).

As observed in S. typhimurium, disruption of H. pylori fliK (hp0906) results in reduced motility and formation of poly-hook structures due to a delay in the switch between export states (Ryan et al., 2005; Williams et al., 1996). Inactivation of fliK in H. pylori and C. jejuni stimulates expression of RpoN-dependent flagellar genes (Kamal et al., 2007; Ryan et al., 2005). Based on the role of FljK and FlhB in substrate switching, we wished to determine how processing-deficient FlhB variants would affect expression of the H. pylori RpoN and Flia regulons. FlhB variants were constructed in which the two residues at the cleavage site, Asn-265 and Pro-266, were replaced with alanine and glycine, respectively, and expressed in H. pylori. In contrast to wild-type FlhB, FlhB N265A and FlhB P266G variants did not undergo detectable autocleavage. FlhB processing was required for wild-type motility and export of filament-type substrates. FlhB processing did not influence flagellar gene expression, as FlhB processing-deficient strains expressed RpoN- and Flia-dependent reporter genes at close to wild-type levels. Disrupting fliK in the strain that produced FlhB N265A inhibited expression of RpoN- and Flia-dependent reporter genes, but this did not occur in the strain producing FlhB P266G. We also observed that flhX was required for optimal expression of the RpoN-dependent reporter gene flaB′′→xyLE in an otherwise wild-type background but not in strains that produced FlhB N265A or FlhB P266G. These findings provide a valuable framework for dissecting the mechanism that couples export apparatus function with flagellar gene expression in H. pylori.
**METHODS**

**Bacterial strains and growth conditions.** *H. pylori* ATCC 43504 was grown on tryptic soy agar supplemented with 5% (v/v) horse serum at 37 °C in a 2% O₂/5% CO₂/95% N₂ atmosphere. When necessary, media were supplemented with 30 μg chloramphenicol ml⁻¹, 30 μg kanamycin ml⁻¹ or 10 μg erythromycin ml⁻¹. *Escherichia coli* DH5α and BL21 pLYS were grown at 37 °C with shaking in Luria–Bertani broth or brain heart infusion (BHI) broth supplemented with 100 μg ampicillin ml⁻¹, 30 μg chloramphenicol ml⁻¹ or 150 μg erythromycin ml⁻¹.

**Motility assay.** *H. pylori* motility was assessed using semisolid medium containing Mueller–Hinton broth supplemented with 0.4% (v/v) Noble agar, 10% (v/v) horse serum and 10 μM FeSO₄. A sterile toothpick was used to stab the cells into the agar, and plates were incubated at 37 °C in a microaerobic atmosphere for up to 7 days.

**Construction of mutants.** *H. pylori* flhB (HP0770), flk (HP0906) and flhX (HP1575) including 500 bp of flanking DNA sequence (see Supplementary Table S1 for primer sequences), were amplified from *H. pylori* strain 26695 genomic DNA using *E. coli* Taq DNA polymerase (Stratagene). Amplicons were incubated with Taq DNA polymerase (Promega) and then cloned into pGEM-T (Promega). The resulting plasmids, pTS45 (flhB), pTS69 (flk) and pTS68 (flhX), were used as templates for inverse PCR with primers that introduced EcoRI sites and annealed to sequences immediately upstream of the translational start or downstream of the translational stop. Amplicons were circularized using T4 DNA ligase and digested with EcoRI, and a 1.1 kb EcoRI fragment containing a chloramphenicol transacetylasel gene (*cat*) was cloned into the vector (Wang & Taylor, 1990). Alternatively, after cutting with EcoRI the overhanging ends were filled in using T4 DNA polymerase, and a 1.1 kb EcoRV fragment containing a *Streptococcus pneumoniae* erythromycin-resistance gene (*ermB*) was cloned into the vector (Stabb & Ruby, 2002). The antibiotic-resistance cassette completely replaced the target gene in the final constructs, which were used as suicide vectors in *H. pylori*. Plasmids were introduced into *H. pylori*, and transformants selected as described previously (Brahmachary et al., 2004). Replacement of the target gene on the chromosome with *cat* or *ermB* was confirmed by PCR.

**Complementation of ΔflhB mutant.** Plasmid pTS45 was used as a template for site-directed mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene). Primers that annealed 11 bp downstream of the translational stop of *flhB* were used to introduce a unique EcoRV site (Supplementary Table S1), into which *ermB* was cloned. The resulting plasmid (pTS50) was introduced into an *H. pylori* ΔflhB:cat mutant to restore the wild-type *flhB* allele. Transformants were selected on solid medium containing erythromycin and screened for chloramphenicol sensitivity. This same strategy was used to introduce *flhB* (N265A) and *flhB* (P266G) alleles. Site-directed mutagenesis was used to introduce the desired substitutions in *flhB* in pTS50 using the primers indicated in Supplementary Table S1, and the resulting plasmids were introduced into *H. pylori* ΔflhB:cat as described above. The *flhB* alleles in selected transformants were PCR-amplified, and amplicons were sequenced at the University of Georgia Integrated Biotechnology Laboratory to confirm that the expected mutations were present and no other mutations had been introduced.

**FLAG-tagged FlgE construction.** The *flgE* gene (HP0870) was amplified from *H. pylori* strain 26695 genomic DNA using *Taq* DNA polymerase and the primers indicated in Supplementary Table S1. The forward primer, containing a *Sal*I site, annealed 100 bp upstream of the translational start, and the reverse primer, containing a *Kpn*I site, replaced the translational stop with a sequence encoding the FLAG epitope (DYKDDDDK). Amplicons were cloned into pGEM-T, confirmed by sequencing, and then subcloned into the shuttle vector pHel3 (Heuermann & Haas, 1998) to obtain pTS14, which was introduced into *H. pylori* strains by natural transformation (Brahmachary et al., 2004).

**Protein export assays.** *H. pylori* strains were grown in BHI (pH 6.5) supplemented with 0.4% (v/v) β-cyclodextran and 30 μg kanamycin ml⁻¹ for strains harbouring pTS14. Cultures were incubated at 37 °C with shaking in a 10% O₂/5% CO₂/10% H₂ atmosphere for 24 h. Before harvesting, cells were vortexed for 10 s to increase shearing of flagella. Cells were separated from the medium by centrifugation for 20 min at ~4000 g. The resulting supernatant was further clarified by centrifugation for 15 min at ~19 000 g, mixed 3:1 with ice-cold 25% (v/v) TCA and incubated at 0 °C for 15 min. Precipitated proteins were collected by centrifugation for 10 min at ~11 000 g. The protein pellet was washed three times with acetone, air-dried, and resuspended in 25 mM Tris-buffered saline (pH 7.4). The cell pellet from the initial centrifugation step was resuspended in 3 ml PBS (pH 7.4) and lysed by two passages through a French press at ~13 800 kPa. Cellular debris was removed by centrifugation for 10 min at ~4000 g and membranes were removed by centrifugation for 60 min at ~100 000 g. Proteins in the resulting supernatant were collected by TCA precipitation as described above. Protein concentrations of the samples were measured using the bichinchonic acid protein assay (Pierce), following the manufacturer’s instructions. Equivalent amounts of protein were analysed for FlgE–FLAG, FlaB and FlaA by Western blotting.

**His-tagged FlhB purification.** A 457 bp fragment encoding *H. pylori* FlhB was PCR-amplified from *H. pylori* 26695 genomic DNA using *Taq* DNA polymerase and the primers indicated in Supplementary Table S1. The resulting product was cloned into pGEM-T, sequenced, and subcloned into pET-28 (Novagen) to generate a hexahistidine-tagged fusion. Recombinant protein was overproduced in *E. coli* BL21 pLYS by inducing expression for 3.5 h with 0.5 mM IPTG in a 1 l culture grown to mid-exponential phase. His-tagged FlhB protein was purified using nickel–NTA (Qiagen) affinity chromatography following the supplier’s instructions. Purified His-tagged FlhB was sent to Cocalico Biologicals to immunize a New Zealand white rabbit.

**Western blots.** Antiserum directed against *H. pylori* FlaB has been described previously (Pereira & Hoover, 2005). Antiserum directed against the FLAG epitope was purchased from Sigma. Antiserum directed against FlhB was affinity-purified by adapting a previously described method (Pereira & Hoover, 2005). Primary antibodies were detected by enhanced chemiluminescence using a peroxidase-conjugated goat anti-rabbit antibody (MP Biomedicals).

**Xyle assays.** Xyle reporter gene fusions have been described previously (Brahmachary et al., 2004; Pereira & Hoover, 2005). Whole-cell Xyle assays were carried out as described previously (Brahmachary et al., 2004). Xyle activity was reported as micromoles product formed per minute per 10⁸ *H. pylori* cells. Xyle activity for each strain was determined from at least 10 technical replicates from two or more biological replicates. Student’s t test was used to determine the standard deviation with 95% confidence intervals (*P*=0.05).

**RESULTS**

**Deletion of fliK or flhB has different consequences for *H. pylori* flagellar gene expression**

Disruption of *H. pylori* fliK results in reduced motility and elevated transcript levels of RpoN-dependent genes (Ryan et al., 2005). Building upon this observation, we examined...
how deletion of flgK influenced expression of flagellar reporter genes in which promoter regulatory regions of selected flagellar genes were fused to a promoterless Pseudomonas putida xylE (encoding catechol dioxygenase) gene. The different constructs included flgI-xylE, which is dependent on RpoD (flgI encodes the P-ring protein); flgB-xylE and hp1120-xylE, which are dependent on RpoN (flaB encodes the minor flagellin; hp1120 encodes a protein of unknown function and is part of an operon with flgK, which encodes hook-associated protein 1); and flaA-xylE, which is dependent on FliA (flaA encodes the major flagellin) (Brahmachary et al., 2004; Pereira & Hoover, 2005). Reporter genes were introduced into H. pylori strains on the shuttle vector pHel3 (Heuermann & Haas, 1998), and XylE activities were measured using a whole-cell colorimetric assay. Consistent with the earlier report (Ryan et al., 2005), we found that the ΔflgK:cat mutation stimulated expression of the RpoN-dependent reporter genes 3.5–6.5-fold compared with the wild-type (Fig. 1). Expression of the flaA-xylE reporter gene in the ΔflgK:cat mutant was similar to that of the wild-type (Fig. 1). In contrast, Ryan et al. (2005) found that disrupting flgK resulted in decreased flaA transcript levels. One possibility for this discrepancy is that the flaA-xylE reporter gene lacks elements that affect flaA transcript stability. This seems plausible, as elements that destabilize flagellin transcripts have been described in other bacteria (Anderson & Gober, 2000). Consistent with the report by Ryan et al. (2005), expression of the RpoD-dependent flgI-xylE reporter gene in the ΔflgK:cat mutant was similar to that in the wild-type strain (Fig. 1).

To investigate the role of FlhB in expression of the flagellar reporter genes, we deleted flhB in H. pylori ATCC 43504. As expected from previous reports (Allan et al., 2000; Wand et al., 2006), the resulting ΔflhB:cat mutant was non-motile (Fig. 2). We tried unsuccessfully to complement the ΔflhB:cat mutant by introducing flhB into the HP0405

**Fig. 1.** Expression of flagellar reporter genes in various H. pylori strains. XylE activities were measured in whole-cell assays for the reporter genes indicated in H. pylori ATCC 43504 (wild-type, black), ΔflgK:cat (white), ΔflhB:cat (dark grey), a strain in which the wild-type flhB allele was restored (flhB restored, light grey), flhB(N265A) (FlhBN265A, striped) and flhB(P266G) (FlhBP266G, stippled). One unit of XylE activity corresponds to 1 μmol product formed min⁻¹ (10⁸ cells)⁻¹. Means are reported for five statistical replicates; error bars indicate 95% confidence intervals (P=0.05) as determined by Student’s t test.
locus (data not shown). HP0405 encodes a NifU-like protein that is not required for motility or flagellar gene expression (Pereira & Hoover, 2005). Complementation in the HP0405 locus may have failed because a cis-acting regulatory element needed for expression was missing, or because contextual factors (Ye et al., 2007) interfered with expression of \( \text{flhB} \) in the HP0405 locus. We replaced the \( \Delta \text{flhB}: \text{cat} \) allele with the wild-type \( \text{flhB} \) allele in its native locus, and motility was restored (Fig. 2), confirming that no other mutations that affected motility had been introduced inadvertently into the original \( \Delta \text{flhB}: \text{cat} \) strain.

Expression of the two RpoN-dependent reporter genes was reduced approximately 10-fold in the \( \Delta \text{flhB}: \text{cat} \) mutant compared with the wild-type (Fig. 1). Disruption of \( rpoN \) or \( \text{flgR} \) resulted in a similar reduction in expression of these reporter genes (data not shown). Expression of the \( \text{flaA}^{9}\text{xylE} \) reporter gene was diminished \( \sim 2.5 \)-fold relative to wild-type in the \( \Delta \text{flhB} \) mutant (Fig. 1). These findings were consistent with an earlier report that disruption of \( \text{H. pylori} \text{flhB} \) results in reduced levels of hook protein and both flagellins (Allan et al., 2000). In contrast to the RpoN- and FliA-dependent reporter genes, expression of \( \text{flgI}^{9}\text{xylE} \) in the \( \Delta \text{flhB}: \text{cat} \) mutant was not inhibited (Fig. 1). Reintroduction of the wild-type \( \text{flhB} \) allele restored expression of the RpoN- and FliA-dependent reporter genes to levels that were similar to those of the wild-type (\(<1.5\)-fold difference), except for expression of the \( \text{flaA}^{9}\text{xylE} \) reporter gene, which for unknown reasons was slightly enhanced (\( \sim 1.7\)-fold) in the \( \text{flhB} \)-restored strain (Fig. 1).

### FliH processing-deficient variants support wild-type expression of RpoN-dependent reporter genes

We postulated that enhanced expression of the RpoN-dependent reporter genes in the \( \Delta \text{flk}: \text{cat} \) mutant resulted from a delay in the switch between export states. Therefore, we reasoned that in \( \text{H. pylori} \) strains producing FliH processing-deficient variants the export apparatus might be locked in the rod-/hook-type conformation and over-express the RpoN regulon. The predicted cleavage site in \( \text{H. pylori} \text{FlhB} \) is between Asn-265 and Pro-266. \( \text{H. pylori} \text{FlhB} \) variants were generated in which Asn-265 was replaced with alanine and Pro-266 was replaced with glycine. These amino acid changes were chosen since equivalent substitutions in \( \text{S. typhimurium} \text{FlhB} \) interfere with cleavage (Fraser et al., 2003). \( \text{H. pylori} \) strains expressing FlhB\( ^{N265A} \) or FlhB\( ^{P266G} \) were reduced in their motility (Fig. 2). These findings were consistent with the decrease in motility observed in \( \text{S. typhimurium} \) expressing the equivalent FlhB variants (Fraser et al., 2003), and were also consistent with the finding that substitutions in the conserved NPTH motif in the \( \text{Yersinia} \) T3SS homologue YscU abolish export of translocators (Sorg et al., 2007). This result, however, contradicts the results of Wand et al. (2006), who reported that the \( \text{H. pylori} \text{FlhB} \) \( ^{P266G} \) variant does not affect motility. This discrepancy is not due to the \( \text{H. pylori} \) strains used. Expressing the FlhB\( ^{P266G} \) variant in \( \text{H. pylori} \) J99 (the same strain used by Wand and co-workers) resulted in similarly reduced motility (data not shown). Wand and co-workers introduced a FLAG tag at the C-terminus of the FlhB\( ^{P266G} \) variant, which may have influenced the activity, conformation and/or processing of this protein in \( \text{H. pylori} \), allowing normal motility. Consistent with this scenario, Wand and co-workers observed that the FLAG-tagged FlhB\( ^{P266G} \) variant was processed at a secondary site when expressed in \( \text{E. coli} \). Those researchers were unable to detect FliH or its variants in \( \text{H. pylori} \) to see if this processing also occurred in \( \text{H. pylori} \) (Wand et al., 2006).

Affinity-purified antibodies that recognize \( \text{H. pylori} \text{FlhB}_{C} \) were used in Western blot assays to examine FliH proteins. In membrane preparations from wild-type \( \text{H. pylori} \), a band with an estimated 34 kDa molecular mass was observed (Fig. 3), which is close to the predicted mass of a protein consisting of the FlhB transmembrane domain and FlhB\( _{C} \) N-terminal subdomain (30 kDa). In some blots a faint band that migrated close to the predicted mass of full-length FliH (41 kDa) was also observed. Neither of these bands was detectable in the \( \Delta \text{flhB}: \text{cat} \) mutant, but both were present in the strain in which wild-type \( \text{flhB} \) had been restored (Fig. 3). These results confirm that \( \text{H. pylori} \) FlhB\( _{C} \) is processed \textit{in vivo} similarly to \( \text{S. typhimurium} \) FlhB (Minamino & Macnab, 2000) and verify previous work that demonstrates processing of \( \text{H. pylori} \) FlhB recombinant proteins in \( \text{E. coli} \) (Wand et al., 2006). In contrast to wild-type FlhB, FlhB\( ^{N265A} \) and FlhB\( ^{P266G} \) were not detectably processed (Fig. 3), indicating that the amino acid substitutions in these FlhB variants inhibit cleavage of the protein. These results are consistent with the previous report that the FlhB\( ^{P266G} \) variant expressed in \( \text{E. coli} \) is deficient in normal processing (Wand et al., 2006).

The FlhB\( _{C} \) subdomain remains associated with the rest of FliH following autocleavage (Minamino & Macnab, 2000). Structural and biochemical studies indicate that the C-terminal subdomain of FlhB T3SS homologues remains intimately associated with the N-terminal subdomain.
following autocleavage (Deane et al., 2008; Riordan & Schneewind, 2008; Zarivach et al., 2008). However, we were unable to detect a cross-reacting protein corresponding to the 
*H. pylori* FlhBCC subdomain (10 kDa) in the membrane preparations. In a similar study with *Yersinia YscU*, the C-terminal subdomain of this FlhB homologue was not detected in cellular extracts of wild-type *Yersinia enterocolitica* (Riordan & Schneewind, 2008). The fate of *H. pylori* FlhBCC in our assay is unknown. FlhBCC may have dissociated from the rest of FlhB in vivo or during the membrane isolation procedure. The affinity-purified antibodies did recognize purified FlhBCC (data not shown), indicating that the inability to detect FlhBCC in the membrane fractions was not due to poor cross-reactivity.

In the strain expressing the FlhBN265A variant, all four reporter genes were expressed at levels slightly higher than wild-type levels (~1.5-fold difference), similar to the levels observed when flhB was restored in the native locus (Fig. 1). In the strain expressing the FlhBP266G variant, only the flaB → xylE reporter gene was expressed at levels that were significantly different from wild-type levels (~1.9-fold difference, Fig. 1). Taken together, these observations indicate that processing of FlhB is not required for normal expression of these representative genes from all three *H. pylori* flagellar regulons. In addition, FlhB processing and FliK apparently mediate different effects on the structure and/or function of the export apparatus, since the FlhB processing-deficient variants did not stimulate expression of the RpoN-dependent reporter genes to the same extent as the ΔfliK: cat mutant (Fig. 1).

### Disruption of fliK in FlhB processing-deficient strains has distinct consequences for flagellar gene expression

Since FliK is a rod-/hook-type substrate (Minamino et al., 1999b), we reasoned that inhibition of the RpoN-dependent reporter genes in the ΔflhB: cat mutant might result from the failure to export FliK. This does not appear to be the case, however, since disrupting fliK in the ΔflhB: cat mutant did not significantly alter expression of the RpoN-dependent reporter genes (Fig. 4). We also disrupted fliK in strains that expressed the FlhB variants. Inactivating fliK in the strain that
produced FlhB<sup>N265A</sup> severely inhibited expression of the RpoN-dependent flaB<sup>−</sup>-xyle reporter gene and inhibited expression of the FliA-dependent flaA<sup>−</sup>-xyle reporter gene approximately twofold compared with the FlhBN265A parenteral strain (Fig. 4). Expression of the RpoN-dependent hp1120<sup>−</sup>-xyle reporter gene was also inhibited in this strain, while expression of the RpoD-dependent flgI<sup>−</sup>-xyle reporter gene was not significantly changed (data not shown). In contrast, disrupting fliK in the strain that produced FlhB<sup>P266G</sup> had little effect on expression of the flagellar reporter genes, with the exception of hp1120<sup>−</sup>-xyle, which was slightly stimulated (Fig. 4, and data not shown). Thus, despite the fact that FlhB<sup>N265A</sup> and FlhB<sup>P266G</sup> are both deficient in autocleavage, these proteins elicit very different effects on the RpoN and FliA regulons in the absence of FliK.

### FlhB<sup>N265A</sup> or FlhB<sup>P266G</sup> variant strains are defective in exporting filament-type substrates

*Helicobacter pylori* strains producing FlhB<sup>N265A</sup> or FlhB<sup>P266G</sup> were tested for the ability to secrete rod-/hook-type and filament-type substrates. For these assays, extracellular fractions from strains grown in liquid medium were analysed by Western blotting for the presence of the hook protein FlgE fused to the FLAG epitope (FlgE–FLAG, a rod-/hook-type substrate) or FlaB and FlaA (filament-type substrates). Flagellar proteins present in the extracellular fraction result from flagella shearing off, and/or failure of flagellar subunits to polymerize with the nascent flagellum. Strains producing FlhB<sup>N265A</sup> or FlhB<sup>P266G</sup> expressed and exported FlgE–FLAG (Fig. 5a) but failed to export FlaA or FlaB, even though both flagellins were present in the soluble cytoplasmic fraction from these strains (Fig. 5b). In this regard the strains producing FlhB variants appeared similar to the ΔfliK:cat mutant in this assay. In the strain producing the FlhB<sup>N265A</sup> in the absence of FliK, FlgE–FLAG, FlaB and FlaA levels were drastically reduced in the soluble cytoplasmic fraction, confirming the results of the reporter gene assays. The phenotype of the strain producing FlhB<sup>P266G</sup> in the absence of FliK appeared similar to that of the other FlhB processing-deficient variants in that FlaA and FlaB were present in the soluble cytoplasmic fraction but were not exported (Fig. 5).

### FlhX is required for optimal expression of flaB in a wild-type background but not in strains producing FlhB processing-deficient variants

To determine whether FlhX (HP1575) influences flagellar gene expression, we constructed a ΔflhX:cat mutant and monitored expression of the RpoN-dependent flaB<sup>−</sup>-xyle reporter gene in the resulting strain. Expression of the reporter gene was inhibited in the ΔflhX:cat mutant ~2 to 8-fold relative to the wild-type (Fig. 6). This result was unexpected, since consistent with the observations of Wand et al. (2006), disruption of flhX had no observable effect on motility (data not shown). The ΔflhX:cat allele was introduced into the strains that expressed FlhB<sup>N265A</sup> or FlhB<sup>P266G</sup> and reporter gene activities were measured in various strains: wild-type, ΔflhB:cat, flaB(N265A), flaB(P266G), ΔfliK:cat, flhB(N265A) ΔfliK:cat and flhB(P266G) ΔfliK:cat. Approximately 15 μg total protein was loaded in each C lane and 20 μg total protein in each E lane. FlgE–FLAG was detected in Western blots with antibodies directed against the FLAG epitope. (b) Western blot analysis of flagellins in soluble cytoplasmic (C) and extracellular (E) fractions. The same protein samples and similar amounts of protein were loaded as in (a). Flagellins were detected in Western blots with antiserum directed against FlaB. The antiserum also cross-reacts with FlaA, which is slightly smaller than FlaB.

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**Fig. 5.** Assay for export of hook and flagellin proteins in various *H. pylori* strains. (a) Western blot analysis of hook protein FlgE with a C-terminal FLAG-tag fusion (FlgE–FLAG) in soluble cytoplasmic (C) and extracellular (E) fractions. The following strains bearing the FlgE–FLAG expression vector pTS14 were analysed: wild-type, ΔfliB:cat, flaB(N265A), flaB(P266G), ΔfliK:cat, flhB(N265A) ΔfliK:cat and flhB(P266G) ΔfliK:cat. Approximately 15 μg total protein was loaded in each C lane and 20 μg total protein in each E lane. FlgE–FLAG was detected in Western blots with antibodies directed against the FLAG epitope. (b) Western blot analysis of flagellins in soluble cytoplasmic (C) and extracellular (E) fractions. The same protein samples and similar amounts of protein were loaded as in (a). Flagellins were detected in Western blots with antiserum directed against FlaB. The antiserum also cross-reacts with FlaA, which is slightly smaller than FlaB.

**Fig. 6.** Effect of flhX deletion on expression of the flaB<sup>−</sup>-xyle reporter gene in *H. pylori* strains with different flaB or fliK alleles. Reporter gene activities were measured in various strains: wild-type, ΔfliK:cat, flaB(N265A) and flaB(P266G) that either possessed or lacked flhX. One unit of Xyle activity corresponds to 1 μmol product formed min<sup>−1</sup> (10<sup>8</sup> cells)<sup>−1</sup>. Means are reported for five statistical replicates; error bars indicate 95% confidence intervals (P<0.05) as determined by Student’s t test.
DISCUSSION

Consistent with earlier studies that show that expression of the H. pylori RpoN and FliA regulons is inhibited by disrupting any one of several genes encoding components of the flagellar protein export apparatus (Allan et al., 2000; Jenks et al., 1997; Niehus et al., 2004; Porwollik et al., 1999; Schmitz et al., 1997), we show here that deletion of flhB inhibits expression of both RpoN- and FliA-dependent reporter genes (Fig. 1). Inhibition of the FliA regulon may result from failure of the ΔflhB:cat mutant to export FlgM via the export apparatus. A sheath contiguous with the outer membrane surrounds the H. pylori flagellum (Geis et al., 1993), but this does not necessarily preclude FlgM secretion, since Vibrio cholerae, which also possesses a sheathed flagellum, secretes FlgM from the cytoplasm via the flagellar protein export apparatus (Correa et al., 2004). The FliA-dependent flaA’–xyle reporter gene was expressed at levels near those of the wild-type in strains that produced the FlhBN265A or FlhBP266G variants, but these strains appear to be deficient in export of filament-type substrates (Fig. 5). Therefore, if the H. pylori export apparatus secretes FlgM, we predict it does so prior to the switch in substrate specificity to filament-type substrates. This is also consistent with the observation that the FliA-dependent reporter gene was expressed at wild-type levels in the ΔfliK:cat mutant.

The molecular basis for the link between the export apparatus and the RpoN regulon is less obvious. The export apparatus possibly secretes a factor that inhibits expression of the RpoN regulon. Alternatively, conformational changes in the export apparatus may be communicated through protein–protein interactions to the RpoN regulon. FlgS is a reasonable candidate for such interactions, since the activity of this sensor kinase could be modulated by interactions with the export apparatus.

Our study confirmed an earlier report that disruption of H. pylori fliK stimulates expression of RpoN-dependent flagellar genes (Ryan et al., 2005). Since fliK is part of the H. pylori RpoN regulon (Niehus et al., 2004), FliK could function as part of a negative feedback loop to downregulate the RpoN regulon. We predict that FliK mediates its effect on gene expression by influencing the conformation and/or activity of the export apparatus. Increased expression of the RpoN regulon in the ΔfliK:cat mutant may result from a delay in the switch in substrate specificity of the export apparatus. This delay could influence the RpoN regulon if, as discussed above, the export apparatus secretes an inhibitor of the RpoN regulon or modulates the activity of FlgS.

To investigate if the phenotype of the ΔfliK:cat mutant resulted from a delay in the substrate-specificity switch, we generated H. pylori FlhB variants that were defective in autolysis (Fig. 3) and appeared unable to export filament-type substrates (Fig. 5). FlhB is part of a family that includes homologues from virulence factor T3SS, and autolysis at the conserved NPTH motif appears to occur universally in FlhB family members (Deane et al., 2008; Riordan & Schneewind, 2008; Zarivach et al., 2008).

Based on studies of similar amino acid substitutions in S. typhimurium FlhB and FlhB T3SS homologues, we predicted the export apparatus in these strains to be locked in the rod-/hook-type conformation. If the enhanced expression of the RpoN-dependent reporter genes in the ΔfliK:cat mutant was due to a delay in the switch in substrate specificity of the export apparatus, we would have expected to observe enhanced expression of these reporter genes in the strains that produced the FlhB processing-deficient variants. Contrary to this prediction, strains producing the FlhBN265A or FlhBP266G variant expressed the flagellar reporter genes at close to wild-type levels (Fig. 1). This expectation of enhanced expression of the RpoN-dependent reporter genes is, however, based on the assumption that the export apparatus only exists in either the rod-/hook-type or the filament-type conformation. The export apparatus might assume additional conformations during flagellar assembly. With this in mind, a significant distinction between the ΔfliK:cat mutant and the FlhB processing-deficient variants is that the export apparatus in the ΔfliK:cat mutant can switch substrate specificity. Polyhooks produced by fliK mutants often have filaments attached (Ryan et al., 2005), while the export apparatus containing FlhBN265A or FlhBP266G appears incapable of switching substrate specificity. Thus, the export apparatus in the ΔfliK:cat mutant may persist in a conformation that stimulates expression of the RpoN regulon, while the export apparatus in strains producing the processing-deficient FlhB variants is unable to assume this conformation.

Two recent studies have reported the crystal structures of the cytoplasmic domains of FlhB T3SS homologues E. coli EspU, S. typhimurium SpaS and Shigella flexneri Spa40 (Deane et al., 2008; Zarivach et al., 2008). These studies reveal the structural changes that take place in FlhB T3SS homologues following autolysis and also further elucidate the mechanism of autolysis. These FlhB homologues contain a central β-sheet surrounded by four α-helices. The NPTH motif is located between the β1 and β2 strands, forming a type II β-turn that undergoes autolysis via a mechanism involving cyclization of the asparagine residue (Ferris et al., 2005; Zarivach et al., 2008). Autolysis does not affect the protein folding but rather generates localized electrostatic and conformational changes that create a unique surface, which is believed to influence interactions with other components of the export apparatus (Deane et al., 2008). Based on the structural
analysis of these FlhB T3SS homologues, the reasons that
H. pylori FlhB<sup>N265A</sup> and FlhB<sup>B266G</sup> were deficient in
processing are that replacing Asn-265 with Ala removes
the reactive asparagine residue and replacing Pro-266 with
Gly disrupts an essential type II β-turn in the NPTH loop
(Zarivach et al., 2008).

Disrupting fliK in the strain that produced FlhB<sup>N265A</sup> resulted
in a dramatic decrease in the expression of the two RpoN-
dependent reporter genes, as well as an approximately twofold
decrease in expression of the flaA<sup>−</sup>−xylE reporter gene. In
contrast, disrupting fliK in the strain that produced FlhB<sup>B266G</sup>
resulted in no change or slightly increased expression of the
RpoN- and FliA-dependent reporter genes (Fig. 4). In the
absence of structural data for the FliK–FlhB interaction, the
molecular basis of the synergistic, negative effect of the
FlhB<sup>N265A</sup> variant and ΔfliK: cat mutation is difficult to
predict. Possibly, FliK interacts with FlhB<sup>N265A</sup> to overcome a
barrier within the export apparatus that prevents expression of
the RpoN and FliA regulons. Similar interactions between
FliK and wild-type FlhB may occur but may no longer be
needed for expression of the RpoN and FliA regulons upon
autocleavage of FlhB. Macnab and co-workers isolated
mutations within flhB that restored motility in an S. typhimurium fliK mutant (Williams et al., 1996). These
mutations occurred at two highly conserved positions (Gly-
293 and Ala-298) within FlhB<sub>CC</sub>, and slowed the rate at which
FlhB was cleaved (Minamino & Macnab, 2000). Structural
studies with Shigella flexneri Spa40<sub>C</sub> suggest that these
substitutions in FlhB disrupt autocleaving of the β2 strand and
α2 helix around the region of the NPTH loop, thus
influencing the ability of the NPTH loop to adopt the
conformation needed for switching substrate specificity of the
export apparatus independently of FliK (Deane et al., 2008).

We observed that disruption of flhX in H. pylori resulted in
decreased expression of the RpoN-dependent flaB<sup>−</sup>−xylE
reporter gene (Fig. 6). This inhibition, however, was not
seen in strains that produced the FlhB<sup>N265A</sup> or FlhB<sup>B266G</sup>
variant. We infer from these results that FlhB<sub>CC</sub> can
dissociate from FlhB<sub>CN</sub> following autocleavage and be
replaced with FlhX, and that association of FlhB<sub>CC</sub> or FlhX
is required for optimal expression of the RpoN regulon. In
the processing-deficient FlhB variants, FlhB<sub>CC</sub> remains
linked to FlhB<sub>CN</sub>, so FlhX is not required for wild-type
expression of the RpoN regulon in strains that produce
these FlhB variants. FliK may facilitate the dissociation of
FlhB<sub>CC</sub> from FlhB<sub>CN</sub>, which could explain why disruption
of flhX in the ΔfliK: cat mutant had less of an effect on
expression of the flaB<sup>−</sup>−xylE reporter gene than in the
wild-type.

Clearly, the molecular mechanisms that govern the function
of the flagellar protein export apparatus and the conforma-
tional changes within the export apparatus that influence
substrate specificity are complex. Further defining the role
that FlhB plays in these processes will be important for
understanding how the export apparatus influences expres-
sion of the H. pylori RpoN and FliA regulons.

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