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

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

Correspondence

Timothy R. Hoover
trhoover@uga.edu

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 σ^{54} (RpoN), employed for expression of genes required midway through flagellar assembly, and σ^{28} (FliA), 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 (FlhB_C). The hook length control protein FliK interacts with FlhB_C to modulate the substrate specificity of the export apparatus. FlhB_C 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 $\Delta flhB$ mutant, disrupting *fliK* did not restore expression of RpoN-dependent reporter genes, suggesting that the inhibitory effect of the $\Delta flhB$ 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 FlhB^{N265A}, 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 FlhB^{P266G}. *H. pylori* HP1575 (FlhX) shares homology with the C-terminal portion of FlhB_C (FlhB_{CC}) and can substitute for FlhB_{CC} in flagellar assembly. Disrupting *flhX* inhibited expression of a *flaB* reporter gene in the wild-type but not in the $\Delta fliK$ mutant or strains producing FlhB variants, suggesting a role for FlhX or FlhB_{CC} 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.

Received 30 July 2008

Revised 17 December 2008

Accepted 22 December 2008

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 ϵ -Proteobacteria, such as *Helicobacter pylori* and *Campylobacter jejuni*, shares some similarities with these established paradigms but also differs signifi-

cantly. 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 σ^{28} (FliA)-RNA polymerase holoenzyme (Kim *et al.*, 1999; Leying *et al.*, 1992), and, as in *C. crescentus*, many *H. pylori* flagellar genes require σ^{54} (RpoN) for their transcription (Spohn & Scarlato, 1999; Suerbaum *et al.*, 1993). Transcription of genes needed early in *H. pylori* flagellar assembly are dependent on σ^{80} (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

Abbreviation: T3SS, type III secretion system.

A supplementary table listing oligonucleotide primers used in this study is available with the online version of this paper.

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 σ^{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, FliO, FliP, FliQ and FliR) 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, FliK. Completion of the mature

hook is communicated by FliK 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 NP_{TH} 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 FliK 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 % CO₂/93 % 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 pLysS 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 % (w/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), *fliK* (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 *PfuTurbo* Hotstart DNA polymerase (Stratagene). Amplicons were incubated with *Taq* DNA polymerase (Promega) and then cloned into pGEM-T (Promega). The resulting plasmids, pTS45 (*flhB*), pTS69 (*fliK*) 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 transacetylase 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 $\Delta flhB$ 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* $\Delta flhB$:*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* $\Delta flhB$:*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 *SalI* site, annealed 100 bp upstream of the translational start, and the reverse primer, containing a *KpnI* 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 % (w/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 % (w/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 bicinchoninic 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_C purification. A 457 bp fragment encoding *H. pylori* FlhB_C 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 pLysS 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_C protein was purified using nickel–NTA (Qiagen) affinity chromatography following the supplier's instructions. Purified His-tagged FlhB_C 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_C 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 statistical 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 *fliK* 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); *flaB'*-*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 $\Delta fliK:cat$ 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 $\Delta fliK:cat$ mutant was similar to that of the wild-type (Fig. 1). In contrast, Ryan *et al.* (2005) found that disrupting *fliK* 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 $\Delta fliK:cat$ 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 $\Delta flhB:cat$ mutant was non-motile (Fig. 2). We tried unsuccessfully to complement the $\Delta flhB:cat$ 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), $\Delta fliK:cat$ (white), $\Delta flhB:cat$ (dark grey), a strain in which the wild-type *flhB* allele was restored (*flhB* restored, light grey), *flhB*(N265A) (*FlhB*^{N265A}, striped) and *flhB*(P266G) (*FlhB*^{P266G}, stippled). One unit of XylE activity corresponds to 1 μmol product formed min^{-1} (10^8 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.

Fig. 2. Motility of *H. pylori* strains in a semisolid medium. *H. pylori* strains shown are wild-type, $\Delta flhB:cat$, a strain in which the wild-type *flhB* allele was restored (*flhB* restored) and strains expressing FlhB^{N265A} or FlhB^{P266G} variants. Bar, 5 mm.

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 *flhB* in the HP0405 locus. We replaced the $\Delta flhB:cat$ allele with the wild-type *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 $\Delta flhB:cat$ strain.

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

FlhB 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 $\Delta flhB:cat$ mutant resulted from a delay in the switch between export states. Therefore, we reasoned that in *H. pylori* strains producing FlhB 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 *H. pylori* FlhB is between Asn-265 and Pro-266. *H. pylori* 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 *S. typhimurium* FlhB interfere with cleavage (Fraser *et al.*, 2003). *H. pylori* strains expressing FlhB^{N265A} or FlhB^{P266G} were severely reduced in their motility (Fig. 2). These findings were consistent with the decrease in motility observed in *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 *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 *H. pylori* FlhB^{P266G} variant does not affect motility. This discrepancy is not due to the *H. pylori* strains used. Expressing the FlhB^{P266G} variant in *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 *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 *E. coli*. Those researchers were unable to detect FlhB or its variants in *H. pylori* to see if this processing also occurred in *H. pylori* (Wand *et al.*, 2006).

Affinity-purified antibodies that recognize *H. pylori* FlhB_C were used in Western blot assays to examine FlhB proteins. In membrane preparations from wild-type *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 FlhB (41 kDa) was also observed. Neither of these bands was detectable in the $\Delta flhB:cat$ mutant, but both were present in the strain in which wild-type *flhB* had been restored (Fig. 3). These results confirm that *H. pylori* FlhB is processed *in vivo* similarly to *S. typhimurium* FlhB (Minamino & Macnab, 2000) and verify previous work that demonstrates processing of *H. pylori* FlhB recombinant proteins in *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 *E. coli* is deficient in normal processing (Wand *et al.*, 2006).

The FlhB_{CC} subdomain remains associated with the rest of FlhB 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

Fig. 3. Western blot analysis of FlhB in *H. pylori* strains. Membrane fractions from strains bearing the following *flhB* alleles were analysed: wild-type, $\Delta flhB:cat$, *flhB* restored, *flhB*(N265A) and *flhB*(P266G). Purified membrane fractions containing approximately 20 μ g total protein were loaded in each lane and proteins were separated by SDS-PAGE. FlhB proteins were detected in Western blots using affinity-purified antibodies directed against FlhB_C. Arrows indicate the positions of full-length and processed FlhB. Locations of protein standards and their molecular masses (kDa) are indicated.

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* FlhB_{CC} 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* FlhB_{CC} in our assay is unknown. FlhB_{CC} may have dissociated from the rest of FlhB *in vivo* or during the membrane

isolation procedure. The affinity-purified antibodies did not recognize purified FlhB_{CC} (data not shown), indicating that the inability to detect FlhB_{CC} in the membrane fractions was not due to poor cross-reactivity.

In the strain expressing the FlhB^{N265A} 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 FlhB^{P266G} 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 $\Delta fliK:cat$ 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 $\Delta flhB:cat$ mutant might result from the failure to export FliK. This does not appear to be the case, however, since disrupting *fliK* in the $\Delta flhB:cat$ 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

Fig. 4. Changes in expression of flagellar reporter genes due to *fliK* disruption in *H. pylori* strains with various *flhB* alleles. XylE activities indicated were measured in strains with the following genotypes: $\Delta fliK:cat$ ($\Delta fliK$, black), $\Delta flhB:cat \Delta fliK:ermB$ ($\Delta flhB \Delta fliK$, white), $\Delta fliK:cat flhB(N265A)$ (FlhB^{N265A} $\Delta fliK$, dark grey) and $\Delta fliK:cat flhB(P266G)$ (FlhB^{P266G} $\Delta fliK$, light grey). Activities of these strains were compared with those of the corresponding parental strain: wild-type, $\Delta flhB:cat$, *flhB*(N265A) and *flhB*(P266G). The fold change is reported as negative if expression decreased when *fliK* was disrupted and positive if expression increased. The dashed lines at 1 and -1 indicate no change in expression between the parental and $\Delta fliK$ strain. Error bars indicate 95% confidence intervals ($P=0.05$) as determined by Student's *t* test.

produced FlhB^{N265A} severely inhibited expression of the RpoN-dependent *flaB'*-*xylE* reporter gene and inhibited expression of the FliA-dependent *flaA'*-*xylE* reporter gene approximately twofold compared with the FlhB^{N265A} parental strain (Fig. 4). Expression of the RpoN-dependent *hp1120'*-*xylE* reporter gene was also inhibited in this strain, while expression of the RpoD-dependent *flgI'*-*xylE* reporter gene was not significantly changed (data not shown). In contrast, disrupting *fliK* in the strain that produced FlhB^{P266G} had little effect on expression of the flagellar reporter genes, with the exception of *hp1120'*-*xylE*, which was slightly stimulated (Fig. 4, and data not shown). Thus, despite the fact that FlhB^{N265A} and FlhB^{P266G} are both deficient in autocleavage, these proteins elicit very different effects on the RpoN and FliA regulons in the absence of FliK.

FlhB^{N265A} or FlhB^{P266G} variant strains are defective in exporting filament-type substrates

H. pylori strains producing FlhB^{N265A} or FlhB^{P266G} 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^{N265A} or FlhB^{P266G} expressed and exported FlgE-FLAG (Fig. 5a) but failed to export FlaA or FlaB, even though both flagellins were present in the soluble cytoplasmic fractions from these strains (Fig. 5b). In this regard the strains producing FlhB variants appeared similar to the $\Delta fliK:cat$ mutant in this assay. In the strain producing the FlhB^{N265A} 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^{P266G} 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 $\Delta flhX:cat$ mutant and monitored expression of the RpoN-dependent *flaB'*-*xylE* reporter gene in the resulting strain. Expression of the reporter gene was inhibited in the $\Delta flhX:cat$ 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 $\Delta flhX:cat$ allele was introduced into the strains that expressed FlhB^{N265A}

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, $\Delta flhB:cat$, *flhB*(N265A), *flhB*(P266G), $\Delta fliK:cat$, *flhB*(N265A) $\Delta fliK:cat$ and *flhB*(P266G) $\Delta fliK:cat$. Approximately 15 μ g total protein was loaded in each C lane and 20 μ g 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'*-*xylE* reporter gene in *H. pylori* strains with different *flhB* or *fliK* alleles. Reporter gene activities were measured in various strains: wild-type, $\Delta fliK:cat$, *flhB*(N265A) and *flhB*(P266G) that either possessed or lacked *flhX*. One unit of XylE activity corresponds to 1 μ mol 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.

and FlhB^{P266G} variants. In contrast to the strain with a wild-type *flhB* allele, disrupting *flhX* in these strains had little effect on expression of the *flaB'*-*xylE* reporter gene (Fig. 6). Disrupting *flhX* in the Δ *fliK*:*cat* mutant inhibited expression of the *flaB'*-*xylE* reporter gene slightly but not to the degree seen with the wild-type background (Fig. 6). Taken together, these data suggest a role for FlhX in *H. pylori* flagellar biosynthesis.

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 Δ *flhB*:*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 FlhB^{N265A} or FlhB^{P266G} 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 Δ *fliK*:*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 Δ *fliK*:*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 Δ *fliK*:*cat* mutant resulted from a delay in the substrate-specificity switch, we generated *H. pylori* FlhB variants that were defective in autocleavage (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 autocleavage 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 Δ *fliK*:*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 FlhB^{N265A} or FlhB^{P266G} 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 Δ *fliK*:*cat* mutant and the FlhB processing-deficient variants is that the export apparatus in the Δ *fliK*:*cat* mutant can switch substrate specificity. Poly-hooks produced by *fliK* mutants often have filaments attached (Ryan *et al.*, 2005), while the export apparatus containing FlhB^{N265A} or FlhB^{P266G} appears incapable of switching substrate specificity. Thus, the export apparatus in the Δ *fliK*:*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* EscU, *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 autocleavage and also further elucidate the mechanism of autocleavage. These FlhB homologues contain a central β -sheet surrounded by four α -helices. The NPTH motif is located between the β 1 and β 2 strands, forming a type II β -turn that undergoes autocleavage via a mechanism involving cyclization of the asparagine residue (Ferris *et al.*, 2005; Zarivach *et al.*, 2008). Autocleavage 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^{N265A} and FlhB^{P266G} 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^{N265A} 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'*-*xylE* reporter gene. In contrast, disrupting *fliK* in the strain that produced FlhB^{P266G} 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^{N265A} variant and Δ *fliK*:*cat* mutation is difficult to predict. Possibly, FliK interacts with FlhB^{N265A} 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_{CC} and slowed the rate at which FlhB was cleaved (Minamino & Macnab, 2000). Structural studies with *Shigella flexneri* Spa40_C suggest that these substitutions in FlhB disrupt packing of the β 2 strand and α 2 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'*-*xylE* reporter gene (Fig. 6). This inhibition, however, was not seen in strains that produced the FlhB^{N265A} or FlhB^{P266G} variant. We infer from these results that FlhB_{CC} can dissociate from FlhB_{CN} following autocleavage and be replaced with FlhX, and that association of FlhB_{CC} or FlhX is required for optimal expression of the RpoN regulon. In the processing-deficient FlhB variants, FlhB_{CC} remains linked to FlhB_{CN}, 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_{CC} from FlhB_{CN}, which could explain why disruption of *flhX* in the Δ *fliK*:*cat* mutant had less of an effect on expression of the *flaB'*-*xylE* reporter gene than in the wild-type.

Clearly, the molecular mechanisms that govern the function of the flagellar protein export apparatus and the conformational 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 expression of the *H. pylori* RpoN and FliA regulons.

ACKNOWLEDGEMENTS

We thank the Department of Microbiology for financial support, Robert Maier and Lawrence Shimkets for use of laboratory equipment, Anna Karls for helpful comments on the manuscript and Lyla Lipscomb for purification of FlhB_C protein. T.G.S. was supported by fellowships from the University of Georgia Graduate School and the Atlanta Chapter of the Achievement Rewards for College Scientists (ARCS) Foundation.

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Edited by: P. W. O'Toole