Insertion mutations in *Helicobacter pylori* flhA reveal strain differences in RpoN-dependent gene expression

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Flagellar biogenesis in the gastric pathogen *Helicobacter pylori* involves a transcriptional hierarchy that utilizes all three sigma factors found in this bacterium (RpoD, RpoN and FliA). Transcription of the RpoN-dependent genes requires the sensor kinase FlgS and response regulator FlgR. It is thought that FlgS senses some cellular cue to regulate transcription of the RpoN-dependent flagellar genes, but this signal has yet to be identified. Previous studies showed that transcription of the RpoN-dependent genes is inhibited by mutations in flhA, which encodes a membrane-bound component of the flagellar protein export apparatus. We found that depending on the *H. pylori* strain used, insertion mutations in flhA had different effects on expression of RpoN-dependent genes. Mutations in flhA in *H. pylori* strains B128 and ATCC 43504 (the type strain) were generated by inserting a chloramphenicol resistance cassette so as to effectively eliminate expression of the gene (ΔflhA), or within the gene following codon 77 (designated flhA77) or codon 454 (designated flhA454), which could allow expression of truncated FlhA proteins. All three flhA mutations severely inhibited transcription of the RpoN-dependent genes flaB and flgE in *H. pylori* B128. In contrast, levels of flaB and flgE transcripts in *H. pylori* ATCC 43504 bearing either flhA77 or flhA454, but not ΔflhA, were ~60% of wild-type levels. The FlhA454 variant was detected in membrane fractions prepared from *H. pylori* ATCC 43504 but not *H. pylori* B128, which may account for the phenotypic differences in the flhA mutations of the two strains. Taken together, these findings suggest that only the N-terminal region of FlhA is needed for transcription of the RpoN regulon. Interestingly, expression of an flaB'-xylE reporter gene in *H. pylori* ATCC 43504 bearing the flhA77 allele was about eightfold higher than that of a strain with the wild-type allele, suggesting that expression of flaB is not only regulated at the level of transcription but also regulated post-transcriptionally.

INTRODUCTION

*Helicobacter pylori* is a member of the Epsilonproteobacteria that colonizes the human gastric mucosa, where it can cause a variety of diseases, including peptic and duodenal ulcers, B-cell mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma (Blaser, 1993; Dunn et al., 1997). *H. pylori* must be motile to colonize the gastric mucosa (Eaton et al., 1989, 1992), which it achieves via a cluster of sheathed polar flagella (Eaton et al., 1992; Goodwin et al., 1985; Schreiber et al., 2005).

The bacterial flagellum is a complex structure, consisting of a basal body, hook and filament. The basal body is embedded in the bacterial cell envelope and consists of a rotary motor, a switch complex that regulates the direction of rotation, a flagellar rod and rings, and a type III secretion system that transports flagellar subunits across the cell membrane (Macnab, 1996; Minamino et al., 2008a, b). The flagellar protein export apparatus consists of six integral membrane proteins (FlhA, FliH, FlbI, Flp, FliQ and FliR), which form an export pore, and three cytoplasmic components, FliI, FliH and FliJ, which deliver protein substrates to the export pore (Macnab, 2003).

Assembly of the bacterial flagellum is a highly ordered process in which the basal body is assembled first, followed by the hook and then the filament. Where it has been examined, flagellar gene expression is controlled by a transcriptional hierarchy that coordinates synthesis of flagellar proteins with...
assembly of the flagellum (Chevance & Hughes, 2008; Smith & Hoover, 2009). The best-studied example of such coordinated gene control is transcription of the Salmonella filament subunit (flagellin) gene, which is dependent on FlIA (σ^28) and is negatively regulated by the anti-σ^28 factor FlgM (Hughes et al., 1993). Upon formation of the mature hook, the export apparatus undergoes a conformational change that results in a switch in substrate specificity from rod-/hook-type to filament-type substrates (Minamino et al., 1999). FlgM is exported as a filament-type substrate from the cell at this point, which results in transcription of the FlIA regulon (Hughes et al., 1993).

H. pylori flagellar genes are organized in multiple regulons which are based on the sigma factor required for their transcription. In general, genes needed early in flagellar assembly are dependent on RpoD (σ^60; primary σ factor in H. pylori) for their transcription, while genes required later in assembly are dependent on RpoN (σ^54), and genes required at the end of the pathway are FlIA (σ^28)-dependent (Collard et al., 2001; Josenhans et al., 2002; Niehus et al., 2004; Spohn & Scarlato, 1999). A two-component regulatory system consisting of the sensor kinase FlgS and the response regulator FlgR is required for transcription of the H. pylori RpoN-dependent flagellar genes (Beier & Frank, 2000; Spohn & Scarlato, 1999). Expression of the H. pylori RpoN regulon is linked with the flagellar protein export apparatus, as mutations that disrupt the H. pylori export apparatus inhibit transcription of RpoN-dependent flagellar genes (Allan et al., 2000; Foynes et al., 1999; Jensk et al., 1997; Niehus et al., 2004; Schmitz et al., 1997). The export apparatus similarly affects expression of RpoN-dependent flagellar genes in Campylobacter jejuni, another member of the Epsilonproteobacteria (Hendrixson & DiRita, 2003).

Although not part of the export apparatus, the hook-length control protein, FlIK, plays a pivotal role in the switch in substrate specificity of the export apparatus (Williams et al., 1996). Disrupting flIK in H. pylori or C. jejuni stimulates expression of RpoN-dependent genes, further suggesting a link between the export apparatus and RpoN regulons in these bacteria (Kamal et al., 2007; Ryan et al., 2005; Smith et al., 2009).

The mechanism by which the export apparatus affects the RpoN regulon in H. pylori and other members of the Epsilonproteobacteria is not known, but it may do so by modulating FlgS activity. In support of this hypothesis, a variant of FlgR that functions independently of FlgS partially restores expression of RpoN-dependent reporter genes in C. jejuni mutants in which flhA, flhB or flIP is disrupted (Joslin & Hendrixson, 2009). It is possible that FlgS responds to conformational changes in the export apparatus during flagellar assembly.

We report here that flhA insertion mutations in two H. pylori strains manifest differences in expression of RpoN-dependent genes. In H. pylori B128, all disruptions in flhA that we examined inhibited transcription of flaB and flgE, two RpoN-dependent flagellar genes. In H. pylori ATCC 43504 (the type strain), however, disruptions within flhA allowed transcription of flaB and flgE. Interestingly, expression of a flaB'-xylE reporter gene in one of the flhA insertion mutants in H. pylori ATCC 43504 was about eightfold higher than that of the wild-type strain, suggesting that flaB is regulated post-transcriptionally, possibly at the level of translation.

### METHODS

#### Bacterial strains and culture conditions.**

H. pylori strains ATCC 43504, 26695 and B128 were cultured routinely at 37 °C in a microaerobic atmosphere (2% O_2/5% CO_2/93% N_2) on trypticase soy agar (TSA) supplemented with 5% horse serum, as described previously (Smith et al., 2009). Where indicated, the medium for culturing H. pylori was supplemented with 30 μg chloramphenicol ml^(-1) or 30 μg kanamycin ml^(-1). *Escherichia coli* DH5α was used for replicating plasmids for routine cloning procedures and was cultured in Luria–Bertani medium supplemented with 100 μg ampicillin ml^(-1), 30 mg kanamycin ml^(-1) or 30 μg chloramphenicol ml^(-1). H. pylori motility was assessed using semisolid Mueller–Hinton medium supplemented with 10% horse serum and 10 μM FeSO_4, as described previously (Smith et al., 2009).

#### Construction of H. pylori mutants.

Genomic DNA from H. pylori 26695 was prepared using the Wizard Genomic DNA Purification kit (Promega). DNA was amplified by PCR using H. pylori 26695 genomic DNA as a template and Taq DNA polymerase (Promega), *Pfu* Turbo Hotstart DNA polymerase (Stratagene) or *i Proof* DNA polymerase (Bio-Rad). When *Pfu* Turbo Hotstart or *i Proof* DNA polymerase was used for PCR, the resulting amplicons were incubated with *Taq* DNA polymerase at 72 °C for 10 min to add single 3′-A overhangs to the ends to facilitate T/A cloning into the vector pGEM-T (Promega). DNA primers used for PCR are indicated in Table 1.

To construct the ΔflhA allele, a ~690 bp DNA fragment which included −90 bp of *rpsL* (encoding ribosomal protein S15; gene is upstream and divergent to flhA), 140 bp of the intergenic region upstream of flhA, and 460 bp of the 5′ end of flhA, was amplified and cloned into pGEM-T to create a plasmid designated pPL120. A suicide vector for introducing the ΔflhA mutant into the chromosome was created by digesting pPL120 with HindIII and Eco47III, filling in the ends of the cut DNA, and then inserting a 1.3 kb Smal fragment containing a chloramphenicol transacylase (*cat*) gene from pSKAT4 (Wang & Taylor, 1990) into the plasmid by blunt-end ligation. The replacement deleted the region corresponding to 90 nt upstream of the predicted start codon of flhA through to codon 76 of flhA. To construct the flhA77 allele, a DNA fragment corresponding to the first 993 bp of flhA was amplified and cloned into pGEM-T to create plasmid pLP78. A 500 bp Eco47III fragment in pLP78 was replaced with the *Smal* fragment from pSKAT4 containing the *cat* cassette by blunt-end ligation, which replaced codons 77–242 of flhA with the *cat* cassette and generated a suicide vector for introducing the flhA77 allele into the chromosome. To construct the flhA545 allele, a 1068 bp DNA fragment corresponding to 968 bp from the 5′ end to 165 bp from the 3′ end of flhA was amplified and cloned into pGEM-T to create plasmid pLP113. The resulting plasmid was used as a template for site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis kit (Stratagene) and the primers indicated in Table 1. The mutagenic primers introduced a unique EcoRI site that overlapped codons 454 and 455 of flhA. A 1.3 kb EcoRI fragment from pSKAT4 containing the *cat* cassette was cloned into the EcoRI site that had been added into pLP113 to create a suicide vector for introducing the flhA545 allele into the chromosome. The resulting

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suicide plasmids were introduced into *H. pylori* ATCC 43504 or B128 by natural transformation, and transformants were selected as described previously (Brahmachary et al., 2004). Replacement of the wild-type *flhA* allele with the mutant alleles was confirmed by PCR.

To construct the A*hp*1042 mutant, *hp*1042 along with 500 bp DNA upstream and 540 bp DNA downstream of the gene were amplified using the *hp*1042 forward and *hp*1042 reverse primers indicated in Table 1. These primers annealed to sequences immediately downstream and upstream of *hp*1042, respectively, and introduced EcoRI sites that was filled in was ligated into the suicide plasmids. Replacement of the *flhA* gene in the shuttle vector pHel3 (Heuermann & Haas, 1998) was described previously (Brahmachary et al., 2004). Insertion of the *hp*1042 cassette that was present in the *H. pylori* ATCC 43504 chromosome was determined from at least six statistical replicates for two or more biological replicates. Student's *t* test was used to determine the standard deviation with 95% confidence intervals (*P*<0.05).

**Detection of FlhA proteins.** A multiple antigen peptide was synthesized in which the first 25 amino acid residues from the *N* terminus of *H. pylori* 26695 FlhA (MANERSKLAFKTFPVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows. A peptide consisting of the first 25 residues of FlhA plus a cytochrome c residue at the C terminus was synthesized (GenScript) and covalently attached to SulfoLink Coupling Resin (Thermo Scientific) following the manufacturer’s protocol that accompanied the SulfoLink Immobilization kit. Two millilitres of antiserum was prepared by diluting with 13 ml PBS, reducing antibodies were dialysed against citric acid-phosphate buffer (55 mM citrate, 55 mM phosphate, pH 5.5) and were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows. A multiple antigen peptide was synthesized in which the first 25 amino acid residues from the *N* terminus of *H. pylori* 26695 FlhA (MANERSKLAFKTFPVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows. A multiple antigen peptide was synthesized in which the first 25 amino acid residues from the *N* terminus of *H. pylori* 26695 FlhA (MANERSKLAFKTFPVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows. A multiple antigen peptide was synthesized in which the first 25 amino acid residues from the *N* terminus of *H. pylori* 26695 FlhA (MANERSKLAFKTFPVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows. A multiple antigen peptide was synthesized in which the first 25 amino acid residues from the *N* terminus of *H. pylori* 26695 FlhA (MANERSKLAFKTFPVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows. A multiple antigen peptide was synthesized in which the first 25 amino acid residues from the *N* terminus of *H. pylori* 26695 FlhA (MANERSKLAFKTFPVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows. A multiple antigen peptide was synthesized in which the first 25 amino acid residues from the *N* terminus of *H. pylori* 26695 FlhA (MANERSKLAFKTFPVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows. A multiple antigen peptide was synthesized in which the first 25 amino acid residues from the *N* terminus of *H. pylori* 26695 FlhA (MANERSKLAFKTFPVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows. A multiple antigen peptide was synthesized in which the first 25 amino acid residues from the *N* terminus of *H. pylori* 26695 FlhA (MANERSKLAFKTFPVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The multiple antigen peptide was used to immunize a New Zealand white rabbit (Cocalico Biologicals), and antibodies that recognized the FlhA peptide were affinity-purified from the resulting antiserum as follows.
citic acid, 50 mM K$_2$HPO$_4$, pH 5.5), followed by Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6), then stored at $-20\, ^\circ\text{C}$.

*H. pylori* membranes were prepared for Western blot analysis as follows. *H. pylori* strains were grown for $72\, \text{h}$ on agar medium and then resuspended in a buffer containing 10% sucrose, 20 mM HEPES and 1 mM EDTA, pH 7.4. Cells were lysed by three passages through a French press at 10 000 kPa. Cellular debris was removed by centrifugation for 15 min at 6000 g. Membranes were separated from cytoplasmic proteins by centrifugation for 60 min at 100 000 g and collected in 0.25 ml of 30% sucrose, 20 mM HEPES and 1 mM EDTA, pH 7.4. The membrane fraction was diluted in 20 mM HEPES and 1 mM EDTA, pH 7.4, and the centrifugation (60 min at 100 000 g) was repeated. Protein concentrations of the membrane fractions were measured using the bicinchoninic acid protein assay (Thermo Scientific) following the manufacturer’s instructions.

The purified *H. pylori* membranes were analysed by Western blotting using the affinity-purified FlhA antibodies as the primary antibody and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Antigen–antibody complexes on the blot were detected by chemiluminescence using SuperSignal West Pico Luminol/Enhancer Solution and SuperSignal West Stable Peroxide Solution (Thermo Scientific). Blots were visualized with a FluorChem E imager (ProteinSimple).

**Detection of exported flagellar proteins.** Plasmid pTS14 is a derivative of pHe3 that carries *H. pylori* flgE under control of its native promoter and a sequence encoding the FLAG tag (DYKDDDDK) fused to the 3' end of *flgE* (Smith et al., 2009). *H. pylori* strains containing pTS14 were cultured in brain heart infusion medium supplemented with 0.4% β-cyclodextrin and 30 μg kanamycin ml$^{-1}$ in sealed serum bottles at 37°C under a microaerobic atmosphere with gentle shaking for 24 h as described by Smith et al. (2009). *H. pylori* extracellular and cytoplasmic proteins were prepared and concentrated using TCA, as described previously (Smith et al., 2009). Protein concentrations were measured using the bicinchoninic acid protein assay. For each sample analysed, approximately the same amount of total protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. A monoclonal peroxidase-conjugated antibody directed against the FLAG epitope (Sigma) and enhanced chemiluminescence were used to detect FlgE–FLAG. Antiserum directed against *H. pylori* FlhA (Pereira & Hoover, 2005), a peroxidase-conjugated goat anti-rabbit antibody (MP Biomedicals) and enhanced chemiluminescence were used to detect *H. pylori* flagellins. *H. pylori* FlhA and FlaA share 58% amino acid identity, and the antiseraum directed against FlhA cross-reacts with FlaA (Brahmachary et al., 2004; Pereira & Hoover, 2005).

**RNA extraction and cDNA synthesis.** *H. pylori* cells were grown on TSA for 18 h before harvesting and resuspended in 1 ml nuclease-free deionized water. Cells were pelleted by centrifugation and then resuspended in 100 μl nuclease-free deionized water. RNA was isolated from the cell pellets using the Aurum Total RNA Mini kit (Bio-Rad). The resulting RNA solutions were treated with the TURBO DNA-free kit (Ambion) to remove contaminating DNA. Single-strand cDNA was synthesized from 200 ng RNA using the iScript cDNA Synthesis kit (Bio-Rad).

**Quantitative RT-PCR.** Transcript levels of *flaA*, *flaB* and *flgE* were monitored by quantitative RT-PCR (qRT-PCR) using the Bio-Rad iCycler iQ System. Transcript levels of gyrA were used as an internal control for the qRT-PCR assays. Primer specificity was confirmed by PCR using genomic DNA. Each qRT-PCR assay, totalling 20 μl, consisted of 10 μl of iQ SYBR Green Supermix, 5 μl of 100-fold diluted cDNA from the cDNA synthesis reaction and 200 nM primer. Experiments were performed in triplicate from three different RNA isolations of each strain. Gene expression levels were quantified by the $2^{-\Delta\Delta C_{\text{T}}}$ method (Schmittgen & Livak, 2008).

**DNA sequencing.** A region of the *H. pylori* 43504 chromosome that included *flgM* (the upstream of *flgM* (shyD) and three genes downstream of *flgM* (a gene encoding a predicted cytotoxic-specific DNA methyltransferase, a potential FlgN homologue and *flgK*, respectively) was amplified using iProof DNA polymerase. After treating the resulting ampiclon with Taq DNA polymerase to add A residues to the 3' ends, the amplified DNA fragment was cloned into pGEMT-Easy and both strands of the cloned DNA were sequenced by Genewiz, Inc., using the primers indicated in Table 1. The DNA sequence from *H. pylori* ATCC 43504 was analysed using the program BLAST. Sequences of the ORFs and intergenic regions from the corresponding regions of the sequenced genomes for *H. pylori* 26695 and B128 were obtained from the Integrated Microbial Genomes database (http://img.jgi.doe.gov/cgi-bin/main.cgi). Nucleotide and predicted protein sequences were aligned using the CLUSTALW2 multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). DNA sequences and predicted amino sequences of *H. pylori* ATCC 43504 flgM and the surrounding genes were deposited in GenBank.

**RESULTS**

**Insertions in flhA affect transcription of RpoN-dependent flagellar genes differently in *H. pylori* strains B128 and ATCC 43504**

*Salmonella* FlhA is 692 aa residues in length and consists of an N-terminal transmembrane domain (FlhATM) predicted to contain six to eight membrane-spanning helices and a large C-terminal cytoplasmic domain referred to as FlhAC (McMurry et al., 2004). The National Center for Biotechnology Information database contains complete sequences for FlhA proteins from over 20 *H. pylori* strains. These FlhA sequences are >98% identical over their entire length of 733 aa residues. The sequences of FlhA from *H. pylori* strains 26695 and B128 were analysed using three different programs for predicting the membrane topology of proteins: TMHMM Server v 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and HMMTOP (http://www.enzim.hu/hmmtop/). The results of this analysis indicated that the predicted membrane topology of *H. pylori* FlhA was similar to that of *Salmonella* FlhA (Fig. 1).

Studies by Niehus et al. (2004) showed that disrupting *flhA* at codon 456 (located within the FlhAC domain) in *H. pylori* N6 or 88-3887 (a motile isolate of strain 26695) with a kanamycin-resistance cassette resulted in reduced transcript levels of both RpoN-dependent and FlhA-dependent flagellar genes, but not RpoD-dependent flagellar genes. To examine further the role of FlhA in flagellar gene regulation, we generated three *flhA* mutant alleles in *H. pylori* ATCC 43504 and B128 by disrupting *flhA* at various positions (Fig. 1) and analysed the phenotypes of the resulting mutants. For one of the *flhA* alleles, the region corresponding to 90 nt upstream of the predicted start codon of *flhA* through to codon 77 of *flhA* was replaced with a cat cassette. Since this mutation should abolish...
expression of the entire \textit{flhA} gene it was referred to as a \textit{ΔflhA} mutation. For the second allele, codons 77–242 of \textit{flhA} were replaced with the \textit{cat} cassette, and this allele was referred to as \textit{flhA77}. The \textit{flhA77} allele has the potential to produce a truncated FlhA which would include the first two membrane-spanning helices of the protein (Fig. 1). A third \textit{flhA} allele was generated by introducing the \textit{cat} cassette between codons 454 and 455, and the resulting allele was designated \textit{flhA454}. The \textit{flhA454} allele could potentially produce a truncated FlhA that would include all of FlhA\textsubscript{TM} plus part of FlhA\textsubscript{C}.

As expected, \textit{H. pylori} strains bearing any of the mutant \textit{flhA} alleles were deficient in motility as assessed using soft agar plates (data not shown). To assess how the \textit{flhA} alleles affected transcription of selected flagellar genes in the \textit{H. pylori} strains, transcript levels of two RpoN-dependent flagellar genes (\textit{flgE}, encoding the hook protein; and \textit{flaB}, encoding a minor flagellin) and one FliaA-dependent flagellar gene (\textit{flaA}, encoding the major flagellin) were determined by qRTPCR. In agreement with Niehus \textit{et al.} (2004), levels of \textit{flaB}, \textit{flgE} and \textit{flaA} derivatives were reduced significantly in \textit{H. pylori} B128 derivatives bearing any of the three \textit{flhA} mutant alleles (Fig. 2a). Levels of \textit{flaB} and \textit{flgE} transcripts in the three \textit{flhA} mutants were <1\% of those in the parental strain. Transcription of \textit{flaA} in the \textit{H. pylori} B128 \textit{flhA} mutants appeared to be affected to a lesser degree, as \textit{flaA} transcript levels in the \textit{flhA} mutants were ~15\% of wild-type levels.

Surprisingly, the flagellar gene transcript profiles for the \textit{flhA} mutants in \textit{H. pylori} ATCC 43504 were strikingly different from those in \textit{H. pylori} B128 (Fig. 2b). Relative to wild-type, the level of \textit{flaA} transcripts in the \textit{H. pylori} ATCC 43504 \textit{ΔflhA} mutant was reduced to a level (~12\%) that was similar to those in the \textit{H. pylori} B128 \textit{flhA} mutants. In the \textit{H. pylori} ATCC 43504 strains bearing the \textit{flhA77} or \textit{flhA454} alleles, however, \textit{flaA} transcript levels were reduced further to ~5\% of wild-type levels. Differences between the \textit{flhA} mutants in \textit{H. pylori} B128 and 43504 were more dramatic with regard to the levels of \textit{flaB} and \textit{flgE} transcripts. As observed in \textit{H. pylori} B128, \textit{flaB} and \textit{flgE} transcript levels in the \textit{H. pylori} ATCC 43504 \textit{ΔflhA} mutant were severely reduced. In contrast to \textit{H. pylori} B128, levels of \textit{flaB} and \textit{flgE} transcripts in \textit{H. pylori} ATCC 43504 strains that carried the \textit{flhA77} or \textit{flhA454} alleles were similar (60–80\%) to those in the parental strain. The \textit{flhA} mutants in \textit{H. pylori} ATCC 43504 were reconstructed, and three clones for each mutation were analysed. The phenotypes of the reconstructed strains were like those of the original strains, suggesting that the phenotypes were not due to variants within the parental population.

Niehus \textit{et al.} (2004) reported that disrupting \textit{flgM} (encoding the anti-σ\textsubscript{28} factor) restored transcription of RpoN-dependent genes in an \textit{flhA} mutant. The molecular basis for this restoration is not known. To determine
whether the unexpected phenotype of the \( H.\ pylori \) ATCC 43504 strains bearing the \( flhA77 \) or \( flhA454 \) alleles might be accounted for by a mutation in \( flgM \), we sequenced the gene and surrounding region in \( H.\ pylori \) ATCC 43504. The predicted amino acid sequences of FlgM proteins from \( H.\ pylori \) ATCC 43504 and \( H.\ pylori \) B128 were identical with the exception of two conservative changes: \( H.\ pylori \) ATCC 43504 FlgM has isoleucine at positions 2 and 4, while \( H.\ pylori \) B128 FlgM has valine at these positions. The ~300 bp intergenic region upstream of \( flgM \) in the two strains was also the same with the exception of three 1 bp insertions and one 2 bp insertion in the \( H.\ pylori \) B128 sequence. Taken together, these findings argue against the idea that the phenotype of the \( H.\ pylori \) ATCC 43504 strains bearing the \( flhA77 \) or \( flhA454 \) alleles is due to a mutation in \( flgM \).

\( H.\ pylori \) flhA mutants are defective in export of flagellar protein substrates

Given that \( H.\ pylori \) ATCC 43504 bearing the \( flhA77 \) or \( flhA454 \) alleles was competent to transcribe RpoN-dependent flagellar genes, we wished to verify that these strains were deficient in secreting protein substrates. To test for deficiencies in export of rod-/hook-type substrates, we monitored secretion of a FlgE–FLAG protein in which the FLAG epitope was fused to the C terminus of the hook protein FlgE (Smith \textit{et al.}, 2009). The FlgE–FLAG protein was expressed from the native, RpoN-dependent \( flgE \) promoter and was carried on the shuttle vector pHeL3. A monoclonal antibody directed against the FLAG tag was used to monitor expression and export of the FlgE–FLAG protein. \( H.\ pylori \) strains harbouring the plasmid for expression of the FlgE–FLAG protein retained the native \( flgE \) allele in the chromosome. To test for export deficiencies of filament-type substrates, antiserum directed against \( H.\ pylori \) FlaB was used to monitor the expression and secretion of flagellins in the \( H.\ pylori \) strains.

Cultures of \( H.\ pylori \) ATCC 43504 and mutant derivatives were grown in serum-free liquid medium, and the extracellular and soluble cytoplasmic proteins from the cultures were analysed by Western blotting. FlgE–FLAG protein was detected in the cytoplasmic fractions of strains bearing the \( flhA77 \) and \( flhA454 \) mutant strains, but not the \( \Delta flhA \) mutant (Fig. 3a). These data were consistent with the results of the qRT-PCR assays showing that \( flgE \) is transcribed poorly in the \( H.\ pylori \) ATCC 43504 \( \Delta flhA \) mutant but is transcribed at close to wild-type levels in strains bearing the \( flhA77 \) or \( flhA454 \) alleles (Fig. 2b). The FlgE–FLAG protein was detected readily in the wild-type extracellular fraction (i.e. TCA-precipitated material from growth medium that had been clarified by centrifugation; this material included flagellar protein subunits as well as sheared flagella), but not those of the \( flhA77 \) and \( flhA454 \) mutants, even though the protein was present in the cytoplasmic fractions of these mutant strains. A small amount of FlgE–FLAG protein was detected in the extracellular fraction of the \( flhA77 \) mutant, which may have been due to cell lysis. Alternatively, the extracellular FlgE–FLAG may have been due to a low level of export activity by the mutant flagellar protein export apparatus. In support of this latter suggestion, Schmitz \textit{et al.} (1997) reported that cells of an \( H.\ pylori \) \( flhA \) mutant displayed an occasional assembled hook structure, indicating that export of rod-/hook-type substrates was possible to a limited extent in the absence of the full-length FlhA.

Likewise, consistent with the results of the qRT-PCR assays, \( H.\ pylori \) ATCC 43504 bearing the \( flhA77 \) or \( flhA454 \) alleles, but not the \( \Delta flhA \) allele, expressed flagellin proteins (Fig. 3b). FlaA and FlaB are difficult to resolve by SDS-PAGE, as they are very similar in size (53 284 and 53 882 Da, respectively), and the antiserum directed against FlaB cross-reacts with both FlaB and FlaA (Brahmachary \textit{et al.}, 2004; Pereira & Hoover, 2005). Thus, the protein band indicated as ‘flagellin’ in Fig. 3(b) may be a mixture of FlaA and FlaB in samples prepared from the wild-type strain. Given the very low levels of \( flaA \) transcript in the \( flhA77 \) and \( flhA454 \) mutants, we infer that most of the flagellin detected in the cytoplasmic fractions of these mutants is FlaB. Flagellin was detected in the extracellular fraction of the wild-type strain, but not in that

\[ \text{Fig. 3. Western blot analyses of hook and flagellin proteins exported from } H.\ pylori \text{ ATCC 43504. (a) Western blot analysis of hook FlgE–FLAG fusion protein in cytoplasmic (C) and extracellular (E) protein fractions. The indicated strains containing the FlgE–FLAG expression vector pTS14 were analysed using 15 \( \mu \)g total protein for each cytoplasmic sample and 20 \( \mu \)g for each extracellular sample. (b) Western blot analysis of flagellins in soluble cytoplasmic and extracellular protein fractions. The same protein fractions and amounts were analysed as in (a).} \]
of the flhA77 and flhA454 mutants. The extracellular fraction of the wild-type strain contained a cross-reacting protein that migrated slightly higher than the cytoplasmic flagellin. This protein was not consistently observed in all of the samples which were analysed (data not shown). H. pylori flagellins are glycosylated (Schirm et al., 2003), and this slower-migrating species could be flagellin that is glycosylated to a greater degree. The results presented here clearly demonstrate that H. pylori ATCC 43504 strains with the flhA77 or flhA454 alleles are capable of expressing RpoN-dependent flagellar genes, but are deficient in exporting the products of these genes.

The product of the flhA454 allele is stably expressed in H. pylori ATCC 43504 but not in H. pylori B128

We wished to address the issue of why the flhA77 and flhA454 alleles enabled transcription of RpoN-dependent genes in H. pylori ATCC 43504 but not in H. pylori B128. We postulated that the truncated FlhA proteins encoded by these flhA alleles were expressed and incorporated into the export apparatus in H. pylori ATCC 43504 but not H. pylori B128. To test this hypothesis, membrane fractions from H. pylori strains were analysed by Western blotting using antibodies that recognized epitopes located within the first 25 aa residues at the N terminus of FlhA. Full-length FlhA was detected in membrane fractions prepared from H. pylori ATCC 43504 and H. pylori B128 in samples that contained as little as 5 mg total protein (Fig. 4a; data shown for H. pylori B128 only). The predicted size of the FlhA454 variant is ~53 kDa, which was determined by sequencing the flhA454 allele (predicted amino acid sequence included FlhA sequence up to Glu-454 plus an additional 27 residues resulting from the cassette inserted between codons 454 and 455 of flhA). A protein that cross-reacted with the purified FlhA antibodies and was ~53 kDa in size was present in membrane fractions prepared from H. pylori ATCC 43504 bearing the flhA454 allele, but not in the H. pylori ATCC 43504 ΔflhA mutant (Fig. 4b). We infer that this protein is the FlhA454 variant. In contrast, we were unable to detect the FlhA454 variant in membrane fractions prepared from H. pylori B128 (Fig. 4b) even when the amount of sample analysed was increased to 80 mg total protein (data not shown). These results were consistent with the hypothesis that the FlhA454 variant is expressed and incorporated into the export apparatus in H. pylori ATCC 43504 but not in H. pylori B128.

The predicted size of the FlhA77 variant is ~18 kDa, as determined from the sequence of the flhA77 allele. Samples from H. pylori B128 bearing either the flhA77 or the ΔflhA alleles contained two cross-reacting bands in the region where a protein of this size would be expected to migrate (Fig. 4c). Two faint bands of similar size were observed for samples from H. pylori ATCC 43504 bearing the flhA77 allele but not in the ΔflhA mutant (Fig. 4c). Given the low intensities of these bands in H. pylori ATCC 43504 bearing the flhA77 allele, together with the observation that bands of similar sizes are present in the membrane fractions prepared from H. pylori B128 bearing either the flhA77 or the ΔflhA alleles, it was not possible to ascertain from the data whether the FlhA77 variant is stably expressed in H. pylori ATCC 43504 but not H. pylori B128.

Expression of an flaB’-’xyLE reporter gene is enhanced in H. pylori ATCC 43504 harbouring the flhA77 or flhA454 alleles

The results of Western blot analysis demonstrated that the RpoN-dependent flagellar genes flaB and FLAG-tagged flgE

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**Fig. 4.** Western blot analysis of wild-type and truncated forms of FlhA. (a) Membrane fractions prepared from H. pylori B128 wild-type and ΔflhA mutant were analysed by Western blotting using affinity-purified antibodies. Lanes: 1 and 4, 5 mg protein; 2 and 5, 10 mg protein; 3 and 6, 20 mg protein. FlhA, which is immediately above a faint band seen in samples from both the wild-type and the ΔflhA mutant, is indicated by the arrow. Approximate positions of molecular mass markers are indicated at the left of the panel. (b) Membrane fractions prepared from H. pylori B128 bearing either the flhA454 or the ΔflhA allele, or H. pylori ATCC 43504 bearing either the flhA454 or the ΔflhA allele, were analysed by Western blotting. Each lane was loaded with 20 mg protein. Approximate positions of molecular mass markers are indicated at the left of the panel. (c) Membrane fractions prepared from H. pylori B128 bearing either the flhA77 or the ΔflhA allele, or H. pylori ATCC 43504 bearing either the flhA77 or the ΔflhA allele, were analysed by Western blotting. Each lane was loaded with 20 mg protein. Approximate positions of molecular mass markers are indicated at the left of the panel.
were expressed in *H. pylori* ATCC 43504 harbouring the flhA77 or flhA454 alleles. As the Western blot assays were not quantitative, we compared expression of an flaB'-xyE reporter gene in the *H. pylori* ATCC 43504 flhA mutants and the parental strain. As expected based on the results of the qRT-PCR assays, expression of the flaB'-xyE reporter gene was significantly reduced in the ΔflhA mutant compared with the wild-type strain (Fig. 5). Surprisingly, expression of the flaB'-xyE reporter gene was upregulated about eightfold in the flhA77 mutant compared with the wild-type strain. Given that flaB transcript levels in the flhA77 mutant were ~60% of those in the wild-type strain (Fig. 2b), these data strongly suggest that transcripts from the flaB'-xyE reporter gene are translated more efficiently in the flhA77 mutant than they are in the wild-type background. Expression of the flaB'-xyE reporter gene was also significantly higher in the *H. pylori* strain bearing the flaA454 allele compared with the wild-type, although to a lesser extent than for the flhA77 mutant. We considered the possibility that the enhanced expression of the flaB'-xyE reporter gene in the flhA77 mutant compared with the wild-type strain was due to polar effects on the downstream gene, hp1042. This does not appear to be the case, however, as disrupting hp1042 in *H. pylori* ATCC 43504 did not result in upregulation of the flaB'-xyE reporter gene (mean XylE activities for the wild-type and Δhp1042: cat mutant were 0.65 ± 0.26 and 0.72 ± 0.30, respectively).

**DISCUSSION**

Assembly of the bacterial flagellum is a highly ordered and sophisticated process that involves the coordinated expression and regulation of dozens of structural, accessory and regulatory genes. Elucidating the mechanism by which the flagellar protein export apparatus controls transcription of RpoN-dependent flagellar genes in *H. pylori* is critical for dissecting the regulatory circuits that coordinate gene expression with assembly of the flagellum in *H. pylori* and related bacteria.

An unexpected finding from the studies reported here is that some mutations in flhA allow expression of RpoN-dependent flagellar genes in *H. pylori* but not in *H. pylori* B128. The results with *H. pylori* B128 are consistent with those reported previously for insertion mutations in flhA for *H. pylori* strains N6 and 88-3887 (Niehus et al., 2004; Schmitz et al., 1997). We found that the FlhA454 variant is stably expressed in *H. pylori* ATCC 43504 but not *H. pylori* B128 (Fig. 4b), which we believe accounts for the phenotypic differences of the flhA mutants in these strains. We were unable to obtain conclusive evidence that the FlhA77 variant was stably expressed in *H. pylori* ATCC 43504 (Fig. 4c). It is possible that the FlhA77 variant can interact with other export apparatus components in *H. pylori* ATCC 43504 without being incorporated into the cell membrane. There is a precedent for such a conjecture, as Barker et al. (2010) showed in *Salmonella* that expression of the cytoplasmic domain of the export apparatus protein FliO was sufficient to restore motility in a ΔfliO mutant.

The phenotypes of the flhA mutants in *H. pylori* ATCC 43504 indicate that only the first 77 amino acids (or less) of FlhA are needed for transcription of RpoN-dependent flagellar genes. This portion of FlhA is predicted to contain a stretch of 25 aa residues at the N terminus of the protein that extends into the cytoplasm, as well as the first two transmembrane helices (Fig. 1). The N terminus of *Salmonella* FlhA, which is similarly predicted to be located on the cytoplasmic side of the membrane, is required for export function, and is believed to interact with the soluble export apparatus protein FliI and other export apparatus components or protein substrates (McMurry et al., 2004; Minamino et al., 2003). We speculate that the N terminus of FlhA may interact with FliG to modulate its activity and thereby affect transcription of the RpoN regulon. Alternatively, the N terminus of FlhA may influence the assembly or conformation of other components of the export apparatus, which, in turn, could modulate transcription of the RpoN regulon.

Not only does FlhA have a role in regulation of RpoN-dependent flagellar genes, but it also functions in regulating FlhA (σ28)-dependent genes in *H. pylori*. Rust et al. (2009) showed that the anti-σ28 factor FlgM interacts with the C-terminal domain of FlhA (FlhAC), and suggested that FlgM function is modulated through these interactions rather than FlgM secretion. The absence of all or part of FlhAC...

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**Fig. 5.** Expression of an flaB'-xyE reporter gene in *H. pylori* ATCC 43504 bearing various flhA alleles. Whole-cell XylE assays were carried out for *H. pylori* ATCC 43504 bearing the wild-type, ΔflhA, flhA77 or flhA454 allele. XylE activity indicated for the y axis is reported in units of XylE activity per 10^8 cells. One unit of XylE activity corresponds to 1 μmol 2-hydroxymerconic semialdehyde produced per minute. Enzyme activities for each strain were determined from at least six statistical replicates for two or more biological replicates. Error bars, 1 SD. Asterisks indicate values that differed significantly from wild-type XylE activity (P=0.05).
may have accounted for the downregulation of the FlhA-dependent fliA gene in the H. pylori flaA mutants examined in this study as well as earlier studies (Niehus et al., 2004).

In addition to the transcriptional control of flagellar genes, flagellar biogenesis in H. pylori involves other levels of regulation. Douillard et al. (2008) reported that H. pylori protein HP0958 (also referred to as FlgZ) binds the fliA mRNA, resulting in decreased stability but enhanced translation of the transcript. Further possible evidence for post-transcriptional control of flagellar genes in H. pylori comes from studies by Xiao et al. (2009), in which they identified a natural antisense transcript complementary to a partial sequence of fliM (encoding a flagellar motor switch protein in the C ring) in H. pylori 26695. Although the authors of this study did not determine whether the antisense RNA affected the translation or stability of fliM transcripts, it seems reasonable to postulate such a regulatory role. Sharma et al. (2010) identified three small non-coding RNAs (sRNAs) in H. pylori 26695 which they predicted to be transcribed from FlhA-dependent promoters. As the only known role for FlhA in H. pylori is flagellar biogenesis, it is reasonable to postulate that these sRNAs have roles in regulating the expression of specific flagellar genes.

Despite having a slightly lower level of fliB transcript than the wild-type strain, the H. pylori ATCC 43504 flhA77 mutant expressed the fliB*-xylE reporter gene at a significantly higher level than the wild-type strain (Figs 2b and 5). These data suggest that the fliB*-xylE transcripts were translated more efficiently in the flhA77 mutant background, and point to a possible regulatory mechanism which controls translation of fliB mRNA. As fliA mRNA levels were significantly reduced in H. pylori ATCC 43504 bearing the flhA77 allele, transcription of other FlhA-dependent genes in this strain is also likely to be depressed. Thus, if any of the potential FlhA-dependent sRNAs inhibit translation of the fliB mRNA, reduced levels of these sRNAs in the H. pylori ATCC 43504 flhA77 mutant may account for the enhanced expression of the fliB*-xylE reporter gene in this strain.

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