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 FlhA). 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 ($\Delta flhA$), 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 $\Delta flhA$, 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'*-*xyIE* 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.

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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; Cover & Blaser, 1992; 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, FlhB, FliO, FliP, 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

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Abbreviation: sRNA, small non-coding RNA.

The GenBank/EMBL/DDBJ accession numbers for the amino acid sequences of *H. pylori* ATCC 43504 *slyD*, *flgM*, *hp1121* homologue, *hp1122* homologue and *flgK* are JQ31766, JQ31767, JQ31768, JQ31769 and JQ31770, respectively.

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^{80} ; 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 (Colland *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; Foyne *et al.*, 1999; Jenks *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* that had the potential to produce truncated FlhA proteins 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 % O₂/5 % CO₂/93 % N₂) 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* DH5 α 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 FeSO₄, 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 iProof DNA polymerase (Bio-Rad). When *Pfu* Turbo Hotstart or iProof 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 Δ *flhA* allele, a ~690 bp DNA fragment which included ~90 bp of *rps15* (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 plasmid pLP120. A suicide vector for introducing the Δ *flhA* mutant into the chromosome was created by digesting pLP120 with *Hind*III and *Eco*47III, filling in the ends of the cut DNA, and then inserting a 1.3 kb *Sma*I fragment containing a chloramphenicol transacetylase (*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 *flhA*77 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 *Eco*47III fragment in pLP78 was replaced with the *Sma*I 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 *flhA*77 allele into the chromosome. To construct the *flhA*454 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 *Eco*RI site that overlapped codons 454 and 455 of *flhA*. A 1.3 kb *Eco*RI fragment from pSKAT4 containing the *cat* cassette was cloned into the *Eco*RI site that had been added into pLP113 to create a suicide vector for introducing the *flhA*454 allele into the chromosome. The resulting

Table 1. Oligonucleotide primers used in this study

Primer	Sequence
$\Delta flhA$ forward	5'-CACTTGCACCTCACAAGAAC-3'
$\Delta flhA$ reverse	5'-CTTCAGTAACCCTAGTAGAGC-3'
<i>flhA77</i> forward	5'-ATGGCAAACGAACGCTCCAAATTAG-3'
<i>flhA77</i> reverse	5'-GCCATGCGATGAATAAAAAGAGAC-3'
<i>flhA454</i> forward	5'-GACTCTCTTTTATTTCATCGCATG-3'
<i>flhA454</i> reverse	5'-CAAGACTTTCATGGCCTTTC-3'
<i>flhA454</i> <i>EcoRI</i> forward	5'-CCCCAACGCATTATGAATTCAAACTTAAAGGCATTGTG-3'
<i>flhA454</i> <i>EcoRI</i> reverse	5'-CACAATGCCTTTAAGTTTGAATTCATAATGCGTTGGGGG-3'
<i>hp1042</i> forward	5'-GTTACATGAAAAATCCCCATTAA-3'
<i>hp1042</i> reverse	5'-GTGTTTGGTTGGGTGGTATGA-3'
<i>hp1042</i> inverse forward	5'-GGATATCAACTAAGCTGTTTAGAAAAACTAA-3'
<i>hp1042</i> inverse reverse	5'-GGATATCTCTCTTTTATCAATTATTTATCC-3'
Seq1_forward	5'-ATGCAAAACCATGATTTAGAGT-3'
Seq1_reverse	5'-TTATTGTTTAATCCCCAATAAG-3'
Seq2_forward	5'-AAGCAGCTATTTGCAAGAAG-3'
Seq2_reverse	5'-CAGGCTCGCCTTCAATATG-3'
Seq3_forward	5'-GCTCTTGATAGGGTAGCTG-3'
Seq3_reverse	5'-GCTCGCTTCATCAATGTCC-3'
Seq4_forward	5'-TATCAAAGCCTTGCAAGAAGC-3'
Seq4_reverse	5'-GTCTCTTATATCGCGCTCG-3'
Seq5_forward	5'-TAGTATTTGCTTGAATACGGC-3'
Seq5_reverse	5'-CCTAAAGGGAATGAAATGG-3'
Seq6_forward	5'-GGGTGCATGATGAGTTTG-3'
Seq6_reverse	5'-GAGAAGTCTCATGCAAGTTG-3'
Seq7_forward	5'-ACGCTCAAAGTGCGAGTC-3'

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 $\Delta hp1042$ mutant, *hp1042* along with 500 bp DNA upstream and 540 bp DNA downstream of the gene were amplified using the *hp1042* forward and *hp1042* reverse primers indicated in Table 1. The amplicon was cloned into pGEM-T, and the resulting plasmid was used as a template for inverse PCR using the *hp1042* inverse forward and *hp1042* inverse reverse primers (Table 1). These primers annealed to sequences immediately downstream and upstream of *hp1042*, respectively, and introduced *EcoRV* sites. Amplicons were circularized using T4 DNA ligase, and a 1.3 kb *EcoRI* fragment from pSKAT4 containing the *cat* cassette that was filled in was ligated into the *EcoRV* sites of the plasmid. The resulting suicide plasmid was introduced into *H. pylori* ATCC 43504 by natural transformation, and transformants were selected as described previously (Brahmachary *et al.*, 2004). Insertion of the *cat* cassette into the *hp1042* locus in the *H. pylori* ATCC 43504 chromosome was confirmed by PCR.

XylE assays. A reporter plasmid bearing an *flaB'*-*xylE* reporter gene on the shuttle vector pHel3 (Heuermann & Haas, 1998) was described previously (Brahmachary *et al.*, 2004). *H. pylori* strains containing the reporter plasmid were cultured for 36 h on agar medium supplemented with kanamycin to maintain the plasmid. Cells were resuspended in 50 mM potassium phosphate buffer, pH 7.0, to OD₆₀₀ 1, which corresponded to $\sim 1 \times 10^9$ c.f.u. ml⁻¹. Reactions were initiated by adding 0.1 ml cell suspension to 0.9 ml of 3 mM catechol in 50 mM potassium phosphate buffer, pH 7.0, which had been warmed for 5 min at 37 °C. Reactions were incubated for 2 min at 37 °C, stopped by adding 0.5 ml of 1 M sodium carbonate, and

clarified by centrifugation at $\sim 16\,000$ g for 2 min. The absorbance of each reaction was measured at 375 nm using a Shimadzu UV-160U spectrophotometer to determine the amount of product (2-hydroxy-muconic semialdehyde) that had been formed. One unit of XylE activity corresponds to 1 μ mol 2-hydroxymuconic semialdehyde produced per minute, and was calculated by multiplying the final A₃₇₅ value for each sample by 22.7. XylE activity levels were expressed as units of XylE activity per 10⁸ cells. XylE activity for each strain 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 (MANERSKLAFKKTFFPVFKRFLQSKD) 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 cysteine 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 the volume to 0.5 ml using an Amicon Ultra-15 centrifugal filter, adding another 13 ml PBS, and then reducing the volume to 0.5 ml again. The buffered-exchanged antiserum was applied to the SulfoLink column; the column was capped and gently rocked for 1 h at room temperature to allow the antibodies to bind to the FlhA peptide immobilized on the resin. The resin was washed five times with 2 ml PBS, and then antibodies were eluted in three 2 ml washes of 0.1 M glycine, pH 2.5. The eluted antibodies were dialysed against citric acid-phosphate buffer (55 mM

citric acid, 50 mM K₂HPO₄, pH 5.5), followed by Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6), then stored at -20 °C.

H. pylori membranes were prepared for Western blot analysis as follows. *H. pylori* strains were grown for ~72 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 FluoroChem E imager (ProteinSimple).

Detection of exported flagellar proteins. Plasmid pTS14 is a derivative of pHel3 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 μ g 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* FlaB (Pereira & Hoover, 2005), a peroxidase-conjugated goat anti-rabbit antibody (MP Biomedicals) and enhanced chemiluminescence were used to detect *H. pylori* flagellins. *H. pylori* FlaB and FlaA share 58 % amino acid identity, and the antiserum directed against FlaB 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 μ l 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 μ l, consisted of 10 μ l of iQ SYBR Green Supermix, 5 μ l 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^{- $\Delta\Delta C_t$} method (Schmittgen & Livak, 2008).

DNA sequencing. A region of the *H. pylori* 43504 chromosome that included *flgM*, the gene upstream of *flgM* (*slyD*) and three genes downstream of *flgM* (a gene encoding a predicted cytosine-specific DNA methyltransferase, a potential *flgN* homologue and *flgK*, respectively) was amplified using iProof DNA polymerase. After treating the resulting amplicon 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/w/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 (FlhA_{TM}) predicted to contain six to eight membrane-spanning helices and a large C-terminal cytoplasmic domain referred to as FlhA_C (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 FlhA_C 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

Fig. 1. Diagram of *H. pylori* FlhA. The FlhA_{TM} and FlhA_C domains of the protein are indicated. White boxes denote the approximate position of predicted transmembrane spanning helices, black boxes indicate putative periplasmic regions of the protein, and grey regions indicate predicted cytoplasmic regions of the protein. The predicted topology of *H. pylori* FlhA is based on a comparison with the predicted topology of *Salmonella* FlhA done by McMurtry *et al.* (2004) along with membrane topology prediction programs as indicated in Results. The heavy black line corresponds to the intergenic region upstream of *flhA*. The corresponding regions of the gene which were replaced with the *cat* cassette in the $\Delta flhA$ and *flhA77* mutants are indicated, as is the approximate location of the *cat* cassette in the *flhA454* mutant.

expression of the entire *flhA* gene it was referred to as a $\Delta flhA$ mutation. For the second allele, codons 77–242 of *flhA* were replaced with the *cat* cassette, and this allele was referred to as *flhA77*. The *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 *flhA* allele was generated by introducing the *cat* cassette between codons 454 and 455, and the resulting allele was designated *flhA454*. The *flhA454* allele could potentially produce a truncated FlhA that would include all of FlhA_{TM} plus part of FlhA_C.

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

Surprisingly, the flagellar gene transcript profiles for the *flhA* mutants in *H. pylori* ATCC 43504 were strikingly different from those in *H. pylori* B128 (Fig. 2b). Relative to wild-type, the level of *flaA* transcripts in the *H. pylori* ATCC 43504 $\Delta flhA$ mutant was reduced to a level (~12 %) that was similar to those in the *H. pylori* B128 *flhA* mutants. In the *H. pylori* ATCC 43504 strains bearing the

flhA77 or *flhA454* alleles, however, *flaA* transcript levels were reduced further to ~5 % of wild-type levels. Differences between the *flhA* mutants in *H. pylori* B128 and 43504 were more dramatic with regard to the levels of *flaB* and *flgE* transcripts. As observed in *H. pylori* B128, *flaB* and *flgE* transcript levels in the *H. pylori* ATCC 43504 $\Delta flhA$ mutant were severely reduced. In contrast to *H. pylori* B128, levels of *flaB* and *flgE* transcripts in *H. pylori* ATCC 43504 strains that carried the *flhA77* or *flhA454* alleles were similar (60–80 %) to those in the parental strain. The *flhA* mutants in *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 *et al.* (2004) reported that disrupting *flgM* (encoding the anti- σ^{28} factor) restored transcription of RpoN-dependent genes in an *flhA* mutant. The molecular basis for this restoration is not known. To determine

Fig. 2. Relative levels of *flaA*, *flaB* and *flgE* transcripts in *H. pylori* strains containing various *flhA* alleles. Transcript levels were determined by qRT-PCR and compared relative to wild-type levels. (a) Relative levels of *flaA*, *flaB* and *flgE* transcripts in *H. pylori* B128 harbouring the $\Delta flhA$ (black bars), *flhA77* (grey bars) or *flhA454* (open bars) allele. (b) Relative levels of *flaA*, *flaB* and *flgE* transcripts in *H. pylori* ATCC 43504 harbouring the $\Delta flhA$ (black bars), *flhA77* (grey bars) or *flhA454* (open bars) allele. Note the differences in scales for the y axes of the two graphs. Error bars, 1 SD.

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 *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 *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 *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

Fig. 3. Western blot analyses of hook and flagellin proteins exported from *H. pylori* ATCC 43504 bearing various *flhA* alleles. (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 μ g total protein for each cytoplasmic sample and 20 μ 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 µg 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 Δ *flhA* 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 µg 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 Δ *flhA* 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 Δ *flhA* 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 Δ *flhA* 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*'-'*xyIE* 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*

Fig. 4. Western blot analysis of wild-type and truncated forms of FlhA. (a) Membrane fractions prepared from *H. pylori* B128 wild-type and Δ *flhA* mutant were analysed by Western blotting using affinity-purified antibodies. Lanes: 1 and 4, 5 µg protein; 2 and 5, 10 µg protein; 3 and 6, 20 µg protein. FlhA, which is immediately above a faint band seen in samples from both the wild-type and the Δ *flhA* 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 Δ *flhA* allele, or *H. pylori* ATCC 43504 bearing either the *flhA454* or the Δ *flhA* allele, were analysed by Western blotting. Each lane was loaded with 20 µg 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 Δ *flhA* allele, or *H. pylori* ATCC 43504 bearing either the *flhA77* or the Δ *flhA* allele, were analysed by Western blotting. Each lane was loaded with 20 µg 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'*-*xylE* 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'*-*xylE* reporter gene was significantly reduced in the Δ *flhA* mutant compared with the wild-type strain (Fig. 5). Surprisingly, expression of the *flaB'*-*xylE* 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'*-*xylE* reporter gene are translated more efficiently in the *flhA77* mutant than they are in the wild-type background. Expression of the *flaB'*-*xylE* reporter gene was also significantly higher in the *H. pylori* strain bearing the *flhA454* 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'*-*xylE* 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'*-*xylE* reporter gene (mean XylE activities for the wild-type and Δ *hp1042: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 circuitries 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* ATCC 43504 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 Δ *fliO* 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 FlgS 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 FliA (σ^{28})-dependent genes in *H. pylori*. Rust *et al.* (2009) showed that the anti- σ^{28} factor FlgM interacts with the C-terminal domain of FlhA (FlhA_C), and suggested that FlgM function is modulated through these interactions rather than FlgM secretion. The absence of all or part of FlhA_C

Fig. 5. Expression of an *flaB'*-*xylE* 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, Δ *flhA*, *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 μ mol 2-hydroxymuconic 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 FliA-dependent *flaA* gene in the *H. pylori* *flhA* 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 *flaA* 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 FliA-dependent promoters. As the only known role for FliA 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 *flaB* transcript than the wild-type strain, the *H. pylori* ATCC 43504 *flhA77* mutant expressed the *flaB'*-*xylE* reporter gene at a significantly higher level than the wild-type strain (Figs 2b and 5). These data suggest that the *flaB'*-*xylE* transcripts were translated more efficiently in the *flhA77* mutant background, and point to a possible regulatory mechanism which controls translation of *flaB* mRNA. As *flaA* mRNA levels were significantly reduced in *H. pylori* ATCC 43504 bearing the *flhA77* allele, transcription of other FliA-dependent genes in this strain is also likely to be depressed. Thus, if any of the potential FliA-dependent sRNAs inhibit translation of the *flaB* mRNA, reduced levels of these sRNAs in the *H. pylori* ATCC 43504 *flhA77* mutant may account for the enhanced expression of the *flaB'*-*xylE* reporter gene in this strain.

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