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Genome-wide analysis of transcriptional hierarchy and feedback regulation in the flagellar system of *Helicobacter pylori*

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Summary

The flagellar system of *Helicobacter pylori*, which comprises more than 40 mostly unclustered genes, is essential for colonization of the human stomach mucosa. In order to elucidate the complex transcriptional circuitry of flagellar biosynthesis in *H. pylori* and its link to other cell functions, mutants in regulatory genes governing flagellar biosynthesis (*rpoN*, *flgR*, *flhA*, *flhF*, HP0244) and whole-genome microarray technology were used in this study. The regulon controlled by RpoN, its activator FlgR (FleR) and the cognate histidine kinase HP0244 (FleS) was characterized on a genome-wide scale for the first time. Seven novel genes (HP1076, HP1233, HP1154/1155, HP0366/367, HP0869) were identified as belonging to RpoN-associated flagellar regulons. The hydrogenase accessory gene HP0869 was the only annotated non-flagellar gene in the RpoN regulon. Flagellar basal body components FlhA and FlhF were characterized as functional equivalents to master regulators in *H. pylori*, as their absence led to a general reduction of transcripts in the RpoN (class 2) and FliA (class 3) regulons, and of 24 genes newly attributed to intermediate regulons, under the control of two or more promoters. FlhA- and FlhF-dependent regulons comprised flagellar and non-flagellar genes. Transcriptome analysis revealed that negative feedback regulation of the FliA regulon was dependent on the antisigma factor FlgM. FlgM was also involved in FlhA- but not FlhF-dependent feedback control of the RpoN regulon. In contrast to other bacteria, chemotaxis and flagellar motor genes were not controlled by FliA or RpoN. A true master regulator of flagellar biosynthesis is absent in *H. pylori*, consistent with the essential role of flagellar motility and chemotaxis for this organism.

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Introduction

More than 40 proteins are involved in the biosynthesis and operation of flagella and their control by the chemotaxis machinery, making motility one of the most complex processes in the bacterial cell. Both the motility organelles and the regulatory pathways involved in their biosynthesis can differ considerably between bacterial genera. Examples of unique flagellar regulons include the flagellar biosynthesis of *Caulobacter crescentus* swarmer cells (Wu and Newton, 1997), the regulation of the polar and lateral flagella of *Vibrio* spp. (McCarter, 2001; Prouty *et al*., 2001) and the FlhCD-dependent flagellar hierarchy of *Salmonella enterica* serovar Typhimurium (Aizawa, 2000). The ∈-proteobacterium *Helicobacter pylori* depends on a functional flagellar motility system in its natural habitat, the human stomach mucus. Mutagenesis of just about any gene of the motility and chemotaxis systems abolishes the ability of *H. pylori* to infect the stomach (Kim *et al*., 1999; Foynes *et al*., 2000; Ottemann and Lowenthal, 2002; Kavermann *et al*., 2003). Despite intensive research about the role of motility in *H. pylori* pathogenesis, the transcriptional network that controls the expression of flagellar genes in *H. pylori* is still incompletely understood. *H. pylori* possesses a small set of obvious regulatory genes, among them three RNA polymerase sigma factors (σ^{80}, σ^{54}, σ^{28}) (Tomb *et al*., 1997; Beier *et al*., 1998; Alm *et al*., 1999), which are all involved in flagellar biosynthesis (Beier *et al*., 1997; Spohn and Scarlato, 1999; Josenhans and Suerbaum, 2001; Spohn and Scarlato, 2001;

Josenhans *et al*., 2002). No flagellar master regulator similar to FlhCD in the Enterobacteriaceae has been found in the *H. pylori* genome (*Supplementary material*, Fig. S1). *H. pylori* FlgR, an RpoN activator (Spohn and Scarlato, 1999), is an orthologue of flagellar master regulators in other bacteria (FleR and FleQ of *Pseudomonas* spp., CtrA and FlbD of *Caulobacter*, FlrA and FlrC of *Vibrio* spp.; Jenal, 2000; Prouty *et al*., 2001; Dasgupta *et al*., 2002), but functional analyses do not support a role for FlgR as a master regulator. The recent characterization of the flagellar anti-σ^{28} factor FlgM of *H. pylori* and the discovery of novel σ^{28}-dependent genes by comprehensive transcript profiling have been major steps towards understanding the flagellar regulon of *H. pylori* (Colland *et al*., 2001; Josenhans *et al*., 2002). Two other known flagellar regulators are RpoN (σ^{54}, encoded by HP0714/jhp652) and FlhA/FlbA (encoded by HP1041/jhp383), an essential membrane component of the flagellar basal body export apparatus (Schmitz *et al*., 1997; Minamino and Macnab, 2000; Fujinaga *et al*., 2001; Kihara *et al*., 2001). One class of *H. pylori* flagellar genes (class 2; middle genes) is supposedly dependent on RpoN, and on the RpoN activator FlgR (Spohn and Scarlato, 1999) and its cognate histidine kinase HP0244 (jhp229; Beier and Frank, 2000), which form a two-component system (TCS), similar to FleS and FleR of *Pseudomonas aeruginosa* (Ritchings *et al*., 1995). FlhA was shown to be required for expression of three flagella-associated genes, which belong to both σ^{28}- and σ^{54}-dependent flagellar regulons in *H. pylori* (Schmitz *et al*., 1997). In a recent report, FlhA of *Bacillus thuringiensis* was involved in both flagellar assembly and the secretion of virulence factors (Ghelardi *et al*., 2002), making it a likely candidate for connecting motility/flagella with other cell functions. A further putative flagellar regulatory gene is *flhF* (HP1035/jhp389), which is not found in the Enterobacteriaceae, but in several other flagellated bacteria. FlhF belongs to the protein family of the signal recognition particle (SRP), which is involved in targeted protein transport across membranes (Keenan *et al*., 2001). *flhF* and the downstream gene (*flhG/fleN*) are involved in polar flagellar placement or regulation of flagellar numbers (Dasgupta *et al*., 2000; Pandza *et al*., 2000).

We and others have recently proposed a hierarchical model of *H. pylori* flagellar regulation (Spohn and Scarlato, 2001; Josenhans *et al*., 2002), which contains elements of both the *Caulobacter* and *Salmonella* flagellar hierarchies. However, many questions remain unsolved. Global flagellar regulators in *H. pylori*, which link motility-associated regulons to other cell functions, have not been found so far, and only a few σ^{54}-dependent genes have been identified. In addition, most flagella-associated genes could not be assigned with defined regulons in *H. pylori*. Mechanisms of transcriptional feedback inhibition in the *Helicobacter* flagellar system have not been explored.

The aims of the present study were to characterize the flagellar regulons, their integration into the global regulatory circuitry and flagellar master regulators in *H. pylori*. Genome-wide transcriptional profiling of *rpoN*, *flhA*, *flgR*, HP0244 and *flhF* mutants, as well as *flhA/flgM* and *flhF/flgM* double mutants, by means of a custom-designed DNA microarray revealed novel flagellar and non-flagellar genes in the σ^{54} regulon or in intermediate regulons in *H. pylori*. Flagellar basal body components FlhA and FlhF are functional equivalents of master regulators in *H. pylori*, as their absence led to a global reduction of transcripts in the FliA, RpoN and intermediate regulons. These effects on class 2 and class 3 flagellar genes were characterized in part as transcriptional feedback mechanisms dependent on FlgM.

Results and discussion

Mutagenesis and characterization of H. pylori *genes involved in flagellar regulation*

Knock-out mutants of two *H. pylori* strains, N6 (Ferrero *et al*., 1992) and 88-3887 (motile 26695 isolate; Josenhans *et al*., 2000) in the genes *rpoN*, *flhA*, *flhF*, HP0244 and *flgR* were constructed by disruption with an *aphA3*′-III kanamycin resistance cassette in direct orientation (genome localization of flagellar genes in *Supplementary material*, Fig. S1). Substantial parts of the 5′ coding regions were deliberately kept intact, such that transcriptional regulation of the mutated gene itself might also be detected by transcriptional profiling of the mutants. *flhA* and *rpoN* are in monocistronic operons with σ^{80}-like promoter sequences (Schmitz *et al*., 1997; Tomb *et al*., 1997; Fujinaga *et al*., 2001). *flhF* is the first gene of a large operon comprising genes HP1035 (*flhF*) to HP1028 and including flagellar switch genes and HP1032 (*fliA*). [The original annotation in both complete genomes (Tomb *et al*., 1997; Alm *et al*., 1999) lists a hypothetical gene with no homologies to database entries, HP1033, which would be oriented in the inverse direction of upstream HP1034 and downstream HP1032. Upon reassessment of the genomic region between HP1035 and HP1028, a new open reading frame (ORF), HP1033b, emerged, which is in direct orientation to the upstream and downstream ORFs. This novel annotation of HP1033b (for sequence, see *Supplementary material*, alignment 1; HP1033b has 49% amino acid similarity to *Campylobacter jejuni* Cj0062c) makes this gene cluster a contiguous putative operon. Co-transcripts between HP1033b and upstream HP1034/HP1035 and downstream HP1032 were detected by reverse transcription polymerase chain reaction (RT-PCR; data not shown).]

The *rpoN*, *flhA*, *flgR* and *flhF* mutants were phenotypically characterized for the presence of flagella (transmission electron microscopy), motility (wet mounts, swarm plates; not shown) and for the expression of the flagellar proteins FlaA, FlaB and FlgE1 (hook protein) in whole-cell lysates as well as in flagellar preparations. All mutants lacked flagella and were non-motile. *rpoN* and *flgR* mutants did not express detectable amounts of FlaA, FlaB or hook protein (data not shown; Beier and Frank, 2000; Fujinaga *et al*., 2001). As described previously, *flhA* mutants expressed hook protein, but no detectable amounts of FlaA or FlaB (Schmitz *et al*., 1997). Complementation *in trans* of the *flhA* mutants was performed, which reconstituted flagellar biosynthesis and motility. Two different *flhF* mutants were constructed (*flhfm1* and *flhfm2*; see *Experimental procedures*). *flhF* mutants had a similar phenotype to *flhA* mutants, but expression of FlgE1 (hook protein) could not be detected in Western blots. Very few *flhF* mutant bacteria displayed one or two short and laterally localized flagella, but the majority of these bacteria were aflagellate.

The RpoN regulon of H. pylori*: σ^{54} plays a central role in flagellar biogenesis and controls a unique set of flagellar genes*

In order to define the RpoN regulon in *H. pylori*, fluorescently labelled cDNAs of wild-type strains and *rpoN* mutants of *H. pylori* N6 and 88-3887 were competitively hybridized on glass slide DNA microarrays, and expression ratios were determined. Two different microarray systems (based on oligonucleotides or PCR products) were used as well as three different biological experiments. To account for possible changes in transcription of the flagellar genes during the different growth phases (Niehus *et al*., 2002), early exponential (OD_{600} = 0.35) and mid-exponential phase (OD_{600} = 0.7) were tested. The transcriptional changes determined by microarray experiments in the *rpoN* mutants versus the wild-type strains (Table 1) were confirmed by semi-quantitative RT-PCR (Table 1; Fig. 1). By these experiments, the *H. pylori* RpoN regulon was characterized on a genome-wide scale for the first time (Table 1). It included four known 'middle' flagellar genes (proposed class 2, Table 1; Spohn and Scarlato, 2001), coding for the flagellar hook subunit (FlgE1), hook–filament adaptor proteins (FlgK, FlgL) and the minor flagellin subunit FlaB. In addition, we identified five genes in four operons as novel members of the σ^{54} regulon that had not been linked to RpoN or flagellar biosynthesis before (Table 1). A single non-flagellar gene in the newly defined RpoN regulon was HP0869 (*hypA*), encoding a nickel-binding protein involved in hydrogenase and urease maturation (Mehta *et al*., 2003), which is co-transcribed with the σ^{54}-dependent gene *flgE1* (HP0870). Three of the novel genes (HP1076, HP1233, HP1154) encode proteins of unknown function, and HP1155 (*murG*) encodes a glycosyltransferase active in peptidoglycan biosynthesis, which has a housekeeping function in all cell wall-possessing bacteria. The protein encoded by HP1154 shows low sequence similarities to UDP-glucuronyl transferases. A weak protein interaction with the FlaA flagellin (Hybrigenics PimRider™ database; Rain *et al*., 2001; http://pim.hybrigenics.com/pimrider) suggests a function in flagellar assembly. Links to flagellar biosynthesis are suspected for the latter four genes, which will be explored in detail in future studies. HP1076, which has no significant amino acid homologies with known flagellar proteins, interacts with the flagellar chaperone FliS (HP0753), and FlgB, a flagellar rod component (Hybrigenics PimRider™). The second novel gene in the RpoN regulon was HP1233, a *Helicobacter*-specific protein of unknown function. A CLUSTALW alignment of the HP1233 protein yielded an overall similarity of 22% (identity 10%) with *Salmonella* FlgJ, the flagellar muraminidase (see *Supplementary material*, alignment 2). Inactivation of HP1076 and HP1233 by mutagenesis led to altered flagellar morphology (shorter and fewer flagella, impaired flagellar sheaths), which indicates a function in flagellar biosynthesis (data not shown). Some operons described previously as possessing σ^{54}-like promoters (Spohn and Scarlato, 1999) could not be confirmed as part of the RpoN regulon in our experiments. Transcription of the gene cluster *flg-x-DE2* (HP0906–HP0908) and the *flgBC* (HP1559–HP1558) flagellar rod operon was not reduced below the cut-off in the microarray experiments (Table 1; Fig. 1). RT-PCR indicated that HP0906, which is preceded by a σ^{54}-like promoter and was shown previously to be under the control of FlgR (Spohn and Scarlato, 1999), and HP1559 (*flgB*) were dependent on RpoN for transcription. HP0907 (*flgD* orthologue; Hirano *et al*., 2003) and HP0908 (*flgE2*), which codes for a flagellar hook FlgE1 orthologue (O'Toole *et al*., 1994) appeared to be independent of RpoN (Fig. 1). Transcription of HP0907/HP0908 was also not reduced in *flgR*, HP0244 or *flhA* mutants compared with the wild type (Table 1 and below) and, by RT-PCR, their transcription was stronger than that of HP0906 (Fig. 1B). HP0908 and HP0907 are likely to be controlled by an additional FliA- and RpoN-independent promoter (σ^{80} consensus-like promoter sequence at position –25 to –59 upstream of the start codon; Vanet *et al*., 2000). So far, *flgE2* genes have only been found in *Helicobacter* and *Campylobacter* species, and their function remains to be clarified. Transcription of all flagellar regulatory genes, among them *rpoN* itself, was not altered in the *rpoN* mutants as shown by microarray and RT-PCR (Table 1). σ^{54}-like promoters could be identified upstream of all genes differentially expressed in the *rpoN* mutants, including the novel σ^{54}-dependent genes (Fig. 2).

Table 1. Selected results of the DNA microarray hybridization experiments with different *H. pylori* strains and flagellar mutants (top). Means of ratios and standard deviations are based on *n* independent competitive PCR product-based microarray hybridizations, including two replicas of every PCR product on each microarray slide (*n*-values are indicated above each lane). Gene names and designations of the loci in strain 26695 (TIGR HP numbers; Tomb *et al.*, 1997) are indicated. A colour code to the left of the gene names indicates the grouping of genes into the regulatory classes 2 and 3, or intermediate classes, which are controlled by more than one promoter (see also Fig. 4). The up- or downregulation of genes as determined by the array experiments is highlighted in the table by red or orange shading (downregulated) and light green shading (upregulated). Regulators investigated in the study are highlighted in grey. RT-PCR was performed for all experiments designated with ° or *: *up- or downregulation, as determined by semi-quantitative RT-PCR; ° no regulation, as determined by semi-quantitative RT-PCR.

	TIGR orf no.	putative gene product (gene)	HpN6 wt / *rpoN* (n=4)	+/-	Hp88-3887 wt / *rpoN* (n=3)	+/-	HpN6 wt / *flgR* (n=2)	+/-	HpN6 wt / HP244 (n=4)	+/-	HpN6 wt / *flhA* (n=4)	+/-	Hp88-3887 wt / *flhA* (n=3)	+/-	HpN6 wt / *flhF* (n=4)	+/-	Hp88-3887wt / *flhF* (n=2)	+/-	HpN6 wt / *flhA/flgM* (n=5)	+/-
class 3	HP0472	outer membrane protein 11 (*omp11*)	° 0.9	0.2	0.9	0.2	1.2	0.1	0.7	0.1	* 15.9	7.1	* 7.9	3.3	* 12.5	10	21.3	10	* 0.4	0.1
	HP0601	flagellinA (*flaA*)	° 0.9	0.2	° 0.7	0.0	° 1.1	0.1	* 0.4	0.1	* 4.4	1.8	* 2.2	1.1	* 4.3	1.2	* 14.1	5.8	* 0.5	0.2
	HP1052	UDP-3-0-acyl N-acetylglycosamine deacetylase (*envA*)	0.9	0.3	0.8	0.1	1.2	0.1	0.8	0.2	3.4	1.1	2.2	0.4	2.0	1.0	4.4	0.8	0.5	0.2
	HP1051	hypothetical protein (operon with *envA*)	0.9	0.2	0.6	0.1	1.4	0.1	0.8	0.1	2.0	0.4	2.6	0.6	1.9	0.2	2.2	0.3	0.5	0.1
intermediate class	HP0751	(*flaG2*)	0.9	0.2	0.6	0.1	1.6	0.2	0.7	0.1	* 3.6	0.8	* 3.9	0.8	2.7	0.7	* 2.9	0.2	* 0.4	0.1
	HP0752	flagellar cap protein (*fliD*)	0.9	0.2	0.6	0.0	1.0	0.1	0.8	0.2	2.4	0.7	* 1.2	0.1	* 1.6	0.4	1.9	0.3	0.4	0.0
	HP0753	flagellar chaperone (*fliS*)	0.9	0.2	0.7	0.1	1.2	0.1	0.8	0.1	* 3.3	0.0	* 2.3	1.2	4.2	0.1	* 1.2	0.0	0.8	0.1
	HP0754	flagellar chaperone (*fliT*)	1.1	0.2	0.8	0.0	1.2	0.1	0.9	0.1	* 1.3	0.0	* 1.1	0.1	* 1.1	0.1	1.0	0.0	* 0.8	0.0
	HP1122	anti-sigma28 factor (*flgM*)	° 0.9	0.1	° 0.9	0.1	1.3	0.1	0.8	0.1	* 2.9	1.3	* 2.3	0.7	1.8	0.4	* 3.4	0.6	N.D.	
	HP1035	flagellar biosynthesis protein of unknown function (*flhF*)	0.9	0.2	1.2	0.1	1.5	0.1	0.8	0.1	1.4	0.1	1.6	0.3	* 1.7	0.4	2.6	0.6	1.4	0.2
	HP1034	ATP-binding protein (*ylxH/flhG*)	1.0	0.4	1.0	0.1	* 2.4	0.3	° 0.8	0.1	° 1.2	0.2	1.7	0.3	* 6.0	2.1	* 15.0	6.7	1.0	0.3
	HP1033	hypothetical protein	1.0	0.2	1.0	0.1	1.6	0.1	0.9	0.0	1.2	0.2	1.4	0.5	* 1.6	0.1	1.4	0.0	1.1	0.2
	HP1032	alternative sigma factor sigma28 (*fliA*)	° 1.1	0.4	° 0.9	0.2	1.7	0.0	0.9	0.1	° 1.2	0.1	1.5	0.5	* 1.9	0.2	* 2.0	0.0	° 0.9	0.2
	HP1031	flagellar motor switch protein (*fliM*)	1.1	0.4	1.0	0.0	1.7	0.1	0.9	0.1	1.2	0.1	1.4	0.5	2.9	1.0	2.1	0.4	1.0	0.1
	HP1030	FliY protein (*fliY*)	1.0	0.5	0.9	0.1	2.0	0.1	0.9	0.3	1.3	0.1	1.5	0.5	2.8	0.5	4.2	2.4	0.9	0.2
	HP1029	hypothetical protein	1.0	0.4	1.1	0.1	2.3	0.1	1.1	0.2	1.4	0.3	2.2	0.1	2.6	0.3	7.5	2.0	0.9	0.2
	HP1028	hypothetical protein	0.9	0.3	0.8	0.0	* 1.9	0.1	° 1.1	0.2	1.5	0.3	1.9	0.4	* 2.5	0.2	* 4.7	0.0	0.5	0.1
	HP1559	flagellar rod protein (*flgB*)	* 1.6	0.3	1.7	0.1	* 2.3	0.1	* 2.3	0.7	1.4	0.4	0.6	0.2	1.6	0.4	1.3	0.1	0.6	0.2
	HP1558	flagellar rod protein (*flgC*)	1.4	0.3	1.3	0.2	1.7	0.1	1.6	0.4	1.1	0.1	0.7	0.1	1.3	0.2	1.2	0.2	0.6	0.2
	HP1557	flagellar basal body protein (*fliE*)	1.0	0.2	1.1	0.2	1.5	0.1	1.3	0.3	1.1	0.3	0.8	0.0	0.9	0.3	1.1	0.2	1.0	0.2
	HP0166	response regulator (*ompR*)	0.9	0.2	1.0	0.2	1.0	0.0	1.1	0.3	* 2.9	0.3	* 1.5	0.2	1.6	0.2	1.1	0.1	* 1.4	0.5
	HP0165	histidine kinase	0.8	0.2	1.3	0.3	1.1	0.0	1.0	0.2	2.6	0.6	2.1	0.3	1.3	0.1	1.2	0.2	1.6	0.3
	HP0367	hypothetical protein	1.2	0.3	1.4	0.3	2.7	0.3	3.5	1.4	1.5	0.2	2.1	1.0	2.0	0.4	3.3	0.7	0.1	0.1
	HP0366	spore coat polysaccharide biosynthesis protein C	1.5	0.6	0.8	0.0	1.1	0.1	3.8	2.1	1.5	0.1	1.2	0.5	1.4	0.5	1.4	0.0	0.5	0.2
	HP0488	hypothetical protein	1.0	0.2	1.0	0.1	1.1	0.1	0.9	0.1	3.0	1.9	1.8	0.2	4.0	2.4	3.9	0.5	0.7	0.3
	HP1440	hypothetical protein	1.0	0.1	0.7	0.0	1.9	0.1	0.7	0.1	* 3.0	1.0	* 1.1	0.1	* 2.8	0.2	5.6	0.0	* 0.6	0.1
	HP0908	flagellar hook protein homolog (*flgE2*)	° 1.4	0.3	° 1.1	0.0	° 1.0	0.1	° 1.3	0.0	° 1.1	0.1	° 0.9	0.2	1.0	0.1	0.9	0.0	° 1.3	0.5
	HP0907	hook assembly protein (*flgD*)	° 1.2	0.2	° 1.5	0.3	° 1.4	0.1	* 2.2	0.7	° 1.1	0.0	° 0.7	0.0	1.0	0.1	1.0	0.2	* 0.6	0.5
class 2	HP0906	hypothetical protein	* 1.5	0.4	* 2.0	0.2	* 1.5	0.1	* 2.3	0.0	* 1.9	0.2	* 1.2	0.1	1.3	0.0	1.3	0.0	* 0.7	0.1
	HP0115	flagellin B (*flaB*)	* 3.4	0.5	* 5.5	2.7	* 2.6	0.4	* 5.6	2.3	* 3.4	1.6	* 2.5	0.3	* 2.8	0.8	* 2.4	0.2	* 0.2	0.1
	HP0114	hypothetical protein	2.9	1.4	2.4	0.4	1.1	0.0	2.2	0.4	1.5	0.4	1.5	0.1	1.4	0.3	1.2	0.4	0.6	0.3
	HP0295	flagellar hook associated protein 3/HAP3 (*flgL*)	* 2.2	0.8	3.2	0.8	5.8	0.7	72	74	8.4	1.4	5.0	4.9	5.6	1.3	6.3	1.6	0.4	0.1
	HP0870	flagellar hook protein (*flgE1*)	* 2.1	0.5	1.7	0.1	1.9	0.1	2.8	0.3	1.8	0.4	1.5	0.1	2.3	0.6	2.2	0.2	0.4	0.1
	HP0869	hydrogenase expression/formation protein (*hypA*)	* 2.0	0.3	1.6	0.1	* 1.7	0.0	* 2.8	0.4	* 2.5	0.2	* 1.7	0.0	* 1.4	0.1	* 1.8	0.3	0.4	0.2
	HP1120	hypothetical protein (operon with *flgK*)	* 8.7	3.3	* 5.2	1.5	* 3.5	1.2	111	127	* 15.9	14	2.1	0.5	* 8.0	3.7	2.0	0.0	* 0.4	0.2
	HP1119	flagellar hook associated protein 1/HAP1 (*flgK*)	* 1.9	0.4	1.7	0.1	2.1	0.1	8.2	6.1	2.4	0.6	2.2	0.6	1.8	0.2	1.5	0.0	0.3	0.0
	HP1076	hypothetical protein	* 5.6	1.4	* 2.9	1.1	* 1.9	0.3	12	8.6	* 2.6	0.5	1.6	0.3	* 2.0	0.8	1.2	0.0	* 0.4	0.2
	HP1154	hypothetical protein (operon with *murG*)	* 2.7	0.8	1.1	0.0	* 3.0	0.2	4.8	1.8	3.1	0.2	1.3	0.1	* 3.0	0.3	1.8	0.1	0.4	0.2
	HP1155	transferase, peptidoglycan synthesis (*mur*G)	* 1.7	0.4	1.7	0.2	* 3.5	0.2	4.2	1.7	2.8	0.6	2.0	0.5	* 3.3	1.2	2.2	0.3	0.5	0.3
	HP1233	putative flagellar muraminidase (*flgJ*)	* 2.2	0.7	2.4	0.5	* 1.7	0.2	3.9	2.3	* 1.6	0.3	* 1.4	0.3	* 1.3	0.1	1.3	0.0	* 0.2	0.0
proposed flagellar class 1 genes	HP0019	CheW/CheY hybrid chemotaxis protein (*cheV1*)	1.2	0.4	1.6	0.4	0.9	0.1	1.2	0.0	0.9	0.1	1.2	0.4	0.6	0.2	1.2	0.2	0.9	0.4
	HP0082	receptor protein of chemotaxis signalling system (*tlpC*)	1.0	0.1	1.0	0.0	0.9	0.0	1.0	0.1	1.1	0.1	1.0	0.1	0.9	0.1	0.9	0.0	1.2	0.2
	HP0099	methyl accepting chemotaxis protein (*tlpA*)	1.1	0.2	0.8	0.2	0.9	0.0	1.0	0.1	° 1.1	0.1	° 1.7	0.1	0.9	0.3	0.9	0.0	° 0.7	0.3
	HP0103	chemotaxis receptor (*tlpB*)	1.2	0.3	0.9	0.1	1.0	0.2	0.8	0.2	0.8	0.1	1.1	0.1	1.0	0.2	1.1	0.2	0.8	0.2
	HP0173	flagellar biogenesis regulatory protein (*fliR*)	1.0	0.2	0.9	0.0	0.9	0.1	1.0	0.1	0.9	0.1	0.8	0.1	0.9	0.1	1.4	0.2	1.1	0.0
	HP0244	histidine kinase specific for *flgR*	° 1.0	0.1	° 0.9	0.0	° 1.1	0.0	1.1	0.0	° 1.2	0.1	° 1.0	0.0	1.1	0.1	° 1.0	0.2	° 0.9	0.1
	HP0246	basal body P-ring protein (*flgI*)	1.1	0.2	1.2	0.1	1.1	0.0	1.0	0.1	1.0	0.1	1.0	0.3	1.0	0.1	1.2	0.2	1.0	0.0
	HP0325	basal body L-ring protein (*flgH*)	1.0	0.1	1.1	0.2	1.1	0.1	1.1	0.2	1.3	0.1	0.9	0.1	1.3	0.2	1.0	0.1	0.6	0.2
	HP0326	CMP-sugar synthetase (*neuA/flmD*)	1.1	0.3	1.0	0.1	1.1	0.1	0.9	0.0	1.6	0.1	0.8	0.1	1.3	0.0	1.2	0.0	1.0	0.2
	HP0327	(*flaG1/flmH*)	1.2	0.4	1.1	0.2	1.5	0.1	1.1	0.1	1.6	0.4	0.9	0.0	1.5	0.1	1.1	0.0	1.3	0.4
	HP0351	basal body M-ring protein (*fliF*)	1.0	0.1	1.3	0.1	0.8	0.0	1.0	0.1	1.4	0.3	0.8	0.1	1.0	0.2	0.8	0.0	0.9	0.1
	HP0352	flagellar motor switch protein (*fliH*)	1.0	0.2	1.0	0.2	1.2	0.1	0.9	0.2	1.0	0.1	1.0	0.2	1.2	0.2	1.1	0.1	0.9	0.0
	HP0391	chemotaxis adaptor protein (*cheW*)	1.3	0.5	0.8	0.0	1.0	0.1	1.2	0.1	1.1	0.2	1.4	0.2	0.7	0.2	1.1	0.0	0.9	0.0
	HP0392	receptor coupled chemotaxis histidine kinase (*cheA*)	1.1	0.4	° 1.1	0.0	0.9	0.1	1.0	0.2	° 1.0	0.1	1.2	0.1	0.9	0.1	1.1	0.2	° 1.0	0.3
	HP0393	hybrid CheW-CheY chemotaxis protein (*cheV3*)	1.0	0.3	1.0	0.0	1.1	0.1	1.1	0.1	1.0	0.0	1.2	0.2	0.8	0.1	1.2	0.3	1.0	0.3
	HP0584	flagellar motor switch protein (*fliN*)	0.7	0.3	0.9	0.2	1.0	0.1	0.9	0.2	1.1	0.1	1.1	0.1	1.2	0.3	1.1	0.2	1.1	0.3
	HP0599	chemotaxis receptor (*tlpB*)	N.D.		N.D.		0.9	0.1	1.4	0.0	1.0	0.0	1.6	0.3	0.8	0.0	1.0	0.0	0.6	0.3
	HP0616	hybrid cheW-CheY chemotaxis protein (*cheV2*)	1.1	0.3	0.9	0.1	1.2	0.1	0.9	0.2	1.2	0.2	0.9	0.0	1.2	0.2	0.9	0.1	1.0	0.2
	HP0684	N-terminal domain of export apparatus gene (*fliP'*)	1.2	0.4	1.0	0.0	1.0	0.1	1.2	0.0	1.0	0.0	1.0	0.0	0.7	0.1	0.7	0.0	1.1	0.3
	HP0685	second part of FliP (*fliP''*)	1.1	0.3	0.9	0.1	1.3	0.2	1.2	0.3	1.6	0.4	1.5	0.1	1.4	0.3	0.8	0.0	1.4	0.5
	HP0703	response regulator (*flgR/fleR*)	° 0.9	0.3	° 1.1	0.0	° 1.5	0.2	0.9	0.0	° 0.9	0.2	° 1.4	0.3	1.0	0.3	° 1.3	0.1	° 0.6	0.1
	HP0714	alternative sigma factor sigma54 (*rpoN*)	° 1.0	0.3	° 0.8	0.2	1.0	0.1	0.8	0.2	° 1.3	0.1	° 1.1	0.0	0.8	0.1	° 1.1	0.2	° 0.9	0.3
	HP0770	flagellar basal body protein involved in export (*flhB1*)	1.0	0.2	0.9	0.2	1.1	0.1	1.0	0.1	1.1	0.1	1.1	0.1	0.9	0.0	1.1	0.0	1.0	0.0
	HP0815	flagellar motor protein A (*motA*)	° 1.0	0.2	0.7	0.1	° 1.2	0.1	0.9	0.2	° 1.2	0.1	° 1.4	0.1	1.5	0.3	° 1.4	0.0	0.8	0.2
	HP0816	flagellar motor protein B (*motB*)	0.8	0.2	0.8	0.0	1.1	0.1	0.8	0.1	0.8	0.1	N.D.		1.4	0.4	1.3	0.2	0.7	0.1
	HP0840	flagella associated protein (*flaA1*)	1.0	0.4	0.9	0.2	0.9	0.1	0.8	0.1	0.8	0.2	0.8	0.0	1.4	0.5	0.9	0.0	1.2	0.2
	HP1041	flagellar basal body protein involved in export (*flhA*)	1.4	0.3	0.8	0.0	1.1	0.1	1.0	0.3	0.8	0.2	1.2	0.5	1.0	0.1	0.9	0.0	0.8	0.1
	HP1067	chemotaxis effector protein / response regulator (*cheY*)	° 1.0	0.2	0.9	0.1	° 0.9	0.1	1.0	0.1	° 1.0	0.1	° 1.0	0.4	0.9	0.1	° 0.7	0.2	° 0.9	0.0
	HP1092	flagellar basal-body rod protein (*flgG*)	1.1	0.2	0.8	0.1	1.1	0.1	1.0	0.2	1.1	0.1	1.2	0.2	1.2	0.0	1.0	0.0	0.9	0.1
	HP1286	putative FliZ protein (*fliZ*)	0.9	0.3	1.1	0.0	1.0	0.1	1.2	0.2	1.4	0.1	1.5	0.3	1.5	0.1	1.4	0.3	0.7	0.3
	HP1419	flagellar basal body protein involved in export (*fliQ*)	0.9	0.2	1.0	0.1	1.1	0.0	1.0	0.2	0.8	0.1	0.9	0.1	1.3	0.1	1.2	0.0	1.1	0.2
	HP1420	flagella-associated ATPase (*fliI*)	0.9	0.3	1.0	0.1	1.1	0.1	0.8	0.1	0.6	0.1	0.9	0.1	1.5	0.2	1.1	0.0	1.0	0.2
	HP1462	secreted protein involved in motility	1.0	0.2	1.1	0.0	1.1	0.0	1.0	0.1	1.0	0.2	0.9	0.1	1.0	0.0	1.2	0.1	1.0	0.0
	HP1575	homolog of FlhB protein (*flhB2*)	1.0	0.3	0.9	0.0	1.5	0.2	0.9	0.2	1.4	0.1	0.9	0.1	1.6	0.4	1.1	0.1	1.0	0.2
	HP1585	flagellar rod protein (*flgG*)	1.1	0.4	0.9	0.1	1.0	0.1	1.1	0.1	0.6	0.2	0.8	0.0	0.8	0.2	0.9	0.0	0.9	0.3
	HP0410	flagellar sheath associated protein paralog (*hpaA2*)	0.7	0.3	0.9	0.2	1.0	0.1	1.0	0.2	* 2.1	0.9	° 1.0	0.0	1.6	0.3	1.1	0.2	0.8	0.2
	HP0492	flagellar sheath associated protein paralog (*hpaA3*)	1.0	0.2	1.6	0.5	0.9	0.2	1.2	0.3	° 1.4	0.4	* 3.2	0.3	0.7	0.2	1.3	0.3	1.2	0.7
	HP0797	flagellar sheath associated protein (*hpaA*)	1.0	0.3	0.9	0.1	0.8	0.1	1.3	0.3	° 1.5	0.5	° 1.2	0.0	0.9	0.2	1.3	0.2	1.1	0.2

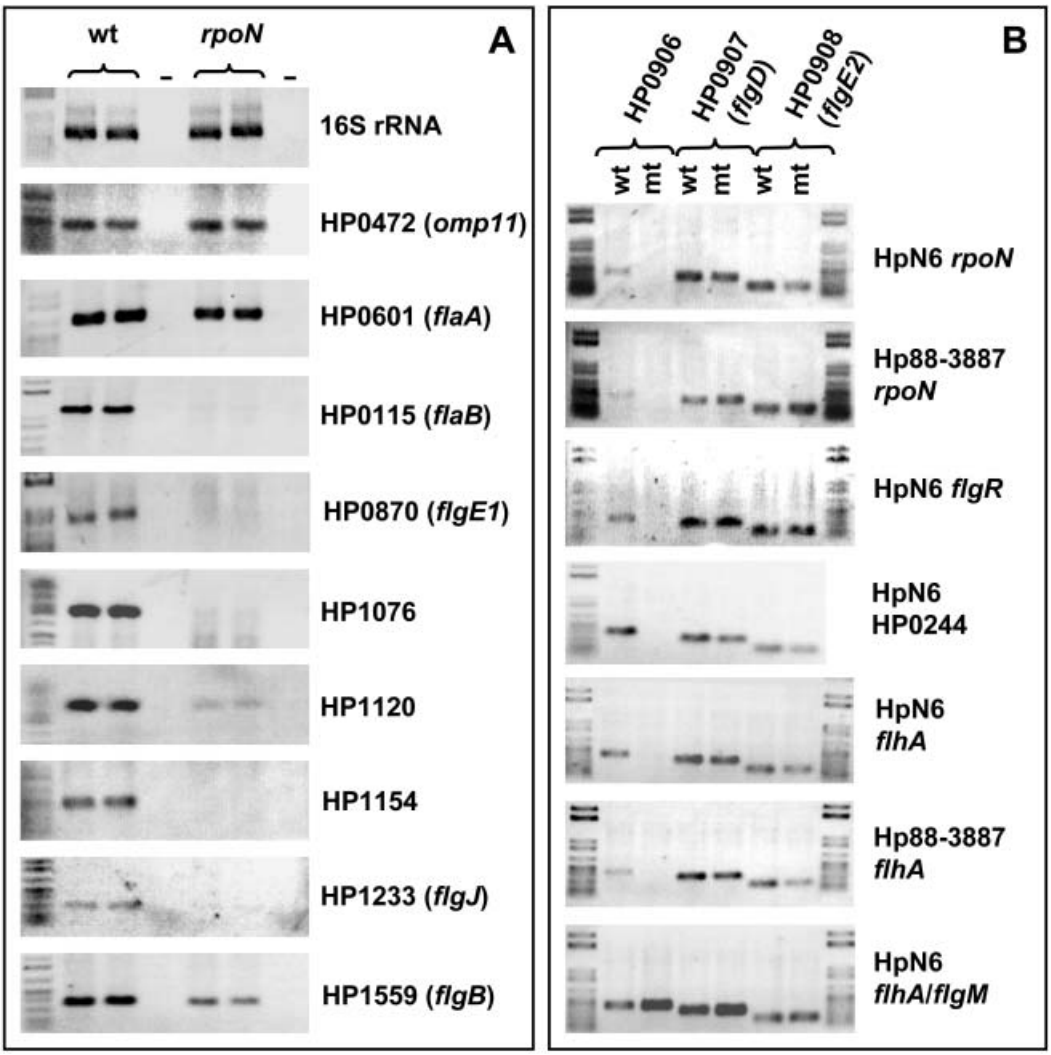


Fig. 1. A. Confirmation of transcriptional changes in selected known and novel RpoN-dependent genes by semi-quantitative RT-PCR. PCRs with two independent cDNA preparations derived from *H. pylori* N6 wild-type strain (wt) and *rpoN* mutant (*rpoN*) and negative controls (–) are shown as indicated above the lanes. Gene-specific products are indicated by ORF number and gene name. The 16S rRNA control and the σ^{28}-dependent *flaA* gene do not show any transcriptional changes in the *rpoN* mutant compared with the wild-type strain. All other genes are downregulated in the *rpoN* strain in concordance with the microarray data (Table 1). HP1559 is downregulated, but appears not to be completely dependent on RpoN.

B. Investigation of the partially σ^{54}-dependent HP906–908 gene cluster by semi-quantitative RT-PCR based on cDNA of two different *H. pylori* strains (HpN6 and 88-3887) and different mutants. PCR with primers specific for HP906, HP0907 and HP0908 was performed based on cDNA of wild-type (wt) and mutant (mt) strains as indicated.

Our present findings suggest that, in *H. pylori*, RpoN is almost exclusively devoted to class 2 (middle) genes of the flagellar system, which may explain the previous finding that RpoN is essential for *in vivo* colonization of the stomach (Kavermann *et al.*, 2003). This is in contrast to other bacteria, such as *C. crescentus*, in which RpoN controls early and middle flagellar genes and may provide a link to metabolism and the cell cycle (Anderson *et al.*, 1995; Buck *et al.*, 2000; Jones *et al.*, 2001; Muir and Gober, 2001; Prouty *et al.*, 2001; Laub *et al.*, 2002). Flagellar regulation and the RpoN regulon of *H. pylori* appear to be similar to *C. jejuni*, which also uses both σ^{54} and σ^{28} for the regulation of middle and late flagellar genes respectively (Jagannathan *et al.*, 2001). In Campylobacter, RpoN-dependent flagellar genes were reported to be under the additional control of FlgS, FlgR, as well as of FlhA and other basal body proteins (Hendrixson and DiRita, 2003), similarly to results obtained for *H. pylori* in the present study. No further detailed information on the *Campylobacter* RpoN regulon is available for comparison, except an *in silico* database that lists putative σ^{54} promoters (PromScan database; Studholme and Dixon, 2003; http://www.promscan. uklinux.net). This database indicates overlaps of the *C. jejuni* and *H. pylori* RpoN regulons.

Fig. 2. Alignment of promoter sequences of four known σ^{54}-dependent genes [HP0115 (*flaB*), HP0295 (*flgL*), HP0870 (*flgE1*) and HP1120] and six novel genes and operons in *H. pylori* that belong to the RpoN regulon. Numbers to the right of the sequences indicate the distance from the putative ATG start codon of the ORFs. The bottom lane shows the derived consensus sequence. The shading was done using the GENEDOC software (http://www.psc.edu/biomed/genedoc). Black boxes indicate 100% conserved residues in all sequences; grey boxes indicate nucleotides conserved in more than half the promoters.

The analysis of the RpoN regulon shows that, in *H. pylori*, in contrast to other bacteria, transcription of late flagellar genes (class 3; FliA dependent; Josenhans *et al*., 2002) is independent of the activity in this regulon. We observed wild-type levels of *flaA* transcription in the *rpoN* mutant, but no FlaA protein, which indicates a post-transcriptional but not a transcriptional feedback. The completed hook structure, encoded by the RpoN-dependent *flgE1*, is the physical gate for the secretion of late flagellar proteins and, in Enterobacteriaceae, provides one checkpoint for transcriptional feedback by allowing for secretion of the antisigma factor FlgM (Karlinsey *et al*., 2000). According to previous studies, the role of FlgM and its interaction with FliA are similar in *H. pylori* (Colland *et al*., 2001; Josenhans *et al*., 2002), but not the transcriptional feedback function of the hook (O'Toole *et al*., 1994).

RpoN accessory proteins, the NtrC-like activator FlgR/FleR and its cognate histidine kinase HP0244 control the same genes as RpoN

The transcription of σ^{54}-controlled genes requires the binding of additional transcriptional activators (Thöny and Hennecke, 1989). In *H. pylori*, the FleR orthologue FlgR (HP0703) was identified as an activator of RpoN (Spohn and Scarlato, 1999). Our finding (Table 1) that the *flgDE2* (HP0907–0908) and *flgBC* (HP1559–1558) genes, previously reported as FlgR dependent, were not or not completely downregulated in the *rpoN* mutants prompted us to do a genome-wide transcript profiling using isogenic *flgR* mutants of *H. pylori* strain N6 (early log phase, OD_{600} = 0.35). All genes dependent on RpoN were also detected as being dependent on FlgR using microarrays and RT-PCR (Table 1). Transcription of HP0906 was under the control of FlgR, while the downstream genes, *flgDE2* (HP0907/0908), were FlgR independent (Table 1; Fig. 1B), which confirmed the results obtained in the *rpoN* mutants. Two differences between the transcript profiles of *flgR* and *rpoN* mutants were observed: a gene cluster containing the *flhF/flhG* and *fliA* genes (HP1035 to HP1028) and the gene HP0367 appeared to be negatively regulated in *flgR* but not in *rpoN* mutants (Table 1). σ^{54}-like promoters were identified upstream of HP0367 and within the HP1035 gene cluster, upstream of HP1032 (Fig. 2), which, however, are probably not the only promoters. The transcription of genes in the operon HP0698 to HP0703 containing *flgR* and of other flagellar regulatory genes was not changed in the *flgR* mutant compared with the wild-type strain, which could be confirmed by RT-PCR (see Table 1). As the subsets of genes downregulated in both *rpoN* and *flgR* mutants were almost identical under the assay conditions used here, it is unlikely that alternative NtrC-like activators for RpoN exist in *H. pylori*, in contrast to other bacteria, such as *C. crescentus* and *Pseudomonas* sp., which both possess more than one RpoN activator.

One factor that could sense a metabolic input signal and contribute to σ^{54} activation to co-ordinate flagellar biosynthesis within the cell cycle is HP0244 (FlgS), an unusual NtrB-like histidine kinase lacking transmembrane domains, which has been shown specifically to phosphorylate its cognate response regulator and σ^{54} activator FlgR (Beier and Frank, 2000). HP0244 mutants are non-motile and do not produce flagellins (Beier and Frank, 2000). The comprehensive transcript profile of HP0244 mutants in early to mid-log growth (OD_{600} = 0.7) was determined using microarrays and proved to be similar to *rpoN* and *flgR* mutants (Table 1). The transcription of HP0366/HP0367 was negatively affected in the HP0244 as in *flgR* mutants. The HP0367 operon has a σ^{54}-like promoter (Fig. 2) and belongs to the regulons dependent on FlhA and FlhF described below. We observed an

increase in *flaA* transcript in the HP0244 mutant (microarray and RT-PCR), similar to previous findings in an *flgR* mutant (Spohn and Scarlato, 1999), indicating that an inverse regulation of late (*flaA*) and middle flagellar genes can occur. The mechanisms and function of this inverse regulation will have to be investigated further under different growth conditions.

The flagellar protochannel component FlhA affects transcription of flagellar class 2 and class 3 genes and non-flagellar genes, and works as a master regulator

We have shown previously that mutations in the flagellar protochannel gene *flhA* negatively affect transcription of selected genes of class 2 (*flaB*, *flgE1*) and class 3 (*flaA*) (Schmitz *et al*., 1997). Therefore, the next aim was to demonstrate whether FlhA might be involved in the global transcriptional regulation of all middle and late flagellar genes. Microarray hybridizations with *flhA* mutants in two different *H. pylori* strains (growth phase early to mid-log, OD_{600} = 0.7) showed that all previously identified members of flagellar gene classes 2 and 3 (class 2, see previous paragraphs; FliA-dependent class 3, see Josenhans *et al*., 2002) were downregulated in the *flhA* mutants (Table 1). In addition, a number of genes (e.g. the *flgM* operon; HP0751–HP0754) that could not be grouped into distinct classes by the analysis of *rpoN* and *fliA* mutants, and that we had suggested to belong to an intermediate group ('class 2 + 3'; Josenhans *et al*., 2002), were also downregulated in the *flhA* mutant (Table 1). This corroborated our previous hypothesis that they might be governed by both RpoN and FliA. Transcription of the flagellar antisigma factor gene *flgM* was not completely suppressed in *flhA* mutants, indicating that, similar to *flgM* regulation in *Pseudomonas*, *H. pylori flgM* may possess a third promoter, in addition to σ^{54} and σ^{28}. Affiliations to regulons other than flagellar classes 3 and 2 probably also exist for *flgBC* (HP1559/HP1558), which were not completely downregulated in the *rpoN*, *flgR*, HP0244 and *flhA* mutants (Figs 1 and 3A). The inactivation of *flhA* led to reduced transcription of additional genes, which might also belong to intermediate regulons (Fig. 4). These included three functionally annotated non-flagellar genes (HP0165/HP0166, HP0869) and three genes of unknown function (HP0488, HP0868 and HP1440). Regulation of these genes could be confirmed by RT-PCR. Conserved σ^{28}-like promoters could be identified upstream of HP0166, HP0488 and HP1440. None of the previously described target genes of the TCS HP0166/HP0165 (Dietz *et al*., 2002; Forsyth *et al*., 2002) were found to be regulated in the *flhA* mutants under the conditions used in this study. A recently suggested effect of *FlhA* on urease (McGee *et al*., 2002) might be provided indirectly by transcriptional changes in *hypA* (HP0869), which is involved in urease maturation (Mehta *et al*., 2003). Transcription of the known flagellar regulatory genes *fliA*, *rpoN*, *flgR*, as well as σ^{80}-dependent early flagellar genes was not altered in the *flhA* mutants (microarray and RT-PCR).

Although most of the transcriptional changes caused by the inactivation of *flhA* were the same in both strains, regulation of some genes, among them genes involved in energy metabolism, appeared to be strain specific (*Supplementary material*, Table S3). One example of strain-specific differential regulation in the *flhA* regulon was the flagellar sheath adhesin *hpaA* gene paralogues HP0410/jhp971 and HP0492/jhp444 (Jones *et al*., 1997; Alm *et al*., 1999). Only HP0410 was downregulated in the N6 *flhA* mutant, whereas only HP0492 was reduced in the strain 88-3887 *flhA* mutant. The respective second gene and the original *hpaA* gene (HP0797/jhp733; Jones *et al*., 1997), which showed high transcriptional activity, remained unaffected in both strains. RT-PCR confirmed the array results and demonstrated that, in addition, the transcript levels of HP0410 and HP0492 differed between both strains (*Supplementary material*, Fig. S2). The nucleotide sequences of the intergenic regions upstream of all three *hpaA* paralogues were determined and compared for the 26695, J99 and N6 strains. They proved to be different, but did not offer an immediate explanation for the differential transcriptional activity. The *hpaA* paralogues were not dependent on RpoN or FliA alone, as confirmed by RT-PCR (*Supplementary material*, Fig. S2).

FlhF is a second global regulator of class 2 and class 3 flagellar gene transcription in H. pylori

The SRP family protein FlhF (Dasgupta *et al*., 2000; Pandza *et al*., 2000) was a further promising candidate for a superimposed regulator of the flagellar system in *H. pylori*. We performed genome-wide transcript profiling of *flhF* mutants of two *H. pylori* strains (non-polar mutant *flhFm2*; OD_{600} of 0.7) using microarrays. *flhF* mutants showed a very similar transcriptional profile to the *flhA* mutants, with a strong decrease in both class 2 and class 3 flagellar gene transcripts (Table 1). One notable exception was that the putative operon HP1035 to HP1028, of which *flhF* is the first gene, was downregulated in the *flhF*, but not in the *flhA* mutants, which was confirmed by RT-PCR (Fig. 3). As one gene in the HP1035ff cluster (HP1032) encodes the regulator FliA, the transcriptional downregulation of FliA-dependent genes in *flhF* mutants might thus be mediated indirectly by a reduced expression of FliA (Fig. 3). Presumed non-flagellar genes that were found to be dependent on both FlhA and FlhF in both *H. pylori* strains included the TCS HP0165/166, HP0488, HP1440 and HP0869 (Table 1). In addition to its influence on tran-

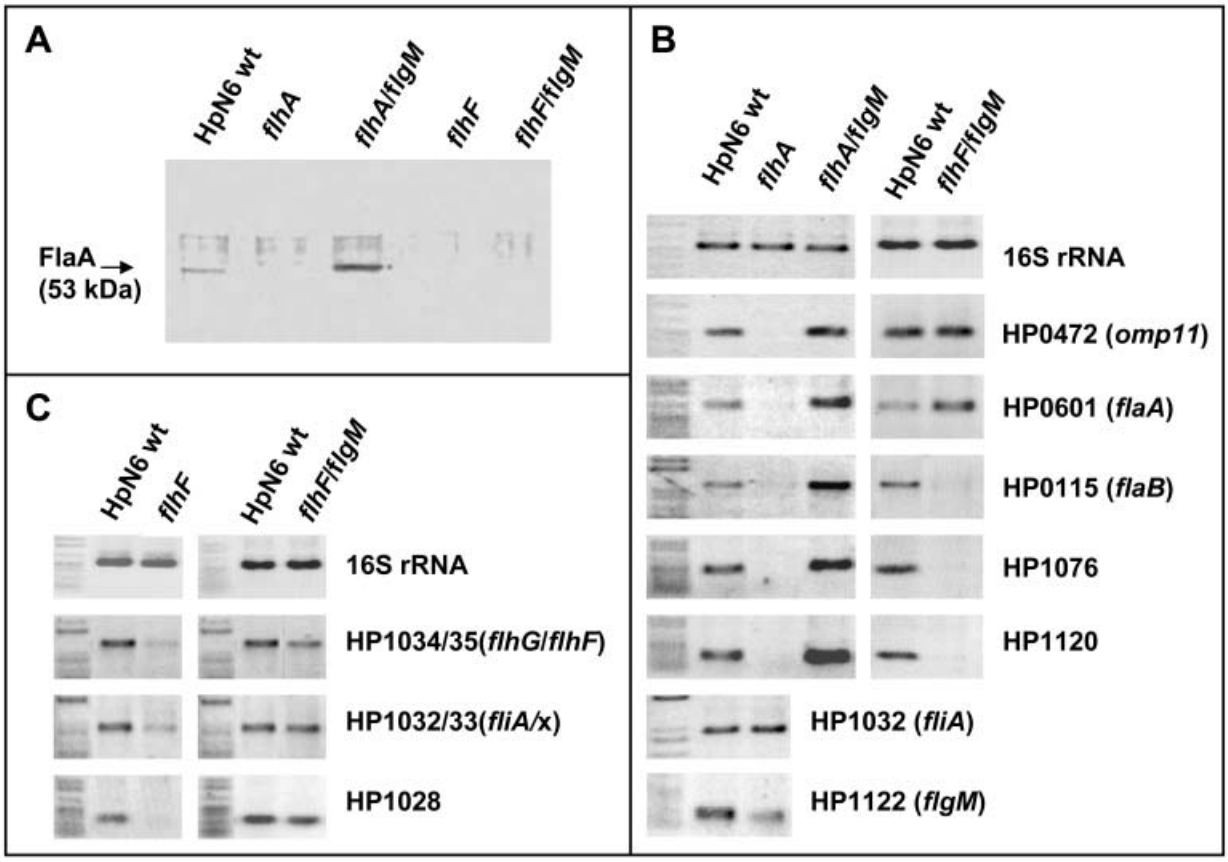
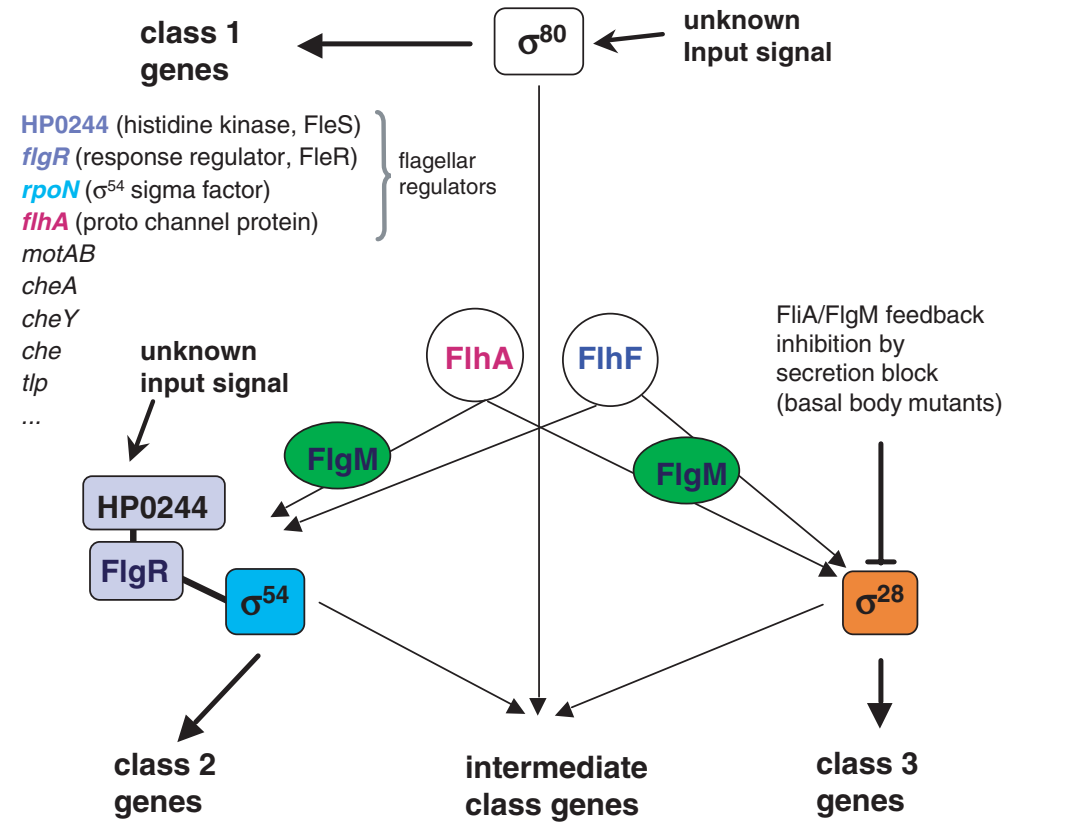


Fig. 3. Characterization of *H. pylori* (Hp) *flhA*, *flhF*, as well as *flhA/flgM* and *flhF/flhM* double mutants to reveal feedback mechanisms in the flagellar hierarchy.
A. Western blot was immunostained using an antiserum raised against recombinant FlaA. *flhA* and *flhF* mutants do not express detectable amounts of FlaA, as well as *flhF/flgM* double mutants. *flhA/flgM* double mutants express and accumulate FlaA in the cytoplasm, in contrast to the *H. pylori flhA* mutants.
B. Characterization of transcriptional feedback mechanisms in *flhA* and *flhF* mutants mediated by *flgM*. Semi-quantitative RT-PCR of two σ^{28}-dependent transcripts (*omp11*, *flaA*) and three σ^{54}-dependent genes (*flaB*, HP1076 and 1120). HpN6 *flhF* mutants (not shown) show the same pattern as HpN6 *flhA*. In both *flhF/flgM* and *flhA/flgM* double mutants, transcription of FliA/σ^{28}-dependent genes (class 3) is recovered. In contrast, σ^{54}-dependent transcripts (class 2) only reappear in *flhA*/f*lgM* double mutants (lane 3). Transcriptional levels of *flgM* and *fliA* in the *flhA* mutants are depicted at the bottom.
C. Semi-quantitative RT-PCR performed on transcripts from the *flhF* (HP1035) to HP1028 gene cluster. Note the recovery of transcripts of this operon in the *flhF*/*flgM* double mutants compared with the *flhF* single mutants.

scription of both class 2 and class 3 genes similar to FlhA, FlhF appeared to be involved in the regulation of its own operon. Transcription of *rpoN*, *flgR* and HP0244, which control the flagellar class 2 regulon, was not changed in the *flhF* mutant, but transcription of *fliA* and *flgM* was reduced in comparison with wild type (Table 1). Similar to the *flhA* mutant, some non-flagellar genes, several of them encoding proteins involved in energy metabolism, were strain-specifically regulated in the *flhF* mutant (*Supplementary material*, Table S3).

Feedback mechanisms that control downregulation of class 2 and 3 genes in flhA *and* flhF *mutants are dependent in part on FlgM*

We next wanted to clarify the mechanisms that mediate the negative feedback on flagellar transcription and biosynthesis, and on a number of non-flagellar genes, in the absence of the structural components of the flagellar secretion machinery, FlhA and FlhF. The inner membrane protein FlhA has no known DNA-binding motifs, and the detailed function of FlhA has not been investigated in any bacterium so far. We asked the question whether the antisigma factor FlgM, a secretion substrate of the flagellar apparatus in Enterobacteriaceae (Karlinsey *et al*., 2000), might be involved in the negative transcriptional effect observed in the *flhA* and *flhF* mutants of *H. pylori*, which are predicted to be secretion incompetent. *fliA* and *flgM* were transcribed in the *H. pylori flhA* and *flhF* single mutants (RT-PCR; Fig. 3B). To clarify the role of FlgM, *flhA/flgM* and *flhF/flgM* double mutants were constructed (nearly complete deletion of *flgM*). The double mutants did



middle flagellar structural genes (rod-hook; flagellin; sheath)

HP0115 *flaB* (flagellin)
HP0295 *flgK* (HAP)
HP0870 *flgE1* (hook)
HP0906
HP1119 *flgL* (HAP)
HP1120
HP1076
HP1154
HP1155 *murG*
HP1233 *flgJ*-like
HP0869 *hypA*

intermediate class: structural and regulatory

HP0751 *flaG'*
HP0752 *fliS* (chaperone)
HP0753 *fliD* (flagellar cap)
HP0754 *fliT* (chaperone regulator)
HP1122 *flgM* (anti-sigma factor)

flhF-*flhG*-HP1033b-*fliA*(σ28)-*fliM*-*fliY*-HP1029-HP1028

HP1558 *flgC* (rod)
HP1559 *flgB* (rod)
HP0907/0908 *flgD/E2* (/hook paralog)
HP0366/367 (regulatory?)
HP0165/166 (TCS)
HP0488
HP1440

late flagellar structure

HP0472 *omp11*
HP0601 *flaA* (flagellin)
HP1051
HP1052 *envA*

Fig. 4. Current model of regulation pathways in flagellar biosynthesis of *H. pylori*. Three different classes of flagellar genes are governed by the housekeeping sigma factor σ^{80} (class 1) and the alternative sigma factors σ^{54} (class 2) and σ^{28} (class 3). A number of intermediate genes controlled by more than one promoter are grouped in a separate category. Class 1 flagellar genes comprise, among others, most of the major regulatory genes of the flagellar system. The list of putative class 1 genes (see Table 1) is not complete, as these regulons have not been investigated in detail. Transcription of class 2 genes is governed by RpoN, assisted by the histidine kinase HP0244 (FlgS) and the response regulator FlgR. The class of RpoN-dependent genes could be extended by a number of novel genes indicated by HP numbers. FlhA and FlhF are both necessary for full transcription of flagellar classes 2 and 3 and the intermediate class. FlgM plays a role in the transcriptional block of class 2 and 3 genes in *flhA* mutants, but only in the feedback block of class 3 genes in *flhF* mutants. A common mechanism of feedback inhibition on class 3 might be mediated by a deficient basal body via FlgM.

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not express flagella and were non-motile like the single mutants. Further phenotypical characterization showed that *flhA/flgM* double mutants, unlike the *flhA* mutants, produced increased amounts of the class 3 flagellar protein FlaA (major flagellin), which accumulated in the cytoplasm (Fig. 3A), whereas FlaB (class 2) was not accumulated. This result suggests that translation of class 3 proteins might be directly coupled to the interaction between FlhA and FlgM. In contrast, *flhF/flgM* mutants did not produce detectable amounts of flagellins (Fig. 3A). Transcriptional regulation in *flhA/flgM* and *flhF/flgM* mutants was monitored by microarray and RT-PCR. Transcription of all class 2 and class 3 genes, which was almost completely switched off in the *flhA* single mutants, was upregulated in the *flhA/flgM* double mutants when compared with the wild-type strains (Table 1; Fig. 3B). Even those operons that were not under the complete control of FliA and/or RpoN (intermediate genes) showed increased transcription in the *flhA/flgM* mutants compared with the wild type (Table 1; Figs 3B and 4). Additional genes upregulated in the *flhA/flgM* double mutants included a large ribosomal protein gene cluster (*Supplementary material*, Table S3), the genes HP0366/HP0367 (reduced in HP0244, *flgR*, *flhF* and *flhA* mutants) and the HP0868 gene, presumably co-regulated with HP0869 (*hypA*). Results of microarray hybridization (not shown) and RT-PCR controls (Table 1; Fig. 3B) of the *flhF/flgM* mutant differed from the *flhA/flgM* mutant. Only class 3 transcripts (e.g. *flaA*) were upregulated in the *flhF/flgM* double mutants (Fig. 3), but class 2 transcripts (such as *flaB*) were not, indicating that negative feedback on class 3 genes is dependent on FlgM, as in the *flhA* mutants, but not the downregulation of class 2 genes. Transcription of the HP1035 operon, which was reduced in the *flhF* single mutants, increased again to wild-type levels in the *flhF/flgM* double mutant (RT-PCR result, Fig. 3C). This suggests that the downregulation of the transcripts downstream of *flhF* (HP1034 to HP1028) in the *flhF* single mutants might result from a defect in flagellar secretion, mediated by FlgM (Fig. 3B). Thus, FliA is probably involved in the transcription of these genes, which is supported by the detection of a σ^{28} consensus promoter (short 14-n spacing) 46 bp upstream of the HP1035 start codon.

Mutagenesis of various other flagellar basal body genes (*fliI*, *fliQ*, *flhB*) of *H. pylori* was reported to lead to a reduction in class 3 gene (*flaA*) transcription as well (Allan *et al*., 2000). The current concept, established in the Enterobacteriaceae, is that, when the flagellar secretion machinery is inactive or the flagellar hook is absent, intracellular accumulation of the secretion substrate FlgM inhibits the transcription of class 3 genes by inactivating FliA (Chilcott and Hughes, 2000; Karlinsey *et al*., 2000). A similar universal mechanism for transcriptional feedback regulation of class 3 genes in *H. pylori*, but occurring before hook formation, is supported by our analysis of *flhA*/*flgM* and *flhF/flgM* mutants (Fig. 4). However, as *Helicobacter* species possess a flagellar sheath (Geis *et al*., 1993), it seems unlikely that FlgM is secreted such as in Enterobacteriaceae. Alternative methods of FlgM inactivation are currently under investigation.

By inactivating either *flhA* or *flhF* genes, negative transcriptional regulation was also exerted on class 2 flagellar genes in *H. pylori*. The transcriptional feedback inhibition on class 2 genes in *flhF* mutants was not dependent on FlgM, as it was not relieved in *flhF/flgM* double mutants (Fig. 3), and its mechanism remains to be elucidated. In contrast, in *flhA*/*flgM* double mutants, an increase in class 2 transcripts, in the absence of detectable accumulation of class 2 proteins, was found in comparison with the wild type and the *flhA* single mutant (Table 1; Fig. 3). This implies that FlgM plays a prominent role in transcriptional feedback inhibition of class 2 flagellar genes in the absence of FlhA in *H. pylori*. Transcription of the *rpoN*, *flgR* and HP0244 genes was not altered in *flhA* and *flhA/flgM* mutants as confirmed by RT-PCR. Therefore, downregulation of class 2 genes in *flhA* mutants is probably not mediated by RpoN or the flagellar TCS. In *Salmonella*, *fliT* and *fliZ* are involved in FlgM-associated inhibition and enhancement, respectively, of transcription from class 2 promoters (Kutsukake *et al*., 1999). By *in silico* analysis, we have now annotated two novel flagellar genes in *H. pylori*, HP0754 (*fliT*) and HP1286 (*fliZ*), both of which might be involved in the negative feedback regulation on class 2 genes in the absence of FlhA (see *Supplementary material*, alignments 3 and 4).

Conclusions

The flagellar regulatory network of *H. pylori* is fundamentally different from other bacteria, despite many similarities (Fig. 4). Three main differences from other bacteria emerged. First, *H. pylori* has no transcriptional master regulator similar to enterobacterial FlhCD, as flagellar biosynthesis, motility and chemotaxis are housekeeping functions and essential *in vivo*. Secondly, in *H. pylori*, genes associated with the flagellar motor (*mot*) and chemotaxis (*che*) are not co-regulated with middle and late flagellar genes (Josenhans *et al*., 2002; present study), unlike in other bacteria, where they are mostly co-regulated with class 3 genes (Eisenbach, 1996). As chemotaxis and motor proteins are indispensable for *H. pylori in vivo* (Foynes *et al*., 2000; Ottemann and Lowenthal, 2002; Kavermann *et al*., 2003), their constitutive expression, partly in operons with housekeeping genes (Beier *et al*., 1997), may be essential. Thirdly, FlhA and FlhF, components of the flagellar basal body, are both required for the transcription of all flagellar genes of

classes 2 and 3 in *H. pylori* and functionally resemble master regulators. Both were also found to control intermediate genes (Fig. 4), which participate in at least two different regulons, and non-flagellar genes. FlhA was different from FlhF in at least two functional aspects: (i) in addition to transcription, translation of class 3 flagellar proteins (FlaA) was also dependent on the interplay between FlhA and FlgM; (ii) FlhA, but not FlhF, cooperates with the antisigma factor FlgM to mediate a feedback regulation of the RpoN-controlled class 2 flagellar genes. FlhA might be a true positive regulator of flagellar genes, as overexpression led to a hypermotile phenotype (own unpublished data). An unambiguous connection of flagellar biosynthesis to the cell cycle, metabolic functions or virulence, which is very obvious in other bacteria (Enterobacteriaceae: Pruss *et al*., 2001; *Caulobacter*: Laub *et al*., 2002) could not be demonstrated under the precise conditions chosen for the present study and will have to be investigated further under different growth conditions. One missing link might be provided by a growth cycle-dependent co-regulation of housekeeping and flagellar genes.

Experimental procedures

Plasmids, oligonucleotide primers and DNA methods

Plasmids used in this work are described below and in *Supplementary material*, Table S1. Sequences of oligonucleotides used for cloning, mutagenesis and RT-PCR are summarized in *Supplementary material*, Table S2. DNA techniques were performed according to standard methods.

Bacterial strains and culture conditions

Helicobacter pylori N6 and 88-3887 (motile derivative of strain 26695; Josenhans *et al*., 2000) were used in the experiments. Bacteria were grown either on blood agar plates or in brain–heart infusion (BHI) broth supplemented with 10% heat-inactivated horse serum and antibiotics as required (Josenhans *et al*., 2002). *Escherichia coli* strains DH5 and MC1061 were used for the cloning experiments. They were grown in Luria–Bertani (LB) medium containing kanamycin (20 mg l^{-1}) or ampicillin (50 mg l^{-1}).

Protein methods

SDS-PAGE, Western blotting, determination of protein concentration and preparations of flagellar components were done as described previously (Josenhans *et al*., 2000). Immunolabelling on Western blots was performed using antibodies raised against native *H. pylori* flagellar components and against recombinantly expressed flagellins (Josenhans *et al*., 2000).

RNA preparation and RT-PCR

RNA was prepared from *H. pylori* bacteria harvested from liquid cultures in different growth phases using the Qiagen RNeasy kit with slight modifications as described elsewhere (Josenhans *et al*., 2002). Semi-quantitative RT-PCRs were done on 2 μg of DNase I-treated RNA samples. Reverse transcription was performed using a random hexamer primer mix and Superscript II TM reverse transcriptase (Invitrogen) at 42°C for 2 h. The cDNA was adjusted to 50 μl with ddH_2O and amplified in different PCRs (including negative controls) on 2.5 μl of cDNA sample with primers specific for the corresponding genes (primers: *Supplementary material*, Table S2). Validation of microarray results by RT-PCR was performed on at least two independent biological experiments.

Construction of H. pylori rpoN*,* flgR*,* flhF*,* HP0244*,* flhA/flgM *and* flhF/flgM *mutants*

Knock-out mutants, combined with the insertion of the *aphA3′*-III kanamycin resistance cassette (Labigne-Roussel *et al*., 1988), in direct orientation into the genes *rpoN*, *flgR*, HP0244, *flhA* and *flhF*, were constructed in two *H. pylori* strains, if not indicated otherwise, N6 (Ferrero *et al*., 1992) and 88-3887 (the motile variant of the sequenced strain KE 26695 (Josenhans *et al*., 2000). *flhA* mutants in both strains were constructed using plasmid pSUS42 as described previously (Schmitz *et al*., 1997). The plasmids for generating each of the novel mutants were constructed as follows (primer sequences and fragment sizes in *Supplementary material*, Table S2; in most cases, small deletions were created in the genes).

RpoN mutant. A PCR fragment including the complete gene was amplified from 26695 DNA using primers OLHPRpoN_1s and OLHPRpoN_2s, cut, ligated into plasmid pILL570 and inverse amplified (primers OLHPRpoN_3s and _4s). The unique *Eco*RI site within the insert was first deleted by digestion, blunt-ending and religation. Subsequently, the *aphA3′*-III kanamycin cassette (1.4 kb) from pILL600 was ligated into the *Eco*RI site created by inverse amplification. The resulting plasmid pSUS1412 contains *aphA3′*-III in the same transcriptional orientation as *rpoN*.

FlgR. A fragment including the complete *flgR* gene of 26695 was amplified using oligonucleotides OLHPFlgR_1s and OLHPFlgR_2s (*Bam*HI sites) and cloned into pUC18/*Bam*HI. The *km* cassette was ligated into the resulting plasmid pSUS1802 using a unique *Eco*RI site created by inverse PCR (OLHPFlgR_3s and _4s).

HP0244.The HP0244 gene of 26695 was amplified using primers OLHP244_1s and OLHP244_2s (*Bgl*II sites) and cloned into pUC18 linearized with *Bam*HI. The *km* cassette was isolated after *Bam*HI digest and inserted after inverse amplification of the resulting plasmid pCJ316 with OLHP244_3s and OLHP244_4s (*Bgl*II sites).

FlhFm1 mutant. The *flhF* gene from 26695 was amplified using primers OLHPFlhF_10s and OLHPFlhF_2s, cut with *Bgl*II and ligated into pUC18/*Bam*HI to give plasmid pCJ94. An inverse PCR using primers OLHPFlhF_11s and _12s was

then performed on pCJ94, purified, cut and ligated with the *aphA3′*-III cassette, resulting in plasmid pCJ301. Because, initially, the comprehensive transcript profiling of *flhF::aphA3′*-III knock-out mutants showed downregulation of transcripts downstream of *flhF*, it was suspected that the mutant might be polar. For this reason, a second *flhF* insertion mutant, *flhFm2*, was constructed, using a more sophisticated non-polar *km* cassette (Menard *et al*., 1993). Upon correct insertion, this cassette leaves downstream transcription completely unaffected and reinitiates translation of the downstream part of the protein-encoding sequence at an ATG start codon in the 3′ end of the cassette. For the insertion of the apolar kanamycin cassette from pUC18k2 (Menard *et al*., 1993), pCJ94 containing the *flhF* gene was again inverse amplified using primers OLHPFlhF_11x and OLHPFlhF_12x, both containing *Xho*I sites. The apolar kanamycin cassette was amplified using primers OLpUC18k2_1x and OLpUC18k2_2x (*Xho*I sites), *Xho*I digested and ligated with the inverse PCR product of pCJ94 to create an in frame construct of the ATG start codon within the 3′ end of the cassette with the downstream part of *flhF* (resulting plasmid pCJ312). In both *flhF* constructs, an ≈300 bp fragment within the *flhF* coding region was deleted. The second *flhF* mutant (*flhFm2*) showed a very similar phenotype and transcript profile to the first mutant. The results obtained for the *flhF/flgM* double mutants, in which transcription of the whole *flhF* operon was reconstituted (Fig. 3), offers evidence that the *flhF* mutants had no polar effect on downstream genes.

Plasmids pSUS1412 (*rpoN::aphA3′*-III), pSUS1802 (*flgR::aphA3′*-III), pCJ301 (*flhF::aphA3′*-III), pCJ319 (HP0244*::aphA3′*-III) and pCJ312 (*flhFm2*) were transformed into both *H. pylori* strains by natural transformation, yielding kanamycin-resistant colonies. The correct double cross-over event in the mutants was verified using PCR and different primer combinations in the *aphA3′*-III gene and in the mutagenized genes. The insertion of a chloramphenicol (Cm) cassette from *Campylobacter coli* into the *flgM* gene for the construction of *flhA/flgM* and *flhF/FlgM* double mutants was performed by ligation of an inverse PCR product (using OLHPFM_3s and OLHPFM_4s, each containing *Bam*HI sites) from plasmid pCJ100 (Josenhans *et al*., 2002), with the Cm cassette, amplified and digested using primers OLcat1s and OLcat2s (each with a *Bam*HI site). The resulting plasmid, pCJ306, from which almost the complete *flgM* gene was deleted, thereby inserting the Cm cassette in the same transcriptional orientation as the *flgM* start codon, was transformed into the *H. pylori* N6 *flhA*$^{-}$ and *flhFm2* mutant strains. Double-resistant mutant colonies were isolated and characterized by PCR to confirm the correct allelic exchange events.

Complementation of the H. pylori flhA *mutant*

The complete *H. pylori flhA* gene (HP1041) was amplified from genomic DNA of strain 85P by PCR using primers OLHPFlbA_30s (*Bam*HI site) and OLHPFlbA_31s (*Kpn*I site), including the 5′ non-coding region that contains the putative partially conserved σ^{80} promoter. The 2.5 kb PCR product was digested *Bam*HI–*Kpn*I and cloned into *H. pylori*–*E. coli* shuttle plasmid pHel2 (selective marker is chloramphenicol resistance; Heuermann and Haas, 1998), which was cut using the same pair of enzymes. The resulting plasmid, pSUS76, was introduced into *H. pylori* strain N6 *flhA* (kanamycin resistant) by natural transformation. Double-resistant colonies (kanamycin and chloramphenicol) were selected, and several clones were characterized by plasmid reisolation and restriction analysis. Subsequently, one correct complementation clone was tested for motility, flagellar morphology and biosynthesis, and for the production of flagellar proteins.

Microarray techniques

Development and validation of H. pylori whole-genome microarray based on PCR products. H. pylori whole-genome PCR product arrays are based on the two sequenced genomes of *H. pylori* 26695 (Tomb *et al*., 1997) and J99 (Alm *et al*., 1999). Oligonucleotides specific for 1655 shared or specific genes of the two strains were generated using the PRIMEARRAY oligonucleotide design software (Raddatz *et al*., 2001). The resulting PCR products covered preferentially the full length of any ORF in both genomes. Universal sequences (primer 1, 5′-GCATGCATCTTGCT CTTCCATG-3′; primer 2, 5′-CGTAGCTCAGGTGCTCTTCG TTA-3′) were added to all primers, so that, in a first round of amplifications, gene-specific PCR products with universal flanking sequences were generated. The PCR products were used as a template for the next rounds of amplification in a PCR with primers complementary to the universal flanking sequences (see above) and containing 5′ amino linkers. Multiple rounds of the universal PCR (conditions available on request) were performed for each template to gain a sufficient amount of DNA for spotting. Amplifications were performed using a pipetting robot (RoboAmp4200P; MWG-Biotech) for reproducibility and in 96-well plates with individually sealed wells to avoid cross-contamination. Quality controls were accomplished by agarose gel electrophoresis of each PCR product. Missing or wrong products were manually reamplified up to a terminal coverage of 96.1% of the *H. pylori* genome (including the shared genes contained in both genomes and the strain-specific genes identified in both complete genomes). The PCR products were adjusted to a concentration of ≈100 ng μl^{-1} in spotting buffer 2 (Quantifoil) and spotted on epoxy-coated glass slides (Quantifoil) in duplicate, using the pin and ring system of an Affymetrix GMS 417™ four-pin microarray spotter. DNA was covalently bound to the slides by heating to 60°C for 1 h. The list of drop-outs and genes not included in the array are available on request. Slides were validated by using cDNA isolated from the *fliA* and *flgM* mutants in competitive hybridization experiments against wild-type cDNA, as described previously (Josenhans *et al*., 2002), and gave the same results. In cases of expected cross-hybridization with other genes for the PCR product array, as a result of high similarities of nucleotide sequences of genes, control experiments were performed with oligonucleotide arrays (MWG *H. pylori* array™) and gene-specific RT-PCR.

Reverse transcription and labelling for microarray analysis. Reverse transcription and fluorescent labelling reactions were performed using 20 μg of RNA for control and experi-

ment as described elsewhere (Josenhans *et al*., 2002). For most mutants (except *flhFm2* and *flgR*), RNA obtained from at least two different biological experiments was used and, for every experiment, a reverse labelling (flip dye) experiment was performed to minimize bias resulting from differential dye bleaching or incorporation of the two Cy dyes during the RT reaction. For most of the mutants, except *flgR* mutants, at least four microarray hybridizations were performed, as indicated in Table 1.

Microarray hybridization and scanning. Competitive microarray hybridizations using *H. pylori* oligonucleotide microarrays (MWG-Biotech) were performed as described previously (Josenhans *et al*., 2002). PCR product microarrays were pretreated according to the slide supplier's instructions (Quantifoil). Cy5/Cy3-labelled cDNAs were purified, dried and resuspended in 30 µl of hybridization buffer (50% formamide, 6× SSC, 0.5% SDS, 50 mM $NaPO_4$, pH 8.0, 5× Denhardt's solution) and denatured for 3 min. Hybridizations were carried out at 50°C for 16 h. After successive washes (10 min each) in 2× SSC + 0.2% SDS, 2× SSC, 0.2× SSC and 0.1× SSC, slides were dried by centrifugation at 1000 *g* for 3 min and scanned immediately. Hybridized slides were scanned using a 418 Affymetrix confocal laser scanner at six different laser intensities for each dye (Cy3, 80% laser power; Cy5, 100% laser power).

Bioinformatic analyses

Bioinformatic analyses on the slide hybridization results of each single experiment were performed as described previously, using IMAGENE™ GENESIGHT software, version 5.5, MAVI 2.3 (MWG), containing a Lowess normalization algorithm and taking into account the saturation levels of hybridized spots at six different scanning intensities, as well as EXPRESSIONIST™ (Genedata) version 3.1.0 for cluster analysis (Josenhans *et al*., 2002). The total number of microarray hybridizations performed per mutant is indicated in Table 1 (*n*-value). An *n*-value >2 indicates that RNA from at least two different biological experiments was used per mutant. Mean values and standard deviations of gene expression ratios based on two spot replicates on each microarray and *n* different hybridization experiments per mutant were calculated in Microsoft EXCEL 2000.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4006/mmi4006sm.htm

Fig. S1. Genome overview of *H. pylori* 26695 with genes relevant for flagellar function.

Fig. S2. Transcription of *H. pylori hpaA* paralogues (HP0797, HP0410, HP0492) in different *H. pylori* mutants of the flagellar regulons; RT-PCR results.

Table S1. Plasmids used in this study.

Table S2. Oligonucleotide primers used for cloning and RT-PCR.

Table S3. Complete microarray results (transcript ratios).

Alignments 1–4. Alignments were generated by CLUSTAL W and shaded using the GENEDOC software (http://www.psc.edu/biomed/genedoc).

Note added in proof

Since the final revision of this manuscript, a microarray study of the flagellar hierarchy in *P. aeruginosa* was published, which provides a rich source for comparative analyses with the *H. pylori* system (Dasgupta, N., Wolfgang, M. C. Goodman, A. L., Arora, S. K., Jyot, J., Lory, S., and Ramphal, R. (2003) A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **50:** 809–824.

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