Functional characterization of FlgM in the regulation of flagellar synthesis and motility in *Yersinia pseudotuberculosis*

Lisha Ding,^{1,3†} Yao Wang,^{1†} Yangbo Hu,^{1,3} Steve Atkinson,² Paul Williams² and Shiyun Chen¹

Correspondence
Shiyun Chen
sychen@wh.iov.cn

¹Wuhan Institute of Virology, The Chinese Academy of Sciences, Wuhan 430071, China

²Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2UH, UK

³Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

We describe here the functional characterization of the *flgM* gene in *Yersinia pseudotuberculosis*. Direct interaction of FlgM with the alternative sigma factor σ^{28} (FliA) was first confirmed. A conserved region in the C-terminus of FlgM was found which included the σ^{28} binding domain. By site-directed mutagenesis, bacterial two-hybrid analysis and Western blotting, the primary FlgM binding sites with σ^{28} were shown to be Ile85, Ala86 and Leu89. A role for FlgM in swimming motility was demonstrated by inactivation of *flgM* and subsequent complementation *in trans*. Transcriptional fusion analyses showed differential gene expression of *flhDC*, *fliA*, *flgM* and *fliC* in the *fliA* and *flgM* mutants compared with the wild-type. *flhDC* expression was not influenced by σ^{28} or FlgM while *fliA* expression was abolished in the *fliA* mutant and considerably reduced in the *flgM* mutant when compared to the wild-type, indicating that both FliA and FlgM can activate *fliA* transcription. Conversely, *flgM* transcription was higher in the *fliA* mutant when compared to the wild-type, suggesting that *flgM* transcription was repressed by σ^{28} . Interestingly, *fliC* expression was markedly increased in the *flgM* mutant, suggesting a negative regulatory role for FlgM in *fliC* expression. The transcription of other σ -dependent genes (*cheW*, *flgD*, *flaA*, *csrA* and *fliZ*) was also examined in *fliA* and *flgM* mutant backgrounds and this revealed that other σ -factors apart from σ^{28} may be involved in flagellar biogenesis in *Y. pseudotuberculosis*. Taking together the motility phenotypes and effects of *flgM* mutation on the regulation of these key motility genes, we propose that the mechanisms regulating flagellar biogenesis in *Y. pseudotuberculosis* may differ from those described for other bacteria.

Received 1 December 2008
Revised 24 February 2009
Accepted 26 February 2009

INTRODUCTION

Flagella-dependent motility is advantageous for bacteria since it allows cells to seek and acquire nutrients and escape unfavourable environmental conditions (Blair, 1995; Wadhams & Armitage, 2004). Flagella are also involved in many important bacterial functions such as adhesion, invasion, colonization and biofilm formation and thereby contribute to virulence (Josenhans & Suerbaum, 2002; Kirov, 2003; Lee *et al.*, 2001; Wood *et al.*, 2006). Flagella also induce innate immune responses in mammalian hosts via Toll-like receptors and are capable of activating defence

mechanisms (Ramos *et al.*, 2004). In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), flagellar gene regulation and assembly have been extensively characterized with the coordinated regulation and expression of over 60 genes arranged into three classes which are expressed in a transcriptional hierarchy (Aldridge & Hughes, 2002; Chilcott & Hughes, 2000; Kutsukake *et al.*, 1990). Once expressed, FlhDC (the master operon of class I) forms a regulatory tetramer required for transcription of class II genes (Givaudan & Lanois, 2000). These class II genes encode the flagellar type III secretion apparatus, the hook-basal body (HBB) structure and σ^{28} (FliA), which are required for transcription of class III genes, including the motor, chemotaxis and flagellin or filament genes (Aldridge *et al.*, 2006; Kutsukake *et al.*, 1990; Liu & Matsumura, 1994). FliA functions as an alternative sigma factor and may also play a role in regulating the initiation of transcription of *flhDC* in conjunction with

†These authors contributed equally to this work.

Abbreviations: FS, flagellar system; HBB, hook-basal body; RNAP, RNA polymerase.

A supplementary table of primers is available with the online version of this paper.

QscB (quorum sensing regulators B and C) in *E. coli* (Clarke & Sperandio, 2005). Furthermore, FliA and FliZ are both expressed from the same overlapping promoters of class II and class III genes at the *fliAZY* locus (Saini *et al.*, 2008).

flgM encodes a negative regulator of flagellin synthesis in *S. typhimurium* (Gillen & Hughes, 1991a) and is expressed from both class II and class III promoters (Gillen & Hughes, 1993). FlgM is produced in the cytoplasm (Karlinsey *et al.*, 2000) and binds to σ^{28} to prevent its association with RNA polymerase (RNAP), thereby inhibiting flagellin gene transcription (Lee *et al.*, 2001; Ohnishi *et al.*, 1992). Once the HBB is completed, FlgM is secreted from the cells, releasing σ^{28} to activate class III genes, and acts as a signal to indicate that HBB construction is complete. This regulatory checkpoint is thought to prevent the production of class III genes until they can be assembled into the flagellar structure (Hughes *et al.*, 1993). Repression of flagellin gene expression by FlgM is observed only in strains that contain mutations in the basal body, switch or hook flagellar genes (Gillen & Hughes, 1991b). A defect in *flgM* resulted in virulence attenuation of *S. typhimurium* and this phenotype was reversed by a mutation in *fliA* (Schmitt *et al.*, 1994), whereas virulence was attenuated in both *fliA* and *flgM* mutants in uropathogenic *E. coli*, revealing a clear link between virulence and regulation of flagellar synthesis (Richardson, 1991).

Genetic and biochemical studies of σ^{28} /FlgM interactions have contributed to the understanding of flagellar regulation. FlgM binds to σ^{28} in a multipartite manner to inhibit σ^{28} activity by destabilizing the σ^{28} -RNAP holoenzyme complex (Chadsey *et al.*, 1998; Chadsey & Hughes, 2001). An increased amount of flagellin in the *flgM* mutant of *Pseudomonas aeruginosa* strain PAK suggested that FlgM negatively regulates transcription of *fliC* by inhibiting the activity of σ^{28} (Frisk *et al.*, 2002). FlgM homologues have also been identified and characterized in the Gram-negative polar flagellate *Helicobacter pylori* (Colland *et al.*, 2001; Josenhans *et al.*, 2002). These studies demonstrated that the FlgM homologues in these bacteria physically interact with σ^{28} and inhibit σ^{28} -dependent transcription. Frameshift mutation analysis suggested that the last 21 amino acids in FlgM were important for the inhibition of σ^{28} activity in *S. typhimurium* (Karlinsey & Hughes, 2006). The crystal structure of the σ^{28} /FlgM complex from *Aquifex aeolicus* has been characterized (Sorenson *et al.*, 2004) and the crystals between σ^{28} and the C-terminal σ^{28} -binding region of FlgM have also been obtained in *E. coli* (Okada *et al.*, 2007).

Little information is available on the role of FlgM in *Yersinia pseudotuberculosis*, a mammalian enteropathogen that causes a self-limiting gastroenteritis in humans. Genome sequence analyses have revealed that yersiniae possess genes homologous to all of the known flagellar genes in *Salmonella* and *E. coli*. The production of flagella

in *Y. pseudotuberculosis* provides a means for motility and a type III secretion system for the export of flagellar and also non-flagellar proteins into the external environment (Young *et al.*, 1999). *Y. pseudotuberculosis* contains two sets of unlinked flagellar genes known as flagellar system 1 (FS1) and flagellar system 2 (FS2). The contribution of FS1 to virulence has been documented (Carniel & Hinnebusch, 2004), but the functions and regulation of the genes constituting FS2 have not been investigated in detail. Both FS1 and FS2 carry copies of *fliA* whereas it is only FS1 which carries a single copy of *flhD* and *flhC*. The biological significance of these observations is not well understood.

In the present study the key amino acids responsible for the interaction between FlgM and σ^{28} in *Y. pseudotuberculosis* strain YpIII pIBI were confirmed. *fliA* and *flgM* isogenic mutants were then constructed to characterize the roles of σ^{28} and FlgM in transcription of the flagellar promoter. Transcriptional fusions to key motility genes (*flhDC*, *fliA*, *flgM*, *fliC* and other σ -dependent genes (*flgD*, *cheW*, *csrA*, *flaA* and *fliZ*) showed differential gene expression in the *fliA* and *flgM* mutants compared with wild-type and complemented strains.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All the mutants and promoter fusions were constructed in flagella-associated genes located in flagellar system 1 (FS1). *Y. pseudotuberculosis* strains were cultured in Yersinia Luria-Bertani (YLB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with appropriate antibiotics where required. *E. coli* strains were grown in Luria-Bertani (LB) broth with appropriate antibiotics. Yersinia selective agar (Oxoid) was used for the screening of mutant strains. Antibiotics were added at the following concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$; kanamycin, 50 $\mu\text{g ml}^{-1}$; tetracycline, 10 $\mu\text{g ml}^{-1}$; chloramphenicol, 30 $\mu\text{g ml}^{-1}$.

Segment and site-directed mutagenesis. Segment mutagenesis was performed by overlapping extension PCR. The primer sets to amplify point mutated genes are named from $M_{\Delta E64T68}F+R$ to $M_{L90}F+R$ (primers are listed in Supplementary Table S1, available with the online version of this paper). Two fragments were obtained by PCR and were used as templates for another round of PCR using primers M-25-F, which generated a *Pst*I site, and M-25-R, which generated a *Kpn*I site, to obtain the mutated genes. Fig. 2(c) shows how the segment mutagenesis was performed. Site-directed mutagenesis was performed with *Pfu* DNA polymerase and primers 70K-F+R and 73I-F+R to generate *flgM*_{K73} and *flgM*_{I70} by using the QuickChange Mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Bacterial two-hybrid assay. These assays were carried out as previously described (Gunton *et al.*, 2007). *flgM* and *fliA* from *Y. pseudotuberculosis* were PCR amplified using primers M-25-F and M-25-R (for *flgM*) and A-18C-F and A-18C-R (for *fliA*) (Table S1). Amplified *flgM* was cloned into pKT25 and *fliA* was cloned into pUT18C, and both were co-transformed into *E. coli* BTH101 by electroporation. The strains were spread on MacConkey plates (IPTG+maltose) and cultured overnight at 37 °C. pKT25-*zip* and pUT18C-*zip* were also introduced into BTH101 as positive controls. This assay was also adopted to test the interaction of FlgM deletion and point mutants with σ^{28} .

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype description	Reference
<i>Y. pseudotuberculosis</i>		
YpIII	Wild-type <i>Y. pseudotuberculosis</i> pIB1, Nal ^r	Rosqvist <i>et al.</i> (1988)
YpIIIΔ <i>flgM</i>	<i>flgM</i> deleted in YpIII	This study
YpIIIΔ <i>fliA</i>	<i>fliA</i> deleted in YpIII	This study
YpIIIΔ <i>flgM</i> V	YpIIIΔ <i>flgM</i> : pJB908	This study
YpIIIΔ <i>flgM</i> C	YpIIIΔ <i>flgM</i> : pJB908- <i>flgM</i>	This study
YpIIIΔ <i>flgM</i> C _W	YpIIIΔ <i>flgM</i> : pJB910- <i>flgM</i>	This study
YpIIIΔ <i>flgM</i> C _{I85}	YpIIIΔ <i>flgM</i> : pJB910- <i>flgM</i> _{I85}	This study
YpIIIΔ <i>flgM</i> C _{A86}	YpIIIΔ <i>flgM</i> : pJB910- <i>flgM</i> _{A86}	This study
YpIIIΔ <i>flgM</i> C _{L89}	YpIIIΔ <i>flgM</i> : pJB910- <i>flgM</i> _{L89}	This study
<i>E. coli</i>		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lac</i> ^q Δ <i>M15</i> Tn10(Tet ^r)]	Stratagene
BTH101	Bacterial two-hybrid system host cells	Karimova <i>et al.</i> (2000)
BTH 114	pKT25- <i>flgM</i> and pUC18C- <i>fliA</i> co-transformed in BTH101	This study
Plasmids		
pDM4	Suicide vector for exchange, T7 promoter, Cml ^r , R6K origin, contains a <i>sacBR</i> gene	Milton <i>et al.</i> (1996)
pDM4-Δ <i>flgM</i>	Δ <i>flgM</i> in pDM4	This study
pDM4-Δ <i>fliA</i>	Δ <i>fliA</i> in pDM4	This study
pDM4- <i>flhDC-lacZ</i>	<i>flhDC-lacZ</i> fusion in pDM4	This study
pDM4- <i>fliA-lacZ</i>	<i>fliA-lacZ</i> fusion in pDM4	This study
pDM4- <i>flgM-lacZ</i>	<i>flgM-lacZ</i> fusion pDM4	This study
pDM4- <i>fliC-lacZ</i>	<i>fliC-lacZ</i> fusion in pDM4	This study
pDM4- <i>cheW-lacZ</i>	<i>cheW-lacZ</i> fusion in pDM4	This study
pDM4- <i>flgD-lacZ</i>	<i>flgD-lacZ</i> fusion in pDM4	This study
pDM4- <i>flaA-lacZ</i>	<i>flaA-lacZ</i> fusion in pDM4	This study
pDM4- <i>csrA-lacZ</i>	<i>csrA-lacZ</i> fusion in pDM4	This study
pDM4- <i>fliZ-lacZ</i>	<i>fliZ-lacZ</i> fusion in pDM4	This study
pKT25	Cloning vector, 15A origin, Kan ^r , <i>lac</i> promoter	Gunton <i>et al.</i> (2007)
pKT25- <i>flgM</i>	<i>flgM</i> in pKT25	This study
pKT25- <i>flgM</i> _{I85}	<i>flgM</i> _{I85} in pKT25	This study
pKT25- <i>flgM</i> _{A86}	<i>flgM</i> _{A86} in pKT25	This study
pKT25- <i>flgM</i> _{L89}	<i>flgM</i> _{L89} in pKT25	This study
pKT25- <i>flgM</i> _{L90}	<i>flgM</i> _{L90} in pKT25	This study
pUT18C	Cloning vector, ColEI origin, Amp ^r , <i>lac</i> promoter	Gunton <i>et al.</i> (2007)
pUT18C- <i>fliA</i>	<i>fliA</i> in pUT18C	This study
pJB908	RSF1010 cloning vector, Amp ^r	Sexton <i>et al.</i> (2004)
pJB908- <i>flgM</i>	<i>flgM</i> in pJB908	This study
pJB910	Amp ^r replaced by Kan ^r in pJB908, plus His tag coding sequence	This study
pJB910- <i>flgM</i>	<i>flgM</i> in pJB910	This study
pJB910- <i>flgM</i> _{I85}	<i>flgM</i> _{I85} in pJB910	This study
pJB910- <i>flgM</i> _{A86}	<i>flgM</i> _{A86} in pJB910	This study
pJB910- <i>flgM</i> _{L89}	<i>flgM</i> _{L89} in pJB910	This study
pGEX6P-1	Expression vector, T7 promoter, GST tag coding sequence, Amp ^r , pBR322 origin, contains a broad-host-range origin of replication <i>oriV</i>	Amersham-Pharmacia
pGEX6P-1- <i>fliA</i>	<i>fliA</i> in pGEX6P-1	This study

Protein detection and Western blotting. PCR primers for amplification of *fliA* and *flgM* are listed in Table S1 and named *fliA*-PD-F+R (generating a 5' *Bam*HI site and 3' *Xho*I site) and *flgM*-PD-F+R (generating a 5' *Bam*HI site and 3' *Sal*I site). A wild-type copy and three *flgM* point mutations (*FlgM*_{I85T}, *FlgM*_{A86D}, *FlgM*_{L89P}) were also amplified by primers *flgM*-PD-F+R, which each generated a 5' *Bam*HI site and 3' *Sal*I site. For co-precipitation, *fliA* was cloned into pGEX6p-1 and the three *flgM* point mutants were cloned into pJB910 and then transformed into *E. coli* XL1-Blue to

express GST-tagged *FliA* and the appropriate His-tagged *FlgM* point mutation. Expression was induced with 1 mM IPTG. Supernatants of sonicated cells were mixed with anti-GST Dynabeads (Novagen), pelleted and eluted in accordance with the manufacturer's instructions. Magnetic beads were separated by SDS-PAGE and probed with anti-His-Tag monoclonal antibody (Shanghai Genomics). The *flgM* upstream region was checked for the presence of consensus σ^{54} (YTGGCACG-N₄-TTGCW) (Barrios *et al.*, 1999) and σ^{28} (TAAAGTTT-N₁₁-GCCGATAA) (Kutsukake *et al.*, 1990) recognition sites.

Mutation, complementation and overexpression of the *flgM* gene. Two fragments up- and downstream of the *flgM* gene were amplified by PCR using two pairs of primers named *flgM*-up-F/*flgM*-up-R and *flgM*-down-F/*flgM*-down-R (Table S1). The DNA fragments were digested with enzymes accordingly and inserted into the suicide vector pDM4 to obtain pDM4- Δ *flgM*, which was introduced into the chromosome of *Y. pseudotuberculosis* as previously described (Atkinson *et al.*, 1999). The *fliA* mutant was constructed similarly. For complementation, the *flgM* gene containing a putative promoter region from *Y. pseudotuberculosis* was amplified by PCR using primers *flgM*oeF + R (Table S1) and ligated onto pJB908 to give pJB908-*flgM* and then introduced into Δ *flgM* to give strain YpIII Δ *flgM*MC, while pJB908 alone was introduced into Δ *flgM* as a control. FlgM was overexpressed by introducing pJB908-*flgM* into *Y. pseudotuberculosis*.

Motility assay and electron microscopy. Swimming motility was assayed on semi-solid agar medium as described by Atkinson *et al.* (1999). Samples for electron microscopy were prepared according to the method of Correa *et al.* (2004).

β -Galactosidase assay. pDM4 derivatives containing *flhDC*, *fliA*, *flgM*, *fliC*, *flgD*, *cheW*, *csrA*, *fliA* and *fliZ* (Table 1) were fused to promoterless *lacZ* and electroporated into the *Y. pseudotuberculosis* parent, *flgM* and *fliA* mutants. Overnight cultures were diluted to 1 % and were grown in YLB broth with antibiotics at 28 °C and harvested at an OD₆₀₀ of 0.2–0.4. Cells were permeabilized with chloroform and SDS, and β -galactosidase activity was assayed as described by Miller (1992). All experiments were performed at least three times.

RESULTS

Characterization of the interaction of FlgM with σ^{28}

When the *Y. pseudotuberculosis* genome was examined for the presence of *flgM* two possible homologues were identified (YPK2427 and YPK0717). By analysing amino acid and sequence information using BLAST, we found that YPK2427 but not YPK0717 was analogous, in sequence, location and apparent function (as outlined in the genome annotation) to FlgM in *Y. pseudotuberculosis* strains Yp IP31758 and Yp IP32953. YPK2427 was also similar to *flgM* family members which belong to flagellar systems in other bacteria such as *S. typhimurium* (Karinsey & Hughes, 2006).

The gene YPK2427 encoding FlgM was identified in the *Y. pseudotuberculosis* genome sequence (GI 170024652) and was predicted to encode a protein of 11.3 kDa. If YPK2427 functions as an anti- σ^{28} factor in *Y. pseudotuberculosis* it is likely to interact physically with the alternative sigma factor σ^{28} (FliA). Bacterial two-hybrid assays were first carried out to examine whether there was a direct *in vivo* interaction between FlgM and σ^{28} and the result indicated that this was indeed the case (data not shown). This is in agreement with the mechanism of action of FlgM in other organisms such as *E. coli* and *S. typhimurium*, where FlgM is known to bind to σ^{28} . Furthermore, β -galactosidase activity using BTH114 (FlgM and σ^{28} co-expressed) induced transcription of *lacZ* due to the interaction between FlgM and σ^{28} to similar levels as the positive control (Fig. 1, columns 1 and 2),

while no activity was detected in the two negative controls (Fig. 1, columns 3 and 4).

Identification of the primary FlgM amino acid residues in contact with σ^{28}

Previous study in *H. pylori* has shown that the C-terminal domain of FlgM is important for the interaction with σ^{28} (Daughdrill *et al.*, 1997; Sorenson *et al.*, 2004). Based on this study, three mutations resulted in frameshifts in FlgM after codon positions 18, 59, and 67. Frameshift mutations after codon 67 suggested that the last 21 amino acids in FlgM are important for σ^{28} interaction. A minimal binding domain has been identified between Glu64 and Arg88 in *S. typhimurium* (Pons *et al.*, 2006). The N-terminal portion of FlgM remains unstructured and may be necessary for recognition by the export machinery (Aldridge & Hughes, 2002). Amino acid alignments of FlgM homologues showed two possible positions (Lys70 and Ile73) where FlgM and FliA may possibly interact (Fig. 2a). However, following mutagenesis of these two sites, the FlgM mutants still retained their activity with σ^{28} (data not shown). We then divided the amino acids of FlgM from Glu64 to Val93 into six parts (Fig. 2b, i), and made subsection mutations via overlapping PCR. Interactions between the newly generated mutants and σ^{28} were examined using the two-hybrid system by introducing the mutants into BTH101 containing pUT18C-*fliA*. Using the bacterial two-hybrid, we first narrowed the pivotal binding domain down to Leu69–Ile73 and Lys84–Val93 since red colonies were only visible in mutants in which there was still an interaction between FlgM and σ^{28} . These amino acids were then divided into two- or three-amino acid segments and secondary overlapping mutations were made (Fig. 2b, ii, iii, iv). These constructs were then transformed into BTH101 containing pUT18C-*fliA* to obtain co-expressed strains. In this case, two regions were obtained capable of interacting with σ^{28} , namely Lys84–Ala86 and Leu89–Leu90. Finally, point mutations in *flgM*

Fig. 1. Interaction of FlgM and σ^{28} by β -galactosidase activity. Positive control (1), FliA and FlgM co-expressed (2), FliA only (3), and FlgM only (4). Assays were performed in triplicate and standard deviations are shown.

Fig. 2. (a) Amino acid alignment of FlgM homologues. The GenInfo Identifier numbers of the FlgM used in the alignments are as follows: *Y. pseudotuberculosis* IP31758 (GI153947903), *Y. pseudotuberculosis* IP32953 (GI51596006), *S. typhimurium* LT2 (GI16764528), *E. coli* K-12 (GI1651526), *Proteus mirabilis* (GI1857441), *Pseudomonas syringae* (GI28869129), *B. subtilis* (GI729520) and *H. pylori* (GI18075990). The σ^{28} binding domain in *Y. pseudotuberculosis* FlgM is indicated by the line at the bottom of the alignments. Locations of Lys70 and Ile73 are marked by asterisks. (b) Point mutations of FlgM by site-directed mutagenesis. The amino acids from Glu64 to Val93 of FlgM were divided into six groups and then narrowed into smaller portions. (c) Outline of the segment and site-directed mutagenesis.

(*flgM*_{I85T}, *flgM*_{A86D}, *flgM*_{A86D} and *flgM*_{L90S}) were cloned into pKT25 by overlapping PCR (Fig. 2c). The *in vivo* results indicated that Ile85, Ala86 and Leu89 were necessary for FlgM interaction with σ^{28} since these mutants were unable to produce red colonies on MacConkey plates (Fig. 3a, segments 3–5). The results were also examined using β -galactosidase assay (Fig. 3b). Almost no β -galactosidase activity was detected in the three FlgM mutants, consistent with the data obtained for the two-hybrid system assays, indicating that these mutants were unable to interact with σ^{28} . Leu90 was a potential site for σ^{28} interaction, but pKT25-*flgM*_{L90S} co-expressed with pUT18C-*flaA* in BTH101 formed red colonies on the plate in the two-hybrid system assay (Fig. 3a) and also showed β -galactosidase activity comparable to the negative control (Fig. 3b, column 6).

Amino acids essential for σ^{28} and FlgM interaction

A pull-down assay and Western blotting were performed to further validate the essential amino acids for the inter-

action of FlgM and σ^{28} *in vitro*. GST-tagged σ^{28} , His-tagged FlgM and His-tagged FlgM Δ were co-expressed from different compatible plasmids (pGEX6p-1 and pJB910, respectively) in *E. coli*. Magnetic metal chelation beads (anti-GST) were then used to precipitate GST- σ^{28} from the *E. coli* lysate. Eluates from the magnetic beads were separated by SDS-PAGE and probed with anti-His antibody. GST- σ^{28} or His-FlgM expressed alone were used as negative controls (Fig. 4, lanes 1–4) whereas the positive controls were GST- σ^{28} and His-FlgM co-expressed within the same cell. Capture of GST- σ^{28} by magnetic beads caused the co-capture of a large amount of His-FlgM (Fig. 4, lanes 5 and 6). The newly generated *flgM*_{I85T}, *flgM*_{A86D} and *flgM*_{L89P} mutants were His-tag cloned into pJB910 and then co-transformed with GST-tagged σ^{28} into *E. coli* XL1-Blue. When GST- σ^{28} and His-FlgM_{I85T} were co-expressed within the same cell, capture of GST- σ^{28} by magnetic beads did not cause the co-capture of His-FlgM_{I85T} (Fig. 4, lanes 7 and 8). GST- σ^{28} co expressed with His-FlgM_{A86D} or His-FlgM_{L89P} also showed the same results (Fig. 4, lanes 9–12). These results demonstrated the interaction between σ^{28} and

Fig. 3. *In vivo* protein interaction of mutated FlgM with σ^{28} . (a) Bacterial two-hybrid assay. The transformants of FliA + FlgM (1), FliA only (2), FliA + FlgM_{I85} (3), FliA + FlgM_{A86} (4), FliA + FlgM_{L89} (5) and FliA + FlgM_{L90} (6) were grown on MacConkey agar. (b) β -Galactosidase activity analysis. Column 1, *E. coli* BTH101 carrying plasmids pKT25-flgM and pUC18C-fliA; column 2, pUT18C carrying *fliA* with the vector control pKT25; columns 3, 4 and 5, pUC18C-fliA with different site-mutated *flgM*; column 6, pKT25-flgM_{L90} and pUT18C-fliA.

FlgM was destroyed when Ile85, Ala86 or Leu89 of FlgM was mutated.

Effect of *flgM* mutation on swimming motility

To further study the function of FlgM, we constructed an in-frame deletion mutant in *flgM*. Swimming motility was analysed in both *Y. pseudotuberculosis* parent and the *flgM* mutant strains. When compared to the parent, Δ *flgM* was non-motile in semi-solid motility plate assays (Fig. 5a, 1 and 2). However, when a functional copy of *flgM* was introduced into the mutant (Δ *flgM*C), motility was restored to levels comparable to the parent whereas the vector control alone (Δ *flgM*V) remained non-motile (Fig. 5a, 3 and 4). The *flgM* mutant was also examined by electron microscopy, which revealed that the parent and complemented *flgM* mutant possess a single polar flagellum (Fig. 5b, panels 1 and 4) whereas the mutant

and pJB908 complement control do not (Fig. 5b, panels 2 and 3).

Interaction of FlgM with σ^{28} influences bacterial motility

The *flgM* mutant showed a significant motility defect in this study but it was unclear whether this was a direct result of disruption of the interaction between FlgM and σ^{28} . To investigate the function of the FlgM- σ^{28} interaction in motility, we inserted *flgM*, *flgM*_{I85T}, *flgM*_{A86D} and *flgM*_{L89P} into the expression vector pJB910 and each plasmid was transformed into the *flgM* mutant. As shown in Fig. 6, the three mutant strains were non-motile when compared to the complemented strain (Δ *flgM* containing pJB910::*flgM*), indicating these three mutations could not restore motility to the *flgM* mutant. To confirm that the three mutants were non-motile in the plate assays, cells

Fig. 4. *In vitro* interaction of FlgM Δ with σ^{28} by Western blotting. Magnetic anti-GST beads were used to capture GST-tagged protein from cell lysates. Then samples were separated on Coomassie-stained polyacrylamide gel and probed with anti-His antibody. Lanes correspond to XL1-Blue cells expressing GST- σ^{28} (lane 1, whole-cell lysate; lane 2, anti-GST bead elute), His-FlgM (lane 3, whole-cell lysate; lane 4, anti-GST bead elute), GST- σ^{28} and His-FlgM (lane 5, whole-cell lysate; lane 6, anti-GST bead elute), GST- σ^{28} and His-FlgM_{I85T} (lane 7, whole-cell lysate; lane 8, anti-GST bead elute), GST- σ^{28} and His-FlgM_{A86D} (lane 9, whole-cell lysate; lane 10, anti-GST bead elute), and GST- σ^{28} and His-FlgM_{L89P} (lane 11, whole-cell lysate; lane 12, anti-GST bead elute). Interactions between FlgM and σ^{28} are reflected in the co-precipitation of both proteins in the presence of anti-His antibody.

Fig. 5. Motility phenotypes and electron micrographs of wild-type *Y. pseudotuberculosis*, the *flgM* mutant and its complemented strains. Cells were inoculated into motility agar and incubated at 22 °C for 3 days. (a) Motility phenotype. 1, *Y. pseudotuberculosis* wild-type; 2, *YpIIIΔflgM*; 3, *YpIIIΔflgMV* ($\Delta flgM$ +pJB908); 4, *YpIIIΔflgMC* ($\Delta flgM$ +pJB908-*flgM*). (b) Electron micrographs of wild-type (1); *YpIIIΔflgM* (2); *YpIIIΔflgMV* (3) and *YpIIIΔflgMC* (4). Bars are equivalent to 1 μ m.

taken from each colony were examined using light microscopy, and only the cells taken from the complemented strain were visibly swimming (data not shown).

Effect of *flgM* mutation on the transcription of key motility genes

To gain further insights into the contribution of FlgM to the regulation of transcription of other components of the flagellar biogenesis pathway, the master flagellar regulator *flhDC*, *fliA*, *flgM* itself and the flagellar structural gene *fliC* were each fused to *lacZ* and transformed into $\Delta flgM$ and $\Delta fliA$. β -Galactosidase activity during exponential growth showed that *flhDC* is transcribed similarly in both the parent and mutants (Fig. 7a). In contrast, *fliA* expression

Fig. 6. Motility phenotype of $\Delta flgM$ and its complemented strains expressing the site-mutated amino acids. 1, *YpIIIΔflgMC_W*; 2, *YpIIIΔflgMC_{I85}*; 3, *YpIIIΔflgMC_{A86}*; 4, *YpIIIΔflgMC_{L89}*. For details of strains, see Table 1.

was reduced considerably in both the *fliA* and *flgM* mutants (Fig. 7b). Interestingly, *flgM* transcription was almost sixfold higher in the *fliA* mutant than in the parent (Fig. 7c), while *fliC* expression was markedly upregulated in the *flgM* mutant (Fig. 7d) but was reduced by more than 50 % in the *fliA* mutant when compared to the parent (29 units and 80 units respectively). This difference is difficult to see in Fig. 7(d) because of the excessively high activity of *fliC* in the *flgM* mutant.

FlgM also affects other σ -dependent transcription

If FlgM functions as an anti- σ^{28} factor, its absence in a $\Delta flgM$ mutant would be predicted to cause an increase in transcription of σ^{28} -dependent transcription. σ^{54} is known to play a role in flagellar biogenesis in *Vibrio cholerae* and *H. pylori* (Correa *et al.*, 2004; Spohn & Scarlato, 1999), both of which are capable of producing polar flagella. *Y. pseudotuberculosis* always produces polar flagella under our experimental conditions, which prompted us to speculate whether σ^{54} or other σ -factors may play a role in flagellar biogenesis in *Y. pseudotuberculosis*. We therefore constructed *cheW*, *flaA*, *flgD*, *csrA* and *fliZ* promoterless-*lacZ* transcriptional fusion plasmids in the wild-type, $\Delta fliA$ and $\Delta flgM$ strains. *cheW* and *flaA* possess σ^{28} -dependent class III promoters in *S. typhimurium* and *H. pylori*, respectively (Josenhans *et al.*, 2002). The *flgD* promoter is a σ^{54} -dependent class III promoter in *H. pylori*, the *csrA* promoter is a σ^A -dependent class III promoter while *fliZ* is a post-translational activator of FlhD₂C₂ (Saini *et al.*, 2008). All strains were grown in liquid culture and harvested at the early exponential phase. As shown in Fig. 8, the lack of FliA (in a $\Delta fliA$ mutant) reduces transcription of *cheW* (Fig. 8a) whereas there is a small increase in transcription of *fliZ* when compared to the wild-type (Fig. 8e). The lack of FlgM (in the $\Delta flgM$ strain) had an approximately twofold stimulatory effect on *cheW* transcription (Fig. 8a) and also caused a small increase in transcription of *flgD* in comparison to the wild-type

Fig. 7. Expression of flagellin promoters in *Y. pseudotuberculosis* wild-type, $\Delta flgM$ and $\Delta fliA$ mutant strains using *lacZ* fusions to *flhDC* (a), *fliA* (b), *flgM* (c) and *fliC* (d). Assays were performed in triplicate and standard deviations are shown.

(Fig. 8b). Oddly, transcription of *flaA* was unaffected by the lack of FlgM or σ^{28} (Fig. 8c) whereas *csrA* transcription was unaffected by the absence of σ^{28} but was reduced in the *flgM* mutant (Fig. 8d).

DISCUSSION

The control of σ^{28} -dependent transcription via FlgM is a fascinating example of how transcription within the bacterial cytoplasm can be temporally controlled by the assembly of an external organelle (Correa *et al.*, 2004). In *S. typhimurium*, FlgM binds to σ^{28} and prevents its association with RNAP until completion of the HBB structure, at which point FlgM is secreted through the nascent flagellum (Hughes *et al.*, 1993). This allows the σ^{28} holoenzyme to transcribe the class III flagellar genes, the products of which are then assembled into the flagellar filament. The bulk of the filament is composed of flagellin subunits, and these are not needed until completion of the HBB structure. Thus, FlgM prevents their expression until an appropriate point in flagellar assembly. *Y. pseudotuberculosis* possesses a single flagellin gene (*fliC*) transcribed by σ^{28} holoenzyme upon completion of the HBB structure, which is also the case in *S. typhimurium* (Karlinsky & Hughes, 2006).

Genetic and biochemical studies of σ^{28} /FlgM interactions have contributed to the understanding of flagellar regulation. We show here that FlgM interacts with σ^{28} in *Y. pseudotuberculosis*. A co-crystal structure of *A. aeolicus* σ^{28} /FlgM has been obtained, and the important domains in σ^{28} and FlgM interactions were defined (Sorenson *et al.*, 2004).

Because the overall amino acid homologies of σ^{28} and FlgM between *A. aeolicus* and *Y. pseudotuberculosis* are low, it is difficult to extrapolate whether the configuration of proteins are significant in *Y. pseudotuberculosis*. In *S. typhimurium*, three mutations resulted in frameshifts in FlgM after codon positions 18, 59 and 67. Frameshift mutation after codon 67 suggests that the last 21 amino acids in FlgM are important for inhibition of σ^{28} activity. Previous studies of FlgM showed that the C-terminus is important for the σ^{28} /FlgM interaction (Daughdrill *et al.*, 1997; Sorenson *et al.*, 2004). Based on these data, we wanted to find the pivotal regions that influence interaction between FliA and FlgM in *Y. pseudotuberculosis*. Initially we made CLUSTAL W amino acid alignments of FlgM homologues to confirm the interactive region. Our target was the segment from Glu64 to Val93. Bacterial two-hybrid analysis of segment mutations of FlgM with σ^{28} demonstrated that amino acid residues Leu69 to Ile73 and Lys84 to Val93 were the pivotal regions. Site-directed mutagenesis then indicated that FlgM probably interacts with σ^{28} through Ile85, Ala86 and Leu89. It is clear that the polar residues that mediate this interaction are different from those reported for *S. typhimurium* (Chadsey *et al.*, 1998). These data were supported by the fact that these three point mutations in FlgM were unable to restore motility to a *flgM* mutant when supplied *in trans*.

σ^{28} is regarded as a type III secretion chaperone for its own anti-sigma factor FlgM in *S. typhimurium* (Aldridge *et al.*, 2006), where the relative cellular levels of FlgM and σ^{28} are tightly controlled (Bleves *et al.*, 2002; Watnick *et al.*, 2001). *S. typhimurium* possesses a single flagellin gene transcribed

Fig. 8. β -Galactosidase activity analyses of different σ -dependent genes in *Y. pseudotuberculosis* wild-type and its *fliA* and *flgM* mutants. (a) *cheW*, (b) *flgD*, (c) *flaA*, (d) *csrA*, (e) *fliZ*. Assays were performed in triplicate and standard deviations are shown.

by σ^{28} holoenzyme upon completion of the HBB structure, and transcription of the *flgM* gene in this strain has been shown to be dependent on both σ^{28} and the master regulator FlhDC (Hughes *et al.*, 1993). We compared Fops of *Y. pseudotuberculosis* and Δ *flgM* by SDS-PAGE, and one protein, which was subsequently identified as flagellin by MS analysis, was absent in the mutant (data not shown). These data demonstrate that in *Y. pseudotuberculosis*, FlgM interacts with σ^{28} , is secreted through the flagellum and can inhibit transcription of some σ^{28} -dependent genes. These functions are consistent with anti- σ^{28} activity. One would therefore expect that deleting FlgM would result in increased σ^{28} -dependent transcription at all of the σ^{28} -dependent flagellin promoters. Fig. 7(d) and Fig. 8(a) show that transcription of *fliC* and *cheW* (σ^{28} -like in *Salmonella*) in the *flgM* mutant is higher than the parent, indicating that, as expected, these class III promoters are activated by free FliA.

The fact that *fliA* expression is considerably reduced in the *flgM* mutant when compared to the parent (Fig. 7b) is intriguing and suggests that FlgM plays a role in the positive regulation of *fliA*. This observation seems paradoxical given that FlgM apparently positively regulates transcription of the σ factor upon which it acts in its role as an anti- σ factor. *fliA* expression is abolished in the *fliA* mutant, suggesting that FliA positively autoregulates its own expression. Conversely, *flgM* expression is sixfold higher in the *fliA* mutant, which suggests that FliA is capable of repressing the expression of its own anti- σ factor.

flgM transcription is sixfold higher in the *fliA* mutant because the β -galactosidase activity units we detected in this study represent free intracellular protein expression and FlgM acts as a chaperone in the parent before HBB construction is completed. In the *flgM* mutant there is clearly a lack of activity on motility plates and flagella are

absent. However, there is also an obvious increase in *fliC* expression; one explanation may be that the *fliC* transcript is subject to post-transcriptional regulation.

We also detected σ^{54} - and σ^A -like promoter activities upstream of *flgM*. For example, potential σ^{54} -dependent transcription of the *flgD* (σ^{54} -like in *H. pylori*) promoters was increased in the absence of FlgM (Fig. 8b), indicating a lack of uniform repressive FlgM activity at not only σ^{28} -dependent promoters. *Yersinia* are usually considered to produce peritrichous flagella, although polar flagella have been described (Chatterjee & Neogy, 1972). Polar flagella production has been considered in terms of σ^{54} -dependent transcription, and under our test conditions we always observed polar flagella as shown in Fig. 5(b). The observation of σ^{54} -dependent transcription of *flgD* and the production of polar flagella suggests that there may also be a σ^{54} -dependent flagellar system in operation in this organism which may determine the number and location of flagella at the cell surface. The transcription of *fliZ*, a post-translational activator of FlhD₂C₂-dependent flagellar gene expression in *Salmonella* (Saini *et al.*, 2008), was also increased in Δ *fliA* but unaffected in Δ *flgM* (Fig. 8e), suggesting that σ^{28} may play a role in activation of *flhDC* transcription in *Y. pseudotuberculosis*. A similar system is in operation in enterohaemorrhagic *E. coli*, in which a feedback loop driven by FliA (σ^{28}) activates the transcription of *flhDC*. It has been suggested that this system may act to sustain and strengthen an environmental response to increase the production of flagella (Clarke & Sperandio, 2005). However in *Y. pseudotuberculosis* it seems unlikely that FliA or FlgM play a direct role in the regulation of *flhDC* since the levels of *flhDC* expression are similar in the parent and mutants (Fig. 7a).

It is difficult to rationalize why Δ *flgM* is non-motile even though *fliC* expression is increased and the *flhDC* expression is unaffected in this strain, but these observations suggest that *fliC* is modified post-transcriptionally. Complex flagella are present in many bacteria with two flagellar systems, but no studies have been performed to link the flagellin composition under different condition to specific attributes of flagella. *Y. pseudotuberculosis* contains two flagella systems (FS1 and FS2) but this study focused on FS1 genes alone. The expression of FS1 genes in *Yersinia* is in part similar to other *Enterobacteriaceae* in that expression is coordinated with flagellar assembly in response to environmental signals, and consists of a hierarchy of three major flagellar gene classes. However, despite the fact that our FS1 *flgM* mutant functions as an anti- σ^{28} factor, our data also reveal that there are differences in the regulatory hierarchies driven by FliA and FlgM when compared to other well-characterized systems such as *E. coli*. Could the system therefore be responsible for cross-talk between FS1 and FS2? A study into the role of FS2 in regulation in the context of growth on surfaces and with respect to the production of polar flagella would be timely.

ACKNOWLEDGEMENTS

This work was funded by grants from the National Natural Science Foundation of China (grants 30570020 and 30770026).

REFERENCES

- Aldridge, P. & Hughes, K. T. (2002). Regulation of flagellar assembly. *Curr Opin Microbiol* 5, 160–165.
- Aldridge, P. D., Karlinsey, J. E., Aldridge, C., Birchall, C., Thompson, D., Yagasaki, J. & Hughes, K. T. (2006). The flagellar-specific transcription factor, σ^{28} , is the Type III secretion chaperone for the flagellar-specific anti- σ^{28} factor FlgM. *Genes Dev* 20, 2315–2326.
- Atkinson, S., Throup, J. P., Stewart, G. S. & Williams, P. (1999). A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Mol Microbiol* 33, 1267–1277.
- Barrios, H., Valderrama, B. & Morett, E. (1999). Compilation and analysis of σ^{54} -dependent promoter sequences. *Nucleic Acids Res* 27, 4305–4313.
- Blair, D. F. (1995). How bacteria sense and swim. *Annu Rev Microbiol* 49, 489–522.
- Blevess, S., Marenne, M. N., Detry, G. & Cornelis, G. R. (2002). Up-regulation of the *Yersinia enterocolitica* yop regulon by deletion of the flagellum master operon flhDC. *J Bacteriol* 184, 3214–3223.
- Carniel, E. & Hinnebusch, B. J. (editors) (2004). *Yersinia: Molecular and Cellular Biology*. Norwich, UK: Horizon Bioscience Press.
- Chadsey, M. S. & Hughes, K. T. (2001). A multipartite interaction between *Salmonella* transcription factor σ^{28} and its anti-sigma factor FlgM: implications for σ^{28} holoenzyme destabilization through stepwise binding. *J Mol Biol* 306, 915–929.
- Chadsey, M. S., Karlinsey, J. E. & Hughes, K. T. (1998). The flagellar anti-sigma factor FlgM actively dissociates *Salmonella typhimurium* sigma28 RNA polymerase holoenzyme. *Genes Dev* 12, 3123–3136.
- Chatterjee, B. D. & Neogy, K. N. (1972). Detection of capsule in *Vibrio parahaemolyticus*. *Experientia* 28, 103–104.
- Chilcott, G. S. & Hughes, K. T. (2000). Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. *Microbiol Mol Biol Rev* 64, 694–708.
- Clarke, M. B. & Sperandio, V. (2005). Transcriptional regulation of *flhDC* by QseBC and sigma (FliA) in enterohaemorrhagic *Escherichia coli*. *Mol Microbiol* 57, 1734–1749.
- Colland, F., Rain, J. C., Gounon, P., Labigne, A., Legrain, P. & De Reuse, H. (2001). Identification of the *Helicobacter pylori* anti- σ^{28} factor. *Mol Microbiol* 41, 477–487.
- Correa, N. E., Barker, J. R. & Klose, K. E. (2004). The *Vibrio cholerae* FlgM homologue is an anti- σ^{28} factor that is secreted through the sheathed polar flagellum. *J Bacteriol* 186, 4613–4619.
- Daughdrill, G. W., Chadsey, M. S., Karlinsey, J. E., Hughes, K. T. & Dahlquist, F. W. (1997). The C-terminal half of the anti-sigma factor, FlgM, becomes structured when bound to its target, σ^{28} . *Nat Struct Biol* 4, 285–291.
- Frisk, A., Jyot, J., Arora, S. K. & Ramphal, R. (2002). Identification and functional characterization of *flgM*, a gene encoding the anti- σ^{28} factor in *Pseudomonas aeruginosa*. *J Bacteriol* 184, 1514–1521.
- Gillen, K. L. & Hughes, K. T. (1991a). Molecular characterization of *flgM*, a gene encoding a negative regulator of flagellin synthesis in *Salmonella typhimurium*. *J Bacteriol* 173, 6453–6459.

- Gillen, K. L. & Hughes, K. T. (1991b). Negative regulatory loci coupling flagellin synthesis to flagellar assembly in *Salmonella typhimurium*. *J Bacteriol* **173**, 2301–2310.
- Gillen, K. L. & Hughes, K. T. (1993). Transcription from two promoters and autoregulation contribute to the control of expression of the *Salmonella typhimurium* flagellar regulatory gene *flgM*. *J Bacteriol* **175**, 7006–7015.
- Givaudan, A. & Lanois, A. (2000). *flhDC*, the flagellar master operon of *Xenorhabdus nematophilus*: requirement for motility, lipolysis, extracellular hemolysis, and full virulence in insects. *J Bacteriol* **182**, 107–115.
- Gunton, J. E., Gilmour, M. W., Baptista, K. P., Lawley, T. D. & Taylor, D. E. (2007). Interaction between the co-inherited TraG coupling protein and the TraJ membrane-associated protein of the H-plasmid conjugative DNA transfer system resembles chromosomal DNA translocases. *Microbiology* **153**, 428–441.
- Hughes, K. T., Gillen, K. L., Semon, M. J. & Karlinsey, J. E. (1993). Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. *Science* **262**, 1277–1280.
- Josenhans, C. & Suerbaum, S. (2002). The role of motility as a virulence factor in bacteria. *Int J Med Microbiol* **291**, 605–614.
- Josenhans, C., Niehus, E., Amersbach, S., Horster, A., Betz, C., Drescher, B., Hughes, K. T. & Suerbaum, S. (2002). Functional characterization of the antagonistic flagellar late regulators FlhA and FlgM of *Helicobacter pylori* and their effects on the *H. pylori* transcriptome. *Mol Microbiol* **43**, 307–322.
- Karimova, G., Ullmann, A. & Ladant, D. (2000). A bacterial two-hybrid system that exploits a cAMP signaling cascade in *Escherichia coli*. *Methods Enzymol* **328**, 59–73.
- Karlinsey, J. E. & Hughes, K. T. (2006). Genetic transplantation: *Salmonella enterica* serovar Typhimurium as a host to study sigma factor and anti-sigma factor interactions in genetically intractable systems. *J Bacteriol* **188**, 103–114.
- Karlinsey, J. E., Tanaka, S., Bettenworth, V., Yamaguchi, S., Boos, W., Aizawa, S. I. & Hughes, K. T. (2000). Completion of the hook-basal body complex of the *Salmonella typhimurium* flagellum is coupled to FlgM secretion and *fliC* transcription. *Mol Microbiol* **37**, 1220–1231.
- Kirov, S. M. (2003). Bacteria that express lateral flagella enable dissection of the multifunctional roles of flagella in pathogenesis. *FEMS Microbiol Lett* **224**, 151–159.
- Kutsukake, K., Ohya, Y. & Iino, T. (1990). Transcriptional analysis of the flagellar regulon of *Salmonella typhimurium*. *J Bacteriol* **172**, 741–747.
- Lee, S. H., Butler, S. M. & Camilli, A. (2001). Selection for in vivo regulators of bacterial virulence. *Proc Natl Acad Sci U S A* **98**, 6889–6894.
- Liu, X. & Matsumura, P. (1994). The FlhD/FlhC complex, a transcriptional activator of the *Escherichia coli* flagellar class II operons. *J Bacteriol* **176**, 7345–7351.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics: a Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Milton, D. L., O'Toole, R., Horstedt, P. & Wolf-Watz, H. (1996). Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J Bacteriol* **178**, 1310–1319.
- Ohnishi, K., Kutsukake, K., Suzuki, H. & Iino, T. (1992). A novel transcriptional regulation mechanism in the flagellar regulon of *Salmonella typhimurium*: an antisigma factor inhibits the activity of the flagellum-specific sigma factor, σ^F . *Mol Microbiol* **6**, 3149–3157.
- Okada, K., Ichihara, H., Takahashi, H., Fujita, N., Ishihama, A. & Hakoshima, T. (2007). Preparation and preliminary X-ray diffraction analysis of crystals of bacterial flagellar sigma factor σ^{28} in complex with the σ^{28} -binding region of its antisigma factor, FlgM. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **63**, 196–199.
- Pons, T., Gonzalez, B., Cecilian, F. & Galizzi, A. (2006). FlgM anti-sigma factors: identification of novel members of the family, evolutionary analysis, homology modeling, and analysis of sequence–structure–function relationships. *J Mol Model* **12**, 973–983.
- Ramos, H. C., Rumbo, M. & Sirard, J. C. (2004). Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. *Trends Microbiol* **12**, 509–517.
- Richardson, K. (1991). Roles of motility and flagellar structure in pathogenicity of *Vibrio-Cholerae* – analysis of motility mutants in three animal models. *Infect Immun* **59**, 2727–2736.
- Rosqvist, R., Skurnik, M. & Wolf-Watz, H. (1988). Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. *Nature* **334**, 522–524.
- Saini, S., Brown, J. D., Aldridge, P. D. & Rao, C. V. (2008). FlhZ is a post-translational activator of FlhD4C2-dependent flagellar gene expression. *J Bacteriol* **190**, 4979–4988.
- Schmitt, C. K., Darnell, S. C., Tesh, V. L., Stocker, B. A. & O'Brien, A. D. (1994). Mutation of *flgM* attenuates virulence of *Salmonella typhimurium*, and mutation of *fliA* represses the attenuated phenotype. *J Bacteriol* **176**, 368–377.
- Sexton, J. A., Pinkner, J. S., Roth, R., Heuser, J. E., Hultgren, S. J. & Vogel, J. P. (2004). The *Legionella pneumophila* PilT homologue DotB exhibits ATPase activity that is critical for intracellular growth. *J Bacteriol* **186**, 1658–1666.
- Sorenson, M. K., Ray, S. S. & Darst, S. A. (2004). Crystal structure of the flagellar sigma/anti-sigma complex σ^{28} /FlgM reveals an intact sigma factor in an inactive conformation. *Mol Cell* **14**, 127–138.
- Spohn, G. & Scarlato, V. (1999). Motility of *Helicobacter pylori* is coordinately regulated by the transcriptional activator FlgR, an NtrC homolog. *J Bacteriol* **181**, 593–599.
- Wadhams, G. H. & Armitage, J. P. (2004). Making sense of it all: bacterial chemotaxis. *Nat Rev Mol Cell Biol* **5**, 1024–1037.
- Watnick, P. I., Lauriano, C. M., Klose, K. E., Croal, L. & Kolter, R. (2001). The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholerae* O139. *Mol Microbiol* **39**, 223–235.
- Wood, T. K., Gonzalez Barrios, A. F., Herzberg, M. & Lee, J. (2006). Motility influences biofilm architecture in *Escherichia coli*. *Appl Microbiol Biotechnol* **72**, 361–367.
- Young, G. M., Schmiel, D. H. & Miller, V. L. (1999). A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc Natl Acad Sci U S A* **96**, 6456–6461.

Edited by: J. G. Shaw