Positive regulation of *flhDC* expression by OmpR in *Yersinia pseudotuberculosis*

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OmpR has been demonstrated to negatively regulate the expression of the flagellar master operon *flhDC* in a wide variety of bacterial species. Here we report the positive regulation of *flhDC* expression by OmpR in *Yersinia pseudotuberculosis*. A σ^{70} -dependent promoter was identified by primer extension analysis and an active region with two conserved OmpR-binding sites around the *flhDC* promoter was confirmed. To confirm the regulation of *flhDC* expression by OmpR, *flhDC* as well as the downstream flagellar genes *fliA*, *flgD*, *flgA*, *flgM*, *fliC* and *flaA* were fused to *lacZ*, and decreased expression of all these genes in an *ompR* mutant ($\Delta ompR$) was detected. Furthermore, $\Delta ompR$ was defective in bacterial motility and flagella synthesis. This defect was due to the low level of expression of *flhDC* in $\Delta ompR$ since overproduction of FlhDC in $\Delta ompR$ restored bacterial motility. The importance of two conserved OmpR-binding sites around the *flhDC* promoter region in the regulation of *flhDC* expression by OmpR was demonstrated by the fact that mutation of either one or both sites significantly decreased the promoter activity in the wild-type but not in $\Delta ompR$. The binding of OmpR to these two sites was also demonstrated by DNA mobility shift assay. The possible mechanism underlying this positive regulation in *Y. pseudotuberculosis* is discussed. To our knowledge, this is the first report to demonstrate that OmpR positively regulates *flhDC* expression.

Received 14 May 2009
Revised 15 July 2009
Accepted 27 July 2009

INTRODUCTION

Flagella, the unique structure for motility, represent an important advantage for bacteria in moving towards favourable conditions or in avoiding detrimental environments. In pathogenic bacteria, flagella are usually considered as virulence factors (Josenhans & Suerbaum, 2002), and they play crucial roles in adhesion, colonization and biofilm formation of many bacteria such as *Pseudomonas aeruginosa* (Toutain *et al.*, 2007), *Escherichia coli* (Pratt & Kolter, 1998), *Vibrio cholerae* (Gardel & Mekalanos, 1996) and *Salmonella typhimurium* (Ciacci-Woolwine *et al.*, 1998). Flagellar gene regulation and assembly have been extensively characterized in *E. coli* and *S. enterica* serovar Typhimurium (Aldridge & Hughes, 2002). Over 60 genes, which are arranged into three classes (class I, class II and class III), are coordinately regulated and expressed in a transcriptional hierarchy (Chilcott & Hughes, 2000;

Kanehisa *et al.*, 2004; Kutsukake *et al.*, 1990). Transcription of the downstream flagellar genes is dependent on a high expression level of some upstream genes. At the top of this hierarchy is the master operon composed of the genes *flhD* and *flhC*. FlhD and FlhC together form a heterohexameric transcriptional activator that binds to the class II operons (Wang *et al.*, 2006).

Due to the key role of *flhDC* in flagella synthesis, the regulation of its expression has been studied at both the transcriptional and post-transcriptional levels (Soutourina & Bertin, 2003). Many regulatory proteins, such as H-NS (Bertin *et al.*, 1994; Soutourina *et al.*, 1999), cAMP-CAP complex (catabolite gene activator protein) (Soutourina *et al.*, 1999), IHF (Yona-Nadler *et al.*, 2003), OmpR (Kim *et al.*, 2003; Shin & Park, 1995) and QseBC (Clarke & Sperandio, 2005), have been demonstrated to participate in this regulation process. Most of these regulators bind to the regulatory region of *flhDC*; this region is not well conserved across different bacterial species, suggesting that the regulatory mechanisms may also be different. For example, the LysR family members LrhA and HdfR, which

Abbreviation: TSS, transcriptional start site.

A supplementary table of primers used in this study and a supplementary figure showing the sequence alignment of OmpR from different bacterial species are available with the online version of this paper.

repress the *flhDC* operon in *E. coli* (Ko & Park, 2000; Lehnen *et al.*, 2002), do not appear to have a significant role in *Proteus mirabilis* swarming motility (Clemmer & Rather, 2007).

In *Yersinia pseudotuberculosis*, an enteric pathogen causing a variety of intestinal and extraintestinal syndromes (Smego *et al.*, 1999), the roles of flagella have attracted increasing attention. Recent studies have shown that regulators important for the synthesis of virulence factors also influence the motility of *Y. pseudotuberculosis* (Heroven & Dersch, 2006). Moreover, mutation of known regulatory genes in the flagellar system, e.g. *flhDC* and *fliA*, resulted in high expression levels of virulence operons in *Yersinia enterocolitica* (Bleves *et al.*, 2002; Horne & Pruss, 2006), suggesting a regulatory relationship between flagella and virulence factors. In *Y. pseudotuberculosis*, the regulation of the *flhDC* operon has not been well studied. A recent study has shown that the YpsRI and YtbRI quorum-sensing systems modulate swimming motility by controlling the expression of *flhDC* (Atkinson *et al.*, 2008). Another report confirmed that *csrA*, the RNA binding regulator, was absolutely required for *Y. pseudotuberculosis* motility, and FlhDC biosynthesis was considerably lower in

the *csrA* mutant compared with the wild-type strain (Heroven *et al.*, 2008). This result is in accordance with earlier studies in *E. coli* showing that the stability of *flhDC* mRNA is controlled by CsrA (Wei *et al.*, 2001). However, whether there are other regulatory proteins that participate in the regulation of FlhDC expression in *Y. pseudotuberculosis* remains unclear. OmpR has been reported to negatively regulate the expression of *flhDC* in several bacteria (Kim *et al.*, 2003; Pruss, 1998; Shin & Park, 1995). Here, we demonstrate, we believe for the first time, that OmpR positively regulates *flhDC* expression in *Y. pseudotuberculosis*, and also confirm that this regulation is through direct binding to the regulatory region of the *flhDC* operon.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* cultures were routinely grown in Luria-Bertani (LB) medium containing 1% tryptone, 0.5% yeast extract and 1% NaCl, or on LB agar (LB medium with 1.2% agar) plates at 37 °C. *Y. pseudotuberculosis* YpIII was grown as described previously (Hu *et al.*, 2009).

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	References
Strains		
<i>E. coli</i>		
S17-1(λ -pir)	λ -pir lysogen of S17-1, <i>thi pro hsdR hsdM</i> ⁺ <i>recA</i> RP4 2-Tc::Mu-Km::Tn7	Simon <i>et al.</i> (1983)
<i>Y. pseudotuberculosis</i>		
YpIII	Wild-type <i>Y. pseudotuberculosis</i> pIB1, Nal ^r	Rosqvist <i>et al.</i> (1988)
Δ ompR	<i>ompR</i> gene deleted in YpIII, Nal ^r	Hu <i>et al.</i> (2009)
Δ ompR-com	Δ ompR carrying plasmid pKT-ompR, Nal ^r Kan ^r	Hu <i>et al.</i> (2009)
Plasmids		
pDM4	Suicide vector, mobRK2, oriR6K (<i>pir</i> requiring), <i>sacBR</i> of <i>Bacillus subtilis</i> , Cm ^r	O'Toole <i>et al.</i> (1996)
pDM400	pDM4 containing promoterless <i>lacZ</i> and 1 kb <i>fliC</i> fragment from YpIII, used for single-copy of promoter activity analysis, Cm ^r	This study
pDM402	<i>flhDCp</i> :: <i>lacZ</i> in pDM400, base pairs (+445 to +250), Cm ^r	This study
pDM404	<i>flhDCp</i> :: <i>lacZ</i> in pDM400, base pairs (+445 to +50), Cm ^r	This study
pDM406	<i>flhDCp</i> :: <i>lacZ</i> in pDM400, base pairs (+445 to -150), Cm ^r	This study
pDM408	<i>flhDCp</i> :: <i>lacZ</i> in pDM400, base pairs (+445 to -350), Cm ^r	This study
pDM410	<i>flhDCp</i> :: <i>lacZ</i> in pDM400, base pairs (+445 to -550), Cm ^r	This study
pDM412	<i>flhDCp</i> :: <i>lacZ</i> in pDM400, base pairs (+445 to -750), Cm ^r	This study
pDM- <i>flhDC-lacZ</i>	<i>flhDC</i> :: <i>lacZ</i> in pDM4, Cm ^r	Ding <i>et al.</i> (2009)
pDM- <i>fliA-lacZ</i>	<i>fliA</i> :: <i>lacZ</i> in pDM4, Cm ^r	Ding <i>et al.</i> (2009)
pDM- <i>flgD-lacZ</i>	<i>flgD</i> :: <i>lacZ</i> in pDM4, Cm ^r	Ding <i>et al.</i> (2009)
pDM- <i>flgM-lacZ</i>	<i>flgM</i> :: <i>lacZ</i> in pDM4, Cm ^r	Ding <i>et al.</i> (2009)
pDM- <i>fliC-lacZ</i>	<i>fliC</i> :: <i>lacZ</i> in pDM4, Cm ^r	Ding <i>et al.</i> (2009)
pDM- <i>flgA-lacZ</i>	<i>flgA</i> :: <i>lacZ</i> in pDM4, Cm ^r	This study
pDM- <i>flaA-lacZ</i>	<i>flaA</i> :: <i>lacZ</i> in pDM4, Cm ^r	This study
pJB908	RSF1010 cloning vector (td Δ i), Amp ^r	Sexton <i>et al.</i> (2004)
pJB908- <i>flhDC</i>	pJB908 carrying <i>flhDC</i> , Amp ^r	This study
pKT100	Cloning vector, p15A replicon, Kan ^r	Hu <i>et al.</i> (2009)

*Amp^r, Kan^r, Cm^r and Nal^r represent resistance to ampicillin (100 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹), chloramphenicol (30 μ g ml⁻¹) and nalidixic acid (15 μ g ml⁻¹), respectively.

Construction of *flhDCp::lacZ* and *gene::lacZ* fusions. All primers used in this study are listed in Supplementary Table S1 (available with the online version of this paper). For *flhDCp::lacZ* fusion construction, a 1000 bp fragment around *fliC*, which was used as the homologous region during the recombination process, was inserted into pDM4, after which a 4.5 kb promoterless *lacZY* was amplified from *E. coli* K-12 and inserted into this recombined pDM4 to obtain pDM400. *flhDC* regulatory region leaders were then inserted into plasmid pDM400 to obtain a series of plasmids named pDM402, pDM404, pDM406, pDM408, pDM410 and pDM412 (Table 1) before being transformed into *E. coli* S17-1(λ -pir). Recombination was carried out as described previously (Atkinson *et al.*, 1999) with the successful reporter insertions being selected on Yersinia selective agar and supplements (Oxoid) containing naladixic acid and chloramphenicol, and confirmed by PCR with primers PflhCp565-F and PpDM4-88R (Supplementary Table S1).

Overlapping PCR was carried out to obtain mutations within the *flhDC* regulatory region using the primers listed in Supplementary Table S1. For the O₁ site mutant, PflhDp1200-SF and PO1R were used to obtain a 3'-end mutation upstream fragment. PO1F and PflhDp-XbaIR were used to obtain a 5'-end mutation downstream fragment, whose 5'-end overlapped with the 3'-end of the upstream fragment. In the next round of PCR, the O₁ mutant of the *flhDC* regulatory region was amplified using these two fragments as template with PflhDp1200-SF and PflhDp-XbaIR as primers. A similar procedure was performed to obtain the O₂ mutant, and the double mutant was obtained by multiple overlap PCR.

To obtain *flgA::lacZ* and *flaA::lacZ* fusions, the reverse primer for each gene was designed at the 3'-end (omitting the stop codon), and the forward primer for each gene was designed 500 bp upstream of the stop codon (all primers are listed in Supplementary Table S1). Each PCR product was inserted between the *Sall* and *SpeI* sites of pDM4-*lacZ* (Hu *et al.*, 2009) to generate plasmids named pDM-*flgA-lacZ* and pDM-*flaA-lacZ* (Table 1).

β -Galactosidase assay. Cells were cultured overnight at 28 °C in YLB and then transferred into fresh YLB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at ratio of 1:100. After incubation at 22 °C for 10 h, β -galactosidase activity was determined as described by Miller (1992). All experiments were repeated at least three times.

Motility assay and transmission electron microscopy of flagella. Swimming motility was assayed on semi-solid agar medium (1% tryptone, 0.5% NaCl, 0.3% Difco Bacto agar) as described previously (Atkinson *et al.*, 1999). For observation by transmission electron microscopy, bacteria were grown on semisolid tryptone medium at 28 °C for 20 h and fixed by whole-cell negative staining (Heroven *et al.*, 2008).

Primer extension reaction. YpIII was grown at 22 °C for 10 h. Total RNA was isolated by using an E.Z.N.A Bacterial RNA kit (Omega-BioTek). The cDNA was synthesized with biotin-labelled primers PY-OF-Rb and PflhDC-50Rb (Supplementary Table S1) using an RNA PCR kit (AMV) ver. 3.0 (TaKaRa). Samples were fractionated through a standard 8% sequencing gel with 8 M urea. Sequencing reactions were performed with a CycleReader DNA sequencing kit (Fermentas) using plasmid pDM412 as template and biotin-labelled DNA oligonucleotides as primers. A LightShift Chemiluminescent kit (Beyotime) was used to detect biotin-labelled DNA fragments.

DNA mobility shift assay. This was carried out as described by Shin & Park (1995) with modifications. Biotin-labelled DNA probes (300 bp Y-OF fragments with or without site mutations) were amplified using primers PflhDp570-SF and PY-OF-Rb (Supplementary Table S1). A 300 bp Y-CF fragment in the *flhDC*

coding region was also amplified as a control using primers named PY-CF-F and PY-CF-R (Supplementary Table S1). The reaction mixture (20 μ l) contained 20 mM Tris/HCl (pH 7.4), 4 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, 10% (v/v) glycerol, 0.5 μ g poly(dI-dC), 100 ng BSA, 100 ng biotin-labelled probes and 0–400 ng OmpR protein. For the phosphorylation of OmpR, 10 mM acetyl phosphate (acetyl-P, Sigma) was added to the reaction mixture. OmpR phosphorylation and binding to probes were performed at 37 °C for 1 h. The samples were then loaded onto a 6.5% native polyacrylamide gel. Electrophoresis was performed in 0.5 $\times$ TBE buffer for 3–4 h on ice.

RESULTS

Primer extension analysis of the *flhDC* transcriptional start site

There is an 1188 bp non-coding sequence upstream of the *flhDC* operon in *Y. pseudotuberculosis* YpIII. In order to identify the transcriptional start site of the *flhDC* operon in YpIII, we performed primer extension analysis. A primer (PflhDC-50Rb, Supplementary Table S1) was designed to anneal approximately 50 bp downstream from the translational start codon of the *flhDC* genes and another primer (PY-OF-Rb, Supplementary Table S1) was designed to anneal about 300 bp upstream of the translational start codon of the *flhDC* genes. We did not detect any extension products ranging from 50 to 400 bp using primer PflhDC-50Rb (data not shown). However, as shown in Fig. 1, the C base 442 bp upstream of the start codon of *flhDC* was mapped to be a transcriptional start site (TSS) using primer PY-OF-Rb, and a σ^{70} -dependent promoter with highly conserved –10 and –35 consensus sequences was identified around this TSS.

Active region for *flhDC* transcription

A series of single-copy *flhDCp::lacZ* transcriptional fusions were constructed (Fig. 2a) and the activities of these promoters were analysed. As shown in Fig. 2(b), the promoter activity was significantly increased in YpIII in the presence of 150 nucleotides upstream of the TSS. However, extension of the DNA fragment beyond the –150 site did not increase promoter activity, which suggests that the DNA fragment containing 150 nucleotides upstream of the TSS is an active region for *flhDC* transcription. To further identify whether any regulatory proteins bind to this active region, this sequence was aligned with conserved binding sequences of known regulators. Two regions around the promoter, named O₁ and O₂ (Fig. 2a), were highly similar to the conserved OmpR-binding sequence reported in *E. coli* (Egger *et al.*, 1997; Yoshida *et al.*, 2006). In the present study, we named the DNA fragment from +150 to –150, which contains both the O₁ and O₂ sites, the Y-OF segment (Fig. 2a).

Positive regulation of *flhDC* expression by OmpR

To test if OmpR could regulate *flhDC* expression, several single-copy *flhDCp::lacZ* fusions were transferred into

Fig. 1. Analysis of the transcriptional start site of *flhDC*. (a) Part of the non-coding sequence upstream of *flhDC* is listed and the transcription start site is marked as *. (b) σ^{70} consensus sequence. The most highly conserved nucleotides are depicted as larger letters and the less conserved nucleotides as smaller letters (James *et al.*, 2004). (c) *flhDC* promoter sequence. Bold letters indicate nucleotides that match the consensus sequences.

YpIII, $\Delta ompR$ (*ompR* mutant) and $\Delta ompR$ -com ($\Delta ompR$ complemented *in trans*). As shown in Fig. 2(b), the activities of promoters containing the Y-OF region decreased to low levels in $\Delta ompR$, while in $\Delta ompR$ -com the promoter activity was restored to the level observed in YpIII. These results suggested that OmpR positively regulates *flhDC* promoter activity and that this regulation is dependent on the Y-OF region. To further confirm the regulation of *flhDC* expression by OmpR, we constructed a *flhDC::lacZ* fusion. A significant decrease in β -galactosidase activity was also detected in $\Delta ompR$ compared to YpIII wild-type and $\Delta ompR$ -com (Fig. 2c). All these results indicated that OmpR positively regulates *flhDC* expression in YpIII.

OmpR controls expression of flagellar genes

Since OmpR regulates the master operon of flagellar genes, we tested the influence of OmpR on the expression of class II and class III genes in flagellar synthesis. Mutation of the *ompR* gene resulted in a two- to three-fold reduction in the expression of *lac* operon fusions to the class II genes *fliA* (Fig. 3a), *flgM* (Fig. 3b), *flgD* (Fig. 3c) and *flgA* (Fig. 3d), which encode σ^{28} factor, anti- σ^{28} factor, structural and assembly proteins required for the biosynthesis of the hook-basal body complex, and in the expression of *lac* operon fusions to the class III genes *fliC* (Fig. 3e) and *flaA* (Fig. 3f), which encode flagellins. These results further confirmed that OmpR positively regulates the expression of flagellar genes.

Influence of OmpR on swimming motility and flagella synthesis

Swimming motility in bacteria is closely related to FlhDC expression (Young *et al.*, 1999). To examine whether OmpR can also influence swimming motility by regulating

flhDC expression, YpIII, $\Delta ompR$ and $\Delta ompR$ -com were carefully spotted onto semisolid tryptone medium. As shown in Fig. 4(a), compared to YpIII, $\Delta ompR$ and $\Delta ompR$ carrying pKT100 both showed motility defects. Introducing the plasmid pKT-*ompR* into $\Delta ompR$ restored the motility phenotype. To further confirm if the influence of OmpR on swimming motility is mediated by FlhDC, we transferred the plasmid pJB908-*flhDC* (*flhDC* transcribed under the *lac* promoter) into $\Delta ompR$. The motility phenotype was also restored in $\Delta ompR$ carrying pJB908-*flhDC*, but was not observed in $\Delta ompR$ carrying the control plasmid pJB908 (Fig. 4a). In accordance with these results, flagella synthesis was also inhibited in $\Delta ompR$ compared to YpIII when viewed under a transmission electron microscope (Fig. 4b). Taken together, these results suggested that OmpR influences motility and flagella biosynthesis by positively regulating the expression of *flhDC*.

Binding of OmpR to the promoter region of the *flhDC* transcript

To further confirm if OmpR binds to the O₁ and O₂ sites in the Y-OF region, full-length *flhDCp::lacZ* fusions with mutations in the O₁ or O₂ sites or in both sites were constructed and β -galactosidase assays were carried out to test the promoter activities. As shown in Fig. 5, mutation of the O₁ or O₂ site or of both the O₁ and O₂ sites distinctly reduced promoter activity in YpIII and $\Delta ompR$ -com but not in $\Delta ompR$. Moreover, lack of OmpR or lack of the OmpR-binding sites resulted in the same effect, since the activity of the promoter with mutations of both the O₁ and O₂ sites in YpIII was the same as that of the full-length promoter in $\Delta ompR$ (Fig. 5), and these effects were not cumulative when the regulator and its binding sites were both missing, as demonstrated by the fact that the activity

Fig. 2. Deletion analysis of the *flhDC* regulatory region. (a) Six different length regulatory regions were fused with a promoterless *lacZ*. Y-OF and Y-CF represent DNA fragments used in the DNA mobility shift assay. Predicted promoter (labelled as p) and OmpR-binding sites (labelled as O₁ and O₂) are shown and the most conserved C base is boldfaced. (b) β -Galactosidase assay of single copies of a series of *flhDCp::lacZ* fusions in YpIII (white) and Δ ompR (black) and its complemented strain (grey). Strains carrying the pDM400 vector were used as negative controls (ck). (c) Expression of *flhDC* in YpIII (white), Δ ompR (black) and its complemented strain (grey).

of the promoter with mutations of both the O₁ and O₂ sites in Δ ompR was the same as that of the full-length promoter. Taken together, these results suggested that both the O₁ and O₂ sites are important for the regulation of *flhDC* transcription by OmpR.

To address whether OmpR functions by binding to both the O₁ and O₂ sites when controlling *flhDC* expression, a DNA-mobility shift assay was carried out. A DNA fragment of about 300 bp (named Y-OF in Fig. 2a) was amplified from the *flhDC* regulatory region of the wild-type strain or the strains with mutations in O₁ or O₂ or both O₁ and O₂,

and incubated with various amounts of phosphorylated OmpR protein (OmpR-p). Another 300 bp DNA fragment located in the *flhDC* operon (named Y-CF in Fig. 2a) was also amplified as a control in this assay. As shown in Fig. 6(a), specific interaction between OmpR and the Y-OF fragment was confirmed by the fact that OmpR retarded the Y-OF fragment but did not retard the Y-CF fragment, and addition of non-labelled Y-OF fragment reduced the amount of bound biotin-labelled probe. Mutation of the O₁ site in the Y-OF fragment slightly reduced its affinity for the OmpR protein, since part of the Y-OF fragment containing the O₁ mutation site remained free in the

Fig. 3. Expression of flagellar genes in YpIII (white), $\Delta ompR$ (black) and its complemented strain (grey) using *lacZ* fusions to the class II flagellar genes *fliA* (a), *flgM* (b), *flgD* (c), *flgA* (d) and the class III flagellar genes *fliC* (e) and *flaA* (f).

Fig. 4. Motility phenotype and electron micrographs of YpIII and $\Delta ompR$. (a) Motility assay of YpIII (1), $\Delta ompR$ (2), $\Delta ompR$ carrying pKT100 (3), $\Delta ompR$ carrying pKT-*ompR* (4), $\Delta ompR$ carrying pJB908 (5) and $\Delta ompR$ carrying pJB908-*flhDC* (6). (b) Flagella of YpIII and $\Delta ompR$ visualized by transmission electron microscopy.

Fig. 5. Roles of the O₁ and O₂ sites in the regulation of *flhDC* expression by OmpR. (a) Full-length *flhDC* promoter (F) or promoters with mutations in O₁ (O_{1m}), O₂ (O_{2m}) or both (O_{1m}O_{2m}) are shown. Mutated sequences are shown in italic and underlined. (b) Activity analyses of the four promoters shown in (a) in YpIII (white), $\Delta ompR$ (black) and its complemented strain (grey).

presence of 400 ng OmpR protein, at which concentration the wild-type Y-OF fragment was completely retarded (compare Fig. 6b with Fig. 6a). Mutation of the O₂ site resulted in a large decrease in its interaction with OmpR but did not abolish its binding because a low level of retarded Y-OF fragment was detected in the presence of 400 ng OmpR protein (Fig. 6c). However, double mutation of both the O₁ and O₂ sites completely abolished the binding of the Y-OF fragment to OmpR protein (Fig. 6d). All these results demonstrated that OmpR binds to both the O₁ and O₂ sites, and that the O₂ site showed greater affinity for OmpR protein than the O₁ site.

DISCUSSION

OmpR is an important global regulator in the up- and downregulation of more than 100 genes (Oshima *et al.*, 2002). Studies of OmpR regulation are mainly focused on

its roles in the regulation of virulence factors and outer-membrane proteins (Brzostek *et al.*, 2007; Yoshida *et al.*, 2006). OmpR has also been shown to regulate flagellar gene regulation. An earlier study in *E. coli* demonstrated that mutation of the *ompR* gene significantly increased *flhDC* expression (Shin & Park, 1995). In *Xenorhabdus nematophila*, inactivation of *ompR* promotes precocious swarming and *flhDC* expression (Kim *et al.*, 2003). However, this is not always the case in other bacteria. OmpR was demonstrated to play no role in either the motility phenotype or *flhDC* expression in *S. typhimurium* and *P. mirabilis* (Clemmer & Rather, 2007; Kutsukake, 1997). Our present study demonstrates that OmpR positively regulates *flhDC* expression and subsequently controls transcription of class II and III genes in flagella biosynthesis.

To address if this difference is due to the sequence specificity of OmpR, amino acid sequences of OmpR from different bacteria were aligned. High sequence similarity exists among these species, and V203, which is important for OmpR binding to DNA (Tran *et al.*, 2000), is conserved (Supplementary Fig. S1). This suggests that the up- or downregulation of *flhDC* transcription by OmpR is not due to dissimilarity of the OmpR protein. Since the regulatory regions of *flhDC* in different bacteria are not similar, we propose that positive or negative regulation of *flhDC* expression by OmpR is dependent on its regulatory region. In *E. coli*, OmpR binds to a region that overlaps the transcription start site of *flhDC* to negatively regulate its transcription (Shin & Park, 1995), while in *X. nematophila*, whether the regulation of *flhDC* by OmpR is direct or indirect has not been illustrated (Kim *et al.*, 2003). In this study, we have shown the binding of phosphorylated OmpR to two regions up- and downstream of the *flhDC* promoter, which suggests that the regulation of *flhDC* expression by OmpR in YpIII is direct but the mechanism is different from that in *E. coli*. It has been shown that binding of OmpR to the *flhDC* promoter in *E. coli* is intensified about 10-fold by phosphorylation of OmpR (Shin & Park, 1995). We also compared the interaction of OmpR and the *flhDC* promoter in the presence and absence of acetyl-P, and a weaker interaction was observed when OmpR was not phosphorylated (data not shown), which suggests that the regulation of *flhDC* expression by OmpR is not only direct but also enhanced by OmpR phosphorylation. This result is consistent with a report showing that the regulatory link between OmpR and outer-membrane proteins is enhanced by OmpR phosphorylation (Yoshida *et al.*, 2006).

OmpR positively regulates the expression of outer-membrane proteins in *E. coli* by directly binding to promoter regions (Yoshida *et al.*, 2006). In addition to DNA binding, OmpR was shown to interact productively with RNA polymerase to effectively regulate its transcription (Bowrin *et al.*, 1994). Analysis of OmpR structure also revealed that the large loop connecting helices $\alpha 2$ and $\alpha 3$ in the N-terminal region was the probable site of interaction with the α subunit of RNA polymerase RpoA

Fig. 6. Interactions of OmpR with the Y-OF and Y-CF fragments by DNA mobility-shift assay. Approximately 100 ng Y-OF fragment (a, white box), Y-OF with mutation at the O_1 site (b, Y-OF- O_{1m}), Y-OF with mutation at the O_2 site (c, Y-OF- O_{2m}) and Y-OF with mutations in both the O_1 and O_2 sites (d, Y-OF- $O_{1m}O_{2m}$) were incubated with varying amounts of OmpR protein (shown at the top of each lane). The positions of bound (B) and free (F) fragments are shown at the right of each picture. The interaction between OmpR and the Y-CF fragment and the influence of adding non-biotin-labelled probes on the interaction between OmpR and the Y-OF fragment are shown in black and grey boxes, respectively (a).

(Martinez-Hackert & Stock, 1997). However, there is no direct evidence for this. To explore the mechanism of OmpR regulation of *flhDC*, an *ompR* gene with mutations in amino acids G₁₉₁, E₁₉₃, A₁₉₆ and E₁₉₈, which are considered to be the active sites for interacting with RpoA (Martinez-Hackert & Stock, 1997), was expressed in $\Delta ompR$ and the expression of *flhDC* was restored (data not shown), suggesting that the upregulation of *flhDC* transcription by OmpR is not achieved by direct interaction with RpoA. It has been reported that, in the positive regulation of OmpC and OmpF by OmpR, the binding sites are always located in the -60 to -100 region upstream of the promoter, which facilitates its binding with RpoA (Aoyama & Oka, 1990). In the present study, we have shown that two OmpR-binding sites are located in the +55 to +75 and -110 to -130 regions (Fig. 2a). This result also indicates that the mechanism of positive regulation of *flhDC* by OmpR is not by direct interaction with RpoA. The details need to be further investigated.

Since the expression of flagellar genes is highly regulated by environmental conditions, regulation of *flhDC* expression is rather complicated (Soutourina & Bertin, 2003). The long non-coding sequence upstream of the *flhDC* transcript also indicates multiple layers of regulation for its transcription. Our current study focused on its positive regulation by the OmpR protein. Some other regulators might also participate in controlling the expression of *flhDC* in YpIII. As shown in Fig. 2(b), promoter activity under the full-length regulatory region was lower than under the -550 site truncated regulatory region, which indicates that some repressors may bind to the region from -550 to -750 bp to downregulate *flhDC* transcription. Further experiments will be conducted to fully characterize the regulation of *flhDC* in *Y. pseudotuberculosis*.

ACKNOWLEDGEMENTS

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

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Edited by: J. G. Shaw