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Evidence that the *Vibrio vulnificus* flagellar regulator FlhF is regulated by a quorum sensing master regulator SmcR

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A single polar flagellum and motility are potential virulence factors of *Vibrio vulnificus*, a foodborne pathogen. In the present study, the functions of FlhF and regulatory characteristics of the *flhF* expression of *V. vulnificus* were investigated. A deletion mutation in *flhF* abolished motility, flagella formation and flagellin synthesis, and introduction of *flhF in trans* complemented the defects. The *flhF* mutant revealed decreased expression of the class III and IV flagella genes, indicating that FlhF is a key regulator for the flagellar biogenesis of *V. vulnificus*. The influence of global regulatory proteins on the expression of *flhF* was examined and SmcR, a LuxR homologue, was found to downregulate *flhF* expression at the transcriptional level. SmcR represses *flhF* expression only in the stationary phase of growth and exerts its effects by directly binding to the *flhF* promoter region. Finally, an SmcR binding site, centred at 22.5 bp upstream of the transcription start site, was identified by a DNase I protection assay. The combined results demonstrate that a quorum sensing master regulator SmcR influences the motility and flagellar biogenesis of *V. vulnificus* through modulating the expression of FlhF in a growth-phase-dependent manner.

INTRODUCTION

Vibrio vulnificus, an opportunistic Gram-negative pathogen, is the causative agent of food-borne diseases such as gastroenteritis and life-threatening septicaemia (Jones & Oliver, 2009). A single polar flagellum provides the bacterium with an effective means of motility and plays a crucial role in adhesion, cytotoxicity, biofilm formation and lethality to mice (Kim & Rhee, 2003; Lee *et al.*, 2004). Recently, upon completion of the genome sequence of *V. vulnificus* MO6-24/O (GenBank accession no. CP002469.1), over 60 genes, presumably involved in flagellar synthesis, were identified. Although the genes are mostly currently uncharacterized, the high level of similarity found in the organization and deduced amino acid sequences (over 70 % identity) of the flagella genes of *V. vulnificus* and *Vibrio cholerae* (GenBank accession no. AE003852) indicate that the genes might perform similar functions in flagellar synthesis.

The functions of the flagella genes of *V. cholerae* and their regulatory mechanisms are well characterized at the molecular level (Correa *et al.*, 2005; Moisi *et al.*, 2009). The flagella genes of *V. cholerae* have been categorized into four classes based on the hierarchy of their transcription. FlrA, an RpoN-dependent activator, is the only class I gene product and activates the expression of class II genes comprising those primarily for structural components of export apparatus, switch and MS (membrane/supramembrane) ring, and those for transcriptional factors including FliA, FlrB, FlrC, FlhF and FlhG. The expression of class III genes encoding the hook, basal body and core flagellin FlaA, and class IV genes encoding additional flagellins and motor components are then regulated by the class II transcription factors (Correa *et al.*, 2005; Moisi *et al.*, 2009).

Among these, FlhF is not found in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, which produces peritrichous, rather than polar, flagella (McCarter, 2001). Disruption and overexpression of *flhF* led to a lack of a polar flagellum in *V. cholerae* and possession of multiple polar flagella in *V. alginolyticus*, respectively (Correa *et al.*, 2005; Green *et al.*, 2009; Kojima *et al.*, 2011; Kusumoto *et al.*, 2006, 2008, 2009), indicating that FlhF is a key regulator conferring the synthesis and number of the polar flagella in *Vibrio* species. Nevertheless, neither the functions of *flhF* nor the regulatory mechanisms for *flhF* expression in *V. vulnificus* have been reported previously. Accordingly, in the present study, the functions of *V. vulnificus flhF* were determined by construction of an *flhF* deletion mutant and comparing its motility and flagellar

Abbreviation: qRT-PCR, quantitative real-time PCR.

Three supplementary figures are available with the online version of this paper.

synthesis with those of the parental wild-type. A single promoter for the expression of *flhF* was mapped and its regulatory characteristics were analysed. It appears that FlhF is essential for the synthesis of the *V. vulnificus* polar flagella and its expression is downregulated by SmcR, a quorum sensing master regulator and a homologue of *V. harveyi* LuxR, at the transcriptional level in a growth-phase-dependent manner.

METHODS

Strains, plasmids and culture conditions. The strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, *V. vulnificus* strains were grown in Luria–Bertani (LB) medium supplemented with 2.0 % (w/v) NaCl (LBS) at 30 °C with aeration. A mobilizable plasmid pKS1101 (Table 1) was constructed by cloning *oriT* originated from pCOS5 into pBAD24 containing an L-arabinose-inducible promoter, as described previously (Nakhamchik *et al.*, 2008). The *flhF* and *smcR* coding regions were subcloned into pKS1101 to result in pKS1102 and pKS1107, respectively (Table 1). For complementation tests, L-arabinose was added to the cultures at a final concentration of 0.02 % (w/v) to induce the expression of recombinant *flhF* (i.e. on pKS1102) or *smcR* (i.e. on pKS1107), as indicated. Bacterial growth was monitored by measuring the optical density of the cultures at 600 nm.

Generation of the *V. vulnificus flhF* deletion mutant. The *flhF* gene was inactivated *in vitro* by deletion (1379 bp of 1494 bp) of the *flhF* ORF using the PCR-mediated linker-scanning mutation method, as described previously (Jeong *et al.*, 2010). Pairs of primers FLHF_5(F) and FLHF_5(R) (for amplification of the 5′ amplicon) or FLHF_3(F) and FLHF_3(R) (for amplification of the 3′ amplicon) were designed using the genome sequence of *V. vulnificus* MO6-24/O (GenBank accession nos CP002469.1 and CP002470.1) (Table 2). The 1379 bp-deleted *flhF* was amplified by PCR using a mixture of both amplicons as the template and FLHF_5(F) and FLHF_3(R) as primers. The resulting 1702 bp DNA fragment containing the deleted *flhF* was ligated with an *Sph*I–*Pst*I-digested suicide vector pDS132 (Philippe *et al.*, 2004) to generate pKS0908. The *E. coli* SM10 *λpir*, *tra* strain (containing pKS0908) (Miller & Mekalanos, 1988) was used as a conjugal donor to *V. vulnificus* MO6-24/O. The conjugation and isolation of the transconjugants were conducted using the methods described previously (Jeong *et al.*, 2003a).

Motility assay and transmission electron microscopy. *V. vulnificus* strains from cultures grown to OD_{600} 0.5 were stabbed into semi solid motility agar (LBS with 0.3 % agar) by using a sterilized toothpick. The plates were incubated for 16 h at 30 °C and photographed by using a digital imaging system (UTA-1100, UMAX Technologies). For transmission electron microscopy, strains were grown to an OD_{600} 0.5, centrifuged and resuspended in PBS. Bacterial cells were adhered to a formvar-coated grid and negatively stained with a 2 % solution of uranyl acetate before microscopy with a JEM1010 transmission electron microscope (JEOL) (Correa *et al.*, 2005).

Preparation of protein samples and Western blot analysis. Bacterial cultures grown to OD_{600} 0.5 were spun down and proteins in the cell-free supernatants were concentrated 10-fold using a 10 kDa cut-off centrifugal filter (Amicon Ultra, Millipore) (Ghelardi *et al.*, 2002). The pelleted bacterial cells were washed and lysed using complete lysis-B buffer (Roche) for 1 min, and residual cell debris was removed by centrifugation. Protein samples from the concentrated supernatants and cell lysates, equivalent to 25 μg total protein, were resolved by using 12 % SDS-PAGE (Sambrook & Russell, 2001). A Western immunoblot of flagellin proteins was performed as described previously (Jeong *et al.*, 2003a) using a rabbit anti-flagellin antiserum purchased from Abcam.

RNA purification and transcript analysis. Total cellular RNA from the *V. vulnificus* strains was isolated using an RNeasy minikit

Table 1. Strains and plasmids used in this study

Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
V. vulnificus		
MO6-24/O	Clinical isolate; virulent	Wright *et al.* (1990)
KS13	MO6-24/O with Δ*flhF*	This study
HS03	MO6-24/O with *smcR*::*nptI*; Km^r	Jeong *et al.* (2003a)
E. coli		
SM10 *λpir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu *λpir*, *oriT* of RP4, Km^r; conjugational donor	Miller & Mekalanos (1988)
Plasmids		
pBAD24	ColE1 *ori*; *araBAD* promoter; Ap^r	Guzman *et al.* (1995)
pCOS5	OriV OriT Ap^r Cm^r *cos*	Connell *et al.* (1995)
pDS132	R6K γ *ori*; *sacB*; suicide vector; *oriT* of RP4; Cm^r	Philippe *et al.* (2004)
pHS104	pRSET C with *smcR*; Ap^r	Jeong *et al.* (2003a)
pKS0908	pDS132 with Δ*flhF*; Cm^r	This study
pKS1002	pRKΩ*lacZ* with 485 bp fragment of *flhF* upstream region; Tc^r	This study
pKS1101	pBAD24 with *oriT* of RP4; Ap^r	This study
pKS1102	pKS1101 with *flhF*; Ap^r	This study
pKS1107	pKS1101 with *smcR*; Ap^r	This study

Table 2. Oligonucleotides used in this study

Oligonucleotide*	Oligonucleotide sequence (5′–3′)†	Location‡	Use(s)
FLHF_5(F)	AGATTTTGGCTTTCTGATCCCGC	VVMO6_00833	Construction of the *flhF* mutant
FLHF_5(R)	TTGCGGCCGCAAAATCGTTTTATTTT	VVMO6_00833	
FLHF_3(F)	TTGCGGCCGCAACCAAAATATTTGAT	VVMO6_00833	
FLHF_3(R)	GCAAACAATTCTCGACCTTCTCGG	VVMO6_00833	
FLEQ_qRT(F)	GGGTTATCGTTGGTTTCGT	VVMO6_00814	qRT-PCR
FLEQ_qRT(R)	TCAGGTTAGGCAGGTGTT	VVMO6_00814	
FLES_qRT(F)	GAGGTGGAAGAGGAGCAA	VVMO6_00815	
FLES_qRT(R)	CATTGACTGGGCGGAAAA	VVMO6_00815	
FLIE_qRT(F)	GATGTTTCTCTTTCCGATGTCA	VVMO6_00817	
FLIE_qRT(R)	ACTGGCATGTTCATAAGGTC	VVMO6_00817	
FLIF_qRT(F)	CGAGGAGATGAAGCAAGTG	VVMO6_00818	
FLIF_qRT(R)	GGTATTTAAGGTCAAGAACACAGA	VVMO6_00818	
FLIG_qRT(F)	ATGGATACACCAGAAGTTGATAT	VVMO6_00819	
FLIG_qRT(R)	TCTAAGTGGCGGATAATGC	VVMO6_00819	
FLAF_qRT(F)	CCATTATGCAGACTGCGGAA	VVMO6_00807	
FLAF_qRT(R)	GAAAGATCACGCATCCTTTG	VVMO6_00807	
FLAA_qRT(F)	CAGTCTTTCCAAATTGGTGC	VVMO6_00809	
FLAA_qRT(R)	TATCTGAACGAAGGTTACCC	VVMO6_00809	
FLAG_qRT(F)	TCAATAAAGGGTTATCTTTT	VVMO6_00810	
FLAG_qRT(R)	TTGGCTTCATAAATCGTCAC	VVMO6_00810	
FLAE_qRT(F)	GAACTCCGATGATGATCGGC	VVMO6_02251	
FLAE_qRT(R)	AAGATGAAGTGGAAAGAATA	VVMO6_02251	
FLAD_qRT(F)	GTTCACCAGCTAGGCTACCT	VVMO6_02252	
FLAD_qRT(R)	CTACAGATCTTTGCTGGTAA	VVMO6_02252	
FLAC_qRT(F)	CTTCACTTACCGATGCGTTG	VVMO6_02255	
FLAC_qRT(R)	GATGATATTGAAGAATTGGC	VVMO6_02255	
SMCR_qRT(F)	CACACACTTCACCACGCTCAATG	VVMO6_00535	
SMCR_qRT(R)	AACATCGCCAACATCACCAACG	VVMO6_00535	
FLHF_qRT(F)	CGGGTTTATTGTGGCAGGAAGTG	VVMO6_00833	
FLHF_qRT(R)	CTGAGACCAAAGCGAGCAAAGC	VVMO6_00833	
FLH_AF(F)	AAGGCGAACCAGCAGTCTTACTCA	−178 to −155	EMSA DNase I footprinting
FLH_AF(R)	TCAATCGCAGCGACGATTTCAACG	+157 to +134	EMSA DNase I footprinting Primer extension

*The oligonucleotides were designed using the *V. vulnificus* MO6-24/O genome sequence (GenBank accession nos CP002469.1 and CP002470.1).
†Regions of oligonucleotides not complementary to *flhF* are underlined.
‡Locus tag numbers are based on the database of the *V. vulnificus* MO6-24/O genome sequence. The oligonucleotide positions are shown as numbers, where +1 is the transcription start site of *flhF*.

(Qiagen). For quantitative real-time PCR (qRT-PCR), cDNA was synthesized by using the iScript cDNA Synthesis kit (Bio-Rad) and real-time PCR amplification of the cDNA was performed by using the Chromo 4 real-time PCR detection system (Bio-Rad). Specific primers used for amplification of the cDNA are listed in Table 2. Relative expression levels of transcripts were calculated by using the 16S rRNA expression level as the internal reference for normalization as described previously (Sultan *et al.*, 2010). The 16S rRNA expression level did not differ between the different time points and strains used in this study.

For the primer extension experiments, an end-labelled 24-base primer FLH_AF(R) complementary to the coding region of *flhF* was added to the RNA (Table 2), and then extended with SuperScript II RNase H^- reverse transcriptase (Invitrogen) as described previously (Jeong *et al.*, 2010). The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders of pKS1002 generated using FLH_AF(R) as a primer. The primer extension products were visualized using a phosphorimage analyser (model BAS1500, Fuji Photo Film).

EMSA and DNase I footprinting. The 335 bp upstream region of *flhF*, extending from residue −178 to +157, was amplified by PCR using ^{32}P-labelled FLH_AF(R) and unlabelled FLH_AF(F) as the primers (Table 2). The expression and purification of the His-tagged SmcR were carried out using pHS104, carrying the *V. vulnificus smcR* gene, as described previously (Jeong *et al.*, 2003a). Binding of SmcR to the labelled DNA and electrophoretic analysis of the DNA–SmcR complexes have already been described (Jeong *et al.*, 2003a).

The same labelled 335 bp DNA was used for the DNase I protection assays. The binding of SmcR to the labelled DNA, and DNase I

digestion of the DNA–SmcR complexes followed the procedure previously described by Jeong *et al.* (2003a). After precipitation with ethanol, the digested DNA products were resolved on a sequencing gel alongside sequencing ladders of pKS1002 generated using FLH_AF(R) as the primer. The gels were visualized as described above for the primer extension analyses.

Data analyses. Means ± SEM were calculated from at least three independent experiments. Data were analysed by Student's *t* test with the SAS program (SAS software; SAS Institute). Significance of differences between experimental groups was accepted at a *P*-value <0.05.

RESULTS

Effects of an *flhF* mutation on motility and flagellar synthesis

The *V. vulnificus flhF* isogenic mutant KS13 (Table 1) was constructed by allelic exchange. Double crossovers, in which the wild-type *flhF* on the chromosome was replaced with the 1379 bp-deleted *flhF* allele, were confirmed using previously described methods (Jeong *et al.*, 2003a) (data not shown). The *flhF* mutant KS13 was non-motile, as determined by its ability to migrate on a semisolid plate surface compared with that of the wild-type (Fig. 1a, b). KS13 cells that were stained and observed by using transmission electron microscopy lacked flagella (Fig. 1c). For complementation of the *flhF* mutant, a recombinant *flhF* (pKS1102) was introduced into KS13. When *flhF* was induced by L-arabinose, the motility was restored to a level comparable to the wild-type level, and a single polar flagellum was produced (Fig. 1). These results suggested that FlhF is required for synthesis of flagella in *V. vulnificus* as was previously noted in *V. cholerae* (Correa *et al.*, 2005).

To extend our understanding of the role of FlhF in flagellar synthesis, flagellin synthesis from the wild-type and *flhF* mutant was examined using Western blot analysis. Flagellin proteins were not detected in the supernatants and cell lysates of the *flhF* mutant and the lack of flagellin synthesis in the *flhF* mutant was restored by the introduction of pKS1102 (Table 1; Fig. 2a). To determine whether FlhF affects the transcription of flagella genes, expression levels of the genes were measured by qRT-PCR analyses. It is noteworthy that the levels of transcripts of the genes classified as class III (*flaC*) and class IV (*flaFAG flaDE*) flagella genes, presumably involved in flagellin synthesis (Klose & Mekalanos, 1998), decreased following the mutation of *flhF* (Fig. 2b). In contrast, levels of the transcripts of class I (*fleQ*) and class II (*fleS*, *fliEFG*) flagella genes were not substantially affected by the mutation of *flhF* (Fig. 2b). The results combined indicate that FlhF is

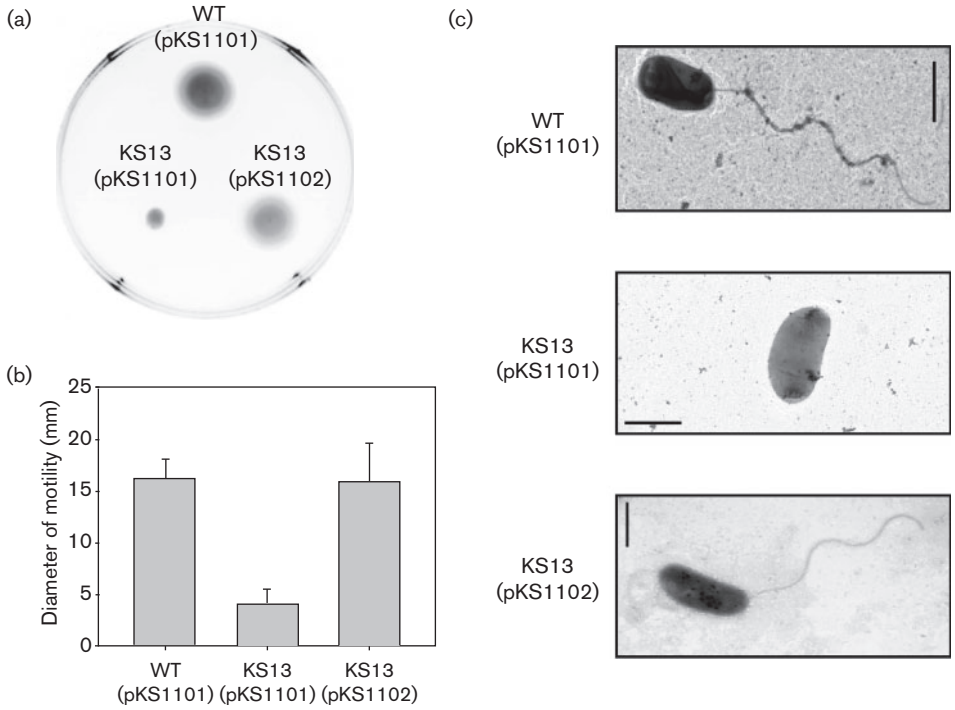

Fig. 1. Motility and electron micrographs of the *V. vulnificus* strains. (a) The areas of motilities of the strains grown for 16 h on LBS plates with 0.3% soft agar. (b) The diameters of motility areas are the mean ± SEM of results from three independent experiments. (c) Liquid-grown cells were negatively stained with 2% (w/v) uranyl acetate and then observed by using a transmission electron microscope. Bars, 1 μm. WT (pKS1101), wild-type; KS13 (pKS1101), *flhF* mutant; KS13 (pKS1102), complemented strain.

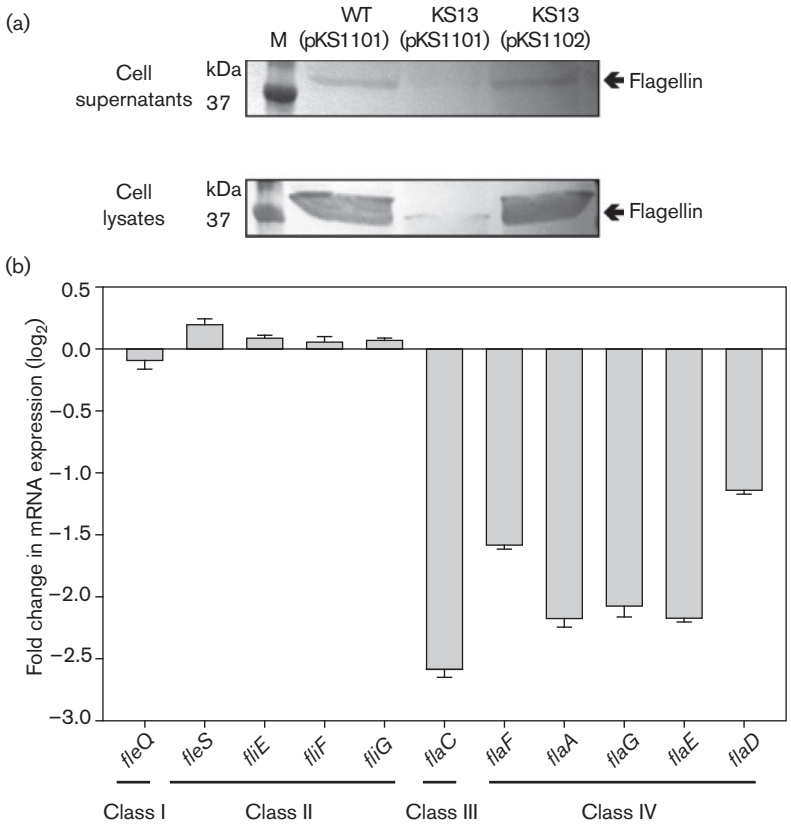

Fig. 2. Effects of the *flhF* mutation on flagellin synthesis and flagellar gene expression. (a) The cell lysates or the concentrated supernatants, equivalent to 25 μg total proteins, were resolved by SDS-PAGE, and flagellin proteins were detected by Western blot analysis using a rabbit anti-flagellin antiserum. M, Protein size markers (Bio-Rad). (b) The relative levels of flagellar gene expression were determined by qRT-PCR analyses. Each column represents the mRNA expression level in the *flhF* mutant relative to that in the wild-type. Gene names are based on the database of the *V. vulnificus* MO6-24/O genome, which was retrieved from GenBank (CP002469.1; CP002470.1). The expression levels of the flagella genes are the mean ± SEM of results from three independent experiments.

essential for flagella synthesis of *V. vulnificus* and positively regulates the transcription of both class III and IV flagella genes.

Effect of an *smcR* mutation on *flhF* transcription

A single reverse transcript was identified from the primer extension of the RNA isolated from the wild-type cells (Fig. 3a). The 5′ end of the *flhF* transcript is located 23 bp upstream of the translation initiation codon of *flhF* and was subsequently designated +1. The putative promoter constituting this transcription start site was named P_{flhF} to represent the *flhF* promoter.

The P_{flhF} activities were compared in the wild-type and mutants which lack transcription factors SmcR, RpoS (Jeong *et al.*, 2003a), CRP (Jeong *et al.*, 2001), ToxRS and LRP (Jeong *et al.*, 2003b) in order to extend our understanding of the regulation of *flhF* expression. The P_{flhF} activity increased in the mutant HS03 which lacks SmcR (Table 1), as determined based on the intensity of

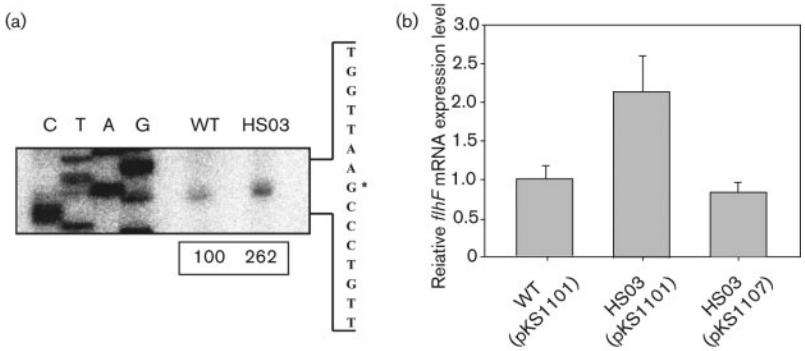

Fig. 3. Effects of the *smcR* mutation on the *flhF* expression. (a) The P_{flhF} activities were determined by primer extension of the RNA derived from each strain grown to stationary phase (OD_{600} 2.0). Lanes C, T, A and G represent the nucleotide sequencing ladders of pKS1002. The asterisk indicates the transcription start site of P_{flhF}. The P_{flhF} activity of the *smcR* mutant HS03 is presented relative to that of the wild-type (WT). (b) The relative levels of *flhF* expression in each strain were determined by qRT-PCR analyses and normalized to the 16S rRNA expression level of the wild-type, normalized to 1. The *flhF* expression levels are the mean ± SEM of results from three independent experiments.

the *flhF* reverse transcript band of primer extension analyses (Fig. 3a). The upregulation of P_{flhF} activity due to the disruption of *smcR* was apparent only when the P_{flhF} activities of the wild-type and HS03 grown to stationary phase (OD_{600} 2.0) were compared (Fig. 3a). In contrast, the P_{flhF} activities did not differ in the wild-type, *rpoS*, *crp*, *toxRS* or *lrp* mutants grown to stationary phase (Fig. S1, available with the online version of this paper).

To confirm the effect of SmcR on the expression of *flhF*, the relative levels of the *flhF* transcript in the same amount of total RNA isolated from the wild-type and *smcR* mutant HS03 grown to stationary phase were compared by using qRT-PCR analyses (Fig. 3b). Consistent with the results of the primer extension analyses, the transcription of *flhF* increased significantly in HS03 (Fig. 3b). The increased *flhF* transcription in HS03 was restored to a level comparable with that of the wild-type by introducing pKS1107 carrying a recombinant *smcR* (Table 1; Fig. 3b). Overall, these results led us to conclude that the expression of *flhF* in *V. vulnificus* is under the negative control of SmcR, at least during stationary phase.

Growth-phase-dependent expression of *flhF* and its effect on motility

The relative levels of the *flhF* transcript in the wild-type were determined at the indicated time intervals by using qRT-PCR. The *flhF* transcript appeared at a maximum level in the exponential-phase cells and decreased on the entry of the *V. vulnificus* into stationary phase (Fig. 4a). The *flhF* transcript level of the stationary-phase cells was about fourfold less than that of the exponential-phase cells. In contrast, the relative levels of the *smcR* transcript in the wild-type, determined by qRT-PCR, increased as the bacterial culture entered stationary phase (Fig. 4a). This result was consistent with our previous observation that the cellular level of SmcR was higher in stationary-phase cells than in exponential-phase cells (Jeong *et al.*, 2003a). This result indicates that the decrease in the level of *flhF* expression in the stationary-phase cells correlated with the increased cellular level of SmcR, and suggests that SmcR plays a major, if not sole, role for the growth-phase-dependent variation of the *flhF* expression. Consistent with this, no significant changes in the level of *flhF* transcript were observed in the *smcR* mutant entering stationary phase (Fig. 4a). It is noteworthy that the levels of *flhF* in the wild-type and *smcR* mutant are not significantly different in exponential phase, supporting the notion that SmcR regulates *flhF* expression in a growth-phase-dependent manner (Fig. 4a).

To examine whether the decreased level of *flhF* transcript by SmcR is associated with the alteration in motility, the *smcR* mutant was tested for its ability to migrate on a semisolid plate surface. As shown in Fig. 4(b), the *smcR* mutant was more motile than the wild-type strain, supporting our previous observation that FlhF function is required for motility of *V. vulnificus* (Fig. 1a).

SmcR binds specifically to the *flhF* promoter region

The 335 bp DNA fragment encompassing the *flhF* promoter region was incubated with increasing amounts of SmcR and then subjected to electrophoresis. As seen in Fig. 5(a), the addition of SmcR at 25 nM resulted in a shift of the 335 bp DNA fragment to a single band with a slower mobility. The binding of SmcR was also specific because assays were performed in the presence of 100 ng poly(dI-dC) as a non-specific competitor. In the EMSA, the *flhF* promoter region did not form any intermediate bands that were chased away to a slower migrating band at higher concentrations of SmcR. This pattern of migration suggests that a single binding site for SmcR is present in the *flhF* promoter region. In a second EMSA, the same, but unlabelled, 335 bp DNA fragment was used as a self-competitor to confirm the specific binding of SmcR to the *flhF* promoter region. The unlabelled 335 bp DNA competed for the binding of SmcR in a dose-dependent manner (Fig. 5b),

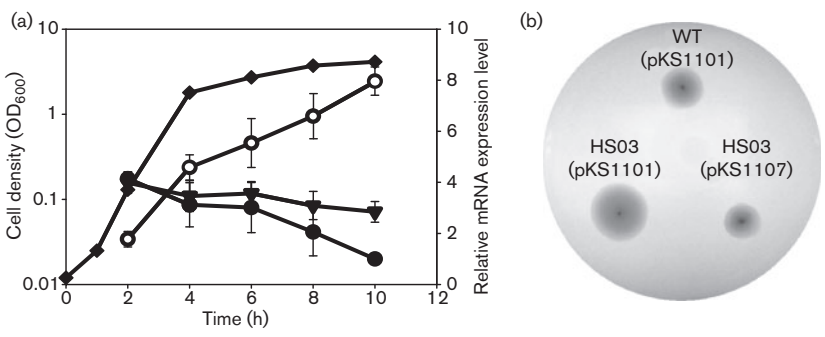

Fig. 4. Growth-phase-dependent expression of *smcR* and *flhF* and motility of the *V. vulnificus* strains. (a) The wild-type and *smcR* mutant HS03 was grown with LBS and samples removed at the indicated time points were analysed for growth (OD_{600}) and expression of *smcR* and *flhF*. The expression levels of *smcR* and *flhF* were determined by qRT-PCR analyses and normalized to 16S rRNA expression level. The relative levels of *smcR* and *flhF* expression are the mean ± SEM of results from three independent experiments. ◆, Cell density of WT; ○, *smcR* mRNA of WT; ▼, *flhF* mRNA of HS03; ●, *flhF* mRNA of WT. (b) The areas of motilities of the strains grown for 16 h on LBS plates with 0.3 % soft agar were photographed.

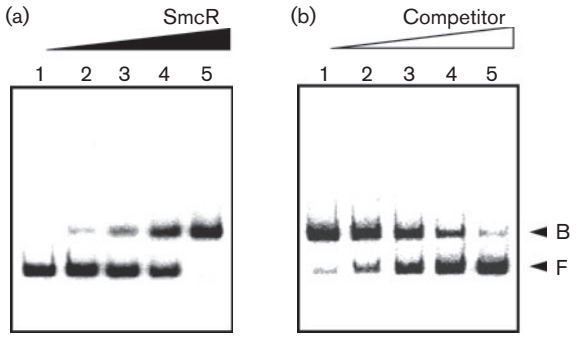

Fig. 5. EMSA for SmcR binding to the P_{flhF} regulatory region. (a) The radiolabelled 335 bp P_{flhF} regulatory region (7 nM) was mixed with increasing amounts of SmcR (0, 25, 50, 100 and 200 nM in lanes 1–5, respectively) and then resolved on a 5% polyacrylamide gel. (b) For a competition analysis, the same but unlabelled 335 bp DNA fragment was used as a self-competitor DNA. The self-competitor DNA (at 0, 15, 50, 125 and 250 nM in lanes 1–5, respectively) was added to the reaction mixture containing the labelled DNA (7 nM) prior to the addition of 150 nM SmcR. B, Bound DNA; F, free DNA.

confirming that SmcR binds specifically to the *flhF* promoter region.

Identification of the SmcR binding site

To determine the precise location of the SmcR binding site in the *flhF* promoter region, a DNase I footprinting experiment was performed using the same 335 bp DNA fragment used for the EMSA. DNase I footprinting revealed a clear protection pattern in the upstream region of *flhF* between −33 and −12 (Fig. 6a). This SmcR binding site is centred 22.5 bp upstream of the transcriptional start site of *flhF*. The sequences for SmcR binding overlap with the sequences of the −35 and −10 regions of P_{flhF}. These results indicate that SmcR bound to the binding site could hinder RNA polymerase binding and thereby repress its activity. This idea supported our earlier observation that SmcR negatively regulates P_{flhF} (Figs 3 and 4). Taken together, these results demonstrate that SmcR represses the *flhF* expression by directly binding to P_{flhF}.

DISCUSSION

Many bacteria monitor their cell population densities through the exchange of diffusible signal molecules (AIs, autoinducers) that accumulate extracellularly. This type of communication, termed quorum sensing, has been recognized as a global regulatory system controlling the expression of numerous genes in bacteria (Fuqua & Greenberg, 2002; Ng & Bassler, 2009). The *Vibrio harveyi* regulation of bioluminescence is frequently used as a model for quorum sensing. LuxR, a transcriptional activator of the luminescence operon, is a quorum sensing master regulator in *V. harveyi* and its cellular level is controlled by the levels of AIs in a cell-density-dependent manner (Waters & Bassler, 2006). LuxR homologues such as *V. vulnificus* SmcR, *V. cholerae* HapR, *V. parahaemolyticus* OpaR and *V. anguillarum* VanT have been identified and proposed to control the genes contributing to survival as well as pathogenesis of the pathogenic *Vibrio* species (Beyhan *et al.*, 2007; Croxatto *et al.*, 2002; Jobling &

Fig. 6. Identification of the SmcR binding site using DNase I protection analysis and sequence analysis of the P_{flhF} regulatory region. (a) The radiolabelled 335 bp P_{flhF} regulatory region (7 nM) was incubated with increasing amounts of SmcR, then digested with DNase I. Lanes: 1, no SmcR added; 2–5, SmcR added at 50, 100, 200 and 250 nM, respectively. Lanes C, T, A and G represent the nucleotide sequencing ladders of pKS1002. The protection and hypersensitivity in the presence of SmcR are indicated by the open box and thick line, respectively. (b) The transcription start site is indicated by a bent arrow (P_{flhF}). The sequences proposed for the binding site of SmcR are presented in a shaded box and the putative −10 and −35 regions are underlined. The consensus nucleotide sequences for the binding of SmcR (Lee *et al.*, 2008) are indicated above the *V. vulnificus* DNA sequence in capital letters. TTG translation initiation codon and putative ribosome-binding site (ACGA) are indicated in bold type. W, A or T; R, A or G; N, any base.

Holmes, 1997; McCarter, 1998; McDougald *et al.*, 2001; Zhu *et al.*, 2002).

There have been a few studies demonstrating that quorum sensing is involved in flagellar biogenesis, which consequently affects the motility of *Vibrio* species (Tian *et al.*, 2008; Zhu *et al.*, 2002). Recently, a genome-wide search using a consensus sequence for SmcR binding predicted that three flagella genes, including *flhF* (VV1_1950, GenBank accession no. AE016795), are under the control of SmcR (Lee *et al.*, 2008). However, until now, no definitive analysis of the role of the LuxR homologues in flagellar gene expression has been reported to our knowledge. Neither the promoter(s) of the quorum-sensing-controlled flagella genes nor LuxR binding sites upstream of the genes has been identified previously. Therefore, the question of whether LuxR directly or indirectly affects flagella production has not been yet addressed. This study has demonstrated that SmcR represses the expression of FlhF, a regulator for flagellin synthesis genes, when *V. vulnificus* enters the stationary growth phase (Figs 3 and 4a). The specific SmcR binding sequences have been determined (Fig. 6), and the assigned sequences for the SmcR binding (TAACTGATCTATTA-ATTAATAA) in the *flhF* promoter region scored 86 % similarity to the consensus SmcR binding sequences that were previously identified by our group (Lee *et al.*, 2008).

The possible benefits that the bacteria can obtain from the stationary-phase-specific repression of the *flhF* expression are not clear yet. However, when the bacteria invade the human gut, increased competition for the specific nutrients imposed by the host cells and endogenous bacterial flora could starve the bacteria and limit their growth to stationary phase. Therefore, stationary-phase-specific repression of *flhF* expression could result in saving the limited nutrients from being used up for flagellar synthesis. The remaining nutrients could alternatively promote the expression of the stationary-phase-specific genes responsible for increased resistance to a range of stresses and thus provide the bacteria with better chance of survival in the adverse environments frequently encountered in hosts.

LuxR homologues, including SmcR, of *Vibrio* species are proposed to sense the point at which their cell densities reach higher than critical levels (Fuqua & Greenberg, 2002; Ng & Bassler, 2009). It is still difficult to define the implications of the quorum sensing downregulation of flagellar synthesis (and thus motility) in the pathogenesis of *V. vulnificus*. Nonetheless, we speculate that during the initial stage of infection, *smcR* expression is repressed because of low cell density, and expression of *flhF* is allowed, leading to flagellar synthesis. The flagellum primes *V. vulnificus* for initial colonization of host intestinal tissue, which is an important step required for the onset of its infectious cycle. In contrast, upon establishing preferred colonization niches with the increase in population density, the motility is superfluous, even detrimental, for a successful infection of hosts by the bacteria. In fact, flagellins of many enteropathogens have been well characterized as a major inducer as well as a target of host innate immune responses (Hayashi *et al.*, 2001; Lee *et al.*, 2006; Smith *et al.*, 2003). It has already been demonstrated that *V. cholerae* integrates flagellar synthesis and quorum sensing regulatory pathways for optimal colonization and disease progression (Liu *et al.*, 2008). In this context, we postulate that the temporally (e.g. stationary-phase-specific) and spatially (e.g. cell-density-dependent) integrated regulation of flagellar synthesis could ensure the overall success of *V. vulnificus* during pathogenesis.

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