INTRODUCTION

*Vibrio vulnificus*, an opportunistic Gram-negative pathogen, is the causative agent of food-borne diseases such as gastroenteritis and life-threatening sepsicaemia (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 FlaA, 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 structure with those of wild-type and a *flhF* complemented strain.
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 \( l^{\text{-}}\)-arabinose-inducible promoter, as described previously (Nakahamchik 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, \( l^{\text{-}}\)-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 \( SphI–PstI \)-digested suicide vector pDS132 (Philippe et al., 2004) to generate pKS0908. The \( E.\ coli \) SM10 \( \lambda \text{pir} \), \( trai \) 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 stained 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 \( \mu \)g 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 RNasey minikit.
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., 2003a). 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).

Table 2. Oligonucleotides used in this study

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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.

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EMS A and DNase I footprinting. The 335 bp upstream region of \( flhF \), extending from residue −178 to +157, was amplified by PCR using \( ^{32}\text{P} \)-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 pH5104, 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.
essential for flagella synthesis of \textit{V. vulnificus} and positively regulates the transcription of both class III and IV flagella genes.

**Effect of an \textit{smcR} mutation on \textit{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 \textit{flhF} transcript is located 23 bp upstream of the translation initiation codon of \textit{flhF} and was subsequently designated +1. The putative promoter constituting this transcription start site was named P\textsubscript{flhF} to represent the \textit{flhF} promoter.

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

**Fig. 2.** Effects of the \textit{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 \textit{flhF} mutant relative to that in the wild-type. Gene names are based on the database of the \textit{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.

**Fig. 3.** Effects of the \textit{smcR} mutation on the \textit{flhF} expression. (a) The P\textsubscript{flhF} activities were determined by primer extension of the RNA derived from each strain grown to stationary phase (OD\textsubscript{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\textsubscript{flhF}. The P\textsubscript{flhF} activity of the \textit{smcR} mutant HS03 is presented relative to that of the wild-type (WT). (b) The relative levels of \textit{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 \textit{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, cpr, 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. 3a). 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.](image)
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 &
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 (TAACTGATCTATTAATTAATA) 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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