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NOTE

Mutations Upregulating the *flhDC* Operon of *Escherichia coli* K-12

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Bacterial motility is governed by the *flhDC* master operon that is under the control of factors like OmpR, LrhA, HdfR, and H-NS. Previously, derivatives of the wild-type MG1655 strain of *E. coli* K-12 with enhanced motility were found to contain insertion sequences (ISs) in the regulatory region of the *flhDC* operon. Here, we report that not only integrations of IS insertion sequences into the regulatory region of the *flhDC* operon, but also a missense mutation in the *lrhA* gene enhances motility by relieving transcriptional repression of the *flhDC* operon. Two novel IS insertions were found upstream of *flhDC*. So far, the relationships between the *trans*-acting factors and the *cis*-acting regulatory sequences associated with the *flhDC* operon have not been clearly established. In this study, it was found that effects of the *cis*- and *trans*-acting mutations were acting in parallel, suggesting their apparently independent regulation of flagellar expression.

***Keywords*:** *flhDC*, *lrhA*, insertion sequence, motility

The *Escherichia coli* flagellar system, consisting of over 40 genes, is required for the assembly and function of flagella, as well as for sensory signaling (Kawagishi *et al*., 1996). Expression of the genes is regulated in a cascade fashion. At the top of the hierarchy is the master operon, *flhDC*, which is essential for the expression of downstream flagellar genes (Komeda, 1982). The downstream genes include *fliA*, encoding the alternative sigma factor σ^F, which controls expression of the class III genes of the flagellar regulon (Arnosti and Chamberlin, 1989). The flagellar motor consists of at least five proteins; MotA, MotB, FliG, FliM, and FliN. The MotAB stator complex functions as a transmembrane proton transporter, and the FliG, FliM, and FliN proteins form a rotor complex which is involved in flagellar assembly, rotation, and switching (Yamaguchi *et al*., 1986; Blair and Berg, 1990; Francis *et al*., 1994; Garza *et al*., 1996).

The regulatory region of the *flhDC* operon is under control of several regulatory proteins. These are CAP, OmpR, H-NS, LrhA, and HdfR (Bertin *et al*., 1994; Shin and Park, 1995; Soutourina *et al*., 1999; Ko and Park, 2000a, 2000b; Lehnen *et al*., 2002). CAP is involved in catabolic repression, and activates the *flhDC* operon (Soutourina *et al*., 1999). OmpR, as a two-component system consisting of OmpR and EnvZ, negatively regulates flagellar expression by sensing either osmolarity or the internal level of acetyl phosphate (Shin and Park, 1995). H-NS-deficient strains are nonflagellated because of reduced transcription of *flhDC* (Ko and Park, 2000b). The positive regulation of H-NS on *flhDC* is mediated by the HdfR repressor, which directly binds to the regulatory region of the *flhDC* operon (Ko and Park, 2000a). LrhA, one of the LysR family, negatively regulates *flhDC* expression and positively auto-regulates itself (Lehnen *et al*., 2002). The binding regions of the proteins in the operator of *flhDC* are presented in Fig. 1.

The insertion sequence (IS) elements contribute to the plasticity of genomic variation (Naas *et al*., 1994, 1995). They do not exert specific function, other than being involved in mobility and nonrandom insertions into the *E. coli* genome (Boyd and Hartl, 1997). To date, many IS elements have been observed to activate expression of adjacent genes, for example, through the formation of hybrid promoters or disruption of transcriptional repression. In *E. coli*, the *bgl* and *ade* operons are well known examples of activation by an IS element inserted upstream or downstream of the promoter (Reynolds *et al*., 1981; Petersen *et al*., 2002). It was found that motile derivatives of MG1655 acquired an IS insertion in the regulatory region of *flhDC*, thereby increasing expression of the *flhDC* operon and down-stream genes (Barker *et al*., 2004). Since the transcription start site of *flhDC* was not altered by an insertion of IS, the upregulation was thought to be due to a relieving of transcriptional repression (Barker *et al*., 2004).

In this study, we isolated motile derivatives of *E. coli* strain MG1655, and characterized the mutations and their phenotypes. Two types of mutations were found to increase *flhDC* expression. One is an insertion of a *cis*-acting IS5 in the regulatory region of *flhDC*, while the other is a missense mutation in the *lrhA* gene, apparently acting *in trans*. We also observed that the *cis*- and *trans*-acting regulations function independently in *flhDC* expression as well as in motility.

We attempted to isolate motile derivatives from the wild-type strain MG1655 as described in a previous study (Barker *et al*., 2004). Overnight cultures of MG1655 in TB was inoculated onto the center of a motility agar plate and incubated at 30°C for 10 h. Outgrowths were observed for MG1655 around the point of inoculation, and the putative motile

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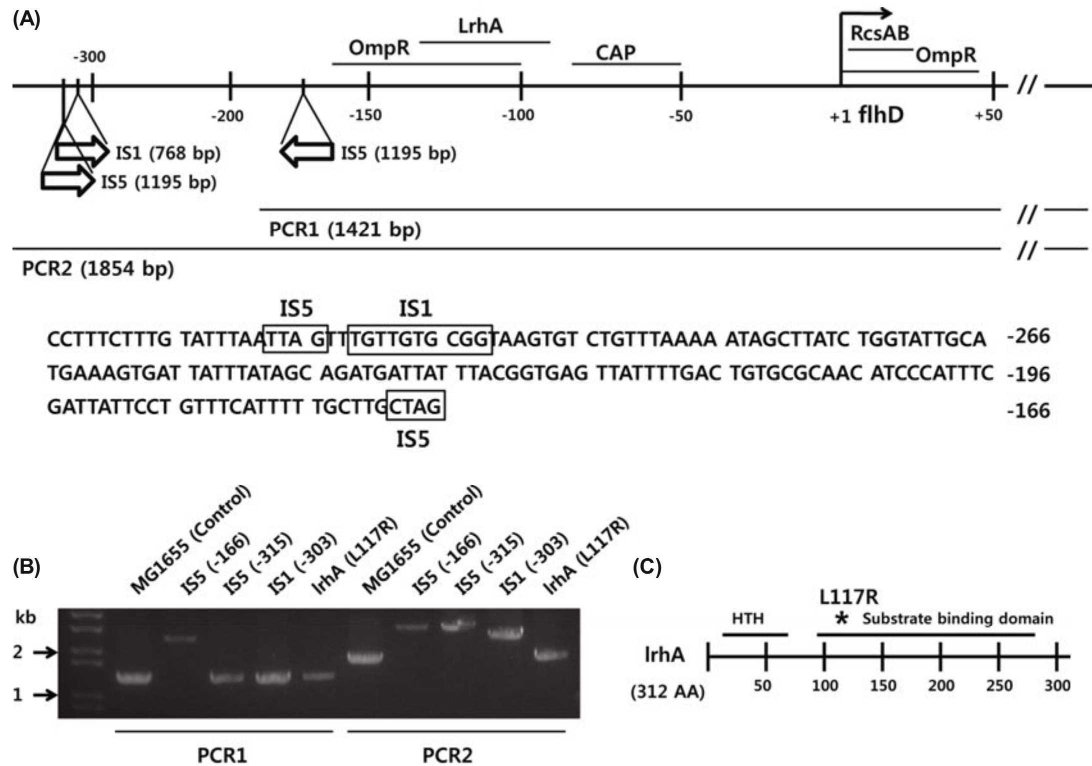

Fig. 1. The regulatory region of *flhDC* with motility-enhancing mutations. (A) Schematic map of the regulatory region of *flhDC*. The binding sites for OmpR, LrhA, CAP, and RcsAB, are indicated (Shin and Park, 1995; Soutourina *et al*., 1999; Lehnen *et al*., 2002; Francez-Charlot *et al*., 2003). The insertions of IS5 are located at -166 and -315, and IS1 is inserted at -303, counting from the transcription start site. The sequences of the regulatory region from -166 to -335 bp were determined, and the insertion sites are marked with boxes. To identify the IS element in the motile derivatives, sizes and locations of PCR products (PCR1 and PCR2) are designated on the map. (B) The PCR products (PCR1 and PCR2) from wild-type MG1655 and motile derivatives were analyzed by agarose gel electrophoresis. Size increase in PCR1 was only observed in the IS5-integrated (-166) motile derivative, although the PCR2 products show an increase in all IS inserted derivatives compared to its parent (MG1655) and to the *lrhA* missense mutant. The PCR primers were PCR1-F (5′-CCTGTTTCATTTTTGCTTGCTAGC-3′), PCR2-F (5′-GTGTAACCGCAACAGCGACA-3′), and PCR-R (5′-GGAATGTTGCGCCTCACCG-3′). (C) LrhA is a member of the LysR family of transcriptional regulators, consisting of 312 amino acids (AA). The N-terminal domain contains a helix-turn-helix (HTH) motif that binds to DNA. The regulatory domain is located in the C-terminal region, where the mutation (L117R) was also found.

subpopulations were isolated. Motilities of these isolates were further tested to confirm their phenotypes.

Previously, Barker *et al*. (2004) isolated motile derivatives from MG1655 and found that IS sequences were integrated into the operator region of *flhDC*. The insertion causes upregulation of *flhDC* and its downstream genes associated with motility (Barker *et al*., 2004). First of all, we sorted out the derivatives containing ISs in the *flhDC* operator by examining the size of the PCR product from the operator and by subsequently sequencing the region of interest (Figs. 1A and 1B). Among ten motile derivatives, four isolates contained an IS in the regulatory region of *flhDC*. Since our PCR screen covered the whole noncoding region between *flhD* and nearby gene, *uspC,* we were able to characterize two new IS insertions in the regulatory region of *flhDC*, IS5 and IS1 at the -315 and -303 position, respectively. The other two IS mutants contain an IS5 insertion at the -166 position, numbering from the transcription initiation site of *flhDC* as previously described (Barker *et al*., 2004).

We further characterized motility-enhancing mutations occurring in *trans*-acting factors, e.g. transcriptional regulators of *flhDC*. Since the *lrhA* gene is known to be the repressor of *flhDC* (Lehnen *et al*., 2002), we scanned the sequence of the *lrhA* gene in the motile derivatives to search for a change, thereby excluding the possibility of IS involvement. Eventually, we found the missense mutation (L117R) of LrhA in one of the motile derivatives (Fig. 1C). The mutation is located in the regulatory region of LrhA. When we compared motilities of the derivatives with that of the parent (Fig. 2), the derivative with IS5 (-166) showed the highest motility on the swarm plate.

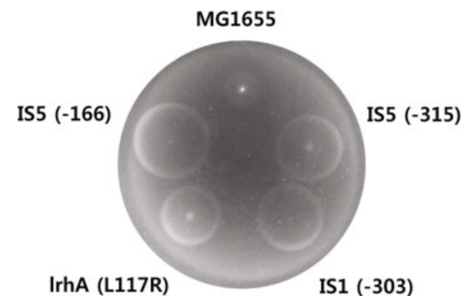

Fig. 2. Swarming phenotype of the motile derivatives. Swarming motility was observed on T-swarm plates. The motile derivatives show enhanced motility relative to that of the parents (MG1655).

Table 1. Bacterial strains

Strains	Relevant genotype[a]	Source or reference
MG1655	F⁻, λ⁻	Lab collection
CH41	MG1655 IS5 (-166 to -169)	This study
CH42	MG1655 IS5 (-315 to -318)	This study
CH43	MG1655 IS1 (-303 to -312)	This study
CH44	MG1655 *lrhA* (L117R)	This study
CH45	MG1655Δ*uvrC*::*tet*	This study
CH46	MG1655Δ*uvrC*::*tet lrhA* (L117R)	This study
CH47	MG1655 Δ*uvrC*::*tet* IS5 (CH41)	This study
CH48	MG1655Δ*uvrC*::*tet lrhA* (L117R) IS5 (CH41)	This study

[a] Insertion sites of IS elements from transcription start site of *flhDC* and missense mutation are described in parentheses.

To confirm the effects of mutations on motility, the IS insertion (IS5 at -166) and point mutation in *lrhA* were transferred to the wild-type MG1655 strain using nearby insertions. Since Tn10 insertions in *uvrC* and *truA* are closely linked to *flhDC* (16 kb, 81% cotransductionally linked) and *lrhA* (30 kb, 63% linked), respectively, these insertions were useful in mutational transfer. The tags were transduced to mutants having the IS or *lrhA* missense mutation, and then P1 phage containing the tag and the mutations were generated. By using phage P1, co-transductants were obtained, and the mutations were confirmed by PCR, showing the size of the *flhDC* operator, or by sequencing the *lrhA* gene. When an IS was transferred, the swarming motility was increased (Table 2; Δ*uvrC* and Δ*uvrC* IS5). Like the IS insertion, the missense mutation of *lrhA*, introduced by using a *truA* tag, conferred enhanced swarming motility (data not shown).

To examine the effect of having both *cis*- and *trans*-acting mutations, the Δ*uvrC lrhA* (L117R) IS5 (-166) strain was constructed and tested for swarming motility (Table 2). Swarming speed of the double mutant (4.38 mm/h) was greater than that of the single IS or *lrhA* mutants, 3.88 and 2.83 mm/h, respectively (Table 2). Similarly, introduction of an *lrhA*-null mutation instead of the missense mutation also enhanced motility of the IS mutant (data not shown). Therefore, the *cis*- and *trans*-acting mutations affect motility independently by activating *flhDC* expression.

Transcriptional and translational expression of *flhDC* and its downstream genes were monitored in the mutant strains, lrhA (L117R), IS5 (-166), and the double mutant (Fig. 3). Transcripts of *flhD* in the motile derivatives were increased 4, 11, and 19 times over that of their parental strain, Δ*uvrC*, as well as of wild-type MG1655 (Fig. 3A). In addition, transcriptional levels of *flhD* in IS5(-315) and IS1(-303) mutants

Table 2. Swarm rates of strains

Strains	Swarm rate (mm/h)[a]
MG1655	1.67±0.08
Δ*uvrC*	1.67±0.21
Δ*uvrC lrhA* (L117R)	2.83±0.43
Δ*uvrC* IS5 (-166)	3.88±0.62
Δ*uvrC lrhA* (L117R) IS5 (-166)	4.38±0.53

[a] To test swarming motility, 2 μl of cells from an overnight culture were inoculated onto T-swarm plates (1% tryptone, 0.25% NaCl, and 0.25% Bacto-agar) and incubated at 30°C. Swarm diameters were measured every hour, and swarming rates were calculated from the linear phase of a graph representing swarm diameter against time (2–5 h).

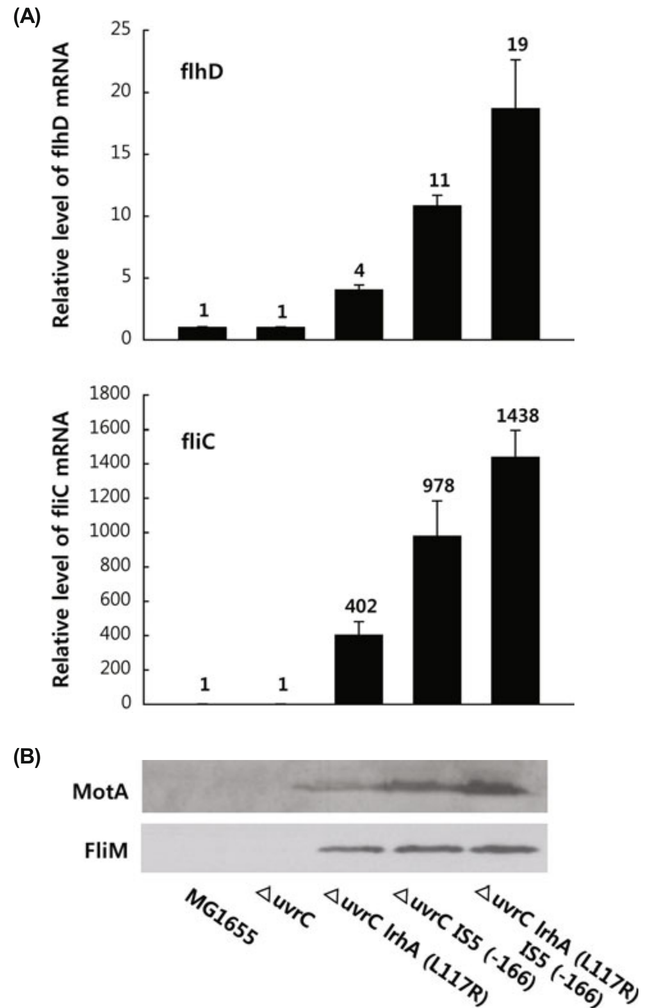

Fig. 3. Transcription and translation levels of *flhDC* and its downstream genes. (A) The transcripts of *flhD* and *fliC* were quantified by real-time qRT-PCR. cDNA was synthesized from RNA isolated from cells. The PCR was carried out with the primers. For *flhD*, flhD-F (5′-CTTCCGCA AATGGTTAAGCTGGCA-3′) and flhD-R (5′-CATTCAGCAAGCGTG TTGAGAGCA-3′), and for *fliC*, fliC-F (5′-CCAGGTTGGCGCAAATGA TAACCA-3′) and fliC-R (5'-TGGCTGCTTCCGTAGAAAGGGTAA-3′). As a reference gene, 16S rRNA (16S rRNA-F; 5′-CTGGTAGTCCACGC CGTAAA-3′ and 16S rRNA-R; 5′-CGAATTAAACCACATGCTCCAC-3′) was used. Bio-Rad CFX-96 was used for the PCR reaction and for detecting the SYBR Green fluorescence change. The ratios of cycle threshold (ct) values, as determined from the Bio-Rad CFX manager software, were compared between samples. (B) Protein expression of MotA and FliM were observed by Western blot using anti-flagellar antiserum from rabbit. The MotA protein expression was increased in motile derivatives, more than 2.2 times in *lrhA* (L117R), 6.0 times in IS5, and 9.5 times in *lrhA* (L117R) IS5 compared to that of the parental strains, wild-type MG1655 and Δ*uvrC*. The FliM protein increased 13.2 times in *lrhA* (L117R), 18.4 times in IS5, and 19.5 times in *lrhA* (L117R) IS5 relative to that of the parents. Band intensity was estimated using the ImageJ program (http://rsbweb.nih.gov/ij/). The cells used to measure the transcription and translation products were cultured at 37°C to an OD_{600} value of 1, in LB medium with agitation

were increased 7 times compared to wild-type. Expression of *fliC*, encoding flagellin, was also significantly increased. The increase in transcription was correlated with the degree of motility enhancement. Protein levels of MotA and FliM, components of the motor complex, were measured by Western blotting (Fig. 3B), which showed enhancement of these motility components. Expressions of MotA in the mutants increased more than 2 times in *lrhA* (L117R), 6 times in IS5, and 9 times in *lrhA* (L117R) IS5 compared to that of the parental strains, wild-type MG1655 and Δ*uvrC*. FliM was increased 13.2 times in *lrhA* (L117R), 18.4 times in IS5, and 19.5 times in *lrhA* (L117R) IS5 strains compared to that of the parents. In addition to transcription, a correlation between the motility and the level of flagellar proteins was observed.

We isolated a number of motile derivatives from the wild-type *E. coli* MG1655 strain. We anticipated three types of mutations, *cis*-acting ones in the regulatory region of *flhDC*, *trans*-acting mutations in the repressors of *flhDC*, and mutations elsewhere in the flagellar regulons. In this study, two types of the mutations were isolated, one with an IS insertion in the operator of *flhDC* (*cis*-acting) and the other with a missense mutation in the *lrhA* repressor of *flhDC* (*trans*-acting). The mutations independently affect the expression of *flhDC* and its downstream genes.

Previously, it was reported that insertion of an IS sequence into the operator region of *flhDC* enhanced motility by increasing expression of *flhDC* and its downstream genes (Barker *et al*., 2004). There are three insertion sites for IS elements in the regulatory region of *flhDC*, -96 bp (IS5), -166 bp (IS5), and -100 bp (IS1) upstream of the transcription start site of *flhDC* (Barker *et al*., 2004). Since the promoter itself was unaltered by the insertions, the increased expression of *flhDC* by IS insertion was not due to the formation of a hybrid promoter (Barker *et al*., 2004). Thus, the upregulation occurred by a derepression involving negative transcriptional regulators, such as H-NS (Wang and Wood, 2011). In this study, we isolated three different IS insertions, IS5 at -166, IS5 at -315, and IS1 at -303. Like other IS insertions into the *flhDC* regulatory region (Barker *et al*., 2004), upregulation of *flhDC* might occur from these new IS insertions. Because the binding sites of LrhA, OmpR, CAP, and RcsAB are not affected by the ISs, some mutations in these genes may also influence *flhDC* expression (Fig. 1A).

As a member of the LysR family, LrhA directly regulates genes involved in motility and chemotaxis by repressing *flhDC* (Lehnen *et al*., 2002). The function of most LysR-type regulators is achieved with effectors (Schell, 1993). However, effectors for LrhA and for the close homologues of HexA (64% identity) and PecT (61% identity) of *Erwinia* have not yet been characterized (Surgey *et al*., 1996; Harris *et al*., 1998). Another protein homolog, CbbR (27% identity) responds to the presence of phosphoenol pyruvate or NADPH (van Keulen *et al*., 1998; Grzeszik *et al*., 2000). However, LrhA does not seem to be affected by these or related compounds (Lehnen *et al*., 2002). The missense mutation could make the LrhA protein unstable. Alternatively, since the missense mutation in LrhA (L117R) is located in the regulatory region, with a putative substrate binding domain (Fig. 1C), it is likely that the mutation renders the LrhA protein inactive even in the presence of a signaling molecule. The consequence of the missense mutation of LrhA needs to be further characterized.

In this study, we found both *cis*- and *trans*-acting mutations, the IS insertions in the *flhDC* regulatory region and an *lrhA* missense mutation, respectively, affecting motility through upregulation of *flhDC*, and these mutations exert their effects independently, suggesting modular and combinatorial regulation modes for *flhDC*. Further, isolation and characterization of diverse mutations conferring enhanced motility may lead to an elucidation of the overall mechanism of flagellar regulation and of the evolutionary diversity of motility regulation in bacterial species.

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