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ORIGINAL ARTICLE

IS*5* inserts upstream of the master motility operon *flhDC* in a quasi-Lamarckian way

Xiaoxue Wang and Thomas K Wood
Department of Chemical Engineering, Texas A & M University, College Station, TX, USA

Mutation rates may be influenced by the environment. Here, we demonstrate that insertion sequence IS*5* in *Escherichia coli* inserts into the upstream region of the *flhDC* operon in a manner that depends on whether the environment permits motility; this operon encodes the master regulator of cell motility, FlhDC, and the IS*5* insertion increases motility. IS*5* inserts upstream of *flhD*$^+$ when cells are grown on soft-agar plates that permit swimming motility, but does not insert upstream of this locus on hard-agar plates that do not permit swimming motility or in planktonic cultures. Furthermore, there was only one IS*5* insertion event on soft-agar plates, indicating insertion of IS*5* into *flhDC* is not due to general elevated IS*5* transposition throughout the whole genome. We also show that the highly motile cells with IS*5* upstream of *flhD*$^+$ have greater biofilm formation, although there is a growth cost due to the energetic burden of the enhanced motility as these highly motile cells have a lower yield in rich medium and reduced growth rate. Functional flagella are required for IS*5* insertion upstream of *flhD*$^+$ as there was no IS*5* insertion upstream of *flhD*$^+$ for *flhD, flgK* and *motA* mutants, and the mutation is stable. Additionally, the IS*5* mutation occurs during biofilm formation, which creates genetic and phenotypic diversity. Hence, the cells appear to 'sense' whether motility is feasible before a sub-population undergoes a mutation to become hypermotile; this sensing appears related to the master transcription regulator, FlhDC.
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Introduction

Bacteria with flagella, such as *Escherichia coli,* have a competitive advantage for moving toward favorable conditions and for avoiding detrimental environments (Fenchel, 2002). The flagellum is a complex organelle that also has an important role in adhesion, biofilm formation and virulence (Pratt and Kolter, 1998; Wood *et al.*, 2006). However, the operation and synthesis of flagella requires significant energy; for example, synthesis involves more than 50 genes at a cost of 2% of the biosynthetic energy in *E. coli* (Soutourina and Bertin, 2003). Hence, coordinating growth and flagella synthesis is necessary. As a result, flagella synthesis is a highly ordered cascade, and on top of the hierarchy, is the master regulator FlhDC required for the expression of all other genes of the flagella regulon (Liu and Matsumura, 1994). The operon that encodes FlhDC, *flhDC*, is one of the most highly regulated loci in the genome and the target for regulation by many environmental factors (Soutourina and Bertin, 2003). The *flhDC* promoter contains many transcription factor-binding sites, including those for global regulatory proteins, such as H-NS and the catabolite gene activator protein complex (Bertin *et al.*, 1994; Soutourina *et al.*, 1999).

Activating genes by insertion of motile DNA elements has been shown to provide benefits to the host under stress by facilitating adaptations to severe environments (Hall, 1998; Petersen *et al.*, 2002; Zhang and Saier, 2009a). As an example, the well-studied *bgl* operon encoding the gene products for the fermentation of β-glucoside depends on the transposition of insertion elements IS*1* or IS*5* to become active (Schnetz and Rak, 1992; Hall, 1998). Also, the glycerol utilization operon (*glpFK*) can be activated by IS*5* when it is inserted upstream (Zhang and Saier, 2009a). For insertion of IS*5* into both the *bgl* and *glpFK* operons, the environment influences the mutation rate. In addition, in biofilms, some cells increase their mutation rate during stress by increasing competence during chronic infections (Ehrlich *et al.*, 2010). Hence, these examples demonstrate a Lamarckian-type evolution (that is, environment-driven mutation) that differs from that of Darwin, which holds that mutation rates should not depend on the environment (Koonin and Wolf, 2009).

In recent years, insertion elements IS*1* and IS*5* have been identified in some *E. coli* strains that

Correspondence: TK Wood, Department of Chemical Engineering, Texas A & M University, 220 Jack E. Brown Building, 3122 TAMU, College Station, TX 77843-3122, USA.
E-mail: Thomas.Wood@chemail.tamu.edu
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activate the promoter of *flhDC* and increase motility (Barker *et al.*, 2004; Gauger *et al.*, 2007); however, these mutations were rejected as cases of directed mutation (Barker *et al.*, 2004). Here, we explored the regulation of *flhDC* by IS*5* hopping under different environmental conditions and found that the environment influences the IS*5* insertion upstream of *flhDC*. Hence, we provide the first example of Lamarckian-type evolution for an active locus.

Materials and methods

Bacterial strains, plasmids and growth conditions
The bacterial strains and plasmids used in this study are listed in Table 1. All experiments were conducted in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) or M9C medium (Rodriguez and Tait, 1983) at 37 °C, except where indicated. Kanamycin (50 μg ml^{-1}) was used for culturing strains with single-gene knockouts from the Keio Collection (Baba *et al.*, 2006), chloramphenicol (30 μg ml^{-1}) was used for maintaining pCA24N-based plasmids, and ampicillin (100 μg ml^{-1}) was used to maintain plasmid pKD46.

Swimming motility assay
Cell motility was examined as described previously on low-salt, soft-agar plates (1% tryptone, 0.25% NaCl and 0.3% agar), where the wild-type BW25113 is motile (González Barrios *et al.*, 2006). High-salt, soft-agar plates were also used, in which wild-type BW25113 is non-motile due to the high salt concentration (Wang *et al.*, 2009).

*Curing IS*5 *from* flhD$^+$ Ω *IS*5 *by a two-step method*
Owing to the stable residence of IS*5* after insertion into the *flhDC* regulatory region, curing IS*5* via natural excision was not successful. Instead, we used a two-step method to replace the *flhDC* regulatory region of *flhD*$^+$ Ω IS*5* with that of the wild-type strain. To facilitate the screening for the right replacement, we first deleted *flhD* from *flhD*$^+$ Ω IS*5* using P1 transduction to transfer the Δ*flhD* Km^R mutation from BW25113 *flhD* (Table 1) to make a non-motile strain, BW25113 *flhD* Ω IS*5*; this strain was verified by its complete lack of motility on low-salt, soft-agar plates. Then, the IS*5* element was removed and the *flhD*$^+$ gene was restored using the one-step inactivation method with pKD46 (Datsenko and Wanner, 2000) by replacing the *flhD* Ω IS*5* region with PCR products amplified from the BW25113 wild-type genomic DNA covering the IS*5* insertion site and the whole coding region of *flhD*$^+$ using primer set PflhDC-4 (PflhDC-F4 and PflhDC-R4) (Table 2). Transformed cells were inoculated into low-salt, soft-agar plates, and cells with restored motility were selected and sequenced to verify the restoration of the wild-type *flhD*$^+$ gene and its promoter region.

Crystal violet biofilm assay
Biofilm formation was assayed in 96-well polystyrene plates using 0.1% crystal violet staining (Corning Costar, Cambridge, MA, USA) (Fletcher, 1977). Briefly, each well was inoculated at an initial turbidity at 600 nm of 0.05 and grown without shaking for 8 h in LB and M9C medium. Biofilm formation was normalized by the bacterial growth for each strain (turbidity at 620 nm). Two independent cultures were used for each strain.

Glass wool biofilms
Overnight cultures of the wild-type strain were inoculated into 250 ml LB with 10 g glass wool (Corning Glass Works, Corning, NY, USA) (Ren *et al.*, 2004) to reach a final turbidity 0.05 (600 nm). The cultures were incubated for 8 h, 12 h, 15 h, 24 h and 39 h at 37 °C during which time the cells formed a biofilm on the surface of the glass wool.

Table 1 *Escherichia coli* bacterial strains and plasmids used in this study

Strains and plasmids	*Genotype/relevant characteristics*	*Source*
BW25113 strains		
wild type	*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$	Baba *et al.*, 2006
flhD$^+$ Ω IS*5*	IS*5* inserted upstream of *flhDC* in the opposite orientation in BW25113	This study
flhD	Δ*flhD* Ω Km^R, replacement of *flhD* coding region with Km^R	Baba *et al.*, 2006
flhD Ω IS*5*	IS*5* inserted upstream of Δ*flhD* in the opposite orientation in Δ*flhD* Ω Km^R	This study
flhD$^+$ Δ IS*5*	IS*5* removed from *flhD*$^+$ Ω IS*5*	This study
flgK	Δ*flgK* Ω Km^R, replacement of the *flgk* coding region with Km^R	Baba *et al.*, 2006
motA	Δ*motA* Ω Km^R, replacement of the *motA* coding region with Km^R	Baba *et al.*, 2006
hns	Δ*hns* Ω Km^R, replacement of the *hns* coding region with Km^R	Baba *et al.*, 2006
hns flhD$^+$ Ω IS*5*	IS*5* inserted upstream of *flhDC* in the opposite orientation in Δ*hns* Ω Km^R	This study
Plasmids		
pCA24N	Cm^R; *lacI*^{q}	Kitagawa *et al.*, 2005
pCA24N-*hns*	Cm^R; *lacI*^{q}, P$_{T5-lac}$::*hns*$^+$	Kitagawa *et al.*, 2005
pKD46	Amp^R, λ Red recombinase expression	Datsenko and Wanner, 2000

Km^R, Cm^R, and Amp^R are kanamycin, chloramphenicol and ampicillin resistance, respectively.

Table 2 Oligonucleotides used for PCR and qPCR in this study

Primer set name	*Primer name*	*Sequence (5′-3′)*	*Source*
PflhDC	PflhDC-F2	CCTGTTTCATTTTTGCTTGCTAGC	Barker *et al.*, 2004
	PflhDC-R3	GGCTGCGATTTTCAATAATGC	This study
PflhDC Ω IS5	PflhDC-F2	CCTGTTTCATTTTTGCTTGCTAGC	Barker *et al.*, 2004
	PflhDC-IS5-R	CCTGTTCTATGGCTCCAGATGAC	This study
PflhDC-4	PflhDC-F4	TCGACCTCACTTGAGGCAATAA	This study
	PflhDC-R4	TTTCGTGCGTCTCAATCTCTTC	This study
purA	purA-F	GGGCCTGCTTATGAAGATAAAGT	Wang *et al.*, 2009
	purA-R	TCAACCACCATAGAAGTCAGGAT	Wang *et al.*, 2009
metG	metG-F	GGTGGAAGCCTCTAAAGAAGAAG	This study
	metG-R	AGCAGTTTGTCAGAACCTTCAAC	This study
IS5-1	IS5-F1	CGTGAATAACATCGCCAGTTGGTT	This study
	IS5-R1	CCGGCAAGGTAAGAACCTTGAAAC	This study
IS5-2	IS5-F1	CGTGAATAACATCGCCAGTTGGTT	This study
	IS5-R2	CAAGAACAAAACGGCCATCAACAT	This study
Pade	Pade-F	ATTTGCGGGTTCACAAAAAC	This study
	Pade-R	GGGAAACGGCTAACAATTCC	This study
Pbgl	PbglGFB-F	GCGATGAGCTGGATAAACTGCTG	Zhang and Saier, 2009a
	PbglGFB-R	ACTTGAGTTAATTCTTTCGCCAGC	Zhang and Saier, 2009a
Pfuc	PfucAO-F	GCTCTGTAATATGACGGCGGTC	Zhang and Saier, 2009a
	PfucAO-R	AGGCAAGTGTCAATAATCTGACGAGC	Zhang and Saier, 2009a
Pglp	PglpFK-F	AAATCCTCGTCCCGATTACC	This study
	PglpFK-R	GGACCTCCACGATGCTTGTA	This study

F indicates the forward primer and R indicates the reverse primer.

At each time point, planktonic cells in contact with biofilms were taken directly from the culture. Glass wool was taken from the culture and was rinsed twice with 100 ml 0.85% NaCl, and biofilm cells were collected after 2 min of sonication of the glass wool with 200 ml 0.85% NaCl in a bath sonicator (Fisher Scientific, Pittsburgh, PA, USA). A population of $\sim 10^8$ cells (OD $\sim$ 1) was inoculated onto a soft-agar plate for the motility assay.

Quantitative PCR (qPCR)

qPCR was performed using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). In all, 100–200 ng of genomic DNA was used for the qPCR reaction using the *Power* SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed using Primer3Input Software (v. 0.4.0; Rozen and Skaletsky, 2000) and are listed in Table 2. The number of chromosomes that lack IS*5* upstream of *flhD*$^+$ was quantified using primer set PflhDC (PflhDC-F2 and PflhDC-R3), and the number of chromosomes with IS*5* upstream of *flhD*$^+$ was quantified using primer set PflhDC Ω IS*5* (PflhDC-F2 and PflhDC-IS5-R). To quantify the total copy number of IS*5* elements in the chromosome under different growth conditions, the chromosome number was quantified using primer sets purA and metG (Table 2), which amplify single-copy genes *purA* and *metG*, respectively, and the copy number of IS*5* was quantified using primer sets IS5-1 and IS5-2 (Table 2), which amplify two different regions of the IS*5* gene. The binding efficiencies of the six sets of primers were tested by varying concentrations of templates to generate a standard curve (Pfaffl, 2001), and the templates used were genomic DNA of the wild-type strain for primer sets PflhDC, purA, metG, IS5-1 and IS5-2, and genomic DNA of the *flhD*$^+$ Ω IS*5* strain for primer set PflhDC Ω IS*5*.

Results

*IS*5 *hops upstream of* flhD$^+$ *only when cells swim on motility plates*

While performing motility assays using a wild-type *E. coli* K12 BW25113 strain (which has low motility), we noticed that a temporal change occurs in the swimming pattern. When the wild-type cells were plated onto soft-agar plates (0.3% agar) after 8–12 h of incubation, a halo of 11–14 mm in diameter formed (Figure 1, Halo 1). Prolonged incubation (after 24 h) led to some cells swimming faster and forming a second halo with less cell density (Figure 1, Halo 2). The two halos were separated by a zone with very low cell density. Cells from Halo 2 were collected and sequenced in the regulatory region of *flhDC*; the sequencing results (GenBank accession JF342359) showed that an IS*5* insertion element (1195 bp, accession J01735) (Kröger and Hobom, 1982) was upstream of *flhD*$^+$ in these cells, but not for cells inside the original halo. IS*5* was inserted in the opposite orientation into a 4-bp target site (5′-TTAA-3′) 96–99 bp upstream of the transcription start site of the *flhDC* operon, the same position described previously for the MG1655 *fnr* strain (Barker *et al.*, 2004). These cells were named *flhD*$^+$ Ω IS*5*. No insertion element was present upstream of *flhD*$^+$ for the culture used to inoculate the soft-agar plate. Hence, on soft-agar plates, IS*5* hops into the promoter region of *flhDC.*

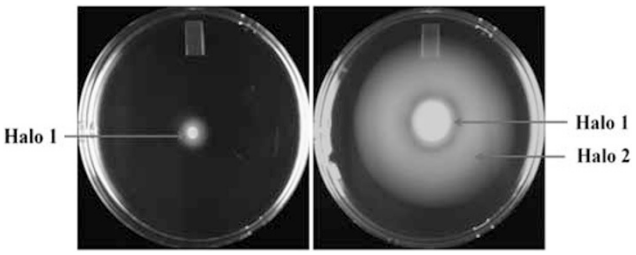

Figure 1 Swimming halo formed after 12 h (left) and 24 h (right) at 37 °C on low-salt, soft-agar plates. Halo 1 refers to the original halo formed by wild-type swimming cells, and Halo 2 refers to the outside halo formed by *flhD*$^+$ Ω IS*5* swimming cells.

To quantify the proportion of cells that have acquired IS*5* during migration on motility plates, qPCR was conducted using a forward primer that binds upstream of the IS*5* insertion site and to one of two reverse primers that bind either to the downstream chromosome region without IS*5* or bind inside IS*5*. The copy numbers obtained from the two separate qPCR reactions were used to estimate the percentage of cells that acquired IS*5* upstream of *flhD*$^+$. Genomic DNA was isolated using cells collected from different regions of the swimming zone, and was used as templates for qPCR. Starting with a BW25113 wild-type colony (lacks IS*5* upstream of *flhD*$^+$), in the first stage of migration (less than 12 h) on soft-agar plates (Figure 1, Halo 1), the proportion of cells with IS*5* upstream of *flhD*$^+$ was very low ($\sim 10^{-7}$), which is similar to that of the culture used for inoculation. During the development of the second halo, $9 \pm 4\%$ cells acquired IS*5* after 24 h, and $21 \pm 1\%$ cells acquired IS*5* after 36 h. Therefore, within 24 h, a substantial fraction of cells within the second halo had IS*5* upstream of *flhD*$^+$.

The frequency of IS*5* hopping was also tested on plates with the same nutrient content as soft-agar plates, but with higher amounts of agar (1.5%), which serves to immobilize the *E. coli* cells, and on regular LB-agar plates with more nutrients (1.5% agar), which also does not support motility (Copeland and Weibel, 2009). In both cases, after 12 h, 24 h, 48 h and 72 h, the frequency of IS*5* hopping upstream of *flhD*$^+$ was unchanged and remained very low ($\sim 10^{-7}$, estimated by qPCR). Thus, there was a 10^4-fold induction of IS*5* hopping upstream of *flhD*$^+$ on soft-agar plates compared with IS*5* hopping on hard-agar plates. In contrast, when cultured in LB liquid medium or in minimal medium, the frequency of IS*5* hopping upstream of *flhD*$^+$ for planktonic cells remained low ($\sim 10^{-7}$) and did not change for 11 days (~ 200 generations in LB and ~ 100 generations in minimal medium). This occurrence of a motile sub-population of cells was also reported for MG1655 *fnr* from an inoculum of poorly motile MG1655 *fnr* on soft-agar plates, and PCR screening confirmed that IS*5* inserted into the *flhDC* regulatory region in the same manner (Barker *et al.*, 2004). The sequenced K12 strain, MG1655, is a relative of BW25113 (Baba *et al.*, 2006), and is highly motile because of a similar IS*1* insertion sequence upstream of *flhD*$^+$ (Blattner *et al.*, 1997; Gauger *et al.*, 2007).

Clearly, IS*5* hopping upstream of *flhD*$^+$ occurred at very different frequencies on plates with different agar concentrations. It is well known that for soft-agar plates (agar $<0.4\%$) the pore size of the gel is larger than the bacterial cells, allowing them to penetrate into the polymer network and swim (Copeland and Weibel, 2009). While on hard-agar plates (agar $>1.5\%$), cells grow into non-motile colonies and retain their planktonic morphology and phenotype. Cells isolated from the edge of the soft-agar plate have distinctively more flagella than cells from a hard-agar plate (Copeland and Weibel, 2009). Increased motility is thus an advantage on soft-agar plates because it allows cells to reach more nutrients. Hence, we conclude that soft-agar plates serve as a type of environmental cue, which promote cell motility by inducing IS*5* hopping into the upstream region of *flhD*$^+$.

*IS*5 *does not hop upstream of* flhD$^+$ *in* flhD *and flagella structure mutants*

To determine whether IS*5* hopping is dependent on the ability to swim, we plated cells with an *flhD* deletion, but which contain the upstream regulatory region and insertion site for IS*5*. As expected, the *flhD* cells did not swim or form a halo on mobility plates after 24 h, 48 h or 72 h incubation. All cells were collected from soft-agar plates and checked for the presence of IS*5* by qPCR, and no cells had IS*5* in the regulatory region of *flhDC*. Hence, motility is required for IS*5* insertion into the regulatory region of *flhDC*.

To determine whether IS*5* insertion relies on FlhD or relies on an active flagella component and rotor, the frequency of IS hopping was also tested for strains that lack essential components of the flagella apparatus. MotA is one of the two membrane proteins that form the stator of the flagellar motor (Dean *et al.*, 1984), and FlgK is a hook-associated protein (Komeda *et al.*, 1978). Critically, after 24 h and 36 h of incubation on low-salt, soft-agar plates, no IS*5* was detected in strains with these two mutations. Hence, we conclude that IS*5* hopping into the regulatory region of *flhDC* requires active motility.

This conclusion is corroborated by results with mutations in the global regulator H-NS (Soutourina *et al.*, 1999). When we plated the *hns* mutant on soft-agar plates, as expected, the *hns* mutant was non-motile due to a reduced expression of *flhDC* and a subsequent lack of flagella biosynthesis (Bertin *et al.*, 1994; Soutourina *et al.*, 1999). However, after long incubation times (24 h) with the *hns* mutant, different from the non-motile *flhD*, *flgK* and *motA* mutants, a halo was formed that contained highly

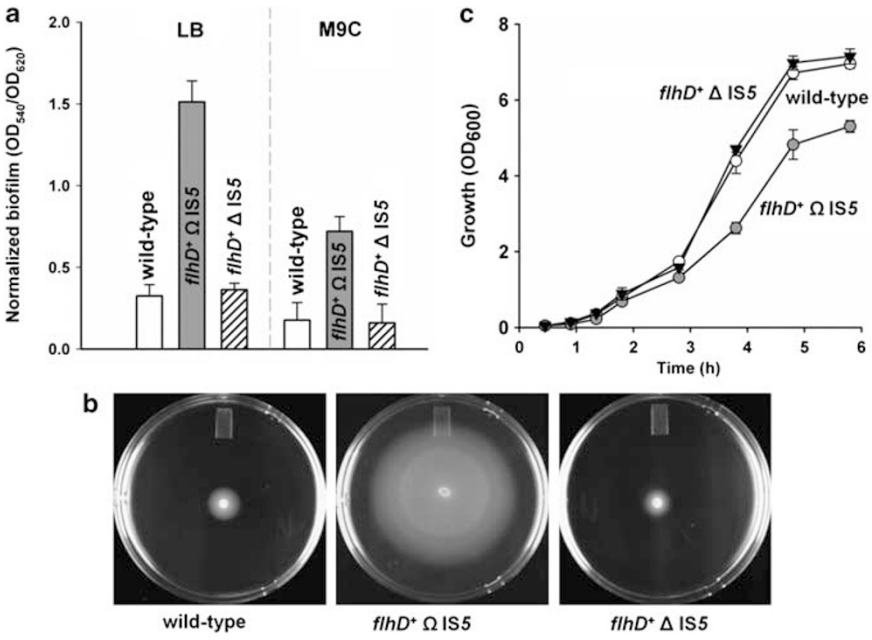

Figure 2 (**a**) Early biofilm formation (8 h) at 37 °C in LB medium and M9C medium for the wild-type strain (white bar), strain *flhD*$^+$ Ω IS*5* that acquired IS*5* upstream of the *flhDC* operon (gray bar), and strain *flhD*$^+$ Δ IS*5* in which IS*5* was cured from strain *flhD*$^+$ Ω IS*5* (slash marks). Data are the average of 10 replicate wells from two independent cultures, and one standard deviation is shown. (**b**) Swimming motility of the three strains on low-salt, soft-agar plates after 12 h incubation at 37 °C. Three independent cultures were tested, and one representative image is shown. (**c**) Growth of the wild-type strain (open white circle), *flhD*$^+$ Ω IS*5* (filled gray circle) and *flhD*$^+$ Δ IS*5* (filled black triangle) in LB medium at 37 °C. Two independent cultures were used.

motile cells. These cells were found to have the IS*5* insertion upstream of *flhD*$^+$. Hence, non-structural mutations in the motility regulon permit IS*5* hopping into the upstream region of *flhD*$^+$ on agar plates that promote motility.

*IS*5 *insertion upstream of* flhD$^+$ *increases motility and biofilm formation while it decreases growth*

The *flhD*$^+$ Ω IS*5* cells formed significantly more biofilm after 8 h than wild-type cells (5 ± 1-fold more, Figure 2a) and have much greater motility (7 ± 2-fold greater more, Figure 2b). All cells from four different spots of the outside halo (Figure 1, Halo 2) had the same IS*5* insertion upstream of *flhD*$^+$ and had the same motility. The specific growth rate of the cells with IS*5* in *flhD* was slightly lower, and the cells had a lower yield (6 ± 1% reduction) (Figure 2c). This result is consistent with an earlier study, which shows that MG1655 (IS*1* inserted upstream of *flhDC*) grew more slowly than MG1655 that lacks IS*1* upstream of *flhDC* due to the reduction in energy available for growth due to the enhanced motility (Gauger *et al.*, 2007). For our work, when IS*5* was cured from the upstream region of *flhD*$^+$, growth, motility and biofilm formation were restored to the wild-type values (Figure 2), which demonstrates that the increase in motility, the increase in early biofilm formation, and the decrease in growth are due to the presence of IS*5* in promoter of *flhDC*.

*IS*5 *insertion upstream of* flhD$^+$ *is stable and heritable*

The stability of IS*5* upstream of *flhD*$^+$ was tested in planktonic cells harboring IS*5* upstream of *flhD*$^+$ using serial dilutions every 24 h for 11 days in LB medium and in minimal medium (M9C). On the basis of qPCR, IS*5* is not removed from the upstream of *flhD*$^+$ after 1, 5 and 11 days. This result indicates that once IS*5* inserted into *flhDC*, it is stable and is not removed (it may make copies and insert into other areas of the chromosome, but it leaves a copy of itself). Moreover, wild-type cells were also assayed using the same conditions, and no cells acquired IS*5* in the *flhDC* regulatory region after 1, 5 and 11 days. These results provide another line of evidence that IS*5* hopping upstream of *flhD*$^+$ occurs only in a motility-inducing environment.

*IS*5 *insertion upstream of* flhD$^+$ *increases diversity in biofilm cells*

Flagella are important for initial attachment in biofilms (Pratt and Kolter, 1998), for the mature structure (Wood *et al.*, 2006), and for the dispersal of biofilms (Kaplan, 2010). Here, we tested whether IS*5* is inserted upstream of *flhD*$^+$ during biofilm development by screening biofilm cells for increases in motility. In the presence of glass wool, planktonic cells were collected from the culture, and biofilm cells were collected from the glass wool by sonicating. Motility results indicated that a small proportion of biofilm cells (as early as 8 h) migrated much faster than the rest of the non-motile cells, which

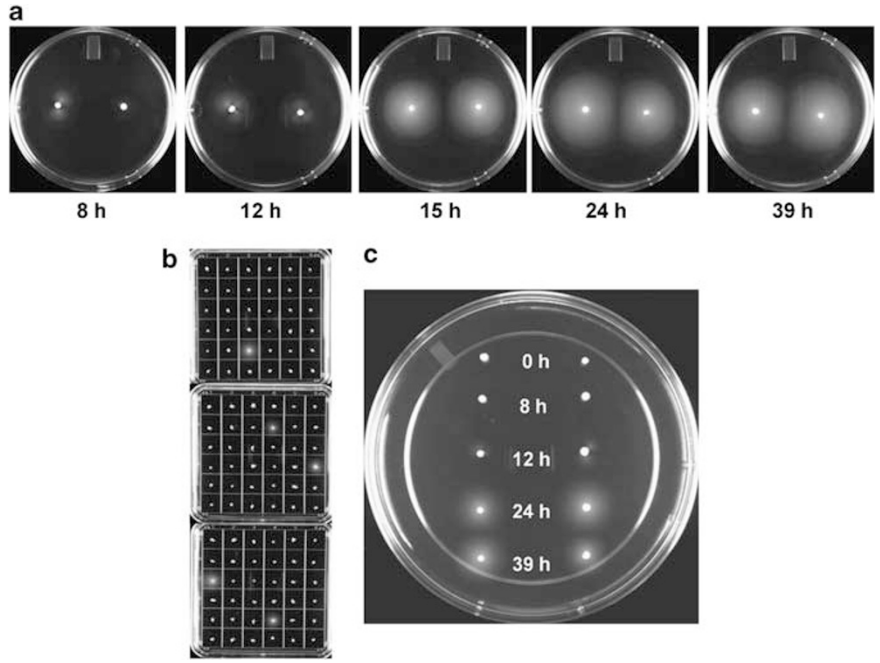

Figure 3 Emergence of highly motile cells (*flhD*$^+$ Ω IS*5*) during biofilm development. (**a**) Biofilm (left) and planktonic cells in contact with biofilms (right) were collected at different time points from cultures with glass wool and plated on high-salt, soft-agar plates and incubated for 12 h. Cells with high motility were identified at these stages by the formation of swimming halos. (**b**) Screening of biofilm cells with high motility after 24 h with high-salt, soft-agar plates where cells were non-motile, unless IS*5* was inserted upstream of *flhD*$^+$. A total of 500 cells were tested, and only 108 cells are shown. (**c**) Emergence of highly motile cells (*flhD*$^+$ Ω IS*5*) in biofilms at different stages in 96-well polystyrene plates. Planktonic cells in contact with biofilms (left) and biofilm cells (right) were collected at different time points and plated on high-salt, soft-agar plates and incubated for 12 h. Cells with high motility were identified at these stages by the formation of swimming halos.

appeared as a bright dot in the inoculum ring (Figure 3a, left side of the plate). For the planktonic cells in contact with biofilms, a small proportion of highly-motile cells emerged after 15 h (Figure 3a); however, these motile cells are probably derived from the biofilms as no highly motile cells arise in shaking flasks without biofilms, and cells frequently detach from biofilms (Kaplan, 2010). After 15 h, 24 h and 39 h of incubation, the proportion of highly motile cells increased for both biofilm and planktonic cells in contact with biofilms. A total of 500 cells from biofilms after 24 h were screened on high-salt, soft-agar plates, and 2.2% of the cells had increased motility (Figure 3b). PCR screening for the insertion element upstream of *flhDC* for these cells showed that IS*5* was inserted upstream of *flhDC*. Similar results were obtained for cells grown statically in 96-well polystyrene plates; cells with higher motility were found after 12 h of incubation (Figure 3c). Thus, we conclude that IS*5* hopping in *flhDC* occurs during biofilm formation and creates a sub-population of cells with increased motility but slower growth.

*IS*5 *hops upstream of* flhD$^+$*, but not into other operons on motility plates and in biofilms*

IS*5* is present in multiple (10–23) copies in the chromosome of *E. coli* K12 (Deonier, 1996), and in K12 strain W3110, which is closely related to BW25113 (Jensen, 1993), IS*5* is present at 23 copies (Umeda and Ohtsubo, 1990). To test whether the transposition of IS*5* upstream of *flhD*$^+$ is specific or due to a general increase in IS*5* transposition during swimming, the copy number of IS*5* in the chromosome was quantified by qPCR and compared with the copy number of *purA* and *metG*, which are present as single copies. For *flhD*$^+$ Ω IS*5* cells collected from outside halos on soft-agar plates, the estimated copy number of IS*5* was 23.6 ± 0.7, and for BW25113 wild-type cells growing planktonically, there were 22.5 ± 0.8 copies of IS*5*. Therefore, the highly motile cells have only one additional IS*5* element.

Moreover, upstream regions of *bgl*, *ade*, *fuc* and *glpFK* operons were also screened for the presence of insertion elements for *flhD*$^+$ Ω IS*5* cells collected from outside halos and for *flhD*$^+$ Ω IS*5* cells collected from 24 h biofilms using PCR screening around the insertion sites (four pairs of primers list in Table 2). Insertion of IS*5* or IS*1* elements into these operons that derepress gene expression have been reported under specific conditions (Hall, 1998; Petersen *et al.*, 2002; Zhang *et al.*, 2010). As expected, based on the qPCR results, no IS element was found in any of these operons that corroborates that IS*5* insertions occurred only upstream of *flhD*$^+$. In addition, we also quantified the copy number of

IS*5* for all BW25113 cells collected from soft-agar plates after 12 h or 24 h incubation to obtain a population average, and the increase in copy number of IS*5* in the population ranged from 0.3 to 1.5 when compared with cells growing planktonically after 24 h in LB, which corroborates again that the IS*5* insertions occurred only upstream of *flhD*$^+$.

*IS*5 *hopping disrupts H-NS binding to the* flhDC *operon* *In vitro* transcription experiments showed that H-NS represses *flhDC* transcription at the AT-rich region that contains the insertion site for IS*5* (5′-TTAA-3′) in cells without IS*5* upstream of *flhDC* (Soutourina *et al.*, 1999). We hypothesized that once IS*5* is inserted into this region, it may lead to a loss of repression of *flhDC* by H-NS due to the disruption of the AT-rich region. As expected, we found that H-NS significantly repressed motility when overproduced in the wild-type strain without IS*5* upstream of *flhD*$^+$, which agrees with earlier studies (Soutourina *et al.*, 1999). However, this repression of motility by H-NS was abolished in cells that had IS*5* upstream of *flhD*$^+$, for example, in *flhD*$^+$ Ω IS*5* and *hns flhD*$^+$ Ω IS*5*. Hence, after its insertion, IS*5* prevents H-NS from repressing motility.

Discussion

Here, we show that IS*5* hopping into the promoter region of *flhDC* occurs at a high frequency only under environmental conditions that promote high flagella activity. It is well known that most transposition is not random, and hot spots have been identified where transposition events occur more frequently. IS*5* has been found to be present in multiple (10–23) copies in the chromosome of *E. coli* K12 (Deonier, 1996) and shows a preference for 5′-YTAR-3′ (Y = C or T) (R = A or G) target sequences (Mahillon and Chandler, 1998). Upstream of the *flhDC* operon, there is one hot spot (5′-TTAA-3′) for IS*5* insertion (Barker *et al.*, 2004). However, we provide evidence that this IS*5* hopping event is not solely due to the presence of a hot spot for IS*5* in the upstream of *flhDC* because prolonged incubation of a mutated strain without *flhD*, *flgK* or *motA* did not increase IS*5* hopping even though the hot spot upstream of *flhDC* remains for these three mutants. Another line of evidence is that prolonged incubation leads to increased IS*5* hopping on soft-agar plates (swimming is allowed), but not on hard-agar plates (swimming is inhibited).

Once inoculated on agar plates, cells replicate, causing an increase in the population and cell density, until nutrients and resources become limited. For cells on soft-agar plates, when the cell density becomes limited by nutrients and wastes, the highly motile cells have an advantage when they migrate further to obtain nutrients and areas with reduced waste concentrations. Hence, for our experiments, a starvation condition led to IS*5* hopping, which allowed cells to obtain a better environment. Thus, we reasoned that the increased transposition occurs when increased motility leads to an increase in fitness, and we propose that transposition of IS*5* upstream of *flhD*$^+$ represents a quasi-Lamarckian phenomenon, because the induced genetic change is beneficial to the organism. Just as Koonin and Wolf (2009) found for the prokaryotic CRISPR-cas system, the lines of evidence for IS*5* hopping in a quasi-Lamarckian way rather than a Darwinian way are as follows: (i) hopping occurs in a motility-driven environment (soft agar) that leads to the insertion of the motile element, (ii) the resulting modification directly affects motility, which is the same cue that caused the modification, and (iii) the modification is adaptive and is inherited by the progeny of the cell that encounters the mobile element.

As determined by qPCR, the number of pre-existing, highly motile mutants in liquid culture was very low (less than $\sim 10^{-7}$) in a total of 10^7–10^8 wild-type cells that were used to inoculate the soft-agar plates. Clearly, selection of pre-existing mutants alone would not be expected to produce 10^4 more cells with high motility, which supports our conclusion that environment-induced mutations contribute more substantially to bacterial evolution under these conditions compared with selection of random pre-existing mutator strains. Moreover, general IS*5* transposition was not induced on the soft-agar plates, as the increase in IS*5* copy number was limited to basically one IS*5* transposition per cell, under these conditions. However, the increase in the proportion of *flhD*$^+$ Ω IS*5* cells from 24 to 36 h on the soft-agar plates may be due to selective amplification of these highly motile cells.

IS*5* has also been shown to behave as a transcriptional enhancer of the otherwise cryptic *E. coli bgl* operon to allow cells to utilize β-glucoside (Schnetz and Rak, 1992). IS*5* is also capable of activating a metabolic operon *glpFK* to allow cells to utilize glycerol in the absence of the catabolite gene activator protein complex (Zhang and Saier, 2009b). In addition, the insertion of IS element in the cryptic *ade* promoter region results in relief of the H-NS-mediated silencing of *ade* operon, which allows the cells to utilize adenine as the sole source of purines (Petersen *et al.*, 2002). However, in contrast to IS transposition into the *ade*, *bgl* and *glpFK* operons, expression of *flhDC* was not silenced before the transposition, and cells were motile in the absence of IS*5* in its promoter (Figure 1, Halo 1); hence, the directed mutation here is fundamentally different in that the transcription of an active gene was increased.

In biofilms, we show that the transposition of IS*5* upstream of *flhD*$^+$ helps to activate *flhD*C expression and generates a proportion of cells with higher motility and slower growth. Consistent with an earlier study with *Pseudomonas aeruginosa*, where

biofilm-grown cells exhibited more variation in swimming motility than did those from the inoculum (Boles *et al.*, 2004; Boles and Singh, 2008), we also observed cells with increased motility and reduced growth arising in *E. coli* biofilms. Genetic diversity has been proposed as a beneficial feature of biofilms and has been reported in various species (Boles *et al.*, 2004; Allegrucci and Sauer, 2007). The differences in swimming motility phenotypes found in *P. aeruginosa* biofilms were heritable and not produced by planktonic growth, and were dependent on RecA function (Boles *et al.*, 2004). Here, we show that increased variation in motility and growth inside biofilms is caused by transposition of insertion elements, which results in a heritable change. Therefore, IS hopping is a novel mechanism for the production of genetic and phenotypic variations in biofilms.

We have shown previously that phage elements have an important role in generating diversity inside *E. coli* biofilms (Wang *et al.*, 2009), and excision of e14, CP4-57, rac and CPS-53 prophages generate isogenetic strains with different phenotypes, including those of motility and growth (Wang *et al.*, 2010). Induction of transposition in prokaryotes under cell-stress conditions is potentially important in creating diversity facilitating adaptation to stressful environments (Hall, 1998; Petersen *et al.*, 2002; Zhang and Saier, 2009a), and IS*5* hopping upstream of *flhD*$^+$ is an adaptive mutation. Moreover, as IS*5* is also found in *E. coli* bacteriophages (Kröger and Hobom, 1982), horizontal gene transfer might influence dissemination of IS*5* elements to different strains of *E. coli*. Hence, horizontal gene transfer appears to be a form of quasi-Lamarckian inheritance (Koonin and Wolf, 2009) in prokaryotes, in which transferred genes confer selective advantages for growth in that environment. Here, we show clearly that cells can become highly motile to adapt to starvation conditions due to IS*5* hopping and perhaps this is facilitated by horizontal gene transfer.

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