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Transposon-mediated Directed Mutations in the *flhDC* Operon Regulatory Region of *Escherichia coli*

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Chika Kukita

Committee in charge:

Professor Milton H. Saier, Chair
Professor Nigel Crawford
Professor James Golden

2015

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Chair

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ABSTRACT OF THE THESIS

Transposon-mediated Directed Mutations in the *flhDC* Operon Regulatory Region of *Escherichia coli*

by

Chika Kukita

Master of Science in Biology

University of California, San Diego, 2015

Professor Milton Saier, Chair

Directed mutation, by definition, is a phenomenon where a mutation occurs at a higher rate when its consequence is beneficial than when neutral or detrimental. Recent work in our laboratory has shown that the *glpFK* operon of *Escherichia coli* is subject to directed mutation under certain specific environmental conditions. The flagellar system in *E. coli* is encoded by more than 50 genes that are expressed under the control of the *flhDC*-encoded master switch. Recently, Wang and Wood (Wang and Wood, 2011)

showed that transposition of IS5 to a site upstream of the *flhDC* operon up regulates transcription of this operon, resulting in overexpression of the whole flagellar cascade.

Here, we show that three IS elements, IS1, IS3, and IS5 transpose to upstream sites within the *flhDC* operon control region, some to previously unidentified sites, to up regulate the flagellar system. Several locations and orientations of insertions were identified for these three IS elements. At lease some of these transposition events occur at tremendously increased rates on soft-agar plates compared to liquid and solid media, allowing the cells to swarm through the agar at increased rates. We also show that at least some of these mutations preferentially occur where the parental cells are entering the stationary growth phase, probably because of nutrient limitation. We thus confirm and extend the results of Wang and Wood, showing that IS insertions upstream of the *flhDC* operon in soft-agar represent a second example of transposon-mediated directed mutation.

I.

Introduction

1. Transposon

A transposon, a jumping gene, hops from one location to another in the genome of an organism. Small transposons can induce mutations, and the consequence can be beneficial or detrimental to the host cell (Zhang and Saier, 2012). Transposition occurs in any of the following four manners; conservative, replicative, excisive and retro. Conservative transposition is known to be "cut and paste"; the original copy will be removed from the original location as it is inserted into a new location. Replicative transposition, on the other hand, is referred to as "copy and paste" as the original copy will remain at the original location while the new copy will be inserted at a novel position. By this method, the total number of copies increases by one. With either of these mechanisms, transposition results in insertion of the sequences into a new target location.

The smallest transposons are ~ 1 kbp long insertion sequences, IS elements. There are several IS elements found in *Escherichia coli,* and they play important roles in molecular and organismal evolution (Zhang and Saier, 2012). Among them, IS5 is the most studied IS element, and there are multiple copies in the *E. coli* chromosome (Wang and Wood, 2011). Several insertion sites have been identified for IS5 in *E. coli* including those upstream of the *fuc, bgl*, *flhDC*, and *glpFK* operons (Zhang and Saier, 2009 a and b, 2010, 2012, and 2014). In their early studies, Zhang and Saier showed that *crp* cells which lack the cyclic AMP receptor protein, Crp, had higher frequencies of IS5 hopping into a single site, and in a single orientation, upstream of the *glpFK* operon when glycerol was present in the medium. The IS5 insertion into that region resulted in tremendous

activation of *glpFK* operon expression. This was the first well documented example of transposon-mediated 'directed mutation'.

2. Directed Mutation

In general, mutations are referred as *adaptive* when their occurrences are random in location but are increased in frequency under specific stress conditions (Zhang and Saier, 2012). Mutations are *directed* if they occur at higher frequencies under the stress condition when the mutations specifically help relieve the stress that promotes mutation. The theory of directed mutation has been controversial, even after publication of the documented example of the transposon-mediated directed mutations in the *E. coli glpFK* operon (Zhang and Saier, 2009a, b).

The *glpFK* operon is one of the five *glp* operons in *E. coli* that together comprise the *glp* regulon. *glpFK* expression is under the control of both the glycerol repressor, GlpR, and the cyclic AMP receptor protein, Crp. *glpFK* expression is required for glycerol utilization. The *crp* strain which lacks Crp cannot utilize glycerol because the complex of Crp and cAMP positively regulates expression of *glpFK*. However, GlpR negatively regulates *glpFK* and the other four operons of the *glp* regulon, so its loss results in high level expression of these operons. IS5 insertion into the upstream activation site of the *glpFK* operon activates the promoter in a *crp* mutant. Zhang and Saier. (2009 a, b) showed that the frequencies of IS5 insertion into this specific site increased substantially when a *crp* mutant was plated on minimal M9 agar plates with glycerol as a sole carbon source. They documented low IS5 insertion frequencies at this site on plates containing other carbon sources, especially glucose, but hopping to other

known IS5 insertion sites was not increased by the presence of glycerol. This indicated that IS5 insertions upstream of the *glpFK* operon were specifically enhanced when the cells were under starvation stress if glycerol was present as a potential growth substrate. This effect of glycerol proved to be mediated by GlpR. Wild type *E. coli* cells specifically exhibit enhanced *glpFK* mutation rates when they are exposed to a glycerol medium in the presence of a non-metabolizable sugar analogue that blocks glycerol utilization (Zhang *et al.,* 2014). Because these mutations could relieve that stress, the phenomenon studied conformed to the definition of "directed mutation."

3. The Flagellar System

Flagellar motility allows bacteria to reach more favorable environments or escape from less favorable environments (Sountourina and Bertin, 2003). Flagellar synthesis and function are energetically expensive; thus, the flagellar system, which consists of more than 50 genes, is highly regulated (Figure 1). Promoters of the *E. coli* flagellar system are categorized into three classes; class I, class II, and class III according to their expression modes and timing (Fitzgerald, Bonocora, and Wade, 2014). In several bacteria including *E. coli*, the transcription factor, FlhDC, is the only class I protein, considered to be the master regulator of the entire flagellar system.

FlhDC activates the middle and late genes directly and indirectly, respectively, and mutants defective in either of *flhD* or *flhC* consequently do not express class II and class III genes. Class II transcripts include proteins for the basal region of the flagellar structure and the transcriptional regulator FlgM as well as an alternative sigma subunit of RNA polymerase, σ^{28} or σ^{F}. σ^{F} is encoded by *fliA* and is required for the transcription of

class III genes. Class III transcripts include motor torque generator subunits, MotA and MotB (genes *motA and motB* are located adjacent to *flhC* on the chromosome), as well as flagellin and chemotaxis proteins.

The transcription of *flhDC* is regulated by many transcription factors including OmpR, CysB, PhoB, NtrC and the cyclic AMP-CPR complex. It is also negatively autoregulated by FlhDC in the absence of σ^F, but positively regulated by FlhDC in the presence of σ^F (Chilcott and Hughes, 2000). Thus, flagellar biosynthesis and function are sensitive to many external and internal conditions.

4. Insertion sequences and their effects on gene activation

There have been several studies showing that IS insertions may cause activation or repression of genes adjacent to the insertion sites. The activation may occur directly or indirectly by up regulating or eliminating the repressor. FNR is known as the primary transcriptional regulator involved in the transition from aerobic to anaerobic growth in *E. coli* through repression of genes for aerobic growth and activation of genes for anaerobic growth (Sawers, 2005). The *nar* operon (*narG*, *narH*, and *narI*), encoding the nitrate reductase, is one of the operons positively regulated by the *fnr* gene product. IS5 insertion into the control region of the *fnr* gene disturbs the negative auto regulation of FNR and causes the increase in transcription of the gene (Bonnefoy, Fons, *et al.*, 1988).

IS insertion also plays a role in antibiotic susceptibility. IS1 insertions were found between the IS5 and *bla*CTX-M genes in several species of *Enterobacteriaceae.* The IS elements interrupted its promoter, causing a reduction in Extended-spectrum ß-lactamase (ESBL) production, lowering the ceftazidime MICs (Fernández, Gil, 2007). Although a

lon mutation alone was not sufficient to generate a multiple-antibiotic-resistant (Mar) phenotype, IS1 insertion in the *acrR* region or IS2 in *marR* caused the overexpression of MarA and resulted in a Mar phenotype (Nicoloff, Perreten, *et al.,* 2007). IS5 also has been found upstream of the *kpgABC* operon, an uncharacterized putative transporter. In *acrAB* mutant cells, this resulted in lowered susceptibility to tigecycline (Nelsen, Snesrud, *et al.*, 2014).

5. Insertion Mutations Upstream of the *flhDC* Promoter

In this study, we focused on transposon-mediated mutations occurring in the region upstream of the *flhDC* promoter. Although the detailed mechanisms remain unclear, *E. coli* K-12 strain BW25113 with any one of the several IS5 elements in the upstream region of the *flhDC* operon is known to have higher motility than wild-type cells (Wang and Wood, 2011, Barker *et al.,* 2004).

Ten identical copies of IS5 have been identified in the chromosome of the *E. coli* K12 BW25113 strain (Figure 2), and its transposition appears to occur by a copy and paste mechanism. IS5 is 1195 bp long and has several insertion sites recognized in *E. coli*. As noted above, IS1 and IS3 can also hop into the upstream region of *flhDC* to upregulate the flagellar system. IS1 is 768bp long with six copies in the *E. coli* strain used, while IS3 is 1258bp long with five copies present. When any one of these three IS elements is transposed into the upstream region of *flhDC*, transcription of *flhDC* is up regulated (Southerina and Bertin, 2003), resulting in the increased transctiption of class II and class III genes of the flagellar system, and consequenctly increasing motility.

6. Environmental Cue for the Directed Mutation

Directed mutation, by definition, is induced by an environmental stress and happens at higher frequencies when its consequence is beneficial (Zhang and Saier, 2012). On solid plates, flagella do not help them swarm, while in agitated liquid media, they do not need flagella to move. Soft agar plates have a low agar concentration (<0.4%), allowing the cells to swim through the gel (Copeland and Weibel, 2009). On solid agar plates (1.5% agar), however, the cells cannot swim through the gel, and they thus form stationary colonies on the surface. In the soft agar plates, they benefit from higher flagellar expression because the cells with more flagella have access to nutrients while non-motile or less-motile cells are trapped near the site of inoculation.

Here we confirm the experimental results of Wang and Wood (2011) and test the IS5 mutation ratio on soft agar plates relative to those with 1.5% or 0% concentrations of agar. We also show that in soft-agar plates, new mutations preferentially arise as growth of the parental cells slows down and ceases.

7. Wang and Wood's Results

In their 2011 paper, Wang and Wood demonstrated the effect of different environmental conditions on the occurrence of IS5 hopping into the upstream sites of the *flhDC* operon. First, they measured IS5 hopping frequencies in soft-agar plates (0.3%). The *E. coli* K12 BW25113 strain, weakly motile without IS5 upstream of the *flhDC* promoter, was inoculated onto soft-agar plates, and centric swarming zones were measured over time. The cells in the inner circle were collected separately from those between the two boundaries. Then, qPCR was performed for each group of cells to reveal

that the outer halo of any one mutant, with a faster swarming rate, had a transposon insertion. They tested the insertion frequencies with solid-agar plates (1.5% agar) as well as in LB and minimal nutrient liquid media. Under both of these conditions, the insertion frequencies remained low compared to the rates observed in soft-agar. The high concentration of agar (1.5%) does not allow *E. coli* to move out from the point of inoculation, while the low concentration (<0.4%) allowed them to move out. Thus, the concentration of agar provides an environmental cue, inducing IS5 insertion into the upstream region of *flhDC*.

Second, they tested for the conditions essential for IS5 hopping. The *flhD*-deleted strain, which had no motility, showed no swarming halo nor IS5 hopping in a qPCR assay. They also tested two other strains, *motA* and *flgK* mutants, which lack motility or the intact flagellar apparatus, respectively, but have an intact *flhDC* operon, and observed that no IS5 hopping had occurred. An *hns* mutant which was poorly motile because of the low expression of *flhDC*, however, showed a swarming halo as well as increased frequencies of IS5 insertions. They concluded that *flhD* and flagellar structural mutants do not generate IS5 insertions upstream of *flhD.*

Third, biofilm formation and growth rate for the strains with or without IS5 upstream of *flhD* were measured in LB and M9 liquid media at 37 °C. The results indicated that the IS5 insertion into the site upstream of *flhD* gained higher motility and an increased capacity for biofilm formation than the wild type strain. The growth rate was also measured in LB medium at 37°C for six hours, indicating slightly slower growth of the IS5 insertion mutant strain.

Fourth, the stability and heritability of the IS5 insertion were tested over 11 days. The wild type strain without the IS5 insertion and the IS5 insertion mutant strain were grown in M9 and LB media for 11 days, and they were diluted every 24 hours. While no wild type cell gained IS5 upstream of *flhD* during the 11-day period, the IS5 remained upstream of the *flhD* gene in the insertional mutant according to the results of qPCR experiments.

Fifth, they showed that IS5 hopping into the upstream region of *flhD* happens during biofilm formation, resulting in a subpopulation of highly motile cells. The swarming halo was detected on soft-agar plates. PCR screening for IS5 insertion into the *flhDC* upstream region was performed. They compared the biofilm cells in glass wool with planktonic cells that had in contact with the biofilm.

Finally they confirmed that IS5 hopping upstream of *flhD* did not result from a general increase in IS hopping into random insertion sites by several means. First, they estimated the total number of IS5 copies in the chromosomes of wild-type cells and the IS5 insertion mutant strain collected from the larger swarming halo on a soft-agar plate, showing there was only one additional copy found in the IS5 insertion mutants. Second, they performed PCR of the upstream regions for various operons other than *flhDC* which are known to have IS insertion sites, but no IS element was detected in any of these regions. Third, they compared the number of IS5 copies from cells grown on soft-agar plates with that from cells grown in LB medium and found that the increase was between 0.3 and 1.5.

II.

Material and Methods

Construction of Bacterial Strains

Strains and DNA oligonucleotides used in this study are described in Table 1 and Table 2, respectively. All the strains were derived from *E. coli* K-12 strain BW25113 (Datsenka and Wanner, 2000). To make the *flhDC* reporter strain, we first fused a promoter-less *cat* (encoding chloramphenicol acetyl transferase) structural gene (plus its own ribosomal binding site) to the 3' end of the *flhC* gene. The resultant operon *flhDC:cat* is under the control of the *flhDC* promoter. To do this, the region containing the *cat* gene and the downstream FRT-flanking kanamycin resistance gene (km^r) in pKD-*cat* (Zhang and Saier, 2009a) was amplified using oligos FlhC.cat-P1 and FlhC.cat-P2 (Table 2). FlhC.cat-P1 is composed of a 20 bp region at the 3' end that is complementary to the beginning of *cat*, and a 50 bp region at the 5' end that is homologous to the 3' end of *flhC*. FlhC.cat-P2 is composed of a 20bp region at the 3' end that is complementary to the FRT-flanking *km* sequence, and a 50 bp region at the 5' end that is homologous to the *flhC/motA* intergenic region. The PCR products (that is "*cat:km*^{r}") were gel purified and electroporated into BW25113 cells expressing the lamada Red proteins encoded by plasmid pKD46 (Datsenka and Wanner, 2000). The cells were applied onto LB + Km plates. Km^r colonies were confirmed for the replacement of the *flhC/motA* intergenic region (-326 to -1 relative to the start codon of *motA*) by the 'cat:km^r' fragment by colony PCR, followed by DNA sequencing. The FRT-flanking km^r gene was removed by first transforming the temperature-sensitive pCP20 plasmid and then growing a transformant at 40 °C overnight. The resultant strain was named Cat.SW- (swarming negative).

As shown in Figure 2, the promoter region of the *motAB* operon is located in the 3' end of *flhC*. The presence of *cat* in the *flhC/motA* intergenic region appears to abolish

expression of the downstream *motAB* operon since its promoter is destroyed. This strain would be expected to be non-motile. To restore motility, the *motAB* promoter region must be added back at the beginning of *motA*. To do this, a DNA fragment (P*motAB*) containing the 3' end of *flhC* and the *flhC/motA* intergenic region was cloned into the *Xho*I/*BamH*I sites of pKDT, yielding pKDT-P*motAB*. Present in this recombinant plasmid, the region (*km*^{r}:*rrnBT*:P*motAB*) carrying *km*^{r}, the *rrnB* terminator (*rrn*BT) and P*motAB* was amplified using the primers PmotAB-P1 and PmotAB-P2 (Table 2). The amplified fragment, *km*^{r}:*rrn*BT:P*motAB*, was electroporated into Cat.SW- cells to replace the region between the 3' end of *cat* and the 5' end of *motA*. The transformants were selected for Km resistance. The *km* colonies were confirmed for such replacement by colony PCR and subsequently by DNA sequencing. The *km*^{r} gene was removed as above. This yielded the reporter strain CAT, in which *flhD*, *flhC* and *cat* formed a single operon that is under the control of the *flhDC* promoter, and *motAB* expression is under the control of its own promoter.

In some cases, we needed to distinguish IS insertional mutants that arose after plating from the insertional mutants initially added. For this purpose, to facilitate isolation of IS insertional mutants, we inserted a *km* marker into the *ycaC/ycaD* intergenic region. The *km* gene was amplified from pKD4 using primers ycaD-P1 and ycaD-P2 (Table 2). The PCR products were purified and then inserted into the *ycaC/ycaD* intergenic region of CAT. The resultant strain that is kanamycin resistant was named CAT_Km^R.

As an alternative, we made a second *flhDC* reporter strain, in which the *flhD/flhC/cat* operon, under the control of the native *flhDC* promoter (P*flhDC-flhDC.cat*), was moved into another chromosomal location. To do this, the region containing the native *flhDC* promoter, *flhD* and the 5' end of *flhC* (but not the 3' end of *flhC* containing the *motAB* promoter region) was deleted. *flhDC.cat* was amplified from the genomic DNA of the first reporter strain CAT using primers PflhDC-Xho-L and Cat-Sal-R (Table 2), digested with *Xho*I and *Sal*I, then ligated into the same sites of pKDT, yielding pKDT-P*flhDC-flhDC.cat.* Using this plasmid as template, the region carrying *km*^{r}*:rrnB*T:P*flhDC-flhDC.cat* was amplified using primers IntC-P1 and IntC-P2. The PCR products were purified and then electroporated into BW25113 cells. The integration of "*km*^{r}*:rrnB*T:P*flhDC-flhDC.cat*" into the *intS/yftG* intergenic region was confirmed by colony PCR and DNA sequencing. The resultant strain was named CAT2, in which the native *flhDC* promoter, *flhD* and the 5' part of *flhC* were deleted, and a new copy of the same promoter driving the *flhDC.cat* operon was located in the *intS/yfdG* intergenic region.

Swarming Rate Measurements

Two wild type strains and 10 IS insertional mutant strains were grown in 3 ml of liquid medium (0.5% tryptone, 0.5% NaCl) at 30°C overnight and diluted with 1X M9 salts solution to an OD of 1.0. Then, 1.5 µl of each culture was used to inoculate the center of a soft-agar plate (0.5% tryptone, 0.5% NaCl, 0.3% agar). The plates were incubated at room temperature, and the diameters of the swarming zones were measured

from 8 to 17 hours after inoculation. Graphs of the swarming diameters were plotted as a function of time, and the swarming rates were calculated.

The experiment was repeated with glucose (0.5%) added to medium. Before inoculating the soft-agar plates, the strains were grown in liquid media (0.5% tryptone, 0.5% NaCl, 0.5% glucose). The soft-agar plates used were made with 0.5% tryptone, 0.5% NaCl, 0.5% glucose, and 0.3% agar. These plates were treated in the same way as for the experiment without glucose. The slopes were used as the swarming rates, and the rates for each strain with and without glucose were determined (Table 3).

Growth Rates

Growth rates of *E. coli* BW25113 *flhDC:cat:'flhC:motAB* (CAT) strain as well as CAT_IS1 and CAT_IS5 were measured in liquid media (0.5% tryptone, 0.5% NaCl, 0% agar), on solid-agar plates (0.5% tryptone, 0.5% NaCl, 1.5% agar) and on soft-agar plates (0.5% tryptone, 0.5% NaCl, 0.3% agar). Each strain was grown in 3 ml of liquid medium (0.5% tryptone, 0.5% NaCl) at 30°C overnight, and the cultures were diluted with about 1X M9 salts solution to an OD of 1.0.

For liquid media, 5-10 µl of the overnight culture was added to 5 ml of medium. The tubes were incubated in a 30 °C shaking water bath for up to 24 hours. Every three hours, 500 µl of the cultures were taken, and the OD_{600} was measured to plot the growth curves.

For the plates, 4 µl of the overnight culture (OD of 1.0) was inoculated in a single streak across the plate and incubated in a 30 °C incubator. At every sample point, the solid agar plates were washed with 3 ml of 1X M9 solution, and the cell slurry was

collected in a microcentrifuge tube. Then the cell culture was diluted with the same buffer to spread on LB plates. These plates were incubated at 37 °C before the colonies were counted.

The soft-agar plates were inoculated in the same way as the solid-agar plates. Every three hours, the entire gel was scraped off into a large test tube, and each plate was washed with 3 ml of M9 buffer. The gel was vortexed for 2 minutes to mix the preparation, and the resultant suspension was centrifuged at 800 rpm for 30 sec to separate the gel from the buffer. Such centrifugation (800 rpm, 30sec) was confirmed not to spin down bacterial cells. The buffer was collected in a microcentrifuge tube and diluted to plate onto LB plates. The plates were incubated at 37 °C to measure the cell populations.

For soft-agar plates, the growth rates of CAT_Km^R (wild type) and CAT_IS5 were measured while these two cell types coexisted in a single culture. The overnight cultures were diluted with M9 buffer, and a mixture of CAT_Km^R (2×10^9 cfu/ml) and CAT_IS5 (2×10^6 cfu/ml) was made. Then, 4 µl of the mixture was inoculated in a single streak across the center of the soft-agar plates and incubated at 30 °C. Every three hours, the plates were washed and diluted with 3 ml of M9 buffer to plate on LB plates, LB + Cm plates (LB with 6 mg/ml chloramphenicol), and LB + Cm + Km plates (LB with 6 mg/ml chloramphenicol and 25 mg/ml kanamycin). These plates were incubated in a 37 °C incubator before counting colonies. The number of colonies on LB plates gave the total population; that on LB + Cm plates gave the total population of insertion mutants, and that on LB + Cm + Km gave the population of newly emerging mutants. The growth

rate of the IS5 insertional mutant that was added at the beginning was calculated by subtracting the newly emerging population from the total insertion mutant population.

Mutant Ratios

Mutant ratios were measured for three media, the same as for the growth rate experiment. The CAT strain was grown in 3 ml of liquid medium in a 30 °C water shaker overnight and diluted with 1X M9 buffer to an OD of 2.0.

For liquid media, 5-10 µl of the overnight culture was inoculated in 5 ml of medium and incubated in a 30 °C shaker. At four different time points, the culture was plated on LB plates (diluted with 1X M9 buffer) and LB + Cm plates (undiluted). The mutant ratio was calculated by dividing the mutant population by the wild type population.

For solid-agar plates, 4 µl of the overnight wild type culture (OD of 2) was plated in a single streak across the plate and incubated in a 30°C incubator. At four different time points, each plate was washed with 3 ml of M9 buffer and plated onto LB plates (diluted with 1X M9 buffer) and LB + Cm plates (undiluted) and incubated in a 37°C incubator. The colonies were counted, and ratios of the total population of wild type to mutant cells were determined.

Soft-agar plates were inoculated and incubated in the same way as solid-agar plates. Every three hours, the agar was collected in a test tube, and the plates were washed with 3 ml of M9 buffer. The collected agar was mixed by vortexing and centrifuged as described above, and the cultures were diluted to plate on LB plates and LB + Cm plates.

Mutant ratios were determined for another wild type strain, CAT2, using these three media at two temperatures, 30 °C and 37 °C. The strain was grown in 3 ml of a liquid medium in a 30 °C and 37 °C shaker, respectively, over night. The overnight culture was diluted with M9 buffer to an OD of 1. Then, the culture was inoculated and incubated as described above. For this strain, a higher concentration of chloramphenicol (LB with 14 mg/ml chloramphenicol) was used for mutant isolation.

Original locations of IS elements on the chromosome

NCBI-BLAST was used to identify the locations and directions of original IS elements on the chromosome of *E. coli* K-12 strain BW25113 (GCA_000750555.1). The positions of the IS element sequences were determined. Then, the relative locations were plotted on the circular diagram of the chromosome. In Figure 3, blue arrow indicates the IS1s; purple, the IS3s, and green, the IS5s. The heads of the arrows indicate the direction of transcription of the transposase gene.

PCR

Polymerase chain reaction (PCR) was performed in the reaction mixtures according to the manufacturer's instructions; all primers were added at a concentration of 1μM. The reaction mixture contained a single colony of a sample in a total volume of 20μl. The forward primer used was PflhDC-Xho-F, and the reverse primer was PflhDC-Rn. The reaction was amplified as follows under the following conditions; initial denaturation at 94 °C for 2 min 30 s, followed by 30 cycles of 94 °C for 1 min, 55 °C for

1 min, and 72 °C for 2 min; a final extension step of 72 °C for 15 min followed. PCR products were analyzed by agarose electrophoresis.

DNA Extraction and Sequencing

Once PCR was run for the reaction mixture of 50µl, total volume, agarose electrophoresis was performed, and the DNA bands were excised. The excised bands were treated with BIOMIGA EZgene™ to extract the DNA, and it was pre-mixed with the reverse primer, PflhDC-Rn to be sequenced by Retrogen, inc.

III.

Results

1. **Swarming rates**

When an IS element was inserted upstream of the *flhDC* promoter, operon expression was increased, causing the cells to acquire higher motility (Wang and Wood, 2011). Including two wild types, *Escherichia coli* K-12 strains BW25113 and CAT, 13 strains were tested for swarming rates (Figure 4) when glucose was absent from the media. All the IS mutants swarmed at higher rate than the wild type with different degrees of increase. IS1(-214 rev), IS1(-469), IS1(-120rev), IS1(-415rev), IS3(-199 rev), IS5(-318), IS5(-318rev), IS5(-99), IS5(-99rev), and IS5(-169) had relative swarming rates of 2.7 mm/h, 2.2mm/h, 2.8mm/h, 2.5mm/h, 2.7mm/h, 2.5mm/h, 2.4mm/h, 2.5mm/h, 2.4mm/h and 2.1mm/h, respectively, while wild type swarmed at 1.2mm/h (Table 3). Thus, the slowest mutant showed a 1.75 fold faster rate while the fastest mutant showed a 2.3-fold increase.

The swarming rates for those strains were measured again with glucose (0.5%) present in the media. In the presence of this nutrient, all of the mutant strains swarmed at faster rates while the wild type strain lost its swarming ability (Figure 5). The mutants increased their swarming rates by 1.5 to almost 2-fold compared to those without glucose in the media (Table 3).

2. **Growth rates for wild type and mutants when separately inoculated**

Three strains, CAT (wild type), CAT_IS1, and CAT_IS5 were inoculated in three different types of media. The media differed only by the concentration of agar, which was 0%, 0.3%, and 1.5% for liquid media, soft-agar plates, and solid-agar plates, respectively.

The growth curves for these three strains overlapped for all three media up to 24 hours, and no significant difference was identified (Figures 6, 7 and 8).

3. Growth rates for wild type and mutant strains when inoculated together

When the mixture of CAT_Km^R (wild type) and CAT_IS5 grew in a medium (0.5% tryptone, 0.5% NaCl, 0.3% agar) together, the IS5 strain showed a slight increase in growth rate where the doubling time of wild type was 62 min and that of the IS5 mutants was 49 min (Table 4). This difference decreased when the tryptone concentration was increased by 5-fold in the medium. The wild type had a doubling time of 57 min and the mutant doubled in 47 min (Table 5). The growth rates were measured using the wild type strain that has a chloramphenicol resistance gene fused to the *flhDC* operon and a kanamycin resistance gene at another location in the chromosome. This allowed us distinguish between the IS5 mutant, CAT_IS5, and the newly emerging insertion mutants. While the added IS5 strain was only resistant to chloramphenicol, the newly derived insertion mutants from CAT_Km^R were resistant to both chloramphenicol and kanamycin. This gave us more accurate growth curves for the wild type and IS5 strains. On both 0.5% tryptone and 2.5% tryptone soft-agar plates, all the strains reached stationary phase around 15 hours after inoculation (Figures 9 and 10).

4. Original locations of IS elements on the chromosome

IS elements are intrinsically present in the *E. coli* chromosome. The number of copies and their locations differ from strain to strain. In this study, *Escherichia coli* K-12 BW25113 was used as a wild type, and the numbers and locations of three IS elements,

IS1, IS3, and IS5, were determined using NCBI-BLAST. There were six copies of IS1 in the chromosome, five copies of IS3, and 10 copies of IS5 (Figure 3). Some of them were in the direct orientation, while others were in the reverse orientation. One IS1 and three IS5s were located near the target *flhDC* promoter, but it is not known which IS element inserted upstream of the promoter.

5. Insertion Sites

The insertion events of IS elements upstream of the *flhDC* promoter did not happen in an identical manner for different IS elements or even for the same IS element. Each IS was found to be inserted into several locations in either one or both orientations. The insertion sites were identified by DNA sequencing of the upstream region of the *flhDC* promoter. Once an insertion mutant was isolated from the soft-agar plates, the part of the upstream region of the *flhDC* operon which includes the insertion site was amplified by PCR. Then, the PCR product was isolated by agarose electrophoresis, and DNA was extracted for sequencing. IS1 was found at four different locations; two in a reversed orientation between -415 and -416 and -214 and -215, and two in a direct orientation between -469 and -470, and -120 and -121 (Figure 11). A single IS3 insertion site was identified between -199 and -200 in the reverse orientation. IS5 was found to insert at three sites, between -318 and -319, -169 and -170, and -99 and -100. At the first and the third of these insertion sites, IS5 was inserted in both orientations.

While multiple insertion sites were identified upstream of the promoter, there were no IS elements found downstream of the transcriptional start site of the *flhDC* promoter.

6. Mutant ratios in liquid media and on solid-agar plates

CAT was used as the wild type strain in this experiment. A chloramphenicol resistance gene was fused at the end of the *flhC* gene so that it was under the control of the *flhDC* promoter and was up regulated whenever the *flhDC* promoter was activated by an IS insertion upstream of *flhDC*. This allowed us to measure the populations of wild type and IS mutants on LB plates and LB + Cm plates, respectively.

In liquid media (0.5% tryptone, 0.5% NaCl, 0% agar), the insertion frequencies remained low (< 5 mutants/10^8 cells) plated after 25 hours (Figure 7). CAT, CAT_IS1, and CAT_IS5 had identical growth rates in liquid media (Figure 7).

On solid-agar plates (0.5% tryptone, 0.5% NaCl, 1.5% agar), the mutation ratio was also low (< 5 mutants/10^8 cells). Both in liquid media and on solid-agar plates, there was no benefit to the cells by up regulating *flhDC* expression. In fact, the biosynthesis and function of the flagellar system would be expected to be detrimental because of the high energy consumption. Wang and Wood, in fact, reported slower growth rates for an IS5 insertional mutant compared to their wild type strain, but we never observed this.

7. Insertion frequencies on soft-agar plates

Soft-agar plates were tested with two different concentrations of tryptone in order to evaluate the effect of nutrient deficiency on mutation rate. While growth of wild type and mutant cells entered the stationary growth phase about 15 hours after inoculation, the emerging insertion mutants only began at about 15 hours and continued to increase for several hours (Figures 9, 10). The mutant population had been low for the first five hours after inoculation, but it increased exponentially thereafter.

The doubling times were compared for the CAT_Km^R (wild type), CAT_IS5, and emerging insertion mutants in 0.5% and 2.5% tryptone soft-agar plates. For 0.5% tryptone soft-agar plates, these three populations showed *apparent* doubling times of 62 min, 49 min, and 62 min, respectively (Table 4). Thus, the IS mutant had a slightly faster doubling time than the wild type, the opposite of that reported by Wand and Wood (2011). Between 15 and 25 hours, growth of both the wild type and IS mutant reached the stationary phase (Figure 9) when the doubling time was 288 min and 866 min, respectively. The mutant population, however, kept increasing as it maintained its *apparent* doubling time of 60 min.

This trend was the same in 2.5% tryptone soft-agar media (Table 5). For the first 15 hours after inoculation, the doubling times for CAT_Km^R, CAT_IS5, and the emerging insertion mutants were 57 min, 47 min, and 60 min, respectively. When both the wild type and IS5 strains reached the stationary phase, the emerging mutant population kept growing with an apparent doubling time of 59 min.

The rate of mutant appearance was also determined with another wild type strain, CAT2 at 30 °C and 37 °C, and the trend was same for these two conditions (Figures 12 and 13). The mutant population remained low for solid-agar and liquid media for the 25-hour period. Mutant cells kept increasing exponentially on soft-agar plates while the wild type cells had reached the stationary phase.

IV.

Discussion

In the reported studies, they found that an IS5 insertion mutation upstream of the *flhDC* operon occurred at a high rate only when its consequence was beneficial. Though the mechanisms of activation of the operon downstream of the insertion sites remain unknown, insertion of the transposons increase motility. In our lab, Dr. Zhang recognized three IS elements, two of which had been demonstrated previously (Baker, Prüss *et al*., 2004, Martinez-Vaz, Xie *et al*., 2005), which have been shown to hop into the regulatory region of the operon and were responsible for increasing expression of the downstream genes. Flagella allow *E. coli* to swim through soft-agar (0.3% agar) but not through solid-agar (1.5% agar), and flagella are not useful in agitated liquid media where the medium is of uniform composition at any one point in time. Of these three media, the overexpression of flagellar genes is beneficial only in soft-agar plates, and only in soft-agar do these mutations arise at high frequency. The cells with more flagella swarm faster through soft-agar and are capable of reaching nutrients ahead of the swarm of cells with fewer flagella. Because these IS elements were found after maintenance in soft-agar, it can be inferred that insertion of all three transposons at *flhDC* activating sites are subject to directed mutation.

Despite different degrees of increase, the IS insertion mutants always swarmed at faster rates than the wild type (Figure 4). Indeed, this was the basis for their selection. Yet, the mechanisms by which the transposition increased expression of the downstream operon is unknown. Activation may be accomplished by one of the following four mechanisms; 1) the IS element could directly activate an existing chromosomal promoter, 2) it could use a promoter already on the transposon to activate expression, 3) it could

create a new hybrid-promoter (e.g., the -35 region on the transposon with the -10 region on the chromosome); and 4) it could inactivate a repressor.

The mutants showed greater swarming rates in the presence of glucose than in the absence of glucose, although glucose repressed flagellar synthesis in the wild type strain (Table 3). The native *flhDC* operon is positively activated by the cyclic AMP receptor protein (CRP) which forms a complex with cAMP to bind to a specific site in the promoter (Soutourina, Kolb *et al.,* 1999). Wild type cells lose their motility when glucose is present, conditions in which cAMP levels are low because the activation mechanism is CRP-dependent.

The *E. coli* chromosome bears several copies of each IS element (Figure 3). We have not determined which IS copy was transposed into which insertion site upstream of the *flhDC* operon. The insertion sites of each IS element were identified by DNA sequencing of the upstream region of the promoter, and no two were the same (Figure 11). Different IS elements inserted into different locations, and a single IS element could localize to different locations with different orientations and still activate *flhDC* expression. No apparent correlation between the insertion sites and swarming rates was seen for IS5, but a possible correlation could be seen for IS1 (Supplementary Figure 1). Although the detailed mechanisms of transposition are unknown, transposition of IS elements to sites upstream of the *flhDC* promoter in soft-agar yielded strains with higher expression, giving rise to increased motility.

For all three media; liquid, solid-agar, and soft-agar, the growth rates for wild type and IS mutants were similar up to 24 hours when each cell type was incubated separately (Figures 6, 7, and 8). In liquid media and on solid-agar plates, the mutant ratio

remained low (< 5 mutants/10^8 cells) up to 25 hours. Under these conditions, up regulation of the flagellar system was not beneficial, and the insertional mutations may have arisen at rates comparable to those of other mutations.

On soft-agar plates, on the other hand, the population of mutants increased to a tremendous rate compared to that in or on other media. In order to take growth rate differences into account, the growth rates of wild type and an IS5 insertional mutant were measured when they were inoculated together in addition to separate inoculation. For both 0.5% and 2.5% tryptone soft-agar plates, the IS5 mutants grew somewhat faster than the wild type (Figures 9, 10) in contrast to the work reported by Wang and Wood (2011). This indicated that higher expression of the flagellar genes gave the cells benefit on soft-agar plates. They could reach a new niche when the nutrients around them were used up.

With the benefit that the cells acquired with higher motility, they grew faster than wild type, contrary to what was reported by Wang and Wood (2011). Yet, the increase in the insertion mutant population was much larger than could be accounted for simply by faster growth. The growth rate was only different by ~20% when cells were in the log phase of growth but the mutant population kept increasing after most cells had reached stationary phase. Between 15 and 25 hours, the wild type and the IS strains did not differ appreciably, but the emerging IS mutants increased by as much as one thousand fold once the cells had approached the stationary phase (Figures 9 and 10). This showed that the insertion mutations were dramatically upregulated on soft-agar plates. This trend was the same for the other wild type strain, CAT2 (Figures 12 and 13).

The mechanism of the IS5 mutation upstream of the *glpFK* promoter provided the first example of directed mutations. By definition, directed mutations are induced by an

environmental stress. Although its detailed mechanisms were still unclear, we showed that IS insertion upstream of the *flhDC* operon provides another example of directed mutation. Transposition of IS elements, specifically to the upstream region of the *flhDC* operon, occurred at higher rates only when its consequence, higher motility, was beneficial to the cells.

In future studies, there are still many aspects of IS-mediated *flhDC* directed mutation that need to be determined. For example, how does each IS element activates the *flhDC* transcription? Which original IS element is transposed to which locations? How do the environmental cues triggers transposition to the upstream sites? Future work will be required to answer these questions.

V.

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VI.

Figures and Tables

Figure 1. Schematic diagram of the *E. coli* flagellar regulatory cascade

Flagellar biosynthesis occurs in an ordered regulated fashion. The flagellar apparatus and associated proteins are made from three classes of genes. FlhDC is the one and only class I product, and it is the master regulator for the entire system. The FlhDC complex activates the σ^{70}–dependent transcription of class II genes, one of which is σ^{F} and its regulator, FlgM. σ^{F} directs transcription of class III genes. Class II genes include the intermediate flagellar structure that comprises the basal region of the flagellum, embedded in the membrane, forming the channel for FlgM and flagellin export. The two competing regulatory proteins, FlgM and FliA (σ^{F} or σ^{28}), are responsible for the expressional timing and transcription of class III genes, respectively. FlgM binds to σ^{F} to prevent premature class III gene transcription until the basal structure is complete; then FlgM is released from the cell when the basal pore-forming structure is completed, freeing σ^{F} for class III transctiprion. Thus, class II genes mainly include the hook-basal body regions of the flagellum while class III genes encode the external filament as well as the *mot* and chemotaxis (*che*) genes.

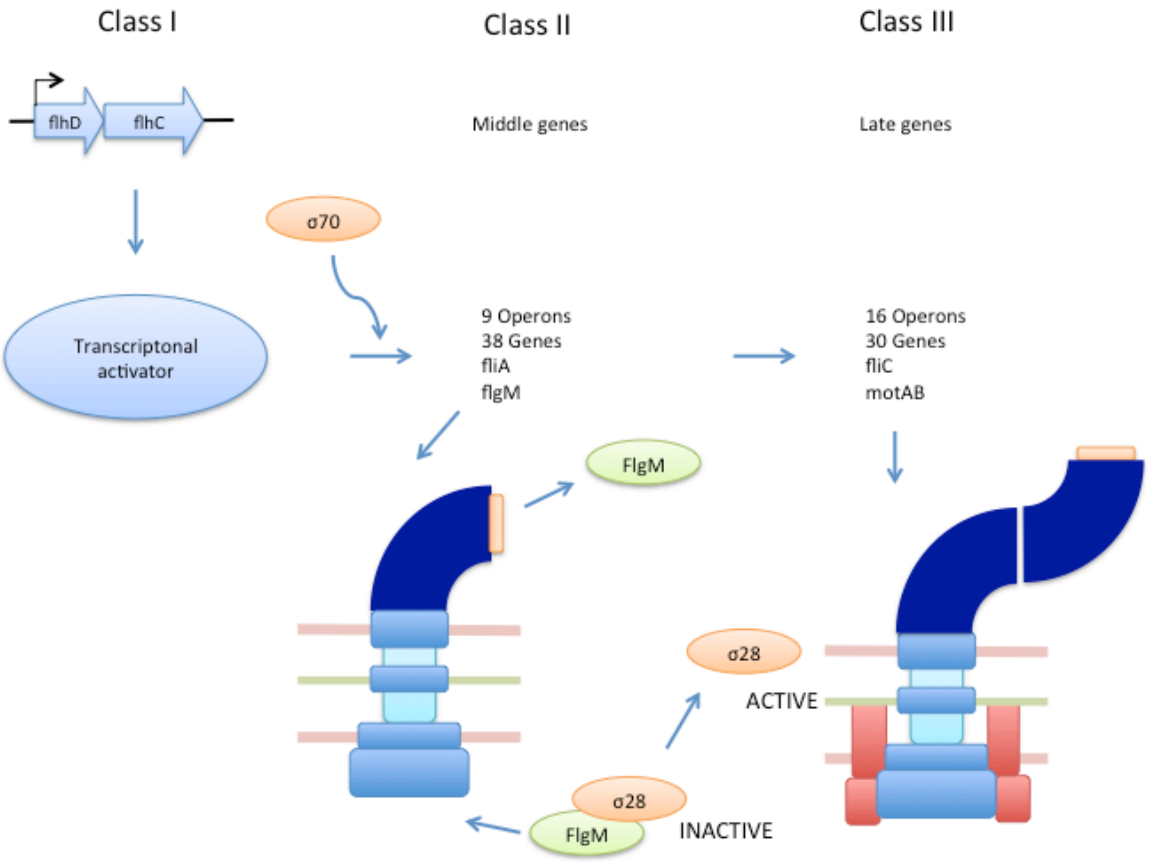
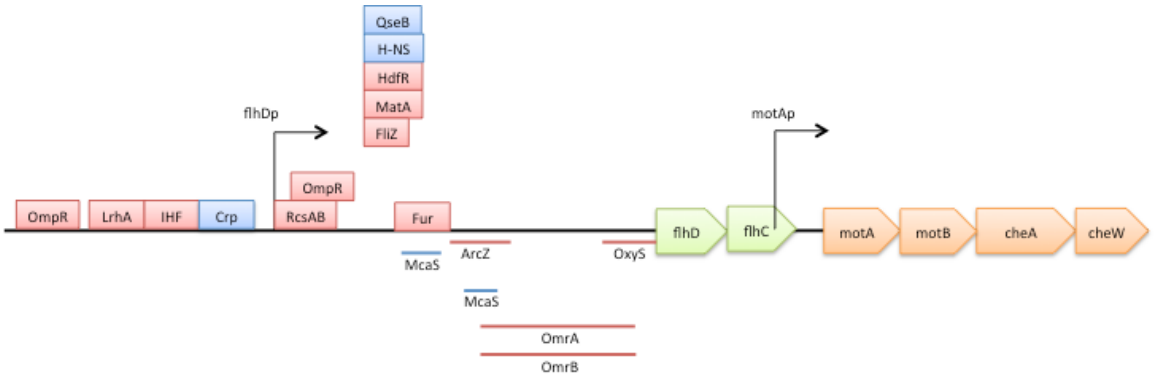
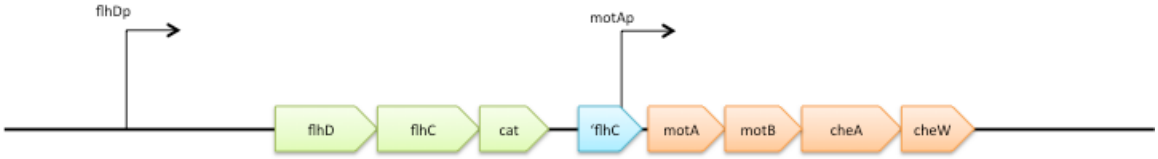

Class I
Class II
Class III
flhD
flhC
Middle genes
Late genes
σ70
Transcriptonal activator
9 Operons
38 Genes
fliA
flgM
16 Operons
30 Genes
fliC
motAB
FlgM
σ28
ACTIVE
σ28
FlgM
INACTIVE

Figure 2. The schematic diagram of the *flhDC* operon

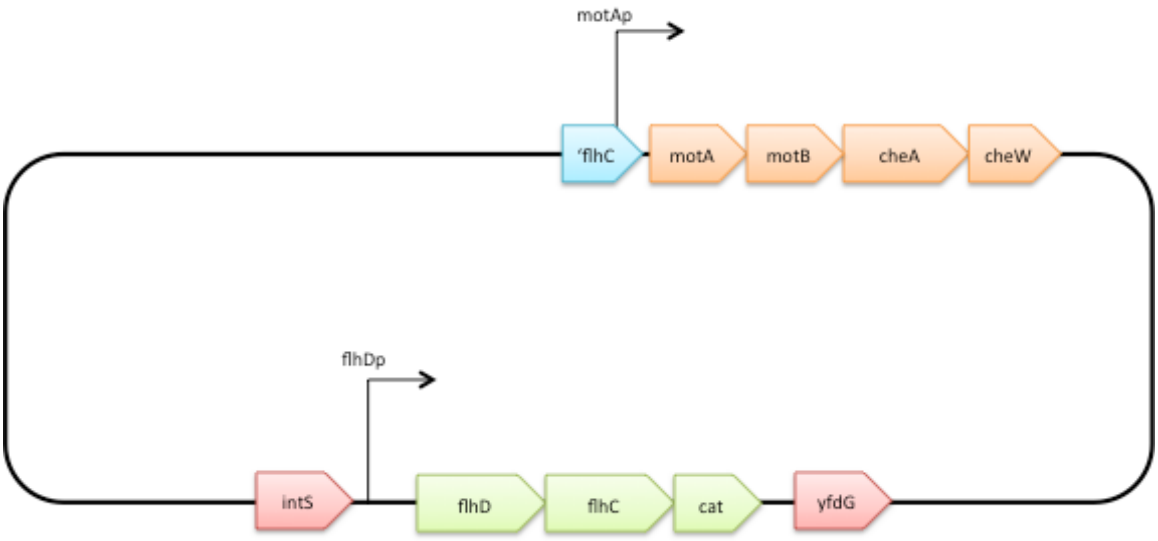
a) Schematic diagram of the wild type *flhDC* operon. The *flhD* and *flhC* genes are transcribed from one promoter while a second promoter is found in the 3' region of *flhC*, in front of *motA, motB, cheA*, and *cheW*. Red rectangles indicate the negative regulators and blue rectangles indicate the positive regulatory proteins. The lines below the gene depiction are binding sites for small RNAs. The two arrows indicate the promoters.
b) Schematic diagram of the constructs used to understand the regulated expression of the *flhDC* operon in the CAT strain. The *cat* gene is fused immediately after the *flhC* gene, and the part of the *flhC* gene within which the *motA* promoter is found was fused to the *motA* gene. This promoter is therefore between the *cat* and *motA* genes, so that the downstream genes are expressed under the control of its native promoter.
c) Schematic diagram of the CAT2 strain. The original *flhDC* promoter, *flhD* and the 5' part of *flhC* (but not the 3' end of *flhC* containing the *motAB* promoter region) was deleted, and the *flhD/flhC/cat* operon was placed under the control of the native *flhDC* promoter. It was moved into the *intS/yfdG* intergenic regio


QseB
H-NS
HdfR
MatA
FliZ
flhDp
motAp
OmpR
OmpR
LrhA
IHF
Crp
RcsAB
Fur
flhD
flhC
motA
motB
cheA
cheW
McaS
ArcZ
OxyS
McaS
OmrA
OmrB

a)


flhDp
motAp
flhD
flhC
cat
'flhC
motA
motB
cheA
cheW

b)


motAp
'flhC
motA
motB
cheA
cheW
flhDp
intS
flhD
flhC
cat
yfdG

c)

Figure 3. The original locations of IS elements on the *E. coli* chromosome
The original locations of IS elements and related genes are indicated on the *E. coli* K-12 BW25113 (GCA_000750555.1) chromosome. The green arrows correspond to IS5, purple to IS3, and blue to IS1. The heads of the arrows indicate the orientations of transcription of the transposase genes. The *flhDC* operon is located between 1972344 and 1973236bp.

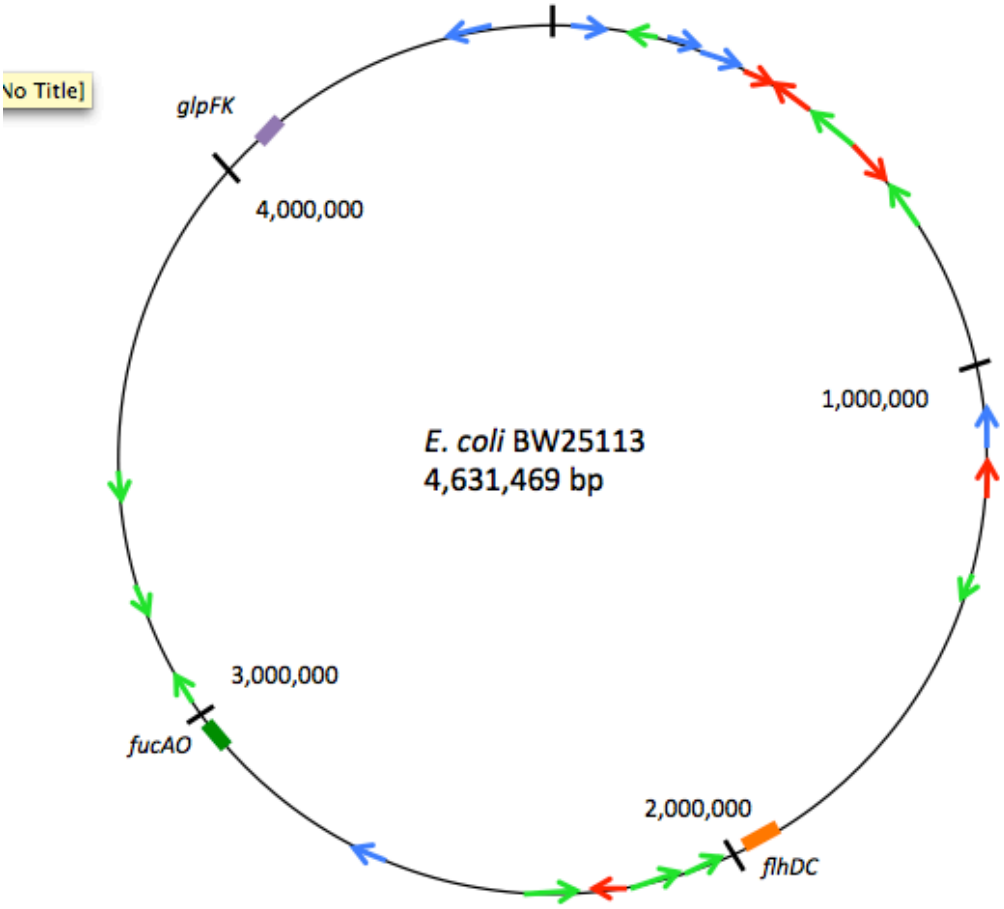

No Title]
glpFK
4,000,000
1,000,000
E. coli BW25113
4,631,469 bp
3,000,000
fucAO
2,000,000
flhDC

Figure 4. Swarming rates of wild type (CAT) and IS mutants without glucose

Diameters of swarming zones of IS mutants and wild type strain when plated on soft-agar without glucose in the medium as a function of time. All the plates were inoculated and incubated at room temperature.

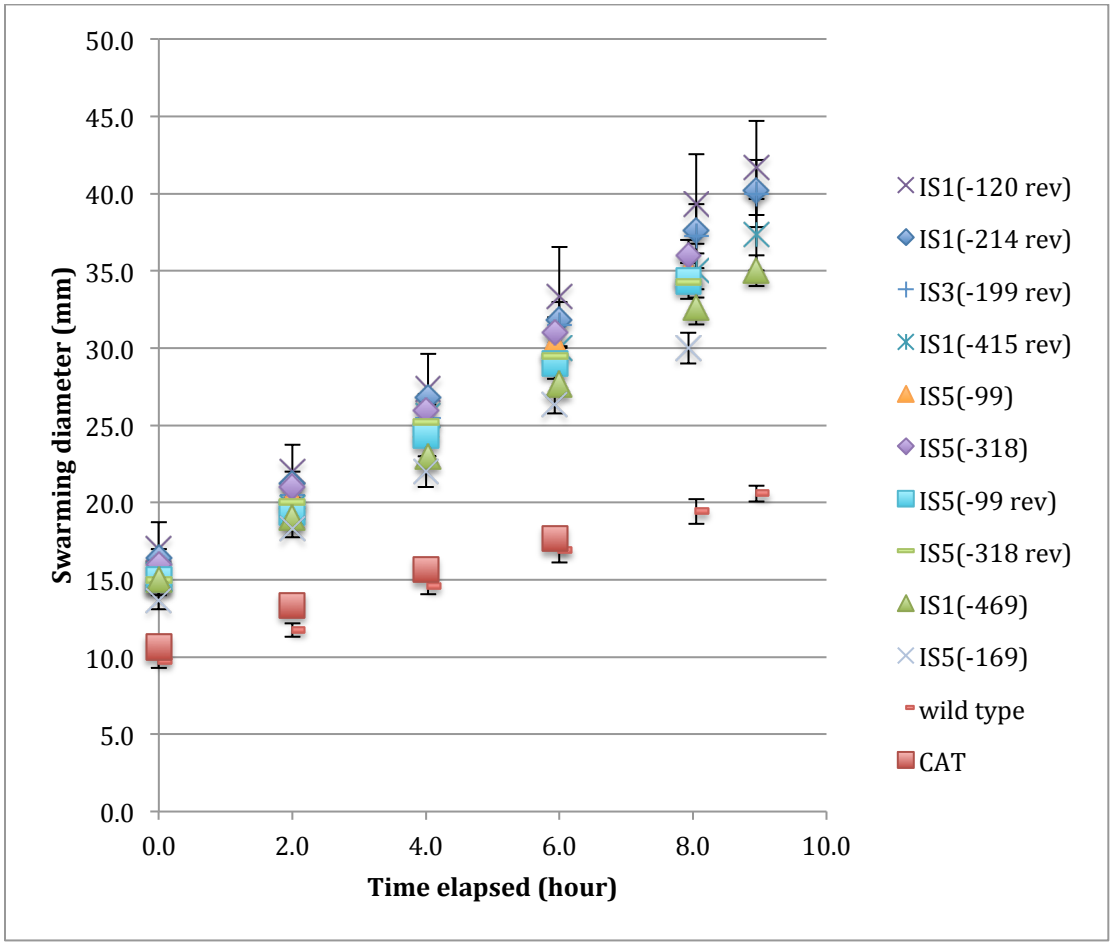

50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Swarming diameter (mm)
0.0
2.0
4.0
6.0
8.0
10.0
Time elapsed (hour)
IS1(-120 rev)
IS1(-214 rev)
IS3(-199 rev)
IS1(-415 rev)
IS5(-99)
IS5(-318)
IS5(-99 rev)
IS5(-318 rev)
IS1(-469)
IS5(-169)
wild type
CAT

Figure 5. Swarming rates of IS mutants with glucose

Diameters of the swarming zones of the IS mutants and the wild type strain (CAT) when plated on soft-agar with glucose (0.5%) in the medium as a function of time. All plates were incubated at room temperature.

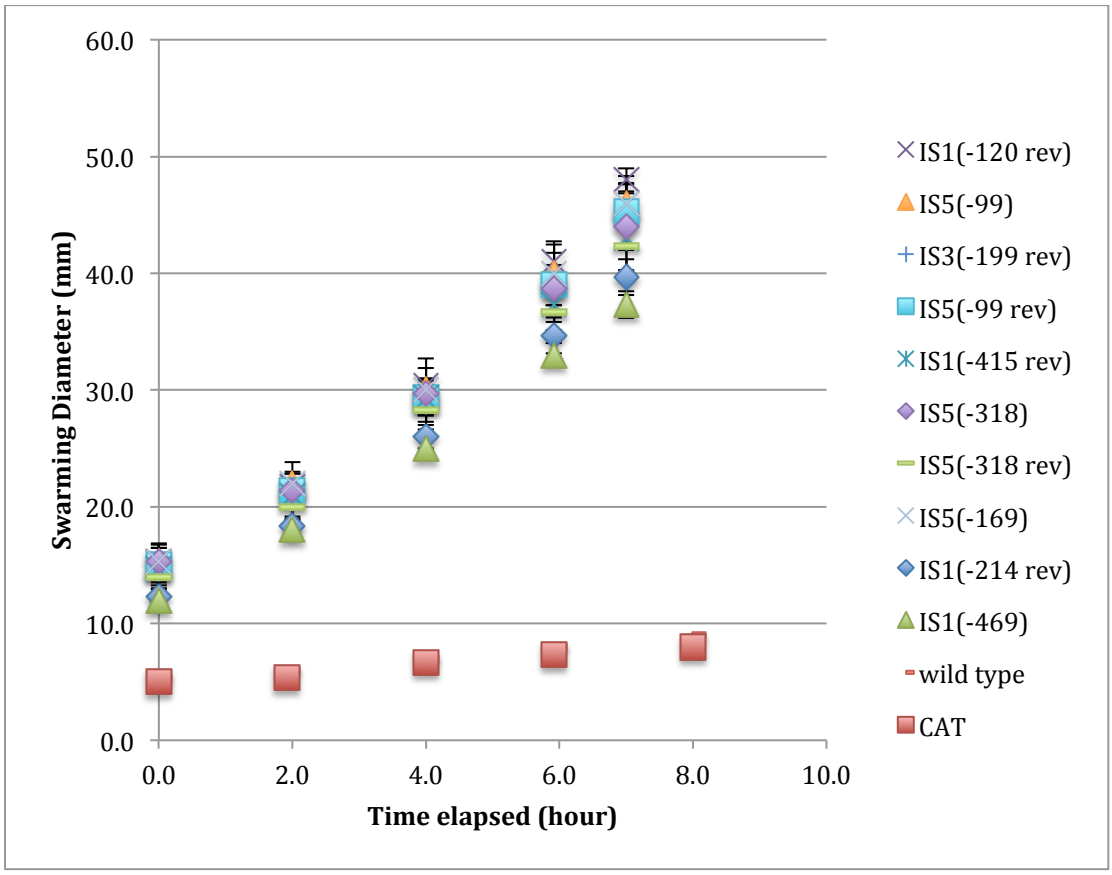

60.0
50.0
40.0
30.0
20.0
10.0
0.0
Swarming Diameter (mm)
0.0
2.0
4.0
6.0
8.0
10.0
Time elapsed (hour)
IS1(-120 rev)
IS5(-99)
IS3(-199 rev)
IS5(-99 rev)
IS1(-415 rev)
IS5(-318)
IS5(-318 rev)
IS5(-169)
IS1(-214 rev)
IS1(-469)
wild type
CAT

Figure 6. Growth curves and mutant ratios on solid-agar plates

a) Growth curves on solid-agar plates. CAT (orange rhombuses), CAT_IS1 (blue squares), and CAT_IS5 (green triangles) were inoculated separately on solid-agar plates (0.5% tryptone, 0.5% NaCl, 1.5% agar) and incubated at 30°C for 24 hours. Cells were washed off the plates, diluted, and plated for colony counting. The growth curves were plotted for each strain as a function of time. These three curves use the left y-axis. b) Insertion frequencies on solid-agar plates. The CAT strain was inoculated on solid-agar plates, and the insertion mutant ratios were determined at four time points throughout the 25-hour period. The ratio used the right y-axis.

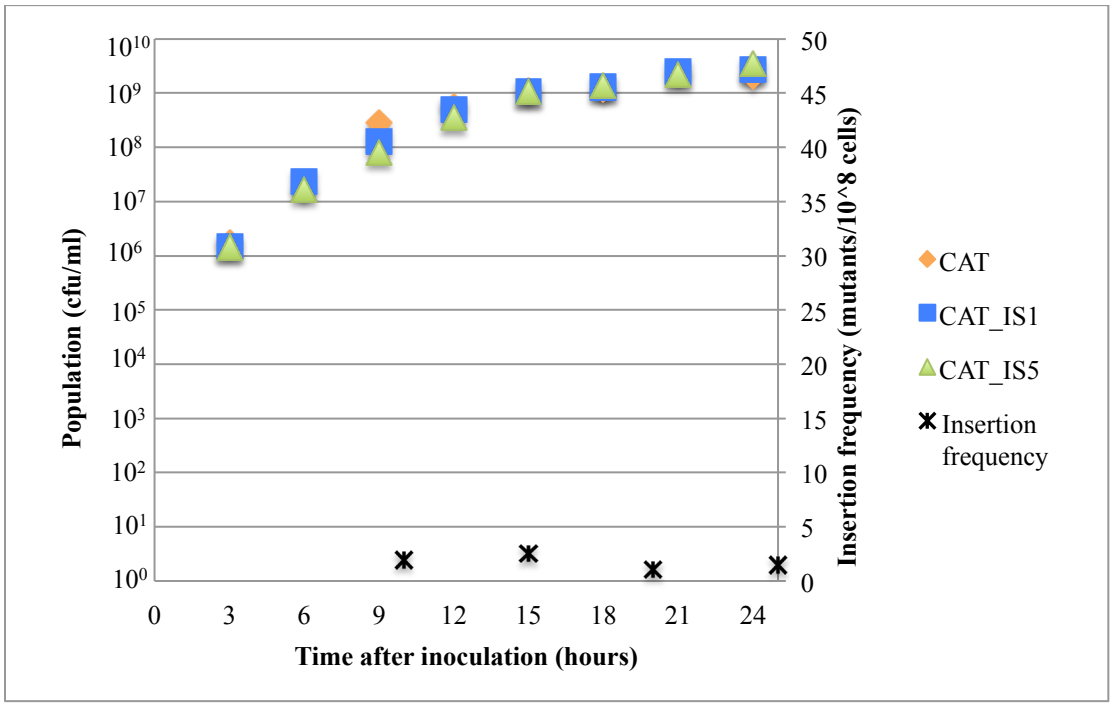

Population (cfu/ml)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Insertion frequency (mutants/10^8 cells)
50
45
40
35
30
25
20
15
10
5
0
0
3
6
9
12
15
18
21
24
Time after inoculation (hours)
CAT
CAT_IS1
CAT_IS5
Insertion frequency

Figure 7. Growth curves and mutant ratios in liquid media

a) CAT (orange rhombuses), CAT_IS1 (blue squares), and CAT_IS5 (green triangles) were inoculated separately into liquid-media (0.5% tryptone, 0.5% NaCl, 0% agar) at 30°C for 24 hours. The growth curves were plotted for each strain. b) Insertion frequencies in liquid media. The CAT strain was inoculated in liquid media, and the insertion mutant ratios were measured at four time points through a 25-hour period. The ratio refers to the right y-axis.

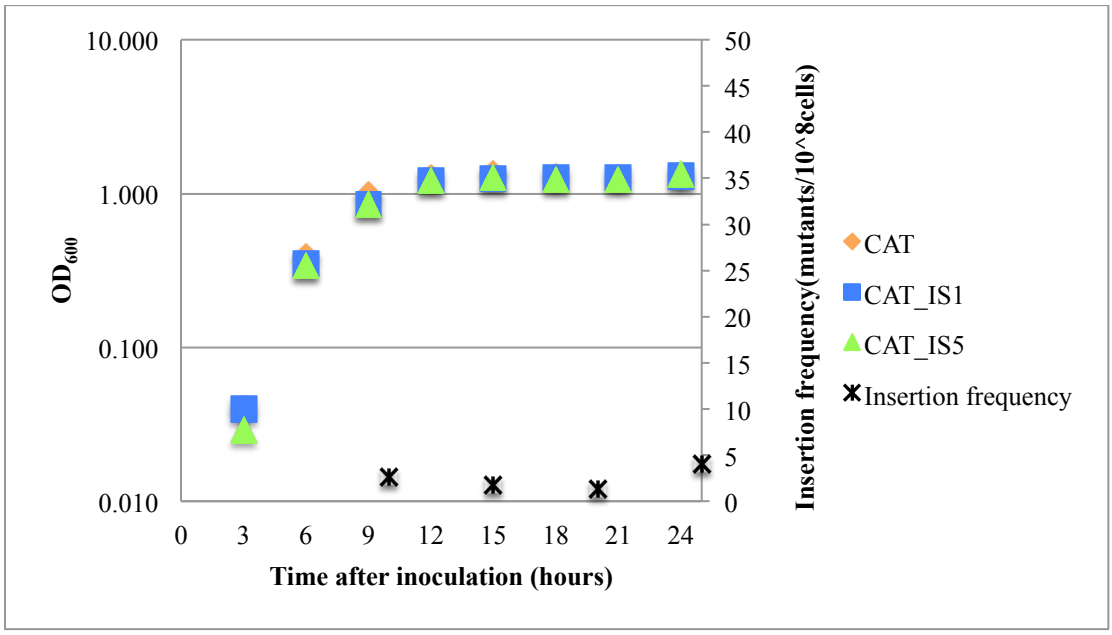

10.000
1.000
0.100
0.010
OD600
0
3
6
9
12
15
18
21
24
Time after inoculation (hours)
50
45
40
35
30
25
20
15
10
5
0
Insertion frequency(mutants/10^8cells)
CAT
CAT_IS1
CAT_IS5
Insertion frequency

Figure 8. Growth curves of wild type and mutants on separate soft-agar plates

CAT, CAT_IS1, and CAT_IS5 were inoculated into soft-agar plates (0.5% tryptone, 0.5% NaCl, 0.3% agar) separately and incubated at 30°C for 24 hours. Each population was measured every three hours.

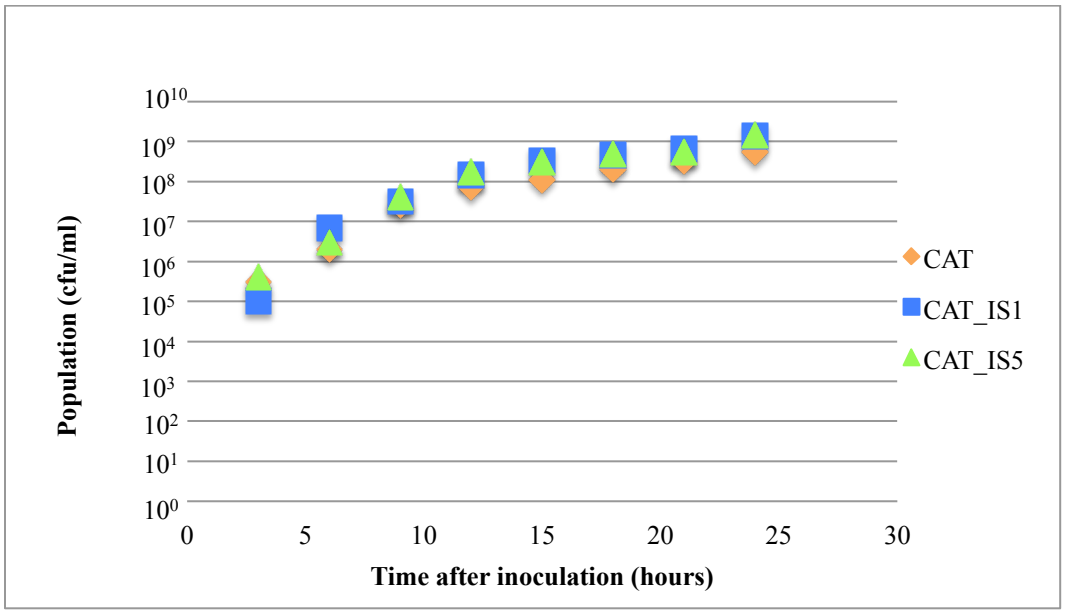

Population (cfu/ml)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
0
5
10
15
20
25
30
Time after inoculation (hours)
CAT
CAT_IS1
CAT_IS5

Figure 9. Growth curves and mutant appearance rates in 0.5% tryptone soft-agar plates.

a) An overnight culture of CAT_Km^R (wild type, $2x10^9$ cfu/ml) and one of the CAT_IS5 mutants ($2x10^6$ cfu/ml) were mixed and inoculated into soft-agar plates (0.5% tryptone, 0.5% NaCl, 0.3% agar) and incubated at 30°C. Every three hours, the cells were collected and plated on LB and LB+Cm plates to measure the two populations. b) The overnight culture of CAT ($2x10^9$cfu/ml) was inoculated on soft-agar plates (0.5% tryptone, 0.5% NaCl, 0.3% agar) and incubated at 30°C. The emerging insertion mutant populations were determined on LB+Cm plates.

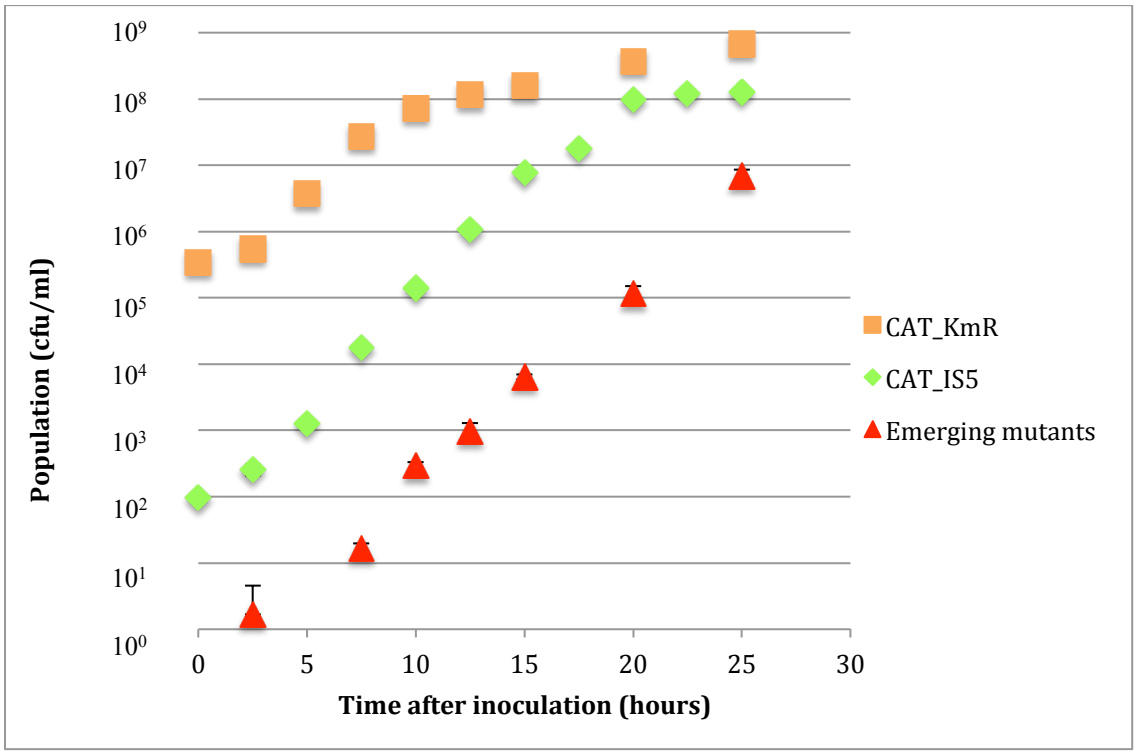

Population (cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
5
10
15
20
25
30
Time after inoculation (hours)
CAT_KmR
CAT_IS5
Emerging mutants

Figure 10. Growth curves and mutant appearance rates in 2.5% tryptone soft-agar plates

a) Overnight cultures of CAT_Km^R (wild type, $2x10^9$ cfu/ml) and CAT_IS5 ($2x10^6$ cfu/ml) were mixed and inoculated in soft-agar plates (2.5% tryptone, 0.5% NaCl, 0.3% agar) and incubated at 30°C. Every three hours, the cells were collected and plated on LB and LB+Cm plates to measure the populations. b) The overnight culture of CAT ($2x10^9$cfu/ml) was inoculated onto soft-agar plates (2.5% tryptone, 0.5% NaCl, 0.3% agar) and incubated at 30°C. The emerging insertion mutant populations were determined on LB+Cm plates.

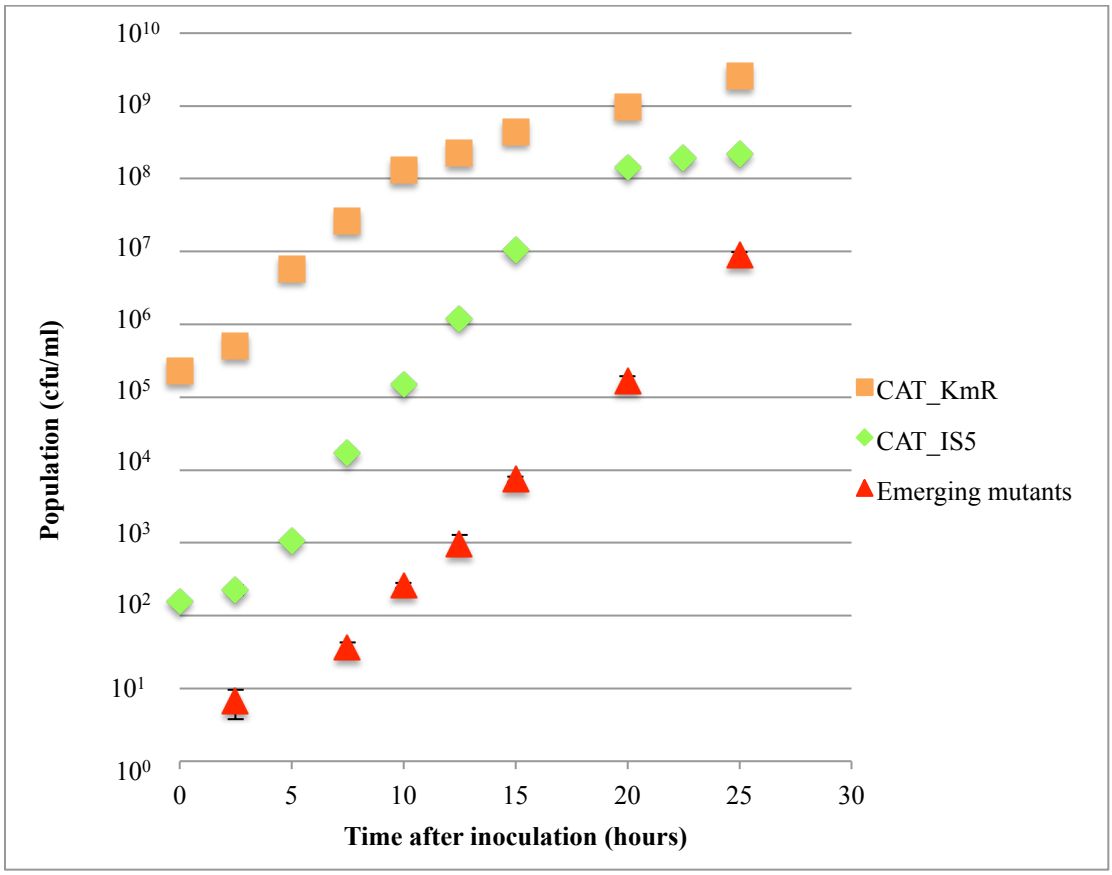

Population (cfu/ml)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
5
10
15
20
25
30
Time after inoculation (hours)
CAT_KmR
CAT_IS5
Emerging mutants

Figure 11. The *flhDC* promoter regulatory region with IS insertion sites

The positions of the insertion sites and their orientations for the IS1, IS3, and IS5 elements in the upstream region of the *flhD* promoter. The transcriptional start site is indicated as +1, and other locations, in parentheses, were assigned relative to +1. The directions of the arrows correspond to the orientations of the insertions. IS1 has four different locations, IS3, two locations, and IS5, three different locations, two of which have the IS element in both orientations.

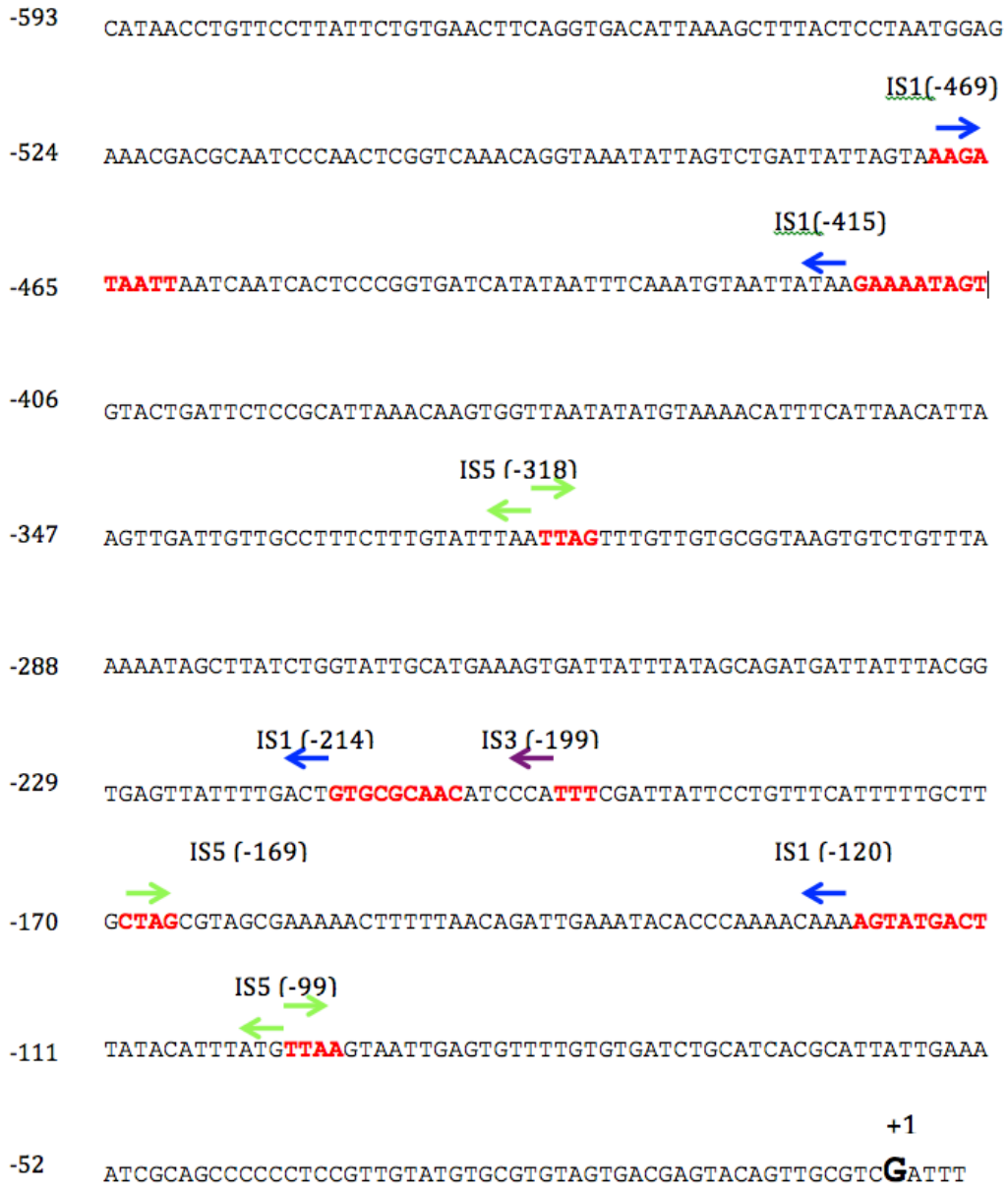
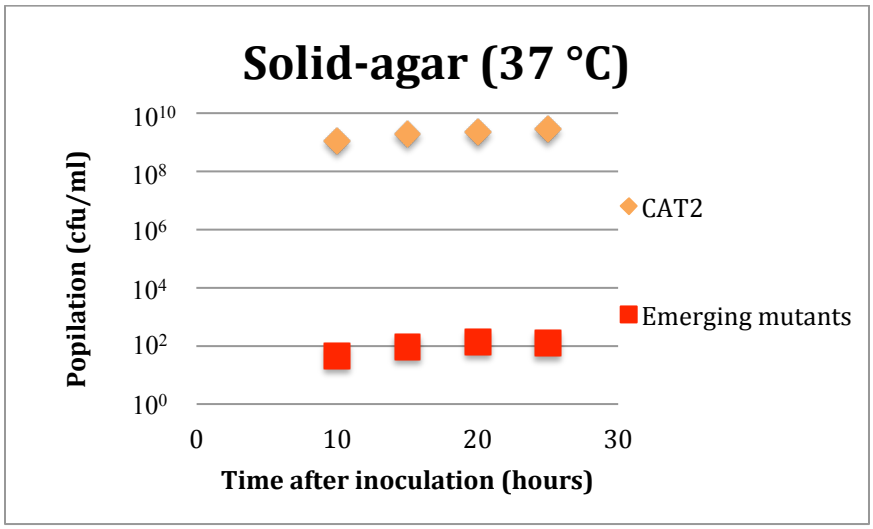
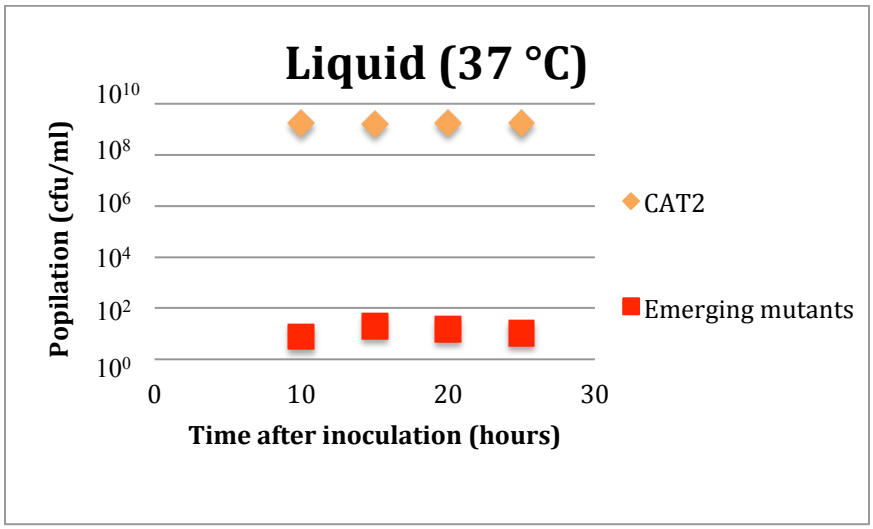

-593
CATAACCTGTTCCTTATTCTGTGAACTTCAGGTGACATTAAAGCTTTACTCCTAATGGAG
IS1(-469)
-524
AAACGACGCAATCCCAACTCGGTCAAACAGGTAAATATTAGTCTGATTATTAGTAAAGA
IS1(-415)
-465
TAATTAATCAATCACTCCCGGTGATCATATAATTTCAAATGTAATTATAAGAAAATAGT
-406
GTACTGATTCTCCGCATTAAACAAGTGGTTAATATATGTAAAACATTTCATTAACATTA
IS5 (-318)
-347
AGTTGATTGTTGCCTTTCTTTGTATTTAATTAGTTTGTTGTGCGGTAAGTGTCTGTTTA
-288
AAAATAGCTTATCTGGTATTGCATGAAAGTGATTATTTATAGCAGATGATTATTTACGG
IS1 (-214)
IS3 (-199)
-229
TGAGTTATTTTGACTGTGCGCAACATCCCATTTCGATTATTCCTGTTTCATTTTTGCTT
IS5 (-169)
IS1 (-120)
-170
GCTAGCGTAGCGAAAAACTTTTTAACAGATTGAAATACACCCAAAACAAAAGTATGACT
IS5 (-99)
-111
TATACATTTATGTTAAGTAATTGAGTGTTTTGTGTGATCTGCATCACGCATTATTGAAA
+1
-52
ATCGCAGCCCCCCTCCGTTGTATGTGCGTGTAGTGACGAGTACAGTTGCGTCGATTT

Figure 12: Mutant appearances from CAT2 (37 °C)

The overnight culture of CAT2 ($1x10^9$cfu/ml) was inoculated onto solid-agar plates (0.5% tryptone, 0.5% NaCl, 1.5% agar), into a liquid medium (0% agar), and onto soft-agar plates (0.3% agar) and incubated at 37 °C. The emerging insertion mutant populations were determined on LB+Cm plate


Solid-agar (37 °C)
Popilation (cfu/ml)
10^10
10^8
10^6
10^4
10^2
10^0
0
10
20
30
Time after inoculation (hours)
CAT2
Emerging mutants


Liquid (37 °C)
Popilation (cfu/ml)
10^10
10^8
10^6
10^4
10^2
10^0
0
10
20
30
Time after inoculation (hours)
CAT2
Emerging mutants

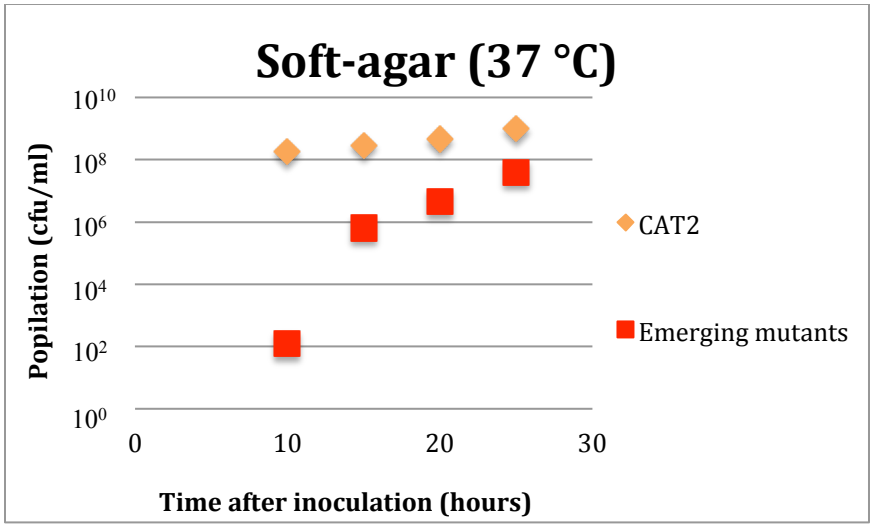
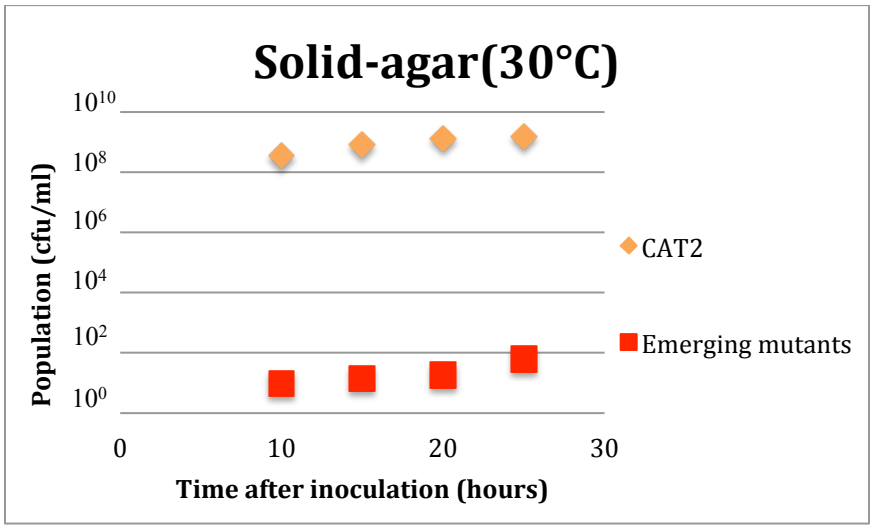
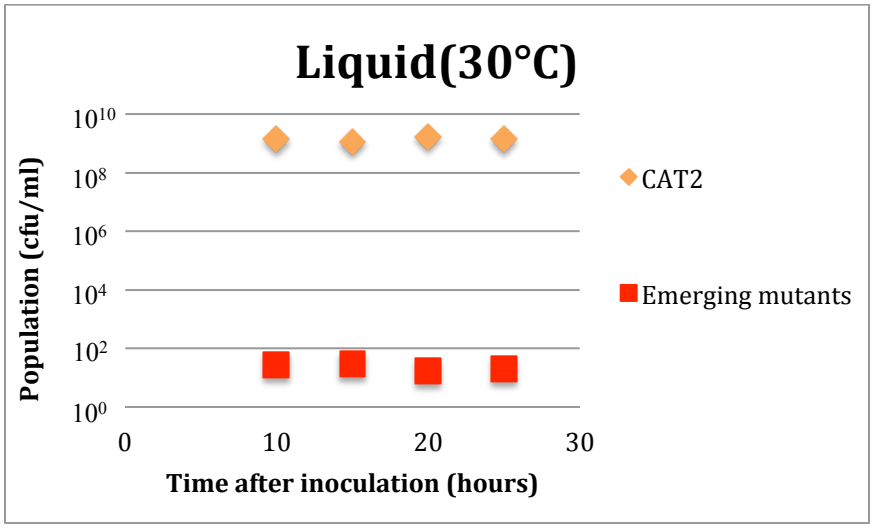

Soft-agar (37 °C)
Popilation (cfu/ml)
10^10
10^8
10^6
10^4
10^2
10^0
0
10
20
30
Time after inoculation (hours)
CAT2
Emerging mutants

Figure 13: Mutant appearances from CAT2 (30 °C)

The overnight culture of CAT2 ($1x10^9$cfu/ml) was inoculated onto solid-agar plates (0.5% tryptone, 0.5% NaCl, 1.5% agar), into a liquid medium (0% agar), and into soft-agar plates (0.3% agar) and incubated at 30 °C. The emerging insertion mutant populations were determined on LB+Cm plates.


Solid-agar(30°C)
Population (cfu/ml)
10^10
10^8
10^6
10^4
10^2
10^0
0
10
20
30
Time after inoculation (hours)
CAT2
Emerging mutants


Liquid(30°C)
Population (cfu/ml)
10^10
10^8
10^6
10^4
10^2
10^0
0
10
20
30
Time after inoculation (hours)
CAT2
Emerging mutants

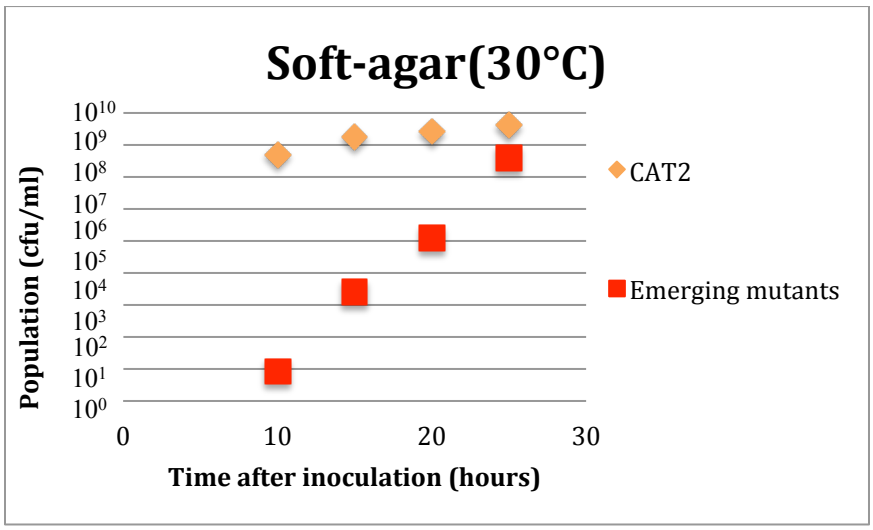

Soft-agar(30°C)
Population (cfu/ml)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
10
20
30
Time after inoculation (hours)
CAT2
Emerging mutants

Table 1. The *E. coli* strains used in this study

Strains	Genotype/relevant characteristics	Source
BW25113 strains		
wild type	BW25113	This study
CAT	*flhDC:cat:'flhC:motA*/Cm^R, used as a wild type	This study
CAT_Km^R	Km^R, in a wild type background	This study
CAT2	Δ*PflhDC-flhD*-5'-*flhC*;*PflhDC*:*flhD*:*flhC*:*cat* located in *intS/yfdG* intergenic region, used as a wild type	This study
CAT_IS1	CAT strain with IS1 upstream of the *flhD* promoter	This study
CAT_IS5	CAT strain with IS5 upstream of the *flhD* promoter	This study
IS1(-120 rev)	IS1 insertion into the -120 bp position relative to the *flhD* start site (+1) in reverse orientation	This study
IS1(-214 rev)	IS1 insertion into the -214 bp position relative to the *flhD* start site (+1) in direct orientation	This study
IS1(-415 rev)	IS1 insertion into the -415 bp position relative to the *flhD* start site (+1) in reverse orientation	This study
IS1(-469)	IS1 insertion into the -469 bp position relative to the *flhD* start site (+1) in reverse orientation	This study
IS3(-199 rev)	IS3 insertion into the -206 bp position relative to the *flhD* start site (+1) in direct orientation	This study
IS5(-99)	IS5 insertion into the -99 bp position relative to the *flhD* start site (+1) in direct orientation	This study
IS5(-99rev)	IS5 insertion into the -99 bp position relative to the *flhD* start site (+1) in reverse orientation	This study
IS5(-169)	IS5 insertion into the -169 bp position relative to the *flhD* start site (+1) in direct orientation	This study
IS5(-318)	IS5 insertion into the -318 bp position relative to the *flhD* start site (+1) in direct orientation	This study
IS5(-318rev)	IS5 insertion into the -318 bp position relative to the *flhD* start site (+1) in reverse orientation	This study