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Insertion Sequence 5 at various upstream locations of the *flhDC* operon causes *Escherichia coli* Hyper Motility

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The Thesis of Jing Wang is approved and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

In recognition of their constant support and inspiration, this thesis is dedicated to my family and to all the members of the Saier lab, in particular Professor Milton Saier, Jr and Zhongge Zhange for their guidance and mentorship.

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ABSTRACT OF THE THESIS

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First discovered in maize by Barbara McClintock, mobile elements inserted in the control regions or coding regions can alter gene activity. Under stress condition, transposons inserted into specific chromosomal sites provide the host with various benefits. The activation of downstream genes can be caused by creating a novel promoter, a hybrid promoter, disruption of repressor binding site, or activation of a native promoter via a transposon. Insertion Sequence 5 was previously found to activate *glpFK*

operon expression (Zhang, *et al*. 2009) by replacing Crp and directly activating the native downstream promoter. When IS5 is inserted upstream of P*flhDC* in various locations and orientations, the mutant increase their motility. This study was conducted with the intension of determining the degree of activation of P*flhDC* and its effector genes, as well as of uncovering the gene activation mechanism for hyper swarming.

Introduction

I. Transposons

Transposons and insertion elements have been found across diverse groups of organisms, and the discovery and engineering of DNA transposons active in vertebrate cells (Miskey *et al.,* 2005) presented the option to conduct research in oncogenes and tumor suppressors. They were first reported by Barbara McClintock in 1951 during the Cold Spring Harbor Symposium in her ground breaking work entitled Chromosome Organization and Genetic Expression. She reported the observation of gene transposition, which requires the presence of an activator. Such behavior does not follow the laws of genetics defined by Mendel. In addition, some transpositions may affect more than one phenotype, and the creation of new phenotypes is sometimes reversible. From the above observations, she deduced that: 1. Reversibility indicated that the insertion of such elements into a gene created an alteration in phenotype, but that the chromatin for the gene remained. 2. Some genes may affect the expression of other genes, or the alteration of genotype can affect not only the phenotype due to expression of that gene, but also multiple phenotypes of the organism. 3. These transpositions can be a source of gene mutation, and play a role in an organism's evolution (McClintock, 1952).

Today, transposition is defined as the relocation of specialized DNA elements within chromosomes or extra chromosomal DNA elements mediated by a transposase or an integrase, the enzyme of transposition. DNA flanked by inverted repeats are the substrates of transposition, and the DNA fragments along with the flanking inverted

repeats are referred to as transposable elements, insertion sequences or transposons. The process of transposition entails three basic steps, 1. transposase binding to the inverted repeat sequences, 2. excision of the transposon from the host DNA, and 3. integration of the transposon into a new locus in the DNA. Integration creates small duplication of DNA at the site of integration. Without the consumption of nucleoside triphosphates, these steps are carried out isoenergetically, and no covalent bond intermediates of transposition are found (Haren *et al*., 1999).

Transposons are classified into three classes based on their mechanism of transposition: Class I, the retrotransposons, Class II, the DNA transposons, and Class III, Miniature Inverted-repeat Transposable Elements or MITEs. Retrotransposons initially copy themselves into RNA, and while being transcribed, the RNA is converted back to DNA by a reverse transcriptase. The product will be later inserted back into the genome. The major distinguishing difference between the two classes of transposons is that Class II conducts their transposition without involving an RNA intermediate while Class I does. Class III was established after recent genome sequencing of rice and C. elegans, where thousands of copies of a single recurring motif were found. They are almost identical throughout the entire stretch of 400 base pairs and are flanked by characteristic inverted repeats (Kimball: see url).

To carry out their catalytic functions, most transposases possess conserved amino acid sequences, for instance, an aspartate-aspartate-glutamate (DDE) motif, which assists metal coordination at the active sites. Transposases related to Tn5 transposases contain an YREK (tyrosine, arginine, glutamate and lysine) signature

element. This group of transposases shows lowered or complete loss of transposition activity as a consequence of YREK mutational alterations. With the availability of crystal structures, mixed β sheets surrounded by α helices were discovered in such enzymes (Davies *et al*., 2000; Haren *et al.,* 1999). With highly distinctive structures and transposing mechanisms, several different pathways of transposition were identified. Based on the type of the system (Dyda *et al.,* 1994; Grindley *et al.,* 2006), the phosphorous atom of a backbone phosphate group in a DNA can be cleaved with different types of nucleophiles: water, activated by enzyme-bound metal ions; a hydroxyl group at the either 5’ or 3’ end of a DNA strand; or a hydroxyl group bearing a serine or tyrosine in the active site of the transposase. In the case where a serine or tyrosine is used, the enzyme becomes covalently boned to the DNA through a phosphoserine or phosphotyrosine or phosphodiester bond.

II. Insertion Sequences

As members of the transposon family, insertion sequences (IS) are the shortest in length and generally encode no functions other than those involved in their mobility (Mahillon and Chandler, 1998). More than 500 such elements have been identified to date in both prokaryotes and eukaryotes, and their lengths are usually defined as less than 2.5 kb (Mahillon and Chandler, 1998). Ester Lederberg, in 1981, initiated the nomenclature of insertion sequences (IS) by simply adding a single number to an IS element, i.e. IS1 (Lederberg, 1981). However, this system was not sufficient to describe the large number of ISs that were discovered. As a result, a second system

was developed which includes the initials of the bacterial species from which it was isolated, i.e., ISRm1 for IS1 from *Rhizobium meliloti*.

Insertion sequences are of great interest due to their capacity to generate mutations upon hopping. They move between plasmids and chromosomes or from one chromosomal location to another, often disseminating antibiotic resistance (Bukhari *et al*., 1977). Our knowledge of the pathogenicity and virulence of bacteria has been greatly influenced by the isolation of the heat stable toxin encoded by IS1 of *E. coli* (So *et al*., 1979). Studies of bacterial pathogenesis over recent years have established an increasing frequency of association between insertion sequences and virulence functions across kingdoms, for example, those in *Bacillus* animal pathogens (Mahillon *et al.*, 1994), *Agrobacterium* plant pathogens (Otten *et al*., 1992), and *Rhizobium* symbionts (Freiberg *et al*., 1997). Many transposons, once integrated into chromosomes result in chromosome rearrangements (Haack and Roth, 1995; Louarn *et al*., 1985; Savic *et al.*, 1983) and participate in plasmid integration (Low, 1996).

Another crucial aspect of transposition is the target site where integration takes place. Ultimately, an IS will not survive cell cycles if inserted into deleterious genomic locations (Guynet *et al.*, 2009). A majority of integration sites appeared to be random, although a few exceptions were noted. Some prefer to insert under the directive of DNA structure such as bent DNA (Hallet *et al.*, 1991; Pribil and Haniford, 2002), single-nucleotide mismatches (Yanagihara and Mizuuchi, 2002), nucleosomes (Pryciak and Varmus, 1992), or specific chromatin-associated proteins such as Sir4 (Zhu *et al.*, 2003). In the case where transposase does not directly recognize the target

site, as in Helicobacter pylori insertion sequence, IS608, the base-pairing interaction with nucleotides within the transposon DNA dictates the site of reaction (Barabas *et al.*, 2008).

Most insertion sequences contain flanking inverted repeats (IR) at both ends, which are between 10 and 40 base pairs in length. IRs generally serve two functions, 1. as the target for transposase TPase binding, and 2. the terminal 2 or 3 base pairs provide a site for cleavage and allow the strand transfer reaction to take place (Derbyshire *et al.*, 1987; Derbyshire *et al*., 1990; Isberg and Syvanen, 1981; Johnson and Reznikoff, 1983). The IS promoter region is found to be partially embedded within the IR upstream of the TPase gene. The above observation leads to the hypothesis of possible modulation of transposition activity or Tpase expression by the presence of IRs (Mahillon and Chandler, 1998).

DNA binding activities of transposases are sequence-specific, and the N-termini of these proteins carry out this function; the C-termini often provide the catalytic function (Machida and Cachida, 1989; Zerbib *et al*., 1990). In prokaryotic species, where transcription and translation are frequently coupled, this arrangement possibly positions the developing protein in close proximity to its substrate target sequence, which allows expression to be coupled to activity (Mahillon and Chandler, 1998). In addition to occurring autonomously, insertion sequences may also occur as parts of composite transposons; in a composite transposon (Figure 1), two insertion sequences flank one or more accessory genes, such as an antibiotic resistance gene

(e.g. Tn10, Tn*5*). Nevertheless, there exists another sort of transposon, called a unit transposon that does not carry insertion sequences at their extremities (e.g. Tn*7*)

III. Insertion Sequence 5

The insertion sequence 5 family is composed of at least 47 distinct members and 21 isoelements including sequences from both bacteria and archaea. The majority of the members of this heterogeneous group carry only a single open frame; thus their lengths range from 850 kb (IS869) to 1643 kb (IS493) with a few exceptions. For example, IS4811 which is 5 kb in length possesses uncharacterized genes (Chen *et al.*, 1992). According to Mahillon and Chandler's 1998 review on Insertion Sequences, this family is divided into six subgroups based on their transposase sequence alignment. One of these groups is the IS5 subgroup, including IS52, ISPsp1, IS5, IS1051, IS1068, IS1169, and IS1194. A peculiar trait of the IS5 subgroup (Figure 2) is that their transposases exhibit spacings between the N3 and C1 domains of an estimated 40 amino acyl residues, a fragment consistent with the canonical DDE motif.

In this study, IS5, of 1195 base pairs is the primary focus. A single insertion sequence site initiates transposase gene transcription at an increased rate under environmental stress conditions as for most members of the IS family. The gene activation response provides the host, for example *E. coli* and its relatives, with a variety of benefits during periods of nutrient depletion.

IV. Transposon Transposition

Originally identified as an extra sequence in the *E. coli* bacteriophage lambda genome, IS5 was shown to be present in up to 23 copies in the chromosomes of various *E. coli* strains although others lack IS5 altogether (Deonier, 1996). The most plausible explanation for the high copy number is that the transposition process takes place at variable frequencies depending on the strain. IS5 and many other insertion elements can be categorized by the transposition mechanism. In the course of integration, transposons often produce spontaneous mutations, DNA rearrangements, and deletions of large DNA segments in both growing and resting cultures of *E. coli* strain (Naas *et al*,. 1995; Faure *et al*,. 2004).

It might be favorable for the IS element to excise and exit the genome once the stress condition is alleviated, as an over expressed gene might create a detrimental disturbance to the bacterium. Some transposable elements exhibit excision behaviors in both precise and imprecise modes of action, with the majority of excision being imprecise (Berg, 1977; Botstein and Kleckner, 1977; Khatoon and Bukhari, 1981). In one study, the imprecise excision of IS5 caused deletion of adjacent regions (Strauch and Beutin, 2006). Until recently, no evidence for precision excision of IS5 had been forth coming (Zhang et al., 2010).

In addition to accuracy, transpositions can be classified by mechanism into replicative and non-replicative. For many prokaryotic IS elements, replication of the transposing segment causes the formation of co-integrates, where the donor and the target molecules are joined by duplicated copies of the element (Grindley and Reed,

1985). The non-replicative pathway is referred to as simple insertion, or cut-and-paste, as the mobile element “hops” from one location to another (Craig, 1996; Haren *et al*., 1999; Mizuuchi, 1992), see Figure 3. The majority of transposons move exclusively by one pathway or the other, with the exception of IS903 and IS1, which have been shown to carry out both simple insertion and replicative transposition (Ohtsubo *et al.*, 1981; Weinert *et al.,* 1984; Turlan and Chandler, 1995). Despite the distinguishing appearance of their products, *in vitro* and *in vivo* experiments have shown that the biochemical steps in both pathways are strikingly similar (Mizuuchi, 1997; Haren *et al.,* 1999). The main difference between the simple and replicative pathways is whether a double-strand break or a single-strand nick occurs at the ends of the transposon before integration (Turlan and Chandler, 2000). A double-strand break clips off the donor DNA without the formation of a co-integrate; in comparison, a single-strand nick maintains the connection of donor and target replicon after integration of the replicated element.

Carrying only the genetic information necessary for their own transposition, most ISs hop alone; however, some are also capable of group-hopping, more commonly referred to as composite transposition (Bartosik *et al*. 2008). Composite transposons are DNA segments that are flanked by 2 IS elements at either end. The IS at either end may or may not be exact replicas. Instead of each IS element moving separately, the entire length of DNA spanning from one IS element to the other is transposed as one complete unit, with the segment between the ISs often carrying one or more genes conferring antibiotic resistance markers. Interestingly, the transposition

of a single IS can also adequately translocate antibiotic resistance situated at a nearby locus (Poirel, *et al.,* 2005; Toleman, *et al.,* 2006).

Movements of a transposon can also mediate efficient and specific chromosomal rearrangements, including deletion, duplication, inversion, the formation of acentric fragments and dicentric bridges during meiosis, translocations and recombination of host genomes (Berg and Howe, 1989; Lim and Simmons, 1994; Saedler and Gierl, 1996). This topic will be further discussed in the next section.

V. Gene Activation by Insertion Sequences

An elevated or repressed neighboring gene expression level is often a byproduct of the presence of insertion sequences, including IS1, IS2, IS5, and numerous others. These ISs, when inserted at appropriate locations with respect to the -10 hexamer by transposition, can create a novel promoter capable of enhancing the expression of neighboring gene (Galas and Chandler, 1989). A common characteristic of many of these insertion sequences is that they have detectable -35 hexamers within the terminal IRs. They include IS21 (Reimmann *et al*., 1989), IS30 (Dalrymple, 1987), IS257 (Leelaporn *et al.,* 1994), IS2 (Szeverenyi *et al.,* 1996), and many more.

Four types of gene activation by transposable elements are listed: (i) Derepression, (ii) Creation of a completely new promoter, (iii) Formation of a hybrid promoter, a especially involving the -35 position, and (iv) Activation of a native promoter via IS5.

One extensively studied example of gene activation by removing native repression was conducted by Schnetz and Rak on IS5 enhancement of *bgl* operon expression. The *bgl* operon expression is necessary for β-glucoside utilization in *E. coli*. With IS5 inserted into various positions, both upstream and downstream of the *bgl* promoter, the enhancement of promoter activity were found in all cases to result in operon activation. Different internal deletions were introduced into IS5, resulting in inability to generate the same enhanced affect in the target promoter, indicating that the activation mechanism is not solely due to the presence of silenced or inactivated inhibitory sequences naturally present to suppress *bgl* promoter activity (Reynolds *et al*., 1981; Schnetz and Rak, 1992). The restoration of activation enhancement in trans intact copies of IS5 led to the identification of *ins5A* as an essential component for transposition function. Likewise, under starvation condition, IS5 induced activation of the normally suppressed fucose/propanediol *fucAO* catabolic operon in *E. coli* (Chen *et al*., 1989; Hacking and Lin, 1976, Zhang *et al.*, 2010). Such behavior allows bacteria to metabolize β-glucosides and propanediol as alternative nutrient sources, respectively.

In the case where a novel promoter is assembled, the endogenous promoter drives the activation of the downstream operon. This hypothesis was first presented to solve the conflicting Tn10 puzzle, where the polar block of Tn10 terminates transcription of downstream genes. In contrast, Tn10 insertions in ribosomal RNA operons permitted continuous transcription of distal sequences (Morgan, 1980). An experiment conducted with deleted host promoter regions P1 and P2 of *Salmonella*

hisH::Tn10 eliminated the possibility of native promoter activation. The resulting evidence implied that transcription initiated within the Tn10 element extended into host sequences adjacent to the insertion site (Ciampi *et al*., 1982).

Another mechanism by which a transposable element may lead to gene activation is by combining a part of the original promoter with a sequence in the mobile element to form a hybrid promoter. Integration of the element was shown to be orientation-dependent. A well studied case was the 20-fold increase in activation of the *amp*C β-lactamase-hyperproducing mutant of *E. coli*. The chromosomal *amp*C gene, which β-lactamase, is normally suppressed by an attenuator. Whenever this enzyme is present, *E. coli* gains resistance to ampicillin and several other antibiotics (Normark and Burman, 1977). As the incorporation of the 1327 bp long transposon, IS2, with designated orientation into the *amp*C promoter, generated a 5 bp sequence duplication. The duplication was found to enhance the transcription of *amp*C, and in turn allowed the bacteria to become resistant to antibiotics in the growth media. After isolation and sequence analysis, Jaurin discovered that the duplications produced resemble the -35 RNA polymerase binding site. This site along with the native -10 region, led to the activation of the *amp*C gene. Transcription initiated by the newly formed hybrid promoter starts at the same position as does the wild type promoter (Jaurin and Normark, 1983).

A novel mechanism of transposon-mediated gene activation was recently discovered in 2009 by Zhongge Zhange, and Milton Saier, who demonstrated that IS5 is capable of activation of the *glpFK* operon, rendering *E. coli* cells able to utilize

glycerol in the absence of Crp. In this case, a part of IS5, which is proximal to the adjacent gene and harbors unique sequences containing A-tracts and an IHF binding site, can functionally replaced Crp and directly activate the native downstream promoter (Zhang and Saier, 2009).

Unlike other modes of gene activation, chromosomal rearrangement requires the presence of two or more similar, if not identical, sequences between strands for the transfer to occur. Such a rearrangement may ultimately lead to the alteration of distal gene activities and genome plasticity. Two possible mechanisms may cause the rearrangement of chromosomal DNA by transposons: (i) indirectly by homologous recombination or (ii) directly by an alternative transposition process (Gray, 2000). The indirect method promotes genomic rearrangement by homologous recombination which can be observed when faulty repair of a double-strand break results in transposable element excision using ectopic homologous sequences as a repair template (Saedler and Gierl, 1996). As for the direct method, only one parental rearranged chromosomal breakpoint contains a mobile element, and some may even be inconsistent with the orientation of the element in the chromosome prior to rearrangement (Lister and Martin, 1989; Lister *et al.*, 1993; Weil and Wessler, 1993). A genomic rearrangement was produced by two IS5 insertion sequences designated IN(*cst*A::IS5-IS5D) (Zinser *et al*., 2003). The process was carried out in two steps: first, an IS5 transposition event inactivated the *cstA* gene, whose product is possibly an oligopeptide permease, and second, an inversion between this IS5 and another IS5 *ca* 60kb away, activated the *ybeJ-gltJKL* four-gene operon (Zinser *et al*., 2003).

VI. Flagella

Micro-organisms that acquired flagella over the course of evolution are able to find most favorable conditions and avoid detrimental environments, thus gaining a competitive advantage over others that lack flagelli (Macnab, 1996). The motility and chemotaxis capabilities allow them to survive under a wide variety of environmental conditions. Additional functions related to this structure include adhesion to substrates, biofilm formation, colonization and virulence (Fenchel, 2002). Due to high energy expenditure upon assembly and activation, and the possible elicitation of a strong immune response in the host organism, expression of flagellar genes is highly regulated by a wide range of environment parameters.

Flagellar motility increases the resources and environmental conditions available to flagellated micro-organisms, allowing such strains to successfully outcompete strains not capable of movement (Soutourina and Bertin, 2003). One example is the chemotaxis in the Fe(III) oxide-reducing bacterium, *Geobacter metallireducens*. This bacterium favors the assorted sedimentary environment, and reaches such environments by utilizing flagella and pili to swim against the insoluble electron acceptor gradient (Childers *et al*., 2002). Additionally, colonization of a host organism or target organ by pathogenic micro-organisms is accomplished by flagellar motility, often considered to be a virulent factor (Ottemann and Miller, 1997; Josenhans and Suerbaum, 2002).

Motility provides a huge advantage, and hence organelles of motility are present in over 80% of bacterial species and many archaea (Moen and Vanderleyden, 1996; Thomas *et al*., 2001). The environment dictates the diversification of flagellar arrangements, which give rise to single polar, multiple polar, and many peritrichous or lateral flagellins (Joys, 1988; Wilson and Beveridge, 1993).

VII. *flhDC*, The Master Switch

E. coli flagellar synthesis is highly regulated via a three level hierarchy where expression of one gene at a given level requires the transcription of another gene (Macnab, 1996). At the top of the hierarchy is the *flhDC* master operon which encodes the gene product $FlhD_2C_2$. This transcriptional regulator activates eight Class II flagellar promoter and 39 non-flagellar promoters (Lewis *et al*., 1994; Fernandez *et al*., 2000; Stafford *et al.*, 2005). Class II flagellar genes encode components of the inner part of flagellum, including the flagellin export system, the flagellar basal body. Class II operons also encode the flagellar σ subunit of RNA polymerase—RNAP, FliA or σ^F and its anti- σ factor, FlgM. FlgM is secreted when the basal body secretion system is functional, followed by FliA release to activate transcription of Class III genes. These Class III genes encode the components of the flagellar filament (flagellin, FliC), as well as hook-associated, motor, and chemotaxis proteins, as well as a number of proteins with unknown functions (Chilcott and Hughes, 2000; Karlinsay et al, 2000; Aldrighe et al., 2006). Class III genes are down regulated by anti-sigma factor, FlgM (Kutsukake *et al.*, 1994; Ohnishi *et al.*, 1992).

The master operon that controls the expression of the entire cascade is *flhDC* in enterobacteria (Komeda, 1982; Komeda, 1986; Kutsukake *et al*., 1990), and such a control system is regulated via transcriptional and posttranscriptional control in *E. coli*. Numerous environmental signals control the expression of *flhDC*, i.e. temperature (Adler and Templeton, 1967), osmolarity, and pH (Li *et al*., 1993; Shi *et al.,* 1993; Soutourina *et al.,* 2002); via global regulatory proteins such as H-NS, integration host factor (IHF) and the catabolite gene activator protein cAMP-CAP (Bertin *et al.,* 1994; Soutourina *et al.,* 1999; Yona-Nadler *et al.,* 2003). In addition, the mRNA produced is stabilized by the presence of the RNA binding regulator, CsrA (Romeo, 1998; Wei *et al.,* 2001). All the sensory inputs are coordinated to ensure the bacteria can reach the most desirable environment.

Motility activation is under close surveillance to preserve the delicate balance between conserving limited energy storage and using the energy-intensive flagella to search out preferable environments. The most common tactic bacteria use under such condition is to minimize the energy-consuming flagellin production, while increase operation of the motor machinery. The usage of energy-intensive flagella could well lead to rapid exhaustion of the energy supply, and hence the term risk-prone foraging (Liu *et al*., 2005; Hastjarjo *et al*., 1990; Houston, 1991).

As osmolarity is crucial factor to bacterial culture growth, the receptor OmpR is an important global regulator in the up- and downregulation of more than 100 genes (Oshima *et al.,* 2002). One of the genes regulated by OmpR is P*flhDC.* A precocious warming and *flhDC* expression level were found in *Xenorhabdus nematophila* when

OmpR is inactivated (Kim *et al.,* 2003). Interestingly, the expression of *flhDC* in S. *typhimurium* and P. *mirabilis* demonstrated no change when OmpR was altered (Clemmer and Rather, 2007; Kutsukake, 1997). In the case of *Yersinia pseudotuberculosis*, OmpR positively regulates *flhDC* expression and subsequently controls transcription of class II and III genes in flagella biosynthesis (Hu *et al*., 2009).

The observation that carbon catabolite cause repression of motility leads to the proposal and confirmation that the relief from the repression plays a key role in motility activation. It was found that the activity level is inversely correlated with the differences in the quality of the carbon sources within a designated range. This regulation is achieved by a complex regulatory circuit involving the cAMP receptor protein, CRP (Crasnier, 1996). However, if the growth condition becomes too harsh, and quantity of the carbon source is no longer sufficient, conservation of precious energy outweighs the potential benefit of reaching nutrients with usable quality. The cell will gradually turn off both synthesis of flagellin and flagellar operation. No more cellular motility or chemotaxis is then observed (Zhao *et al.,* 2007).

In *E. coli* and numerous other bacteria, a novel type of global regulatory system called the carbon storage regulatory system (Csr) was found to influence *flhDC* activation (Wei *et al*., 2001). As the carbon storage level fluctuates, a small RNA-binding protein called CsrA is produced, which acts as the effector and binds to the *flhDC* promoter. The presence of the protein represses gluconeogenesis, glycogen biosynthesis and catabolism, and activates glycolysis and acetate metabolism (Romeo and Gong, 1993; Romeo *et al.,* 1993; Sabnis *et al*., 1995; Yang *et al*., 1996; Wei *et al*.,

2000). The down regulation of the carbon converting pathways may also cause a change in *flhDC* expression level.

Some components of flagellar function are a type III secretion system (TTSS). Enteropathogenic and enterohaemorrhagic *E. coli* (EPEC and EHEC) contain an additional type III secretion system coded by locus of enterocyte effacement (LEE) (McDaniel and Kaper, 1997). This secretion system mediates virulent factor injection into mammalian cells, and it is positively regulated by the Ler regulator (Sperandio *et al.,* 2000; Sanchez-SanMartin *et al.,* 2001). Integration host factor (IHF) directly activates the expression of Ler (Friedberg *et al.,* 1999) and represses the flagellar expression in EPEC (Yona-Nadler *et al.,* 2003). This repression is unique to EPEC and is not found in all *E. coli* strains.

The effects of proton concentration on flagellar biosynthesis and motility are unknown. Flagellar rotation is driven by the proton motive force, enhanced by a low ambient pH as long as the cytoplasmic pH remains high. Exception to the former conclusion is observed when permeable chemotactic repellents, such as acetate and benzoate, are present. They alter the internal pH, diminish the proton motive force and impair rotation of the flagellar motor (Khan and Macnab, 1980; Minamino *et al.,* 2003; Slonczewski *et al*., 1982). A low pH elicits negative chemotaxis, and with a pH not higher than 8.3, elicits a positive response (Repaske and Adler, 1981). Evidence has shown that alkaline conditions suppress flagellar motility and chemotaxis (Maurer et al., 2005). Acid induction activates *flhDC* via two separate pathways, 1. via adenylate cyclase, *CyaA*, 2. via *DnaK-DnaJ-GrpE* (Kutsukake, 1997; Shi et al., 1992).

An alternative model is that pH regulation of *flhDC* is mediated by proteolysis, as in the case of ClpXP proteolysis of FlhD and FlhC (Tomoyasu *et al.*, 2003).

The H-NS histone-like nucleoid structuring protein upregulates the flagellar regulon, in spite of the fact that the majority of these genes are affected by it negatively (Bertin *et al.*, 1994). After careful examination of the *flhDC* gene activation, Ko and Park proposed that the H-NS dependent regulation of the flagellar master operon is mediated by a LysR family protein (Ko and Park, 2000), which represses *flhDC* transcription. LysR is encoded by the *hdfR* gene, and expression of this gene is negatively regulated by H-NS, which fits the observation that H-NS acts as a transcription silencer.

In addition to the complex transcriptional regulation, the concentration of *flhDC* mRNA is modulated sharply during differentiation and consolidation in the cell cycle (Furness *et al.*, 1997; Claret and Hughes, 2000a). The concentrations of the FlhD and FlhC monomers is further controlled by proteolysis, resulting in half-lives of about 2 to 6 minutes. Those compounds that cause degradation of FlhD and FlhC belong to a group of potent regulators referred to as timing proteins which also play a role in the whole system (Claret and Hughes, 2000a).

Although homodimers can be formed from FlhD and FlhC separately, it is the heterotetramer $FlhD_2C_2$ that functions as the activator of the class II σ^{70} promoters (Liu and Matsumura, 1996; Claret and Hughes 2000b). DNA gel footprinting conducted *in vitro* confirmed that the *E. coli* $FlhD_2C_2$ binding site is centered 50-60 bp 5' of the transcriptional start sites of the class II genes such as *fliA* (Liu and

Matsumura, 1994). No obvious DNA-binding motif was found either in the 13.3 kDa FlhD nor in the 21.5 kDa FlhC; however, FlhD increases the specificity of FlhC for DNA recognition, and this provide enhancement to the affinity and the stability of the $FlhD_2C_2$/DNA complex (Claret and Hughes 2000b).

Inorganic polyphosphate is also required for motility. A principle enzyme is polyphosphate kinase PPK, is responsible for the synthesis of inorganic polyphosphate from ATP. The mutant with the *ppk* gene knocked out are impaired in motility on semisolid agar plates, and upon restoration of the *ppk* gene in *trans,* the defect was corrected (Rashid *et al*, 2000). The above research consolidate that the linear polymer, composed of hundreds of orthophosphate residues linked by high-energy phosphoanhydride bonds, is essential for bacteria to be optimally motile (Francez-Charlot *et al*, 2003).

The activation of *flhDC* is also coordinated with growth stage. There is a life style change from motile planktonic to adhesive-sedentary when *E. coli* is transitioned from late-exponential to early-stationary phase (O'Toole et al, 2000; Hall-Stoodley et al, 2004). With complex growth media, cells gradually increase theirs motility during the post-exponential growth phase (Adler and Templeton, 1967; Amsler *et al*., 1993; Zhao *et al*. 2007). Once the cells enter stationary phase, motility is then replaced by induction of adhesive curli fimbriae, since the sticky adhesive apparatus is considered counterproductive to be present at the same time as swimming activities (Pesavento et al. 2008). When IS5 is incorporated upstream of *flhDC*, it appears to permit higher

motility and consequently an upsurge in species vitality by allowing the organism to reach a more nutrient rich settings (Barker *et al.*, 2004).

VIII. *fliA* and *flhB* activation

fliA encodes σ^f or σ^{28} and is a Class II gene in the flagellar control cascade. Transcription of *fliA* is initiated by sigma 70 and the product of *flhDC.* Sigma 28 carries out the function of activating Class III chemotaxis and structural components of flagella. One of these Class III genes that is activated with the assistance of the above minor σ factor is FlhB, which along with FlhA and FliOPQR, reside in the membrane-embedded MS ring. It is essential for flagellar export (McMurry *et al*. 2004). Both *fliA* and *flhB* are considered downstream effectors of *flhDC,* and alteration in the degree of activation is here studied to further understand the flagellar master operon's properties.

Objective

The goal of this study is to screen new hyper-motile mutants by incubating a wild type strain onto soft agar plates for over a 24 hour period. Four distinct mutants became hyper-motility due to transposition of IS5 and were selected. All of the insertions occurred upstream of P*flhDC*. Among the four mutants, one has an insertion in the reverse orientation with respective to *flhDC*, and the rest were inserted directly. The elevated motility levels were studied and correlated with their observable phenotypes with swarming activity assays. Gene activation levels via LacZ fusion were determined the demonstrating increase in gene activation in all three levels of the motility regulatory cascades.

Method

Escherichia coli strains and plasmids

Strains and oligonucleotides used in this study are described in Table 1. The *flhDC::lacZ, fliA::lacZ, and flhB::lacZ* mutants were derived from *E. coli* K-12 strain BW25113. IS5-169 *PflhDC* mutant was supplied by Barker's lab (Barker *et al*., 2004).

Motility Assays

Compound media were used for swarming assays. Sterilized media used in swarming assays with *E. coli* consisted of 0.35% Fisher BioReagents Agar (Molecular Genetics Graulated) with 4g/liter Difco Nutrient Broth. Three variations of the above compound media were tested, including nutrient broth with no additional carbon source, nutrient broth with 0.5% glucose, and nutrient broth with 0.5% glycerol. Swarming agar was typically allowed to solidify and dry in a 30˚C incubator for one hour before use.

Defined media were also used for swarming assays. M9 minimal medium was used after vacuum filtration sterilization. Carbon source as 0.5% glycerol or glucose was added after filter sterilization. No observable activities found in either medium.

Chromosomal *lacZ* Fusions

P*flhDC* activity of mutants with IS5 inserted 318, 169, or 99 base pairs upstream of the +1 transcriptional start site of P*flhDC*. For the insertion at position 99,

mutants with both direct and inverted IS5 orientations were studied. These mutants were then fused with *lacZ* to monitor *flhDC* expression levels. In addition, *lacZ* fusions were constructed for study of class II *PfliA* and class III *PflhB* gene activation.

β-Galactosidase Assays

β-galactosidase activity was assayed by the use of o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. It was added to a mixture of 0.2ml of culture taken from exponentially growing cells along with 0.8ml of Z buffer. The reaction was terminated by adding 0.5ml of sodium carbonate once a color change from clear to yellow was observed.

P1-transduction

Strains to be used as recipients in P1 transduction were grown to saturation in steriled LB Broth (EMD) by suspending 25g in 1 liter of deionized water. After mixing the phage, buffer and bacterial culture, the tubes were incubated at 37˚C for 30 minutes. Reaction was terminated by addition of sodium citrate, and cells were plated onto LB-Km plates before being incubated at 37˚C over night.

Results

1. Selection of Swarming Mutants

The wild-type BW25113 strain has no IS element present in its *flhDC* promoter region and is poorly motile. To select swarming mutants with higher motility, an overnight culture in LB medium was streaked (10 ul cells/9-cm streak) and inoculated onto the surface of Nutrient Broth soft agar (0.35%, wt/vol) in a straight line. The soft agar plates were then incubated in a humid 30°C incubator. A pan of water was placed on a shelf directly underneath the plates to ensure constant humidity. After 24 hour of incubation, the plates were examined for the occurrence of swarming in the shape of a swarming loop by the outgrowths of motile subpopulations on both sides of the bacterial streak. Cells that were furthest away from the original streak were re-inoculated from these swarms and stabbed onto fresh Nutrient Broth motility agar for purification. After incubation at 30°C for approximately eight hours, a small amount of cells from the margins of the swarming zone were cultured in liquid LB medium for storage and subsequent analyses.

2. Identification of Swarming Mutants

Barker *et al* (2004) first showed that several native motile *E. coli* strains contain an insertion sequence element upstream of the *flhDC* promoter region. To determine if this was the case for the swarming mutants isolated in our lab, the *flhDC* regulatory region was amplified by polymerase chain reaction using primers PflhDC-Xho and PflhDC-BamH (Table 1).

3. Motility Assay

A portion of overnight cultures (1ml) were frozen at -80°C in 40% glycerol stocks to preserve the seed for each isolated mutant. 1μl of the remaining sample was then inoculated onto motility agar with Nutrient Broth (50%) at 30°C as a stab. The same motility tests were repeated for media with different types of carbon sources to determine whether IS5 alters the sensitivity of *flhDC* activation. The M9 minimal media with additional 0.5% glucose or 0.5% glycerol showed no swarming activity within the first 24 hour incubation period. Instead of the swarming diameter, the areas covered by the swarming colonies were recorded to increase accuracy. The diameter of swarming colonies was plotted against time to demonstrate the swarming rate as the slope of the linear regression trend line.

Results of Motility Assay

3.1 Wild type

As shown in Figure 4, the wild type, with no insertion sequence present, swam best when no additional carbon source was present. The swarming rate was 0.15mm/hour. The data collected from tests with addition of 0.5% glucose had a swarming rate of 0.086 mm/hour, roughly half of the rate for Nutrient Broth medium. 0.5% glycerol gave a swarming rate of 0.34 mm/hour, a 2-fold increase compare to Nutrient Broth medium.

3.2 *IS5-318 PflhDC*

A mutant with IS5 inserted 318 basepairs upstream of the *flhDC* promoter transcription start site behaved rather similarly to the wild type strain. The highest motility rate for this mutant was observed in Nutrient Broth (NB) medium, with a 0.83mm/hour. In 0.5% glucose-Nutrient Broth medium, the rate dropped to one-fourth of that observed for the previous medium, at 0.28 mm/hour. With 0.5% glycerol-Nutrient Broth medium, the cell swarmed 2.5-fold faster than in the Nutrient Broth medium, at 1.8 mm/hour, as shown in Figure 5.

3.3 *IS5-169 PflhDC*

This mutant revealed highest swarming rate in Nutrient Broth medium with 0.5% glucose with a swarming rate of 0.37 mm/h, a 4-fold increase with respective to Nutrient Broth medium where swarming occurred at 0.09mm/h. The addition of 0.5% glycerol produced a 2-fold increase compared to Nutrient Broth medium, at 0.18 mm/hour (see Figure 6).

3.4 *IS5-99 PflhDC;*

The mutant with IS5 inserted in the direct orientation at 99 bp upstream of the *flhDC* transcription start site generated similar data compare to wild type and IS5-318 *PflhDC* strains. The highest motility rate was observed in Nutrient Broth medium with 0.5% glycerol at 0.25mm/h. Nutrient Broth medium came in second place with a

0.2mm/h swarming rate. Nutrient Broth with 0.5% glucose swarmed the slowest, at only 0.05mm/h (Figure 7).

3.5 *IS-99(inverted insertion)PflhDC*

Similar to the strain with direct orientation of IS5 at -99 (see Figure 8), the inverted orientation also showed lowered motility in Nutrient Broth medium with 0.5% glucose, and high motility in Nutrient Broth medium. The plate with 0.5% glycerol revealed a similar activity level as the plate with 0.5% glucose, and unlike the wild type, *IS5-318 PflhDC, and IS5-99 PflhDC,* it did not show increased motility in Nutrient Broth with 0.5% glycerol.

3.5 Medium effects on swarming with different strains

As shown in Figure 9, a mutant with IS5 inserted 318 bp upstream of the *flhDC* transcription start site had an 8-fold increase in swarming rate compared to the wild type with no insertion when assayed on 0.35% soft agar plate made with 50% nutrient broth (motility plates were made with the above agar and Nutrient Broth concentrations). A 6-fold increase was observed in Nutrient Broth with addition of 0.5% glycerol. This also one of the faster swarming mutants in Nutrient Broth with 0.5% glucose, a ~3.5-fold increase compared to the wild type strain.

Mutant IS5-169 *PflhDC* showed no difference in swarming diameter in NB and NB+0.5% glycerol media compare to wild type. However, it had a 4.5 fold increase when 0.5% glucose was added to NB.

Direct insertion of IS5 at -99 bp upstream of the *flhDC* transcription start site revealed a 2-fold increase in motility compared to the wild type strain in NB medium. It appeared to have similar or slightly lowered swarming activities in NB with 0.5% glucose and glycerol.

The IS5-99 (inverted orientation) *PflhDC* mutant showed elevated levels of swarming in NB and NB+0.5% glucose media, with a 4-fold and a 2-fold increase compare to the wild type strain. No significant increase in motility was noted when growing in NB+0.5% glycerol medium with respective to the wild type strain.

4. LacZ Activity Assay

lacZ fusions provide an accurate technique to determine levels of gene activity. This is especially true when the target gene does not produce a quantitative phenotype. The reaction of β-galactosidase acting on ONP- β-galactoside produces a bright yellow color, which is distinguishable by the naked eye and can be easily quantitated using a spectrophotometer.

4.1 P*flhDC-lacZ*

The wild type strain exhibited strong repression of *flhDC* in both compound and minimal media upon the addition of glucose compared to the same media without the addition of glucose as shown in Figure 9-1(a). The addition of glycerol, on the other hand, stimulated gene expression. This observation was also true for mutant

strain IS5-318 *PflhDC-lacZ* (Figure 9-1b). In Figure 9-1c, IS-169 P*flhDC-lacZ* reproduced the same pattern for compound media, where the addition of glucose resulted in lowered gene activation, and glycerol did the opposite. This mutant did not behave as expected in minimal media. The two mutants with IS5 inserted directly (Figure 9-1d) and inversely (Figure 9-1e) at position -99 also gave similar result.

4.2 *PfliA-LacZ*

We now focus on Class II gene activation. These genes are directly activated by the gene products of the *flhDC* operon. As shown in Figure 10 a-e, out of 4 mutants and the wild type studied, mutant IS5-318 *PflhDC PfliA-lacZ*, IS5-99 *pflhDC PfliA-lacZ, and* IS5-99 inverted *PflhDC PfliA-lacZ* produced similar results. They all revealed glucose repression and stimulation by glycerol presumably by providing supplementary energy.

Wild type showed elevated activity in M9 with 0.5% glucose medium, but the activity was strongly repressed when the same amount of glucose was added to LB medium. The data collected in M9+0.5% glucose, LB, and LB +0.5% glycerol are analogous to the rest of the mutants.

4.3 P*flhB-lacZ*

All four mutants generated comparable data, where LB+0.5% glycerol promoted the highest gene expression level, and the readings for all the others is reduced to about one-fifth of this value. IS5-169 *PflhDC PflhB-lacZ* showed little

growth in the M9 media, and the data deviated slightly from the rest. Wild type, however, had high levels of activity in all the media tested except M9 +0.5%glycerol (Figure 11a-e).

Discussion

Swarming

Due to strong carbon catabolite repression of the *flhDC* promoter, 0.5% glucose with Nutrient Broth medium produced the slowest swarming rate among the 3 types of media investigated for wild type, *IS5-318PflhDC, IS5-99PflhDC, IS5-99* (inverted insertion)*PflhDC* strains. As mentioned in the Introduction, one of the regulations of *flhDC* is achieved by a complex regulatory circuit involving the cAMP receptor protein, CRP (Crasnier, 1996). Mutant strain *PflhDC IS5-169* showed little or no carbon catabolite repression, and the swarming rate turned out to be the fastest when glucose was present, presumably due to the additional energy provided. All of the four strains studied showed increased motility when 0.5% glycerol was added to the NB medium. Since glycerol is a much weaker catabolite repressing carbon source compare to glucose, it is possible that the energy it provides outweigh the repression of gene expression generated, and hence increases swarming activities.

Mutant *IS5-169PflhDC* also revealed an increase in its motility when 0.5% glycerol was added compare to NB medium, and this observation can be explained with the same theory as the other strains. Interestingly, it showed a 4.5 fold increase in swarming rate as 0.5% glucose was added to NB medium. It is likely that the insertion of IS5 at -169 interrupted the cAMP-CRP dependent, or decrease the binding affinity to the regulatory region of *flhDC* so that the repression is diminished.

LacZ activity tests

PflhDC-lacZ

Wild type and mutant IS5-318 *PflhDC-lacZ* strains demonstrated the strong repression of *flhDC* in both compound and minimal media in comparison to the same media without the addition of glucose as shown in Figure 9-1(a-b). This observation confirms the well known phenomenon of carbon catabolite repression on motility and flagellin production. Glycerol promotes little or no repression of the target promoter, and presumably provides additional energy for cellular activities, hence the elevated level of gene expression. The same theory may explain why IS-169 *PflhDC-lacZ* reproduced the same pattern in compound media. For minimal media, the mutant revealed a prolonged doubling period, which resulted in a lower cell density compared to the other strains. As the number of cells decrease, proteins produced by individual cells tend to increase. Glucose catabolite repression and the extra energy activation produced the same effects on the mutant with IS5 inserted directly and inversely at position -99.

PfliA-lacZ

For mutant IS5-318 *PflhDC PfliA- lacZ,* IS5-99 *PflhDC PfliA- lacZ, and* IS5-99 inverted *PflhDC PfliA- lacZ,* the results coincide with our hypothesis. They all exhibit glucose repression and glycerol stimulation. The latter presumably due to the supplementary energy. For wild type cells, it is plausible that glucose provides enough

energy to the flagella, resulting in increased motility, allowing the cells to seek more favorable environments. In M9 medium with glycerol, this compound may provide energy, thereby increasing motility. With the LB+0.5% glucose medium, with enriched nutrients in the LB allows full glucose repression. IS5-169 *PflhDC PfliA-lacZ* once again shows little or no glucose repression, which agrees with the results gathered from swarming activity assays.

PflhB- lacZ

The explanation for the hypersensitivity of mutants with IS5 with respect to gene expression of *flhB*, a Class III gene in the flagellar cascade, is not understood. One hypothesis is the glycerol and Nutrient Broth together might stabilize the σ^F that transcribes this gene by prolonging its half life. Alternatively, it may provide assistance by increasing the affinity of the sigma factor for binding to the gene regulatory site. It might also counter an innate repressing agent. The wild type strain, on the other hand, behaved strangely in M9+0.5% glycerol media. The cells with a *lacZ* fused to *PflhB* did not propagate properly, and growth was extremely slow. It is not known why this strain grew slowly. It could be due to secondary mutation. More interestingly, the results could be derived from both mutations. However, the activity levels in the 4 other media ranged from 5-7mu, which is low compared to the mutants (activities up to a few hundred mu). This indicates that the *flhB* gene, by its nature, may not be activated under conditions of rapid energy consumption. Mutant IS5-169 , also revealed no carbon catabolite repression when glucose was present.

In summary, wild type *E. coli* strains lack mobile elements in the *flhDC* regulatory region. 0.35% soft agar might facilitate and selects for cells with increased motility due to the hopping of insertion sequences upstream of the operon. One particular mobile element, IS5, its effect on (1) motility under various conditions, (2) expression of the *flhDC* operon (stage I) and the expression of its downstream genes (*fliA*; stage2 and *flhB*; stage 3) were studied. IS5 appears to activate the master switch as well as the downstream machineries for swarming, all by activation of *flhDC* gene expression. Mutant IS5-169 *PflhDC* revealed little or no carbon catabolite repression. These studies open up the field for detailed mechanistic studies.

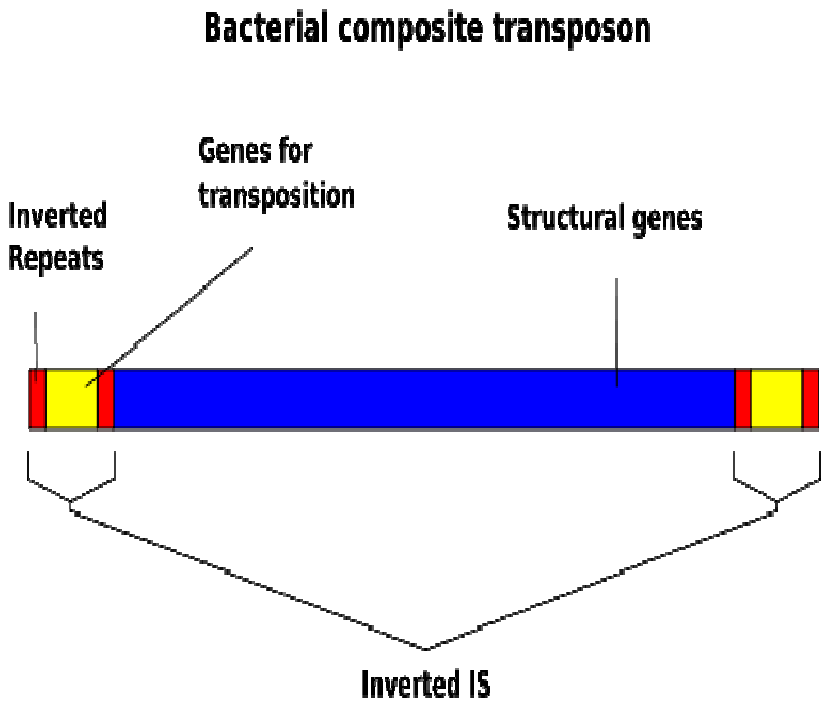

Figure 1: Composition of a composite bacterial transposon where two IS elements flank the structural genes. The complete structure behaves as a single transposon.

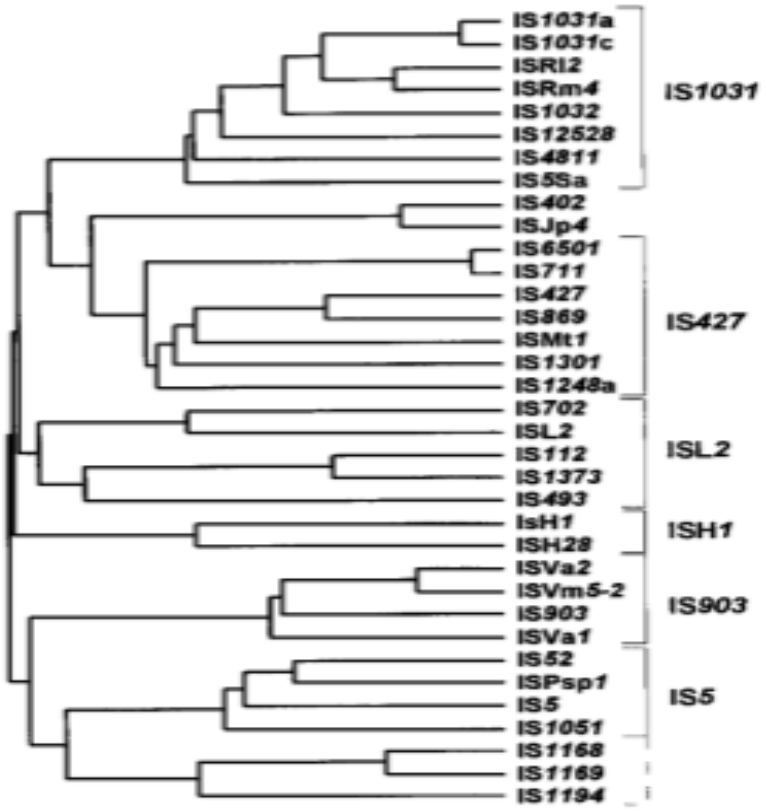

Figure 2: The IS5 family. The dendrogram, based on the alignment of various transposase sequences, shows the division of the transposase families into various subgroups (Mahillon and Chandler, 1998)

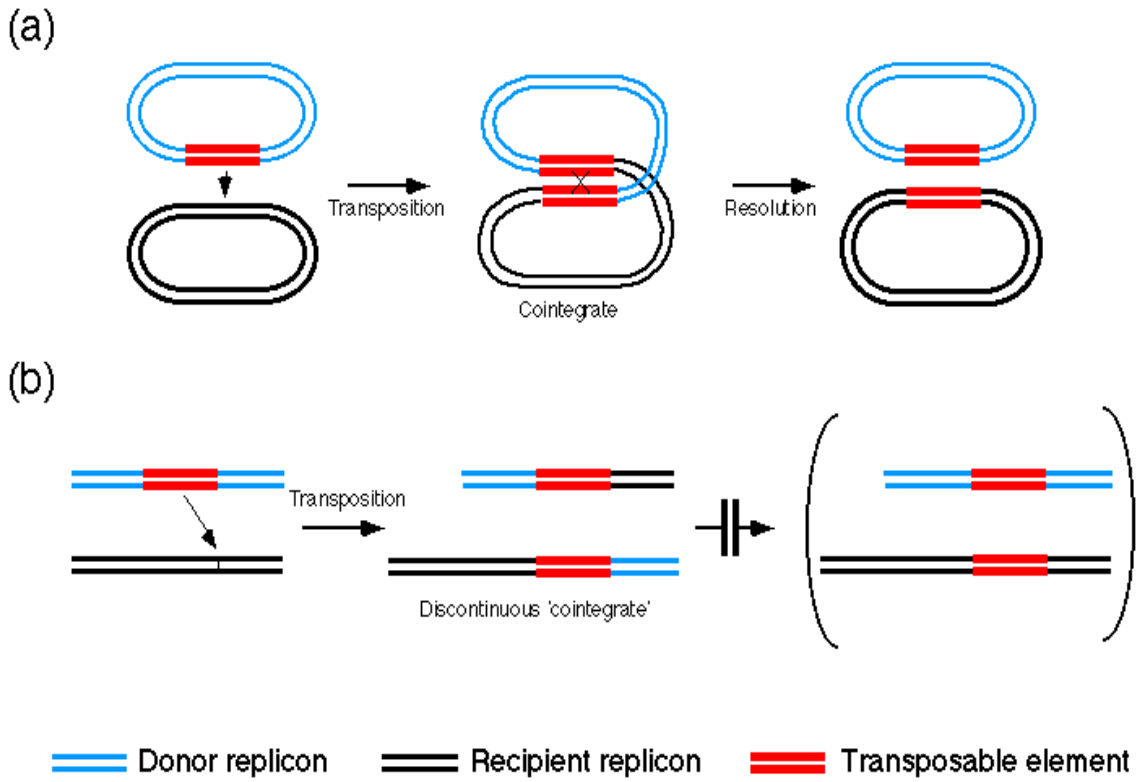

Figure 3: Replicative transposition and replicon topology (a) Circular replicons. Replicative transposition gives rise to a cointegrate, in which the two replicons are fused by the duplicated copies of the transposable element. The element-specific resolvase aligns the *res* sites and resolves the two replicons by site-specific recombination. The continuity of the cointegrate molecule is important for the resolution reaction. (b) Linear replicons. Replicative transposition creates two recombinant replicons, each with a copy of the transposable elements at the junction of exchange. This discontinuous 'co-integrate' may be the final product, because resolution is unlikely (shown in parentheses) in the absence of continuity between the two molecules, according to the model.

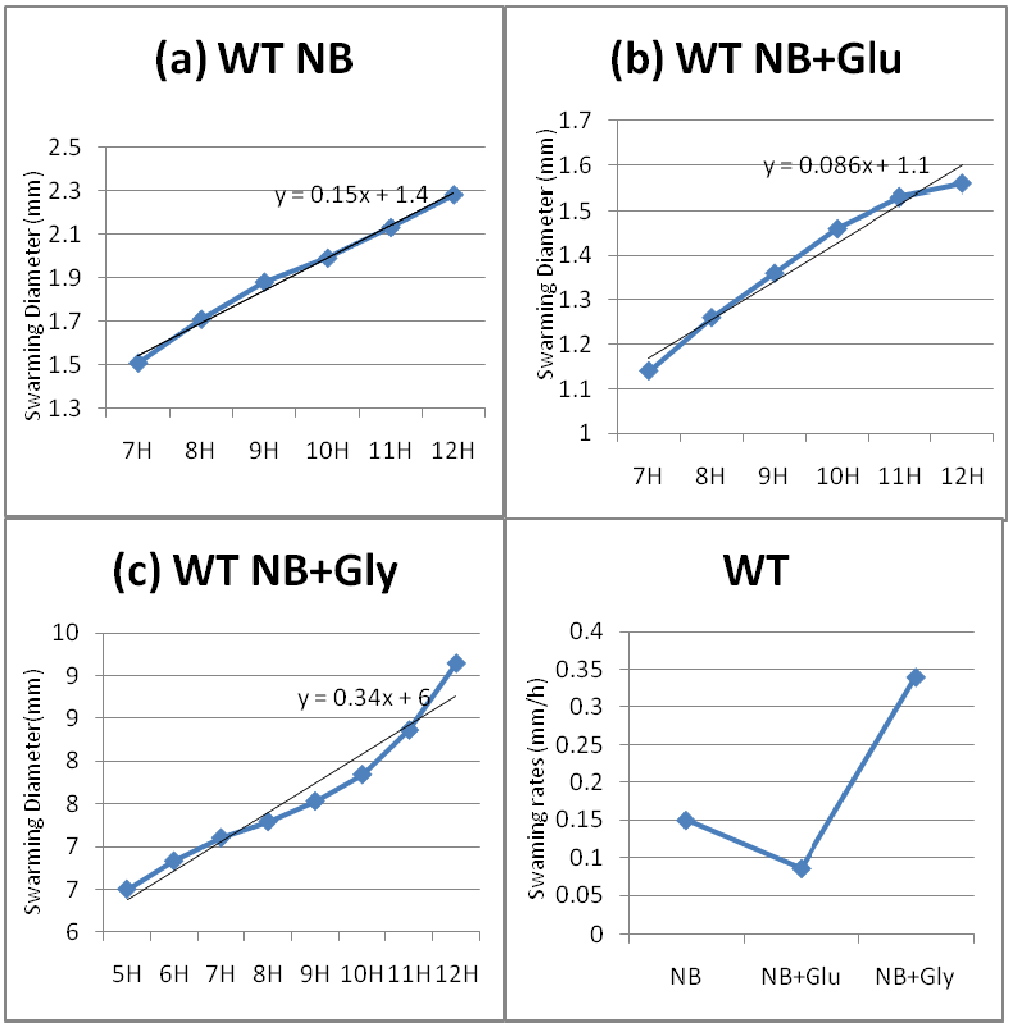

Figure 4: Swarming data and linear regression slopes representing the tentative swarming rate for the wild type strain inoculated into 0.35% motility agar containing different nutrients (a) Nutrient Broth, (b) Nutrient Broth with 0.5% glucose, (c) Nutrient Broth with 0.5% glycerol, (d) the overall estmated rates for all 3 types of media.

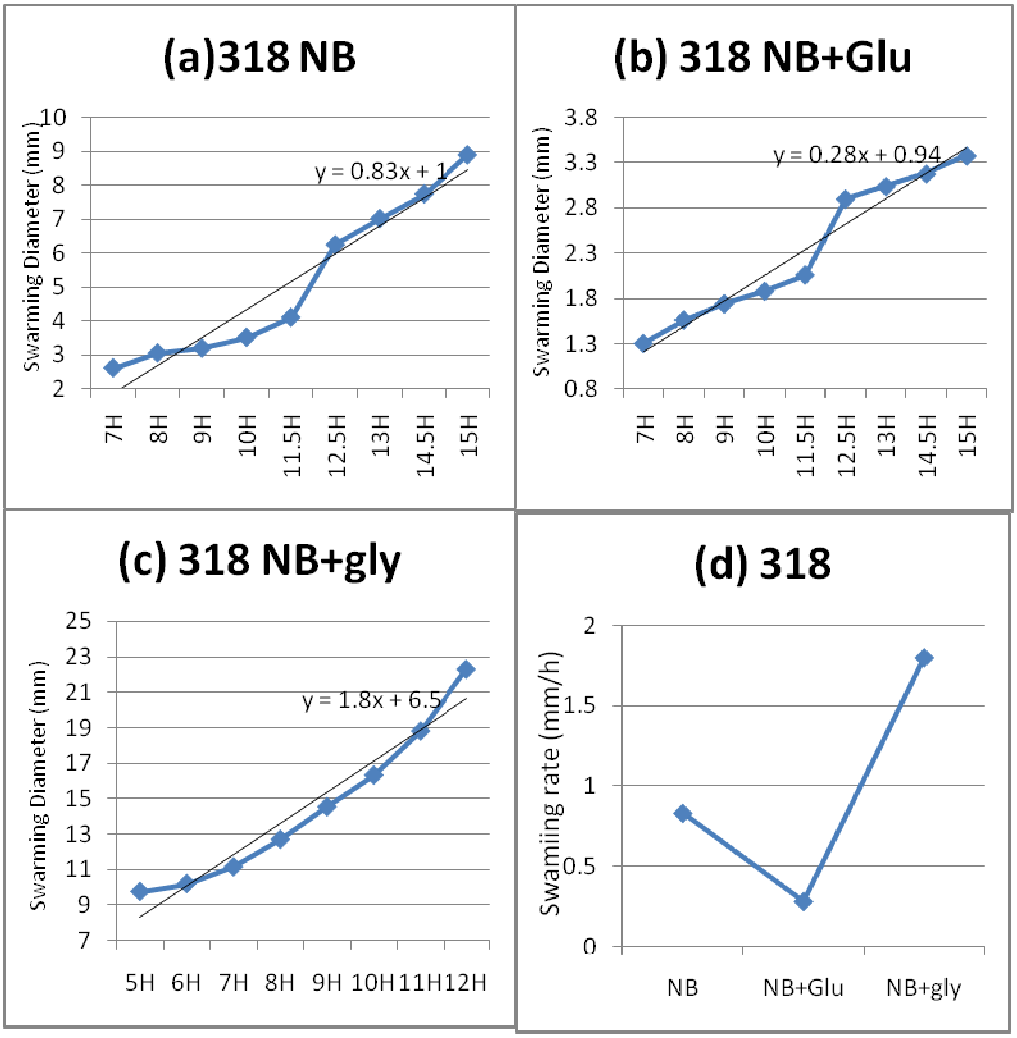

Figure 5: Swarming data and linear regression slope representing tentative swarming rates for the *IS5-318 PflhDC* strain inoculated onto 0.35% motility agar containing different nutrients (a) Nutrient Broth, (b) Nutrient Broth with 0.5% glucose, (c) Nutrient Broth with 0.5% glycerol, (d) the overall trend for these 3 media.

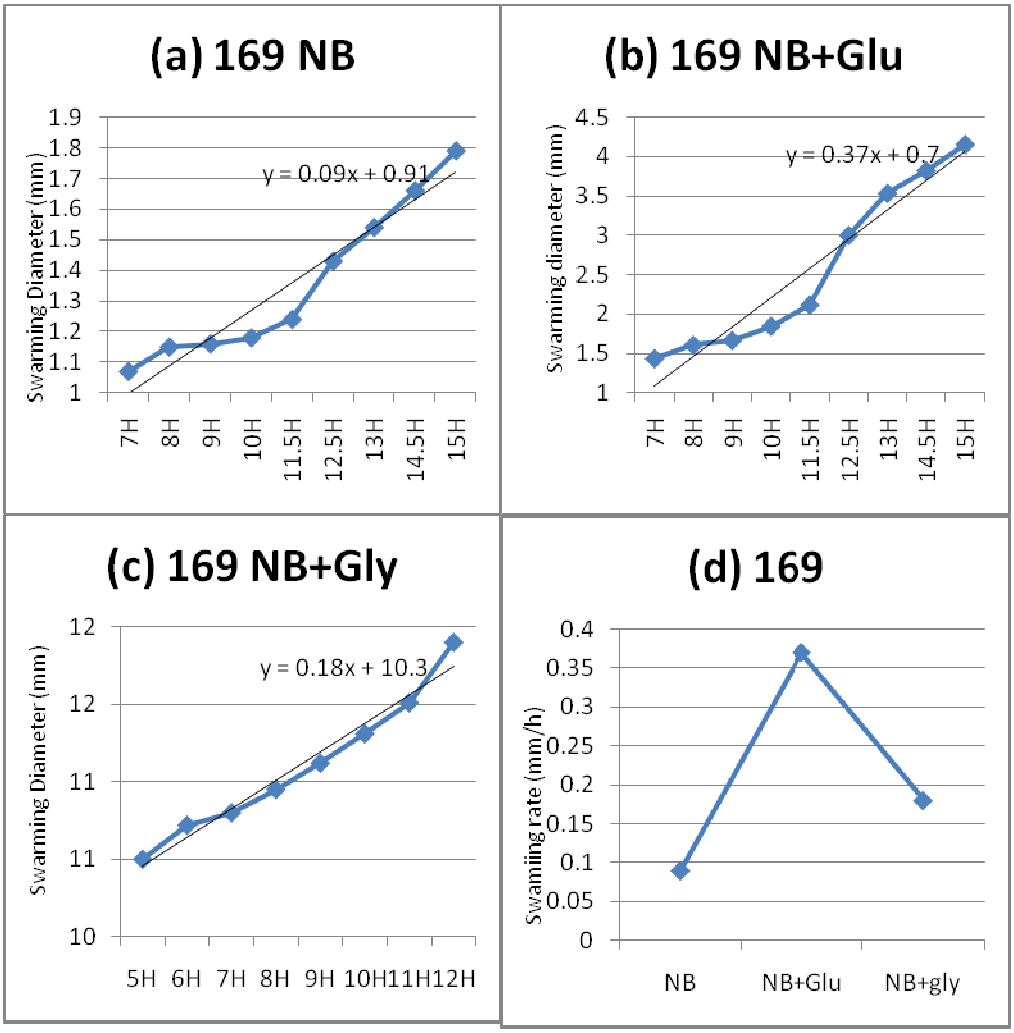

Figure 6: Swarming data and linear regression slope representing tentitive swarming rates for *IS5-169 PflhDC* strain inoculated onto 0.35% motility agar containing different nutrients (a) Nutrient Broth, (b) Nutrient Broth with 0.5% glucose, (c) Nutrient Broth with 0.5% glycerol, (d) the overall estimated swarming rates for these 3 media.

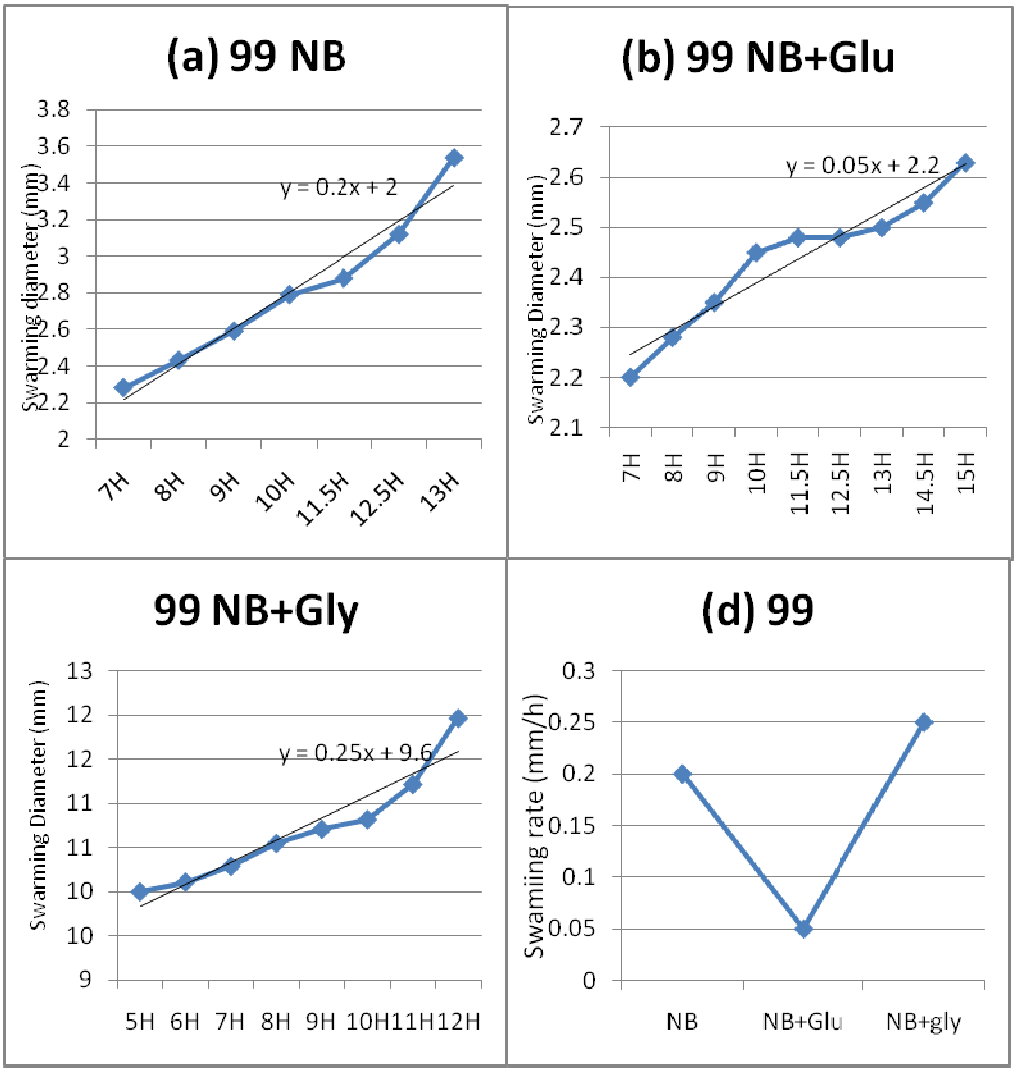

Figure 7: Swarming data and linear regression slope representing tentitive swarming rates for *IS5-99 PflhDC* strain inoculated onto 0.35% motility agar containing different nutrients (a) Nutrient Broth, (b) Nutrient Broth with 0.5% glucose, (c) Nutrient Broth with 0.5% glycerol, (d) the overall estimated rates for these 3 media.

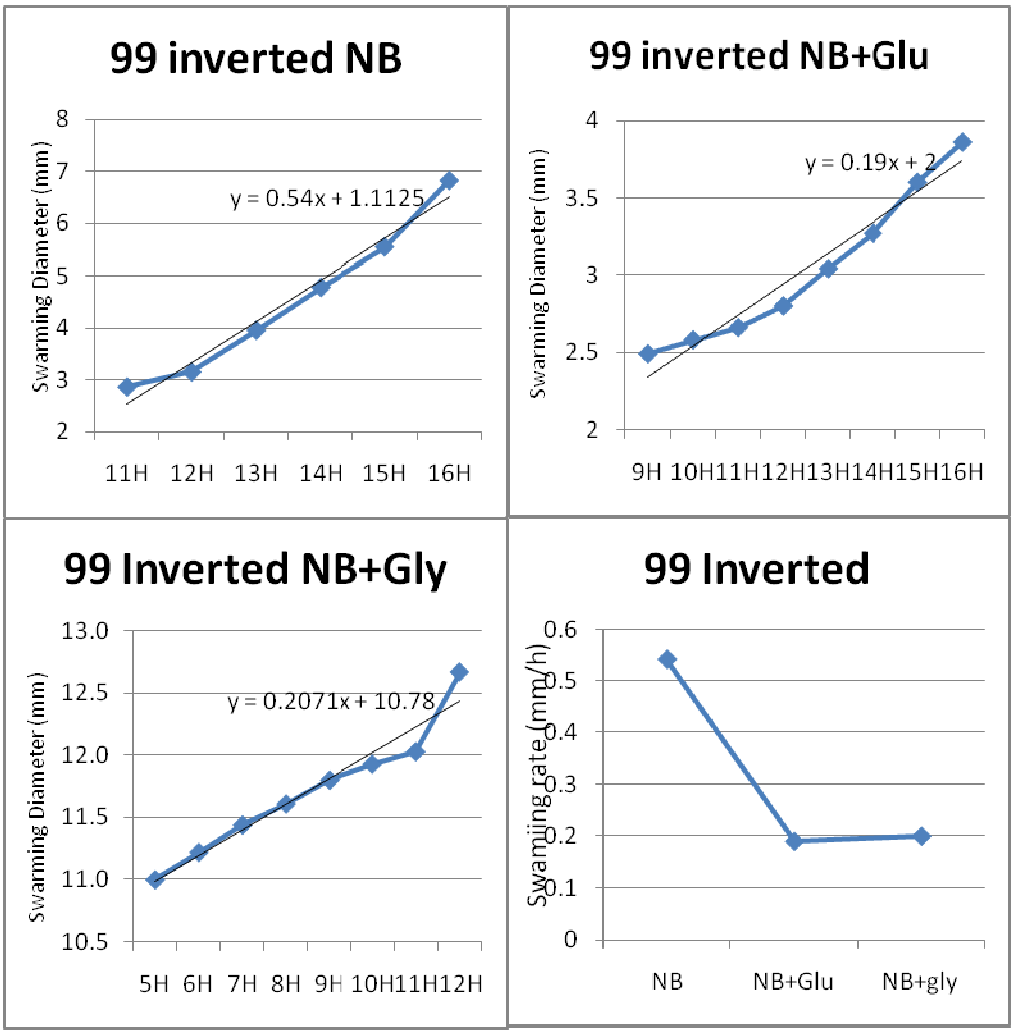

Figure 8: Swarming data and linear regression slope representing tentitive swarming rates for inverted *IS5-99 PflhDC* strain inoculated onto 0.35% motility agar containing different nutrients (a) Nutrient Broth, (b) Nutrient Broth with 0.5% glucose, (c) Nutrient Broth with 0.5% glycerol, (d) the overall estimated rates for these 3 types of media.

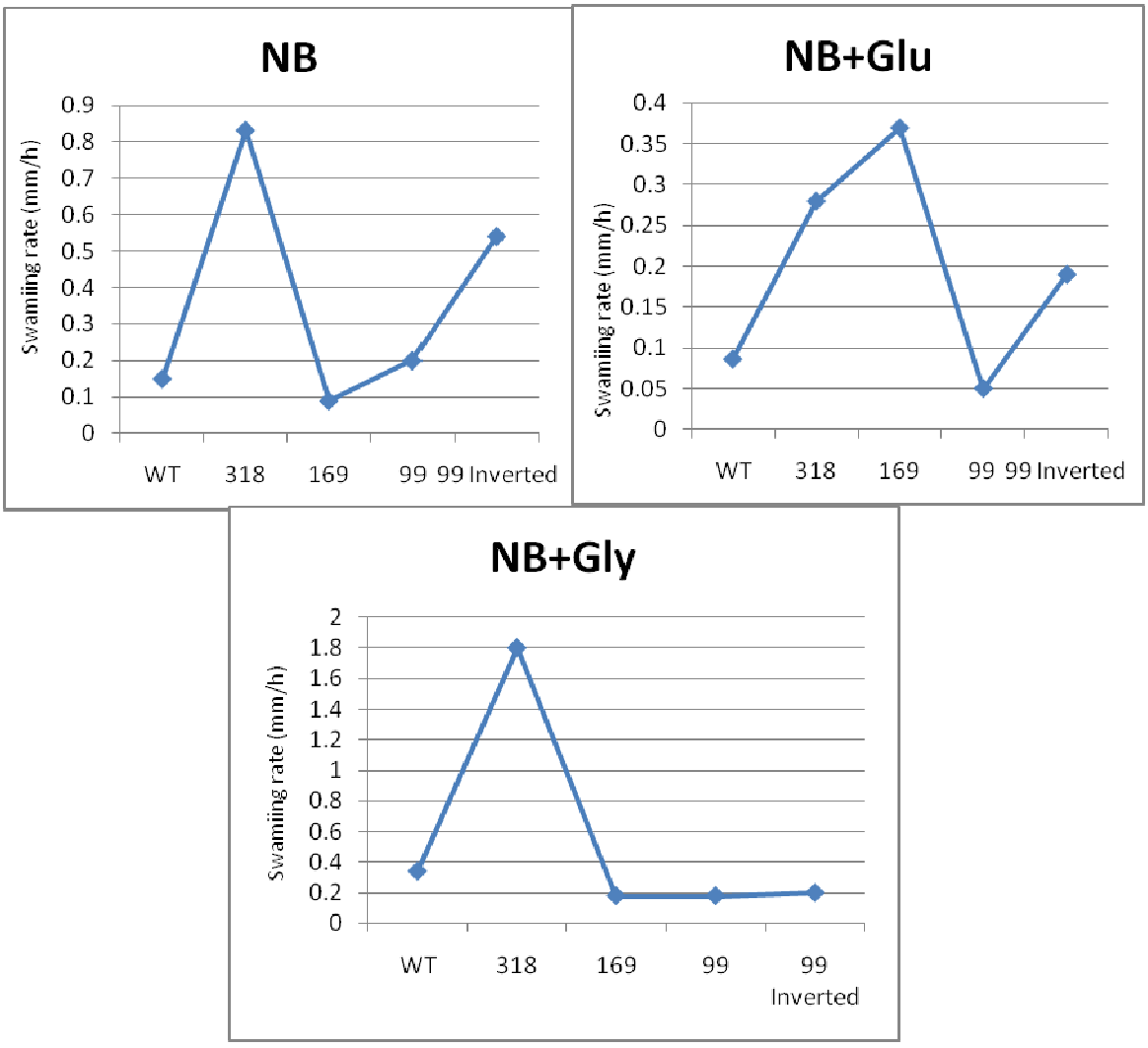

Figure 9: Swarming rates: calculated by plotting the swarming diameter against time for each strain tested in the same medium. (a) Nutrient Broth, (b) Nutrient Broth with 0.5% glucose, (c) Nutrient Broth with 0.5% glycerol.

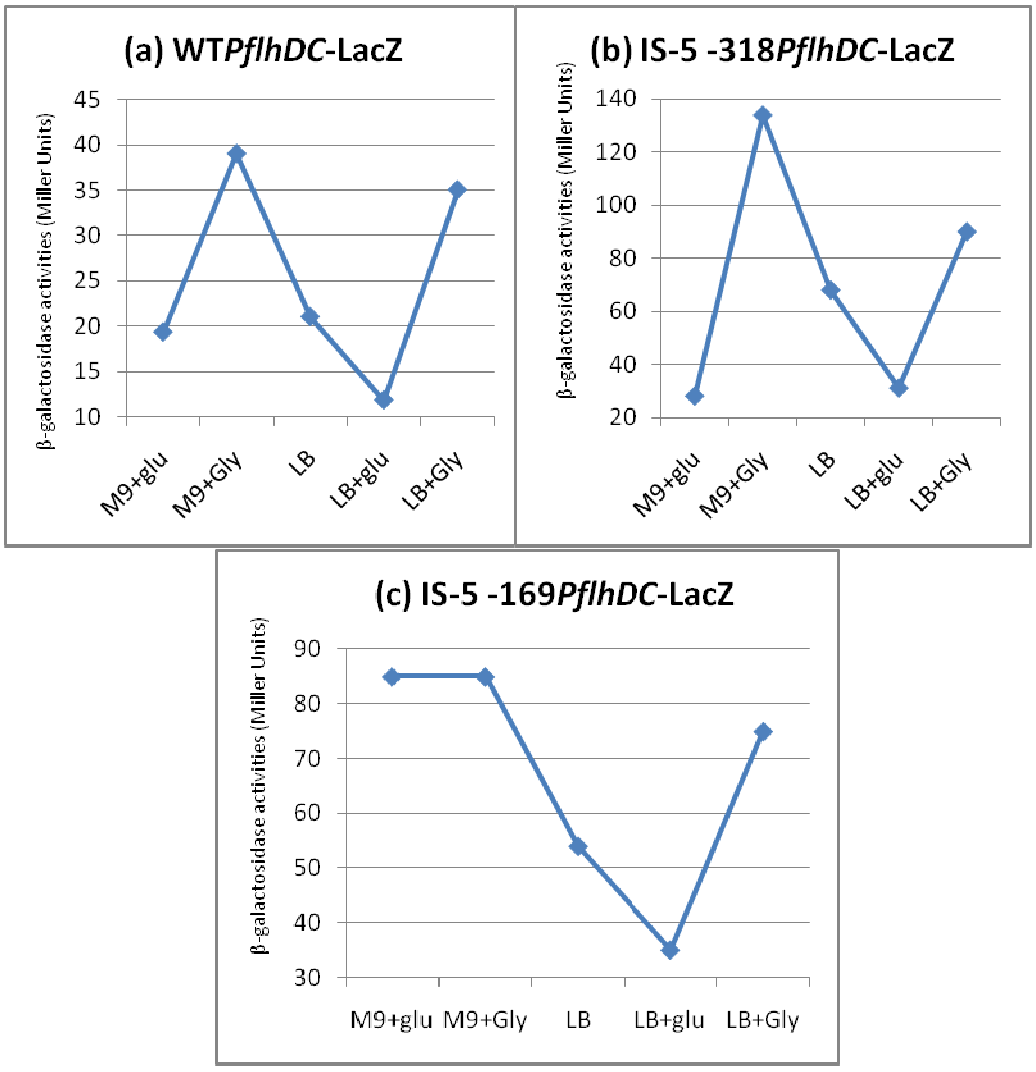

Figure 10-1: β-galactosidase activity tests for wild type strain and various *PflhDC-lacZ* constructs. (a) Wild type in 5 different types of media, M9 minimal media with 0.5% glucose, M9 minimal medium with 0.5% glycerol, LB medium, LB medium with 0.5% glucose, LB medium with 0.5% glycerol, (b) Mutant IS5-318 *PflhDC- lacZ,* (c) Mutant IS-169 *PflhDC- lacZ* in the same 5 media as listed above. The y-axis shows the enzyme activity expressed in Miller Units; The x-axis reveals the different media used.

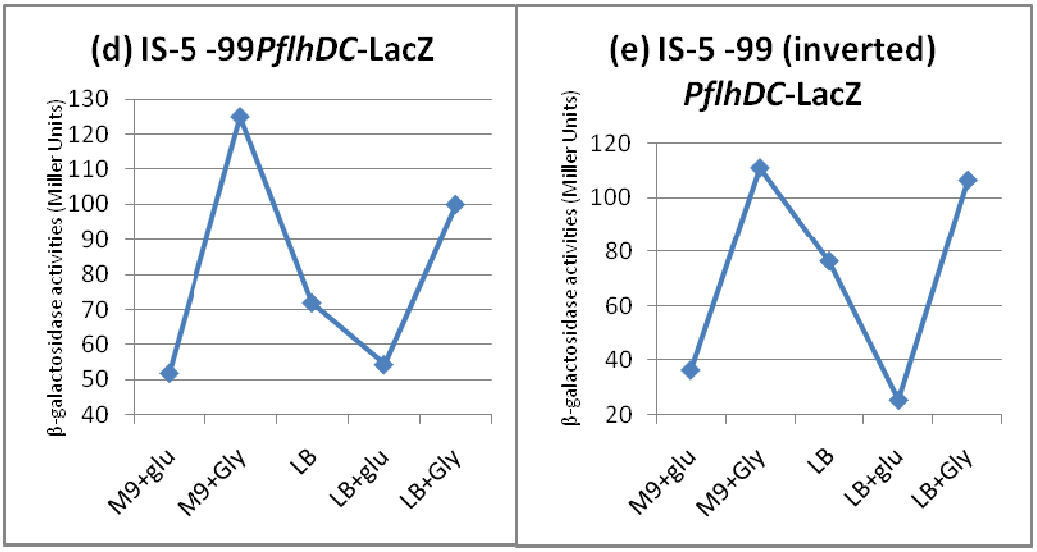

Figure 10-2: (d) Mutant IS5-99 *PflhDC- lacZ* direct insertion and (e) Mutant IS5-99 *PflhDC- lacZ* inverted insertion of IS5. Enzyme activities were measured in the above mentioned 5 media.

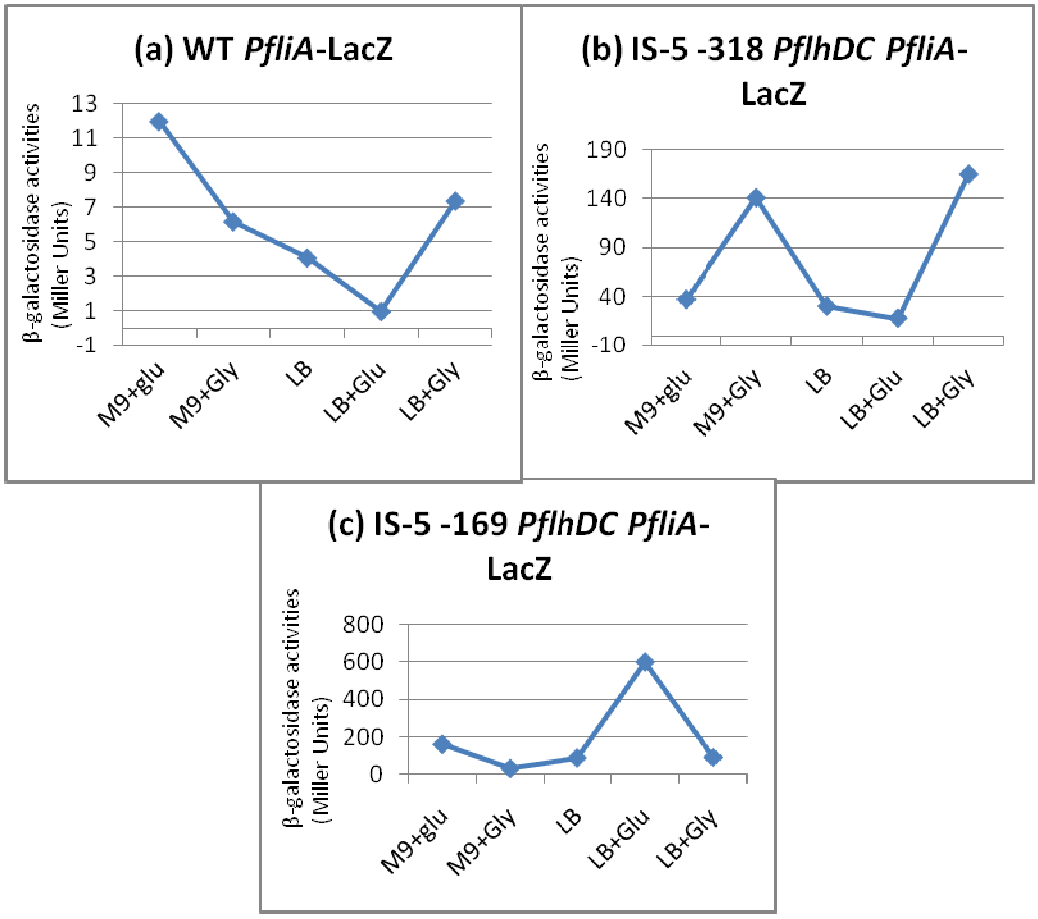

Figure 11-1: β-galactosidase activity test for the *PfliA-lacZ* constructs. (a) Wild type enzyme activity in 5 different type of media, M9 minimal with 0.5% glucose, M9 minimal with 0.5% glycerol, LB medium, LB medium with 0.5% glucose, LB medium with 0.5% glycerol. (b) Mutant IS5-318 *PflhDC PfliA- lacZ,* (c) Mutant IS-169 *PflhDC PfliA- lacZ* enzyme activities in the same 5 media as listed above. The y-axis shows the enzyme activities expressed in Miller Units; the x-axis reveals the different media used.

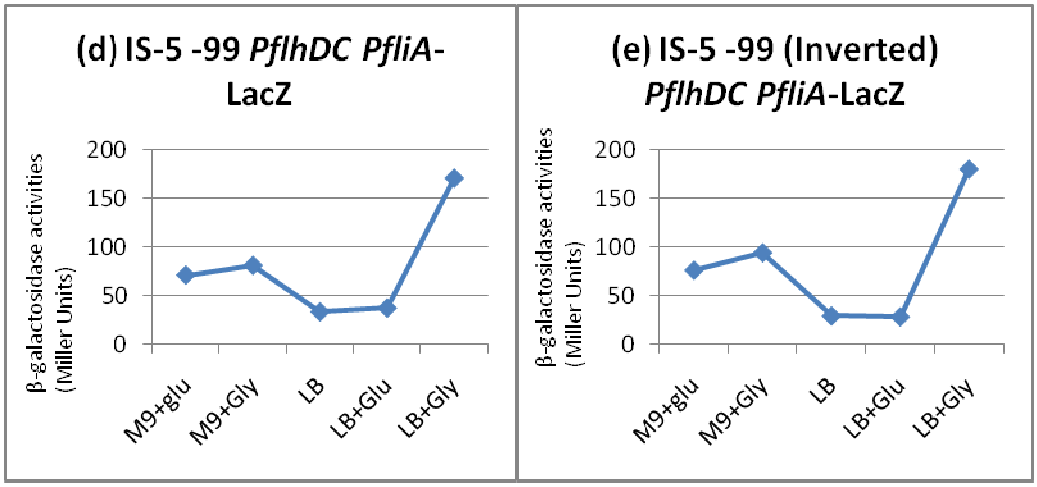

Figure 11-2: (d) Mutant IS5-99 *PflhDC PfliA-lacZ* direct orientation and (e) Mutant IS5-99 *PflhDC PfliA- lacZ* inverted orientation of IS5. Enzyme activity was measured as mentioned in the above 5 media.

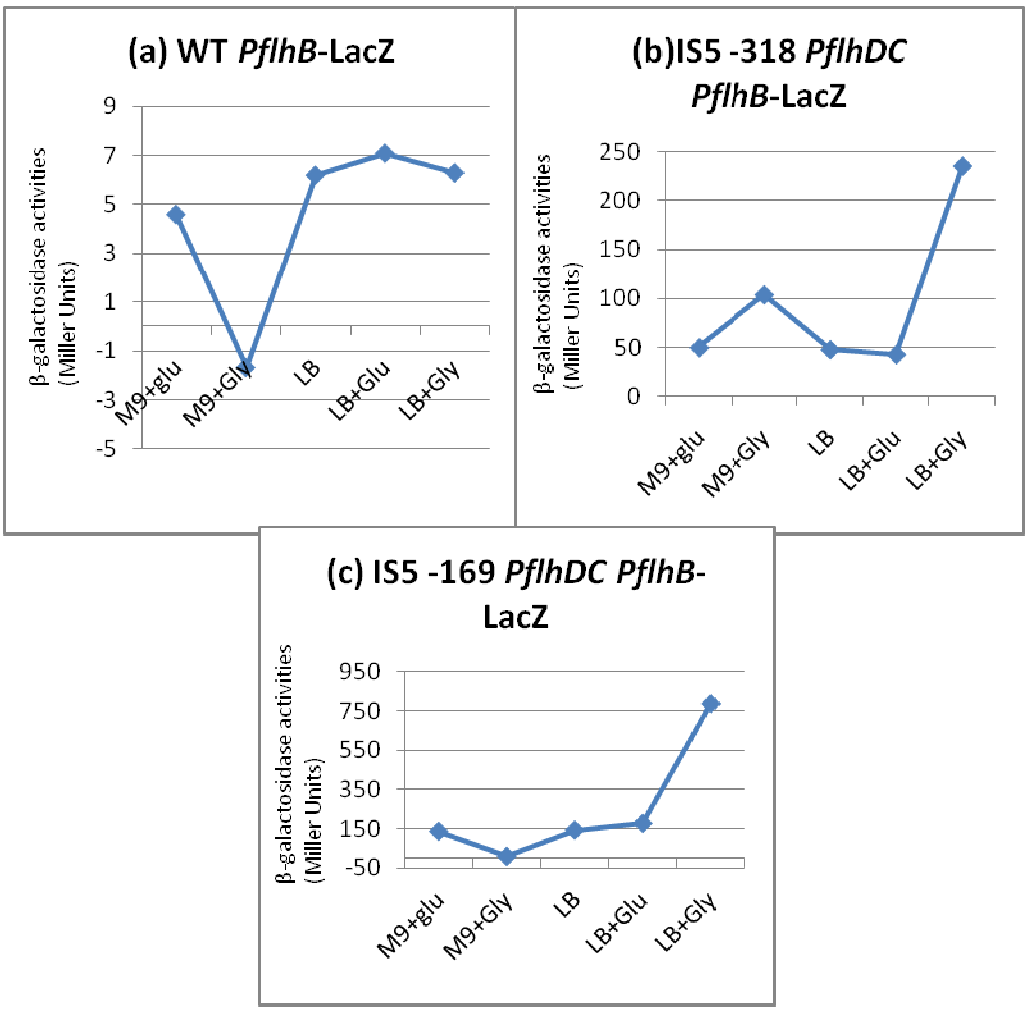

Figure 12-1: β-galactosidase activity tests for *PflhB-lacZ* construct. (a) Wild type enzyme activity in 5 different types of media, M9 minimal medium with 0.5% glucose, M9 minimal medium with 0.5% glycerol, LB medium, LB mudium with 0.5% glucose, LB medium with 0.5% glycerol. (b) Mutant IS5-318 *PflhDC PflhB- lacZ*, (c) Mutant IS-169 *PflhDC PflhB- lacZ* enzyme activities in the same 5 media are presented as listed above.

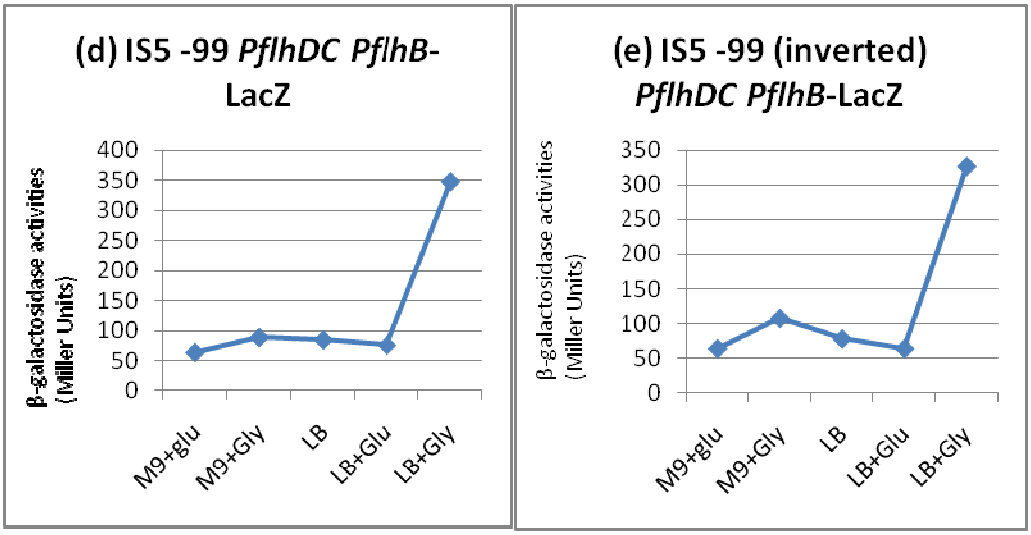

Figure 12-2: (d) Mutant IS5-99 *PflhDC PflhB- lacZ* direct *orientation* and (e) Mutant IS5-99 *PflhDC PflhB- lacZ* inverted *orientation* of IS5. Enzyme activities in the above mentioned 5 media. The y-axis shows the enzyme activities in Miller Units; the x-axis reveals the different media used.

Table 1: DNA oligonucleotides used in this study

Name	**Sequence**	**Use**
PflhDC-Xho-F	tatctcgagcattaagttgattgttgcctttc	Amplification of *flhDC* regulatory region
PflhDC-Bam-R	aatggatcccatagcggacgctttgtcctgaac	Amplification of *flhDC* regulatory region
pKD-ver-F	ctgcggactggctttctacgtg	Verification of PflhDC in pKD13
pKD-ver-R	ctcgctttgtaacggagtagag	Verification of PflhDC in pKD13
PflhDC1-P1	gcatttacgttgacaccatcgaatggcgcaaaac ctttcgcggtatgtgtaggctggagctgcttc	Chromosomal integration of P*flhDC*:*lacZ* fusion
PflhDC2-P2	ccagtcacgacgttgtaaaacgacggccagtga atccgtaatcatggtcattattcccacccagaata accaac	Chromosomal integration of P*flhDC*:*lacZ* fusion
LacZα-R	aaagagctcttaaccgtgcatctgccagtttgag	Verification of Chromosomal P*flhDC*:*lacZ* fusion

Table 2. Various swarming mutants of *E. coli* strain BW25113 that are caused by insertion of IS elements upstream of the *flhDC* promoter region

Mutant type	**Number of mutants**	**Insertion site**	**Location**	**Orientation**
IS1	3	CATTTATG	-107	Reverse
IS1	3	GTGCGCAA	-214	Reverse
IS1	6	AAGATAATT	-469	Direct
IS3	6	TTT	-199	Reverse
IS3	6	CAT	-206	Reverse
IS5	3	TTAA	-99	Direct
IS5	9	TTAA	-99	Reverse
IS5	3	TTAG	-318	Direct

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