SWITCHING IN BACTERIAL GENE EXPRESSION NETWORKS

BY

SANTOSH KOIRALA

DISSERTATION

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Doctoral Committee:

Professor Christopher V Rao, Chair
Professor James M Slauch
Associate Professor Mary L Kraft
Associate Professor Brendan A Harley

ABSTRACT

An ability of a bacterium to appropriately respond to its environmental cues ultimately decides its fate. Bacteria deal with the fluctuating environment as a population instead of individual cells. By allowing individual cells to stochastically switch between multiple phenotypes, the cell population can make sure some cells are always fit for the environmental change. The underlying genetic circuitry plays a key role in eliciting multiple phenotypes by an isogenic population of bacteria. Understanding the underlying mechanism requires careful and systematic approach. In this study, we investigated two very well-known systems: the motility in *Salmonella enterica* serovar Typhimurium and the sugar utilization in *Escherichia coli*.

Many bacteria are motile only when nutrients are scarce. By contrast, *Salmonella enterica* is motile only when nutrients are plentiful, suggesting this bacterium uses motility for purposes other than foraging, most likely host colonization. We investigated how nutrients affect motility in *S. enterica* and found that nutrients tune the fraction of motile cells. In particular, we observed co-existing populations of motile and non-motile cells, where the distribution was determined by the concentration of nutrients in the growth medium. Interestingly, *S. enterica* does not respond to a single nutrient but apparently a complex mixture of them. We investigated the mechanism governing this behavior and found that it results from two antagonizing regulatory proteins, FliZ and YdiV. We further demonstrated that the response is bistable: namely, that genetically identical cells can exhibit different phenotypes under identical growth conditions. We further characterized the differences within class 2 and class 3 gene expression and showed that a secretion-dependent feedback loop involving flagellar specific sigma factor, σ^{28}, is responsible for partitioning cells into two fractions. Together, these results uncover a new facet to the regulation of the flagellar genes in *S. enterica* and further demonstrate how bacteria employ phenotypic diversity as general mechanism for adapting to change in their environment.

We then investigated the sugar utilization system in *E. coli*. Glucose is known to inhibit the transport and metabolism of many sugars in *Escherichia coli*. This mechanism leads to its preferential consumption. Far less, however, is known about the preferential utilization of non-glucose sugars in *E. coli*. One notable exception is arabinose and xylose. Previous

studies have shown that *E. coli* will consume arabinose ahead of xylose. Selective utilization results from arabinose-bound AraC binding to the promoter of the xylose metabolic genes and inhibiting their expression. This mechanisms, however, has not been explored in single cells. Both the arabinose and xylose utilization systems are known to exhibit a bimodal induction response to their cognate sugar, where mixed populations of cells either expressing the metabolic genes or not are observed at intermediate sugar concentrations. This suggests that arabinose can only inhibit xylose metabolism in arabinose-induced cells. To understand how crosstalk between these systems affects their response, we investigated *E. coli* during growth on mixtures of arabinose and xylose at single-cell resolution. Our results show that mixed, multimodal populations of arabinose and xylose-induced cells occur at some intermediate sugar concentrations. We also found that xylose can inhibit the expression of the arabinose metabolic genes and that this repression is due to XylR. We further found that xylose-bound XylR binds to the divergent promoter region of the regulator *araC* and the arabinose metabolic genes and inhibit expression. These results demonstrate that a strict hierarchy does not exist between arabinose and xylose as previously thought and this may aid in the design of *E. coli* strains capable of simultaneous sugar consumption.

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CHAPTER 1
BACKGROUND

GENETIC SWITCHING IN BACTERIA

Bacteria face an array of challenge from external factors such as shortage of nutrients, temperature shock, pH change, predation, anti-microbial agents and host immune response (1). Bacteria employ a number of different strategies for responding to changes in their environment. An epigenetic mechanism to cope with the environmental challenges is genetic switching. Change in environmental condition triggers a set of complex gene regulatory cascade which enables bacteria to appropriately respond (2). However, in many cases only a fraction of bacterial population adapts to these changes, which is often hypothesized as a bet-hedging strategy (3). By committing only a portion of population to the change, the bacterial population is fitter to respond to unknown changes in the future. Heterogeneity in an isogenic cell population is achieved in various ways such as genetic rearrangement (4), DNA modification (5) or feedback architecture of genetic networks (2).

Genetic rearrangement is a mechanism of producing reversible and high-frequency genetic changes at a certain locus of a chromosome (4, 6, 7). Most studied of these loci contain genes involved in virulence such as adhesin, lipopolysaccharides, lipoproteins, pilli and flagella (4). Genetic rearrangement can be achieved by various mechanisms such as slipped-strand mispair (6), site-specific DNA rearrangement (8) and DNA shuffling (4). The loci consisting capsule biosynthesis gene *siaD* of *Neisseria meningitidis* (9), M9/pMGA haemagglutinin genes of *Mycoplasma gallisepticum* (10), adhesin genes *hmw1* and *hmw2* of *Haemophilus influenza* (11) and *uspA1* of *Moraxella catarrhalis* (12) are controlled by slipped-strand mispairing. Expression of flagellum of *Salmonella Typhimurium* (13), type 1 fimbriae of *Escherichia coli* (14) and type IV pilli of *Moraxella lacunata* (15) are controlled by site-specific DNA rearrangement. Expression of type IV pilli of *Neisseria gonorrhoeae* (16), surface-exposed lipoprotein VlsE of *Borrelia burgdorferi* (17), surface protein Vmp of *Borrelia hermsii* (18) and haemagglutinin VlhA of *Mycoplasma synoviae* (19) are controlled by DNA shuffling. Heterogeneity achieved

through genetic rearrangement are usually associated with phenotypic variation which prevents a fraction of pathogenic bacteria being recognized by host immune system (20).

DNA modification is another strategy for epigenetic regulation. DNA methylation is a very common mechanism of DNA modification in bacteria (21). It controls DNA replication, repair, transfer and portioning of chromosome to daughter cells (22-25). Additionally, most adhesin genes in *Escherichia coli* are controlled by DNA methylation (26, 27). One of the most studied system controlled by DNA methylation is Pyelonephritis-associated pilli (Pap Pilli) of *E. coli* (21, 28). Methylation of two GATC sites close to the promoter region of *papBA* by DNA adenine methylase (Dam) controls Pap pilus expression (28). Selective methylation of these two sites leads to heterogeneous population of cells with only a fraction of them expressing Pap pilli (29).

Phenotypic variation does not always have to be a result of DNA rearrangement or modification. There is a significant variation in expression of genes among individual cells in a bacterial population because of random fluctuations in transcription and translation. These fluctuations, referred to as noise (30), lead to normal distribution of gene expression (31). However, these differences in levels of gene expression are not sufficient to produce mixed phenotypes in an isogenic cell population. In order to generate phenotypic heterogeneity, two (or more) subpopulations, each with a normal distribution of gene expression levels, need to be present in a cell population. These discrete levels of gene expression are referred to as state (31). Gene networks can exhibit two (or more) stable states resulting in a bistable (or multistable) bacterial population (2), each with a distinct phenotype. Cells switch between these states stochastically and the switching is usually reversible (31). This study primarily focuses on stochastic genetic switching that arises from feedback architecture of gene networks and does not depend on DNA rearrangement or modification.

BISTABILITY IN GENE EXPRESSION

Bistability refers to the coexistence of two subpopulations, each with a normal distribution of gene expression levels in an isogenic cell population (2). The earliest example of bistable population was all-or-none induction of an enzyme that regulates lactose utilization in *E. coli* (32). In 1957, Novick and Weiner demonstrated that when cells induced by gratuitous inducer thiomethyl-β-D-galactoside (TMG) at low levels of enzyme activity are diluted and recultured, two subpopulations of cells, one with high enzyme activity (all) and another with no enzyme activity (none), are observed. In 1961, Monod and Jacob, speculated that components of the regulatory network may be responsible in producing bistability (33). Since then, it has been one of the most studied gene networks in bacteria (34).

It has been established that a bistable system need to exhibit kinetic behavior where output is not linear to input (31). For a gene regulatory system this implies a non-linear response to the concentration of a regulator or inducer. One of the most observed mechanism is to require a threshold concentration of the regulator to produce a measurable response (35). In the case of transcriptional regulators, non-linear kinetics can also be achieved by multimerization, cooperativity in DNA binding, or phosphorylation of certain amino acid residues of regulators (31).

In addition to non-linear kinetics, it has been shown that, specific feedback within the gene network is required for bistability (2). The simplest example of feedback is autostimulation in which a regulator activates its own expression. It has been experimentally shown that an addition of autoregulatory feedback loop can convert a graded response (response increases monotonously with regulator/inducer concentration) into bistable one (36). Due to noise in gene expression, some cells will reach the threshold and initiate a positive feedback which results in some cells expressing at low levels (off state) and others at high levels (on state), giving rise to bistable population (37, 38). Similarly, a gene network containing two regulators that activates each other's expression can also give rise to bistability (31).

Bistability, however, is not limited to systems with positive feedbacks. A gene network containing two regulators that represses each other's expression, known as genetic toggle switch, can also exhibit bistability (39). In the case of genetic toggle switch, both regulators

are indirectly enhancing their own expression by repressing their repressor. The two states correspond to reciprocal states of the two regulator. In other words, one state correspond to low levels of regulator A and high state of regulator B (A off and B on) and another state corresponds to high state of regulator A and low state of regulator B (A on and B off). The strength of regulatory interactions determines the fractions of cells in each state. Moreover, if one of the regulator is much stronger than another, the system will exhibit only one state where the expression of stronger regulator is on (31). Thus, a gene network that contains atleast one positive feedback loop or even number of negative feedback loops (resulting in net positive feedback) are capable of exhibiting bistability. However, existence of positive feedback does not guarantee bistable response.

A unique characteristic of a bistable system is that they exhibit hysteresis. In another word, bistable systems possess memory. Hysteresis can be observed by comparing the response when cells switch between the two states. The response is different when switching from off state to on state compared to the response when switching from on state to off state, suggesting that cells remember their previous state. In the case of *lac* operon in *E. coli*, Novick and Weiner observed that when cells grown with high concentration of TMG are subcultured and grown in media with low concentration of TMG, they continued to express β-galactosidase enzyme at high level (32). This phenomenon was later characterized in detail by Ozbudak and co-workers (40). They were also able to show that bistability is not observed during growth in lactose. When the non-metabolizable inducer TMG is replaced with lactose, it eliminates the autoinducing mechanism essential for bistability. Recently, Afroz *et al*. (41) investigated the response of eight inducible metabolic pathways in *E .coli* and found that four pathways (D-xylose, L-arabinose, L-rhamnose and D-gluconate) exhibit a bistable response and four of them (D-lactose, D-galactose, N-acetylglucosamine and N-acetylneuraminic acid) exhibit a graded response. They further characterized L-arabinose and D-xylose pathways and showed that they exhibit hysteresis.

SALMONELLA

Salmonella is a gram negative, rod shaped pathogenic bacteria which belongs to *Enterobacteriaceae* family (42). It is a facultative anaerobe that doesn't form spores and is motile in its dominant form. They can infect a broad range of warm-blooded hosts and are capable of causing different types of diseases. They are divided into different subspecies according to their host preference and specificity. Extensive body of literature is available for *Salmonella* morphology, physiology, genetics and its interaction with its host.

Salmonella are causative agents of a food borne disease, salmonellosis. Gastrointestinal tract of animals is their primary habitat (42). Most of the infections results from consuming contaminated foods from animal origins or fruits and vegetables contaminated with animal faeces (43, 44). In human, salmonellosis usually takes the form of self-limiting gastroenteritis, but occasionally could lead to systemic infection (enteric fever), bacteremia and other complications (42, 45). It accounts for 26% of hospitalizations (15,000 per year) and 31% of deaths (400-600 per year) caused by the food borne diseases in the United States (43, 46). Non-typhi *Salmonella* infection is also a major public health problem among children under the age of 5 in developing countries (47). Although vaccines are readily available for typhoid fever, no vaccines are available for non-typhoidal salmonellosis (45). Understanding *Salmonella*'s interaction and survival inside the host, through continued research in this area, is paramount for developing new vaccines and drugs.

Although only capable of causing self-limiting gastroenteritis in human under normal conditions, *Salmonella enterica* serovar Typhimurium **(Figure 1)** is capable of causing systemic infection that resembles human Typhoid fever caused by *Salmonella enterica* serovar Typhi (42). An array of classic and modern genetic tools is readily available to manipulate *Salmonella* strains. The mouse infection model and the ease of manipulation make *Salmonella enterica* serovar Typhimurium ideal for studying rather complex host-pathogen interactions.

CHEMOTAXIS

An ability of a cell to appropriately respond to its environmental cues ultimately decides its fate. Cells have to be able to sense chemicals in their environment and decide to move towards or away from them. This process in which cells move towards chemical attractants and move away from chemical repellants is called chemotaxis and has been studied extensively in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, two closely related bacterial species. Cells swim in a liquid environment and drift along surfaces by assembling and rotating flagella (48).

The demonstration that bacteria move to a capillary tube filled with chemical attractant was first done by a German botanist Wilhelm Pfeffer. It was later quantified by Adler by counting the number of bacteria that accumulated in the capillary and is well-known in the field of chemotaxis as cap-assay. Alder also developed a positive chemotaxis assay by using low concentration agar plates (49). When bacteria are deposited at the center of the plate they create local gradient by consuming the attractant and move further away in search of more attractant. This motility assay is very common in the field as it gives the phenotype with quick and easy experiment. He further theorized that bacteria must have chemosensors which allow them to detect attractants or repellants.

Contemporarily, Howard C. Berg and Douglas A. Brown, using three dimensional tracking, showed that bacteria chemotaxed randomly in series of straight "runs" and "tumbles" (50). In isotropic environment both run and tumble are equal. However, when the concentration gradient is present, the runs are elongated and the frequency of tumbles decreases **(Figure 2)**. Silverman and Simon showed that each flagellar filament is capable of spinning in both clockwise (CW) and counter-clockwise (CCW) direction (51). Simon and colleagues further demonstrated that CCW rotations of helical filaments causes a flagellar bundle formation and propels the cell along a more or less smooth trajectory called a "run", whereas CW rotations disperses the bundle resulting in uncoordinated filament action with rapid somersaulting called a "tumble" (52). Thus bacterial chemotaxis is a random walk, albeit it is biased to efficiently move up the gradient of attractant or down the gradient of repellants.

FLAGELLA

A flagellar structure consists three individual parts: a basal body, a hook and a filament **(Figure 3)**. In *Escherichia coli* and *Salmonella*, flagella are long (several body lengths) thin helical filaments, usually 5-10 per cell, assembled at random sites on their body. Purification of intact flagella from *E. coli* was first done in 1971 by DePamphilis and Adler (53). Using dark field light-microscopy, Kamiya and Asakura showed that *Salmonella* flagella were lefthanded helices with a pitch of 2.3 μm at neutral pH. Flagellar filaments can take 12 possible conformations, 2 of which are straight and 10 are helical (54, 55). An atomic model of the bacterial flagellar filament was built by Namba group using electron cryomicroscopy (56).

The basal body which anchors the flagellum to the cell has a complex structure embedded in the bacterial membranes. The flagellar hook-basal body (HBB) of *Salmonella* was purified and characterized in Yamaguchi lab which provided the evidence for its structural basis and its morphology embedded in the membrane(57). A complete picture of HBB structure and how they embed in a multilayer cell wall of gram negative bacteria was produced by averaging images taken with electron microscope (58-60)**.** The filament is a polymer of falgellin (FliC) and is connected to the hook, a polymer of hook protein (FlgE), by hook-associated protein (HAPs) FlgK and FlgL. Hook is anchored to the cell body by a distal rod (FlgG), L-ring (FlgH), P-ring (FlgI)and MS-ring which are embedded in the cell wall. The P-ring and the L-ring act as bushing for a hollow rod that is built onto the MS-ring and spans the periplasmic space. A proximal rode made up of FlgB, FlgC and FlgF connects distal rod with MS-ring and spans the distance between peptidoglycan layer and cytoplasmic membrane. The cytoplasmic face of the MS-ring anchors C-ring and type III secretion apparatus that delivers majority of the protein subunits through a central channel within the growing flagellar structure. C-ring is inside the cytoplasmic membrane and is surrounded by stator proteins MotA and MotB spanning the cytoplasmic membrane (61, 62). The torque produced by the motor is transmitted to the filament by a flexible joint, the hook. At the center of each C-ring is a knob which comprises the main body of transport apparatus (63, 64). Flagellar motors are driven by a proton-motive force (65-68).

Flagellar assembly is sequential which begins with the formation of the basal body inside out along the membranes and concludes with the formation of filament (69-71). The

flagellar biogenesis starts with the assembly of MS-ring in the cytoplasmic membrane. After the completion of MS-ring, C-ring and FliG are added to it (72). To continue the process of flagellar assembly, a type III transport apparatus capable of translocating other structural proteins is assembled using proteins FlhA, FlhB, FliH, FliO, FLiP, FliQ and FliR (73-77). Components of proximal rod (FlgB, FlgC, FlgF) are the first component to be transported by the export apparatus assembled on top of MS-ring. Then, P-ring and L-rings are assembled whose components are secreted into the periplasmic space by sec-pathway. (78, 79). Although the assembly of hook can begin before L-rings and P-rings are assembled, the construction is halted until they are assembled (80, 81). The hook cap (FlgD) is then assembled which allows for the assembly of hook (FlgE) and it is discarded (82). The hook associated proteins (HAPs) are added to the hook which allows for the polymerization of FliC subunits under a rotating cap (FliD) to form the helical filament (83). Finally the stator is assembled using MotA and MotB proteins expressed together (84).

In *Salmonella enterica* serovar Typhimurium, more than 50 genes divided among at least 17 operons are involved in making chemotactic decisions. Among these, about 10 genes are involved in detecting and processing sensory cues and the rest encode for flagellar subunits and a number of regulators that synchronizes gene expression with the assembly process (85, 86). These operons are divided into three classes forming an organized hierarchical gene cascade (87). Flagellar assembly is initiated by products of a single operon controlled by P_{class1} promoter, consisting *flhDC* genes, and is therefore called the master operon (88). The environmental signals and sensory cues manifest into the flagellar gene expression hierarchy through the master operon by the action of different global regulators on P_{class1} promoter, which allows the cells to determine whether to be motile or not. When motility is induced, $FlhD_4C_2$ hexaheteromeric complex, products of the master operon, binds to the P_{class2} promoter region and initiates the transcription by σ^{70} RNA polymerase. These promoters control the expression of genes encoding hook basal body (HBB) proteins and an array of regulatory proteins (89, 90). Among the regulatory proteins encoded from class 2 operons, FlgM and FliA (σ^{28}) play major roles in enforcing HBB checkpoint (91-94). The σ^{28} alternate sigma factor controls the transcription of class 3 operons encoding for late genes. Before HBB completion, FlgM binds to FliA and stops

it from activating P_{class3} promoters. However, after HBB completion, FlgM is secreted out of the cells allowing σ^{28} to initiate the transcription of class 3 operons (92, 95, 96). However, mere presence or absence of functional HBBs doesn't determine the expression of flagellar genes. Class 3 gene expression is controlled by the rate of FlgM secretion mediated by the σ^{28}-FlgM regulatory circuit (97, 98).

In addition, flagellar morphogenesis has also been shown to be regulated by two other flagellar proteins, FliT and FliZ (99). FliT, encoded in the *fliDST* operon, is the secretion chaperone for the filament cap protein FliD, and negatively regulates the class 2 gene expression by binding to $FlhD_4C_2$ complex and hence inhibiting its activation of P_{class2} promoters. Class 2 gene expression has been shown to increase in a *fliT* mutant (99). FliZ, encoded in the fliAZY operon, is a positive regulator of class 2 gene expression (99). It was shown to be $FlhD_4C_2$-dependent activator of P_{class2} activity and to participate in a positive-feedback loop that induces a kinetic switch in class 2 operon expression (100, 101). Recently, a non-flagellar protein YdiV was reported to negatively regulate class 2 expressions (102). YdiV has a weak homology to EAL domain proteins, which are known to be involved in regulation of cyclic-di-GMP, a second messenger molecule (103, 104).

ARABINOSE AND XYLOSE UTILIZATION IN *ESCHERICHIA COLI*

Plant biomass is a renewable and low-cost feedstock for biofuel and many value-added compounds. Lignocellulosic feedstock derived from plant biomass consist of three primary components: cellulose, hemicellulose and lignin (105). These feedstock are mechanically degraded and pretreated with acid and further broken down enzymatically to obtain a mixture of sugars which can be fermented to biofuel and other valuable chemicals by engineered microorganisms (106-108). After glucose, xylose and arabinose are the next most abundant sugars in plant-derived hydrolysates (109). To make the fermentation process economic and efficient, it is necessary to engineer microorganisms able to utilize all of these sugars and ideally at the same time. Numerous previous studies have proposed various strategies to enable simultaneous sugar utilization in *Escherichia coli* as well as other bacteria and yeast (110-113). Most of these studies have focused on the co-utilization of glucose and another sugar (114). Only a few studies have been directed towards the co-utilization of non-glucose sugar (110, 113, 115, 116).

E. coli cells are unable to simultaneously consume multiple sugars. Rather, they are consumed in a defined hierarchy. This process of ordered sugar consumption is known as carbon catabolite repression and has been studied in many species of bacteria (117-121). These studies have principally focused on the mechanisms governing the preferential consumption of glucose, which in the case of *E. coli* is known to involve the regulation of specific genes and metabolic fluxes (122-124). Far less is known about the mechanisms governing the preferential consumption of sugars other than glucose. One notable exception is the growth of *E. coli* on mixtures of (L-)arabinose and (D-)xylose (113, 125-127).

Arabinose and xylose metabolic pathways are shown in **Figure 4**. Enzymes required for metabolism of arabinose are encoded in a single *araBAD* operon. *araA* encodes for arabinose isomerase that converts arabinose into ribulose. *araB* encodes for ribulokinase that phosphorylates ribulose into ribulose-5-phosphate at the expense of one ATP. *araD* encodes for ribulose-5-phophate-epimerase that converts ribulose-5-phophate to xylulose-5-phosphate. Similarly, enzymes required for metabolism of xylose are encoded on a single *xylAB* operon. *xylA* encodes for xylose isomerase that converts xylose into xylulose. *xylB* encodes for xylulokinase that phosphorylates xylulose into xylulose-5-phosphate also the

expense of one ATP. Xylulose-5-phosphate can enter the central metabolism through pentose phosphate pathway. The products of pentose phosphate pathway, glyceraldehyde-3-phosphate and fructose-6-phosphate, can enter glycolysis to produce pyruvate. Pyruvate can be fermented to ethanol and acids or converted to other value added chemicals.

Arabinose is transported into the cells by arabinose specific transporters AraE or AraFGH (128). AraE is a low-affinity arabinose/proton symporter. AraFGH is a high-affinity transporter of ATP binding cassette (ABC) transporters superfamily (129). AraF is an arabinose binding protein, AraG is an ATPase subunit and AraH is a transmembrane subunit responsible for translocation of arabinose (130, 131). In a similar manner, xylose is transported into the cells by xylose specific transporters XylE or XylFGH (132). XylE is a low affinity D-xylose/proton symporter of the major facilitator superfamily (MFS) (133). XylFGH is a high-affinity transporter of ATP binding cassette (ABC) transporters superfamily. XylF is a periplasmic xylose binding protein (134), XylG is an ATPase subunit and XylH is a transmembrane subunit responsible for translocation of xylose (132). However, *E coli* cells are able to metabolize arabinose or xylose even in the absence of their cognate transporters which suggest that an alternate, though less efficient, mechanisms for the transport of these sugars exist (125, 132).

Regulation of arabinose (**Figure 5**) and xylose (**Figure 6**) metabolic and transporter genes are very similar to each other. The arabinose metabolic and transporter genes are activated by arabinose bound AraC (129, 135-137). This initiates two competing feedback mechanisms that affects the concentration of arabinose inside the cells: increased concentration of transporters AraE and AraFGH increases the rate of arabinose uptake whereas increased concentration of enzymes AraB, AraA and AraD decreases the concentration of arabinose by actively metabolizing it (135). Similarly, the xylose metabolic and transporter genes are activated by XylR when xylose binds to it (138). Analogous to arabinose, this activates two feedback loops that affect the concentration of xylose inside the cells: increased concentration of transporters XylE and XylFGH increases the rate of xylose uptake whereas increased concentration of enzymes XylA and XylB decreases the concentration of xylose by actively metabolizing it. These competing feedback loops have been believed to impart bistable phenotypes: one in which cells can transport and metabolize sugar and the other in which they cannot (41).

FIGURES FOR CHAPTER 1

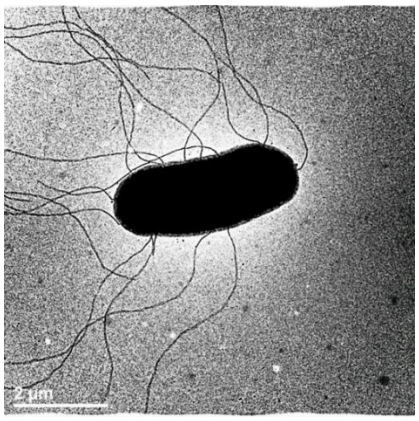

Figure 1. Electron micrograph of *Salmonella enterica* serovar Typhimurium. It is a gram negative, rod shaped pathogenic bacteria which belongs to *Enterobacteriaceae* family (42). The long thin filaments attached to the body are flagella.

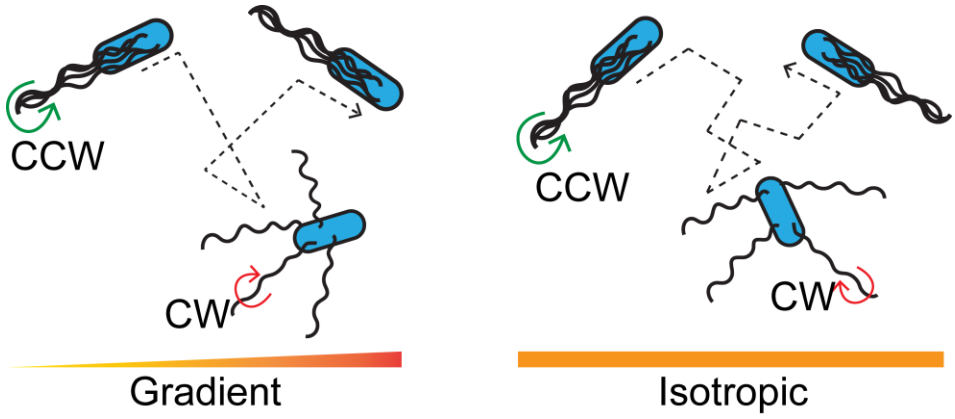

Figure 2. Flagellated bacteria employ run/tumble strategy for chemotaxis (85). Counter-clockwise rotation of flagella creates a bundle that causes cells to propel forward (run). Clockwise rotation of flagella unwinds the bundle that causes cells to somersault (tumble). In the presence of gradient, cells bias their runs towards higher concentration of attractant. In an isotropic solution, there is no bias.

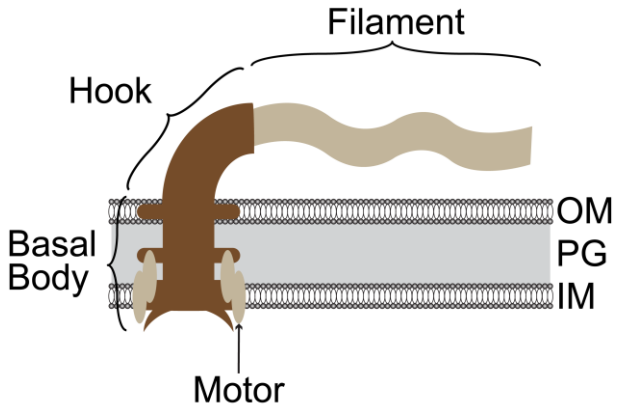

Figure 3. Bacterial flagella can be divided into three broad subunits: the basal body, the hook and the filament. The basal body that anchors the flagella to the cells has a complex structure embedded in the inner membrane (IM), peptidoglycan (PG) and outer membrane (OM) (139). The flagellar hook is a molecular universal joint that transmits the torque from the motor to the filament (57). The filament is a helical propeller attached to the hook (56).

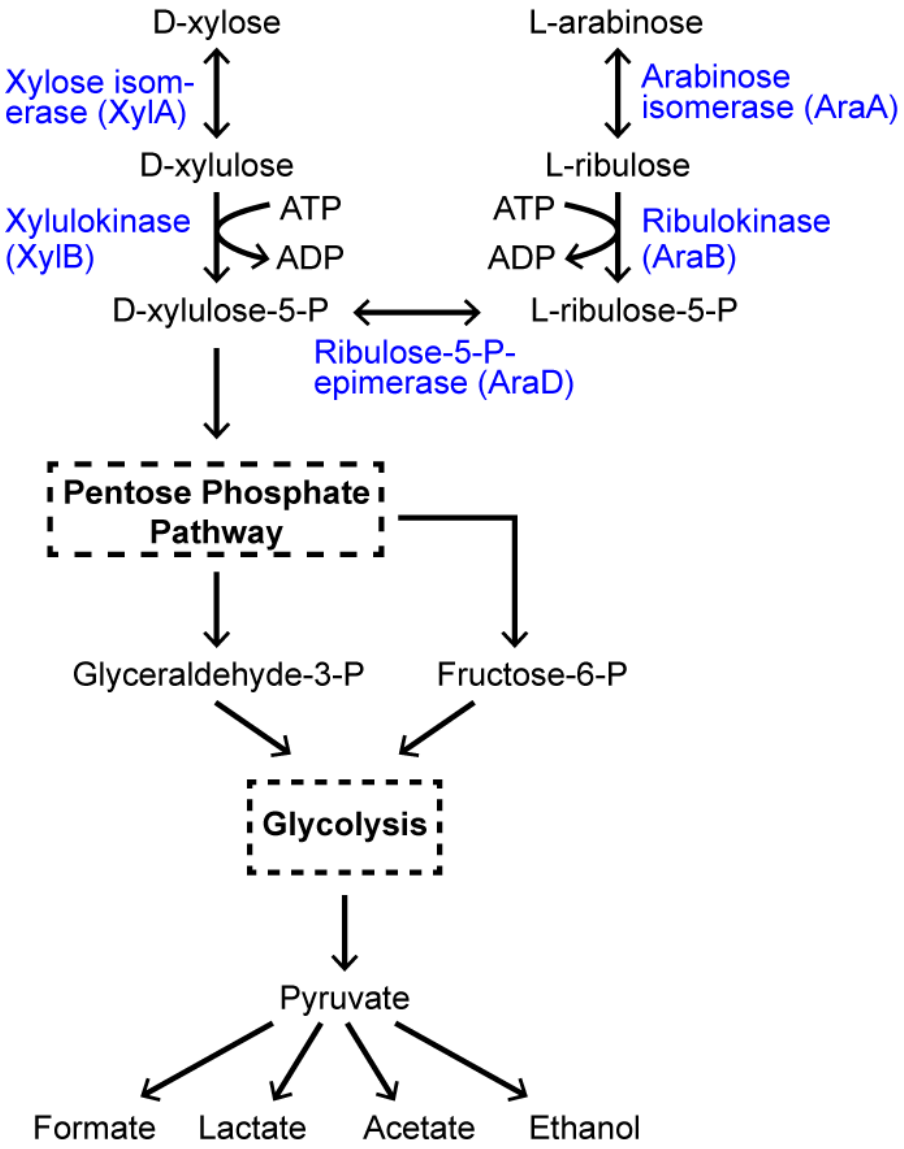

Figure 4. Pathways for L-arabinose and D-xylose metabolism in E. coli. They enter the central metabolism through the pentose phosphate pathway. The products of pentose phosphate pathway can be converted to pyruvate through glycolysis and further fermented into ethanol and other acids. Enzymes that catalyze the reactions are shown in blue.

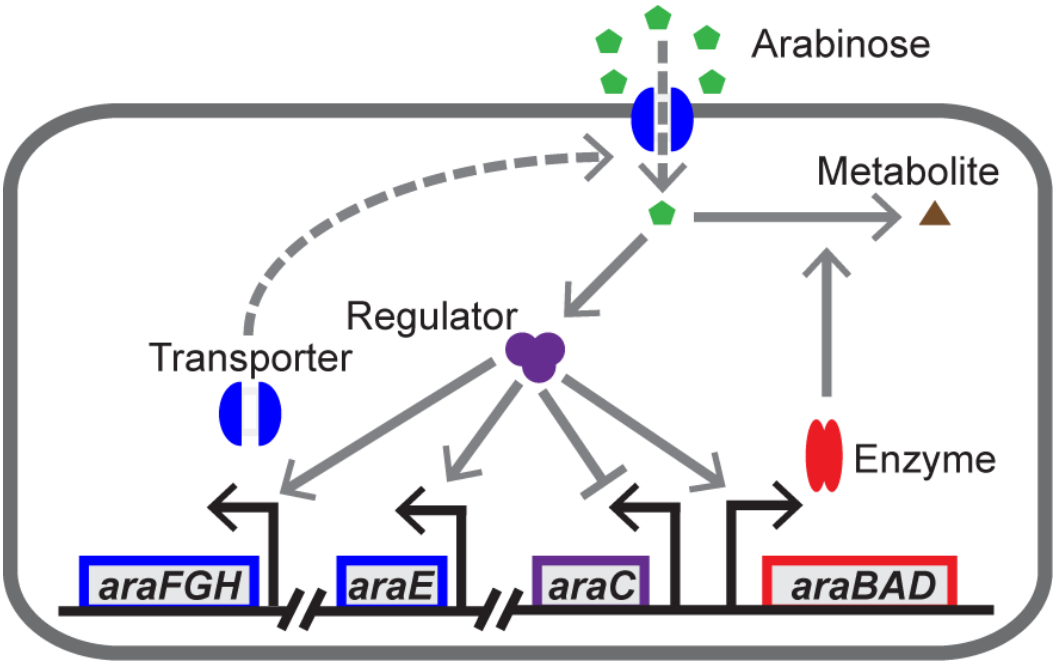

Figure 5. The arabinose metabolic and transporter genes are activated by AraC when arabinose binds to it. Transporters AraE and AraFGH increases the rate of arabinose uptake by actively importing arabinose. Catabolic enzymes AraB, AraA and AraD decreases the concentration of arabinose by actively metabolizing it.

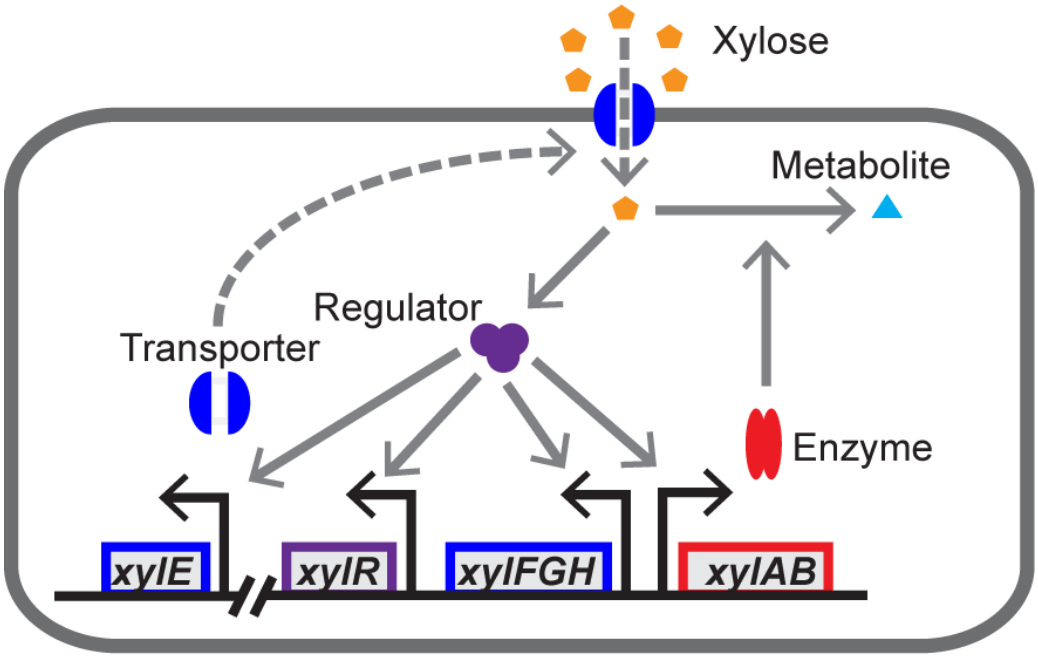

Figure 6. The xylose metabolic and transporter genes are activated by XylR when xylose binds to it. Transporters XylE and XylFGH increases the rate of xylose uptake by actively importing xylose into the cells. Catabolic enzymes XylA and XylB decreases the concentration of xylose by actively metabolizing it.

CHAPTER 2
MATERIALS AND METHODS

STRAINS

All *Salmonella* strains **(Table 1)** used in flagellar studies and *E. coli* strains **(Table 2)** used in sugar utilization studies are isogenic derivatives of *Salmonella enterica* serovar Typhimurium (ATCC 14028) and *Escherichia coli* MG1655 (CGSC 6300) respectively. All the plasmids used in this study are given in **Table 3**. Oligonucleotide primers are listed in **Table 4**. Gene deletions were performed using Datsenko and Wanner method (140). Plasmids were constructed using traditional cloning method (restriction digestion and ligation) as well as ligase cycling reaction method (141). Plasmids were integrated into the chromosome using CRIM method (142). Promoter replacement with tetRA element was done using the method described by Karlinsey (143).

MEDIA AND REAGENTS

Luria-Bertani (LB) (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) and tryptone broth (TB) (10 g/l tryptone and 5 g/l NaCl) were used as rich media whereas M9 glycerol medium (6.8 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.5 g/l NaCl, 2 mM $MgSO_4$, 100 µM $CaCl_2$, 0.4% glycerol, and 0.001% thiamine hydrochloride) and Vogel-Bonner (144) Minimal E medium (200 mg/l $MgSO_4.7H_2O$, 2 g/l citric acid monohydrate, 10 g/l anhydrous K_2HPO_4 and 3.5 g.$NaNH_4PO_4$) supplemented with 0.2% (w/w) glucose (MG media) were used as poor media for cell growth. Agar plates were prepared by adding 15 g/l Bacto agar and antibiotics at following concentrations: ampicillin at 100 µg/ml, chloramphenicol at 20 µg/ml, tetracycline at 12.5 µg/ml and kanamycin at 40 µg/ml.

EBU plates used to clean up phage during P22 transduction were prepared as follows. 5 g tryptone, 2.5 g yeast extract, 2.5 g NaCl, 1.25 g glucose, 7.5 g Bacto agar and 450 ml water were added to a 1 liter flask. In a separate 250 ml flask 1.25 g K_2HPO_4 was added to 50 ml water. Both flasks were autoclaved, cooled to 55°C and mixed. 625 µl of sterile

solution of 1% Evans Blue and 1.25 ml of sterile solution of 1% Uranine were added to the mixture. Plates were poured and stored in dark at 4°C.

Polymerase, ligase, restriction enzymes, buffers, reagents and other kits were used per manufacturer's recommendation. Phusion® high-fidelity polymerase, restriction enzymes and T4 polynucleotide kinase were purchased from New England BioLabs (NEB). GoTaq green master mix and Promega LigaFast™ rapid DNA ligation system were purchased from Fisher Scientific. Ampligase thermostable ligase and Ampligase buffer were purchased from Epicentre. Dimethyl sulfoxide (DMSO) was purchased from VWR. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) was purchased from Life Technologies. Anhydrotetracycline (aTc) was purchased from Clontech. BD LB broth, BD yeast extract, BD tryptone, BD agar and agarose were purchased from Fisher Scientific. ATP, betaine and other chemicals were purchased from Sigma Aldrich. Zymo Spin I (gel recovery) and Zymo Spin II (PCR clean-up) kits were purchased from Zymo Research. GeneJET plasmid miniprep kit was purchased from Fisher Scientific.

LIGASE CYCLING REACTION

Ligase cycling reaction (LCR) method was used to clone multiple fragments of DNA into a plasmid (141). DNA fragments were PCR amplified using Phusion® high-fidelity polymerase (New England BioLabs) using genomic DNA or plasmids as templates. Bridging oligos were designed to have melting temperature of 65-70°C. The PCR products were phosphorylated in a reaction containing 55 ng/kb of each PCR fragment, 5 µl of 20 mM ATP, 2 µl of 10× Ampligase buffer and 1 µl of 10 U/µl T4 polynucleotide kinase in a total volume of 20 µl. The reaction mixture was incubated at 37°C for 1 hour and the enzymes were deactivated by incubating at 65°C for 20 minutes in a thermal cycler.

The phosphorylated PCR products were ligated in a LCR containing 16.7 µl of phosphorylation reaction mixture from the previous step, 2 µl of 100% dimethyl sulfoxide (DMSO), 2 µl of 5M Betaine, 0.5 µl each of 1.5 µM bridging oligos (final concentration of bridging oligos being 30 nM), and 1 µl Ampligase in a total volume of 25 µl. Thermal cycler for LCR was programmed as follows: i) 94°C for 2 minutes ii) 94°C for 10 seconds iii) 55°C for 30 seconds iv) 66°C for 60 seconds v) repeat steps ii-iv for 50 cycles vi)

incubate at 4°C. 2 µl of the LCR mixture was transformed into chemical competent cells (145). Colonies were screened using colony PCR.

QUANTITATIVE REAL TIME PCR (QRT-PCR)

Cells were grown for 4 hours in LB media. Total RNA was isolated from these cells using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. cDNA was generated from the RNA using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Primers were designed using the Primer3Plus software (146). PCR amplification of cDNA was done using the HotStart-IT SYBR Green qPCR Master Mix with UDG (Affymetrix) and a MiniOptican Real-Time PCR system (Bio-Rad). Standard curves were prepared by PCR amplifying known amounts of genomic DNA. All results were quantified using threshold PCR cycle-numbers. *S. enterica mreB* gene was used as a control for normalizing differences in total DNA or RNA quantities.

GENE DELETION AND REPLACEMENT

Gene deletions were performed using homologous recombination method described by Datsenko and Wanner (140). Plasmid pKD46, expressing λ-Red recombinant proteins gamma, exo and beta, was transformed into the host cell in which gene deletion was desired. Plasmid pKD46 has a temperature sensitive origin of replication and ampicillin resistance gene. Cells harboring plasmid pKD46 were grown overnight in LB medium supplemented with ampicillin at 30°C. The overnight culture was diluted to optical density (ABS_{600}) 0.05 and grown at 30°C in LB medium supplemented with ampicillin and 10mM arabinose for 3 hours. Cells were harvested by centrifugation at 3,000 ×g for 10 minutes and made electrocompetent by washing three times with ice-cold 10% glycerol. DNA fragments containing antibiotic cassette and homology region to the flanking regions of gene of interest were amplified using polymerase chain reaction (PCR) and plasmids pKD3 (chloramphenicol resistance cassette) or pKD4 (kanamycin resistance cassette) as templates. 200 ng of PCR products were transformed into 50 µl cells by electroporation. Cells were allowed to recover at 37°C for 2 hours and plated on selective media. Colonies with correct recombination were identified using colony PCR. They were streaked on LB plates and incubated at 42°C twice to cure from plasmid pKD46. The antibiotic cassette

were removed by transforming the cells with plasmid pCP20 expressing Flp recombinase from yeast (147). Plasmid pCP20 also has a temperature sensitive origin of replication and ampicillin resistance gene. Colonies with correct recombination were screened using colony PCR. They were streaked on LB plates and incubated at 42°C twice to cure from plasmid pCP20. Promoter replacement with tetRA element was done using the method described by Karlinsey (143) following this protocol.

PLASMID INTEGRATION

To minimize the artifacts associated with plasmid copy numbers, plasmids were integrated into the chromosome when possible using CRIM method (142). CRIM plasmids require pir^+ host for replication. Genes or promoters of interest were cloned into these plasmids using pir^+ E. coli strain BW23474 as a host. These plasmids can be integrated into *pir*$^-$ strains of E. coli and *S. enterica* by expressing corresponding phage Int protein. In this work plasmids were integrated into λ-attachment sites or φ_{80}-attachment site. Helper plasmids pInt and and pAH123 express λ Int and φ_{80} Int proteins respectively. The strain in which a CRIM plasmid was desired to be integrated was transformed with a corresponding helper plasmid and grown overnight in LB supplemented with ampicillin at 30°C. The overnight culture was diluted to optical density (ABS_{600}) 0.05 and grown at 30°C in LB medium supplemented with ampicillin for 3 hours. Cells were harvested by centrifugation at 3,000 ×g for 10 minutes and made electrocompetent by washing three times with ice-cold 10% glycerol. 200 ng of the CRIM plasmid was transformed into 50 µl cells by electroporation and allowed to recover in LB at 37°C for 1 hour. After an hour the cell culture was moved to 42°C for further recovery and high level expression of the Int protein. 200 µl of the cell culture was plated on selective media. Colonies with correct integration were screened using colony PCR. They were streaked on LB plates and incubated at 42°C twice to cure from helper plasmids.

P22 TRANSDUCTION

When P22 phage infects a cell, occasionally the P22 nuclease cuts at chromosomal sites which are homologous to P22 *pac* sites and packages 48Kb fragments of chromosomal DNA into P22 phage heads. The P22 particles carrying chromosomal DNA can inject this DNA into a new host. The DNA can then recombine into the chromosome by homologous recombination. P22 can transfer DNA fragments form all regions of the chromosome. Hence the process is called Generalized Transduction (148, 149).

P22 transduction was used to transfer integrated plasmids and genomic mutations between *S. enterica* strains to ensure spurious mutations were not propagated. Transductions were usually done following gene mutations and knockouts or integration of CRIM plasmids. Transductions were performed in a two-part process. First, lysate was prepared from the strain with the marker or phenotype that was desired to be transferred. Then, the lysate was used to transduce the marker or phenotype into a recipient strain without the marker or phenotype.

To avoid cross-contamination, all the work involving phage was done using glass pipettes. Donor cells were grown overnight at 37°C in LB. 40 µl of the overnight culture and 3 drops of wild-type P22 lysate were added to 2 ml fresh LB and grown for 8-16 hours in a disposable test tube. The cells debris was pelleted by centrifuging at 18,000 ×g for 5 minutes. Supernatant containing the donor lysate was carefully pipetted into a clean centrifuge tube. 3 drops of chloroform was added to the lysate and vortexed for 30 seconds. Chloroform was allowed to settle down in the tube and the lysate was used for transduction or stored at 4 °C for future use.

The donor lysate was diluted 1:10 and 1:100 in LB. 100 µl each of the overnight recipient cell culture and donor lysate were mixed and incubated at 37°C for 30-45 minutes. 100 µl of the mixture was plated on selective plates. Cells only and lysate only control were also plated on selective plates to ensure no cross contamination. The colonies were streaked on EBU plates to clean up phage. Pseudolysogens are stained by the Evans Blue dye and form blue colonies while phage-free colonies are white/light-green. The Fluorescein enhances the color distinction. Phage-free colony was picked from EBU plate and streaked on LB plate to get the final strain.

P1 TRANSDUCTION

P1 transduction was used to transfer integrated plasmids and genomic mutations between *E. coli* strains to ensure spurious mutations were not propagated (150). Transductions were usually done following gene mutations and knockouts or integration of CRIM plasmids. Transductions were performed in a two-part process. First, lysate was prepared from the strain with the marker or phenotype that was desired to be transferred. Then, the lysate was used to transduce the marker or phenotype into a recipient strain without the marker or phenotype.

To avoid cross-contamination, all the work involving phage was done using glass pipettes. Donor cells were grown overnight at 37°C in LB. 20 µl of the overnight culture and 10 µl of 1M $CaCl_2$ (P1 phage requires $CaCl_2$ for its activity) were added to 2 ml fresh LB and grown for an hour or until mildly turbid in a disposable test tube. 1 drop of stock sterile wild type lysate was added to the cell culture and allowed to grow for additional 2-4 hours. The cell culture should be clear because of cell lysis. 50 µl of chloroform was added to the lysate and vortexed for 30 seconds. Chloroform was allowed to settle down in the tube for 30 minutes and supernatant containing the donor lysate was carefully pipetted into a clean centrifuge tube. The lysate was used for transduction or stored at 4°C for future use.

500 µl of overnight recipient cell culture was gently pelleted by centrifuging at 4000 ×g and resuspended in 100 µl of P1 salts (10 mM $CaCl_2$, 5 mM $MgSO_4$). 3 drops of the donor lysate was mixed with 100 µl of the recipient cells and incubated at 30°C for half an hour. 1 ml LB and 100 µl of 1M sodium citrate was added to the mixture and incubated for 1 hour at 37°C. Sodium citrate chelates the calcium necessary for P1 activity. The cells were gently pelleted by centrifuging at 4000 ×g and washed twice with LB. The cells were resuspended in 100 µl LB and plated on selective media with 5 mM sodium citrate. Cells only and lysate only control were also plated on selective plates to ensure no cross contamination. The colonies were streaked on LB plates with 5mM sodium citrate to clean up phage. Phage-free colony was picked and streaked on LB plate to get the final strain.

MOTILITY ASSAY

Motility plates were used to quantify the swimming ability of *S .enterica* strains. Motility plates contained the media of choice (LB, TB or Vogel Bonner minimal media E with various amount of yeast extract) and 0.25% of Bacto agar. For swimming assay, 1 µl overnight culture of the strain of interest was spotted on to the center of the plate and incubated for 8-24 hours. The plates were imaged and the diameters of the ring formed by motile cells were measured to quantify the swimming ability of cells. Motility plates were always prepared fresh to keep the percentage of agar consistent.

CELL TRACKING

Cell tracking was used to quantify the percentage of motile and sessile cells. Cells were grown overnight at 37°C in MG media supplemented with 0.2% final concentration of yeast extract. Cells were subcultured to an OD_{600} of 0.05 in fresh MG media supplemented with 0%, 0.2%, 0.5%, 1%, and 2% yeast extract. After subculture, the cells were then allowed to grow at 37°C for 5 hours before harvesting. Glass slides and coverslips were soaked in 1 M KOH for 15 minutes and washed with deionized water prior to use. A 5 µl volume of appropriately diluted sample, such that there would be roughly 50 cells in the view frame, was put on glass slide, covered, and sealed with epoxy. Cells were tracked by phase contrast using a Zeiss standard RA microscope equipped with a Hyper HAD B&W video camera. The movie was then analyzed using custom Matlab software. The algorithm ignores all the cells that are stuck on the glass slides and only analyses those cells swimming (motile) or drifting in the liquid (sessile).

BULK FLUORESCENCE MEASUREMENT

Transcriptional and translational fusions to fluorescent proteins were used as indirect measure of gene and protein expression at various experimental and growth conditions. For bulk end-point fluorescence measurement, 150 µl of each sample was transferred to a 96-well microplate (black with clear bottom). Then the fluorescence (excitation 515 nm and emission 528 nm for Venus; excitation 488 nm and emission 530 nm for SGFP; excitation 435 nm and emission 475 nm for CFP; excitation 587 nm and emission 610 nm for mCherry) and optical density (ABS_{600}) of the samples were measured using Tecan Infinite

M1000 Pro microplate reader. The fluorescence readings were normalized with optical density measurement to account for variable cell density. For time course measurements, overnight culture was diluted to optical density (ABS_{600}) of 0.02 in fresh media. 200 µl of each sample was then transferred to 96-well microplate (black with clear bottom) and covered with Breathe-Easy® sealing membrane (Sigma). The microplate reader was programmed to maintain constant 37°C temperature and take fluorescent and optical density (ABS_{600}) measurements every 15 minutes. Relative fluorescence normalized with optical density was plotted as a function of time to obtain dynamic expression results.

FLOW CYTOMETRY

Flow cytometry was used to analyze single-cell behavior at various experimental and growth conditions. Throughout the experiments cell samples were collected and centrifuged at 3200 ×g for 10 minutes, resuspended in PBS containing 50 µg/ml chloramphenicol and kept in ice. In cases where DAPI staining was desired, cells were pelleted by centrifuging at 3200 g for 10 minutes and resuspended in DAPI staining buffer with 14.3 µM DAPI and 50 µg/ml chloramphenicol. The cells were then incubated at room temperature for half an hour. The cells were then analyzed using BD LSR II or BD LSR Fortessa (used for strains expressing mCherry fluorescent proteins) flow cytometer. Fluorescence values were recorded using Pacific Blue channel (excitation: 405 nm; emission: 450/50 nm) for DAPI, FITC channel (excitation: 488 nm; emission: 530/30 nm) for Venus, Alexa Fluor 430 channel (excitation: 405 nm; emission: 525/50 nm) for cyan fluorescent protein (CFP) and PE-Texas Red channel (excitation 561 nm; emission 610/20 nm) for mCherry fluorescent proteins. Cells stained with DAPI were gated using DAPI channel whereas cells not stained with DAPI were gated according to side scatter (SSC) and forward scatter (FSC) channels. Data extraction and analysis for the FACS experiments were done using FCS Express Version 4 (De Novo Software). The data was exported to Microsoft Excel (2010) and further processed in Origin Pro 9.0 to obtain the data for fluorescence and relative density distributions.

PROTEIN PURIFICATION

The pET-28(a) vector expressing 6× his-tagged gene of interest was transformed into overexpression strain BL21(DE3) expressing T7 RNA polymerase and grown overnight in LB supplemented with 40 µg/ml kanamycin. The overnight culture was diluted 1:33 in fresh LB and grown to optical density (ABS_{600}) of 0.6 before inducing with 1mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Cells were grown for an additional 4 h at 37°C and harvested by centrifugation at 5,000 × *g* for 15 min. The cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) containing 1 mg/ml lysozyme and incubated on ice for 30 minutes. The cells were then sonicated (8 cycles of 10 second pulse and 2 min interval) on ice. The cell lysate was separated from cells debris by centrifugation at 9,000 × *g* for 30 min. The lysate was then mixed with half volume of Ni-NTA agarose (QIAGEN) and gently mixed on a rotary shaker at 4°C for 1 hour. The mixture was then centrifuged at 1,000 × *g* for 5 minutes and supernatant was discarded. The resin was washed twice with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8). Finally, the protein was eluted with 2 volumes of 0.5 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8). The eluate was analyzed by SDS-PAGE.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Electrophoretic mobility shift assay was used to investigate protein-DNA interaction. DNA fragments were ^{32}P-labeled in a phosphorylation reaction mixture containing 5 pmol DNA, 2 µl T4 polynucleotide kinase 10× buffer, 1 µl T4 polynucleotide kinase and 2 µl of ATP [γ-^{32}P] in a final volume of 20 µl. The mixture was incubated at 37°C for 20 minutes. The phosphorylation reaction was then quenched by adding 2 µl of 0.5 M ethylenediaminetetraacetic acid (EDTA). The binding reaction was performed by mixing 0.5 pmol ^{32}P-labeled DNA, 0.5 µg salmon sperm DNA (Invitrogen), 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 100 µg/ml bovine serum albumin (BSA), 10% glycerol, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, and 100 ng of purified protein in a total volume of 50 µl. The mixture was incubated at room temperature for 30 minutes. The electrophoresis was carried out on a 5% native polyacrylamide gel in TBE buffer (10.8 g/l Tris, 5.5 g/l boric acid, 0.37 g/l EDTA disodium salt). The gel was vacuum-dried on a filter paper and

placed on a phosphor-screen overnight. The phosphor-screen was scanned using Storm 840 PhosphorImager.

TABLES FOR CHAPTER 2

Table 1. *Salmonella enterica* serovar Typhimurium strains used in this study

Strain	Relevant Characteristics
14028	Wild-type serovar Typhimurium
SK74	Δ*fliT*
SK75	ΔP_{flhDC}::tetRA
SK181	Δ*ydiV*
SK184	ΔP_{flhDC}::tetRA Δ*ydiV* attλ::pVenus::P_{flhB}-venus
SK186	Δ*fliZ* Δ*ydiV* attλ::pVenus::P_{flhB}-venus
SK190	Δ*fliA*
SK192	Δ*flgM*
SK258	Δ*fliZ*
SK286	attλ::pVenus::YdiV-SGFP
SK328	Δ*flgM* attλ::pVenus::P_{flhB}-venus
SK330	Δ*fliA* attλ::pVenus::P_{flhB}-venus
SK397	attλ::pVenus::P_{flhB}-venus
SK398	Δ*fliZ* attλ::pVenus::P_{flhB}-venus
SK399	Δ*ydiV* attλ::pVenus::P_{flhB}-venus
SK405	attλ::pVenus::P_{fliC}-venus
SK406	*ΔfliZ* attλ::pVenus::P_{fliC}-venus
SK407	*ΔydiV* attλ::pVenus::P_{fliC}-venus
SK419	*ΔflgM* attλ::pVenus::P_{fliC}-venus

Table 1 (cont.)

SK435	ΔP*flhDC*::tetRA attλ::pVenus::P*flhB*-venus
SK437	ΔP*flhDC*::tetRA attλ::pVenus::P*fliC*-venus
SK447	*ΔydiV ΔflgM* attλ::pVenus::P*flhB*-venus
SK448	*ΔydiV ΔflgM* attλ::pVenus::P*fliC*-venus
SK483	*ΔfliZ ΔflgM* attλ::pVenus::P*flhB*-venus
SK485	*ΔfliZ ΔflgM* attλ::pVenus::P*fliC*-venus
SK510	attλ::pVenus::P*fliC*-venus araB::[cm P*flhB*-mcherry]
SK520	attλ::pVenus::P*flhDC*-venus
SK521	ΔP*flhDC*::tetRA Δ*fliZ* attλ::pVenus::P*flhB*-venus
SK522	ΔP*fliA*:: tetRA attλ::pVenus::P*flhB*-venus
SK523	ΔP*fliA*:: P*flhB* attλ::pVenus::P*flhB*-venus
SK524	*ΔfliZ* attλ::pVenus::P*fliC*-venus *araB*::[*cm* P*flhB*-mcherry]
SK525	ΔP*flhDC*::tetRA *ΔfliZ* attλ::pVenus::P*flhB*-venus
SK526	ΔP*flhDC*::tetRA *ΔfliZ* attλ::pVenus::P*fliC*-venus

Table 2. *Escherichia coli* strains used in this study

Strain	Relevant Characteristics
MG1655	λ *rph-1* (wild-type)
DH5αZ1	*E. coli* cloning strain
BW23474	*E. coli* pir-116 cloning strain
SK76	Δ*xylR*::FRT
SK517	Δ*xylAB*::FRT
SK459	attλ::[*kan* P_{araB}-*Venus oriR6K*]
SK463	attφ80::[*cm* P_{xylA}-mCherry *oriR6K*]
SK504	attλ::[*kan* P_{araB}-*Venus oriR6K*] attφ80::[*cm* P_{xylA}-mCherry *oriR6K*]
SK518	Δ*xylR*::FRT attλ::[*kan* P_{araB}-*Venus oriR6K*]
SK519	Δ*xylAB*::FRT attλ::[*kan* P_{araB}-*Venus oriR6K*]
SK527	attλ::[*kan* P_{araC}-*Venus oriR6K*]
SK528	attλ::[*kan* P_{araE}-*Venus oriR6K*]
SK529	attλ::[*kan* P_{araF}-*Venus oriR6K*]

Table 3. Plasmids used in this study

Plasmid	Relevant Characteristics	Source
pKD46	*bla* P_{araBAD} *gam bet exo* pSC101 *ori (ts)*	(140)
pKD3	*bla rgnB* FRT *cat* FRT *oriR6K*	(140)
pKD4	*bla rgnB* FRT *aph* FRT *oriR6K*	(140)
pCP20	*bla cat cI857* $\lambda P_{R'}$*-flp* pSC101 *ori (ts)*	
pBAD30	*bla araC* P_{araBAD} *ori*M13 *p15A ori*	(151)
pBAD30-fliA	pBAD30*::fliA* (*S. enterica*)	
P_{ydiV}-CFP	*cm* (*S. enterica*)P_{ydiV}*-cfp* pSC101* *ori*	
pVenus	*kan att*λ venus *oriR6K*	(152)
pLC153	*cm att*φ80 *oriR6K*	(153)
pInt-ts	*bla Int oriR6K* (helper plasmid for *att*λ integration)	(142)
pAH123	*bla Int oriR6K* (helper plasmid for *att*φ80 integration)	(142)
pKW669	*cm* $P_{LtetO-1}$*-mCherry colE1*	(153)
pmCherry	*cm att*φ80 *mCherry oriR6K*	
pSK376	*cm att*φ80 (*E. coli*)P_{xylA}*-mCherry oriR6K*	
pSK459	*kan att*λ (*E. coli*)P_{araB}*-Venus oriR6K*	
pET-28(a)	*kan lacI* P_{T7}-(*his*)$_6$ *t7 pBR322*	Novagen
pSK451	*kan lacI* P_{T7}-(*his*)$_6$*-xylR* (*E. coli*) *t7 pBR322*	

Table 4. Oligonucleotide primers used in this study

Primer	Sequence	Description
SK006F	taaactgccaggaattgggg	pPROBE Check Forward
SK006R	atgttgcatcaccttcaccc	pPROBE Check Reverse
SK016F	gagcacatcagcaggacgca	proTetE Check Forward
SK016R	tctagattaattaattaagc	proTetE Check Reverse
SK021F	ttgtcggtgaacgctctcct	pVenus λ_{att} cloning Check Forward
SK021R	atgttgcatcaccttcaccc	pVenus λ_{att} cloning Check Reverse
SK027F	taatgacctcagaactccatc	CRIM Integration Check forward
SK027R	acttaacggctgacatgg	CRIM Integration Check reverse
SK028F	tttaagttgctgatttatat	CRIM Integration Check forward
SK028R	ccgtgttccggctgtcagcg	CRIM Integration Check reverse
SK031F	ggcataatagcaatgtactggcgt	λatt check S. typhimurium forward
SK031R	gcgtctctggcacgatatcgcaaa	λatt check S. typhimurium reverse
SK037F	taaaagcttaattagctgag	pVenus Forward
SK037R	cgagctcggtacccggggat	pVenus Reverse
SK057F	cggataacaattgacattgtgagc	pZE12 Check Forward
SK057R	gtattaccgcctttgagtgagc	pZE12 Check Reverse
SK063F	atctgttgtttgtcggtgaacg	pAH68/153 MCS Check Forward
SK063R	gctgtgctttcagtggatttcg	pAH68/153 MCS Check Reverse
SK072F	actggatggcgaatagcgccctaaccatgg gactggcgtagtgtaggctggagctgcttc	ydiV knockout forward
SK072R	agacggttaatcaccggttaaacaccggcaaa cagaaaggcatatgaatatcctccttag	ydiV knockout reverse
SK073F	gaatattggtttataatcag	ydiV knockout check forward
SK073R	gggtaaaagcgcggtatacg	ydiV knokout check reverse
SK074F	ggg ggtacc aatttaacctcgcagacg	ydiV Promoter Forward KpnI
SK074R	ggg gaattc gaagcaatcattacgccagtc	ydiV Promoter Reverse EcoRI
SK107F	ggg ggtacc catttatgtcaggcaggaattg	nlpC promoter Forward KpnI
SK107R	ggg gaattc ctgcaactgatcgttcagacc	nlpC promoter Reverse EcoRI

Table 4 (cont.)

SK108F	gggggtacctacggtttgcgctttcgacg	nlpC promoter Forward KpnI
SK108R	ggggaattcaaaacgcatgccgcaacaatc	nlpC promoter Reverse EcoRI
SK109F	gggggtaccacgatataaattttatagtc	ydiV promoter Forward KpnI
SK109R	ggggaattcgaagcaatcattacgccag	ydiV promoter Reverse EcoRI
SK110F	atgtttaatatccgcaatacacaaccttct	14028 sspH1 gene Forward
SK110R	tcagttaagacgccaccgggctgtcagata	14028 sspH1 gene Reverse
SK113F	ggtcagaatgcagctctgtgaacacgatat	LT2 stm3256 gene Forward
SK113R	ctaattaaacaatcgagatagccaactgcg	LT2 stm3256 gene Reverse
SK133F	acttaacggctgacatgg	pAH125 integration check Forward
SK133R	acgagtatcgagatggca	pAH125 integration check Reverse
SK134F	ttgtcggtgaacgctctcct	pAH125 cloning check Forward
SK134R	aagttgggtaacgccagg	pAH125 cloning check Reverse
SK135FI	ctaactaaagattaactttataaggaggaaaa acatatgcgtaaaggcgaagagctgttc	Super folder Forward I
SK135FII	gctcgaattccctaactaactaaagattaact ttataaggaggaaaaacatatgcgtaaa	Super folder Forward II EcoRI
SK135RI	cgatctcgagtaattaagctcatcatttgta cagttcatccataccatgcgtg	Super folder Reverse XhoI
SK135RII	cgataagctttaattaagctcatcatttgtac agttcatccataccatgcgtg	Super folder Reverse HindIII
SK151Fi	cccgtcgactttgttcaatcggataatcc	flhD promoter Forward SalI
SK151Fii	ggggtcgacgacggcgcaggcggcggaac	flhB promoter Forward SalI
SK152Fi	atcgtcgacagtggtgctggacgccacgg	fliC promoter Forward SalI
SK159F	accgaattcaactaaagattaactttataag	cypet/ecfp Forward
SK159R	ggggctagcctttgagtgagctgatacc	cypet/ecfp Reverse
SK160Ri	cgtttacgtcgccgtccagc	pECFP λ_{att} cloning Check Reverse
SK160Rii	ttaacatcaccctctaattc	pCypet λ_{att} cloning Check Reverse

Table 4 (cont.)

SK162F	gtgaaagttggaacctcttacg	proTetE tetR Check Forward
SK162R	tcactttacttttatctaatctagac	proTetE tetR Check Reverse
SK164F	ttgtcggtgaacgctctcct	pAH143/153 Check Forward
SK164R	aggatgcgtcatcgccatta	pAH143/153 Check Reverse
SK165F	taatcatgccgataactcatttaacgcagg gctgtttatcgtgtaggctggagctgcttc	fliA knockout forward
SK165R	atacgttgtgcggcacttttcgggtgcgat catgcgcgaccatatgaatatcctccttag	fliA knockout reverse
SK166F	tcttttatagccttattccttcgatag	fliA knockout check forward
SK166R	tcatgagaactcctggtagtc	fliA knockout check reverse
SK179F	gggctcgag gccgatgaacagtctcgatg	ydiV Forward with native promoter
SK179R	gggaagcttttattatcgctgaacgagtttaatg	ydiV Gene Reverse
SK180F	ggggaattcaaggaggaaaaacatatgattgc ttcacttgatgagc	ydiV Gene Forward
SK180R	gggaagcttttattatcgctgaacgagtttaatg	ydiV Gene Reverse
SK181F	aaccaactgctgtcgatgagttaatacaggaca ttttatggtgtaggctggagctgcttc	flgG-J gene knockout forward
SK181R	cgcatcgctgctgtccggcgttttcggtatggc tttgcgccatatgaatatcctccttag	flgG-J gene knockout reverse
SK182F	gacccgtcaattcgcattatg	flgG-J gene knockout check forward
SK182R	caaatacgttatagctgggttc	flgG-J gene knockout check reverse
SK192F	gccgataacaacgagtattgaaggattaaa aggaaccatcgtgtaggctggagctgcttc	flgKL gene knockout forward
SK192R	aaaacatatccagtttcgtgatatgtttcaaa aagaggtgcatatgaatatcctccttag	flgKL gene knockout reverse
SK193F	gtaaattgaccagcatgattc	flgKL gene knockout check forward
SK193R	gacaatatgaccactaactc	flgKL gene knockout check reverse

Table 4 (cont)

SK222F	agactcgaggccgatgaacagtctcgatg	nlpC promoter Forward XhoI
SK222Ri	ctcttcgcctttacgcataccagaaccacc tcgctgaacgagtttaatg	ydiV Gene Reverse w/o stop codon, GGSG, 20bp of SGFP
SK222Rii	gaaaagttcttctcctttactcataccagaac cacctcgctgaacgagtttaatg	ydiV Gene Reverse w/o stop codon, GGSG, 24bp of Venus
SK223Fi	cattaaactcgttcagcgaggtggttctggta tgcgtaaaggcgaagag	SGFP Forward, GGSG, 20bp of ydiV
SK223Fii	cattaaactcgttcagcgaggtggttctggta tgagtaaaggagaagaacttttc	Venus Forward, GGSG, 20bp of ydiV
SK223R	caaa gctagc ttggattctc	SGFP/Venus Reverse NheI
SK227Fi	cattaaactcgttcagcgaggcgccggcgc cggcgccatgcgtaaaggcgaagag	SGFP Forward, GAGAGA, 20bp of ydiV
SK227Fii	cattaaactcgttcagcgaggcgccggcgc cggcgccatgagtaaaggagaagaacttttc	Venus Forward, GAGAGA, 20bp of ydiV
SK227R	caaa gctagc ttggattctc	SGFP/Venus Reverse NheI
SK243F	aaacaaaaaagaatttggtgttgacgtaccc ctattcagcagagtagggaactgcca	PflhD::tetRA forward
SK243R	gtgcgacgtagccgcaccccgtgatgtcgc cgggaaggcc ctaagcacttgtctcctg	PflhD::tetRA knockout reverse
SK244F	gctgtgacgagattaattaataacg	PflhD::tetRA check forward
SK244R	atgctttgtcctggacgatc	PflhD::tetRA check reverse
SK245F	taatcatgccgataactcatttaacgcagggct gtttatcgtgtaggctggagctgcttc	fliA gene knockout forward
SK245R	cgttgtgcggcacttttcgggtgcgatcatgcg cgaccta catatgaatatcctccttag	fliA gene knockout reverse
SK246F	tgctcttttagccgctaaaaag	fliA gene knockout check forward
SK246R	aagtctttaagatagcggctc	fliA gene knockout check reverse
SK247F	ggggaattcacaaaaataaagt tggttattctggatg	flhDC Gene with native RBS Forward

Table 4 (cont.)

SK247R	ggggtcgacttattaaacagcctgttcgatctg	flhDC Gene Reverse
SK248F	ggggaattcaaggaggaaaaacatatgatg ctgaacagaaaaga	flhDC Gene with canonical RBS Forward
SK248R	ggggtcgactta ttaaacagcctgttcgatctg	flhDC Gene Reverse
SK249F	ggggtcgacaactcgctccttgattgcaag	flhDpromoter Forward SalI
SK249R	ggggaattccggaacatcgcagatgctttg	flhD promoter Reverse EcoRI
SK258F	aga ctcgag gccgatgaacagtctcgatg	ydiV promoter Forward XhoI
SK258Ri	tcgcccttgctcaccataccagaaccacctc gctgaacgagtttaatg	ydiV Gene Reverse w/o stop codon, GGSG, 20bp of CFP
SK258Rii	tcgcccttgctcaccatggtgctggtgctggtg cttcgctgaacgagtttaatg	ydiV Gene Reverse w/o stop codon, GAGAGA, 20bp of CFP
SK260F	atggtgagcaagggcga	CFP Forward
SK260R	gggaagctttttacttgtacagctcgtccatg	CFP Reverse HindIII
SK353F	gaccgaattctaaaaggaggagaaaatg	mCherry Forward with RBS from pProtet.E-mCherryfix EcoRI
SK353R	atggctagcctttgagtgagctgataccgctc	mCherry Reverse with terminator from pProtet.E-mCherryfix NheI
SK354F	ggggtcgacgattacgatttttggtttatttctt gatttatgaccg	xylA Promoter Forward SalI
SK354R	cctccttataaagttaatctttagttgaattcgg tcataatcaggtaatgccgcgggtg	xylA Promoter Reverse and CFP Forward EcoRI
SK380F	agagaggtcgacacttttcatactcccaccattc	araB Promoter MG1655 Forward SalI
SK380R	agagaggaattccatccaaaaaaacgggtat ggag	araB Promoter MG1655 Reverse EcoRI
SK388F	agtgaaataacccttcttttatagcc	fliA knockout check forward -100bp of start
SK388R	tctttaagatagcggctcaaag	fliA knockout check reverse +100 bp of stop

Table 4 (cont.)

SK394F	cgcagaccagaagacagacg	hin knockout check forward -200bp
SK394R	ctggtagtgttttgagcgatg	hin knockout check reverse +200bp
SK399F	attagcatttttgtccataag	integrate pProtetE in ara operon check forward
SK399R	atggcagtttggcttcggtattc	integrate pProtetE in ara operon check reverse
SK400F	actgtttctccatacctgtttttctggatggag taagacggacggcgcaggcggcggaac	integrate PflhB-mCherry in ara operon forward
SK400R	ccatacttcataattatcaaaaatcgtcattgt cgtgtcctctagggcggcggatttgtc	integratePflhB-mCherry in ara operon reverse
SK413F	cagcgctagctttactaaacgtcaccgcatc	xylR gene forward NheI
SK413R	gctcctcgagttattactacaacatgacct cgctatttac	xylR gene reverse XhoI
SK414F	gctagttattgctcagcggtgg	pET28a Check, Forward
SK414R	taatacgactcactataggggaattgtgag	pET28a Check, Reverse
SK417F	agctgagtcgaccgtcaggaggagagggg	CRP reporter Forward SalI
SK417R	agctgagaattcagctgtttgcagtgtgaaattc	CRP reporter Reverse EcoRI
SK432F	agctgaggtacccgtgaattcactgtataccgc	fliA gene Forward KpnI
SK432R	agctgaaagcttttattactataact tacccagtttggtgc	fliA gene ReverseHindIII
SK446F	aacgaagagatggcaaacac	flhD gene RT-PCR forward (+124)
SK446R	cgttgaaagcatgatacctg	flhD gene RT-PCR reverse (+303)
SK447F	aaaagcattgttcaggaag	flhC gene RT-PCR forward (+10)
SK447R	tgaatattttgctcccagg	flhC gene RT-PCR reverse (+215)
SK450F	caaggcatcgtattgaatg	mreB gene RT-PCR forward (+85)
SK450R	tttcggtcacaaagaagtc	mreB gene RT-PCR reverse (+265)

CHAPTER 3

A NUTRIENT-TUNABLE BISTABLE SWITCH CONTROLS CLASS 2 FLAGELLAR GENE EXPRESSION[1]

INTRODUCTION

Motile bacteria move from less favorable environments to more favorable ones. This process has been studied extensively in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, two closely related bacterial species. These bacteria move by rotating left-handed helical flagellar filaments (139). Their motility systems, including chemotaxis pathways that govern them, are nearly identical. They principally differ in how the associated genes are expressed in response to different cellular and environmental cues (154). Motility is not constitutive in these bacteria but rather is induced in response to specific signals. How these bacteria respond to these signals presumably reflects differences in how they employ motility.

Nutrients provide one example. In *E. coli*, nutrients inhibit the expression of the motility genes (155). The mechanism is governed in part by the cyclic AMP (cAMP) receptor protein (CRP) involved in carbon catabolite repression (156). The cAMP-CRP complex positively regulates the transcription of the *flhDC* operon, which contains the genes encoding the master flagellar regulator, $FlhD_4C_2$ (157). When glucose concentrations are high, *flhDC* expression is repressed, as cAMP levels are low. Conversely, when glucose concentrations are low, *flhDC* expression is enhanced, as cAMP levels are high. In *S. enterica*, nutrients enhance the expression of the motility genes (104). The mechanism involves the protein YdiV, which binds $FlhD_4C_2$ and prevents it from activating its target promoters. In addition, YdiV promotes the degradation of $FlhD_4C_2$ through the protease ClpXP (158). Nutrients repress the expression of YdiV, in part, through the action of the mRNA-binding protein CsrA, which is involved in regulating central carbon metabolism (159). When the nutrients level is high, YdiV expression is repressed, leading to enhanced expression of the motility genes. Conversely, when the nutrients level is low, YdiV

expression is enhanced and expression of the motility genes is repressed. The cAMP-CRP complex also regulates the transcription of the *flhDC* operon in *S. enterica* (88), although YdiV apparently masks the effect, at least under the conditions where these experiments were performed.

YdiV participates in a double-negative feedback loop involving the flagellar regulator FliZ. FliZ directly represses *ydiV* transcription, and YdiV indirectly represses *fliZ* transcription through $FlhD_4C_2$ (**Figure 7**) (160). FliZ and YdiV have also been shown to influence the population dynamics of flagellar gene expression. In particular, multiple studies have observed co-existing populations of motile and non-motile cells (101, 161-163); however, these co-existing populations are not observed in Δ*fliZ* (101) or Δ*ydiV* (162) mutants. Based on these results, FliZ and YdiV have been hypothesized to function in a genetic on-off switch, causing some cells to be motile and others not (164).

Although this mechanism is appealing, it has yet to be proven. Moreover, we previously found that these co-existing populations are transient, so that the entire population eventually becomes motile (101). However, these experiments were performed in rich media. One aspect that has yet to be explored is the role that nutrients play in shaping this dynamic response.

In this study, we investigated how nutrients tune flagellar gene expression dynamics in *S. enterica*. Our results demonstrate that nutrients tune the fraction of motile cells. While co-existing populations are observed at all nutrient concentrations, they persist only at intermediate nutrient concentrations. We further investigated the mechanism that governs this tunable response. We found that FliZ and YdiV are necessary for the response. In addition, we found that the positive feedback loop involving FliA is required, although its role appears solely to enhance FliZ expression. Together, these results reveal a new facet of motility and flagellar gene regulation in *S. enterica*.

RESULTS

Nutrients tune the fraction of motile cells. Previous experiments investigating the dynamics of flagellar gene expression in *S. enterica* were performed in rich Luria-Bertani (LB) medium. Based on the recent discovery that nutrients tune YdiV expression (104), we hypothesized that nutrients may also tune the fraction of motile cells. To test this hypothesis, we grew cells in Vogel-Bonner medium E (144) supplemented with 0.2% (w/w) glucose and various concentrations of yeast extract. The cells were harvested during late exponential phase, and their swimming behavior was analyzed by video microscopy. Consistent with our hypothesis, we found that nutrients, specifically yeast extract, tune the fraction of motile cells (**Figure 8A**). We also performed growth experiments. Except in its complete absence, we found that the concentration of yeast extract does not strongly affect the growth rate (**Figure 9**). These results show that the response to yeast extract at the concentrations tested is not determined by the growth rate but is instead regulated by nutrient availability.

Nutrients tune the fraction of cells expressing flagellar genes. We next investigated whether regulation occurs at the level of gene expression. Flagellar genes can be divided into a transcriptional hierarchy comprising three classes (**Figure 7**). We used flow cytometry to measure the expression from a representative promoter from each hierarchical class, using single-copy, chromosomally-integrated transcriptional fusions to the fluorescent protein Venus (165). The class 1 P_{flhDC} promoter was active in all cells irrespective of yeast extract concentrations (**Figure 8B**). However, we found that the class 2 P_{flhB} and class 3 P_{fliC} promoters were active in only a subpopulation of cells at intermediate yeast extract concentrations, giving rise to a bimodal distribution (**Figure 8C-D**). The fraction active increased with yeast extract concentrations in a manner consistent with the video microscopy experiments (**Figure 8E-F**). We note that nutrients increased the relative expression of the P_{flhB} and P_{fliC} promoters in those cells where the promoters were active, though the effect is minor. As the P_{flhB} and P_{fliC} promoters are nearly identical in their response to yeast extract, our remaining investigations focus on the class 2 P_{flhB} promoter.

In dynamic gene expression experiments, co-existing populations of cells with active and inactive promoters were transiently observed at all yeast extract concentrations (**Figure**

10). However, the population with inactive promoters persisted only at low yeast extract concentrations. At the higher concentrations, the promoters in all cells eventually become active. These results are consistent with our previous findings, in which we observed transient heterogeneity in nutrient-rich media (101). At the lower concentration of yeast extract, the co-existing populations persist for many hours with no significant change in their distribution. These results suggest that the observed response is bistable: namely, genetically identical cells can exhibit different phenotypes that persist under identical growth conditions.

Multiple nutrients activate flagellar gene expression. Yeast extract is a complex mixture containing many nutrients. We tested a number of different compounds for their ability to activate the P_{flhB} promoter (**Table 5**). Among the compounds tested, we found that amino acids were able to activate the P_{flhB} promoter, although not to the same degree that yeast extract was able to. Analysis of individual amino acids suggested that most were able to activate the P_{flhB} promoter weakly, whereas a combination of all twenty was able to activate the P_{flhB} promoter to roughly half the level of yeast extract. These results indicate that the activating signal is not a simple compound but rather a mixture of them, of which amino acids are a subset. We also note that previous studies have shown that that the RNA-binding protein CsrA, which is involved in carbon storage regulates YdiV translation, though YdiV is still subject to nutritional regulation in a Δ*csrA* mutant (104). CsrA is regulated by CsrB and CsrC, two non-coding RNAs that are transcriptionally regulated by the BarA/SirA two-component signal transduction system (166, 167). One study found that formate and acetate regulate CsrB transcription through BarA and SirA, respectively (168). We also tested the ability of formate and acetate to activate the P_{flhB} promoter and found that neither was able to do so (**Table 5**). As we were unable to isolate a single activating compound, we employed yeast extract in the remainder of our studies.

YdiV and FliZ are necessary for bimodal flagellar gene expression. Both YdiV and FliZ have previously been shown to affect single-cell gene expression dynamics (101, 162). To test how these two proteins contribute to the nutritional response to yeast extract, we measured flagellar gene expression in Δ*ydiV* and Δ*fliZ* mutants. In a Δ*ydiV* mutant, the P_{flhB} promoter was strongly active in most cells, irrespective of yeast exact concentration (**Figure 11A**; see also **Figure 12A**). In fact, yeast extract had no substantive effect on P_{flhB}

promoter activity. We note that there is a tail in the distribution, indicating that the P_{flhB} promoter is weakly active in a small population of cells. This population was observed at all yeast extract concentrations and was also present in the wild type, even at the highest concentration of yeast extract employed (**Figure 8C-D**). In contrast, the Δ*fliZ* mutant exhibited a homogeneous nutrient response consisting of a single population (**Figure 11B**; see also **Figure 12B**). We also found that *ydiV* was dominant, so that a Δ*ydiV* Δ*fliZ* double mutant was indistinguishable from the Δ*ydiV* single mutant (**Figure 12C**).

YdiV and FliZ are known to repress each other, with FliZ directly repressing *ydiV* transcription and YdiV indirectly repressing *fliZ* transcription via $FlhD_4C_2$ (**Figure 7**) (160). To observe this competitive interaction at single-cell resolution, we employed two-color flow cytometry to measure simultaneous expression from the P_{ydiV} and P_{flhB} promoters. Expression from the P_{ydiV} promoter was measured using a transcriptional fusion to the cyan fluorescent protein (CFP). Note, the P_{ydiV}-*cfp* fusion was active only when expressed from a low-copy (pSC101* origin of replication) plasmid: single-copy transcriptional fusions, like those employed for the P_{flhB} and P_{fliC} promoters, were not sufficiently active to measure a response for the P_{ydiV} promoter.

Figure 11C shows a comparison of P_{flhB} and P_{ydiV} promoter activities as a function of yeast extract concentrations. In the absence of yeast extract (0%), a single population was observed in which the P_{ydiV} promoter is active and P_{flhB} promoter is inactive. At intermediate concentrations of yeast extract (0.2-1%), two populations were observed: one in which the P_{ydiV} promoter was active and the P_{flhB} promoter was inactive and the other in which the reciprocal pattern occurred. As yeast extract concentrations were increased, the relative number of cells occupying the population where P_{flhB} promoter was active increased. At high yeast extract concentration (>1%), only the P_{flhB} active population was observed, although transcription from the P_{ydiV} promoter was still detectable. These results suggest that the heterogeneous nutrient response arises from the mutual repression of YdiV and FliZ.

We note that a previous study did not report any significant changes in *ydiV* transcription in response to nutrients. Instead, only changes in the level of YdiV protein were observed, suggesting that nutrients principally regulate YdiV via a post-transcriptional mechanism (104). We, on the other hand, found that yeast extract decreased

expression from the P_{ydiV} promoter, indicating that the mechanism involves a significant transcriptional component. One possible explanation for this discrepancy is that the decrease in *ydiV* transcription is due to FliZ, which is more strongly expressed at high yeast extract concentrations and which is known to bind the P_{ydiV} promoter. We found, however, that yeast extract also decreased expression from the P_{ydiV} promoter in a Δ*fliZ* mutant (**Figure 13A**). In both the wild type and the Δ*fliZ* mutant, we observed more than a two-fold decrease in promoter activity at high concentrations of yeast extract. When YdiV expression was measured using a translational fusion to SGFP, we observed similar decreases in its expression (**Figure 13B**), indicating that YdiV is regulated at the transcriptional level.

Flagellar gene expression is bistable and exhibits hysteresis. Our data suggest that flagellar gene expression is bistable. As bistable systems exhibit hysteresis, which reflects history dependence (37), we wished to determine how cells transition between different states of flagellar gene expression. To test whether flagellar gene expression exhibits hysteresis, we first replaced the native P_{flhDC} promoter with a tetracycline inducible one, as described previously (100). We then grew cells either in the presence or absence of 10 ng/mL anhydrotetracycline (aTc) – hereafter referred to as the on or off state, respectively – prior to subculturing into fresh media containing different aTc concentrations. We fixed the yeast extract concentration at 0.2%, as bistability is most pronounced at this concentration. If flagellar gene expression is bistable, then the response should be different.

Consistent with a bistable response, we observed that cells exhibited different patterns of P_{flhB} promoter activity depending on whether they were initially in the on or off state (**Figure 14A**). In general, P_{flhB} promoter activity was lower when cells were initially in the off state relative to those cells initially in the on state. Bimodality was not observed when cells transition from an on to off state (**Figure 14B-C**). We performed similar experiments using the Δ*ydiV* and Δ*fliZ* mutants. For both mutants, no hysteresis was observed: the response was the same irrespective of whether they were previously induced with aTc or not (**Figure 14A**; see also **Figure 16**). These results demonstrate that YdiV and FliZ are essential for the hysteresis response.

Similar experiments were performed at different concentrations of yeast extract, and hysteresis was again observed (**Figure 15**). However, we cannot directly compare these

experiments with one another as the response to yeast extract and aTc are not orthogonal: as yeast extract concentrations increase, expression from the tetracycline-inducible P_{tetA} promoter (169) decreases, for unknown reasons (**Figure 15D**). Despite this crosstalk, the conclusions that flagellar gene expression is bistable and exhibits hysteresis do not change.

The FliA positive feedback loop is necessary for bistability. The flagellar network possesses two feedback loops in addition to the ones involving FliZ and YdiV. One is a negative feedback loop involving FliT. Expressed from a hybrid class 2/3 promoter (170), FliT binds to $FlhD_4C_2$ and prevents it from activating its cognate class 2 promoters (171, 172). The second is a positive feedback loop involving FliA and FlgM. The *fliAZ* operon is under the control of both class 2 and class 3 promoters (89). The class 2 promoter functions in a double-negative feedback loop involving FliZ and YdiV; the class 3 promoter functions in an autogeneous loop involving the alternate sigma factor FliA. Note that the latter loop is not directly autocatalytic, as FliA is inefficiently translated from the class 3 transcript (173). Rather, positive feedback is indirect, such that FliA activates FliZ expression and FliZ indirectly activates FliA expression by repressing the expression of YdiV.

An additional facet of the regulation involves FlgM, which regulates FliA activity by binding to it and preventing it from activating its cognate class 3 promoters (174). FliA and FlgM function in a developmental checkpoint involving protein secretion (175). In addition, they are believed to function in a regulatory circuit involved in controlling the number of flagella produced per cell (94, 97, 98).

To determine whether these regulatory loops contribute to bistability, we examined the activity of the P_{flhB} promoter in Δ*fliT*, Δ*fliA*, and Δ*flgM* mutants. In the case of the Δ*fliT* and Δ*flgM* mutants, the response to yeast extract was similar to that of wild-type cells (**Figure 17A-B**), demonstrating that neither gene product is required for bistability. The Δ*fliA* mutant, on the other hand, exhibited a homogenous response to yeast extract (**Figure 17C**) in a manner equivalent to a Δ*fliZ* mutant. We also tested a P_{fliA}::P_{flhB} promoter mutant, where the hybrid class 2/3 P_{fliA} promoter was replaced with a pure class 2 promoter (98), and found that it also exhibited a homogeneous response to yeast extract (**Figure 17D**). These results demonstrate that both FliA and the class 3 component of the P_{fliA} promoter are essential for bistability.

DISCUSSION

The findings presented here show that the fraction of motile cells in a population of *S. enterica* is determined, at least in part, by nutrient availability. YdiV and FliZ control two feedback loops with opposing activities that govern this response, as previously proposed (164). Although these feedback loops are necessary for bistability, they are not sufficient; a positive feedback loop involving FliA is also required. This third loop is not strictly autoregulatory in the sense that FliA does not directly enhance its own expression.

FliZ is expressed from both class 2 and class 3 promoters. The features of nutrient regulation raise the question of why *fliZ* is transcribed from a hybrid class 2/3 promoter when expression from a strong class 2 promoter would suffice to generate bistability. One potential explanation is that the FliA feedback loop couples the switch to the completion of assembly of the hook-basal body (HBB) complex. A developmental checkpoint involving FliA and FlgM couples class 3 flagellar gene expression to flagellar assembly (175). Prior to completion of the hook-basal body, FlgM binds to FliA and prevents it from activating class 3 promoters. Upon completion of the HBB complex, FlgM is secreted from the cell by the flagellar export apparatus, freeing FliA to transcribe from class 3 promoters. Mutations that inhibit formation of the hook-basal body are deficient in FlgM secretion and thus prevent transcription from class 3 promoters. This checkpoint likely ensures that cells do not switch to the on state for class 3 transcription until they are able to make functional hook-basal body complexes. In support of this prediction, we discovered that gene expression is homogenous in hook-basal body mutants (101) and in a Δ*fliA* mutant. Moreover, *fliA and fliZ* reside in the same operon in most bacteria that possess *fliZ* (176). Thus, coupling of *fliZ* and *fliA* may be necessary for the full function of FliZ to be realized.

In our work, we used yeast extract as the nutrient to tune flagellar gene expression. How yeast extract regulates the P_{ydiV} promoter is not known. Our work demonstrates that amino acids are able to activate the P_{ydiV} promoter, but they are unlikely to be the only compounds that do so. One additional possibility is that the P_{ydiV} promoter responds to the growth rate of the cell. Although our experiments provide no support for this possibility, a caveat is that we used relatively high concentrations of nutrients that supported approximately equal growth rates. If flagellar gene regulation also responds to the growth

rate of cell, as one might expect, then our experiments would not have detected this phenomenon, as our conditions were chosen to keep the growth rate nearly constant.

The present study raises the questions as to why nutrients control bistable flagellar gene expression in *S. enterica.* One possible explanation derives from the fact that motility is intimately coupled to virulence and host colonization in *S. enterica*. Numerous studies have shown that flagellar gene expression is coupled to the expression of the invasion genes associated with the type III secretion system encoded within *Salmonella* pathogenicity island 1 (SPI-1) (177-182). Of note, FliZ positively regulates the expression of the SPI-1 invasion genes. In addition, flagellin activates the innate immune response (183). The nutrient response likely ensures that flagellin is expressed at specific sites within the host, as previously shown (161). Bistability, as argued by Steward and Cookson (164), may enable a division of labor and a degree of bet hedging, where motile cells are invasive and non-motile cells non-invasive. Non-motile cells could thus avoid the inflammatory environment of the intestinal epithelium and serve as a reservoir for the next phases of colonization. Our results extend this model by showing that nutrients control these respective fractions of motile and non-motile cells.

Nutrients repress motility in *E. coli,* a form of regulation that is consistent with *E. coli* employing motility as a foraging mechanism. Only when starved are these bacteria motile. However, recent results suggest that motility in *E. coli* is also employed for host colonization. For example, the bacterial quorum sensing signal AI-2 and interkingdom signaling molecule norepinephrine control motility in *E. coli* (184-187). These results indicate that both *S. enterica* and *E. coli* employ motility for multiple purposes.

We note that the *ydiV* gene is also present in *E. coli* (188). Although, the gene is transcriptionally active, it is poorly translated (173). However, the *E. coli ydiV* gene is efficiently translated in *S. enterica*, suggesting that some factor represses its translation in its native host. These results suggest that the flagellar gene networks in *E. coli* and *S. enterica* are quite plastic, in the sense that small changes in the expression of individual genes can result in significantly different responses to nutrients and perhaps to other environmental cues. Such plasticity in the regulatory pattern of the flagellar gene network may reflect the disparate roles motility plays in these two closely related organisms and enable them to adapt readily to new environments in which these roles differ.

TABLES FOR CHAPTER 3

Table 5. Relative P_{flhB} promoter activity with different supplements to MG media

Supplement	Conc(mM)	Fluorescence (A.U.)
2% Yeast Extract		100
All Amino Acid‡		53
No amino acid		13
Glycine	10	22
Alanine	10	15
Serine	10	22
Threonine	10	29
Cysteine	0.8	28
Valine	10	13
Leucine	10	18
Isoleucine	10	22
Methionine	10	28
Proline	10	30
Phenylalanine	10	16
Tyrosine	2	28
Tryptophan	10	20
Aspartic acid	10	17
Glutamic Acid	10	18
Asparagine	10	17
Glutamine	10	24
Histidine	10	20
Lysine	10	20
Arginine	10	16
Succinate	25	24
Citrate	25	24
Lactate	10	16

Table 5 (cont.)

Formate	10	12
Acetate	10	10
Propionic Acid	10	22
Butyric Acid	10	25
Indole	1	20

‡All amino acids with the concentrations listed below in this table

FIGURES FOR CHAPTER 3

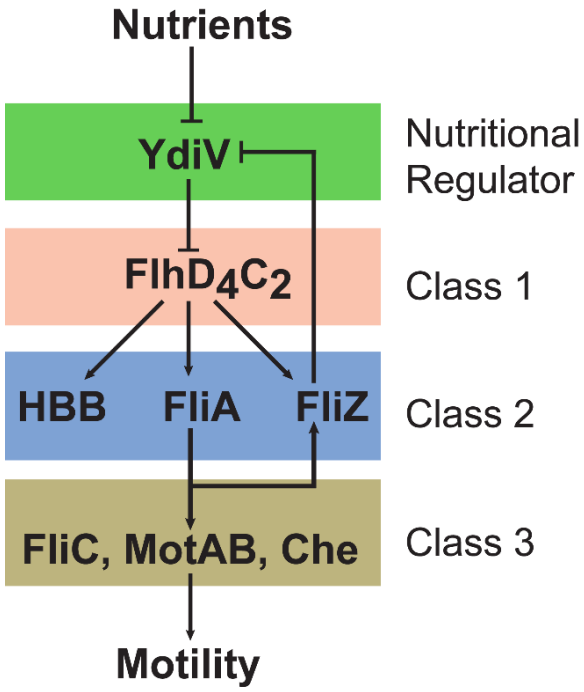

Figure 7. Schematic of flagellar gene network. The flagellar genes can be arranged into three classes based on how they are transcriptionally regulated (86, 87). The sole class 1 operon encodes the $FlhD_4C_2$ master regulator. $FlhD_4C_2$ activates the expression of class 2 operons, which encode the hook-basal body proteins. In addition, $FlhD_4C_2$ activates the expression of the alternate sigma factor, FliA (also known as σ^{28}) and FliZ. FliA, in turn, activates the expression of the class 3 operons, which encode the motor proteins (MotAB), flagellar filament (FliC), and chemotaxis pathway (Che). YdiV binds $FlhD_4C_2$ and prevents it from activating class 2 promoters (104). In addition, YdiV promotes the degradation of $FlhD_4C_2$ via ClpXP (158). Both nutrients and FliZ repress the expression of YdiV (160).

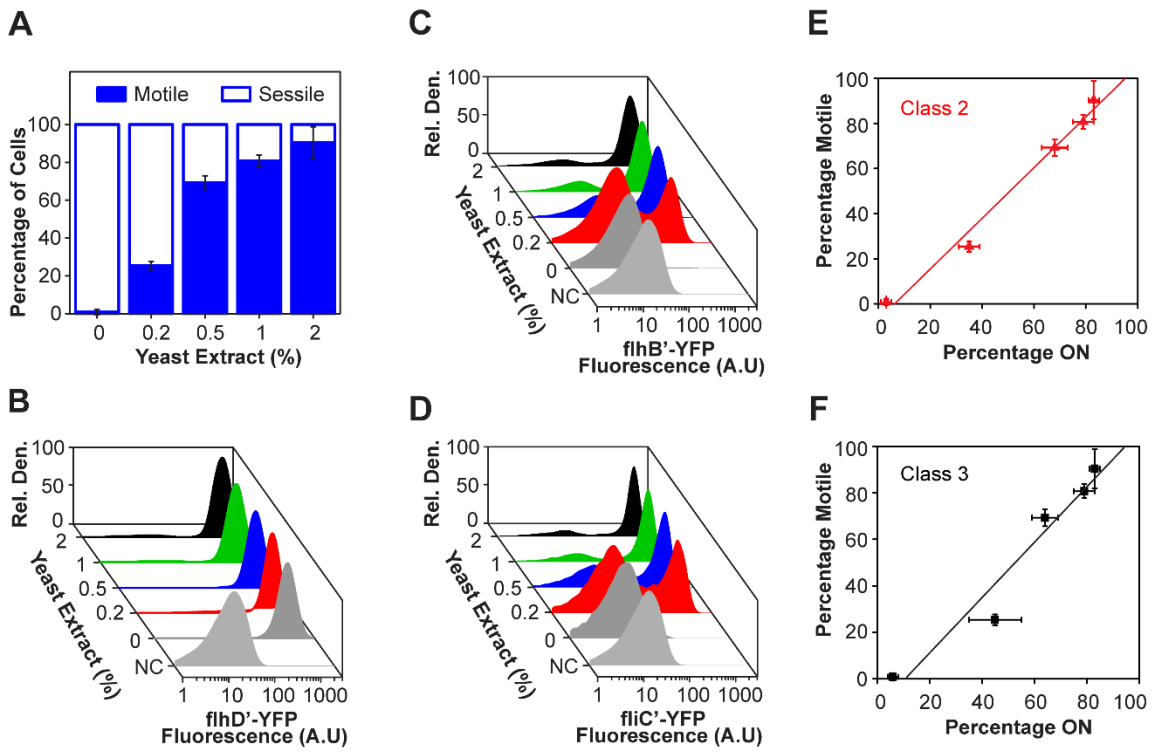

Figure 8. Nutrients tune the fraction of motile cells in S. enterica. **A.** Fraction of motile cells as a function of nutrient concentrations as determined by video microscopy. Data presented is an average of three independent repeats and error bars indicate standard deviations. **B-D.** Flagellar gene expression as determined using single-copy transcriptional fusions to the fluorescent protein Venus for representative class 1 (P_{flhD}), class 2 (P_{flhB}) and class 3 (P_{fliC}) promoters. The negative controls (NC) are Δ*flhDC* mutant. **E-F.** Scattered plot of percentage of motile cells versus percentage of cells expressing class 2 and class 3 flagellar genes. Straight lines are linear fits to the data. Error bars indicate standard deviations of three independent repeats.

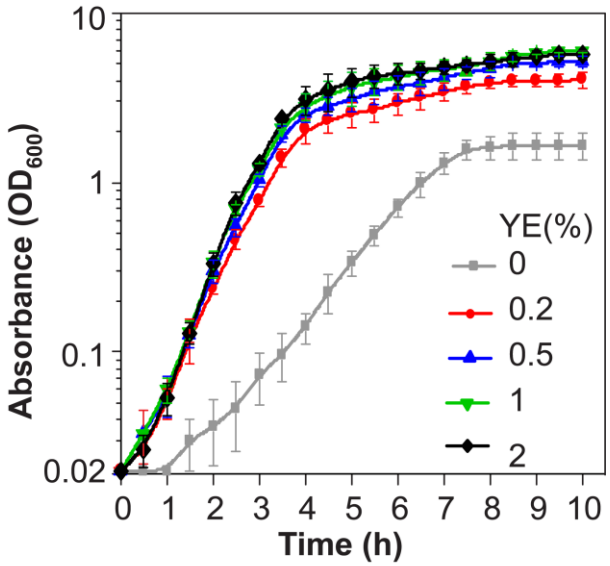

Figure 9. Growth curves of wild type cells at different concentration of yeast extract. Error bars indicate standard deviations of three independent repeats.

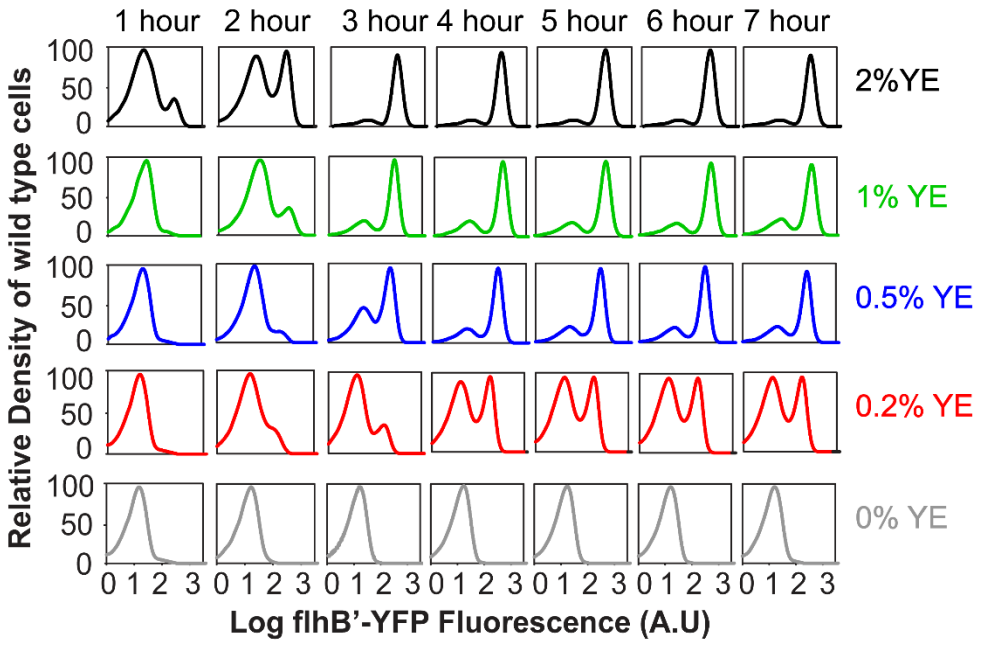

Figure 10. Dynamic activity of a representative class 2 (P_{flhB}) promoter, presented as a function of time and yeast extract concentration, as determined by flow cytometry in wild type cells.

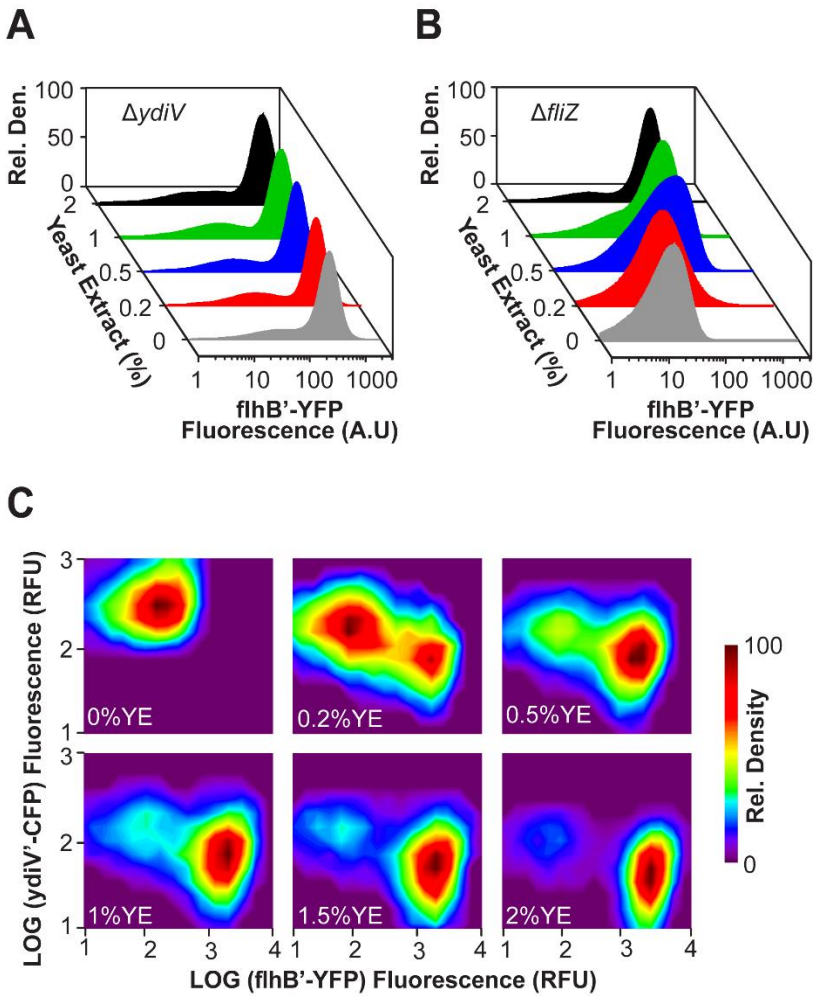

Figure 11. FliZ and YdiV are necessary for bimodal gene expression. **A-B.** Class 2 gene expression as determined using single-copy transcriptional fusions to the fluorescent protein Venus in Δ*fliZ* **(A)** and Δ*ydiV* **(B)** mutants. **C.** Simultaneous measurement of representative class 2 (P_{flhB}) and YdiV promoters, as determined using two-color flow cytometry.

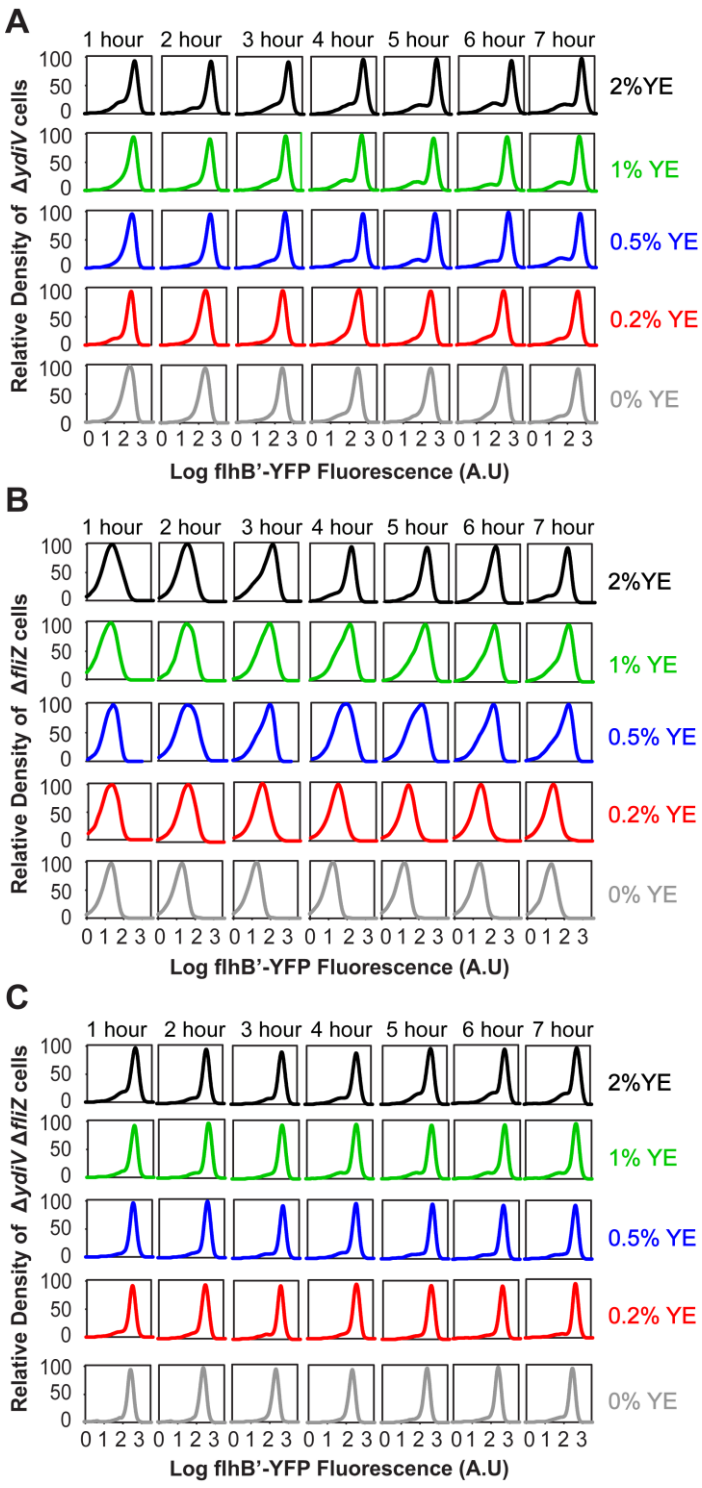

Figure 12. Class 2 gene expression profile is unimodal in the absence of two antagonizing proteins YdiV and FliZ. **A-C.** Class 2 PflhB promoter activity as a function of time and yeast extract concentration as determined by flow cytometry in Δ*ydiV*, Δ*fliZ* and Δ*ydiV*. Δ*fliZ* mutants.

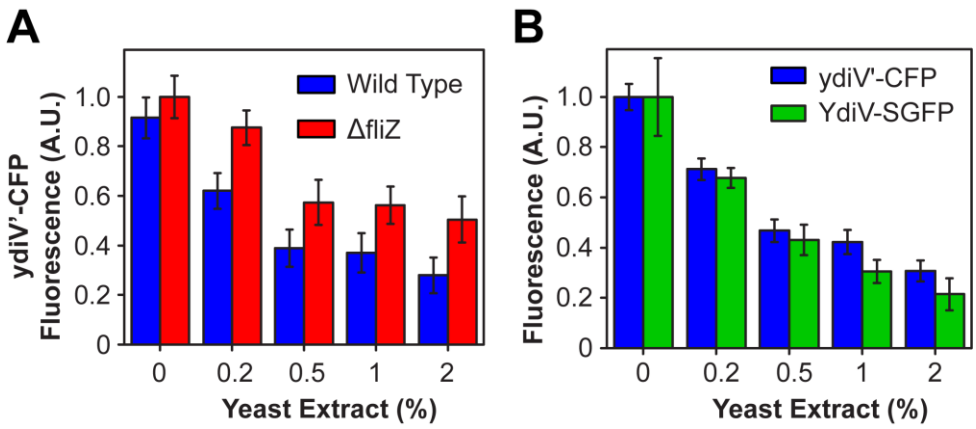

Figure 13. A. YdiV transcription is enhanced under nutrient limited condition, and its transcription is repressed by FliZ. P_{ydiV} promoter activity as a function of yeast extract in wild type and ΔfliZ strains. **B.** YdiV is regulated at the transcriptional level. Comparison of YdiV transcriptional (*ydiV*'-CFP) and translational (YdiV-SGFP) fusions. Note, the YdiV-SGFP translational fusion is unable to repress $FlhD_4C_2$. Error bars indicate the standard deviations for three independent repeats.

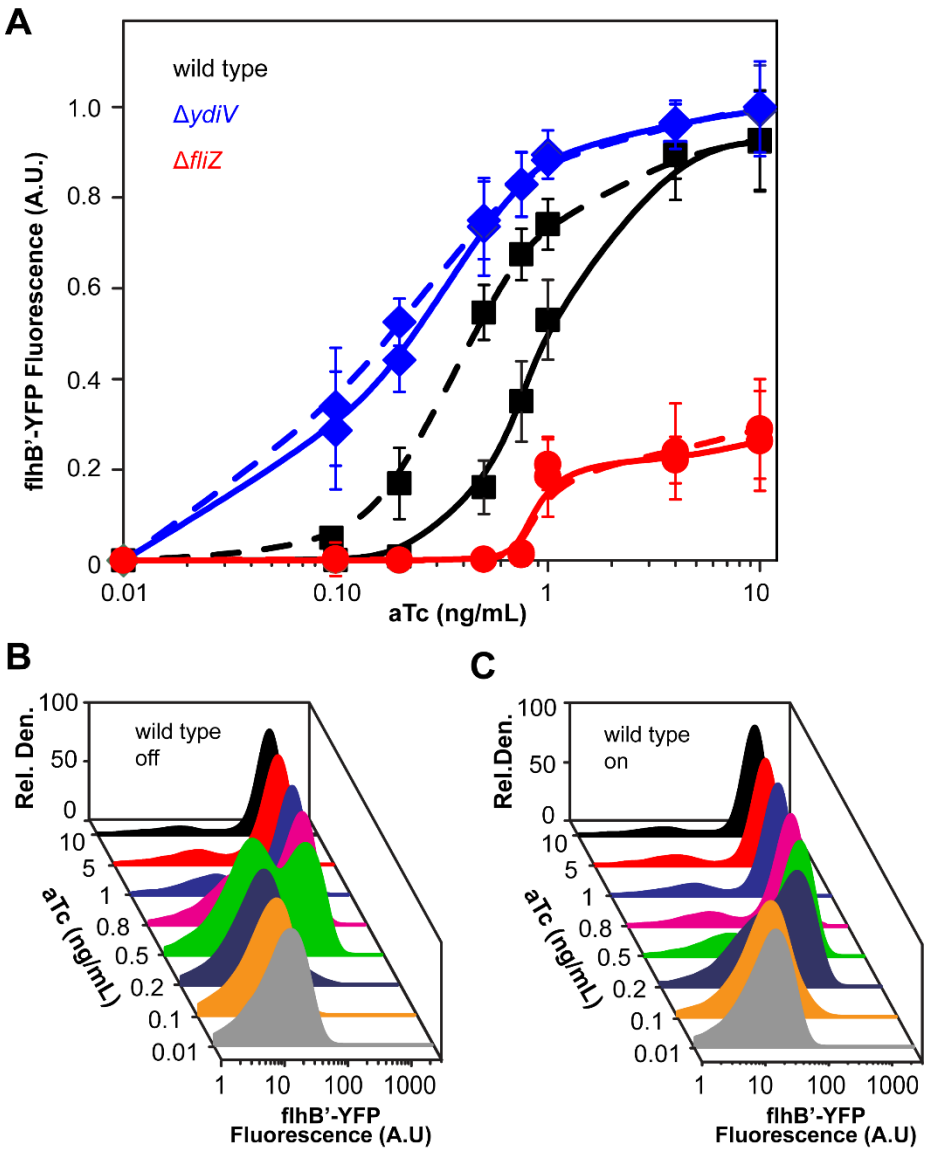

Figure 14. Flagellar gene expression exhibits hysteresis. **A.** Experiments were performed in a strain where the native P_{flhDC} promoter was replaced with aTc-inducible one (P_{tetRA}). Cells were grown in presence (on) or absence (off) of aTc and then subcultured at intermediate aTc concentrations. Experiments were performed in 0.2% yeast extract; results for other concentrations are provided in **Figure 15**. Data presented is an average of three independent repeats and error bars indicate standard deviations. **B-C.** Data presented in terms of distributions. Note that bistability is not observed during the on-to-off transition. Equivalent plots for Δ*fliZ* and Δ*ydiV* mutants are given in **Figure 16**.

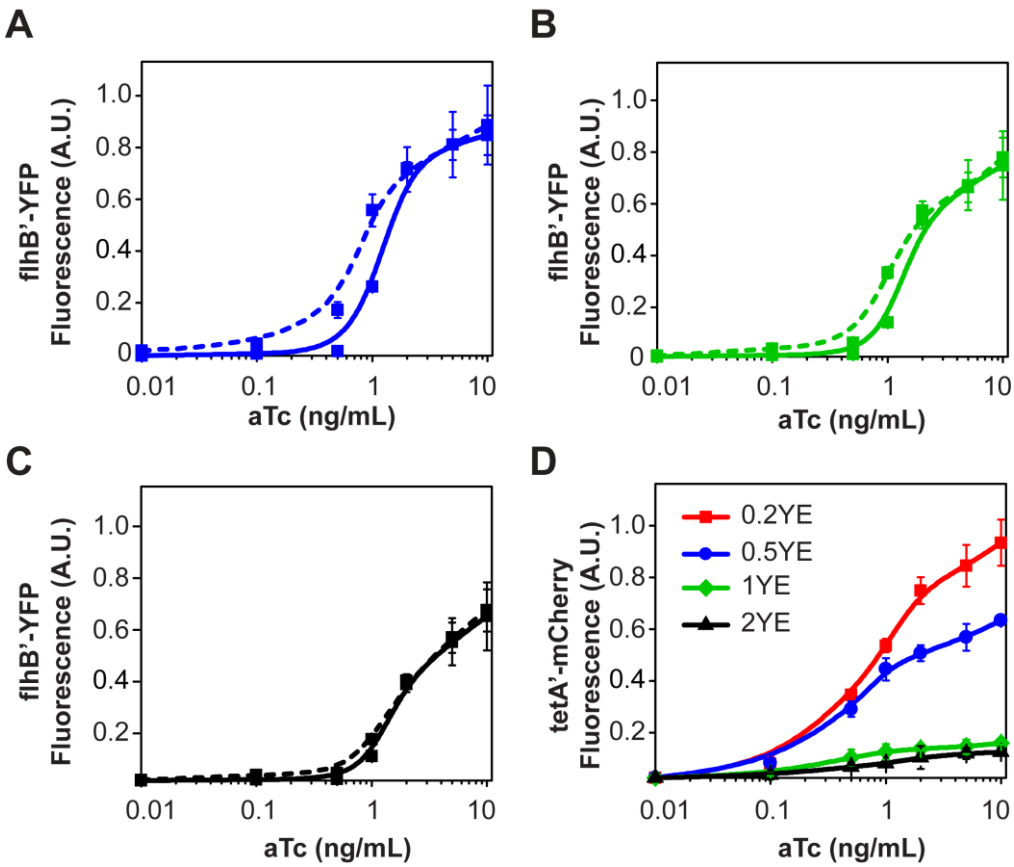

Figure 15. Flagellar gene expression exhibits hysteresis even at higher concentrations of yeast extract. However, these experiments cannot be compared against each other as the response to yeast extract and aTc are not orthogonal: as yeast extract concentrations increase, expression from the aTc-inducible P_{tetA} promoter decreases for reasons unknown. **A-C.** Class 2 P_{flhB} promoter activity as a function of anhydrotetracycline concentration (measure of $FlhD_4C_2$ concentration inside the cells) in a P_{flhDC}::*tetRA* strain, initially off (solid lines) or initially on (dashed line), grown with 0.5% yeast extract, 1% yeast extract and 2% yeast extract. The data were normalized relative to the experiments using 0.2% yeast extract. **D.** P_{tetA} promoter activity as a function of aTc and yeast extract measured using mCherry transcriptional fusion in a strain where repressor TetR is produced independently from P_{tetR} promoter (169). Data presented is an average of three independent repeats and error bars indicate standard deviations.

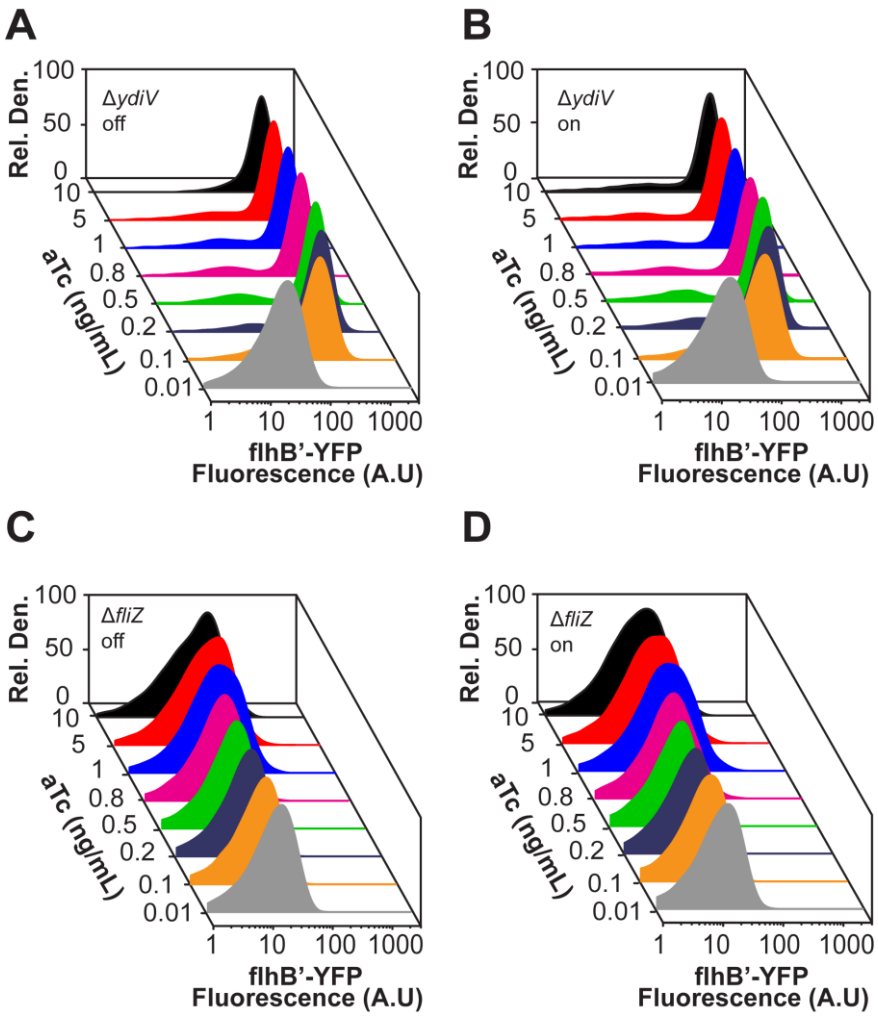

Figure 16. The flagellar gene circuit does not exhibit hysteresis in Δ*ydiV* and Δ*fliZ* mutants. Class 2 P_{flhB} promoter activity as a function of anhydrotetracycline concentration (measure of $FlhD_4C_2$ expression inside the cells) in a P_{flhDC}::*tetRA* Δ*ydiV* mutant initially off **(A),** P_{flhDC}::*tetRA* Δ*ydiV* mutant initially on **(B),** P_{flhDC}::*tetRA* Δ*fliZ* mutant initially off **(C)** and P_{flhDC}::*tetRA* Δ*fliZ* mutant initially on cells **(D)** determined using flow cytometry.

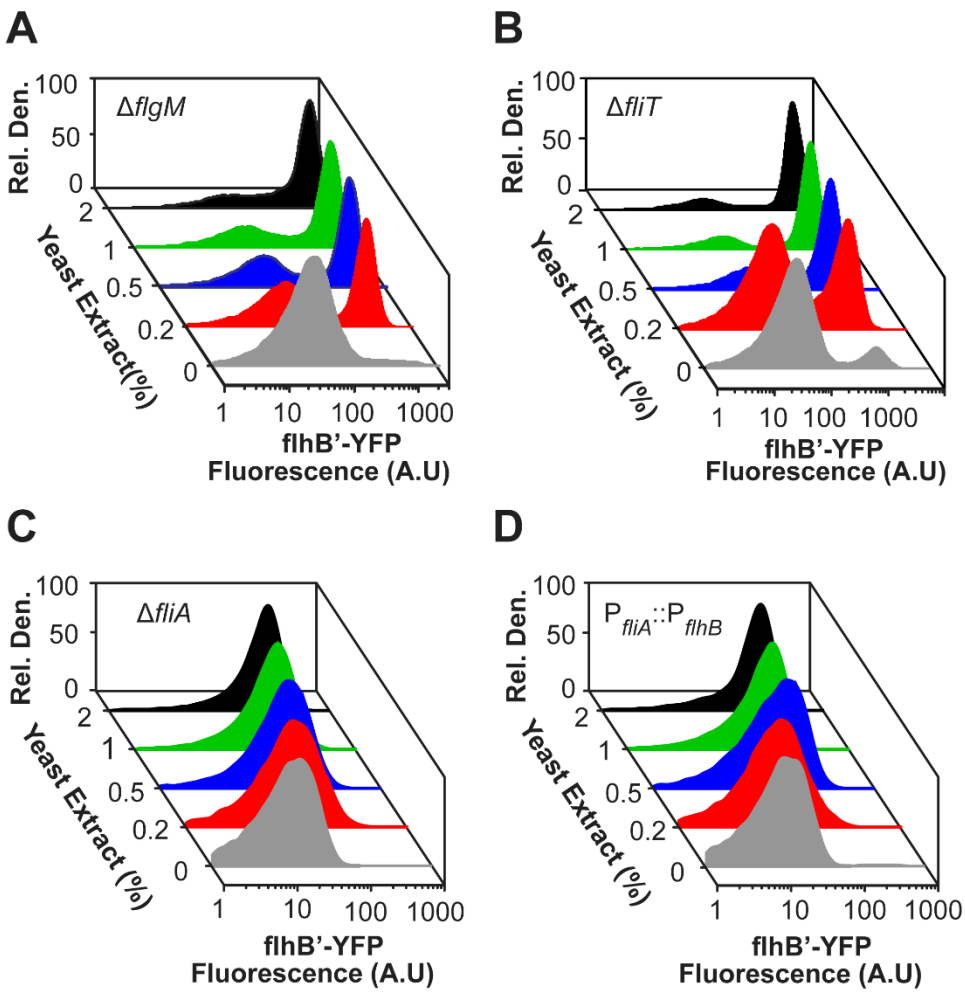

Figure 17. FliA positive feedback is necessary for bistability but FlgM and FliT are not. Class 2 gene expression as determined using single-copy transcriptional fusions to the fluorescent protein Venus in a Δ*flgM* (**A**), Δ*fliT* (**B**), Δ*fliA* (**C**) and P_{fliA}::P_{flhB} (**D**) strains.

CHAPTER 4
A SECRETION-DEPENDENT SWITCH CONTROLS CLASS 3 FLAGELLAR GENE EXPRESSION

INTRODUCTION

Flagellar biogenesis in *Salmonella enterica* serovar Typhimurium begins with MS ring at the base and concludes with the filament at the top (189). During assembly, structural components required at the distal end are transported through a central channel using type III secretion apparatus assembled at the cytoplasmic interface (190). Flagellar genes are expressed in temporal hierarchy mirroring the sequential flagellar assembly process (5, 191). The promoters controlling the expression of the flagellar operons are divided into three classes (86). The P_{class1} promoter controls the expression of the *flhDC* master operon. Multiple global transcriptional regulators control its activity that is critical for cells to determine whether to be motile or not (6, 88). Under motility inducing conditions, $FlhD_4C_2$, product of the master operon, initiates the transcription from all the P_{class2} promoters (90). These promoters control the expression of genes encoding for hook-basal body (HBB) and regulatory proteins FliA and FlgM (7). FliA is an alternate sigma factor, σ^{28}, which controls the expression from P_{class3} promoters (91). The P_{class3} promoters control the expression of genes encoding for filaments, motors and chemotaxis proteins (86). Before HBB assembly, FlgM binds to σ^{28} and represses the transcription from the P_{class3} promoters (95, 96). After successful completion of HBB, FlgM is secreted out of the cells and the repression of σ^{28} is relieved which initiates the transcription from the P_{class3} promoters (175).

In *S. enterica*, motility is repressed under low-nutrient condition (8, 104, 192). It is mediated by a non-flagellar protein, YdiV, which binds to the master regulator $FlhD_4C_2$ and prevents it from activating the the P_{class2} promoters. Additionally, YdiV releases $FlhD_4C_2$ bound to the P_{class2} promoters and accelerates $FlhD_4C_2$ degradation by ClpXP protease (158). Nutrients enhance the expression of class 2 genes by repressing the expression of YdiV (8, 104, 192). Moreover, YdiV participates in a double negative feedback loop involving flagellar regulator FliZ. FliZ is encoded in the *fliAZ* operon and

has been shown to enhance the P_{class2} promoter activity (89, 99-101). FliZ directly represses the transcription of *ydiV* gene and YdiV indirectly repress the transcription of *fliZ* gene through repression of $FlhD_4C_2$ (160). This mutual repression plays a key role in partitioning cells into motile and sessile fractions (101, 161, 162, 164, 192).

In chapter 3, we showed that nutrients tune the fractions of cells expressing flagellar genes in *Salmonella enterica* (192). We found that both class 2 P_{flhB} and class 3 P_{fliC} promoters were nearly identical in their response, at least in wild type cells, to yeast extract and focused our analysis on the class 2 P_{flhB} promoter. We further demonstrated that the response is bistable at intermediate yeast extract concentrations and the double negative feedback loop involving FliZ and YdiV is responsible for the bistable response. However, during a previous investigation done in rich LB media, we had shown that although class 2 and class 3 promoters behave similarly in wild type cells, they behave differently in a Δ*fliZ* mutant (101). In particular, FliZ regulates class 2 promoter gene expression dynamics but not the class 3. A more recent study by Stewart and Cookson has shown that FliZ is not required for bistable class 3 *fliC* expression (193). These findings show that class 2 and class 3 genes are regulated in a different way. The mechanism by which class 2 and class 3 gene expressions differ, however, has not been investigated.

In this study, we investigated the underlying molecular mechanism that independently controls the flagellar class 2 and class 3 gene expression **(Figure 18)**. We found that σ^{28}-FlgM regulatory circuit play a crucial role in partitioning cells into *fliC*-OFF (P_{fliC} promoter inactive) and *fliC*-ON (P_{fliC} promoter active) population at intermediate expression of class 2 genes. Moreover, the bistability is governed by auto activation of σ^{28} which enhances its own expression by activating the transcription from *flhDC* operon.

RESULTS

Flagellar class 2 and class 3 gene expression is symmetrical. To elucidate the mechanism by which class 2 and class 3 flagellar gene expression differs in *S. enterica*, we followed our previous protocol (192) to measure expression from class 2 P_{flhB} and class 3 P_{fliC} promoters using transcriptional fusion to fluorescent protein Venus **(Figure 19A-B)**. Consistent with our previous observation (192), both class 2 P_{flhB} and class 3 P_{fliC} promoters exhibit bimodal response at intermediate yeast extract concentration in wild type cells. Next we wanted to understand how class 2 and class 3 gene expression are correlated: specifically are there any cells that express class 2 genes but not class 3 genes? To answer this, we measured expression from class 2 P_{flhB} and class 3 P_{fliC} promoters in a single strain that harbors chromosomally integrated transcriptional fusion of the P_{flhB} and P_{fliC} promoters to the fluorescent proteins mCherry (2) and Venus (4) respectively. As shown in **Figure 19C**, we observed near perfect symmetry between class 2 and class 3 gene expression. The observation that cells with uninduced class 2 promoter also having class 3 promoter inactive is not surprising as the product of class 2 operon, σ^{28}, is required for transcription of class 3 operons (91). However, we also observed that all the cells with active class 2 promoter also have class 3 promoters active. These results suggest that once the decision to be motile is made, cells commit to that decision and expresses all the required genes, given that all assembly checkpoints are met.

FliZ is required for heterogeneous response from class 2 but not class 3 promoters. We have previously shown that FliZ is required for heterogeneous response from class 2 promoter (192). Moreover, when cells are grown in rich media class 2 and class 3 promoters elicit different dynamic response in a Δ*fliZ* mutant (101). To investigate the role of FliZ in heterogeneous response, we measured expression from class 2 P_{flhB} and class 3 P_{fliC} promoters in Δ*fliZ* mutants at various yeast extract concentration **(Figure 20A-B)**. Consistent with our previous observation, class 2 P_{flhB} elicited homogenous response at all yeast extract concentrations. On the other hand, we observed that class 3 P_{fliC} promoter exhibits heterogeneous response at intermediate yeast extract concentration. To further elucidate the difference between class 2 and class 3 response, we measured expression from class 2 P_{flhB} and class 3 P_{fliC} promoters in a single Δ*fliZ* mutant strain that harbors chromosomally integrated transcriptional fusion of the P_{flhB} and P_{fliC} promoters to the

fluorescent proteins mCherry (2) and Venus (4) respectively. We observed that as class 2 gene expression increases homogeneously, class 3 genes are partitioned into active and inactive fractions **(Figure 20)**. Moreover, the class 3 active fractions increases with yeast extract concentration. These results suggest that at intermediate homogenous expression of class 2 genes, class 3 genes are expressed only in a fraction of cells.

We also measured class 2 and class 3 gene expressions in a Δ*ydiV* mutant. Consistent with our previous findings (192), the class 2 P_{flhB} promoter is strongly active in most cells, irrespective of yeast extract concentration **(Figure 21Figure 20A)**. Additionally, we observed that the class 3 P_{fliC} promoter is also strongly active in most cells, irrespective of yeast extract concentration **(Figure 21B)**. These observations indicates that when class 2 gene expression is strong, so is class 3 gene expression in all the cells. This observation also holds in the case of wild type and Δ*fliZ* cells grown in 2% yeast extract.

FlgM is required for heterogeneous class 3 gene expression. Flagellar genes in *Salmonella enterica* are expressed in temporal hierarchy mirroring the flagellar assembly process (86, 87). Particularly, genes required for filaments and motors are not expressed until a successful completion of hook basal body (HBB). This developmental check-point is mediated by FlgM (92). Before HBB assembly, FlgM binds to alternate sigma factor, σ^{28}, required for transcription from class 3 promoters. After successful completion of HBB, FlgM is secreted out of the cells and the repression of σ^{28} is relieved which initiates the transcription from class 3 promoters. Moreover, σ^{28}-FlgM regulatory circuit is implicated in continually sensing the HBB assembly process and regulating class 3 gene expression and number of flagella in response (97, 98, 172). In other words, cells use the secretion rate of FlgM as a cue for successfully completed HBBs and modulate the gene expression. We hypothesized that σ^{28}-FlgM regulatory circuit also controlled bistable class 3 gene expression.

To determine the role of FlgM, we measured class 2 P_{flhB} and class 3 P_{fliC} promoter activity in single-cell resolution in a Δ*flgM* mutant. As shown in **Figure 22A-B**, class 2 P_{flhB} and class 3 P_{fliC} promoters were nearly identical in their response to yeast extract. This result is consistent with the response observed in wild type cells: the fraction of cells with inactive class 2 P_{flhB} promoter has inactive class 3 P_{fliC} promoter and the fraction of cells with active class 2 P_{flhB} promoter has active class 3 P_{fliC} promoter. Next, we measured class

2 P_{flhB} and class 3 P_{fliC} promoter activity in a Δ*flgM* Δ*fliZ* double mutant. We used Δ*flgM* Δ*fliZ* double mutant so that we could measure class 3 gene expression at intermediate expression of class 2 genes in the absence of FlgM. As shown in **Figure 22C**, class 2 P_{flhB} promoter exhibited a homogenous nutrient response consisting of a single population in Δ*flgM* Δ*fliZ* double mutant which is the same response seen in Δ*fliZ* mutant. In contrast to the response in Δ*fliZ* mutant, the class 3 P_{fliC} promoter exhibited a homogenous nutrient response in Δ*flgM* Δ*fliZ* double mutant (**Figure 22D**). Also, the class 3 P_{fliC} promoter is strongly active even when the class 2 P_{flhB} promoter is only intermediately active (0.2% ≤ yeast extract ≤ 1%) except the case where the class 2 P_{flhB} promoter is completely inactive in all cells (0% yeast extract) in which case the class 3 P_{fliC} promoter is also completely inactive in all cells.

We also measured class 2 P_{flhB} and class 3 P_{fliC} promoter activity in a Δ*flgM* Δ*ydiV* double mutant. The class 2 P_{flhB} and class 3 P_{fliC} promoter are strongly active in all cells, irrespective of yeast extract concentration **(Figure 22D-E)**. This response is similar to Δ*ydiV* mutant that when class 2 gene expression is strong, so is class 3 gene expression in all the cells. These results suggest that σ^{28}-FlgM regulatory circuit plays a crucial role in partitioning cells into *fliC*-OFF (P_{fliC} promoter inactive) and *fliC*-ON (P_{fliC} promoter active) population at intermediate expression of class 2 genes. Moreover, the fraction of *fliC*-ON cells is determined by the relative strength of class 2 gene expression.

FliA enhances its own and other class 2 gene expression by directly activating class 1 promoter. Our data suggest that flagellar class 3 gene expression is also bistable, albeit governed by a separate switch than class 2 genes. A typical feature of bistable switch is positive feedback. The *fliAZ* operon is under the control of both class 2 and class 3 promoters (89). The class 2 promoter functions in a double-negative feedback loop involving FliZ and YdiV; the class 3 promoter functions in an autogeneous loop involving the alternate sigma factor FliA. The latter loop is not directly autocatalytic, as FliA is inefficiently translated from the class 3 transcript (173). Rather, positive feedback is indirect, such that FliA activates FliZ expression and FliZ indirectly activates FliA expression by repressing the expression of YdiV (**Figure 18**). In the absence of FliZ, the FliA-FliZ-YdiV loop is not autocatalytic. However, class 3 gene expression is still bistable

in a Δ*fliZ* mutant. One possible mechanism where FliA can enhance its own activity in the absence of FliZ is if FliA directly activated the transcription of class 1 genes.

We tested this hypothesis by measuring class 2 P_{flhB} promoter activity in wild type and Δ*fliZ* mutants with FliA overexpressed from an arabinose-inducible pBAD30 (9) plasmid. As controls we also measured class 3 P_{fliC} promoter activity in wild type and Δ*fliZ* mutants. As shown in **Figure 23A**, class 2 P_{flhB} promoter activity is enhanced in both wild type and Δ*fliZ* cells. The control experiments show significant activation of class 3 P_{fliC} promoter by FliA. These results suggest that FliA enhances its own expression from class 2 operon. To test if this activation is a result of direct activation of P_{fliC} promoter by FliA, we measured class 2 P_{flhB} and class 3 P_{fliC} promoter activity in a strain in which native class1 P_{flhDC} promoter is replaced with tetracycline-inducible one **(Figure 23B)**, as describe previously (100). In the absence of inducer anhydrotetracycline (aTc), both class 2 P_{flhB} and class 3 P_{fliC} promoters were inactive. In the presence of arabinose, only class 3 P_{fliC} promoter was active which indicates FliA cannot activate class 2 promoter directly. In the presence of aTc or both arabinose and aTc both promoters were active. Interestingly, in the case of Δ*fliZ* mutants, there was no effect of FliA overexpression on class 2 P_{flhB} promoter. We did, however, observe enhanced activity of class 2 P_{flhB} promoter activity in wild type cells. This is consistent with the autocatalytic nature of the FliA-FliZ-YdiV loop. The controls experiment show significant activation of class 3 P_{fliC} promoter in both wild type and Δ*fliZ* mutants. We further confirmed the FliA activation of *flhDC* transcription by measuring the *flhD* and *flhC* mRNA levels using reverse transcription polymerase chain reaction (RT-PCR). As shown in **Figure 24**, when FliA overexpression is induced, both *flhD* and *flhC* mRNA levels increase by nearly three folds. These results show that FliA activates class 2 gene expression by activating the transcription from *flhDC*, flagellar master, operon.

DISCUSSION

We have shown that when class 2 promoters are inactive in all cells, class 3 promoters are also inactive in all cells (wild type and other mutants except Δ*ydiV* grown in 0% yeast extract); when class 2 promoters are active in all cells, class 3 promoters are also active in all cells (wild type and other mutants grown in 2% yeast extract or Δ*ydiV* mutant grown in all concentrations of yeast extract). However, at intermediate level of class 2 promoter activity in all cells (Δ*fliZ* mutant grown in 0.2% ≤ yeast extract ≤ 1%), class 3 promoters are active only in a fraction of cells. These results suggest that flagellar regulon is not under a sole control of the nutrient-responsive bistable switch involving FliZ and YdiV. We further demonstrated that a secretion-dependent switch involving alternate sigma factor, σ^{28}, and its antagonist FlgM controls the partitioning of cells into *fliC*-OFF (P_{fliC} promoter inactive) and *fliC*-ON (P_{fliC} promoter active) population at intermediate expression of class 2 genes. Moreover, the bistability is governed by auto activation of σ^{28} which enhances its own expression by activating the transcription from *flhDC* operon.

Stewart and Cookson (1) also showed that FliZ is not required for bistable *fliC* expression. However, they did not address the need for a comprehensive model for bistable class 3 gene expression. Moreover, they did not differentiate between the circuits that controls the expression of class 2 and class 3 genes. In this study, we have proposed a comprehensive model that explains these differences. A natural question is what is the need for two switches in flagellar regulon? A simple explanation is, the nutrient dependent class 2 and class 3 switches respectively govern bistability before and after HBB completion (86).

Kutsukake and Iino (94) had shown that when overexpressed from a plasmid, FliA was able to enhance the expression of all the flagellar class 2 and class 3 genes as measured by *lac* fusion. Our findings are consistent with these observations. However, they did not observe any change in *flhD-lac* activity with overexpressed FliA. The strain they used was *flhDC::lac* allele and did not express native *flhDC* genes. P_{flhDC} promoter is a very complex promoter with multiple transcription sites and binding sites for various global regulators (11, 88). It is challenging to obtain an accurate measure of transcriptional activity of this promoter using *lac* fusions or fluorescent reporter fusions. Thus, we used RT-PCR to

directly measure the *flhDC* mRNA levels. Our data shows that FliA directly enhances the transcription from P_{flhDC} promoter.

fliA and fliZ are co-transcribed from a hybrid class 2/3 promoter (89). However, why these two positive regulators of flagellar genes are expressed in the same operon is not clear. The developmental checkpoint involving FliA and FlgM couples class 3 flagellar gene expression to flagellar assembly (175). Mutations that inhibit formation of the hook-basal body are deficient in FlgM secretion and thus prevent transcription from class 3 promoters. This checkpoint likely ensures that cells do not switch to the on state for class 3 transcription until they are able to make functional hook-basal body complexes. FliA works in two positive feedback loops ($FlhD_4C_2$-FliA autocatalytic loop and FliA-FliZ-YdiV-FlD_4C_2 loop) to enhance transcription of flagellar genes. Similarly, FliZ works in two positive feedback loops ($FlhD_4C_2$-FliZ-YdiV double negative feedback loop and FliA-FliZ-YdiV-FlD_4C_2 loop) to enhance transcription of flagellar genes. One of these loops are common between them. These scenarios suggest that they may need each other to realize their full potential and explain why they are transcribed from the same operon.

We conclude by noting that flagellar gene expression is also bistable in *Bacillus subtilis* (194), although the mechanism governing bistability is quite different than the one in *S. enterica* (195). In *B. subtilis*, the flagellar-specific sigma factor SigD resides at the end of a large operon containing thirty-one flagellar genes. Presumably, SigD expression is weak or non-existent in many cells because of incomplete transcription of the full operon. In those cells in which the entire operon is transcribed and expression of SigD exceeds some threshold, it can further enhance its own expression through a SigD-dependent promoter that resides in the middle of the operon. This positive feedback mechanism, which involves the stochastic triggering of the loop, generates the observed bistability in *B. subtilis* motility. Indeed, moving the *sigD* gene upstream in the operon increases the fraction of motile cells (195). Whether the bistable response in *B. subtilis* is tuned by external factors to the same degree as in *S. enterica* is not known. Nonetheless, the bistable response in these two distantly related bacteria suggests that heterogeneous expression of flagellar genes is a general phenomenon and may reflect a widespread strategy for deploying motility as adaptational response to the environment.(104)

FIGURES FOR CHAPTER 4

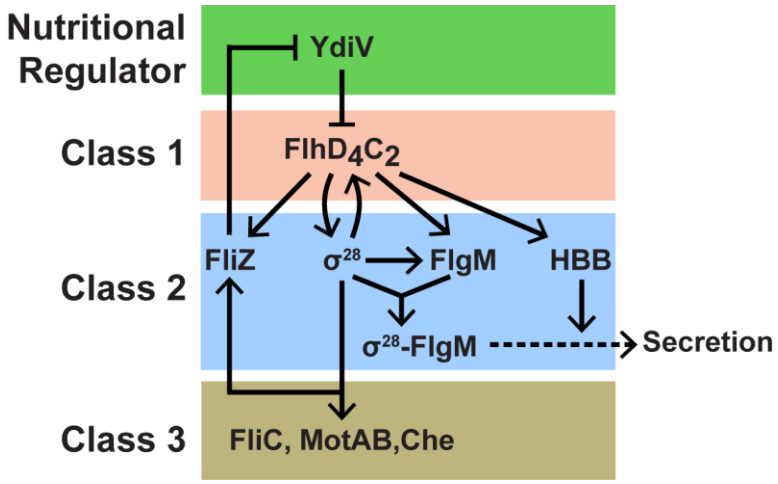

Figure 18. Schematic of flagellar gene network. The flagellar genes can be arranged into three classes based on how they are transcriptionally regulated (86, 87). The sole class 1 operon encodes the $FlhD_4C_2$ master regulator. $FlhD_4C_2$ activates the expression of class 2 operons, which encode the hook-basal body proteins. In addition, $FlhD_4C_2$ activates the expression of the alternate sigma factor, FliA (also known as σ^{28}) and FliZ. FliA, in turn, activates the expression of the class 3 operons, which encode the motor proteins (MotAB), flagellar filament (FliC), and chemotaxis pathway (Che). YdiV binds $FlhD_4C_2$ and prevents it from activating class 2 promoters (104). In addition, YdiV promotes the degradation of $FlhD_4C_2$ via ClpXP (158). Both nutrients and FliZ repress the expression of YdiV (160). FlgM is anti-sigma factor which binds to σ^{28} and stops it from activating class 3 operons (92). Once HBB is complete, FlgM is secreted out of the cells which relieves its σ^{28} repression and expression of the class 3 operons are activated (93). Additionally, σ^{28} takes part in an autocatalytic loop where it enhance its own expression from class 2 promoter by directly activating expression the expression of class 1 operon.

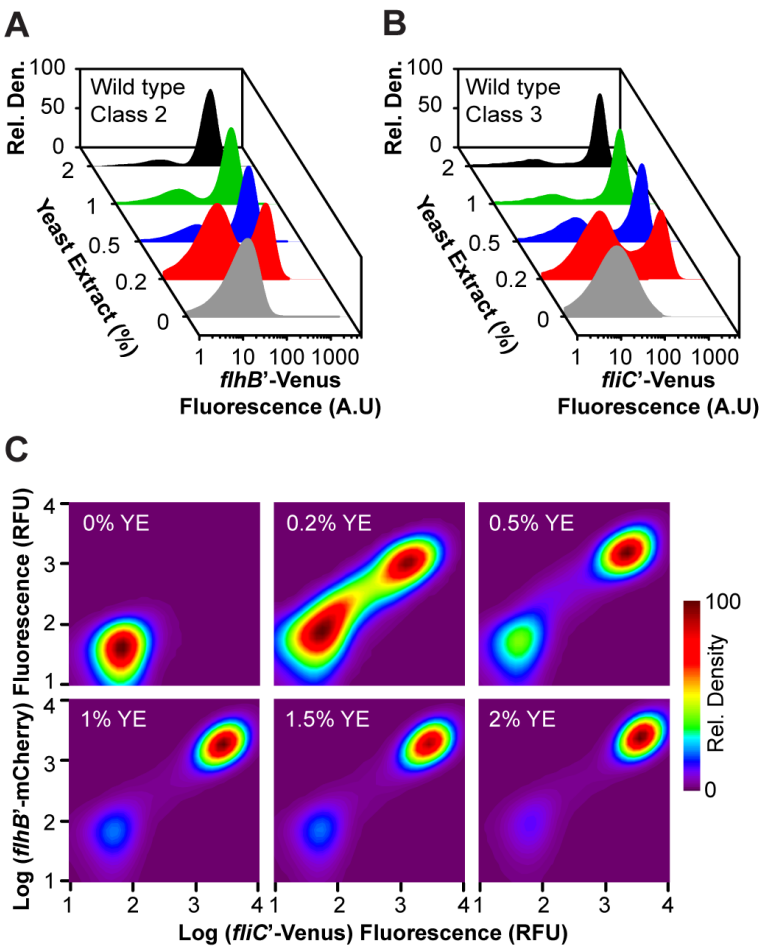

Figure 19. Flagellar class 2 and class 3 gene expression is heterogeneous and symmetrical in wild-type cells. Class 2 P_{flhB} **(A)** and class 3 P_{fliC} **(B)** promoter activity measured with transcriptional fusion to fluorescent protein Venus in wild type strains. **(C)** Class 2 P_{flhB} and class 3 P_{fliC} promoter activity measured in a single wild type strain using transcriptional fusion to fluorescent protein Venus and mCherry respectively.

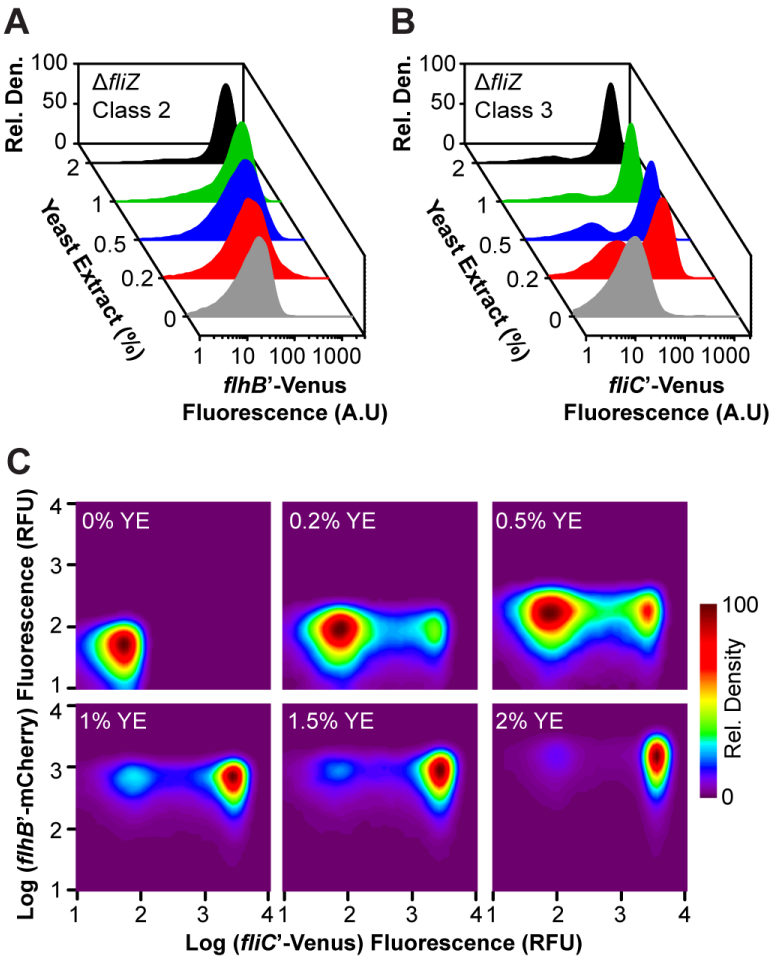

Figure 20. Flagellar class 2 gene expression is homogenous but class 3 gene expression is heterogeneous in Δ*fliZ* mutants. Class 2 P_{flhB} **(A)** and class 3 P_{fliC} **(B)** promoter activity measured with transcriptional fusion to fluorescent protein Venus in Δ*fliZ* mutants. **(C)** Class 2 P_{flhB} and class 3 P_{fliC} promoter activity measured in a single in Δ*fliZ* mutant strain using transcriptional fusion to fluorescent protein Venus and mCherry respectively.

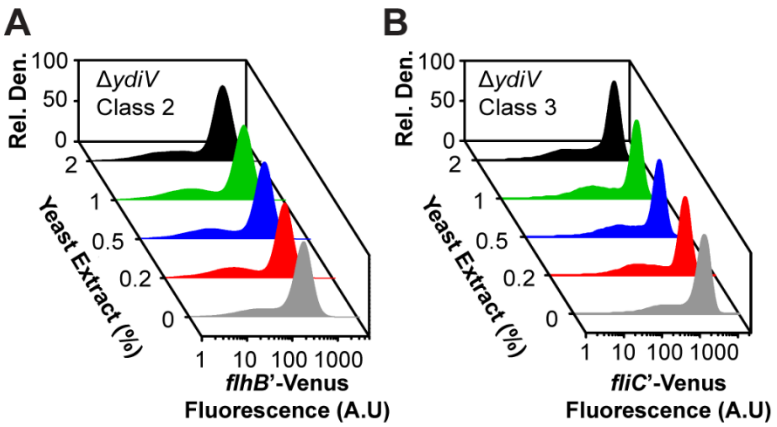

Figure 21. Flagellar class 2 and class 3 gene expression is homogenous and strongly activated in Δ*ydiV* mutants. Class 2 P_{flhB} **(A)** and class 3 P_{fliC} **(B)** promoter activity measured with transcriptional fusion to fluorescent protein Venus in Δ*ydiV* mutants.

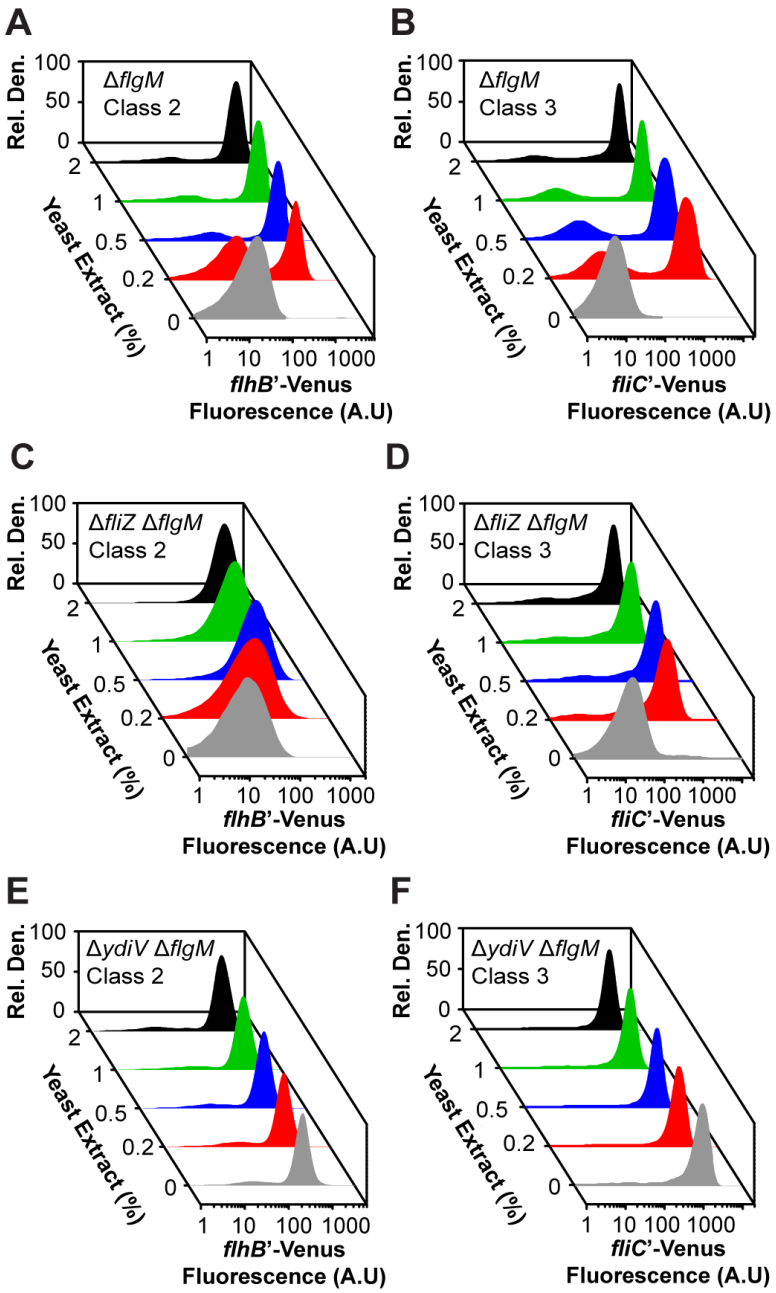

Figure 22. FlgM is required for heterogeneous class 3 gene expression. Class 2 P_{flhB} and class 3 P_{fliC} promoter activity measured with transcriptional fusion to fluorescent protein Venus in Δ*flgM* **(A-B)**, Δ*fliZ* Δ*flgM* **(C-D)** and Δ*ydiV* Δ*flgM* **(E-F)** mutants.

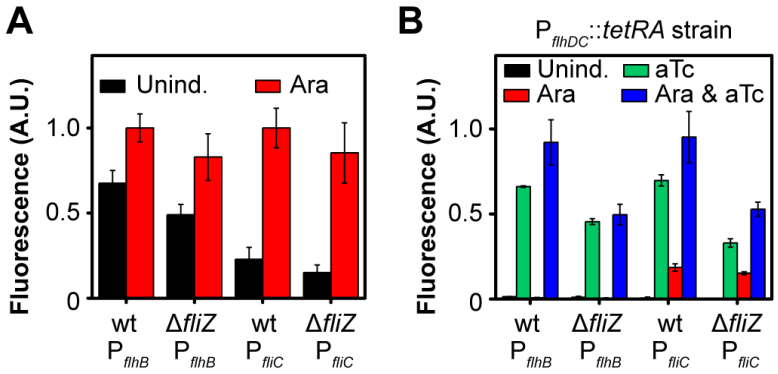

Figure 23. FliA induces class 2 expression by directly activating class 1 P_{flhDC} promoter activity. **(A)** Class 2 P_{flhB} and class 3 P_{fliC} promoter activity in wild type and Δ*fliZ* cells with FliA overexpressed from pBAD30 plasmid. **(B)** Class 2 P_{flhB} and class 3 P_{fliC} promoter activity in P_{flhDC}::*tetRA* and P_{flhDC}::*tetRA* Δ*fliZ* cells with FliA overexpressed from pBAD30 plasmid. PBAD30 plasmid was induced with 10mM arabinose (ara) and P_{flhDC}::*tetRA* was induced with 1 ng/ml anhydrotetracycline (aTc).

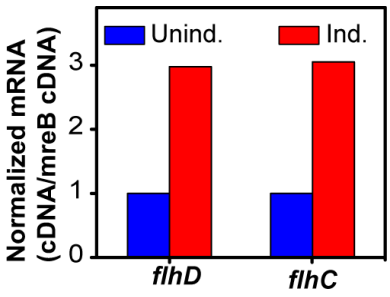

Figure 24. FliA induces class 1 expression by directly enhancing its transcription. mRNA concentration were measured using qRT-PCR. Expression of *mreB* gene was used as an internal control.

CHAPTER 5

RECIPROCAL REPRESSION OF ARABINOSE AND XYLOSE METABOLISM IN *ESCHERICHIA COLI*

INTRODUCTION

The *Escherichia coli* K-12 genome is predicted to contain more than four thousand protein-coding genes (196). However, not all of these genes are constitutively expressed. Rather, many genes are expressed in response to the cellular and growth environment. One such example is the expression of sugar transport and metabolism genes. Many sugar transport and metabolic genes are only expressed in the presence of their cognate sugar or a downstream intermediate. These inducible utilization pathways are believed to have evolved to optimize the resources available to the cell (197). Most of these gene regulatory networks employ positive and/or negative feedback loops to achieve inducible expression (41). Gene networks that contain both positive and negative feedback loops have been reported to show unique features not identified in the network with either feedback loop alone. These features include stable oscillations (198-201), excitability (202) and bistability (192, 202, 203).

E. coli K-12 sub-strain MG1655 possesses many well-characterized metabolic pathways featuring inducible transporters and catabolic genes (41, 204). Not all of them exhibit a similar response to their cognate inducer. A previous study, for example, has shown that the D-lactose, D-galactose, N-acetylglucosamine and N-acetylneuraminic acid utilization pathways exhibit homogenous population of cells at all concentration of inducers whereas the L-arabinose, D-xylose, L-rhamnose and D-gluconate pathways exhibit heterogeneous population of cells (41). This study, however, only dealt with single sugars. When bacteria are grown on a mixture of sugars, they will often consume them one at a time in a defined hierarchy. The classic example is growth of *Escherichia coli* on glucose and lactose, where the cells will first consume the glucose and then the lactose. This process of ordered sugar utilization is known as carbon catabolite repression and has been studied in many species of bacteria (117-120). These studies have principally focused

on the mechanisms governing the preferential utilization of glucose, which in the case of *E. coli* is known to involve the regulation of specific genes and metabolic fluxes. This repression is achieved by keeping the ratio of phosphoenolpyruvate (PEP) to pyruvate low inside the cells by complex interactions of a number of regulatory molecules including cyclic AMP (cAMP), cyclic AMP receptor protein (CRP), adenylate cyclase (AC) and EIIA from the PEP:glucose phosphotransferase system (PTS) (117, 205). Far less is known about the preferential utilization of sugars other than glucose. One notable exception is the growth of *E. coli* on mixtures of L-arabinose and D-xylose (113, 125, 126). After glucose, arabinose and xylose are two most abundant sugars found in plant biomass. Understanding growth on mixtures of these sugars is necessary to develop bacteria capable of producing renewable fuels and chemicals from plant biomass.

Kang and coworkers first demonstrated that *E. coli* selectively consumes arabinose before xylose (126). They further demonstrated that the xylose metabolic genes were repressed in the presence of arabinose. More recently, Desai and Rao investigated the mechanism for this hierarchy (125). Both the arabinose and xylose utilization pathways involve similar regulatory mechanisms (**Figure 25**). The genes for these pathways are only expressed when their cognate sugar is present. AraC positively regulates the transcription of the arabinose metabolic and transporter genes in response to arabinose. Likewise, XylR positively regulates the transcription of the xylose metabolic and transporter genes in response to xylose. In addition, the cyclic AMP receptor protein (CRP) regulates both sets of genes, which explains why growth of glucose inhibits their expression (118, 132, 206). Desai and Rao found that arabinose-bound AraC binds to xylose promoters and inhibits their expression likely by competitive mechanism (125). In support of a competitive mechanism, Groff and coworkers found that over-expression of XylR enabled the co-consumption of the two sugars, presumably by outcompeting arabinose-bound AraC (115).

Expression of both the arabinose and xylose genes are known to exhibit a bimodal/heterogeneous response to their cognate sugar, where they are expressed (induced) in only a fraction of cells at intermediate sugar concentrations (41, 207). This response, however, has never been investigated in mixtures of arabinose and xylose. An open question then is if induction is bimodal, will catabolite repression be as well. In other words, if only a fraction of cells are induced by arabinose then is other population capable

of being induced by xylose? And, will populations of arabinose and xylose-induced cells co-exist at some intermediate concentrations of the two sugars?

To answer these questions, we investigated the single-cell response of *E. coli* during growth on arabinose and xylose using fluorescent protein reporters. Our results show that mixed populations of arabinose and xylose-induced cells occur at some sugar concentration. During the course of these studies, we also found that xylose inhibits the expression of the arabinose genes. We also demonstrated that this repression occurs through XylR. We were also able to show that xylose-bound XylR binds to the promoter regions of arabinose metabolic genes *in vitro* and competitively inhibits the activation of arabinose metabolic genes by AraC. Collectively, these results demonstrate the catabolite repression is reciprocal during growth on arabinose and xylose.

RESULTS

Arabinose and xylose utilization pathways exhibit bimodal response during growth in single sugars. A previous study by Afroz and coworkers investigated the expression of the arabinose and xylose metabolic pathways in *E. coli* in response to their cognate sugar (41). Using transcriptional fusions to the green fluorescent protein, they measured expression from the P_{araB} and P_{xylA} promoters at single-cell resolution using flow cytometry. They found that both pathways exhibited a bimodal response to their cognate sugar at intermediate concentrations. Specifically, they found that the promoters were active in some cells but inactive in others. In the case of xylose, the response was "all-or-nothing", where increasing the concentrations of sugar simply increased the number of induced cells where the P_{xylA} promoter was active. In the case of arabinose, the response was "all-or-nothing" at low sugar concentrations and then graded at higher concentrations. In other words, the promoter activity in arabinose-induced cells increased with sugars concentrations but did not in xylose-induced cells.

As arabinose is known to inhibit the expression of the xylose metabolic genes, we sought to determine how this crosstalk would affect the single-cell response of cells grown in a mixture of arabinose and xylose. In particular, would we observe mixed, multimodal populations of arabinose and xylose induced cells or would we observe some cells induced for arabinose but not xylose and other cells induced for xylose but not arabinose? To simultaneously measure the expression of the arabinose and xylose metabolic pathways in single cells, we constructed transcriptional fusions of the P_{araB} and P_{xylA} promoters to respective fluorescent proteins Venus and mCherry. These constructs were then integrated single-copy in the chromosome using the λ and φ80 phage attachment sites. This design enabled us to measure the simultaneously the activity of the P_{araB} and P_{xylA} promoters in single cells using flow cytometry. Following the protocol of Afroz and coworkers, the cells were grown for 20 hours in M9 minimal media supplement with 0.4% glycerol and varying amounts of arabinose and xylose starting with a very low OD to ensure that cells were harvested during exponential growth.

We first investigated the response to a single sugar to validate our strains. As shown in **Figure 26**, the cells exhibited a bimodal response to both sugars. In particular, co-existing populations of induced (promoter active) and uninduced (promoter inactive) cells were

observed at intermediate sugar concentrations. We also found that the xylose pathway exhibited an "all-or-nothing" response whereas the arabinose pathway exhibited a more complex response, "all-or-nothing" at low concentrations and graded at higher ones (>1 μM). These results are entirely consistent with those of Afroz and coworkers.

Arabinose and xylose utilization pathways exhibit multimodal response during growth in a mixture of sugars. We next investigated the response to mixtures of sugars. Here we investigated two concentrations of arabinose – 0.5 μM associated with a mixed population (**Figure 28A**) and 1 μM associated with a nearly induced population (**Figure 28B**) and a range of xylose concentrations. At low concentrations (>0.5 μM), xylose did not have an effect, consistent with the single-sugar response (**Figure 26B**). At intermediate xylose concentrations (0.5-1 μM) and 0.5 μM arabinose, we observed four distinct populations: one where both *araB* and *xylB* promoters were inactive, one where only *araB* promoter was active, one where only *xylA* promoter was active, and one where both promoters were active (**Figure 28A**). The same behavior was also observed at 1 μM arabinose (**Figure 28B**) though the results are less pronounced as the population where only the *araB* promoter was active predominates, presumably due to repression of the xylose genes. These results indicate the cells exhibit a mixed, multimodal response to arabinose and xylose with some cells capable of consuming both sugars. At high xylose concentrations (>1 μM), the *xylA* promoter was active in all cells.

Xylose represses the expression of arabinose genes. We made an interesting observation from our promoter activities data. The fraction of cells expressing the *araB* promoter decreased with increased xylose concentration, suggesting that xylose can inhibit the expression of the arabinose pathway. This inhibition becomes more apparent when expression from the P_{araB} is averaged over the entire population. As shown in **Figure 29A**, the P_{araB} promoter activity decreases with increasing concentration of xylose. We note, however, that inhibition by xylose is much weaker than inhibition by arabinose (**Figure 29B**). Arabinose is known to repress the xylose genes (125). However, this is the first report of repression of the arabinose genes by xylose.

Repression of arabinose metabolism is XylR dependent but does not involve any xylose metabolic intermediate. Desai and Rao previously found that arabinose represses the xylose genes through AraC, where arabinose-bound AraC competitively inhibits

activation of the P_{xylA} promoter (125). To determine if the reciprocal mechanism occurs with xylose, namely that xylose-bound XylR competitively inhibits activation of the P_{araB} promoter, we measured the response of the P_{araB} promoter in a Δ*xylR* mutant. No repression by xylose was observed (**Figure 31A**). The results suggest that repression is XylR-dependent as opposed to xylose somehow inhibiting AraC. One caveat is that the xylose utilization genes are not expressed in Δ*xylR* mutant, suggesting that xylose may not be able to enter the cells due to the xylose transporters not being expressed. However, numerous studies have shown that the arabinose transporters are promiscuous and capable of uptaking xylose (125, 132, 208). We also measured the response of the P_{araB} promoter in a Δ*xylAB* mutant to determine whether xylose was inhibiting the arabinose genes or some downstream metabolite. As shown in **Figure 31B**, repression of the arabinose genes by xylose still occurs in the absences of xylose metabolism.

XylR binds to the P_{araC}/P_{araB} promoter region. Based on the results above, XylR likely binds and competitively inhibits the arabinose promoters. To test this mechanism, we performed the electrophoretic mobility shift assay with purified XylR and a DNA fragment containing the divergently arranged P_{araC} and P_{araB} promoters. We also tested binding to the P_{xylA} promoter as a positive control and P_{flgB} promoter from the flagellar regulon as a negative control because the latter was unlikely to be bound by XylR. As shown in **Figure 33**, XylR binds both the P_{xylA} and P_{araC}/P_{araB} promoters in a xylose-dependent manner. In particular, a shift is observed only in the presence of XylR and xylose. Not surprisingly, we observed no shift with the P_{flgB} promoter. These results demonstrate that xylose-bound XylR binds the divergent P_{araC}/P_{araB} promoter.

We next used the FIMO program (209) to search for likely XylR binding sites within the P_{araC}/P_{araB} promoter using the consensus motif derived from known XylR binding sites within the P_{xylA} and P_{xylF} promoters (210). One putative XlyR binding site was identified ($P<0.005$) overlapping the P_{araC} promoter (**Figure 34**). These results suggest that XylR does not directly repress the P_{araB} promoter but rather indirectly represses it by inhibiting expression of AraC. To test this mechanism, we measured expression from the P_{araC}, P_{araE}, and P_{araF} promoters using single-copy transcription fusions to the Venus fluorescent protein at different concentration of xylose. With all three promoters, xylose was found to weakly inhibit their expression (**Figure 35**). Taken together, these results suggest that

xylose-bound XylR binds to P_{araC} promoter and competitively inhibits expression of AraC, which in turn reduces expression from the P_{araB}, P_{araE}, and P_{araF} promoters.

DISCUSSION

The original motivation for this study was to understand how individual *E. coli* cells behave when grown in mixtures of arabinose and xylose. Both sugar utilization systems exhibit bimodal responses (41, 207, 211-214), and our initial goal was to understand how catabolite repression affected the response of individual cells. Our data show that the response to the two sugars is multimodal and that the shape of this distribution is determined by the reciprocal regulation of these two sugar utilization systems. Multiple studies have previously shown that arabinose inhibits the utilization of xylose in *E. coli* by repressing the expression of the xylose metabolic genes (125, 126). The key finding in the present study is that xylose can also inhibit, albeit weakly, the expression of the arabinose metabolic genes. These results demonstrate that the hierarchy between arabinose and xylose is not fixed as previously believed but rather determined by their respective sugar concentrations. An immediate question is why.

The repression of xylose utilization by arabinose ostensibly makes sense because *E. coli* grows at a faster rate on arabinose than it does on xylose due to differences in the route of transport employed (215). In particular, arabinose is primarily transported through the low-affinity AraE proton-symporter whereas xylose is transported through the high-affinity XylFGH ATP-dependent transporter (215). This behavior fits the general pattern observed with hierarchical sugar consumption and presumably reflects the efficient allocation of metabolic resources by the cell (127). If xylose is in excess, however, then it makes sense for the cells not to ignore it and to allocate their metabolic machinery in proportion to the availability of this alternate growth substrate. Indeed, both arabinose and xylose are principally derived from hemicellulose hydrolysates, where xylose is the more predominant sugar (108). Alternatively, mutual repression of these two sugar utilization systems could reflect a division-of-labor strategy, where some cells grow on arabinose and others grow on xylose. As our data show (**Figure 28**), these mixed populations are observed during growth at intermediate sugar concentrations. Furthermore, we observe fewer cells induced for growth on both sugars than would be expected if the pathways operated independently of one another (**Table 6**). For either strategy, be it be proportional allocation or division-of-labor one would expect repression by arabinose to be stronger because arabinose is the better growth substrate. In other words, when the concentrations

are equal, we would expect more cells to be adapted to growth on arabinose than xylose. This in fact is what we observe. Likely, individual cells can employ either strategy, because we observe some adapted to grow on just one sugar and others adapted to grow on both.

While both the arabinose and xylose metabolic genes exhibit a bimodal response to their cognate sugar, the nature of this response is different. As first documented by Afroz and coworkers, the xylose response is all-or-nothing at all sugar concentrations whereas the arabinose response is all-or-nothing at low sugar concentrations and graded at high sugar concentrations. While the physiological significance of these differences is not known (at least in response to a single sugar), they potentially explain why repression by arabinose is significantly stronger than repression by xylose – the arabinose genes exhibit a greater range of expression levels in individual cells than the xylose genes. Presumably, the degree of repression by arabinose-bound AraC also exhibits greater range than xylose-bound XylR.

While transcriptional crosstalk provides one mechanism to explain catabolite repression among non-glucose sugars, other mechanisms have also been proposed. Aidelberg and coworkers recently investigated the selective consumption of six non-PTS sugars (127). They observed a hierarchy in the expression of the genes associated with lactose, arabinose, xylose, sorbitol, rhamnose, and ribose metabolism in *E. coli*. The ordering of hierarchy, with lactose at the top and ribose at the bottom, matches the ordering of the growth rates supported by these sugars. In other words, a sugar is preferred to another if it supports faster growth. These results demonstrate that catabolite repression is widespread among non-PTS sugars. In addition to discovering this hierarchy, they also found that the CRP-cAMP complex differentially activates the promoters for these metabolic genes, where the relative degree of activation follows the same hierarchy. This observation is significant, because previous studies have demonstrated that cAMP synthesis is inversely proportional to the growth rate of the cell (216-218). Thus a cell growing on lactose will produce less cAMP than one growing on any one of the other five sugars. Based on these findings, they proposed an alternate model for catabolite repression among non-PTS sugars based on sequential activation as opposed to one based on competitive inhibition. According to this model, the metabolic genes for the less preferred sugar are not expressed because cAMP concentrations are too low to induce their

expression due to the faster growth supported by the preferred sugar. While this model is appealing, it does not explain the selective utilization of arabinose and xylose. For one, the present study demonstrates that the hierarchy between these two sugars is not fixed but rather is determined by their relative concentrations. Furthermore, Desai and Rao demonstrated that arabinose still inhibits xylose gene expression in a mutant (Δ*araBAD*) unable to metabolize arabinose (125). Moreover, we found in the present study that xylose inhibits arabinose gene expression in a mutant (Δ*xylAB*) unable to metabolize xylose (**Figure 31B**). These results clearly demonstrate that catabolite repression is not due to differential activation by CRP-cAMP but instead transcriptional crosstalk – how else can xylose (or arabinose) repress arabinose (or xylose) gene expression when xylose (or arabinose) is not being metabolized? Our results, however, do not invalidate their general model as only two sugars were considered in the present study. In addition, the relative degree of activation of the arabinose and xylose genes by CRP-cAMP, as reported by Aidelberg and coworkers, is small and substantially less than many other sugars investigated in their work. It is unlikely to result in the hierarchical expression of these two sets of metabolic genes. Whether the differences are sufficiently great to govern the hierarchical expression of other sugar genes is unknown.

In addition to identifying a new facet to the co-regulation of arabinose and xylose metabolism, the present work may also aid efforts to produce chemical and fuels from plant biomass. Plant biomass is a renewable and low-cost feedstock for many value-added compounds. Fermentation of the constituent sugars using engineered microorganisms provides one promising route for the conversion of plant biomass to chemicals and fuels (106, 107). After glucose, xylose and arabinose are the next most abundant sugars in plant-derived hydrolysates. For the fermentation process to be economic and efficient, the microorganisms need to be able to use all of these sugars and ideally at the same time. Not surprisingly, numerous design strategies have been proposed to enable simultaneous sugar utilization in *E. coli* as well as other bacteria and yeast. Most of these efforts have focused on the co-utilization glucose and another sugar (114). Less effort has been directed towards the co-utilization of non-glucose sugar (110, 113, 115, 116). In the context of this work, the work of Groff and coworkers (115) is notable. They engineered an *E. coli* strain capable of simultaneously consuming arabinose and xylose by over-expressing XylR. Presumably,

the increased concentration of XylR counterbalances inhibition by arabinose-bound AraC, thus enabling the expression of both sets of metabolic genes. In the course of designing this strain, they found that arabinose utilization was inhibited when they over-expressed XylR from too strong of a promoter. They hypothesized that high levels of XylR repress expression of the arabinose metabolic and transport genes. The present work validates their hypothesis and furthers shows that xylose can repress arabinose gene expression even under native conditions. It also shows that regulation of these two sugar utilization systems is not as simple as previously believed and further engineering will be required to design optimal co-utilizing strains. An open question concerns how the individual cells of the strain engineered by Groff and coworkers actually behave. Are they in fact simultaneously consuming the two sugars or are there two balanced populations selectively consuming both sugars? The present work demonstrates that both scenarios are possible. An additional question is whether one scenario would be preferred over the other in the context of industrial fermentations; in particular, are generalists preferred to specialists? Nature seems to prefer both. More work is required to resolve these issues.

TABLES FOR CHAPTER 5

Table 6. Population distribution at various concentrations of arabinose and xylose obtained using quadrant gate in FCS Express version 4 (De Novo Software)

Arabinose (µM)	Xylose (µM)	Both Uninduced (%)	P_{araB} Induced (%)	P_{xylA} Induced (%)	Both Induced (%)
0	0	99	1	0	0
0.1		98	2	0	0
0.25		72	27	0	0
0.5		45	55	0	0
0.75		29	71	0	0
1		24	76	0	0
10		3	97	0	0
100		3	97	0	0
0	0	99	1	0	0
	0.1	99	1	0	0
	0.25	98	1	1	0
	0.5	78	0	22	0
	0.75	72	0	28	0
	1	59	0	41	0
	10	17	0	83	0
	100	0	0	99	1
0.5	0	38	62	0	0
	0.1	35	64	0	0
	0.25	36	62	1	1
	0.5	34	54	6	6
	0.75	33	50	10	8
	1	31	47	12	10
	10	14	6	53	27
	100	0	0	86	14
1	0	23	77	0	0
	0.1	19	81	0	0
	0.25	19	79	0	2
	0.5	21	69	3	7
	0.75	19	64	6	12
	1	18	60	8	14
	10	7	5	40	48
	100	0	0	81	19

FIGURES FOR CHAPTER 5

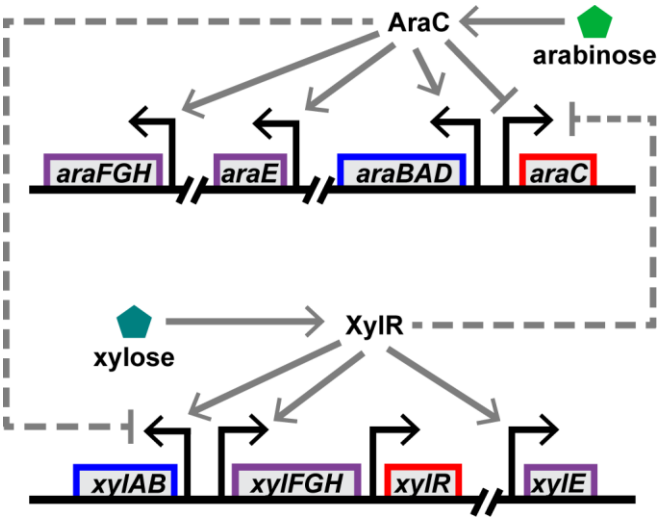

Figure 25. Regulation of the arabinose and xylose sugar utilization systems. Both systems are induced by their cognate sugar. Arabinose-bound AraC induces the expression of the arabinose metabolic (*araBAD*), high-affinity transporter (*araFGH*), and low-affinity transporter (*araE*) genes (135). In addition, arabinose-bound AraC represses its own expression. Xylose-bound XlyR induces the expression of the xylose metabolic (*xylAB*), high-affinity transporter (*xylFGH*), and presumably low-affinity transport (*xylFGH*) genes (138). Xylose-bound XylR does not appear to regulate the P_{xylR} promoter though it may induce its expression due to transcription from the upstream P_{xylF} promoter. In addition, the two systems are subject to transcriptional crosstalk. Arabinose-bound AraC represses the expression of the xylose metabolic genes (125) and, as demonstrated in this study, xylose-bound XylR represses the expression of the arabinose metabolic genes. Furthermore, the arabinose and xylose systems are both repressed by glucose.

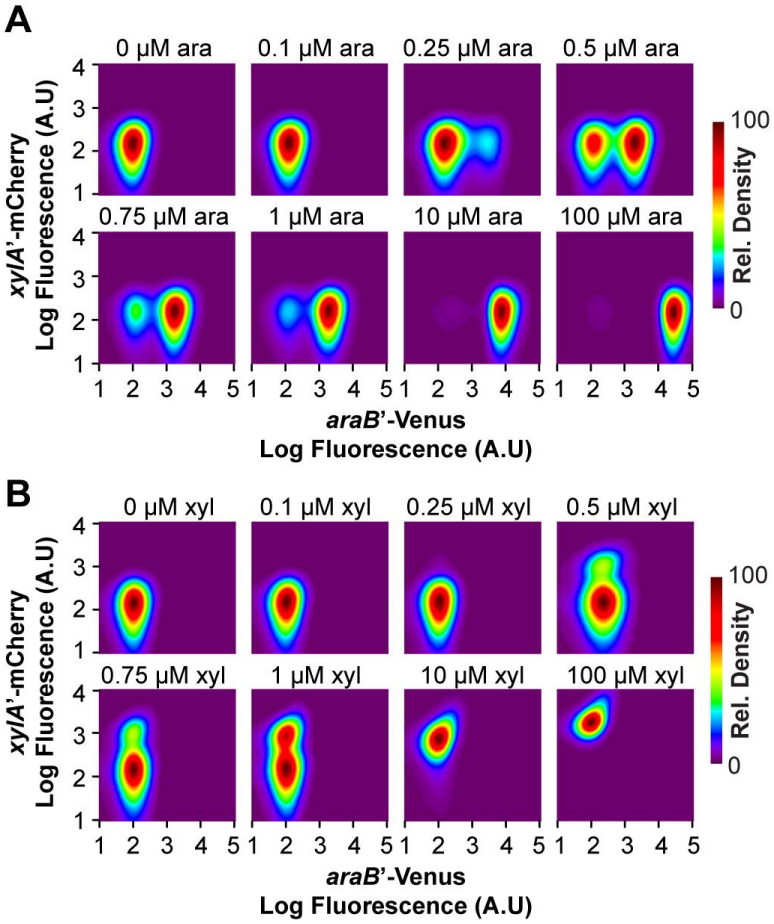

Figure 26. Both the arabinose and xylose systems exhibit a bimodal response to their cognate sugar. The response was measured in a single strain containing single-copy, chromosomal transcriptional fusions of the P_{araB} promoter to the Venus fluorescent protein and the P_{xylA} promoter to the mCherry fluorescent protein. The cells were grown in M9 minimal media containing 0.4% glycerol and varying concentrations of arabinose (**A**) or xylose (**B**) as noted in the figure panels. **Table 6** lists the fraction of cells in the uninduced and induced states.

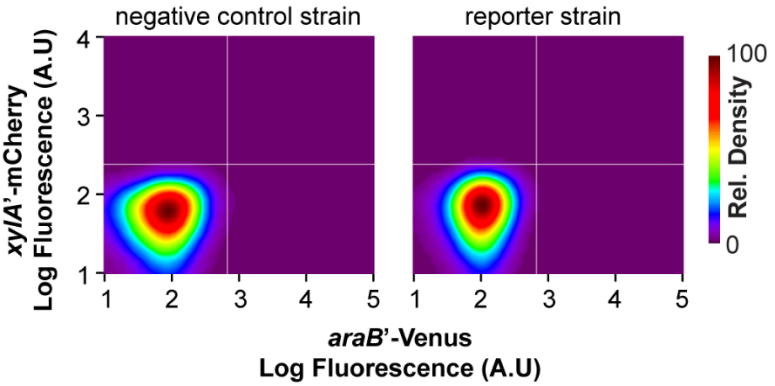

Figure 27. Cells grown without any sugar provide the same response as the negative control strain. The observed fluorescence level of the negative control strain was used as a reference for uninduced population of the reporter strain. The reporter strain contains single-copy, chromosomal transcriptional fusions of the P_{araB} promoter to the Venus fluorescent protein and the P_{xylA} promoter to the mCherry fluorescent protein. The negative control strain lacks both fluorescent proteins.

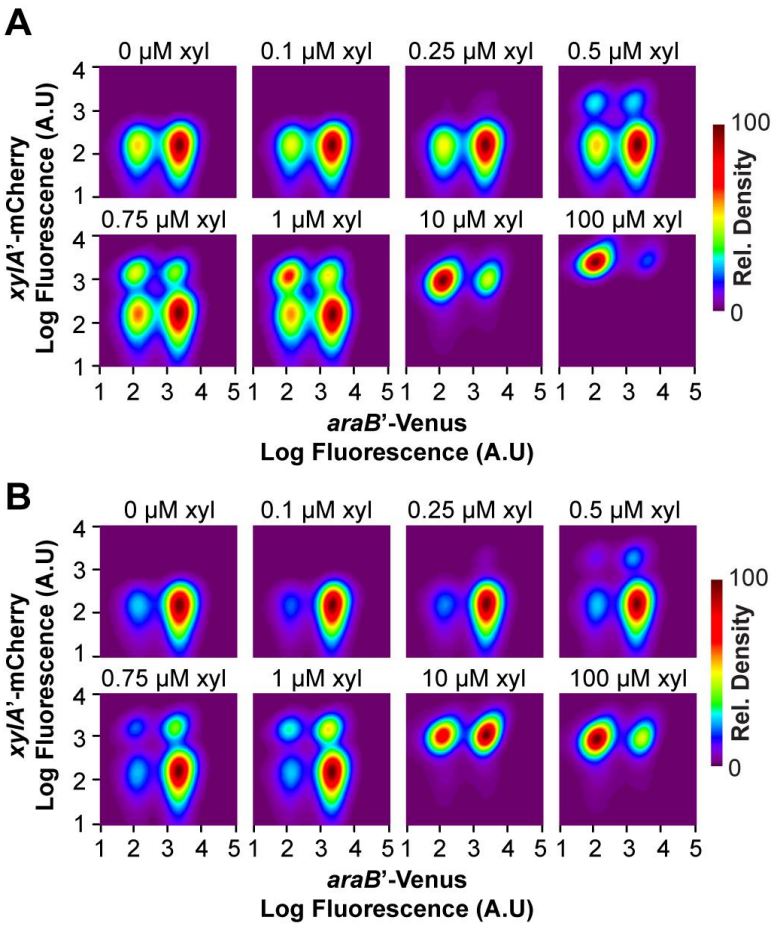

Figure 28. The arabinose and xylose systems exhibit a multimodal response to two sugars. The response was measured in a single strain containing single-copy, chromosomal transcriptional fusions of the P_{araB} promoter to the Venus fluorescent protein and the P_{xylA} promoter to the mCherry fluorescent protein. The cells were grown in M9 minimal media containing 0.4% glycerol, 0.5 μM (**A**) or 1 μM (**B**) arabinose, and varying concentrations of xylose as noted in the figure panels. **Table 6** lists the fraction of cells in the uninduced and induced states.

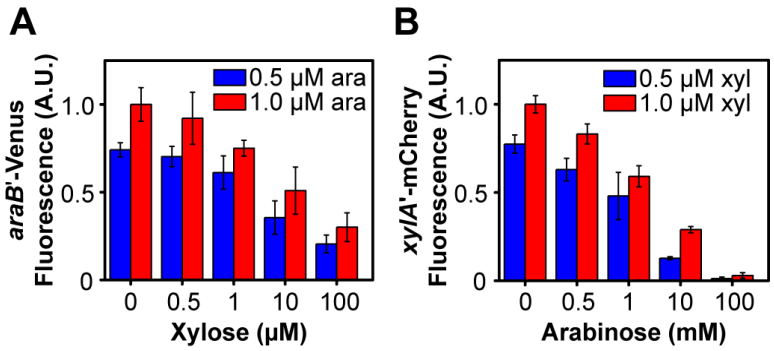

Figure 29. The arabinose and xylose systems are subject to transcriptional crosstalk. **A**. Xylose represses expression of the arabinose metabolic genes. **B**. Arabinose represses expression of the xylose metabolic genes. The response was measured in a single strain containing single-copy, chromosomal transcriptional fusions of the P_{araB} promoter to the Venus fluorescent protein and the P_{xylA} promoter to the mCherry fluorescent protein. The cells were grown in M9 minimal media containing 0.4% glycerol and the specified concentrations of arabinose and xylose. Note that repression by arabinose is stronger than repression by xylose. Fluorescence values are averaged from single-cell, flow-cytometry data. Errobars denote the standard deviation from three experiments performed on separate days. Histograms are provided in **Figure 30**.

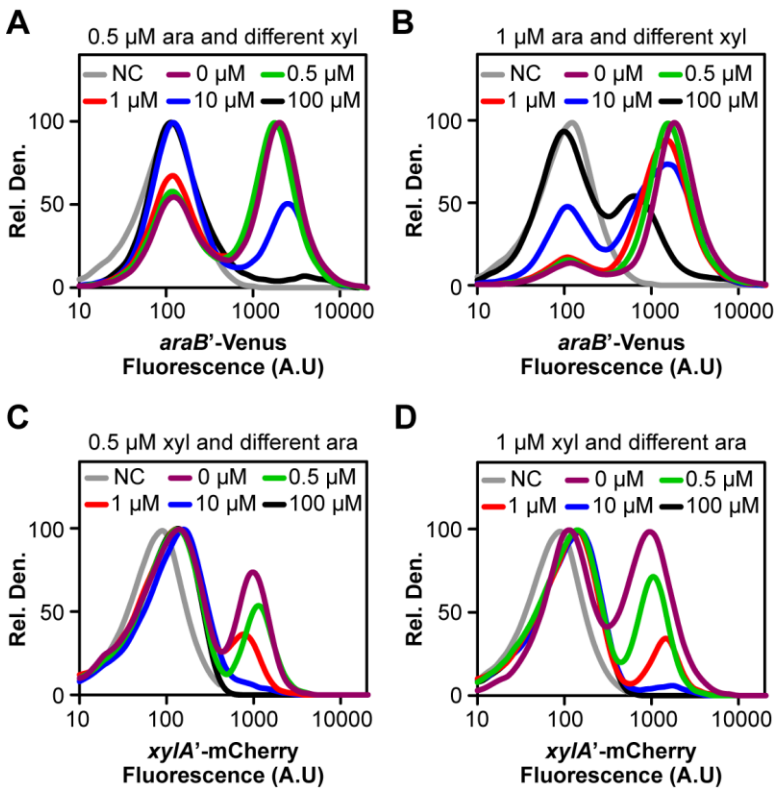

Figure 30. Single-cell data for Figure 29. NC is negative control strain with no reporters.

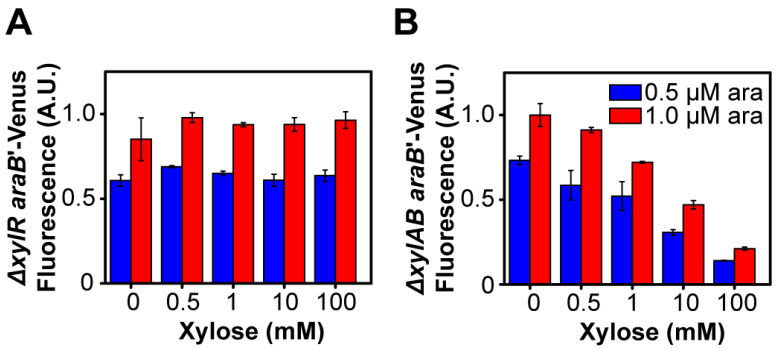

Figure 31. Xylose does not repress arabinose gene expression in the absence of (**A**) XylR (Δ*xylR*) but does in the absence of (**B**) xylose metabolism (Δ*xylAB*). The response was measured in the specified strain containing single-copy, chromosomal transcriptional fusions of the P_{araB} promoter to the Venus fluorescent protein. The cells were grown in M9 minimal media containing 0.4% glycerol and the specified concentrations of arabinose and xylose. Fluorescence values are averaged from single-cell, flow-cytometry data. Errobars denote the standard deviation from three experiments performed on separate days. Histograms are provided in **Figure 32**.

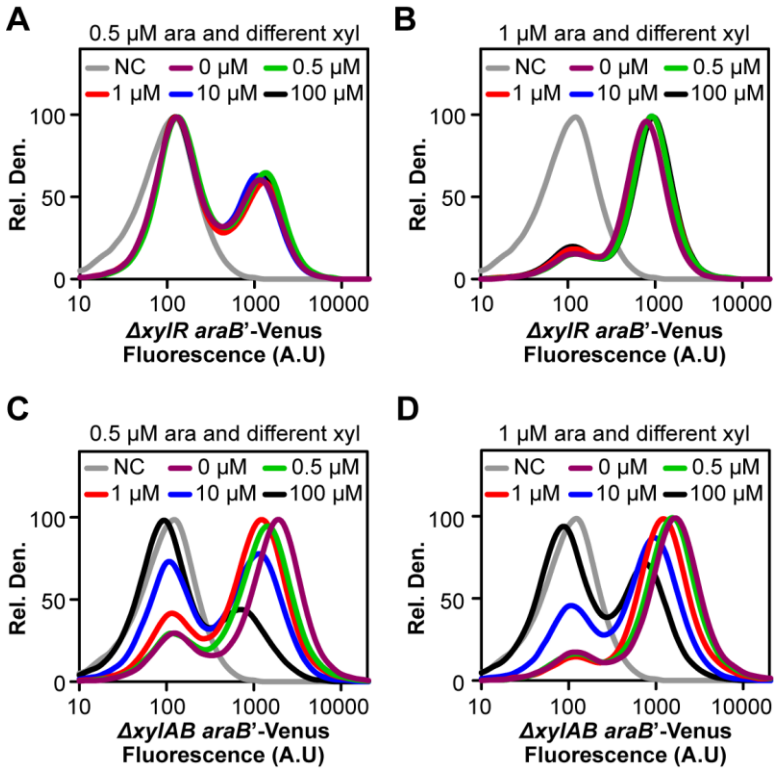

Figure 32. Single-cell data for **Figure 31**. NC is negative control strain with no reporters.

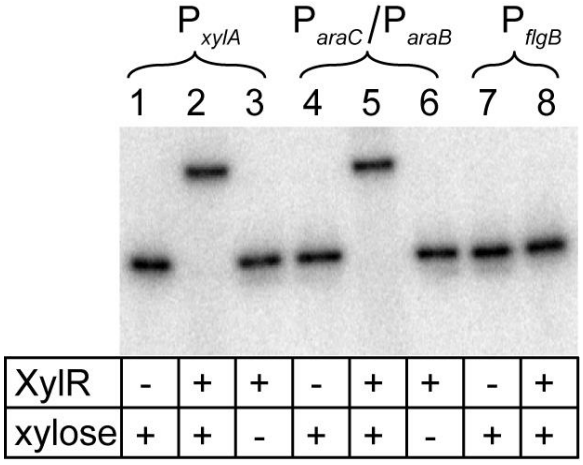

Figure 33. XylR binds the P_{araC}/P_{araB} promoter in a xylose-dependent manner as determined using the electrophoretic mobility shift assay. The P_{xylA} promoter was included as a positive control and the P_{flgB} promoter as a negative control.

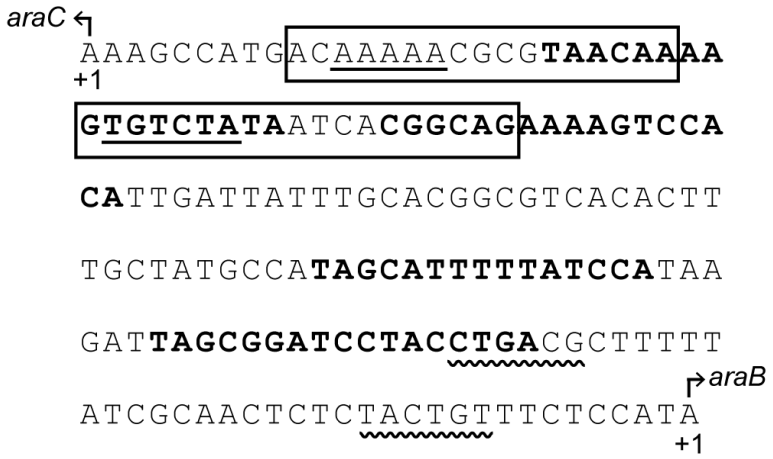

Figure 34. Nucleotide sequence of P_{araC}/P_{araB} promoter region. The -10/-35 region of P_{araC} and P_{araB} promoters are shown with straight and wavy underlines respectively. The AraC binding sites are shown in bold face. These annotations were taken from RegulonDB (210). The putative XylR binding sites, as determined through sequence analysis, are boxed.

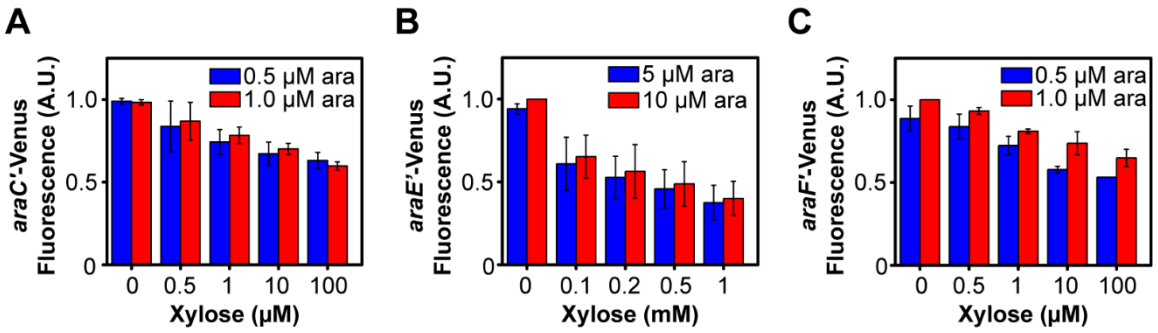

Figure 35. Xylose represses expression of arabinose regulator *araC* **(A)** and transporters *araE* **(B)** and *araFGH* **(C)**. The response was measured in strains containing single-copy, chromosomal transcriptional fusions of the P_{araC}, P_{araE} or P_{araF} promoter to the Venus fluorescent protein. The cells were grown in M9 minimal media containing 0.4% glycerol and the specified concentrations of arabinose and xylose. Fluorescence values are averaged from single-cell, flow-cytometry data. Error bars denote the standard deviation from three experiments performed on separate days. Histograms are provided in **Figure 36**.

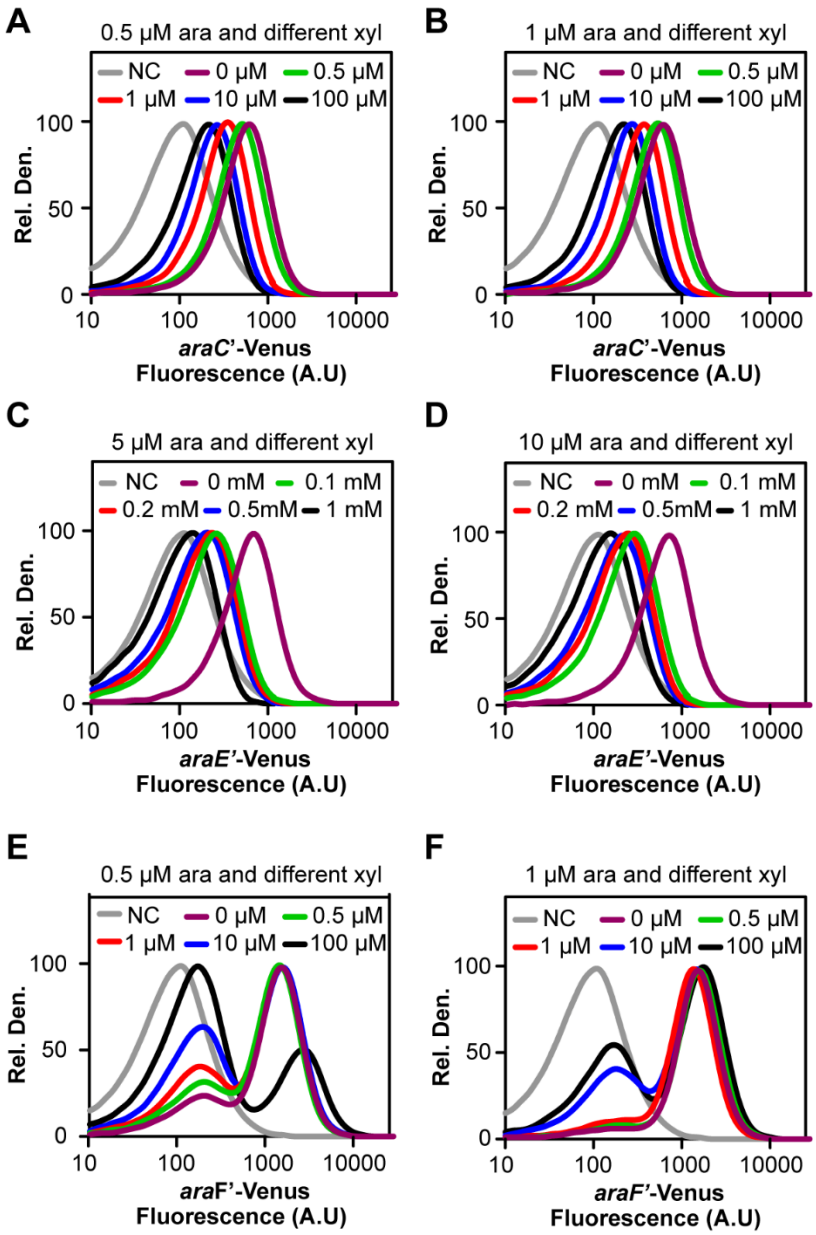

Figure 36. Single-cell data for **Figure 35**. NC is a negative control strain with no reporters.

CHAPTER 6
CONCLUSION AND FUTURE DIRECTION

SIGNIFICANCE

Many bacteria employ flagella for motility. These bacteria are often not constitutively motile but become so only in response to specific environmental cues. The most common is nutrient starvation. Interestingly, in *S. enterica*, nutrients inhibit the expression of flagella, suggesting that motility is used for purposes other than foraging. In this work, we investigated how nutrients affect motility in *S. enterica* and found that nutrients tune the fraction of motile cells within a population. We determined the mechanism governing this tunable response. These results uncover a new facet to motility in *S. enterica* and demonstrate that nutrients serve not only to guide where bacteria move but also the fraction that do so.

Glucose, xylose and arabinose are the most abundant sugars in plant biomass. Developing efficient fermentation processes that convert these sugars into chemical and fuels will require strains capable of co-utilizing these sugars. *Escherichia coli* natively consumes the glucose first, arabinose second, and xylose third. While much is known about the preferential utilization of glucose, less is known about the preferential utilization of arabinose. Previous studies found that arabinose represses the expression of the xylose metabolic genes. In the present study, we found that xylose also represses the expression of the arabinose metabolic genes, leading to mixed populations of cells capable of utilizing arabinose and xylose. These results further our understanding of mixed-sugar utilization and may aid strain design.

FUTURE DIRECTION

Effects of growth rate on flagellar gene expression in *Salmonella enterica*. Yeast extract is a complex mixture of various compounds. In our effort to isolate a single compound that activates transcription from class 2 promoters, we tested various components of yeast extract. However, we were unable to isolate a single compound that was capable of repressing YdiV expression and inducing flagellar genes as strongly as yeast extract does. One additional possibility is that the P_{ydiV} promoter responds to the

growth rate of the cell (172, 219). Although our experimental data provided no support for this possibility, a caveat is that we used relatively high concentrations of nutrients that supported approximately equal growth rates except in the case where yeast extract is completely absent. If flagellar gene regulation also responds to the growth rate of cell, as one might expect, then our experiments would not have detected this phenomenon, as our conditions were chosen to keep the growth rate nearly constant.

To get the complete picture of flagellar regulation in *S. enterica*, a comprehensive study is needed to investigate the effects of growth rate on flagellar gene expression. A valid hypothesis is that the P_{ydiV} promoter responds to the growth rate of the cells and acts as a valve to tune flagellar gene expression in response to the growth rates. Chemostat can be used to control the growth rate. Since steady state growth-rate (μ) is equal to the dilution rate (D) in a chemostat, the growth can be precisely controlled. To monitor *ydiV* and flagellar gene expression simultaneously, a two color reporter strain in which activities of both the P_{flhB} promoter and the P_{ydiV} promoter can be measured can be used.

A previous study did not report any significant changes in *ydiV* transcription in response to nutrients. Instead, only changes in the level of YdiV protein were observed, suggesting that nutrients principally regulate YdiV via a post-transcriptional mechanism (104). The growth rate can be speculated to have similar effects on YdiV translation but not transcription. We, on the other hand, found that yeast extract decreased expression from the P_{ydiV} promoter, indicating that the mechanism involves a significant transcriptional component. To investigate at which level growth rates affect YdiV expression, P_{ydiV} fused to CFP and YdiV fused to SGFP in a single strain can be employed to measure both transcriptional and translational effects. In general, cell can be grown in a chemostat with various dilution rates. The samples collected at various dilution rate can analyzed using flow cytometer. These experiments will elucidate the role of growth rates on flagellar gene expression and also the role of YdiV in tuning these responses.

Effects of cell growth on switching dynamics in the metabolism of two sugars: arabinose and xylose in Escherichia coli. A genetic circuit in a cell is not an isolated system. In addition to the regulation by products of the specific gene regulatory network, they are under global regulation that depends on the physiological state of the cell. Physiological state of a cell may be represented by the abundance of DNA and RNA

polymerases, ribosomes and expression of specific sigma factors which affects the machinery of DNA replication, transcription and translation (219). Only in an ideal environment where the state of the cell remains unchanged is it possible to treat a gene regulatory network as an isolated system. In a real environment, the physiological state of a cell is constantly changing. The gene expression from a specific gene circuit may also affect global regulation. The coupling of physiological state of a cell and gene expression complicates the quantitative studies of individual gene circuits.

The physiological state of an exponentially growing bacteria, to a large extent, has been shown to be growth rate dependent (219-222). Growth rate of a bacteria in a batch culture depends on the nutrient-content of the medium. As an example, net doubling time of *E coli* in a batch culture varies from 20 minutes to many hours. By controlling the growth rate of bacteria, it is possible to closely mimic a specific physiological state of cells.

Previous study by You *et al* characterized the effects of growth rates on catabolism of various carbon sources and found that the catabolic gene expression decreased linearly with increasing growth rate for carbon limiting conditions whereas the opposite was found to be true for non-carbon limiting conditions (nitrogen and sulfur limitations) (216). This effect was demonstrated to be a consequence of cAMP-dependent signaling: concentration of cAMP decreased with increasing growth rate for carbon limiting conditions and vice versa for non-carbon limiting conditions. These experiments utilized transcriptional fusion to *lacZ* reporter to quantify gene expression in bulk. How growth rates affect gene expression at the single-cell level and what role it plays in phenotypic switching in sugar utilization system is still not well understood. The effects of growth rates on utilization of two pentose sugars: arabinose and xylose can be investigated further to answer these questions.

In a study, Fritz *et al* characterized single cell gene expression dynamics of the arabinose utilization system and found that cells switch to on-state (phenotypic state where arabinose utilization genes are active) with various time delay and this variation was tuned by the inducer concentration (207). A similar experiment can be performed to gauge the dynamics of switching within arabinose and xylose operons in the presence of both sugars. Live cell imaging can be utilized to track the dynamics of switching.

Role of transcriptional cross talk on hierarchical utilization of other sugars. Aidelberg and coworkers recently investigated the selective consumption of six non-PTS

sugars (127). They observed a hierarchy in the expression of the genes associated with lactose, arabinose, xylose, sorbitol, rhamnose, and ribose metabolism in *E. coli*. As discussed in chapter 5, our observations for arabinose and xylose did not follow their model. Our results, however, do not invalidate their general model as only two sugars were considered in our study. Further investigation of transcriptional cross talk between these sugar utilization systems will advance our understanding of these systems. Simple experiments can be carried out to measure the expression of metabolic genes of each sugar in the absence and presence of other sugars using a combinatorial approach. Transcriptional fusions to fluorescent protein Venus can be employed to measure promoter activities. Our preliminary results have shown that lactose represses the utilization of xylose but not arabinose. Moreover, sugar utilization can be measured using high pressure liquid chromatography (HPLC) to observe the order of their consumption.

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