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Regulation and Function of the Swarming Inhibitor *disA* in

Proteus mirabilis

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Doctor of Philosophy

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Regulation and Function of the Swarming Inhibitor *disA* in *Proteus mirabilis*

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Bree Ann Szostek Barker
Bachelor of Science, Molecular Biology, University of Texas at Dallas, 2008
Cum Laude and Collegium V Honors

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By: Bree Ann Szostek Barker

Abstract

Proteus mirabilis is a Gram negative bacterium best known for its form of multicellular surface motility, termed swarming. Three to four hours after encountering a solid surface *P. mirabilis* differentiates from a vegetative rod into an enlongated, aseptate, polyploidy, hyperflagellated swarmer cell. Swarmer cells interact with one another to migrate away from the central inoculum. After migration the cells undergo a process of consolidation where swarmer cells return to their vegetative form to prepare for another onset of swarming. While a great deal is known about the regulation and initiation of differentiation and migration, very little is known about how the cell undergoes consolidation. Our lab previously identified a decarboxylase inhibitor of swarming, *disA*, which completely abolishes swarming when overexpressed. Homology to aromatic amino acid decarboxylases and an increase in *disA* transcription over the swarm cycle led us to hypothesize DisA may produce a consolidation signal, particularly phenethylamine. We have further characterized the *disA* locus by identifying the transcriptional start site as a guanine residue seventy basepairs upstream of the open reading frame. Site-directed mutagenesis also identified an extended -10 promoter element. Further work identified mutation of *umoB*, the *P. mirabilis* homologue of *igaA*, increased *disA* expression and demonstrated the upregulation is mediated by the Rcs phosphorelay. Additionally, exogenous addition of phenethylamine decreased *disA* expression. We have also demonstrated overexpressing *disA* is able to inhibit motility in other Gram negative organisms known to swarm, but does not affect organisms with *flhDC* independent motility. Significant work has been performed to identify the product and substrate of DisA. Metabolomic analysis of *disA* mutant and overexpressing strains suggests DisA may be involved in regulating swarming by modifying the levels of central metabolic intermediates. Supplementation of media with 10mM succinate, fumarate or malate, three intermediates of the tricarboxylic acid cycle (TCA), increased swarming motility of a *disA* mutant strain under anaerobic conditions. Tested TCA cycle intermediates had no effect on motility of the *disA* mutant during aerobic growth. Wild-type motility was also unaffected by all tested metabolites under aerobic and anaerobic growth. These data suggest that DisA may function to regulate swarming by altering the levels of central metabolites in the cell, particularly fumarate and aspartate.

The Regulation of the Swarming Inhibitor *disA* in *Proteus mirabilis*

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Chapter 1: Introduction

Proteus mirabilis

Proteus mirabilis is a Gram-negative member of the family *Enterobacteriaceae*, an opportunistic pathogen, and best known for its form of multicellular surface motility termed swarming (107). The *Proteus* species is found in several different environments, including soil, water, the mammalian gastrointestinal tract (116, 136), and as an opportunistic pathogen in the mammalian urinary tract (71, 111). Five species of the *Proteus* genus have been identified: *P. mirabilis* and *P. vulgaris*, both studied by Gustav Hauser, as well as *P. penneri, P. myxofaciens,* and *P. hauseri* as well as three unnamed genotypes (111).

Originally identified by Hauser in 1885, the organism was given the genus *Proteus* in reference to the "Greek god of the ocean who took many shapes to escape questioning" (54). The morphological changes in cell structure that Hauser noted nearly 130 years ago still fascinate scientists today. The morphology phenomena Hauser described is now known to be a differentiation process necessary for *Proteus* to undergo swarming motility. In order to swarm over solid surfaces, *Proteus* must differentiate from vegetative, peritrichously flagellated, rod-shaped swimmer cells into elongated, aseptate, multi-nucleate, hyper-flagellated swarmer cells (124). This process is discussed in depth throughout this chapter and Chapter 2.

I. Disease

In healthy individuals *P. mirabilis* is non-pathogenic (110). However, unlike many of its fellow *Enterobacteriaceae* and urinary pathogens, *P. mirabilis* is most frequently isolated from urinary tract infections (UTI) in individuals undergoing urinary catheterization (156, 157). *Proteus* accounts for 12% of complicated UTI and is the second leading cause of catheter-associated urinary tract infection (CAUTI) in patients requiring long term catheterization (137). It is believed that self-contamination of the urinary tract from the natural reservoir in the human gastrointestinal tract is the main source of *Proteus* based CAUTI (28).

P. mirabilis infections account for 44% of all UTIs from long-term catheterization (157). Most organisms responsible for CAUTI are fecal contaminants or part of the normal flora that gain access to the urinary tract during the catheterization process (28). While most CAUTIs are asymptomatic, symptomatic disease can range from mild fevers and cystitis to severe bacteremia, pyelonephritis and renal scarring (82, 104). If untreated urosepsis and death are possible. Frequently these infections, particularly in *Proteus* associated cases, are recurrent due to the inability of the body to fully void the bladder during catheterization, providing the bacteria a reservoir for continued survival (52). Additionally, the catheter provides a surface for bacterial adhesion, allowing the formation of biofilms which provide the bacterium protection from host defenses and antibiotic treatment (34). *Proteus* produces a myriad of virulence factors that permit colonization and disease progression once present in the urinary tract (110).

a. Urease

The enzyme urease, encoded by *ureABC*, is a multimeric nickel metalloenzyme and an important factor in allowing *P. mirabilis* to maintain infection within the bladder and kidneys (66, 103). Expression is stimulated by the presence of urea, the target molecule of urease. Urease hydrolyzes urea to form ammonia and carbon dioxide, creating an alkaline pH in the bladder (109). An alkaline environment in the bladder permits the sedimentation of divalent cations leading to the accumulation of struvite (magnesium ammonium phosphate) and apatite (calcium phosphate). Formation of salt compounds leads to the production of bladder stones and permits the development of crystalline biofilms, which frequently assemble on the catheter (49, 139). The presence of crystalline biofilms makes catheter removal difficult, often leaving surgical removal as the only recourse while both the biofilm and stone formation provides *P. mirabilis* a protective niche against antibiotic therapies and host antimicrobials (32, 82).

The increase in pH due to urease activity serves an additional purpose beyond stone formation. The accumulation of ammonia due to urea degradation damages host tissue, providing a source of carbon and nitrogen, both of which are limiting in the urinary tract (32). Studies in the ascending mouse model of *Proteus* infection have demonstrated that urease negative strains have no deficit in colonization but are attenuated at later time points in the bladder and kidneys (65). Transcription of *ureABC* increases during swarming, suggesting a correspondence between motility and virulence (4, 41, 65, 67). However, it has been demonstrated that cells do not need to be motile in order to produce increased levels of urease (67).

b. IgA Protease

The *Proteus* IgA protease, ZapA, is a member of the Serralysin family of zinc dependent metalloproteases (158). Unlike many other IgA proteases, ZapA is capable of fully degrading IgA as well as cleaving IgG and additional proteins involved in the complement system, cytoskeleton assembly, cell matrix integrity and cationic antimicrobial peptides (14, 74, 129). A conserved motif at the C-terminus suggests that ZapA may be exported by an ABC transporter (158). Alkaline pH (pH 8) is optimal for ZapA function, which is congruous with the knowledge that *P. mirabilis* frequently finds itself in alkaline environments during infection due to the activity of urease (discussed above) (book cit).

Like many other virulence factors, IgA protease activity is increased during swarming and mutants in *zapA* are attenuated in the mouse model of ascending infection (4, 153). While the primary role of IgA protease is immune evasion, a secondary role has been proposed where ZapA degrades host proteins for amino acid acquisition.

c. Haemolysin

The *Proteus* haemolysin is encoded by *hpmAB*, where HpmA serves as the calcium-independent protoxin while HpmB cleaves HpmA into its active form and aids in its secretion from the cell (150, 162). HpmA functions by forming pores in host cells leading to an efflux of sodium and consequently lysis. It is hypothesized that haemolysin may aid *P. mirabilis* in spreading to the kidneys as hemolytic activity is correlated with invasiveness (32, 122, 126). Like IgA Protease and urease, haemolysin production is

overexpressed in swarmer cells (4, 118). However, the importance of haemolysin in infection is unclear in the literature. One study reports hemolytic mutants are not deficient in urinary tract colonization (141) while another demonstrated non-hemolytic strains are less virulent and have higher lethal doses via intravenous inoculation (121).

d. *Proteus* Toxic Agglutinin

Proteus toxic agglutinin (Pta) represents the second toxin virulence factor identified in *P. mirabilis*, the first being haemolysin. Encoded on the pathogenicity island ICE*Pm1* (integrative and conjugative element of *P. mirabilis* 1) Pta is unusual in that it demonstrates bifunctionality, acting as both an adhesin and a toxin. Its outer-membrane domain mediates cell-cell aggregation but also contains an α-domain that functions as an alkaline protease, causing the lysis of kidney and bladder cells (2, 43). The possession of an alkaline protease domain is consistent with the possibility of induction due to the increased pH caused by urease activity within the bladder. Strains mutant in *pta* cause reduced pathology and have colonization defects in the bladder, kidney and spleen (1, 2). It's worth noting that strains with mutantions in both *pta* and *hpmA* have a more severe reduction of cytotoxicity than either single mutant, demonstrating an additive effect of Pta and HpmA (1). This may explain the why *hpmA* mutants do not demonstrate a decrease in pathogenicity as Pta is able to compensate for the lack of haemolysin.

e. Glutamate Dehydrogenase

Glutamate dehydrogenase (GdhA) mediates the conversion of ammonia and α-ketoglutarate to glutamine and oxaloacetate. During infection *P. mirabilis* upregulates

gdhA while downregulating glutamine synthetase, suggesting that GdhA may be upregulated to utilize the ammonia produced by urease to form glutamate. The decreased colonization phenotype in the bladder, kidneys and spleen of a *gdhA* mutant during infection demonstrates the importance of this pathway during infection (120). The importance of glutamate and glutamine in swarming motility will be discussed later in this chapter.

f. Iron Chelation

P. mirabilis was originally believed to lack siderophores, however recently at least 21 putative iron acquisition systems have been identified (8, 119). Proteobactin and the *nrp* operon are two that have been recently well characterized. Proteobactin is encoded by *pbtABCDEFGHI* (59). The *nrp* operon is located on the ICE*Pm1* pathogenicity island (43), upreguated in iron limited environments (45) and has homology to the Yersiniabactin systhesis genes but is functionally distinct (59). Upregulation of both systems has been demonstrated *in vivo* and is more prevalent in isolates from UTI than non-UTI isolates (59, 120). Mutation in both siderophores demonstrated that both are important for colonization of the bladder but mutation of the Proeobactin system had a negative impact on overall fitness (59).

g. Fimbriae

Proteus mirabilis has 17 different fimbriae encoding operons that span five classes (119). While fimbriae are typically involved in attachment to assist in establishing infection little is known about many of the fimbriae encoded by *P. mirabilis* with the greatest

research focused on the MR/P, mannose-resistant *Proteus*, family. Generally flagella and fimbriae regulation are inversely related given that fimbriae promote adhesion and flagella promote motility. The MR/P fimbriae operon encodes MrpJ, which is able to repress flagella production during fimbrial expression (12, 81, 117). When *P. mirabilis* is collected from the CBA mouse model of ascending infection MR/P fimbriae are expressed, thus we know MR/P fimbriae are activated *in vivo* (10, 11). Expression of MR/P is regulated by phase variation. *mrpI* encodes a site-specific recombinase that can excise a regulatory region upstream of the operon and alter its orientation, turning expression on or off (80, 164).

Li et. al. demonstrated that MR/P fimbriae have a role in attachment in the kidneys and bladder and to be involved in initial biofilm formation in the bladder (80). When expressed MR/P fimbriae facilitate attachment to the luminal surface of the bladder uroepithelial cells, permitting biofilm formation and allowing attachment to the renal tubular epithelial cells in the kidneys. Mutants locked in MR/P phase-off were outcompeted by wild-type in co-infection and had reduced colony counts in the urine, bladder, kidney and caused less severe disease. However, when inoculated alone phase-off mutants established infection equal to wild-type but failed to colonize the kidneys. Phase-on mutants outcompeted wild-type during co-infection, demonstrating that MR/P fimbriae are important in establishing infection but are most significant in kidney infection initiation (80).

MN/K fimbriae (mannose-resistant fimbriae *Klebsiella*-like) are less studied in *P. mirabilis* but have been shown to facilitate catheter adhesion, suggesting these too have a role in establishing infection and may assist in biofilm formation (132). Uroepithelial cell adhesion/non-agglutinating fimbriae also possess the ability to adhere to uroepithelial cells and some kidney lines but also resemble fimbriae involved in intestinal colonization (6, 78, 132). This homology suggests that fimbriae may be involved in establishing the *Proteus* reservoir in the gastrointestinal tract.

An additional adhesin has recently been discovered, the autotransporter AipA. Characterized by Alamuri *et.al.* AipA has been shown to mediate adhesion to human kidney and bladder epithelial cell lines and *in vivo* work demonstrates that AipA contributes to colonization of the kidneys and spleen in the mouse model of ascending UTI (2). However, an *aipA* mutant is still able to colonize the bladder and kidneys at high levels, likely due to the function of multiple other virulence factors and adhesins (2).

II. Flagella

a. Structure and Assembly

The assembly of the bacterial flagella has been extensively studied (reviewed in Macnab (88)). The first component assembled is the MS-Ring. Embedded in the inner membrane, it acts as the base for hook-basal body assembly. Following MS-Ring assembly the C-Ring attaches to the cytoplasmic side of the MS-Ring. This complex forms a type three secretion system allowing for the transport of additional structural proteins to the periplasm. All additional proteins involved in flagella assembly are transported by the

secretion system with the exception of the P-Ring and L-Ring, which associate with peptidoglycan and the outer membrane, respectively. An ATPase complex associates with the C-ring in the cytoplasm and assists in the secretion of flagellar proteins. The proximal and distal rods also assemble in the periplasm. Once the periplasmic components of the flagellum are assembled, components for hook construction are secreted followed by secretion of FlaA, the building block of the flagellar filament. The hook, rod and filament have capping proteins; these serve to control assembly size as well as protein export.

In a fully assembled flagella a motor complex is associated with the C-ring in the cytoplasm and powers flagellar rotation by utilizing the proton motive force. The MS-ring generates torque, leading to rod rotation (19). The hook acts as a flexible connection between the filament and rod proteins, allowing the flagella to rotate at different angles than the rods. Bacterium can have several different patterns of flagellation including a single flagellum at a cell pole (monotrichous), several flagella at a pole (lophotricous), flagella at both poles (amphitricous), or flagella distributed across the cell body (peritrichous). *P. mirabilis* is a peritrichously flagellated bacterium and contains a bi-directional flagellar rotor, allowing for clockwise and counter-clockwise rotation of the flagella that results in tumbling or running, respectively (18). Whether running or tumbling occurs depends on the nutrient gradient cells are exposed to and the methylation state of the chemotaxic proteins, discussed later in this chapter.

b. Regulation

Regulation of flagellar synthesis is broken down in to a hierarchy of three gene classes: Class I, II, and III (reviewed in (27)). Class I is composed solely of the two-gene operon *flhDC*, encoding the master regulator of swarming $FlhD_4C_2$. Once assembled the heterohexamer acts as a transcription factor to facilitate the activation of Class II genes. Class II is more expansive than Class I and consists of many structure-encoding genes including the components of the hook-basal body structure of the flagella. Additionally Class II contains *fliA*, encoding the swarming sigma factor, σ^{28}, and *flgM*, encoding the cognate anti-sigma factor (114). Class III genes are activated by σ^{28} and include late structural genes, such as *flaA* which encodes the flagellar filament, the motor and torque proteins responsible for flagellar rotation, as well as chemotaxis genes (113).

The temporal regulation of the expression of the three gene classes is imperative for proper assembly of the flagella. Genetic regulation is exceptionally important in assuring proper synthesis, as is physical regulation based on substrate sequestration and structure formation. As discussed above, many components of the flagella are secreted via the hook-basal body type three secretion system. The early and mid-phase secreted proteins are used to assemble the hook-basal body apparatus. How the cell senses when the hook-basal body is complete is not fully understood, but it is known that at least two proteins, FliK and FlhB, are involved. FlhB is part of the export apparatus involved in assembling the hook-basal body while FliK measures the hook length (130). Once the hook is fully assembled FliK interacts with FlhB, the autocatalytic domain of FlhB is activated and the

type three secretion system changes substrates (97). It is not until this secretion system is fully assembled that FlgM, the anti-sigma factor, is secreted to permit activation of Class III genes by σ^{28} (62, 76). Capping proteins also exist for the rod, hook and filament structures to selectively permit the secretion of some proteins either for export or polymerization of the flagella. This system is highly conserved between *P. mirabilis*, *Escherichia coli* and *Salmonella typhimurium*.

c. *flhDC* Regulation

The *flhDC* operon is regulated by many different pathways and can be affected either at transcriptional or post-translation levels. There are several transcriptional regulators involved in both *flhDC* repression and activation, which will be highlighted in this and later chapters (Chapter 2, (107)). Leucine-responsive regulatory protein (Lrp) activates transcription of *flhDC* during the swarm cycle. A mutation in Lrp abrogates swarming due to the strongly decreased transcription of *flhDC* (55). Given that swarming is an energy intensive process, Lrp may act to regulate swarming based on nutrient availability. Many repressors of *flhDC* have been characterized, including RcsB (30, 107).

RcsB acts as the response regulator in the Rcs Phosphorelay, a highly conserved and well-studied signal transduction system in Enterobacteria (61, 90). The role of RcsB in *flhDC* regulation has been demonstrated in both *Escherichia coli* and the *Salmonella* genus (24, 44, 75, 155). A hyper-swarming phenotype is observed when *rcsB* as well as other members of the Rcs phosphorelay are mutated (15, 30, 83, 84). While binding of RcsB upstream of *flhDC* has not been demonstrated in *P. mirabilis*, there is a near

consensus (13/14 basepairs) Rcs box upstream of *flhDC*, suggesting there is direct RcsB regulation of *flhDC* (160). A more in depth review of *flhDC* regulation is available in Chapter 2.

d. Chemotaxis

Chemotaxis refers to the method bacteria use to alter their direction of motility in response to the levels of nutrients and repellents in the environment (reviewed in (87, 151 and 152)). Chemotaxis primarily refers to modulation of swimming motility where the bacteria will continue to swim forward, or run, providing the nutrient concentration does not fall below a threshold and no repellents are encountered. If nutrients are present at too low of a concentration or a repellent is sensed, the bacteria will begin randomly swimming in a different direction, this is referred to as tumbling (148). If the new direction has a favorable nutrient gradient the bacteria will begin a run in this direction, if the new direction is unfavorable the bacteria will tumble again and continue this process until a favorable gradient is identified. Bacterial chemotactic dependent motility is composed of a succession of runs and tumbles (87, 151, 152).

Whether a bacterium runs or tumbles is determined by the presence or absence of nutrients and repellents. The bacteria use a somewhat modified two-component system to sense the presence of nutrients and repellents to determine if the cell should run or tumble. Flagella naturally rotate in a counter clockwise direction to permit forward movement. In order to change direction or tumble the flagella must switch to clockwise rotation; this change is regulated by chemotaxis. Tumbling is induced either by an

increasing repellent gradient or a decreasing nutrient gradient. It is the chemotaxis system that senses these gradients and alters flagellar rotation appropriately (87, 151, 152).

A class of proteins known as Methyl-Accepting Chemotaxis Proteins (MCPs) form as dimers at a pole of the plasma membrane and act as a component of a sensor kinase complex (89). MCPs in conjunction with CheA, an autokinase, sense the abundance of nutrients and repellents (56). The methylation state of the MCP acts as a gauge for measuring the level of attractants and repellents in the environment and whether the bacteria is moving toward favorable or unfavorable conditions. The MCPs have increased repellent binding at high methylation states (70). Two proteins are responsible for regulating the methylation state of the MCP: CheR is continuously present and methylates the MCP while the amount of active demethylase, phosphorylated CheB, is dependent upon the presence of attractants and repellents (70, 79, 135).

When repellents are sensed, MCP conformation is altered and CheA autophosphorylates. CheA transfers it's phosphate to either CheY or CheB (58, 79). CheY is phosphorylated more readily than CheB and acts as an effector protein (58, 79). Phosphorylated CheY is able to bind FliM, a component of the flagella motor, and induces the flagella to switch from counter clockwise rotation (running) to clockwise rotation (tumbling) (101, 161). This change in rotation direction induces the bacteria to tumble, allowing it to begin swimming in a different direction that may have a more favorable gradient (152). Throughout this process CheR is continuously methylating the MCP while

phorphorylated CheB is slowly removing methyl groups from the MCP (135). This balance of methyl addition and removal allows the bacteria to sense if repellent levels are increasing or decreasing based on the rate of methyl addition and removal by altering the methylation state of the MCP complex.

A similar but opposite process occurs when moving along a nutrient gradient. Nutrient interaction with the MCP inhibits CheA phosphorylation, decreasing the amount of active CheY and CheB in the cell. This allows the flagella to continue counterclockwise rotation and swim without tumbling. The lack of phosphorylated CheB leads to the accumulation of methyl groups on the MCP, permitting CheA phosphorylation and CheB activation to begin demethylation. This "resets" the MCP complex so it can continue to sense attractants (20, 87). Even under high attractant conditions eventually phosphorylated CheY will accumulate and cause tumbling, allowing the bacteria to begin down a different gradient.

The role of chemotaxis in swarming motility is poorly understood. While multiple studies have demonstrated swarming defects in chemotactic mutants in multiple organisms, such as *E. coli* (22), *S. enterica* (93), *V. parahaemolyticus* (127) and *S. marcescens* (112) the results conflict as to whether it is the disruption of chemotactic sensing or the loss of the machinery that causes altered motility (46). Thus far we know that functional CheA and CheW are required for swarming motility in *P. mirabilis* though, again, whether this is a reliance on the machinery or the signaling pathway is unknown (13, 21).

III. Bacterial motility

There are several forms of motility bacteria can employ to maneuver through their environment to find nutrients and escape toxic conditions. Broadly, forms of motility can be broken down in to two classes: Flagella dependent and flagella independent. Flagella dependent motility includes swimming and swarming (57). Flagella independent motility can be further divided into forms dependent on extracellular appendages and those that are appendage independent. Twitching is a form of flagella independent motility that relies on Type-IV pili extension and retraction for movement (96) while sliding/gliding and darting motility are powered by the cell's growth, expulsion of fluid from the cell to facilitate movement, and/or slime secretion to aid in migration (102, 163). *P. mirabilis* possess both swimming and swarming motility, depending upon its growth conditions. Below we discuss swarming in some prominent Gram-negative organisms and review the process by which *P. mirabilis* swarms.

A. Swarming

i. *E. coli* and *Salmonella*

Swarming in *E. coli* and *Salmonella* differs substantially from *P. mirabilis* in several areas. *E. coli* and *S. enterica* both form elongated, polyploid cells and double their flagella number but neither demonstrate consolidation during their outward migration (51, 143, 149). *E. coli* demonstrates swarming motility when on 0.5-0.8% Eiken agar, while *S. enterica* can swarm on Difco agar as well as Eiken agar. *P. mirabilis* is able to swarm on both agar types but at higher percentages (1.5% Difco). *flhDC* is responsible for regulating swarming in *P. mirabilis* and functions similarly in *E. coli*; however,

Salmonella swarmer cells exhibit an increase in flagella without increasing *flhDC* transcription (154).

Lipopolysaccharide (LPS), oligopolysaccharide (OPS) and enterobacterial common antigen (ECA) have been shown to be important in *E. coli* and *S. enterica* swarming (46, 63, 154). This is likely due to the role of these outer-membrane components in affecting surface wettability. Studies of the fluid collected from surfaces of *S. enterica* swarms demonstrated that an osmotic agent, hypothesized to be LPS, acts to increase the wettability of the non-polar surface to permit swarming (26). It was proposed that reversal of the flagellar motor leads to stripping LPS from the exterior of the cell and its accumulation on the swarming surface, permitting LPS to act as an osmotic agent (94). This hypothesis is supported by the observation that chemotaxis machinery, but not chemotaxis itself, is required for swarming in *Salmonella.* A *cheY* mutant (discussed earlier) is deficient in swarming motility while a constitutively active CheY permits swarming motility even in the presence of other chemotactic mutants (93). This demonstrates that reversing flagellar motor direction is absolutely essential for swarming in *S. enterica*. LPS, and more specifically O-antigen, have been shown to serve a sensory role in swarming regulation. O-antigen acts to sense that the bacteria has encountered a solid surface and leads to modification of *flhDC* transcription via the Rcs phosphorelay (105). This is further discussed in a later section.

Like *P. mirabilis*, these organisms form swarming rafts to migrate away from the central inoculum. Raft formation is dependent on collision with neighboring cells to correctly

align the cells for migration (35, 149). The flagella of swarming cells form bundles to propel the rafts forward. Cells at the exterior of the colony appear to have a different function from interior cells. Exterior cells have been shown to have decreased migration rate despite flagellar rotation (33. 35). It is hypothesized this rotation without migration allows wetting agents to be deposited on new surfaces to permit motility by the raft (33, 35).

An increase in iron acquisition is required for *E. coli* and *S. typhimurium* swarming (63, 154). Changes in cellular metabolism have also been observed (72). For example, in *Salmonella* it was shown that swarmer cells increase de novo biosynthetic pathways and both *E. coli* and *Salmonella* require at least portions of the Krebs Cycle to sustain swarming due to the energy expensive nature of the process (72).

ii. *Vibrio* and *Pseudomonas*

The *Vibrio* and *Pseudomonas* species represent a unique form of swarming motility. Most Gram-negative swarmers are peritrichously flagellated even during swimming; however *V. parahaemolyticus* and *P. aeruginosa* both possess a single, sheathed polar flagellum in swimming form. Upon induction of swarming *V. parahaemolyticus* elongates, increasing its cell length by 5-20 fold and becomes peritrichiously flagellatedv (98, 99). This conversion from a monotrichious to a peritrichious organism can occur because *V. parahaemolyticus* contains two separate types of flagella. Encoded by the *laf* genes, the peritrichious lateral flagella utilized for swarming are produced in response to a few environmental stimuli (98, 99) including inhibition of polar flagellar rotation and

binding by anti-flagellar antibodies (15, 98). This signaling demonstrates the function of the polar flagella as a mechanosensor to detect whether the environment is swimming or swarming permissive (16, 69, 98). The concept of flagellar rotation acting as a differentiation signal for swarming bacteria is a well-accepted hypothesis by motility researchers and it was the initial characterization of the *V. parahaemolyticus* mechanoreceptor that lent support to this idea. Two additional signals, low iron concentration and calcium ion levels, have been shown to regulate *laf* gene expression and promote swarming (48). Similar to *P. mirabilis*, *V. parahaemolyticus* is able to swarm on a range of agar concentrations, with the highest permissive concentration exceeding 2% agar and produces distinct terraces in its swarming pattern (100).

P. aeurogenosa also has a polar flagellum during swimming like *V. parahaemolyticus* but upon differentiation a second polar flagella is assembled (73, 123). The signal stimulating differentiation remains unknown. Unlike *P. mirabilis* and *V. parahaemolyticus*, *P. aeurogenosa* can only swarm on agar levels between 0.5-0.7% and exhibits a unique pattern of swarming (73, 123). The formation of tendrils on the swarming plate is characteristic of *Pseudomonas* swarming; these tendrils radiate outward from a central inoculum but never intersect (23). *Pseudomonas* produces a family of extracellular glycolipids called rhamnolipids. It has been demonstrated that rhamnolipids are responsible for tendril formation during *P. aeurogenosa* swarming (23, 36, 73, 115, 123, 147). Formation of rhamnolipids is mediated by two genes; *rhlA* produces e-(e-hydroxyalkanoyloxy) alkanoic acid (HAA) (36) while the protein encoded by *rhlB* mediates the addition of TDP-L-rhamnose to HAA (73, 147).

Chemotactic machinery is required for *P. aeurogenosa* swarming motility; however a classical chemotactic sensing system is not. Of the two flagellar stators encoded by *P. aeurogenosa* only one, MotCD, is required for swarming motility (146). However, a chemotaxis system is responsible for guiding this organism's motility. Rhamnolipids can exist in both mono and di forms. The mono-rhamnolipids act as a wetting agent while di-rhamnolipids act as an attractant (147). HAA molecules act as repellents in this chemotactic system, promoting movement outward from the central inoculum (147). Rhamnolipid production is regulated by nutritionally dependent quorum sensing with regulation occurring on succinate but not glutamate (131).

iii. *Serratia*

Members of the *Serratia* genus also demonstrate swarming motility. Like *P. mirabilis*, *Serratia* swarming cells are elongated, aseptate, hyper-flagellated cells (39). However, unlike *P. mirabilis*, *Serratia* has some distinct conditions required for swarming. Swarming motility has only been demonstrated for *Serratia* at 30ºC, not at 37ºC (3, 86). Additionally, *Serratia* cannot swarm on agar higher than 0.9% (3). While *Serratia* do form swarming rafts, there is no consolidation step during swarming, leading to the absence of terraces (40).

Two *Serratia* species, *S. marcesens* and *S. liquefaciens*, have been observed to swarm but differ in how they regulate swarming. *S. marcens* best mirrors *P. mirabilis* in swarming regulation, demonstrating an increase in *flhDC* transcription that correlates with increased flagellar production (134). In *S. liquefaciens flhDC* transcription does not increase,

suggesting there may be post-translational regulation (144). The same studies showed that over-expression of *flhDC* in each strain also yields very different results. In liquid media over-expression of *flhDC* will lead to differentiation in both strains, but on solid plates high levels of *flhDC* causes hyperswarming in *S. marcescens* but completely inhibits swarming in *S. liquefacians* (134, 144). The role of temperature in *S. marcescens* swarming can be attributed to *flhDC* regulation as well since it has been demonstrated that *flhDC* expression is decreased at 37°C (86).

Surfactants are required for swarming in *Serratia* and the genus produces a unique version termed serrawettins (95). Serrawettins are cyclic lipopeptides whose production is regulated via quorum sensing (85). Furthermore, surface sensing has been implicated in *Serratia* swarming due to the observation that provision of the quorum sensing signal in liquid media does not cause serrawettin production to the same level as is observed on a surface (85).

iv. *Proteus mirabilis*

a. Environmental Requirements

P. mirabilis demonstrates both forms of flagella mediated motility: swimming and swarming. Which form of motility the cell utilizes is dependent upon the current environment of the cell. Similar to the patterns observed during *Salmonella* and *Serratia* swarming, *P. mirabilis* swarms outward from the central inoculum in all directions. The process of differentiation, migration and consolidation gives *P. mirabilis* its characteristic bulls-eye pattern on an agar plate. Unlike many of Gram-negative swarmers, *Proteus*

does not require specialized media to promote swarming. *P. mirabilis* swarming can be observed on standard Luria-Bertani (LB) plates with agar percentages greater than 0.6% and less than 2.5%. Above 2.5% *P. mirabilis* is unable to swarm. Additionally, *P. mirabilis* exhibits swarming motility at all growth permissive temperatures (25°C, 30°C and 37°C) as well as under both aerobic and anaerobic conditions.

Surface Sensing

In liquid culture or on a low concentration of agar *Proteus* is found as a short, peritriouchously flagellated bacilli, this is the organisms' vegetative or swimming form (13). *P. mirabilis* will switch to swarming when placed on a solid agar plate. This change in mode of motility requires *Proteus* to sense its environment and modify gene regulation accordingly to accommodate a unique form of motility.

Inhibition of flagellar rotation on a solid surface was proposed as a signal to begin differentiation. The precedence for flagella acting as mechanoreceptors has been set in *Vibrio parahaemolyticus* and is discussed above. This hypothesis was proven in *Proteus* by demonstrating that strains co-incubated with anti-flagellar antibodies in liquid media differentiated (17). Swarmer cells can also be induced in liquid culture through genetic mutation and overexpression. For example, overexpression of *wosA* causes hyper-swarming and differentiation in liquid (53). Furthermore mutation in *fliG* or *fliL*, encoding the motor-switch complex and an unknown component of the flagella, respectively, are able to differentiate in liquid (17). FliL is suggested to interact with the motor complex to relay the torque the flagella experience when on a solid surface;

mutations in these components are thought to mimic the torque experienced by the cell when the flagella attempts to rotate on a solid surface. Thus it was proposed that the first step in swarming initiation is sensing the solid surface and that flagella are required for this process. Mutants that do not produce flagella are unable to differentiate, adding further evidence in support of this hypothesis (13).

However, a second outer membrane structure has been demonstrated to be required for surface sensing in *P. mirabilis*. Morgenstein *et.al.* have demonstrated that LPS, and more specifically O-antigen, acts as a surface sensor. The authors proved that the absence of O-antigen prevented the derepression of *flhDC* and, consequently, swarming (106). Furthermore, this work demonstrated the Rcs phosphorelay was responsible for coupling surface sensing to genetic regulation (106).

The Rcs phosphorelay is extensively studied in *Escherichia coli* and *Salmonella erterica* serovar *Typhimurium* (25, 29, 42, 44, 77, 90, 91, 94, 142, 155). While considered a two component system (TCS), the Rcs phosphorelay is more complex than the canonical TCS. There are four components of the Rcs phosphorelay: RcsC (the sensor kinase), RcsB (the response regulator), RcsD (a phospho-transfer protein), and RcsA (an accessory DNA binding protein). RcsB, when phosphorylated and in conjunction with RcsA, has been shown to repress *flhDC* transcription (44). When in liquid culture RcsC is able to autophosphorylate, leading to the phosphorylation of RcsB and the repression of *flhDC*. However, on a solid surface, RcsC is prevented from phosphorylating leading

to derepression of *flhDC* and swarming. It has been demonstrated that another protein, UmoB, is responsible for inhibiting RcsC autophosphorylation on a solid surface (106).

UmoB was initially discovered in *P. mirabilis* during a search for mutations able to suppress the swarming defect of a *flgN* (a flagellar chaperone) mutant and is the *P. mirabilis* homologue of *igaA* (24, 38, 145). Mutation of *umoB* leads to a decrease in *flhDC* expression and a non-swarming strain. While the exact function and mechanism of UmoB is unknown, the protein resembles an integral membrane protein suggesting it is localized to the cytoplasmic membrane (37). The Rcs phosphorelay is able to respond to stress on the outer or periplasmic membrane, such as surface growth, peptidoglycan stress and osmotic changes (42, 50, 77, 133). It is hypothesized that UmoB mediates the transmission of these membrane stresses to the Rcs phosphorelay by inhibiting RcsC autophosphorylation during surface growth (106).

Density Dependence

Initiation of swarming in *P. mirabilis* is dependent on the cells reaching a certain density. The reliance on density suggests quorum sensing may be involved in swarming regulation. Quorum sensing is a form of bacterial intercellular communication used to regulate gene transcription within the population dependent upon the amount of bacteria present (reviewed in (159)). The individual bacteria are able to sense the density of their population by synthesizing two molecules, an autoinducer (AI) produced by a LuxI-family protein and a transcriptional regulator encoded by a LuxR-family protein. The autoinducer molecule is produced by each cell in the population and is permeable across

the membrane. The greater the number of cells present the more AI is produced and the higher the probably it will be sensed by the cell and lead to a modification in regulation. Two mechanisms have been identified to activate LuxR proteins and promote transcriptional regulation. Some LuxR family proteins are phosphorylated in response to AI while others bind the AI directly. Regardless of the mechanism of activation of the LuxR protein, the AI must accumulate to a certain concentration in order to successfully alter transcriptional regulation in the majority of the population. Thus the cells must reach a high enough density, or a quorum, to change the genetic profile of the population (reviewed in (64, 108)).

Quorum sensing is often utilized to regulate changes in gene expression that are only advantageous and efficient when a large population of bacteria is present, such as the expression of virulence factors or population dependent motility systems. Hence, swarming motility is a good candidate for quorum sensing regulation given that it does not occur before the population reaches a certain density. There are three canonical quorum sensing systems in the Gram-negative bacteria: LuxIR encoding an acyl-homoserine lactone autoinducer (AI), LuxMN encoding AI-1, and LuxSQ encoding AI-2 (159). According to whole genome sequencing *P. mirabilis* does not contain homologues of *luxI* or *luxM* though a homologue of *luxS* was identified (119). However, a *luxS* mutant had no effect on swarming (128). While *P. mirabilis* may not use any of the canonical quorum sensing systems to regulate swarming this does not discount quorum sensing regulation completely. *P. mirabilis* may utilize an alternative molecule for intercellular communication, the best candidate thus far is putrescine (discussed below).

b. Regulation by Molecules

Putrescine

P. mirabilis has two pathways of putrescine production. The primary pathway requires the genes *speA* and *speB* while the secondary pathway utilizes *speC*. SpeA and SpeB, an arginine decarboxylase and agmatine ureohydrolase, respectively, work in concert to convert arginine to putrescine. Strains with a mutantaion in either gene are delayed in differentiation by two to three hours, though swarming does eventually occur at a reduced level due to *speC*-dependent putrescine synthesis. If both the primary and secondary putrescine production pathways are non-functional, the strains fail to differentiate though flagella production increases, as is characteristic for swarming cells. Thus it is hypothesized putrescine regulates the genes involved cell elongation (140).

Central Metabolism Intermediates

Recent studies have demonstrated that concentrations of central respiratory metabolites may act as internal standards for regulating the swarm cycle (5, 7). An intermediate in the Tricarboxylic Acid (TCA) cycle, fumarate is a central metabolite for aerobic respiration of the cell and shown to be important for swarming motility. Alteri *et.al.* demonstrated that altering fumarate metabolism causes a change in the periodicity of swarming under aerobic conditions (5). Furthermore, the authors demonstrated a fumarase metabolism mutant produces reduced levels of acid fermentation products dependent on the available carbon sources and oxygen conditions. Provision of alternative carbon sources under certain oxygen conditions was able to restore fermentation and, consequently, swarming motility. Similar results were observed with

succinate dehydrogenase mutants. Mutations in pathways responsible for glycolysis, pentose phosphate metabolism and gluconeogenesis also affected the appearance of the swarm cycle. Taken together, these findings implicate central metabolites in swarming regulation and demonstrate that swarming motility is dependent upon having an intact aerobic and anaerobic respiratory pathway (5).

Amino Acids

Three common amino acids: arginine, glutamine and histidine along with ornithine have been demonstrated to promote swarming on otherwise non-permissive medias. The effect observed by the exogenous provision of these four compounds is dependent on intact putrescine biosynthesis and amino acid metabolism pathways. Additionally glutamine has been shown to be an essential requirement for swarming in normally permissive media as well as an inducing signal in non-permissive media (7). These data suggest the concentration of basic nutrients in the environment may act as a measure for determining if the surroundings are swarming permissive (7). The decarboxylated form of phenylalanine, phenethylamine, has also been implicated in swarming regulation, its effect is discussed later in this chapter (138).

c. Differentiation and Elongation

Approximately three to four hours after encountering a solid surface *P. mirabilis* can be found in its swarmer cell form. The conversion of swimming cells into swarmer cells is termed differentiation. One aspect that distinguishes a differentiated swarmer cell is the 10-40 times increase in length compared to a swimmer cell. Swarmer cells also diverge

from the standard bacillus in that they are aseptate and multinucleate. This suggests regulation of cell division and chromosome replication genes is crucial for swarmer cell formation in that the ratio of cell size to DNA is unaltered in a swarming cell (9, 47). However, the mechanism of this regulation and the target genes have yet to be identified. Another key characteristic of the swarmer cell is hyper-flagellation. A swarmer cell has greater than 50 times the number of flagella than its swimming counterpart. Thus, a huge increase in flagellar synthesis occurs during differentiation. This is largely mediated by increased expression of the Class I flagellar regulatory genes, the *flhDC* operon.

d. Migration

Once differentiation is completed swarmer cells are able to interact with neighboring cells to form multicelluar bodies termed swarming rafts. These rafts are critical for motility as mutations affecting raft formation result in non-swarming strains, regardless of flagellar production. To form a raft, swarmer cells align themselves pole to pole and electron micrographs suggest adjacent cells' flagella become intertwined (68). Multiple rows of these associated cells interact to form a mesh-like structure identifiable as a raft. Once rafts form migration can occur. It is worth noting the swarm front does not represent one raft expanding outward but multiple adjacent rafts migrating independently. As swarm cycles accumulate each successive migration occurs on top of the previously visible swarm ring. Thus, each swarm zone is created by multiple rafts, has a rough, rather than smooth, edge and is composed of multiple layers of cells (60, 125).

e. Consolidation

Markedly little is known about how and why *P. mirabilis* undergoes a de-differentiation process after swarming, nor have consolidation factors or signals been conclusively identified. However, two mutants produced interesting phenotypes that may suggest roles in consolidation.

***flhDC* intergenic region**

Clemmer *et.al.* identified two transposon insertion mutants that initiated swarming 1.5 hours earlier than wild-type and failed to consolidate at any point during swarming (31). The absence of consolidation created a thin swarm layer lacking any terrace formation. These mutations were identified to be 325 and 740 basepairs upstream of the *flhDC* operon and analysis of the transposon insertion demonstrated that no new promoters were formed. It appears these mutations interrupt regulatory binding sites as demonstrated by the observation that *flhDC* mRNA levels do not decrease at hours five and six as observed in wild-type (31). Furthermore, introducing the region upstream of *flhDC* on a multicopy plasmid to wild-type cells caused an increase in swarming motility. This result suggests that these sites are involved in *flhDC* repression and that over-expression of the site in *trans* can reduce levels of free repressor via titration, leading to hyperswarming due to increased expression from the chromosomal *flhDC* alleles. Importantly, cells harboring transposon insertions 325 or 740 basepairs upstream of *flhDC* remain undifferentiated in liquid though they do exhibit increased swimming motility on soft

agar (31). Still, the absence of differentiation in liquid suggests these sites are essential for regulating consolidation during swarming.

disA

The decarboxylase inhibitor of swarming was initially identified by Stevenson *et.al.* as a mutation that restored motility to a strain incapable of producing putrescine (138). They observed that *disA* over-expression completely abolished motility and mutation of *disA* caused a modest but reproducible hyper-swarming effect. Due to the apparent homology of *disA* to an aromatic amino acid decarboxylase, the decarboxylated forms of amino acids were added exogenously to LB to determine if any of the compounds would be able to inhibit swarming motility to the extent observed in a *disA* over-expressing strain. The authors demonstrated the addition of 25mM phenethylamine (PEA), the decarboxylated form or phenylalanine, inhibited swarming motility (the reported value of 4 mM is a miscalculation) (138). It has been hypothesized that DisA decarboxylates phenylalanine producing PEA and accumulation of PEA acts to inhibit swarming or promote consolidation.

disA expression increases over the course of the swarm cycle, peaking at hour three. Consequently, DisA is maximally expressed at an appropriate point in the swarm cycle to have a role in initiating consolidation. However, *disA* over-expression significantly decreases the expression of Class II and III flagellar regulatory cascade genes without altering the transcription of *flhDC* or the half-life of FlhD and FlhC. Consequently it was

proposed that the product of DisA, presumably PEA, exerts a post-translational effect on $FlhD_4C_2$ (138).

Relatively little is known about phenylalanine specific decarboxylases. While other Gram-negative Enterobacteria have conserved flagellar regulation hierarchies and *flhDC* systems, *disA* homologues have not been identified. Traditionally phenylalanine decarboxylase activity is a secondary characteristic of tyrosine decarboxylases, presumably due to the structural similarities of the two amino acids. To date only one primary phenylalanine decarboxylase has been identified and conclusively validated in the Enterobacteria, *Enterococcus faecium* RM58 accession number AJ83966; this decarboxylase also demonstrates activity for tyrosine (92). The highest homology result for *disA* using NCBI BLAST returns an Enterococcus like tyrosine decarboxylase. It is important to note that tyramine, the decarboxylated form of tyrosine, does did not inhibit swarming to a level comparable to *disA* over-expression, thus we do not believe DisA functions primarily as a tyrosine decarboxylase. DisA appears to have homology to several other enzymes, including glutamate decarboxylase, aspartate decarboxylase and aspartate aminotransferase superfamily fold type I. These other families will be discussed in greater length in Chapter 6.

Given the stark effect on swarming motility observed in a *disA* over-expressing strain and its transcription profile during the swarm cycle, characterizing the regulatory mechanism of *disA* was of interest. *disA* represented a novel and *Proteus* specific mechanism for regulating swarming motility with no explanation for how its expression is regulated and

very little known about its mechanism of action. The next three chapters describe in more detail how swarming is regulated in *P. mirabilis*, what effect *disA* expression has on other Gram-negative bacteria that both harbor and lack *flhDC*, and how *disA* is regulated in *P. mirabilis*. Chapters 5 and 6 will provide unpublished data and a discussion on what is now known about *disA*, what remains to be investigated, and how this knowledge has altered our understanding of the swarm cycle in *Proteus mirabilis*.

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Chapter 2: Regulation of gene expression during swarmer cell differentiation in *Proteus mirabilis*

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Abstract.

The gram-negative bacterium *Proteus mirabilis* can exist in either of two cell types, a vegetative cell characterized as a short rod and a highly elongated and hyperflagellated swarmer cell. This differentiation is triggered by growth on solid surfaces and multiple inputs are sensed by the cell to initiate the differentiation process. These include the inhibition of flagellar rotation, the accumulation of extracellular putrescine and O-antigen interactions with a surface. A key event in the differentiation process is the upregulation of $FlhD_2C_2$, which activates the flagellar regulon and additional genes required for differentiation. There are a number of genes that influence $FlhD_2C_2$ expression and the function of these genes, if known, will be discussed in this review. Additional genes that have been shown to regulate gene expression during swarming will also be reviewed. Although *P. mirabilis* represents an excellent system to study microbial differentiation, it is largely understudied relative to other systems. Therefore, this review will also discuss some of the unanswered questions that are central to understanding this process in *P. mirabilis.*

Introduction.

When cultured on agar plates, *P. mirabilis* exhibits a striking form of motility, termed swarming, that results in the formation of motility waves forming distinct terraces on agar plates, Fig. 1A (Mobley and Belas, 1995; Rauprich et al, 1998; Rather, 2005). This distinctive swarming behavior allows for the rapid identification of *P. mirabilis* in clinical microbiology labs by the characteristic bulls-eye pattern of agar grown colonies. Swarming in *P. mirabilis* was originally described in 1885 by Gustav Hauser, who gave this organism the name *Proteus* based on its ability to change shape. It is now known that this shape change is the result of a complex differentiation process that converts vegetative cells, with a morphology typical of gram-negative members of the *Enterobacteriaceae,* to highly elongated swarmer cells (Fig. 1B) (Rather 2005). Swarmer cells express levels of flagellin, encoded by the *flaA* locus, that are 10-fold higher than vegetative cells (Belas, 1994). The process of swarming requires that swarmer cells align together to form multicellular rafts that translocate across solid surfaces (Jones et al, 2004). The migration of swarmer cells is a transient process and after a period of migration, typically 1-2 hours at 37^{o}C, cells de-differentiate back to the vegetative form and movement ceases in a process termed consolidation. After a period of growth in the consolidated state, the vegetative cells differentiate back to swarmer cells and a new round of migration initiates. This cycle can repeat multiple times, resulting in the formation of distinct terraces that represent a period of swarming and consolidation and appear as a bulls-eye colony phenotype on agar plates (Fig. 1A). A number of theories have been put forward to explain the cyclic nature of swarming and the onset of

consolidation, including changes in gene expression and mathematical models involving changes in population density or water activity at the periphery of the expanding cells (Rauprich et al, 1996; Esipov & Shapiro, 1998; Medvedev et al, 2000; Matsuyama et al, 2000; Arouh, 2001, Lahaye et al, 2007). The nature of consolidation is without question a fascinating area of study, but is outside the scope of this review, which will focus on the regulation of gene expression during swarming.

As a human pathogen, *Proteus mirabilis* is well known for its ability to cause urinary-tract infections (Mobley and Belas, 1995; Rozalski et al., 1997). These infections primarily occur in patients undergoing extended periods of catheterization, such as the elderly or patients with spinal cord injuries. Some aspects of virulence are associated with the swarmer cell state. For example, swarmer cells express virulence factors such as urease, IgA protease and hemolysin at higher levels than vegetative cells (Allison *et al.,* 1992; Walker *et al.,* 1999; Fraser *et al.,* 2002). Studies have demonstrated that swarmer cells are more invasive of uroepithelial cells than vegetative cells (Allison, 1992). In addition, in mouse models of virulence, intravenously injected motile, but non-swarming mutants of *P. mirabilis* were impaired in killing (Allison 1994). In a separate study, a non-motile *flaD* mutant exhibited a 100-fold decrease in colonization in a mouse model of urinary tract infection (Mobley et al, 1996). However, in the above study, a *flaD* mutant would also be unable to swim. Therefore, it is difficult to separate the roles of swimming and swarming in virulence. Taken together, these results suggested that swarmer cells were more virulent than vegetative cells. However, subsequent studies have indicated that the relationship between swarmer cells and virulence is less clear.

For example, strains lacking flagella are capable of causing human infections (Zunino 1994, 1996; Legani-Fajado, 1996). In addition, using *P. mirabilis* cells tagged with green fluorescent protein (GFP), it was shown that swarmer cells are rarely observed in the urinary tract during infection (Jansen, 2003). One possibility is that the role of swarmer cells in virulence and colonization is tissue specific. Further studies will be required to clarify the role of swarmer cells in the pathogenicity of *P. mirabilis.*

Requirements for the initiation of swarming.

Surface Sensing. *P. mirabilis* only forms swarmer cells when grown on a solid surface; therefore, the ability to sense surfaces is the first requirement for swarmer cell differentiation. Since *P. mirabilis* is a gram-negative, peritrichoulsy flagellated bacteria that expresses flagella in undifferentiated vegetative cells, it was proposed by Belas and co-workers that inhibition of flagella rotation was a physical signal for swamer cell differentiation in a manner possibly similar to that described for *Vibrio parahemolyticus* (Alavi and Belas, 2001, McCarter & Silverman, 1990). In liquid media, flagella are able to freely rotate. However, on a solid surface, it is proposed that the rotation of flagella is inhibited leading to activation of swarmer cell differentiation by an unknown mechanism. Several lines of data support the role of flagellar inhibition in swarmer cell differentiation. First, the addition of a thickening agent (polyvinylpyrrolidone) to liquid media resulted in the formation of swarmer cells (Belas and Suvanasuthi, 2005). The addition of anti-flagellar antibody was also able to cause differentiation in liquid media, presumably by interfering with flagellar rotation (Belas, 2005). Mutations in genes involved in construction of the flagella (secretion), or in the expressed copy of *flaA,*

encoding flagellin, result in the inability to differentiate (Belas, 1994, Belas, 2005). Interestingly, some mutations in the flagellar complex lead to differentiation in liquid, a non-inducing condition. For example, mutations in FliG (motor switch complex) and FliL (unknown) lead to elongation in liquid, while being defective in swarming (Belas and Suvanasuthi, 2005). The *fliL* mutation results in non-flagellated cells that constitutively express genes normally up-regulated only during swarming. It is not known how FliL works or how it affects swarming, however it is proposed that FliL helps to stabilize the motor complex and the absence of FliL causes stimulation similar to flagella inhibition (Belas and Suvanasuthi, 2005).

Another cellular component that is exposed to a surface is the outer membrane. Previous studies have implicated LPS in *P. mirabilis* swarming (Armitage, 1979, Belas, 1995, Gue, 2001, Armitage, 1982), but the exact role for LPS in swarming is unclear. Upon contact with a solid surface, the LPS undergoes biochemical changes where swarmer cells have a higher proportion of lipid bilayer in the outer-membrane than swimmer cells, along with more long chain O-antigen units (Armitage et al, 1979, Armitage, 1982). More recent techniques have been used to show that there are changes in the LPS during differentiation (Gue et al, 2001). Genetic approaches have also succeeded in showing the importance of LPS. Mutants have been found in *cld* (*wzz*) (O-antigen chain length determinant), along with genes required for inner core synthesis (*waaD, waaC*) that are impaired in swarming motility (Belas et al, 1995). Recent studies suggest a more direct role for O-antigen in transmitting surface contact to changes in transcription (Morgenstein et al, 2010). In addition to the outer membrane, an acidic polysaccharide

designated Cmf is required for efficient swarming, but not for swarmer cell differentiation (Gygi et al, 1999).

Regulation of the flagellar gene cascade in *P. mirabilis.* After surface contact and growth, one of the hallmark events associated with the initiation of swarmer cell differentiation is the upregulation of flagellin (FlaA) expression. The regulatory proteins that control flagellin expression in *P. mirabilis* appear to be conserved with those in *Escherichia coli* and *Salmonella typhimurium* and the flagellar regulatory pathway in these organisms are composed of Class 1, 2 and 3 genes (Chilcott and Hughes, 2000; Pearson et al, 2008). The primary Class 1 gene, *flhDC,* encodes the $FlhD_2C_2$ complex, a heterotetrameric transcriptional regulator. The $FlhD_2C_2$ complex is central to swarmer cell differentiation and is required for the copious amounts of flagellin produced in swarmer cells. $FlhD_2C_2$ also likely regulates additional genes required for swarmer cell differentiation and null alleles in *flhDC* prevent swarmer cell differentiation in *P. mirabilis* (Claret and Hughes, 2000). The expression of *flhDC* is regulated by a variety of environmental conditions and regulatory genes and these are discussed in subsequent sections. During the initiation of swarmer cell differentiation, the levels of *flhDC* expression increase 10-fold and $FlhD_2C_2$ activates the promoters for Class 2 genes in the flagellar cascade that encode the flagellar basal body and hook proteins and the sigma factor s^{28}(Claret, L. and C. Hughes 2000). The expression of s^{28} allows RNA polymerase to transcribe the Class 3 genes, which include genes required for flagellar assembly and the flagellin structural gene, designated *flaA* in *P. mirabilis* (Belas 1994).

The regulation of *flhDC* is central to swarmer cell differentiation. In synchronously differentiating cells on agar plates, the levels of *flhDC* typically rise 10-fold at a time point approximately 3 to 4 hours after cells have been plated. Then, after 6 to 7 hours of growth, the levels decrease significantly during the process of consolidation. Mutations have been isolated that result in the failure to decrease *flhDC* expression during consolidation. These mutations are defined by transposon insertions located at –325 or -740 bp upstream of the start site for *flhDC* transcription (Clemmer and Rather, 2007). There are no obvious open reading frames disrupted by these insertions and they do not change the start site of transcription. These insertions appear to be *cis*-acting mutations that alter the binding of one or more regulatory proteins that serve to downregulate *flhDC* during consolidation.

Regulation of gene expression during swarming

Two-component systems. Two-component systems (TCS) are one of the most common ways bacteria control gene expression (Stock et al, 2003). The canonical TCS is made up of a sensor kinase, which senses stimuli and a response regulator, a DNA binding protein that transcriptionally controls gene expression and is activated by the sensor kinase. *P. mirabilis* has sixteen predicted two-component systems (Pearson et al, 2008), yet as discussed below, only two (Rcs and Rpp) have been shown to directly play a role in swarming.

The RcsCDB phosphorelay. The Rcs system is a complex regulatory system consisting of the RcsC sensor kinase, the response regulator RcsB, and RcsD, a protein that serves

as an intermediate in the transfer of phosphate to the response regulator RcsB. Studies in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium have revealed that the system is activated upon surface growth and membrane or peptidoglycan stresses (Hagiwara, et al, 2003; Laubacher and Ades, 2008; Sledjeski and Gottesman, 1996; Ferrières and Clarke 2003). An additional protein in the Rcs phosphorelay is RcsF, an outer membrane lipoprotein that influences RcsC phosphorylation, possibly by transmitting stress signals from the outer membrane (Castanie-Cornet, 2006; Majdalani et al, 2005).

In *P. mirabilis,* mutations in the Rcs system result in a hyper-swarming phenotype. This was first shown with *rcsC* and *rcsD (rsbA)* mutants (Belas et al, 1998, Liaw et al, 2001), but has more recently been shown for *rcsB* mutants (Clemmer and Rather, 2008). This hyperswarming phenotype is most likely due to an increased expression of *flhDC,* although direct binding of RcsB to the *flhDC* promoter has not been established in *P. mirabilis.* An interesting phenotype of mutations in the Rcs system is an elongation phenotype in liquid media, a condition normally non-permissive for elongation. Mutations that cause over-expression of *flhDC* do not result in a similar phenotype, suggesting that the Rcs regulon may include additional genes involved with cellular elongation (Clemmer and Rather, 2008).

RppAB. The *rppA* gene encoding a response regulator was found in a screen for transposon insertions that decreased polymyxin B resistance (Wang et al, 2008). Encoded adjacent to *rppA* is a gene designated *rppB,* encoding a gene product similar to

members of the histidine sensor kinase family. A null allele in *rppA* exhibited a hyperswarming phenotype and the levels of *flhDC* were elevated approximately 2-fold at the initiation of swarming (Wang et al, 2008). In addition, the swarmer cells from an *rppA* mutant were longer than wild-type, possibly due to the increased amounts of *flhDC*. There was also a concomitant increase in hemolysin, a toxin normally upregulted during swarming. The presence of polymyxin B is able to repress flagellin expression and swarming and this repression was less apparent in the *rppA* mutant. This suggests that polymyxin B may be sensed by the RppB sensor kinase.

Additional regulators of *flhDC*.

Umo proteins. The *umo* loci in *P. mirabilis* were identified as genes that when overexpressed could suppress the swarming defect of a *flgN* mutant, defective in a flagellar chaperone (Dufour et al, 1998). Four *umo* genes were identified, *umoA, umoB, umoC* and *umoD,* and each was capable of increasing the expression of *flhDC* when overexpressed. The UmoA and UmoC proteins appear to be unique to *P. mirabilis.* However, the UmoD protein is similar to YcfJ of *E. coli* and UmoB is similar to YrfF in *Escherichia coli* and IgaA in *Salmonella typhimurium* (Dufour et al, 1998; Cano et al, 2001). In. *S. typhimurium,* IgaA has been shown to interact with the Rcs system in a negative manner, possibly by inhibiting the kinase activity of RcsC. In *S. typhimurim,* mutations in *igaA* result in the overexpression of genes in the Rcs regulon and this phenotype is lethal (Wang et al, 2007). In *P. mirabilis,* a mutation in the *igaA* ortholog, *umoB,* results in a non-swarming phenotype that is likely due to loss of *flhDC* expression (Dufour et al, 1998). The role of the other Umo gene products in swarming is less clear.

A mutation in the *umoD* gene results in loss of swarming, but mutations in *umoA* and *umoC* have little effect on swarming (Dufour et al, 1998). It is currently unknown how the Umo proteins upregulate *flhDC* expression.

Mrp, a family of proteins controlling adherence or motility. Bacteria can be either motile or sessile, with the genes for one lifestyle expressed when those for the other are repressed. *P. mirabilis* makes a variety of fimbriae, with potentially 17 different fimbrial operons representing 5 different types, present in the genome (Pearson and Mobley, 2008). While flagella mediate motility, fimbriae are required for adhesion. Because adhesion is the opposite phenotype to motility (swimming or swarming) it makes sense that when fimbriae are upregulated, flagella synthesis is down-regulated. The MR/P fimbriae in *P. mirabilis* is encoded in a nine-gene operon (Bahrani and Mobley, 1994) with a transcriptional regulator, MrpJ, encoded at the end of the operon (Li et al, 2001). MrpJ directly binds the *flhDC* promoter region to repress expression (Pearson and Mobley, 2008). This allows the cell to express either adhesion or motility genes, but not both at the same time. The *mrpJ* gene was also found to have 14 paralogs, 12 of which repressed motility when overexpressed and a subset of these were shown to repress flagellin expression as well (Pearson and Mobley, 2008). Like MrpJ, direct binding of the paralog UcaJ to the *flhDC* promoter region has been demonstrated (Pearson and Mobley, 2008).

WosA. The *wosA* gene was identified by the hyperswarming phenotype conferred by overexpression (Hatt and Rather, 2008). Interestingly, in *wosA* overexpressing strains,

the initiation of swarming was similar to wild-type, which resulted in the designation wos (wild-type onset with superswarming). The WosA protein is 321 amino acids and predicted to have a cytosolic C-terminus and a transmembrane domain near its N-terminus. There are no obvious WosA homologs in other bacteria and its function is unknown. Overexpression of WosA also resulted in differentiated swarmer cells in liquid culture. The hyperswarming phenotype of *wosA* is characterized by increased velocity during a shortened swarm cycle and less time spent in consolidation before entering the next cycle. However, the onset of swarming is unaffected. WosA overexpression increases the expression of *flhDC* to varying levels, depending on the point in the swarm cycle. The levels of *flhDC* are also increased in liquid culture, but to a lesser extent. Expression of *wosA* is growth phase dependent, with expression elevated during early stationary and continuing to increase into late stationary phase. *wosA* expression is also increased in more viscous media. This increase in expression is partially dependent upon the expression of *flaA*, the flagellar filament, since mutants in *flaA* failed to maximally produce WosA in viscous media. Additionally, mutation of *fliL* (discussed earlier) causes a constitutive increase in WosA expression. Thus, it has been proposed that WosA is involved in a signaling cascade, possibly in conjunction with FliL, to increases the expression of *flhDC* when it senses the presence of the bacteria on a solid surface through inhibition of flagellar rotation (Hatt and Rather, 2008).

Leucine-Responsive Regulatory Protein (Lrp). Lrp is a transcriptional regulatory protein highly conserved within the *Enterobacteriaceae* (Freidberg, 1995). In *Escherichia coli* Lrp is involved in regulating several pathways including, amino acid

synthesis, pilin synthesis, and peptide transport (Calvo & Matthews, 1994; Newman & Lin, 1995). Lrp responds to amino acids and this may reflect the requirement for amino acids in swarming. The *P. mirabilis* Lrp homologue bears 97% identity to the *E. coli* K-12 protein (Freidberg, 1995). However, despite this homology, there are functional differences between Lrp proteins, particularly in the target genes (Lintner et al, 2008). In wild-type *P. mirabilis, lrp* is expressed in differentiating cells and reaches maximal levels approximately 3.5 hours into the swarm cycle (Hay et al, 1997). Thus, maximum *lrp* expression is achieved before peak *flhDC* expression. Mutation in *lrp* substantially decreases expression of *flhDC* as well as the flagellar filament (*flaA*) and *hpm* (hemolysin) (Hay et al, 1997). Consequently, a mutation in *lrp* results in a non-swarming phenotype. Due to its role as a global regulator, it is possible that mutation in *lrp* makes it impossible for the cell to sustain hyperflagellation. However, over-expression of *flhDC* was able to rescue both of these phenotypes, restoring swarming to twice the wild-type rate and rescuing the production of hyperflagellated cells (Hay et al, 1997). There is some conflicting data suggesting that Lrp may affect *flhDC* and *flaA* in liquid cultures also, but this remains to be conclusively shown (Hay et al, 1997).

Repressor of Secondary Metabolites (RsmA). RsmA is a homolog of the *E. coli* CsrA protein, a member of a critical global regulatory system that controls the expression of a variety of stationary-phase genes, such as glycogen biosynthesis, catabolism, and biofilm formation (Romeo, 1998; Romeo, 1993) by affecting the stability of mRNA (Liu, 1995; Liu, 1998). In several *Enterobacteriaceae,* a counterpart of RsmA, RsmB, has been identified. RsmB is an untranslated regulatory RNA that binds and neutralizes RsmA (Liu, 1998). CsrA and RsmA are found in many Gram-negative bacteria, including

Erwinia carotovora subsp. *carotovora, Serratia marcescens* and *Proteus mirabilis,* and have been linked to swarming regulation and virulence factor expression in these organisms (Liu, 1998, Cui, 1995, Mukherjee, 1996, Wei, 2001, Liaw, 2003). In *Proteus mirabilis,* RsmA is a 62 amino acid, 6.8 kDa protein with 96% and 94% identity to the *E. coli* CsrA and *E. carotovora* subsp. *carotovora* RsmA, respectively (Romeo, 1998). *P. mirabilis* RsmA also contains the KH motif characteristic of proteins associated with RNA (Siomi, 1994) and conserved within other homologs. It has been shown that *P. mirabilis rsmA* is able to reduce the over-production of glycogen in an *E. coli csrA*$^{-}$ strain to near wild-type levels, supporting the suggestion that *Proteus'* RsmA is a functional homolog of CsrA. However, CsrA and RsmA do not have the same regulatory affects in all strains and this may be due to differences in the swarming process in various bacteria. In *E. coli* CsrA positively regulates swarming and *flhDC* expression (Wei, 2001), while in the *Erwinia* species RsmA represses swarming and virulence factor expression (Liu, 1998, Cui, 1995, Mukherjee, 1996). Increasing the expression of RsmA in *P. mirabilis* inhibits swarming, differentiation of swarmer cells, and the expression of virulence factors, including haemolysin, protease, urease, and flagellin. Haemolysin mRNA was shown to be fully degraded at 8 minutes post rifampicin treatment in *P. mirabilis* over expressing *rsmA*, suggesting that RsmA functions by affecting mRNA stability in *P. mirabilis,* as it does in other Gram-negatives. Highly over-expressing *rsmA* in *Proteus* leads to complete growth inhibition. Furthermore, over-expressing *rsmA* from *Erwinia* or *Serratia* in a *P. mirabilis* strain lacking *rsmA* induces the same inhibition of swarming, differentiation, and virulence factor expression as over-expressing the native *P. mirabilis rsmA* (Liaw, 2003). Finally, over-expressing *rsmA* in a strain mutant for *rsbA (rcsD)*, a

protein required for RcsB phosphorylation and subsequent repression of *flhDC* (Liaw, 2001), suppresses the hyperswarming phenotype of the *rsbA* mutant (Liaw, 2003). This observation suggests that the *rsmA/B* system may interact with the Rcs regulatory pathway.

Regulation of $FlhD_2C_2$ Activity.

DisA, a decarboxylase inhibitor of swarming. The *disA* gene was identified by transposon insertion that suppressed the swarming defect in a strain deficient in the production of putrescine (Stevenson and Rather, 2006). However, this suppression is independent of putrescine since both over-expression and mutation of *disA* has no effect on putrescine levels. In addition, insertions in *disA* have been isolated in wild-type cells based on the resulting hyperswarming phenotype. Mutation of *disA* induces cells to undergo early swarming initiation, increases the distance migrated each cycle by 30-35%, and causes cells to leave the consolidation phase at least 1 and half hours before wild-type (Stevenson and Rather, 2006). However, swarmer cell morphology is unchanged in *disA* mutants and expression of Class 1 genes, specifically *flhDC,* is largely unaffected (1.4-1.5 fold). Conversely, a significant increase in Class 2 and Class 3 genes is seen in a *disA* mutant, with a 16 -32 fold increase in *flaA* (Class 3) mRNA. Over-expression of *disA* in high or medium copy number leads to total inhibition of swarming and differentiation, and completely blocks mRNA synthesis of Class 2 and 3 genes without significantly reducing *flhDC* mRNA levels. However, *disA* over-expression in a strain also over expressing *flhDC* still results in complete swarming inhibition. DisA is

homologous to amino acid decarboxylases and most closely resembles those involved in phenylalanine and tyrosine decarboxylation. Due to the similarity of DisA to amino acid decarboxylases, the effects of different decarboxylated amino acids on swarming were tested. The decarboxylated product of phenylalanine, phenethylamine, was able to inhibit swarming by 50% when present at a concentration of 1 mM and completely abolished swarming at 4 mM (Stevenson and Rather, 2006). Additionally, phenethylamine also inhibited the expression of Class 2 and Class 3 genes while having little affect on Class 1 expression, a phenotype consistent with over-expression of *disA*. It is proposed that DisA is a phenylalanine decarboxylase and that phenethylamine inhibits swarming by affecting the expression of Class 2 and Class 3 genes. The fact that *flhDC* mRNA levels are not altered by the overexpression or absence of *disA* when Class 2 and Class 3 genes are affected suggests that inhibition mediated by DisA must occur downstream of *flhDC* transcription. Based on this data, it has been proposed that DisA targets FlhD and/or FlhC, either by preventing the assembly of the heterotetramer or by inhibiting binding of the heterotetramer to DNA. However, these hypotheses remain to be proven. These models, in conjunction with the fact that DisA expression and swarmer cell development are concomitant, suggest that DisA acts to decrease the expression of genes involved in swarming in preparation for the next cycle of de-differentiation (Stevenson and Rather, 2006).

Lon protease. Lon is an ATP-dependent protease that is highly conserved in bacteria (Tsilibaris et al, 2006). In *P. mirabilis,* a role for the Lon protease in the regulation of gene expression during swarming was revealed by the hyperswarming phenotype of a

mini-Tn*5* transposon insertion in the *lon* gene (Clemmer and Rather, 2008). In addition, the *lon* mutation resulted in the formation of swarmer cells in liquid, which is normally non-permissive for differentiation. At least one target of the Lon protease appears to be the FlhD protein. The half-life of this protein increased from 8 minutes in wild-type cells to 32 minutes in the *lon* mutant and the increased levels of FlhD likely account for the increased flagellin expression in the *lon* mutant. However, the ability to differentiate in liquid is probably due to the accumulation of another protein, since mutants that overexpress FlhDC to levels that are higher that the *lon* mutant do not differentiate in liquid (Clemmer and Rather, 2008). The *lon* mutation also increased the expression of the virulence genes *zapA* and *hmpBA* encoding an IgA protease and hemolysin, respectively. These genes are under FlhDC control (Allison *et al.,* 1992; Walker *et al.,* 1999; Fraser *et al.,* 2002).

Cell-cell signaling and the regulation of swarming.

Role of AHL signals and AI-2. The regulation of gene expression by the secretion of small chemical signals is a process termed quorum sensing (Waters and Bassler, 2005). In several types of bacteria, quorum sensing is required for swarming motility (Daniels et al, 2006; Lindum et al, 1998). The roles of cell-cell signaling in the swarming process of *P. mirabilis* are just beginning to be addressed. Studies employing a *luxS* mutant of *P. mirabilis* revealed that the extracellular signal AI-2 did not have a role in the process of swarming (Schneider et al, 2002). In addition, the recently completed sequence of the *P. mirabilis* genome revealed that the canonical LuxI or LuxM proteins that produce N-acyl homoserine lactone signals are not encoded in the *P. mirabilis* genome (Pearson et al,

2008). Based on this information, it is unlikely that N-acyl homoserine lactone signaling molecules are produced by *P. mirabilis.*

Glutamine. When grown on minimal media, *P. mirabilis* is unable to differentiate into swarmer cells. However, the presence of glutamine allows swarming to initiate on minimal media (Allison et al, 1993). Interestingly, the swarming behavior on minimal media with glutamine is very different than on rich media and is characterized by an absence of the concentric rings seen with swarming on rich media. The mechanisms by which glutamine restores swarming on minimal media is unknown. In addition, this effect may be strain specific, as the swarming of PM7002 is not rescued by glutamine on minimal media (P. Rather unpublished).

Fatty Acids. Liaw and colleagues have shown that swarming behavior can be modified by external fatty acids, such as oleic acid, which stimulated swarming and lauric acid and myristic acid, which inhibited swarming (Liaw et al, 2004). Interestingly, some of these signals were dependent on a functional *rsbA* (*yojN, rcsD*) gene for the inhibitory effect, suggesting that these fatty acids may be sensed by the RcsCDB phosphorelay. However, the role of fatty acids as signals for cell-cell communication has not been established in *P. mirabilis* and the physiological role of fatty acids in swarming remains to be determined.

Putrescine. A role for putrescine in the regulation of swarmer cell differentiation was revealed by the isolation of mutations in the *speA* and *speB* genes that act in a pathway to

produce putrescine (Sturgill and Rather, 2004). The SpeA protein is arginine decarboxylase and SpeB is agmatine ureohydrolase that function together to convert arginine to putrescine. Mutations in either *speA* or *speB* resulted in a 2-3 hour delay in differentiation to swarmer cells. This swarming delay was rescued by adding putrescine to the media or by extracellular complementation via adjacent cells (Sturgill and Rather, 2004). The residual swarming that was present in the *speA* or *speB* mutant was likely due to low-level production of putrescine by the SpeC-dependent pathway. Consistent with this, a *speA/speC* double mutant is unable to swarm (unpublished data).

The mechanism by which putrescine regulates swarming is currently unknown. However, the requirement for putrescine appears to be after activation of the flagellar cascade, as flagellin expression is activated in a normal manner in a *speA/speC* double mutant during swarmer cell differentiation. Therefore, the putrescine-regulated genes may have a direct role in the cell elongation process.

Conclusions.

Over the past 10 years, our understanding of the control of gene expression during swarming in *P. mirabilis* has grown significantly. A summary of the genes identified to date that either directly regulate or influence gene expression during swarming are shown in Fig. 2. A central regulatory event during swarmer cell differentiation is the activation of *flhDC* expression during growth on surfaces. As discussed in this review, a variety of gene products have been identified that influence *flhDC* expression, both positively (Lrp, Umo, WosA) and negatively (RcsCDB, RppAB, MrpJ, RsmA, Lon, DisA). Mutations

that uncouple swarmer cell differentiation from growth on solid surfaces have been identified and these mutants differentiate to swarmer cells in liquid, a normally non-permissive condition. These mutations include *rcsC, rcsD, rcsB, lon* and *fliL,* although in the case of *fliL,* the differentiated cells do not swarm due to non-functional flagella. This suggests that the Rcs pathway functions to repress *flhDC* and additional genes for differentiation and this repression is relieved during growth on surfaces. Incorrect swarmer cell differentiation in liquid media is also triggered by overexpression of the WosA protein (Hatt and Rather, 2008). A common feature of both *wosA* overexpression and mutations in *rcs* or *lon* is that they increase *flhDC* expression 5 to 20-fold during swarming. However, this alone cannot account for the differentiation in liquid, as other mutants that overexpress *flhDC* do not have this phenotype, including those that overexpress *flhDC* to far greater levels than the *rcs* or *lon* mutations (Clemmer and Rather, 2007, 2008). Therefore, additional targets of RcsB and Lon likely include those directly involved with cell elongation and/or inhibition of cell division.

Surface sensing. An additional important question that remains unanswered is how growth on a solid surface triggers *flhDC* activation and additional genes required for cell elongation/inhibition of cell division. The actual sensing of surfaces likely involves multiple mechanisms. Clearly, one aspect of surface sensing involves inhibition of flagellar rotation, however, the regulatory target(s) that are triggered by this inhibition are unknown. Moreover, recent data indicates that a *motA* mutant, which is unable to rotate its flagella, is still able to activate the *flhDC* operon during surface growth. This suggests

that at least one additional pathway exists for surface sensing (Morgenstein and Rather, unpublished).

A second mechanism for surface sensing may involve O-antigen contact with surfaces. A mutation in O-antigen ligase (*waaL*) or the O-antigen chain length determinant (*wzz*) results in the inability to up-regulate *flhDC* on solid surfaces and a failure to swarm (Morgenstein and Rather, in press). Interestingly, *waaL* mutants are able to swim normally, suggesting that the requirement for WaaL is specific to solid surfaces. We propose that O-antigen is acting separately from flagella inhibition as a surface sensor to control *flhDC* expression and flagella inhibition acts on a different part of the differentiation pathway. How O-antigen can control *flhDC* expression is not known, but it may work through the Rcs phosphorelay.

How and why do cells consolidate during swarming? An additional aspect of swarming that is largely unexplored is how and why cells decide to stop swarming and undergo de-differentiation back to vegetative cells during the process of consolidation. One class of regulatory mutants that control *flhDC* expression has provided some information regarding this issue. Transposon insertions upstream of the *flhDC* promoter at positions –325 or –740 result in a novel swarming phenotype, where the mutants fail to consolidate and swarm as a rapidly spreading thin film that does not contain the characteristic concentric rings (Clemmer and Rather, 2008). Interestingly, in these transposon insertions, the expression of *flhDC* fails to shut down after 6 to 7 hours of growth on surfaces like wild-type cells. From this information, it can be inferred that a

critical step in the consolidation process is the decreased expression of *flhDC*. A second mechanism contributing to consolidation may be the expression of DisA, a putative phenylalanine decarboxylase. DisA is activated during swarming and the DisA catalyzed production of phenethylamine acts as an intracellular inhibitory signal that decreases FlhDC activity by an undefined mechanism.

Additional mechanisms are likely to control the timing of consolidation, including changes in cell density (Rauprich et al, 1996; Esipov & Shapiro, 1998; Medvedev et al, 2000; Matsuyama et al, 2000; Arouh, 2001). An appealing mechanism proposed by Harshey and colleagues is that swarming on solid surfaces is controlled, in part, by the accumulation of extracellular signals that trigger differentiation (Toguchi et al, 2000, Harshey 2003). This model invokes a buildup of extracellular carbohydrates and other components (slime) during growth, which then act as a differentiation signal. As cells move out in swarming rafts, extracellular slime is depleted during the movement and eventually cells are unable to maintain the differentiated state and de-differentiate back to vegetative cells. Upon re-growth, slime builds up again and differentiation/swarming proceeds for the second cycle. This model remains to be verified in *P. mirabilis.* However, extracellular carbohydrates, such as colony migration factor (Gygi et al, 1995), accumulate during swarming and may have a role in modulating the swarming cycle. Although putrescine could also be considered a candidate extracellular signal for mediating the cycle of differentiation and consolidation, the addition of putrescine to agar plates had little effect on the timing or extent of the differentiation and consolidation cycles (Sturgill and Rather, 2004)

In summary, *P. mirabilis* is an attractive model system to study microbial differentiation and the regulatory mechanisms that are involved, because unlike other bacteria, *P. mirabilis* swarming is highly coordinated with easily visible periods of swarming and consolidation. The recent availability of genome sequences for *P. mirabilis*, along with the use of technologies for global analysis of gene expression should facilitate these studies. As a better understanding of gene expression during swarmer cell differentiation is compiled, there will be likely novel aspects of gene regulation that are revealed. This information may serve as a framework for other systems that involve complex differentiations.

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Figure legends.

Figure 1. Swarming phenotype of *P. mirabilis.* Panel A shows the swarming phenotype of *P. mirabilis* on a 1.5% agar plate. A small drop of a overnight culture was placed in the center of the plate and incubated at 37^{o}C overnight. The arrows designate individual terraces that represent one cycle of differentiation and swarming followed by consolidation and de-differentiation. In panel B. the cell morphology of vegetative cells and swarmer cells is shown by phase contrast microscopy. The vegetative cells were obtained from broth grown cells and the swarmer cells were obtained from the outermost part of a fresh swarming ring.

Figure 2. Key regulators of gene expression during swarming. A summary of the genes that are known to regulate gene expression during swarming in *P. mirabilis* is shown. The predicted location of each gene product in the outer membrane (OM), inner cytoplasmic membrane (IM) or cytoplasm is shown.

Figure 1.

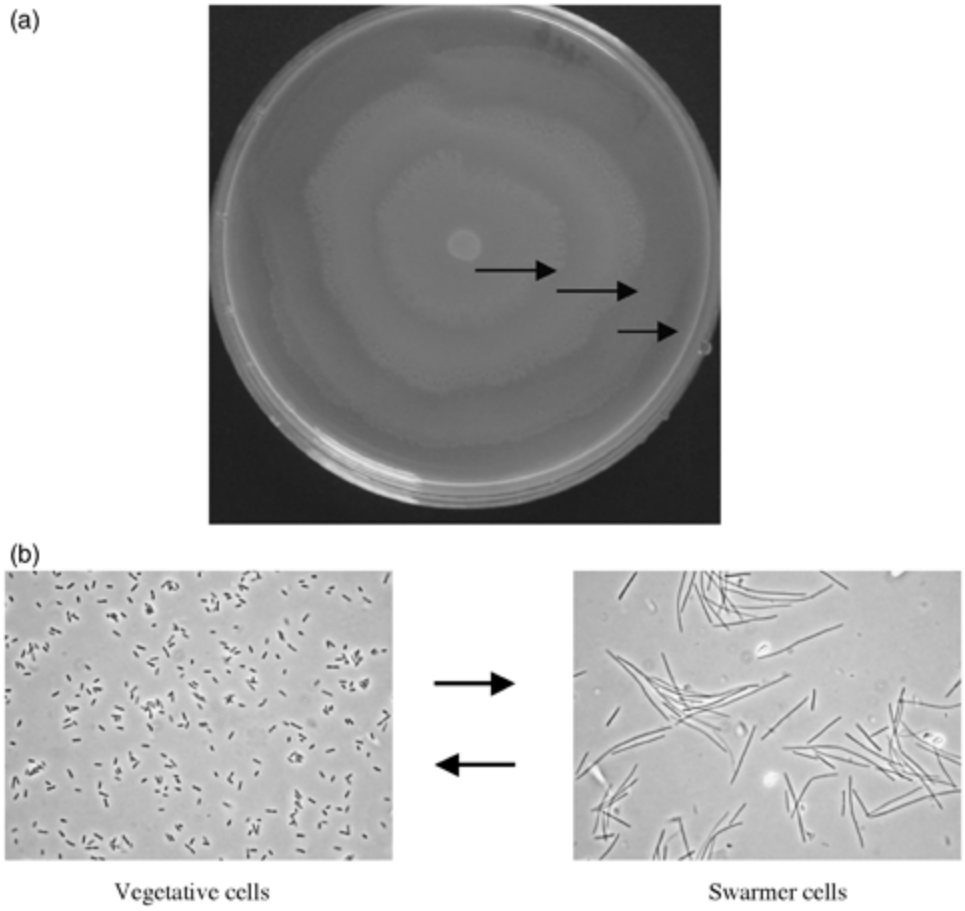
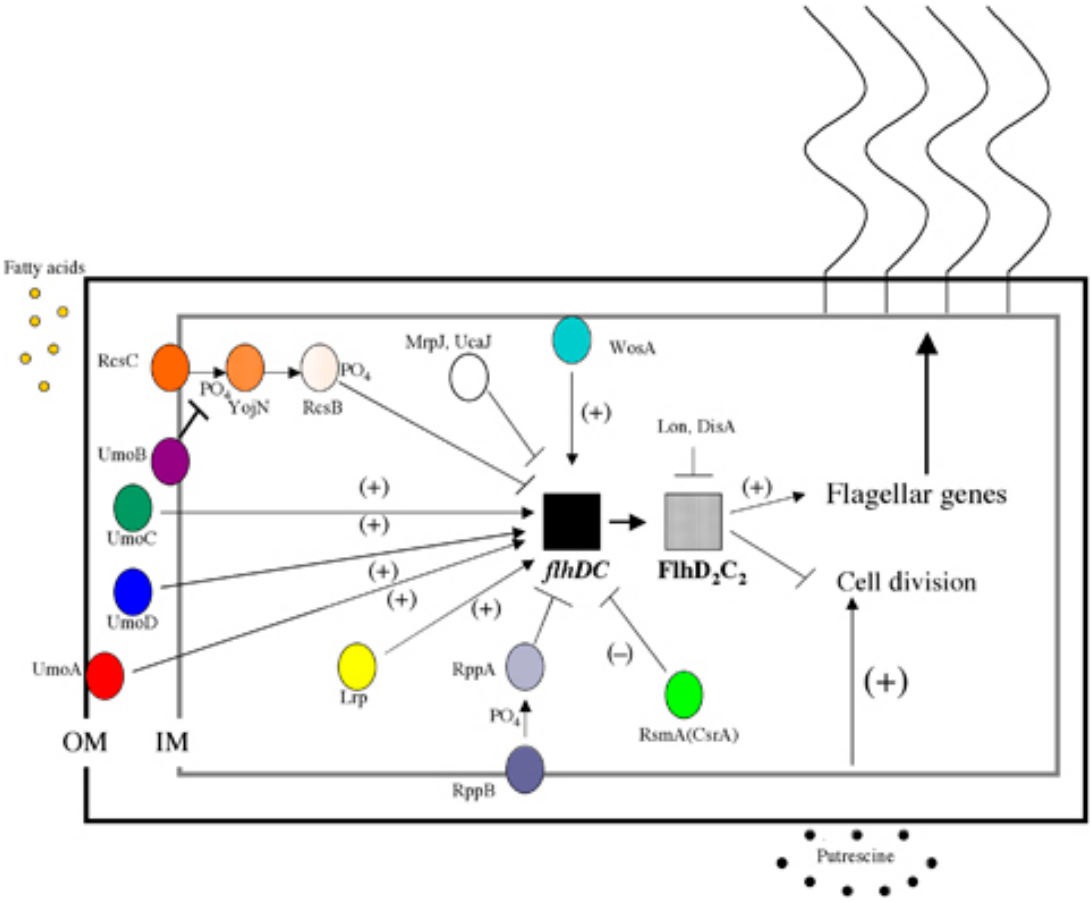


Figure 2.


Fatty acids
RcsC
PO4
YojN
PO4
RcsB
MrpJ, UcaJ
WosA
(+)
Lon, DisA
UmoB
UmoC
UmoD
UmoA
(+)
(+)
(+)
(+)
(+)
flhDC
FlhD2C2
(+)
Flagellar genes
Cell division
(+)
Lrp
RppA
PO4
(–)
RsmA(CsrA)
OM
IM
RppB
Putrescine

Chapter 3. Expression of the DisA amino acid decarboxylase from *Proteus mirabilis* inhibits motility and class 2 flagellar gene expression in *Escherichia coli*.

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Abstract.
In *P. mirabilis*, a putative phenylalanine decarboxylase (DisA) acts in a regulatory pathway to inhibit class 2 flagellar gene expression and motility. In this study, we demonstrate that DisA expression in *E. coli* blocked motility and resulted in a 50-fold decrease in the expression of class 2 (*fliA*) and class 3 (*fliC*) flagellar genes. However, the expression of *flhDC* encoding the class I activator of the flagellar cascade was unchanged by DisA expression at both the transcriptional and translational level. Phenethylamine, a decarboxylation product derived from phenylalanine was able to mimic DisA overexpression and decrease both motility and class 2/3 flagellar gene expression. In addition, both DisA overexpression and phenethylamine strongly inhibited biofilm formation in *E. coli*. DisA overexpression and exogenous phenethylamine could also reduce motility in other enteric bacteria, but had no effect on motility in non-enteric Gram-negative bacteria. It is hypothesized that phenethylamine or a closely related compound formed by the DisA decarboxylation reaction inhibits the formation or activity of the $FlhD_4C_2$ complex required for activation of class 2 genes.

Introduction

Bacterial motility, mediated through the use of flagella, plays an important role in locomotion, host colonization, adhesion, and biofilm formation (Giron et al., 2002; Lane et al., 2005). Two types of flagellar-mediated motility, swimming and swarming, have been described (reviewed in Harshey, 2003, Jarrell and McBride, 2008). In *E. coli*, swimming motility occurs in liquid medium where the predominant cell type is a short vegetative rod with few flagella. Swarming motility also requires flagella and occurs in Gram-negative bacteria such as *Proteus mirabilis, Serratia marcescens, Escherichia coli,* and *Salmonella typhimurium* when cells contact a solid surface (Allison et al., 1992; Kim and Surette, 2003; Rauprich et al., 1996). During a swarming state, cells become elongated, aseptate, multinucleate and an increase in flagellar gene expression results in a hyperflagellated swarmer cell. Swarmer cells act in a coordinated manner that may involve cell-cell signaling to move across a solid surface (Sturgill and Rather, 2004). In *E. coli,* the analysis of defined mutants in the Keio collection has revealed 216 genes that are specifically required for swarming (Inoue at al, 2007). These genes encode diverse functions including: carbon metabolism, DNA replication/repair, ion transport, protein chaperones, amino acid transport, signal transduction and synthesis of cell envelope components.

Motility in *E. coli* as well as other motile enteric bacteria is regulated via a hierarchical system of gene expression. Details of the regulation of flagellar expression have been extensively reviewed (Aldridge and Hughes, 2002; Fraser and Hughes, 1999; Soutourina and Bertin, 2003). The apex of this hierarchy is composed of the *flhDC* operon (class 1)

that is activated by a number of regulatory proteins, some of which respond to environmental stimuli (Aldridge and Hughes, 2002; Soutourina and Bertin, 2003). The *flhDC* operon encodes a heteromeric transcriptional activator, $FlhD_4C_2$, necessary for activation of a second class of genes designated class 2 (Wang et al, 2006). Class 2 genes include flagellar hook and basal body motor components as well as the flagella specific sigma factor *fliA* (Sigma 28). Sigma 28 is necessary for transcription of class 3 genes such as *fliC* (flagellin) which complete the flagella as well as additional motor and chemotaxis proteins (Soutourina and Bertin, 2003).

Both *P. mirabilis* and certain strains of *E. coli* possess the ability to colonize either host tissue or implanted devices such as catheters and produce a biofilm community (Jones et al, 2005; Warren, 1997). The association of cells within a biofilm provides a barrier to antibiotics as well as the host immune system (Jesaitis et al, 2003; Patel, 2005). While some strains of flagella deficient or non-motile *E. coli* exhibit decreased or altered biofilm formation (Pratt and Kolter, 1998, Wood et al, 2006), non-motile (*fliC* mutant) enteroaggregative *E. coli* forms a biofilm indistinguishable from wild type on glass and plastic surfaces (Sheikh et al, 2001) . Additionally, overproduction of curli can compensate for lack of flagella (Prignet-Combaret et al, 2001).

In a screen for mutants of *P. mirabilis* with enhanced swarming motility, a putative amino acid decarboxylase (DisA) was isolated (Stevenson and Rather, 2006). When complementing the mutation, it was observed that *disA* in multicopy resulted in a complete inhibition of swarming motility. Similarly, swimming motility in *P. mirabilis*

was enhanced by a mutation in *disA* and abolished by overexpression. Analysis of flagellar gene transcript levels in swarmer cells of *P. mirabilis* demonstrated that class 2 (*fliA*), as well as class 3 (*flaA,* encoding flagellin) gene expression was undetectable when *disA* was present in multicopy. However, class 1 (*flhDC*) expression was not significantly altered. Since $FlhD_4C_2$ is necessary for activation of *fliA,* these results suggest that DisA effects flagellar regulation at a point after *flhDC* transcription and prior to *fliA* transcription. We propose that in *P. mirabilis,* DisA effects the post-translational regulation of protein subunits FlhD or FlhC through altering heterotetramer ($FlhD_4C_2$) interaction, or $FlhD_4C_2$ DNA binding.

The putative DisA decarboxylase from *P. mirabilis* has homology to tyrosine decarboxylases from *Carnobacterium divergens* and *Lactococcus lactis* as well as a phenylalanine decarboxylase from *Enterococcus faecium*. In addition, the only members of the Enterobacteriaceae with a putative DisA homolog are the *Providencia spp*. Amino acid decarboxylases catalyze the enzymatic removal of the α-carboxyl group of an L-amino acid releasing CO_2 and forming an amine. Tyrosine decarboxylases catalyze the conversion of L-tyrosine to tyramine while phenylalanine decarboxylase produces phenethylamine (Sandmeier et al, 1994). We propose that the decarboxylated product resulting from a DisA mediated decarboxylase reaction may regulate flagellar gene expression and motility. In fact, we have shown that in *P. mirabilis* phenethylamine produces a similar pattern of flagellar gene regulation to that seen with *disA* in multicopy (Stevenson and Rather, 2006).

In this work, we use *E. coli* and other members of the Enterobacteriaceae to demonstrate that inhibition of motility due to DisA overexpression can be extended to bacteria that do not contain the *disA* gene. As was observed in *P. mirabilis*, swimming motility was abolished in *E. coli* strains when *disA* is present in multicopy and the defect was due to decreased expression of class 2 and class 3 genes, but not the class 1 (*flhDC*) genes. Moreover, we show that the decrease in flagellar production when DisA is overexpressed can be rescued by *flhDC* in multicopy. Exposure of *E. coli* cells to exogenous phenethylamine, the putative product of the DisA decarboxylase, decreased motility and also reduced the expression of class 2/3 genes in the flagellar regulon. Both DisA overexpression and phenethylamine significantly reduced biofilm formation in *E. coli.* Finally, the expression of DisA or the presence of phenethylamine significantly reduced motility in other enteric bacteria, such as *S. marcescens, Enterobacter cloacae,* and *Citrobacter koseri*, but did not decrease motility of *Acinetobacter baumannii,* a non-enteric Gram-negative bacterium that is missing the flagellar gene regulatory cascade (Clemmer and Rather, 2011).

Materials and Methods.

Bacterial strains and plasmids. All bacterial strains and plasmids are shown in Table 1. All bacterial strains were electroporated by growing cells to mid-log phase in LB broth and harvesting 25 ml by centrifugation at 4,000 x g. Cell pellets were washed twice with ice cold 10% glycerol and resuspended in ice cold 10% glycerol at $1/100^{th}$ the original volume for electroporations using 0.2 cm gap length cuvettes (Bio-Rad).

Motility assays. Swimming motility assays were done using LB broth containing 0.2% Eiken agar for *E. coli* strains and 0.3% for all other bacteria and incubated at 37°C except for *S. marcescens* which was incubated at 30°C. Depending on the plasmids present, the media was supplemented with ampicillin (100 mg/ml) or chloramphenicol (25 mg/ml). Swarming assays were carried out by spotting equal density cultures onto LB plates with 0.5% Eiken agar and 0.5% glucose. Plates were incubated at 37°C for 16 to 24 hours. Phenethylamine (Acros) was prepared by adding to media followed by adjusting to pH 7.0. For plates, the pH was adjusted to 7 and monitored using pH paper. Bacterial cultures were grown to mid-log phase and 1ul was spotted onto either LB or LB containing phenethylamine plates. Plates were incubated for 16 hours at 37°C.

Construction of *E. coli* strains for β-galactosidase assays. Transcriptional fusion plasmids pVS175 (*fliC-lacZ*), pVS177 (*filA-lacZ*), and pVD182 (*flhD-lacZ*) (Sperandio et al, 2002) were electroporated into *E. coli* MC1061 containing vector control pACYC184 or pAC.DisA and selected on chloramphenicol (25μg/ml) and ampicillin (150μg/ml). In addition, pVS175, pVS177 and pVS182 were electroporated into MG1655 *Dlac* for b-

galactosidase assays in the presence of phenethylamine. For β-galactosidase assays strains were grown overnight and diluted 1:000 in LB with antibiotics. Cells were collected at an OD_{600} of 0.5 representing mid log phase cultures. β-galactosidase assays were carried out as previously described (Miller, 1972).

Genes in high copy that can restore motility to MG1655 pAC.DisA. Genomic DNA from *E. coli* PB103 was partially digested with *Sau*3A and fragments were ligated into *Bam*HI digested vector pET21a. The plasmid library was electroporated into *E. coli* MG1655 pAC.DisA. The resulting transformants were spread in a line on a 0.3% LB agar plate containing ampicillin (150μg/ml) and chloramphenicol (25 μg/ml) and incubated at 30°C. Transformants with restored swimming motility migrated away from the line of growth and were purified by streaking onto fresh agar. Plasmids which restored swimming upon retransformation to MG1655 pAC.DisA were sequenced using a T7 primer.

Biofilm assays. MG1655 pSK or pSK.DisA was grown to an OD_{600} of 0.5 in LB containing ampicillin (150μg/ml) and then diluted 1:100 in fresh LB with ampicillin (150μg/ml). Similarly, MG1655 was diluted in LB or LB containing 3mM and 6 mM phenethylamine adjusted to pH 7.0 with HCl. Dilutions were added to 6 wells (200ul/well) of a polystyrene 96 well microtiter plate (Fisher Scientific) and plates were incubated at 30°C for the time indicated. Before removal of non-adherent cells, the OD_{600} was read from each well to measure bacterial growth. Plates were washed 3X in water to remove non-adherent cells before staining with 1% crystal violet for 20 min. Crystal

violet was removed by washing in water until control wells were clear. Plates were dried prior to solubilizing the crystal violet with 33% acetic acid and absorbance was measured at 580 nm.

Results

DisA inhibits motility in *E. coli.* We have previously reported that a putative amino acid decarboxylase (DisA) from *Proteus mirabilis* inhibited swimming and swarming motility when overexpressed from a plasmid vector (Stevenson and Rather, 2006). Flagellar systems of *P. mirabilis* and *E. coli* have homologous protein components and are likely regulated by similar mechanisms (Claret and Hughes, 2000; Furness et al, 1997). Also, detailed examinations of motility systems and flagellar regulation in *E. coli* have been completed (Chilcott and Hughes, 2000). Therefore, we chose *E. coli* as a model organism to test if the effect of DisA on motility was observed in other bacteria. *E. coli* strain MG1655 was transformed with a high-copy plasmid, pBluescript.SK, containing the *disA* gene expressed from its native promoter (pSK.DisA) or with the vector only. As seen in Fig. 1A, swimming motility was significantly reduced on motility plates in cells containing pSK.DisA. The block in motility was not due to a decrease in growth rate, as growth was unaffected by the presence of pSK.DisA (data not shown). In addition, a similar loss of motility was observed when *disA* was present on the medium copy plasmid pACYC184 (data not shown). Moreover, the presence of pSK.DisA blocked motility in other *E. coli* strains, indicating that this effect was not specific to MG1655 (data not shown).

Isolation of multicopy suppressors that restore motility to DisA-overexpressing strains. To determine the pathway by which DisA inhibits motility, we initially attempted to isolate second-site suppressors that restored motility to *E. coli* overexpressing DisA. Despite repeated attempts, we were unable to obtain such suppressors. Therefore, we attempted to isolate multicopy suppressors of the motility defect in cells overexpressing DisA. Presumably, if DisA was blocking a key component of flagellar gene expression, then overexpressing this protein should bypass any DisA mediated inhibition. A partial *Sau*3A *E. coli* genomic library generated in the vector pET21a was electroporated into MG1655 pAC.DisA and transformants were screened for restoration of swimming motility. In this plasmid library, there is no expression from the T7 promoter, but inserts with their own promoter can overexpress gene products due to their presence on a multicopy plasmid.

After screening approximately 6,600 transformants, two classes of plasmids were found to restore motility of MG1655 pAC.DisA. Twelve of these isolates contained the same approximately 12 kb insert which included the flagellar master operon *flhDC* as well as other motility and chemotaxis genes. A second class of plasmids that restored motility contained an approximately 4kb insertion with only the *flhDC* operon.

Class 2 and 3 flagellar gene expression in *E. coli* is blocked by DisA expression. To determine if DisA overexpression inhibited motility by targeting the flagellar gene cascade, a panel of *lacZ* fusions to class 1 (*flhDC*), class 2 (*fliA*) and class 3(*fliC*) genes were assayed. The expression of β-galactosidase from a plasmid-based *flhD-lacZ*

transcriptional fusion (pVS182) was examined in the presence of the compatible plasmid pACYC184 (vector only) or pAC.DisA (Table 2). In both cases, *flhDC* expression was similar (Table 2). The expression of a single-copy translational FlhDC-LacZ fusion was also similar in the presence or absence of DisA, indicating that DisA does not alter transcription or translation of *flhDC* (data not shown). In contrast, the expression of multicopy transcriptional *lacZ* fusions to class 2 (*fliA*) and class 3 (*(fliC*) genes were reduced 56 and 55-fold, respectively, when *disA* was present in high copy (Table 2).

Phenethylamine inhibits motility and expression of class 2 and class 3 genes in *E. coli.* The DisA decarboxylase shares strongest homology with tyrosine and phenylalanine decarboxylases. In *P. mirabilis*, phenethylamine, but not tyramine, specifically altered class 2/3 gene expression (Stevenson and Rather, 2006). Therefore, the effect of phenethylamine on motility was examined with *E. coli* MG1655, which exhibited a strong inhibition of swimming motility at 12 mM phenethylamine (Figure 1B). Since DisA overexpression specifically altered class 2 (*fliA*) and class 3 (*fliC*) gene expression, the effect of phenethylamine on the expression of these genes was also examined. In the presence of 12 mM phenethylamine, the expression of *fliA-lacZ* was reduced (3-fold) and a *fliC-lacZ* fusion was reduced (2.2-fold). As a control, phenylalanine at 12 mM had no significant effect on *fliA* or *fliC* expression (data not shown).

DisA overexpression and phenethylamine decrease biofilm production in *E. coli* MG1655. To determine if DisA overexpression affected biofilm formation, a time course was carried out with biofilm measurements taken at 14, and 24 hours post inoculation.

Biofilm was inhibited by DisA at each time point, with a 30% decrease at 14 hours and a 60% decrease at 24 hours (Fig. 3). Given that both DisA and phenethylamine inhibited flagellar gene expression in a similar pattern, biofilm formation was examined in the presence of phenethylamine. At 24 hours, in 3 mM and 6mM phenethylamine, biofilm formation was decreased 48 and 49%, respectively. These concentrations did not significantly alter growth rate (data not shown).

DisA overexpression can inhibit motility in other members of the Enterobacteriaceae. To determine if the inhibition of $FlhD_4C_2$ activity by DisA could be extended to other members of the Enterobacteriaceae, strains of *Serratia marcescens, Enterobacter cloaceae, Citrobacter koseri* were transformed with high copy plasmids containing the *disA* gene. In all cases, the presence of *disA* in multicopy resulted in a significant inhibitory effect on swimming motility Fig. 4. Each of these bacteria contains a flagellar regulatory system similar to that of *E. coli* with FlhD and FlhC proteins that are highly similar. In each of these bacteria, motility was also inhibited at least 70% by 12 mM phenethylamine (data not shown).

To investigate if the inhibitory effect of DisA overexpression on motility could be observed in bacteria that do not contain the FlhD and FlhC proteins, the *disA* gene was transformed into *Acinetobacter baumannii,* a motile Gram-negative bacterium. In contrast to the previous bacteria, the overexpression of DisA in *A. baumannii* did not reduce motility (Fig. 4A).

Discussion.

In this study it has been demonstrated that a putative phenylalanine decarboxylase (DisA) isolated from *P. mirabilis* could alter flagellar mediated motility of *E. coli* and other members of the Enterobacteriaceae that contained the FlhD and FlhC proteins. The overexpression of DisA also decreased biofilm formation, which can involve flagellar mediated motility. Through the use of flagellar gene fusions in *E. coli*, it was shown that expression of class 2 and class 3 but not class 1 gene expression was decreased in the presence of DisA. This corresponds to previous work in *P. mirabilis* where a similar pattern of gene regulation was observed by Northern Blot analysis of genes in the flagellar regulon (Stevenson and Rather, 2006). These data suggest that a conserved mechanism for the action of DisA is present in both organisms. In an attempt to identify multicopy suppressors of the DisA-mediated inhibition of motility, an *E. coli* library was screened for the restoration of swimming motility to MG1655 pAC.DisA. These plasmids all contained the *flhDC* operon. The use of *lacZ* fusions demonstrated that *disA* in multicopy did not inhibit *flhDC* transcription or translation. In addition, the stability of the FlhD or FlhC proteins is not altered by DisA expression (Stevenson and Rather, 2006). Therefore, it is suggested that FlhDC is regulated by DisA at the post-translational level by altering the formation of heterotetramers or affecting the ability of $FlhD_2C_2$ to bind DNA.

Based on our previous studies in *P. mirabilis* (Stevenson and Rather, 2006), we propose that phenethylamine or a closely related molecule formed by the DisA decarboxylation reaction is directly mediating the inhibition of FlhDC activity at the post-translational

level. Although exogenous phenethylamine did not inhibit class 2 flagellar gene expression to the same extent as DisA overexpression, this may be due to the inability to achieve the same intercellular concentration of decarboxylated product. For example, DisA overexpression may generate higher intracellular amounts of this product than can be transported when it is supplied exogenously, possibly due to inefficient uptake into the cell. It is also possible that phenethylamine is not the actual product of the reaction catalyzed by DisA, but sufficiently similar in structure to have partial activity.

The finding that flagellar gene regulation and motility of *E. coli* expressing DisA is similar to that in *P. mirabilis* indicates that both organisms have a conserved target for the DisA product. *E. coli* does not encode an obvious DisA ortholog, however, a functionally similar enzyme may be present in *E. coli* with limited similarity to DisA. In addition, amino acid decarboxylation products such as phenethylamine are found in the human digestive tract and may influence bacterial attachment to intestinal mucosa by their effects on flagellar mediated motility (Lyte, 2004, Fischer, et al, 2010). In support of this, *E. coli* possesses genes which convert phenethylamine to phenylacetic acid, suggesting the possibility that cells may encounter phenethylamine in the environment (Hanlon et al, 1997). This study reveals a potentially important application of phenethylamine for the inhibition of motility in the Enterobacteriaceae. For example, the development of urinary tract catheters impregnated with phenethylamine could significantly reduce both motility and biofilm formation on catheter surfaces and reduce the incidence of catheter associated urinary tract infections.

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Table 1. Bacterial strains and plasmids

Strain	Relevant motility genotype	Source or Reference
Escherichia coli MG1655	Motile	Lab strain
Escherichia coli MC1061	Motile	Lab strain
Citrobacter koseri	Motile	Lab strain
Serratia marcescens	Motile	Lab strain
Enterobacter cloacae	Motile	Lab strain
Acinetobacter baumannii M2	Motile	Lab strain

Plasmid	Relevant features	Source
pBluescript.SK	High-copy, Ap^R	Stratagene
pBC.SK	High-copy, Cm^R	
pACYC184	Medium copy, Cm^R, Tc^R	
pAC.Dis	pACYC184 containing *disA* as a XbaI-SalI fragment	
pSK.DisA	pSKwith *disA* expressed form its own promoter	Stevenson & Rather, 2006
pBC.DisA	pBC.SK with *disA* expressed from its own promoter	Stevenson & Rather, 2006
pQF1266	pQF50 containing a 1.3 kb fragment with an *A. baumannii* origin of replication, Amp^R	
pQF.disA	pQF1266 with *disA* as a XbaI-SalI fragment expressed from its own promoter.	This study

Table 2. Effects of DisA overexpression on flagellar gene expression.

Fusion	Plasmid	b-galactosidase[a]	Fold-reduction
pVS182 (*flhD-lacZ*)	pACYC184	22498 ± 962	
pVS182 (*flhD-lacZ*)	pAC.DisA	21863 ± 3891	1
pVS177 (*fliA-lacZ*)	pACYC184	30214 ± 183	
pVS177 (*fliA-lacZ*)	pAC.DisA	537 ± 115	56
pVS175 (*fliC-lacZ*)	pACYC184	16052 ± 521	
pVS175 (*fliC-lacZ*)	pAC.DisA	293 ± 19	55

a. Determined in Miller units from cells harvested at an $O.D._{600} = 0.5$. Values represent quadruplicate samples from two independent experiments.

Figure legends.

Figure 1. Effect of DisA overexpression and phenethylamine on swimming motility in *E. coli.* In panel A, *E. coli* MG1655 containing pSK or pSK.Dis were grown to an OD_{600} of 0.6 and 1 ul was spotted onto 0.2% motility agar for swimming assays. Plates were grown at 37° for 16 hours. In panel B**,** *E. coli* MG1655 cultures were grown to an $O.D._{600}$ of 0.6 and 1ul was spotted onto 0.2% motility agar containing either LB (A) or phenethylamine at a concentration of 12 mM (B). Plates were incubated at 37° for 16 hours.

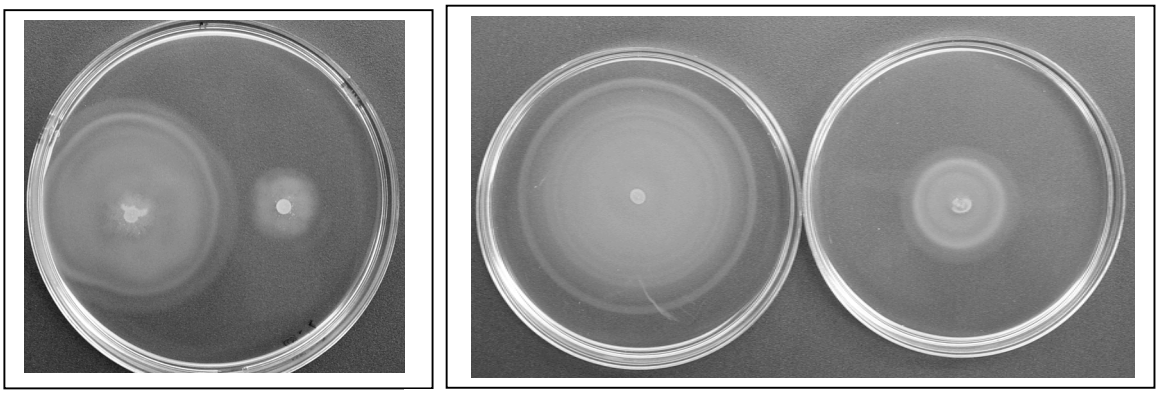
Figure 2. Effect of DisA overexpression on biofilm formation. Biofilm formation was measured by crystal violet staining of *E. coli* MG1655 containing vector (pSK) or *disA* in multicopy (pSK.DisA). Cultures of equal density were diluted 1:100 in LB containing ampicillin and aliquoted into 96 well polystyrene plates. Density was measured at 14 and 24 hours after incubation at 30° prior to washing and staining with crystal violet for biofilm formation. The average of 6 wells per strain is shown. The growth rates of cells containing pSK and pSK.DisA were similar. P-values for the biofilm assays were less than 0.05 between MG1655/pSK and MG1655/pSK.DisA for all conditions.

Figure 3. Effect of phenethylamine on biofilm formation. *E. coli* MG1655 was diluted 1:500 in LB only or LB containing various concentrations of phenethylamine (PEA) and aliquoted into 8 wells per sample of a 96 well polystyrene microtiter plate. Biofilm formation was measured after 24 hours by crystal violet staining of adherent cells. P-

values were less than 0.05 between LB and the two concentrations of phenethylamine tested. The growth rate of cells in either concentration of phenethylamine was not significantly different than in LB only.

Figure 4. Effect of DisA overexpression on motility in additional enteric and non-enteric bacteria. The indicated strains were grown overnight to stationary phase in LB plus the appropriate antibiotics (ampicillin 200 mg/ml for enterics, 800 mg/ml for *A. baumannii* or chloramphenicol (100 mg/ml) and a 1 ml drop was applied to 0.3% LB agar plates. Plates were incubated for 12-24 hours depending on the strain used.

Figure 1.



pSK pSK.DisA 0.2% LB 0.2% LB + PEA

Figure 2.

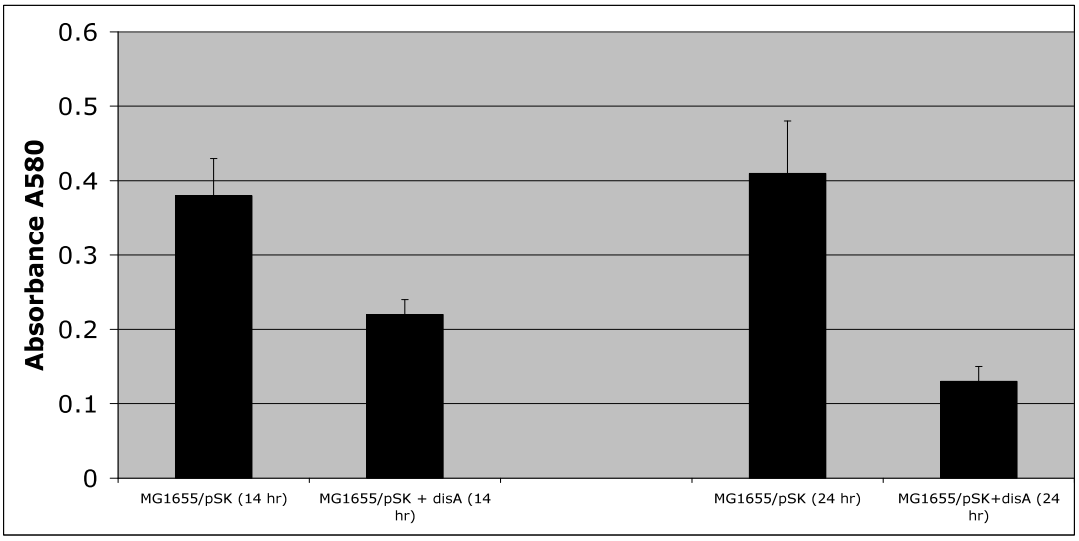

Absorbance A580
0.6
0.5
0.4
0.3
0.2
0.1
0
MG1655/pSK (14 hr)
MG1655/pSK + disA (14 hr)
MG1655/pSK (24 hr)
MG1655/pSK+disA (24 hr)

Figure 3.

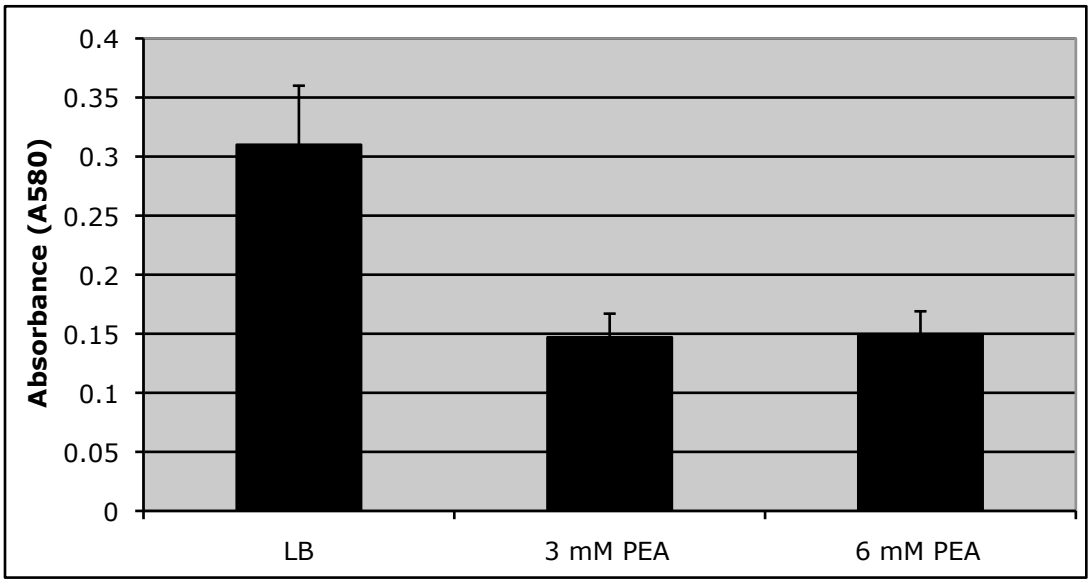
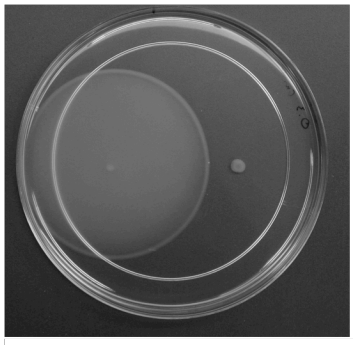
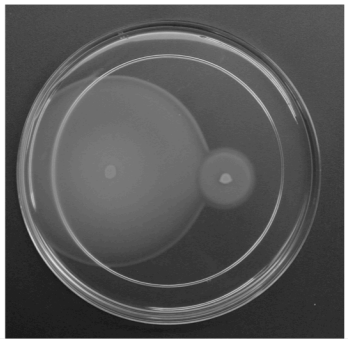


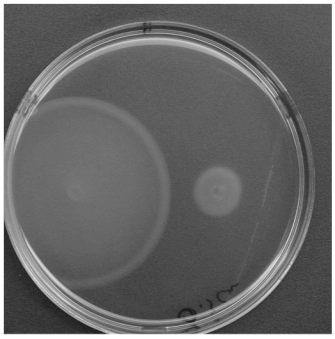
Figure 4.

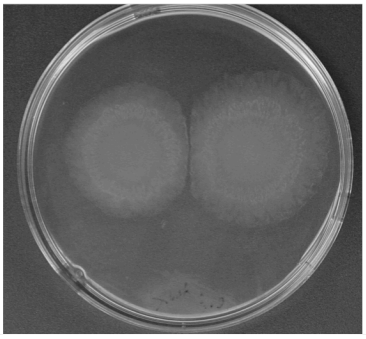




S. marcescens
pBC pBC.DisA

E. cloacae
pSK pSK.DisA





C. koseri
pBC pBC.DisA

A. baumannii
pQF1266 pQF1266.disA

Chapter 4. Regulation of the swarming inhibitor *disA* in *Proteus mirabilis*

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Abstract

The *disA* gene encodes an amino acid decarboxylase that inhibits swarming in *Proteus mirabilis*. The use of 5'RACE and deletion analysis allowed us to identify the *disA* promoter. The use of a *disA-lacZ* fusion indicated that $FlhD_4C_2$, the class I flagellar master regulator, did not have a role in *disA* regulation. The putative product of DisA, phenethylamine, was able to inhibit *disA* expression indicating a negative regulatory feedback loop was present. Transposon mutagenesis was used to identify regulators of *disA* and revealed that *umoB (igaA)* was a negative regulator of *disA*. Our data demonstrate that the regulation of *disA* by UmoB is mediated through the Rcs phosphorelay.

Introduction

Proteus mirabilis is a Gram-negative bacillus and a causative agent of urinary tract infections in patients with abnormal urethras or requiring long-term catheterization (1-3). *P. mirabilis* is also known for its ability to swarm, a form of flagellar-mediated surface motility (4, 5). In liquid media, *P. mirabilis* exists as a vegetative, peritrichously flagellated swimming cell. However, 3 to 4 hours after being placed on a solid surface the vegetative swimming cells differentiate into elongated, multinucleate, aseptate, hyperflagellated swarmer cells (reviewed in (6). The swarmer cells aggregate to form multicellular rafts and move concentrically away from the central inoculum for approximately 1 to 2 hours before de-differentiating back to vegetative swimming cells (7). This cycle of differentiation and consolidation gives *P. mirabilis* its characteristic bulls-eye appearance on agar plates (4).

Flagellar biogenesis is tightly controlled in *P. mirabilis* through a hierarchically tiered regulatory cascade consisting of class I, II, and III gene clusters (reviewed in (8)). Class I consists solely of the flagellar master regulator, *flhDC*. The $FlhD_4C_2$ heterohexamer is the master swarming regulator and activates transcription of Class II genes (9-11). Class II is comprised of genes needed to form the hook-basal body structure of the flagella as well as *fliA*, encoding the swarming sigma factor σ^{28}, and *flgM*, the correspondent anti-sigma factor. σ^{28} is responsible for transcribing Class III genes, including genes involved in chemotaxis, and the structural genes of the flagellar filament and motor. The energy expenditure to fully flagellate a swarmer cell as well as the cyclic aspect of swarming requires that swarming be a tightly regulated process. Several signals inducing

differentiation, such as the inhibition of flagellar rotation, accumulation of putrescine, and O-antigen contacts with a solid surface have been identified; however the signals responsible for consolidation are poorly understood (12-15).

A novel regulator of swarming, *disA*, that bears homology to aromatic amino acid decarboxylases was discovered by Stevenson *et. al.* (16). Disruption of the *disA* gene resulted in a hyperswarming phenotype, whereas overexpression completely abolished swarming (16). Currently, the mechanism by which DisA inhibits swarming is unknown, but it inhibits $FlhD_4C_2$ at the post-translational level, possibly by interfering with multimer formation (16). In addition, the mechanism of DisA mediated inhibition is conserved in other Gram-negative enterics, where DisA overexpression inhibited motility (17). The actual biochemical function of DisA is currently unknown and metabolomic analysis of both *disA* mutant and overexpressing strains did not reveal significant changes in the cellular levels of decarboxylated amino acids. In addition, the use of purified DisA and all possible amino acids did not reveal any products. However, the strong homology of DisA to tyrosine/phenylalanine decarboxylases, together with the fact that phenethylamine, but not tyramine, mimics the effect of DisA overexpression, suggests that DisA is a phenylalanine decarboxylase.

This study further defines the *disA* locus by identifying the transcriptional start site and begins the process of elucidating the regulation of *disA*. 5′ RACE analysis and transcriptional *lacZ* fusions demonstrate that *disA* transcription begins at a thymine residue 70 base pairs upstream of the DisA start codon. Use of a *disA-lacZ* fusion

demonstrated that $FlhD_4C_2$ does not have a significant role in *disA* expression. Transposon mutagenesis was used to identify UmoB as a negative regulator of *disA*. The *umoB* gene product is a negative regulator of the Rcs phosphorelay and has been previously implicated by our lab and others in swarming regulation (15, 18-25). Our data indicate that the effect of the *umoB* mutation on *disA* expression is dependent upon the Rcs phosphorelay system. Taken together, our data indicate that a complex network is responsible for regulation of *disA*, allowing the cell more precise control over the energy intensive process of swarming.

Materials and Methods

Bacterial growth conditions. The bacterial strains and plasmids utilized are listed in Table 1. *P. mirabilis* and *E. coli* were grown in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g sodium chloride per liter) at 37°C with shaking at 250 rpm. For plate growth, *E. coli* and non-swarming *P. mirabilis* strains were grown on 1.5% agar; swarming strains of *P. mirabilis* were plated on 3% agar to inhibit motility. Concentrations of antibiotics for selection for *E. coli* were as follows: 25 μg/mL for streptomycin and chloramphenicol, 20 μg /mL kanamycin, 100 μg /mL ampicillin. Concentrations of antibiotics for selection for *P. mirabilis*: 35μg /mL streptomycin, 100 μg /mL chloramphenicol, 300 μg /mL ampicillin, 20 μg /mL kanamycin, 15 μg /mL tetracycline. 12 μg/mL 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) was used to observe blue and white colonies unless otherwise stated.

Construction of plasmids and strains. All plasmids were introduced by electroporation into PM7002 as follows. PM7002 was grown in 30 ml of LB to an OD_{600} of 0.4-0.6 and cells were harvested by centrifugation at 3000 X g for five minutes at 4°C. Pellets were washed and resuspended in cold 10% glycerol with a final resuspension volume of 60 μl per electroporation. Cells were electroporated in cold cuvettes (Bio-Rad Gene Pulser Cuvette, 0.2 cm) using a Bio-Rad MicroPulser electroporater set to the E2 value for bacterial electroporation. Cells were recovered from the cuvette using 200 μl of pre-warmed LB broth and incubated shaking at 37°C for a minimum of three hours for all plasmids harboring ampicillin resistance and a minimum of one hour for all other

antibiotics. Cells were plated on LB with appropriate antibiotic and agar concentrations and incubated at 37°C to allow growth of transformants.

For PCR amplification of selected genes, Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific) was used. Validation of vector constructs as well as identification of transposon insertion sites was done through Beckman Coulter QuickLane sequencing, according to their recommendations.

To construct pBB1, primers PdisA Fwd and PdisA Rev were used to amplify the 1245 bp region upstream of *disA*. The product was ligated into the SmaI site of pQF50, verified and used to transform PM7002. Plasmid pBB2 was constructed by annealing 10 μg disPro Full Fwd and disPro Full Rev at 72°C for 10 minutes. The product was digested with SalI and BamHI and ligated into pQF50 digested with the same enzymes. The plasmid was validated by sequencing before transformation into PM7002. Plasmid pBB3 was constructed by performing site-directed mutagenesis on pBB1 using primers dis-10Fwd and dis-10Rev. The QuikChangeII Site-Directed Mutagenesis Kit (Agilent Technologies) was used to mutate the eight base pairs at the putative -10 region upstream (TATATCAT to CGCCGGCC) as described in the user manual with the following modifications: 54 ng of pBB1 isolated from XL1 was used as dsDNA template. 18 rounds of amplification were performed at 68°C with one-minute extension per 500 bp (16.5 minutes). 1 μL of 10 μM dis-10Fwd and dis-10Rev primers were used in the reactions. EC100D was transformed with 1 μl of the mutagenesis product and plated on 1.5% LB Agar plates containing 100 μg/mL ampicillin. Plasmid pBB4 was constructed by digesting pBC + *flhDC* with EcoNI, which digests once in the middle of *flhC* gene.

The product was blunt ended with T4 Polymerase (Roche) as described by Promega. The blunt ended vector was ligated to itself and digested a second time with EcoNI to enrich the sample for plasmids that had been successfully re-ligated in the first reaction. This product was transformed into XL1 and selected by plating on chloramphenicol. Sequencing validated the presence of the single base pair insertion in *flhC* that resulted from religation. pBC + *flhC*$^-$ was digested with PvuII and SalI and the excised product was ligated to the suicide vector pKNG101 digested by SmaI with SalI. The proper *flhC* mutation in pBB4 was validated by sequencing before transformation into PM7002.

Strain BB1 was obtained by transposon mutagenesis of PM7002/pBB1 as described in the Transposon Mutagenesis section. BB4 was constructed by conjugating PM7002 with SM10 harboring pBB6. Exconjugants were plated on tetracycline and streptomycin to select for *P. mirabilis* containing a Campbell type insertion of pBB4. A colony was grown in LB broth in the absence of antibiotics and plated on 10% sucrose to identify colonies that had successfully excised pKNG101. Recombinants containing the *flhC*$^-$ allele were validated by Southern blot and transformed with pBB1. Strain BB1 was cured of pBB1 and then mated with SM10 containing pRcsB or pRcsC to obtain the *umoB, rcsB* and *umoB, rcsC* double mutants. Mutants were transformed with pBB1 to yield BB2 and BB3.

5′ Rapid Amplification of cDNA Ends (5′ RACE). 5′ RACE was performed on 5 μl PM7002 RNA according to the 5′ RACE System for Rapid Amplification of cDNA ends, Version 2.0 (Invitrogen) methods section with a few modifications. cDNA was

synthesized according to the Alternative Protocol for first strand cDNA synthesis. Nested PCR was performed using the AUAP primer provided and GSP3 in 40 cycles with 30 second annealing at 59°C and 45 second extension using Phusion Hot Start II High-Fidelity DNA Polymerase. DNA was ligated to the EcoRV site of pBC and transformed into DH5α for blue/white screening. White colonies were cultured and insertion of 5′ RACE products was verified by restriction digestion. Clones harboring inserts were sequenced using the universal T7 primer to identify potential transcriptional start sites. pBB2 and pBB3 were constructed based on the sequence of the cloned 5′ RACE products to determine the transcriptional start site.

Transposon mutagenesis. Strain BB1 was transformed by electroporation with EZ-Tn5™ <Kan-2> Tnp Transposome™ (Epicentre). Transformants were selected on 3% LB containing ampicillin, kanamycin, and X-gal (60 μg /mL). Colonies with increased or decreased blue color were cultured as described above and assayed for β-galactosidase activity (see below). Southern blotting was performed to identify the segment of the chromosome where the transposon inserted. This region was subcloned into pBC and sequenced using primers provided with the transposome to identify the specific site of insertion.

Construction of an *umoB* disruption by a Campbell-type insertion. An *umoB* disruption was constructed in PM2199 *disA*::mini-Tn*5lacZ* by cloning a PCR derived fragment internal to the *umoB* coding region obtained using the following primers:

5’CGTCATCTAGAGCGGTAGAGA

TCCATATTCC and 5'- CGTCAGGATCCGGCCCTTGCTTGATAACATG into the suicide plasmid pKNG101 (26). The construct was then mobilized into PM2199 by a filter mating with *E. coli* SM10 containing the plasmid. Exconjugants were selected on LB plates containing 35 μg/ml streptomycin and 15 μg/ml of tetracycline. The correct disruption of *umoB* was verified by Southern blot analysis.

β-Galactosidase Assays. Overnight cultures were grown and optical densities (ODs) were normalized to the lowest density culture. 200 μl of normalized culture was spread on a 1.5% LB plate and incubated at 37°C for two or four hours, these time points were chosen to assess expression before (T2) and at the peak (T4) of swarming. Cells were collected from plates either by resuspension in 1 ml of fresh LB (two-hour harvest) or by washing one quarter of the plate with 500 μl of fresh LB (four-hour harvest). The OD_{600} was recorded and a portion of the culture was pelleted and frozen at -20°C overnight. Pellets were lysed by chloroform/SDS treatment and assayed as previously described (27). 25mM phenethylamine (PEA) plates were made using appropriate volume phenethylamine (Sigma-Aldrich Cat No. 241008-50ml or Acros Organics Cat No. 156491000) and pH was adjusted to 7.

Results

Identification of the *disA* promoter. The 5'end of the *disA* transcript was identified by 5′ RACE performed on total RNA harvested from wild-type PM7002. Sequencing of the 5′ RACE PCR products returned two potential transcriptional start sites, one located 8 base pairs upstream of the *disA* open reading frame (Figure 1A, open arrow, designated -8) and a second site located 70 base pairs upstream of the *disA* ORF (Figure 1A, closed arrow, designated -70). To determine if active promoters were present upstream of these potential start sites, various fragments were cloned into pQF50 (28) to create transcriptional *lacZ* fusions. These constructs were transformed into PM7002 and assayed for β-galactosidase activity (Fig. 2).

Plasmid pBB1 contains a fragment extending from –1206 to +39 relative to the ATG start of the *disA* gene (Table 1; Fig. 1B). This fragment contains both potential transcriptional start sites as well as part of the *disA* and PMI1208 open reading frames (Fig. 1B). The expression of β-galactosidase from pBB1 was 816-fold and 50-fold higher than in cells containing the pQF50 vector with no promoter inserted at two and four hours after plating, respectively (Fig. 2). PM7002 containing plasmid pBB2 with the *disA* region from –69 to –1 exhibited no β-galactosidase activity, indicating the absence of a promoter upstream of the –8 transcriptional start site (Fig. 1C and Fig. 2). This indicated that the functional *disA* promoter region was upstream of the transcriptional start site originating 70 base pairs upstream of the *disA* ATG start codon. To verify this, site directed mutagenesis was used to change base pairs at the –10 sequence from TATATCAT to CGCCGGCC. The resulting plasmid pBB3 contains these altered base pairs in the

context of the full-length *disA* region present in pBB1. Plasmid pBB3 exhibited a 4.3-fold reduction and a 10.7-fold reduction in β-galactosidase activity two and four hours after being plated on agar surfaces, respectively, when compared to pBB1, indicating that altering nucleotides in the –10 region severely decreased the overall promoter activity.

$FlhD_4C_2$ does not regulate *disA*. The class I activator $FlhD_4C_2$ has a central role in activating gene expression during swarming and our lab and others have previously demonstrated that *disA* expression increases during swarming (16). To address the role of $FlhD_4C_2$ in regulating *disA* expression, a null allele in *flhC* was constructed as described (Materials and Methods) resulting in strain BB4. As expected, BB4 was unable to swarm (data not shown). The loss of *flhC* did not have a statistically significant effect on the expression of a *disA-lacZ* fusion (pBB1) when cells were assayed either 2 or 4 hours after plating on agar surfaces (data not shown). Furthermore, when *flhDC* was overexpressed from a medium copy plasmid, *disA* expression was not altered in a statistically significant manner (data not shown). These data indicate that $FlhD_4C_2$, the master regulator of swarming, does not have a role in regulating *disA* expression.

Role of phenethylamine and autoregulation in *disA* expression. The predicted product of the DisA decarboxylase is phenethylamine (PEA) and previous work demonstrated that exogenous PEA inhibited swarming and flagellar gene expression in a manner similar to *disA* overexpression (16). We assessed the effect of PEA on *disA* expression in PM2199, containing a single-copy transcriptional *disA-lacZ* fusion generated by the insertion of mini-Tn*5lacZ1* into the chromosomal copy of *disA*. This strain was used

because the *disA* gene is inactivated thereby reducing the intracellular levels of the putative product phenethylamine and allowing for a more sensitive assessment of the effects of exogenous phenethylamine. The presence of varying concentrations of phenethylamine decreased *disA* expression in a dose dependent manner at T_4, with 3.1 fold repression seen at 25 mM, 2.7 fold repression at 16 mM, 1.7 fold repression at 8 mM, and 1.3-fold repression at 4 mM (Fig. 3A). The presence of phenethylamine had little effect in cells at T_2 (Fig. 3). To determine if *disA* expression was subject to autoregulation, the *disA* gene was overexpressed in *trans* (Figure 3B). The overexpression of *disA* in PM2199 decreased *disA-lacZ* expression 1.3-fold at four hours after plating on agar, but had no effect at 2 hours.

UmoB is a negative regulator of *disA*. To identify potential regulators of *disA*, transposon mutagenesis was used to create random mutations in PM7002/pBB1 and colonies were screened on X-gal plates for those with increased expression from the *disA-lacZ* fusion. This yielded an insertion in the *umoB (igaA)* gene encoding an integral membrane protein that has been shown in members of the *Enterobacteriaceae* to act as an inhibitor of the Rcs phosphorelay (reviewed in (29)). The transposon insertion in *umoB* resulted in a 2.5 to 3-fold increase in *disA* expression (Figure 4). The levels of *disA* expression were reduced to wild-type in the presence of a plasmid containing the cloned *umoB* gene. An *umoB* mutation was also independently constructed in PM2199 where the *disA-lacZ* is in single-copy and a similar 2.5-fold increase in expression was observed (Fig. 4).

The *umoB* mutation alters *disA* expression via the Rcs phosphorelay. The effect of UmoB on *disA* expression, as well as its established role as an inhibitor of the Rcs phosphorelay, led us to investigate if the Rcs phosphorelay was involved in regulating *disA*. Single mutations in *rcsB* and *rcsC*, the Rcs response regulator and sensor kinase, as well as *rcsB, umoB* and *rcsC, umoB* double mutants were constructed, designated BB5, BB6, BB2 and BB3, respectively (Table 1). Activity of *disA* was measured in samples harvested two hours and four hours after plating by β-galactosidase assays, as described earlier. The data demonstrate that an *rcsB* or *rcsC* single mutant had no effect on *disA* expression; however, the *umoB, rcsB* and *umoB, rcsC* double mutants mitigated the effect of an *umoB* single mutant, returning *disA* expression to wild-type levels (Fig. 5).

Discussion

In this study, regulation of the *disA* locus was characterized by using both single-copy and plasmid based transcriptional *lacZ* fusions, which allowed us to: (i) identify the promoter region, (ii) determine that $FlhD_4C_2$ does not have a role *disA* regulation, (iii) address the role of autoregulation via phenethylamine and *disA* overexpression, and (iv) identify a regulatory mutation in *umoB* that alters *disA* expression via the Rcs phosphorelay. Interestingly, the levels of β-galactosidase from the single copy *disA-lacZ* fusion in PM2199 were higher than the *disA-lacZ* fusion in multicopy (pBB1), compare Figs. 2 and 3. There are several possible explanations for this. First, the *lacZ* gene is translated from different ribosome binding sites in each construct, the RBS in mini-Tn*5lacZ* is from the *trp* operon of *E. coli* and in pBB1 (pQF50 vector), it is derived from *lpp* gene. Second, there may be *cis*-acting regulatory sequences that are missing from the promoter region cloned into pBB1 that are required for full expression.

Mutations in *rcsB* and *rcsC* in conjunction with an *umoB* mutation demonstrated that the increased *disA* expression observed in an *umoB* mutant background was completely abrogated when the Rcs phosphorelay was non-functional. The observation that *disA* expression was not decreased in either an *rcsB* or *rcsC* single mutant was unexpected, but may be due to the presence of the *disA-lacZ* fusion in pQF50, where the effect of the Rcs phosphorelay and specifically the RcsB response regulator on *disA* expression may be masked by the multicopy nature of the *disA-lacZ* fusion. The increased RcsB activity in the *umoB* mutant may be enough to still see regulatory changes in the multicopy *disA-lacZ* fusion. In support of this, recent data from our lab has identified *disA* as an RcsB

activated gene by RNA-Seq analysis (unpublished data). The fact that RcsB activated $FlhD_4C_2$, yet $FlhD_4C_2$, did not have a role in *disA* regulation indicates that RcsB may directly bind *disA* to mediate regulation. Future studies will address this possibility.

Previous research and sequence homology led to the prediction that phenethylamine, the decarboxylated form of phenylalanine, or a similar molecule was the product of DisA (16). We have provided evidence that a negative feedback loop is present for *disA* regulation. First, *disA* expression was decreased in PM2199 when *disA* was overexpressed (Fig. 3). Second, the addition of exogenous phenethylamine inhibited *disA* expression (Fig. 3). However, the magnitude of the repression differed under each condition, 3-fold with phenethylamine at 25 mM versus 1.4-fold with *disA* overexpressed. This may be due to differences in the intracellular levels of phenethylamine, with higher levels present during growth with 25 mM versus potentially lower levels when *disA* is overexpressed. Consistent with this, a strong dose dependent effect on *disA* repression was observed with phenethylamine concentrations ranging from 4 to 25 mM (Fig. 3).

One purpose of this negative feedback loop could be to downregulate *disA* expression after the peak levels of expression have been reached at three to four hours into the swarming cycle. The subsequent decrease in DisA activity would then prepare cells for the next cycle of swarming by relieving the inhibition of $FlhD_4C_2$. It is also possible that extracellular phenethylamine encountered in the environment could also have a role in regulating *disA* expression. It has been proposed that phenethylamine concentrations in

the low millimolar range could be encountered in the intestinal tract and this could influence swarming in the intestine (30). A similar mechanism for the control of *disA* expression could exist in the urinary tract. However, this is highly speculative because, to our knowledge, the levels of phenethylamine in the urinary tract are unknown. The ability to fine tune disA expression by both a negative feedback loop and the Rcs phosphorelay would provide a more precise control of *disA* expression required for the transition between swarming and the consolidation phases.

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Table 1.

Strains or plasmid	Genotype	Source or reference
Strains		
E. coli		
XL1	*endA1 gyrA96*(Nal^r) *thi-1 recA1 relA1 lac glnV44* F' *hsdR17*(r_K^- m_K^+)	Laboratory stock
CC118	*araD139 Δ(ara-leu)7697 ΔlacZ74 phoAΔ20 galE galK thi rpsE rpoB argE*(Am) *recA1 λpir*	(31)
SM10 λ*pir*	*thi thr leu tonA supE recA* RP4-2Tc::Mu Km^r *λpir*	(32)
EC100D	TransforMax EC100D *pir*$^+$ Electrocompetent *E. coli: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (Sm^R) nupG pir$^+$(DHFR)*	Epicentre
DH5α	F$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF'*)*U169 endA1 recA1 hsdR17*(r_K^- m_K^-) *deoR thi-1 supE44* λ^- *gyrA96 relA1*	Laboratory stock
P. mirabilis		
PM7002	Wild type; Tc^r	ATCC
PM2199	PM7002 *disA*::mini-Tn5-*lacZ*; Km^r	Laboratory stock
BB1	PM7002 *umoB*::mini-Tn5-Km^r/pQF50 + P_{disA} *–1206 to +39-lacZ*	This study
BB2	PM7002 *umoB*::mini-Tn5-Km^r *rcsB*::Strep^r/pQF50 + P_{disA} *–1206 to +39-lacZ*	This study
BB3	PM7002 *umoB*::mini-Tn5-Km^r *rcsC*::Strep^r/pQF50 + P_{disA} *–1206 to +39-lacZ*	This study
BB4	PM7002 *flhC*$^-$/pQF50 + P_{disA} *–1206 to +39-lacZ*	This study
BB5	PM7002 *rcsB*::Sm^r/pQF50 + P_{disA} *–1206 to +39-lacZ*	This study
BB6	PM7002 *rcsC*::Sm^r/pQF50 + P_{disA} *–1206 to +39-lacZ*	This study
Plasmids		
pQF50	Low copy; Amp^r	(28)
pACYC184	Low copy; Cm^r	(33)
pFDCH1	pACYC184 + *flhDC*	(34)
pKNG101	R6K-derived suicide vector; Sm^r	(26)
pBB1	pQF50 + P_{disA} *–1206 to +39-lacZ*	This study
pBB2	pQF50 + P_{disA} *–69 to -1-lacZ*	This study
pBB3	pQF50 + P_{disA} *–1206 to +39(Δ-10)-lacZ*	This study

pUmoB	pACYC184 + *umoB*	Laboratory stock
pBB4	pKNG101 + *flhC*⁻ (frameshift mutation)	This study
pUBK	pKNG101 + *umoB*::Km^r	(15)
pRcsB	pKNG101 + *rcsB*::Sm^r	(15)
pRcsC	pKNG101 + *rcsC* (internal fragment)	(15)
pDisA	pKNG101 + *disA* (internal fragment)	Laboratory stock
pMDA	pACYC184 + *disA*	Laboratory stock

Table 2.

Name	Sequence (5′-3′)	Use
GSP1	GGATGACGTGCAATCGCCATCGGCAG	5′ RACE
GSP2	GCTGATAATGTTTTTAAC	5′ RACE
GSP3	CCAGCAGCTAATGAATAA	5′ RACE
disPro Full Fwd	CCCAAGCGTCGACCGTAAAATAAACT CAATTCTGATTAAAATTGATAACAAA AATTTATATATGGATCCTTGGC	$P_{disA}^{-}{}_{69-}{}^{-}{}_{1:}lacZ$
disPro Full Rev	GCCAAGGATCCATATATAAATTTTTG TTATCAATTTTAATCAGAATTGAGTTT ATTTTACGGTCGACGCTTGGG	$P_{disA}^{-}{}_{69-}{}^{-}{}_{1:}lacZ$
PdisA Fwd	ATCAAGGATCCATGAAGATATCGCTT TACCG	$P_{disA}^{-}{}_{1206-}{}^{+}{}_{39:}lacZ$
PdisA Rev	ATCAAGCGTCGACCTGCAGCACTCAG ACAGG	$P_{disA}^{-}{}_{1206-}{}^{+}{}_{39:}lacZ$
dis-10Fwd	GATTCTTACTCATCATGTAGCGCCGG CCTTAAAAAGAGACTAATTATT	$P_{disA}^{-}{}_{1206-}{}^{+}{}_{39\ \Delta-10:}lacZ$
dis-10Rev	AATAATTAGTCTCTTTTTAAGGCCGG CGCTACATGATGAGTAAGAATC	$P_{disA}^{-}{}_{1206-}{}^{+}{}_{39\ \Delta-10:}lacZ$

Figure 1. Construction of transcriptional *lacZ* fusions. In panel A, the region upstream of *disA* is shown with the non-coding region comprised of 511 base pairs, designated -511 to +1 with the *disA* ORF beginning at +1. The bent arrows indicate potential transcriptional start sites identified by 5′ RACE. The black arrow (-70) is located 70 base pairs upstream of the *disA* ORF. The open arrow (-8) is located eight base pairs upstream of the *disA* ORF. Plasmid pBB1 contains a region extending from 39 bp into the *disA* gene to 1206 bp upstream of the *disA* gene (including 695 bp from the PMI1208 gene) fused to a promoterless *lacZ* gene in pQF50 (Panel B). Plasmid pBB2 contains the region from –69 to –1 fused to *lacZ* in pQF50 (Panel C). Plasmid pBB3 is identical to pBB1 but contains an eight bp substitution at the putative -10 region, changing the sequence from TATATCAT to CGCCGGCC (see Materials and Methods; Table 1) (Panel D). Panel E shows the sequence upstream of the *disA* open reading frame. The start codon is underlined, the putative transcriptional start sites located 8 bp and 70 bp upstream of the ATG start codon are shown in bold font. The proposed -10 promoter element is shaded and italicized. Three Rcs binding sites with homology to the consensus sequence (TAAGAATAATCCTA) are underlined and mismatched bases are in grey.

Figure 2. Transcriptional activity of promoter fragments. 5′ RACE returned two potential transcriptional start sites: ¯70 and ¯8 (Figure 1). Various promoter fragments were cloned upstream of a promoterless *lacZ* gene in pQF50 as shown in Fig. 1. LB agar plates were inoculated with an overnight culture of each strain that were adjusted to identical optical densities and cells were harvested off plates at 2 and 4 hours after

plating. The data shown are representative of two independent experiments with samples assayed in triplicate.

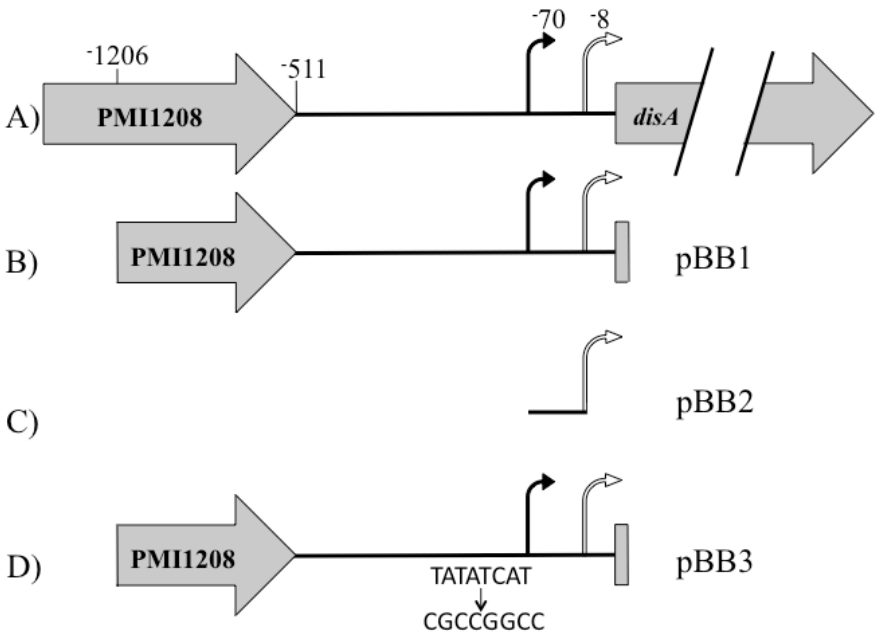
Figure 3. Role of autoregulation in *disA* expression. In panel A, the effect of phenethylamine on *disA* expression was assayed in PM2199 containing a single copy *disA-lacZ* fusion generated by an insertion of mini-Tn*5lacZ1* into the *disA* coding region. In this strain, the *disA* gene is inactivated by the transposon insertion. In panel B, *disA-lacZ* expression is measured in PM2199 cells containing either the vector pACYC184 or pACYC184 + *disA*. Cells were harvested off plates as described for Fig. 2. Data shown are representative of two independent experiments with samples assayed in triplicate.

Figure 4. *disA* expression in *umoB* mutants In panel A, the expression of *disA-lacZ* was monitored from plasmid pBB1 in wild-type PM7002 and an *umoB::Km* mutant. In panel B, the effect of the *umoB* mutation on a single-copy *disA-lacZ* fusion was examined in PM2199 or the corresponding *umoB::Sm* mutant. The expression of *disA* was measured by β-galactosidase expression in cells harvested 2 and 4 hours after plating on agar plates as described for Fig.2. Data shown are representative of two independent experiments with samples assayed in triplicate.

Figure 5. Regulation of *disA* via UmoB is through the Rcs phosphorelay. The expression of *disA* was measured by β-galactosidase expression from plasmid pBB1 in wild-type PM7002, *umoB::Km* mutant (BB1), an *rcsB::Sm* mutant (BB5), an *rcsC::Sm* mutant (BB6) and *rcsB, umoB* and *rcsC, umoB* double mutants (BB2 and BB3,

respectively). Data shown are representative of at least two independent experiments with samples assayed in triplicate.

Figure 1.



E) E

E)

GACTATGGTAAAAATATTATAATAAGTTTTGAA
AAATAGCATAAATCAAAATTGATAATGAAATT
TATAAACTAATTAATAAAACTAAATCATTTATC
ATGAAAAAGTAAAAACTTTAGAACGAACTTGG
AAAATCTTATCTGATATCAATAAAATAAAGAA
GATTCTTACTCATCATGTAG*TATATCAT*TTAAAA
AGA**G**ACTAATTATTTACGTAAAATAAACTCAA
TTCTGATTAAAATTGATAACAAAAATTTATATA
TAGGTGCGATG

Figure 2.

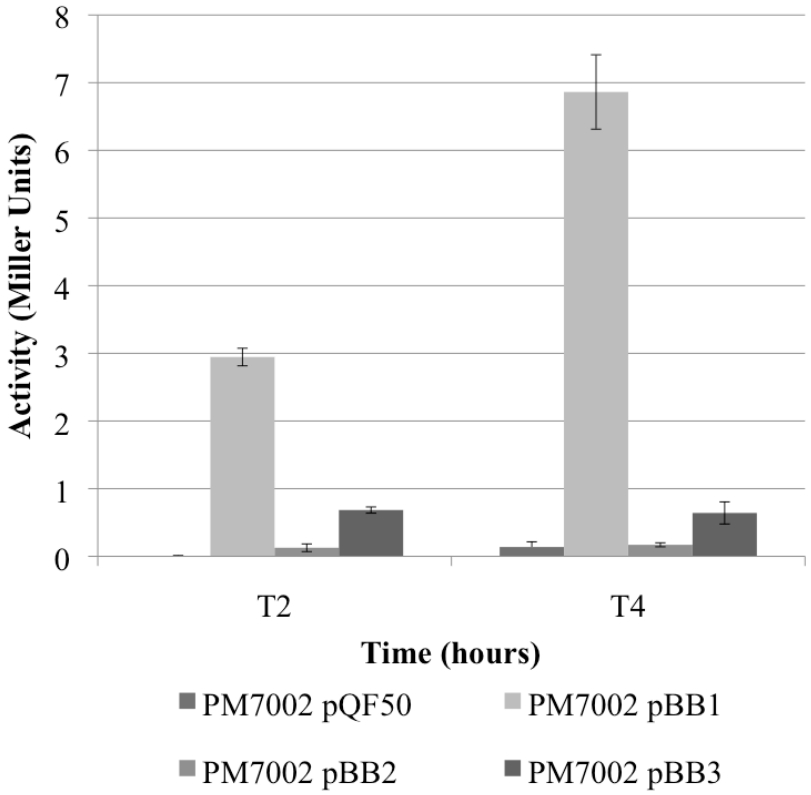

8
7
6
5
4
3
2
1
0
Activity (Miller Units)
T2
T4
Time (hours)
PM7002 pQF50
PM7002 pBB1
PM7002 pBB2
PM7002 pBB3

Figure 3.

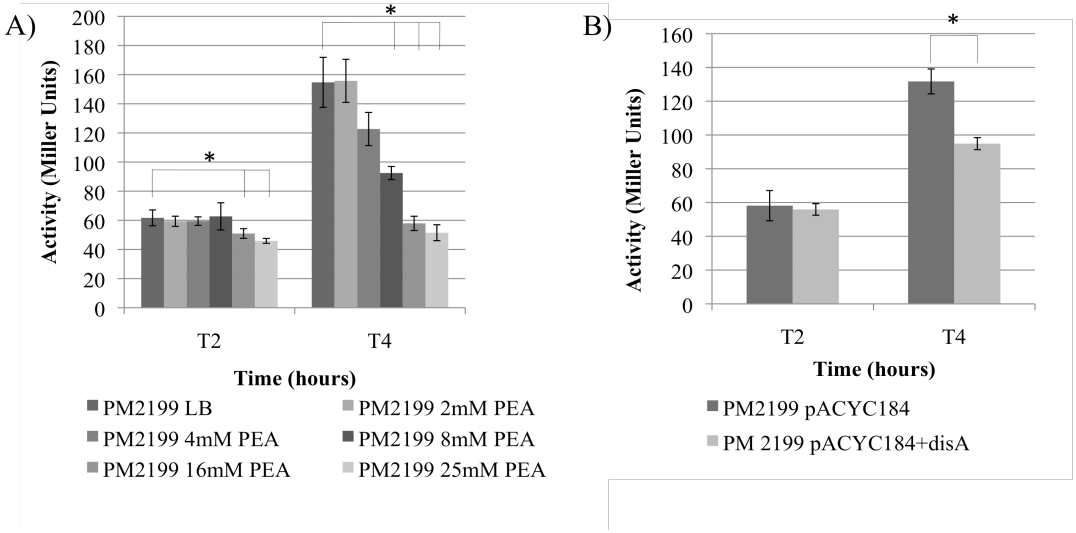

A)
Activity (Miller Units)
200
180
160
140
120
100
80
60
40
20
0
*
*
T2
T4
Time (hours)
PM2199 LB
PM2199 2mM PEA
PM2199 4mM PEA
PM2199 8mM PEA
PM2199 16mM PEA
PM2199 25mM PEA
B)
Activity (Miller Units)
160
140
120
100
80
60
40
20
0
*
T2
T4
Time (hours)
PM2199 pACYC184
PM 2199 pACYC184+disA

Figure 4.

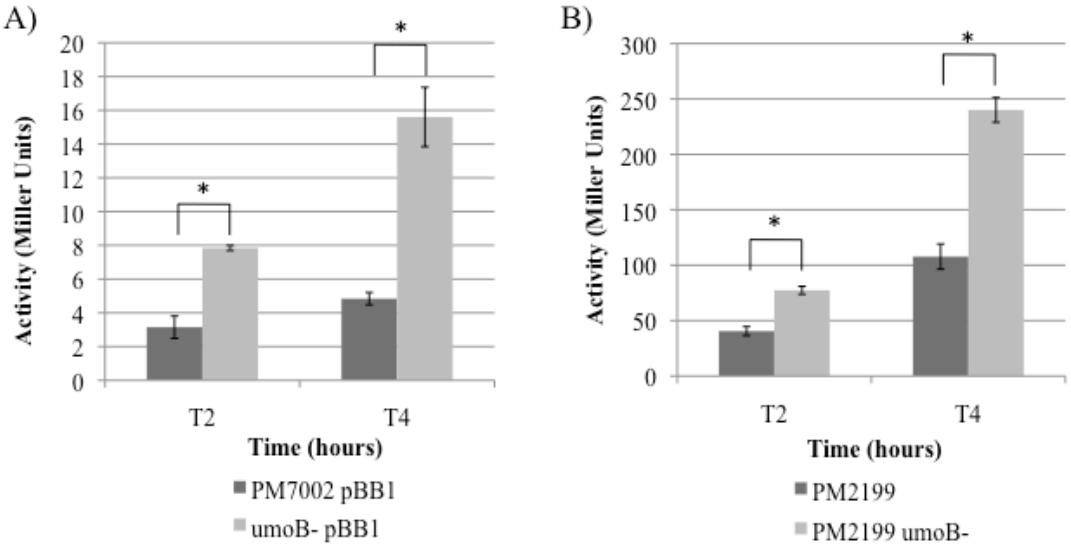
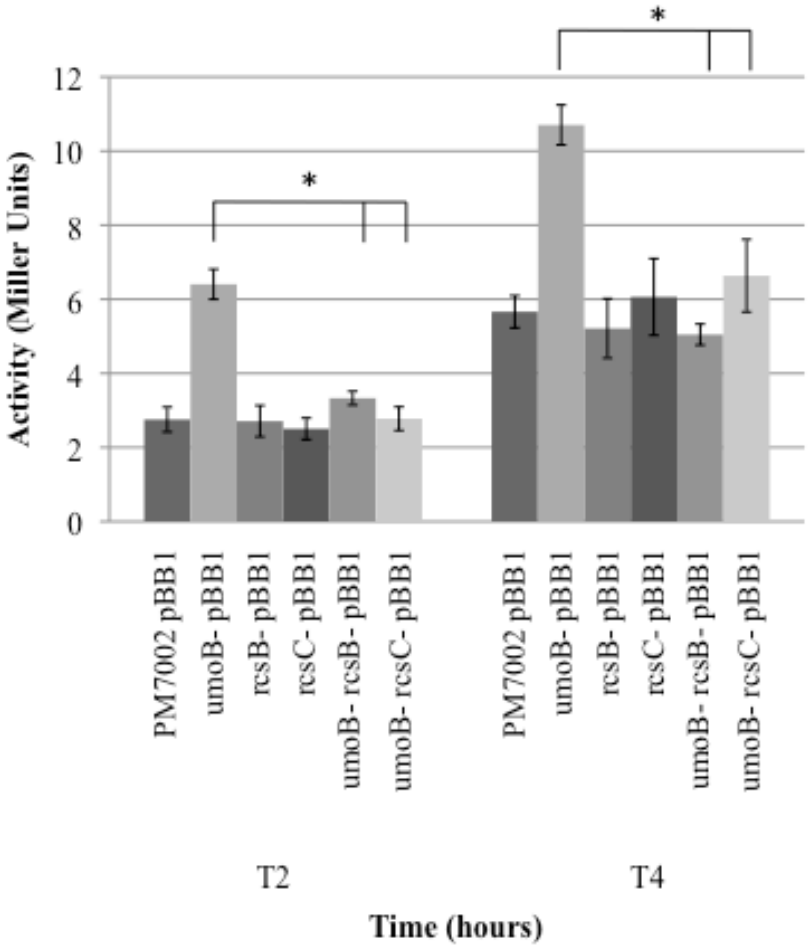

A)
Activity (Miller Units)
20
18
16
14
12
10
8
6
4
2
0
*
*
T2
T4
Time (hours)
PM7002 pBB1
umoB- pBB1
B)
Activity (Miller Units)
300
250
200
150
100
50
0
*
*
T2
T4
Time (hours)
PM2199
PM2199 umoB-

Figure 5.


*
*
12
10
8
6
4
2
0
Activity (Miller Units)
PM7002 pBB1
umoB- pBB1
rsB- pBB1
rsC- pBB1
umoB- rsB- pBB1
umoB- rsC- pBB1
PM7002 pBB1
umoB- pBB1
rsB- pBB1
rsC- pBB1
umoB- rsB- pBB1
umoB- rsC- pBB1
T2
T4
Time (hours)

Chapter 5: Supplementary Data

I. Identification of a spontaneous mutant repressing *disA* expression.

A. Methods

i. Determining the presence of a spontaneous mutant

PM7002 had been previously transformed with pBB1 (pQF50+P_{disA}::*lacZ*) and mutagenized with Ez-Tn5™ <Kan-2> Tnp Transposome™ (Epicentre) as described in the Materials and Methods section of Chapter 4. A strain harboring the transposon demonstrated a 13-fold reduction in *disA* expression at T4 (Figure 1). Subcloning and sequencing determined that the transposon had inserted in the gene *cpsF*, previously shown to encode capsular polysaccharide glycosyl transferase (4). To construct an independent *cpsF* mutation, a 523 bp interior region of *cpsF* was cloned into the suicide plasmid pKNG101 (primers cpsFint 5'-CGTCAGGATCCGGAACA TTAGCAGCAAG and cpsFrev 5'-CGTCATCTAGAGCCCAAGCAGATTGTATAG) and transformed into *E. coli* SM10 for conjugation with PM2199 (*disA::*Tn5<*lacZ*>) and PM7002 pBB1. After 7 hours, exoconjugants were plated on 3% agar LB $Strep_{35,}$ Tet_{15} with Amp_{300} for PM7002 pBB1 exogonjugants. Mutation of *cpsF* via a Campbell-type insertion was validated by Southern blotting. *disA* activity was measured by β-galactosidase assays as described in Materials and Methods Chapter 4.

B. Results

The β-galatosidase expression of *disA* in wild-type (PM7002) pBB1 and a *cpsF* mutant at two hours and four hours after growth on an agar plate is shown in Figure 1A. To

validate the decrease observed in *disA* expression in PM7002 *cpsF*⁻ was due to mutation in the chromosome and not a mutation in the *lacZ* reporting vector the strain was cured of pBB1 and retransformed. β-galactosidase assays in the newly transformed strain confirmed the abrogation of *disA* expression was due to mutation in the chromosome and not the reporter vector (data not shown). *cpsF* has been previously characterized to act as a capsular polysaccharide glycosyl transferase and mutations in *cpsF* inhibit swarming. The observations that mutation of *cpsF* in PM2199 does not decrease *disA* expression suggested *cpsF* may not be responsible for the decrease in *disA* observed in PM7002 pBB1 *cpsF*::Tn5 and that a second site mutation may have occurred (Figure 1B). To verify the discrepancy in *disA* regulation by *cpsF* was not the result of the *lacZ* reporter fusion being in *cis* in PM2199 (compared to in *trans* im PM7002 pBB1), we also reconstructed the mutant in PM7002 pBB1. PM7002 pBB1 *cpsF*::pKNG101 did not generate the same decrease in *disA* expression observed in the PM7002 pBB1 *cpsF*::Tn5 strain (compare Figure 1A and C). All of our *cpsF* mutant strains were not swarming, as previously shown for *cpsF* mutants. Complementation of the *cpsF* allele was impossible given that it is located in a large operon. Both transposon mutagenesis and disruption with pKNG101 create polar mutations thus the regulatory effect of *cpsF* on *disA* should be observed even if the responsible gene were downstream of *cpsF*. While all three *cpsF* mutants are genotypically identical and all demonstrate swarming negative phenotypes, *disA* expression is not decreased in PM2199 or *cpsF*::pKNG101. These data strongly suggest a second site mutation, not *cpsF*, is responsible for the observed decrease in *disA* in PM7002 pBB1 *cpsF*::Tn5. The second site mutation has thus been renamed as *disI*, *disA* inhibitor though its location in the chromosome remains unknown.

II. Purification of DisA and biochemical analysis of decarboxylation of phenylalanine.

A. Methods

i. Protein Purification

Induction: 500 mL LB Amp_{150} was inoculated with 1.3 mL of an overnight BL21 pET21a+*disA* culture and grown to an OD_{600} of 0.4-0.5 shaking at 25ºC. Cultures were induced at 25ºC for 3 hours with 1 mM IPTG. Induced cells were spun down for 20 minutes at 5520 x rcf at 4ºC, the supernatant was discarded and pellets were frozen overnight at -20ºC.

Lysis: All steps occured at 4ºC to maintain native structure of the protein unless otherwise stated. Pellets were resuspended in 10 mL of chilled Equilibration Buffer (EqB, 50mM $NaPO_4$, 300mM NaCl, pH7) with 5 µl DNaseI (Epicentre) and 1 tablet protease inhibitor (Roche complete mini EDTA-free Protease Inhibitor cocktail tablet) dissolved and french pressed in a cooled pressure cell at 600 psi twice. The lysate was centrifuged at 10000 x rcf for 20 minutes and the supernatant was applied to a ClonTech His60 Ni^{2+} Superflow Resin equilibrated as follows:

Binding: 2mL of resin (giving a bed volume of 1mL) was resuspended and spun at 700 x rcf for 2 minutes. Storage liquid was removed without disturbing the resin pellet and the resin was resuspended in 10 mL EqB and spun at 700 x rcf for 2 minutes, the EqB wash was done twice. Cell lysate and resin were mixed on a tube roller for 90 minutes before being spun down at 700g for 5 minutes. Resin was washed twice with 10mL EqB in tube inverter for 10 minutes and centrifuged at 700g for 5 minutes. Resin bound by protein

was resuspended in 2mL EqB, added to the gravity column and allowed to settle before elution.

Elution: The EqB present from loading the column was allowed to flowthrough before adding 10 mL of EqB to wash the column. Next 10 mL of a 20mM imidazole (in EqB) wash was added followed by 10 mL of a 50 mM imidazole wash (in EqB) and then 2 mL of 200 mM imidazole elution buffer (in EqB). These steps were all performed at 4°C and the eluted material was stored at 4°C. Fractions were visualized on a 12% SDS-PAGE gel strained with coomassie blue to identify purified fractions containing DisA.

Dialysis: SpectrumLabs Spectra/Por Float-A-Lyzer G2 MWCO 20kDa was filled with and submerged in UltraPure ddI water for 30 minutes at room temperature to condition the dialysis membrane. The tubing was transferred to 100 mL of dialysis buffer (50 mM $NaPO_4$, 100 mM NaCl, pH 7) and the ddI water was from the tubing using a Pasteur pipette. The purified elution fractions were added to dialysis membrane and incubated at 4ºC with gentle stirring. The dialysis buffer was changed at hours 3-4 and 8-10 and allowed to dialyze overnight.

Concentration of protein: To concentrate the dialyzed protein eluent we used SpectrumLabs Spectra/Gel absorbent. Dialysis membrane was placed back in the plastic container it was originally shipped in and gel absorbent was poured to surround the membrane. The top of the container was parafilmed, incubated at 4ºC for 90 minutes and remaining eluent volume was assessed every 15 minutes beyond the initial incubation time. Sample was reduced to approximate 400 µl of 1.3 mg/mL of protein. 100% glycerol was added to a final concentration of 50% and purified protein was stored at -20ºC.

ii. Biochemical decarboxylation

This assay was originally designed by Osterman *et. al.*, modified by Smithson *et. al.* and was altered for our purposes as follows (5, 6). Briefly, our experiments were carried out under normal atmospheric conditions at 25ºC or 37ºC. Absorbance at OD_{340} was measured every 2 minutes in a plate reader over the course of 70 minutes. All reagents were stored as recommended by the manufacturer and equilibrated to either room temperature or 37ºC for ten minutes prior to whole cell lysate or enzyme addition to reduce background absorbance. Final volume of reactions was 61.8µl using the following concentrations of reagents: 58% Infinity CO_2 Liquid Stable Reagent (Thermo Scientific), 0.5 units LDC (Sigma), 2.5 mM Tyrosine, 200 mM Lysine, 200 mM Alanine, 24.3 mM Phenylalanine, 30.5 mM Phenylalanine, 5 mM Pyridoxal 5' Phosphate (PLP), 0.5-5 µl 1.27 mg/mL Purified DisA.

B. Results

The theoretical basis of the assay was to observe the functionality of an amino acid decarboxylase by linking the availability of CO_2 to the consumption of NADH by malate dehydrogenase and measure the depletion of NADH by a decrease in absorbance at OD_{340}. The assay, initially designed for ornithine decarboxylase, uses CO_2 as the limiting reagent in the reaction. As decarboxylation occurs, more free CO_2 is available to be converted to bicarbonate. Phosphoenolpyruvate carboxylase (PEPC) uses bicarbonate to convert phophoenolpyruvate to oxaloacetate. Malate dehydrogenase reduces oxaloacetate to malate in an NADH dependent pathway. Thus, as the amount of free CO_2

in the system increases the rate of NADH oxidation increases and absorbance at 340 nm decreases. For our purposes we modified the assay to function with lysine decarboxylase as a means to generate a positive control (Figure 2).

A functional positive control demonstrated we had successfully modified the assay to function under normal atmospheric conditions (the assay was previously developed under an inert nitrogen atmosphere to increase sensitivity and reduce background (6)) and now needed to optimize the reaction for DisA. In this venture we added pyridoxal 5'-phosphate to the reaction given its prediction to be a necessary co-factor for DisA decarboxylation. We used multiple varying conditions to try to identify the functional parameters for DisA. These included the concentrations listed above in addition to pH 5-9, the addition of whole cell lysate in case of denaturation during purification or requirement of an unknown co-factor and reaction temperatures of 25ºC and 37ºC, a complete list of tested conditions is available in Table 1. Our data indicate decarboxylation activity by DisA is unobservable using this assay in our hands (data not shown).

III. Metabolomic Analysis of PM7002 pSK, PM2199 (*disA::*EzTn5*lacZ*) pSK and PM7002 pSK+*disA*.

A. Methods

i. Growth and harvest of strains

Strains were grown in 3mL LB Amp_{300} to maintain the pSK plasmid shaking at 37ºC overnight. Optical densities were normalized the following morning and 200µl of each

culture was plated on to LB 1.5% agar plates in sextuplet. Cultures were incubated at 37ºC for four hours to allow cells to reach the peak of swarming and then harvested. OD_{600} was measured and harvested cells were normalized to an OD_{600}= 1.6. One mL of the normalized cultures was spun down for 10 minutes at 21130 x rcf and stored at -20ºC until being sent for metabolic analysis.

ii. Bacterial metabolomics

Metabolite Extraction from Pellet: Methods for this section were kindly provided by James Cox, PhD, Director of the Metabolomics Core Facility at the University of Utah. Cell pellets were extracted using a modified method derived from Canelas *et. al*. (3). To each cell pellet was added 5 mL of boiling 75% EtOH (aq) followed mixing by vortex then incubated at 90°C for five minutes. Cell debris was removed by centrifugation at 5000 g for three minutes. The supernatant was removed to new tubes and dried *en vacuo*.

GC-MS Analysis: All GC-MS analysis was performed with a Waters GCT Premier mass spectrometer fitted with an Agilent 6890 gas chromatograph and a Gerstel MPS2 autosampler. Dried samples were suspended in 40 µL of a 40 mg/mL O-methoxylamine hydrochloride (MOX) in pyridine and incubated for one hour at 30°C. To autosampler vials was added 25 µL of this solution. Ten microliters of N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) was added automatically via the autosampler and incubated for 60 minutes at 37°C with shaking. After incubation 3 µL of a fatty acid methyl ester standard solution was added via the autosampler then 1 µL of the prepared sample was injected to the gas chromatograph inlet in the split mode with the inlet temperature held at 250°C. Two GC-MS runs were performed, one at a 10:1 split ratio to

detect low level metabolites and a second at 100:1 split ratio to accurately measure high concentration metabolites which saturate the detector at the 10:1 split ratio. For the 10:1 split ratio analysis the gas chromatograph had an initial temperature of 95°C for one minute followed by a 40°C/min ramp to 110°C and a hold time of 2 minutes. This was followed by a second 5°C/min ramp to 250°C, a third ramp to 350°C, then a final hold time of 3 minutes. For the 100:1 split ratio analysis the gas chromatograph had an initial temperature of 95°C for one minute followed by a 40°C/min ramp to 110°C and a hold time of 2 minutes. This was followed by a second 25°C/min ramp to 330°C. A 30 m Phenomex ZB5-5 MSi column with a 5 m long guard column was employed for chromatographic separation. Helium was used as the carrier gas at 1 mL/min.

Analysis of GC-MS data: Data was collected using MassLynx 4.1 software (Waters). A two-step process was employed for data analysis, a targeted followed by non-targeted analysis. For the targeted approach, known metabolites were identified and their peak area was recorded using QuanLynx. These data were transferred to an Excel spread sheet (Microsoft, Redmond WA). For the non-targeted approach, peak picking and analysis was performed using MarkerLynx. Principle component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) analysis was performed using SIMCA-P 12.0 (Umetrics, Kinellon, NJ). Potential metabolite biomarkers were further investigated by manually recording the peak area into the original Excel file followed by performing t-tests. All metabolites that were found at high concentrations in the 10:1 analysis, particularly phosphate and glucose, the 50:1 data set was used to record accurate data. These data were normalized for extraction efficiency and analytical variation by mean centering the area of D4-succinate.

Metabolite identity was established using a combination of an in house metabolite library developed using pure purchased standards and the commercially available NIST library. When reporting each metabolite, those with absolute identity are not qualified, those that are identified using a NIST library are noted using a percentage of certainty produced by the NIST software.

Metabolites which are completely unknown are labeled as unRT_m/z where un=unknown, the RT=retention time and m/z=mass to charge ratio. An example of this is an unknown metabolite that elutes at 15.22 minutes and has a characteristic mass of 247 would be written as un15.22_247. Only those unknowns that are statistically relevant are recorded.

Not all metabolites are observed using GC-MS. This is due to several reasons, one being that they present at very low concentrations. A second is they are not amenable to GC-MS due to either being too large to volatilize, are a quaternary amine such as carnitine, or just don't ionize well. Metabolites that do not ionize well include oxaloacetate, histidine and arginine. Cysteine is observed depending upon cellular conditions as it often forms disulfide bonds with proteins and is at low intracellular concentration.

Data was further analyzed for statistical significance using MetaboAnalyst 2.0 (7). The raw data was normalized to D4-succinate to account for analytical variation. These data were then filtered using mean intensity to remove low quality data. To account for biological variation from cell culture the data was row normalized by the total sum of the

row. To account for the large variation in concentration between different metabolites the data was scaled using the autoscaling method.

B. Results

Non-targeted metabolic analysis of PM7002 (wild-type), PM2199 (*disA* mutant) and the *disA* overexpressing strain PM7002 pSK+*disA* indicated 12 metabolites were differentially regulated: Five metabolites were decreased in abundance when *disA* was overexpressed compared to the levels observed in PM7002 and/or PM2199, seven metabolites were present at increased levels when *disA* was overexpressed compared to PM7002 and/or PM2199, and one metabolite was decreased when *disA* was mutated compared to PM7002 (Figure 3). All metabolites reported had a p-value < 0.05 (noted with asterisks), those with a p-value < 0.01 are noted with †. The metabolites which differ with the highest confidence based on abundance in the cell, p-values and volcano blots in the comparison of $disA^+$ to PM2199 are sucrose-trehalose, glycersol and aspartic acid. Glycerol and aspartic acid also have the highest confidence ratings of the metabolites altered in $disA^+$ versus PM7002.

IV. Aerobic and Anaerobic Growth of PM7002 pSK, PM2199 (*disA::*EzTn5*lacZ*) pSK and PM7002 pSK+*disA* with Metabolic Intermediates.

A. Method

i. Growth of strains

Strains were grown shaking at 250 rpm at 37°C overnight in 3mL LB cultures with Amp_{300} in order to maintain pSK. The optical densities of the strains were normalized at 600 nm before 200 µl of each strain was plated. Cultures were spotted in the middle of a

1.5% agar LB plate containing Amp_{300} and plates containing 10 mM succinate, fumarate or malate as previously described (1). Plates were incubated at 37°C in aerobic conditions until swarming reached the exterior of the plate (~10 hours for PM2199 and PM7002). For anaerobic growth plates were incubated at 37°C for 16 hours, anaerobic conditions were achieved using GasPak™ EZ Anaerobic Container System (BD).

B. Results

Our metaboloic data indicated that two TCA intermediates, citric acid and succinic acid, were altered by *disA* overexpression. Furthermore, recent publications demonstrated that TCA cycle intermediates and glutamine have a role in regulating swarming during both aerobic and anaerobic growth (1,2). Taken together, these observations prompted us to investigate if providing TCA intermediates exogenously would alter swarm patterns under aerobic or anaerobic conditions. We observed no change in the swarming pattern of PM7002 pSK, PM2199 (*disA*$^-$) pSK, or PM7002 pSK+*disA* during anaerobic growth with succinate, fumarate or malate compared to LB alone (Figure 5). At the end of 16 hours of anaerobic growth on LB PM7002 exhibited a small swarm zone off one side of the central inoculum, the distance this zone (termed a spray for our purposes) extended from the inoculum increased when incubated with the studied TCA intermediates though the spray remained localized to one side. We observed a more drastic change in PM2199 motility, reported in Table 2.

Supplementaing plain LB with succinate, fumarate or malate did not result in terrace formation by wild-type (PM7002) *P. mirabilis*. Both succinate and fumarate produced a spray that added 16 cm to the migration distance. Providing malate created a spray that increased migration distance by 6 cm. Given that terraces were not formed when metabolites were added to the media, it is unlikely that the sprays are a result of media supplementation but may indicate that a mutation occurred enhancing motility at one portion of the inoculum. Whether this mutation occurred in response to metabolite supplementation is unclear. As shown in Table 2, the addition of metabolites had a far greater effect on PM2199 (*disA*$^{-}$) than wild-type. On unsupplemented LB, PM2199 showed a single uneven swarm ring after 16 hours of anaerobic incubation. Succinate provided the smallest change in motility, producing two distinct swarm rings but not altering the distance migrated. Malate increased the migration distance by 10 cm and permitted the development of three distinct terraces. Fumarate exhibited the greatest effect on swarming in PM2199, increasing the migration distance by 35 cm and allowing the production of four distinct terraces. These data, presented in Table 2, are from one experiment but are representative of multiple independent repetitions. The implications of these data are discussed in the following chapter.

References

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Figure Legends

Figure 1. A spontaneous mutant is responsible for decreased *disA* expression.

A) β-galactosidase assay of PM7002 pBB1 and PM7002 pBB1 *cpsF*::Tn5. B) β-galactosidase assay of PM2199 and PM2199 *cpsF*::pKNG101. C) β-galactosidase assay of PM7002 pBB1 and PM7002 pBB1 *cpsF*::pKNG101

Figure 2. Optimization of a biochemical decarboxylation assay.

A) Schematic of the chemical reaction leading to decreased NADH observed at 340 nm. In this system, Lysine is decarboxylated by Lysine Decarboxylase (LDC) releasing CO_2 which is utilized to convert phosphoenolpyruvate to oxaloacetate by phosphoenolpyruvate decarboxylase. Malate dehydrogenase can then reduce oxaloacetate to malate in an NADH-dependent reaction. NADH is observed at an absorbance of 340nm, thus as decarboxylation occurs the rate of reaction increases as indicated by a decrease in absorbance. Modified from Smithson et.al. (5). B) Proof of principle graph demonstrating optimization of the assay for LDC at pH6. Solution-only samples indicate the presence of all reagents except for LDC. Decarbox samples had 0.5units of LDC added after 10 minutes of incubation at 37°C (black arrow) and then were allowed to proceed for an additional hour. The sharp decrease in absorbance observed after addition of LDC indicates the enzyme functioned to decarboxylate Lysine, thereby increasing the amount of CO_2 available in the system and increasing the oxidation of NADH to NAD^+.

Figure 3. Comparative fold change of cellular metabolites in PM7002 pSK, PM2199 pSK, and PM7002 pSK+*disA*.

Twelve metabolites were shown by non-targeted bacterial metabolomics to be differentially regulated in a *disA* overexpressing strain (PM7002 pSK+*disA*) when compared to wild-type (PM7002) and a *disA* mutant strain (PM2199) and/or when PM2199 is compared to PM7002. Data should be read such that in "strain A vs strain B" a positive fold-change indicate that strain A has x-fold more of a given metabolite when compared to strain B, a negative fold-change shows that strain a has x-fold less of a metabolite compare to strain B. * denotes a p-value <0.05, † denotes a p-value <0.01, statistical analysis by 2-tailed student T-test.

Figure 4. The TCA cycle.

A diagram of the classical aerobic TCA Cycle with the addition of side reactions that may utilize intermediates of aerobic cellular respiration to affect cellular levels of Aspartic Acid.

Figure 5. Addition of succinate, fumarate or malate does not alter swarming during aerobic growth.

200 µl of over-night, normalized PM7002 (wild-type) and PM2199 (*disA*$^{-}$) were spotted on to LB plates with 1.5% agar containing either 10 mM succinate, 10 mM fumarate or 10 mM malate and incubated anaerobically at 37°C for 16 hours. No changes in motility were observed for any of the assayed strains on the chosen metabolites.

Table 1. Experimental conditions for a biochemical decarboxylation.

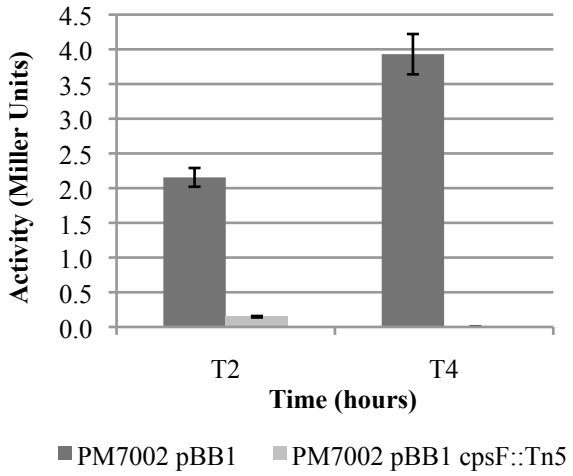
Summary of conditions used in the modified biochemical decarboxylation assay. Conditions were tried in different combinations over the course of twenty-five independent experiments. [a] Concentration of substrates was limited due to solubility. [b] Lysates were harvested from 90 minute inductions of *disA* with 1 mM IPTG in either 30 mL or 100 mL LB flasks. Cells were lysed as described in Chapter 5 Section II.A.i. Debris was removed by centrifugation and pellets were resuspended in 1 mL for 30 mL flasks and 5 mL for 100 mL flasks.

Table 2. Addition of succinate, fumarate and malate affects the swarm pattern of PM2199 during anaerobic growth.

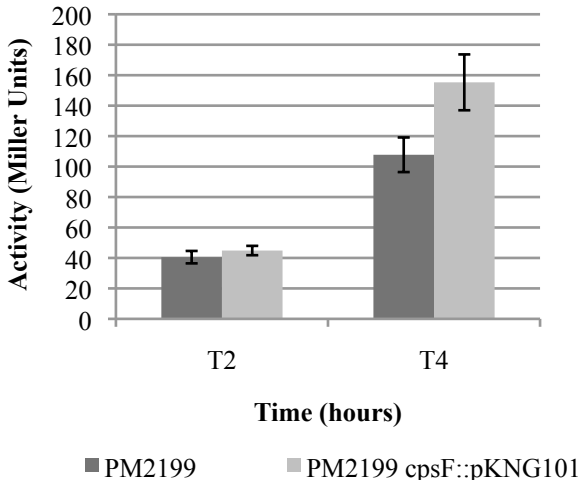
Cells were spotted on LB plates with 1.5% agar as well as plates supplemented with 10 mM succinate, fumarate, or malate and grown anaerobically for 16 hours at 37°C. The number of swarm rings formed was counted and the maximum distance between the swarm front and central inoculum was measured. [a] S denotes the swarm pattern was in a "spray", only one portion of the inoculum swarmed creating motility on one portion of the plate but not a full swarm terrace. Measurement shown represents the distance from the outer end of the spray to the central inoculum.

Figure 1.

A)


4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Activity (Miller Units)
T2
T4
Time (hours)
PM7002 pBB1
PM7002 pBB1 cpsF::Tn5

B)


200
180
160
140
120
100
80
60
40
20
0
Activity (Miller Units)
T2
T4
Time (hours)
PM2199
PM2199 cpsF::pKNG101

C)

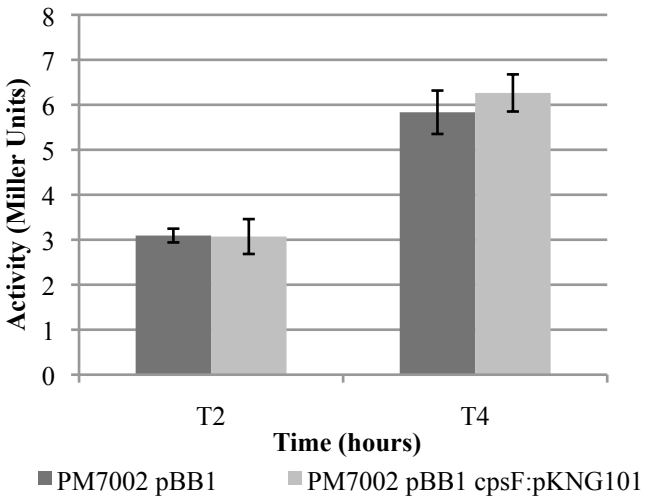
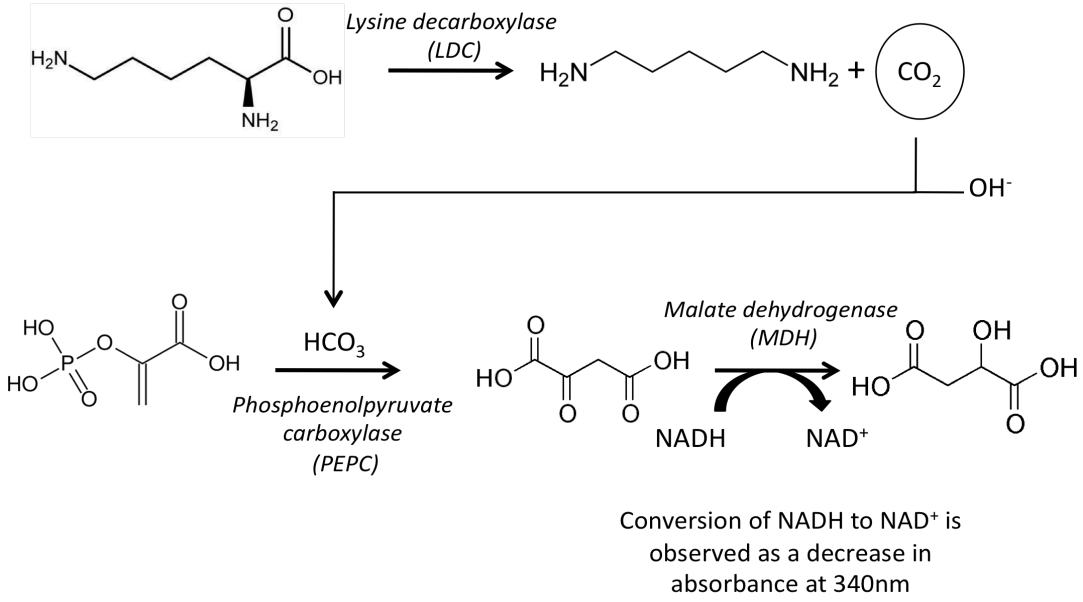

8
7
6
5
4
3
2
1
0
Activity (Miller Units)
T2
T4
Time (hours)
PM7002 pBB1
PM7002 pBB1 cpsF:pKNG101

Figure 2.

A.


Lysine decarboxylase
(LDC)
H_2N
OH
NH_2
H_2N
NH_2 +
CO_2
OH^-
HO
HO
O
OH
HCO_3
Phosphoenolpyruvate
carboxylase
(PEPC)
Malate dehydrogenase
(MDH)
NADH
NAD^+
Conversion of $NADH$ to NAD^+ is observed as a decrease in absorbance at 340nm

B.

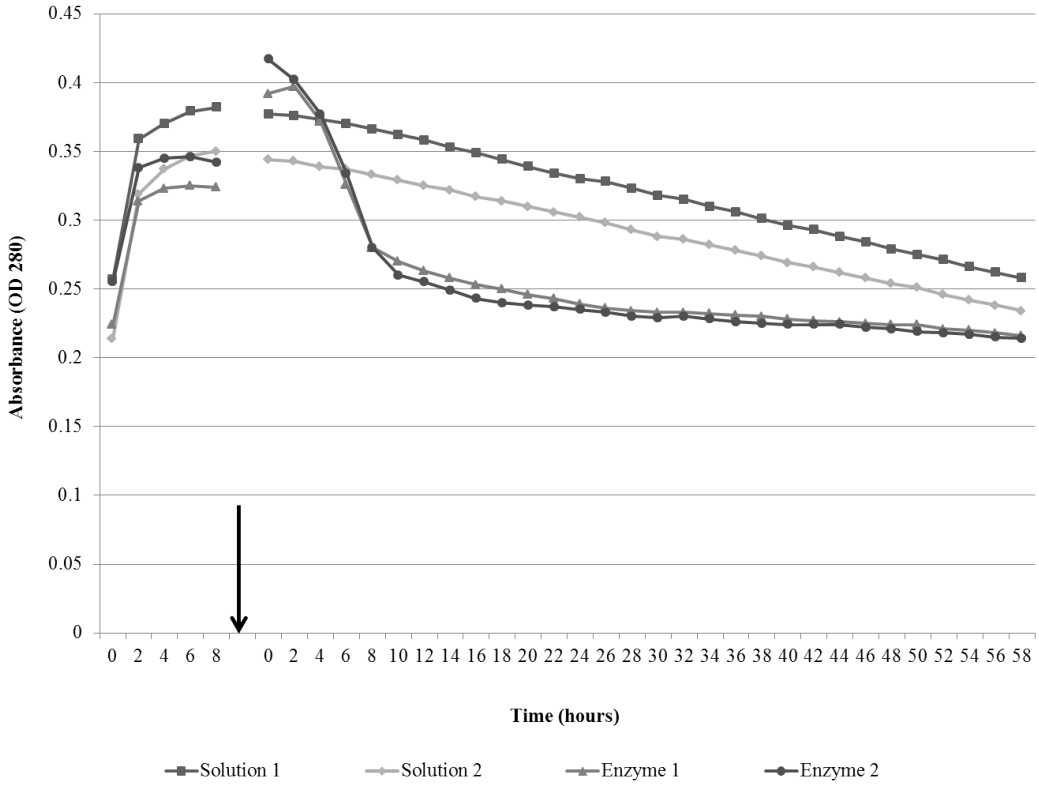

Absorbance (OD 280)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 2 4 6 8 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58
Time (hours)
Solution 1
Solution 2
Enzyme 1
Enzyme 2

Figure 3.

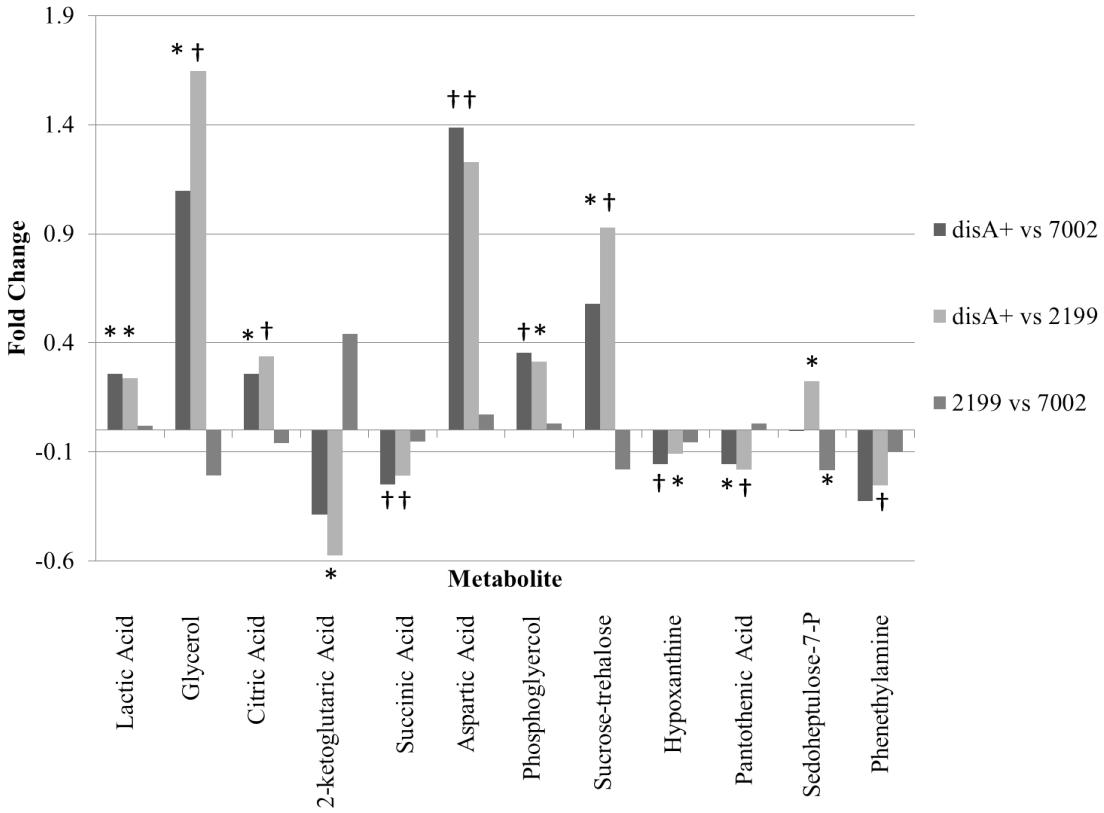

1.9
1.4
0.9
0.4
-0.1
-0.6
Fold Change
* *
* †
* †
*
††
††
* †
†*
* †
†*
* †
*
*
†
Metabolite
Lactic Acid
Glycerol
Citric Acid
2-ketoglutaric Acid
Succinic Acid
Aspartic Acid
Phosphoglycercol
Sucrose-trehalose
Hypoxanthine
Pantothenic Acid
Sedoheptulose-7-P
Phenethylamine
disA+ vs 7002
disA+ vs 2199
2199 vs 7002

Figure 4.

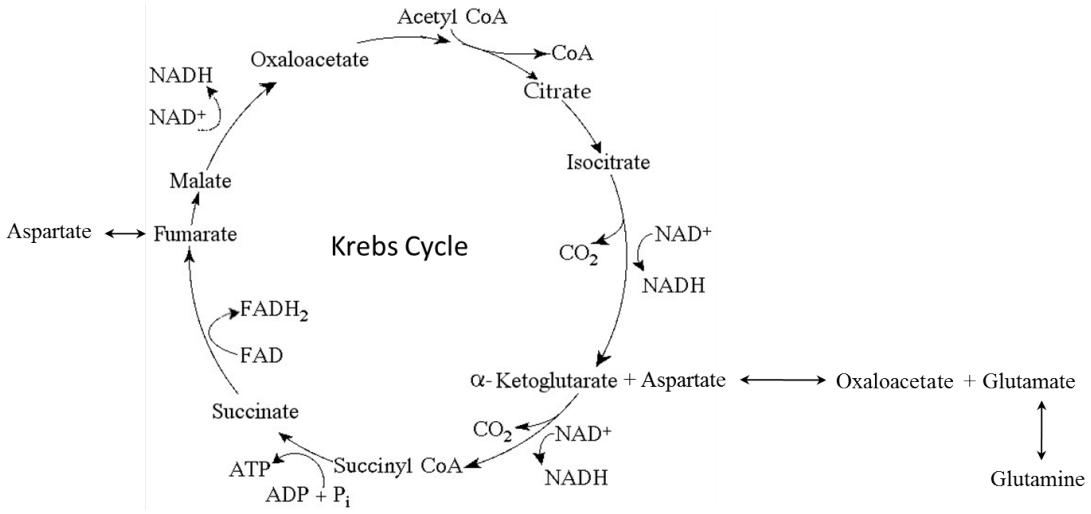

Acetyl CoA
CoA
Citrate
Isocitrate
NAD+
NADH
CO2
α-Ketoglutarate + Aspartate
Oxaloacetate + Glutamate
Glutamine
NAD+
NADH
CO2
Succinyl CoA
ATP
ADP + Pi
Succinate
FADH2
FAD
Fumarate
Aspartate
Malate
NADH
NAD+
Oxaloacetate
Krebs Cycle

Figure 5.

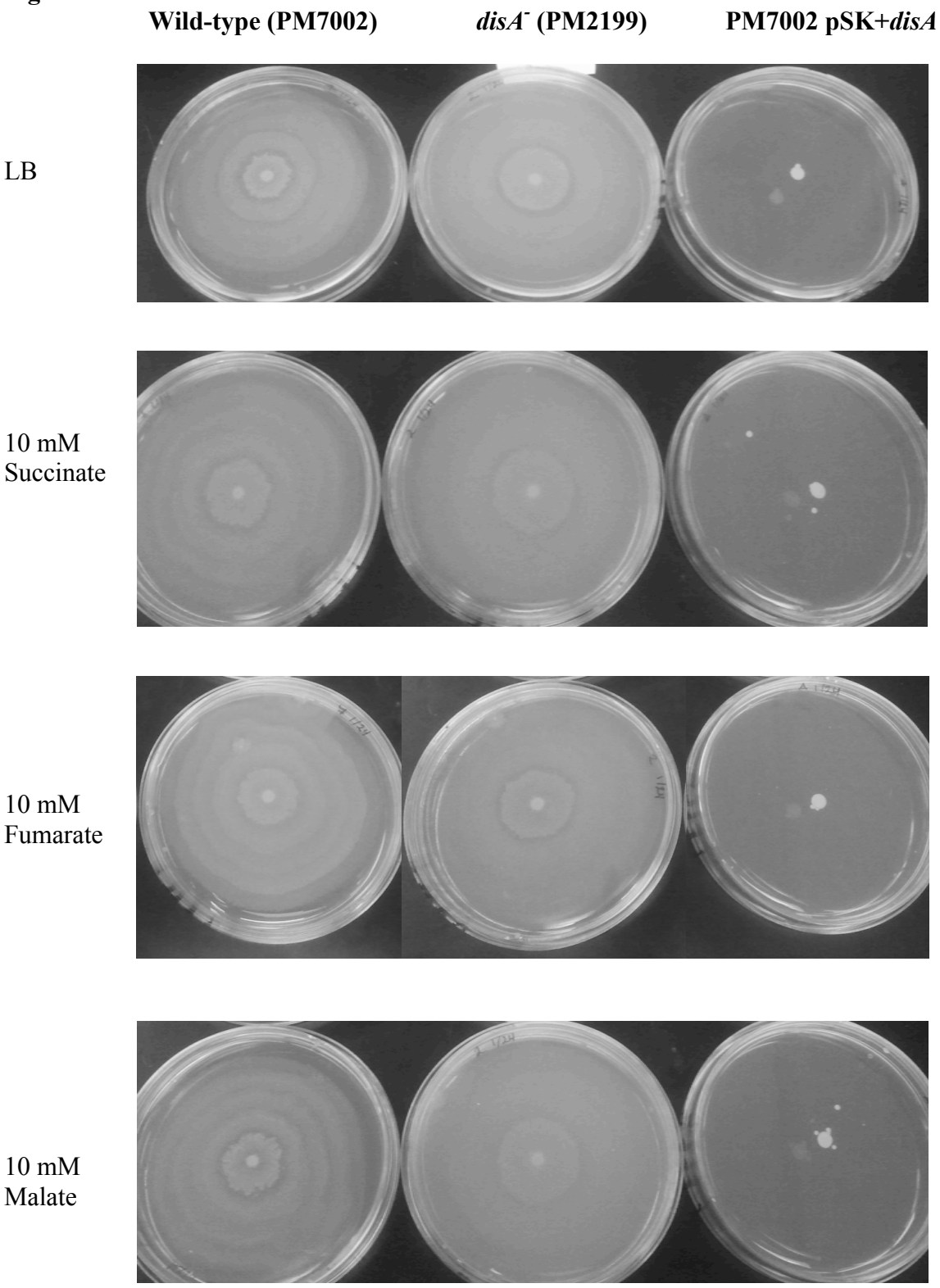

Wild-type (PM7002)
disA⁻ (PM2199)
PM7002 pSK+*disA*
LB
10 mM Succinate
10 mM Fumarate
10 mM Malate

Table 1.

Parameter	**Conditions Tested**
Substrate[a]	Phenylalanine – 30.5 mM, 34.75 mM, 100 mM Tyrosine – 2.5 mM
Temperature	25ºC, 37ºC
pH	5.3, 5.5, 6, 7, 8, 9
Time	1 hour, 2 hours
Reducing Agent	None, 1 mM DTT, 2.3 mM DTT
Protease Inhibitor	None, 1-2 tablet EDTA-free complete protease inhibitor
Co-factor	No PLP, 1.5mM PLP
Enzyme	Crude whole cell lysate[b] – 1 µl, 5 µl, 10 µl Purified – 0.635 µg, 1.27 µg, 3.175 µg ,6.35 µg ,12.7 µg
Total Assays Performed[c]	25

[a] Concentration of substrates was limited due to solubility.

[b] Lysates were harvested from 90 minute inductions of *disA* with 1 mM IPTG in either 30 mL or 100 mL LB flasks. Cells were lysed as described in Chapter 5 Section II.A.i. Debris was removed by centrifugation and pellets were resuspended in 1 mL for 30 mL flasks and 5 mL for 100 mL flasks. [c] Number refers to assays performed after LDC optimization.

Table 2.

Strain	Metabolite (10 mM)	Migration Radius (cm)	No. of Rings[a]
Wild-type	None	4	None
Wild-type	Succinate	20	S
Wild-type	Fumarate	20	S
Wild-type	Malate	10	S
disA$^-$	None	15	1
disA$^-$	Succinate	15	2
disA$^-$	Fumarate	50	4
disA$^-$	Malate	25	3

[a] S denotes the swarm pattern was in a "spray", only one portion of the inoculum swarmed creating motility on one portion of the plate but not a full swarm terrace. Measurement shown represents the distance from the outer end of the spray to the central inoculum.

Chapter 6: Discussion

P. mirabilis is a Gram-negative, rod-shaped bacterium and a member of the *Enterobacteriaceae*. While *P. mirabilis* is an effective pathogen harboring a myriad of virulence factors and the leading cause of catheter associated urinary tract infections, the organism is best studied for its form of multicellular surface motility, termed swarming (12). Originally described by Hauser in 1885 the swarming motility of *P. mirabilis*, observed over a century ago, still beguiles scientists today (8).

While several Gram-negative organisms swarm, swarming in *P. mirabilis* is unique in its cyclical nature and distinctive pattern (swarming of Gram-negatives reviewed in (20)). Swarming occurs in three basic stages: differentiation, migration and consolidation. Differentiation occurs approximately 3-4 hours after the cells encounter a solid surface (reviewed in (13)). At this point the swimmer cells, differentiate into elongated, aseptate, multinucleate, hyper-flagellated swarmer cells. Following differentiation, the cells collide with one another to form multicellular motile bodies termed rafts. Held together by co-bundled flagella, these rafts migrate away from the central inoculum; this is the migratory process that will create the first swarm terrace on an agar plate. After migration, the cells undergo a process of de-differentiation, or consolidation, where they return to their vegetative form and prepare to repeat the process. It's the cycle of these phases that gives *P. mirabilis* its characteristic terrace, or bulls-eye, pattern on an agar plate.

Swarming is an energetically expensive process, thus each phase of swarming is highly regulated to ensure the cell does not commit to swarming in undesirable conditions. Differentiation is regulated by several signaling pathways, including a proposed quorum sensing mechanism mediated by putrescine levels (17) as well as surface sensing mediated by O-antigen and the Rcs phosphorelay (10). It was previously shown that O-antigen senses the solid surface by imposing membrane torque (10). This torque activates an integral membrane protein, UmoB (a homologue of *E. coli* IgaA) which is well characterized to interact with the Rcs phosphorelay (11, 19) (Figure 1). As a non-canonical two component system, the relay's sensor kinase, RcsC is unable to autophosphorylate when UmoB is active, leading to an increase in dephosphorylated RcsB, the response regulator, in the cell (9). Phosphorylated RcsB (RcsB~P) has been shown in *E. coli* to repress the master regulator of swarming, *flhDC* (7) (Figure 1B). It is proposed RcsB~P represses *flhDC* in *P. mirabilis* as well due to the highly conserved nature of the system among the Enterobacteria. While many other regulators of *flhDC* have been identified and characterized in both *E. coli* and *P. mirabilis* (reviewed in (12, 13)), the Rcs phosphorelay is of greatest significance to this work.

While a great deal is known about regulating differentiation, very little is known about the regulation of consolidation during swarming. Approximately 5 hours post-plating, cells undergo the consolidation process marked by the return to vegetative morphology and a decrease in transcription of *flhDC* and flagellar components. How this quick genetic change occurs is unknown. To date two mutations affecting consolidation have been identified, both by our laboratory. The first is a transposon insertion at either 325

and 720 basepairs upstream of the *flhDC* operon in a region devoid of open reading frames (5). These mutations completely abolish consolidation, leaving the cells in a perpetual hyperswarming state and creating a thin, fast spreading, singular swarm ring on an agar plate. The second was identified and initially characterized by Stevenson *et. al.* and is known as the decarboxylase inhibitor of swarming A, *disA* (16). The work presented here has focused on increasing our understanding of the regulation and function of this enzymatic swarming inhibitor.

When initially studied very little was determined about *disA* with the exception of its swarming phenotypes. Stevenson *et. al.* demonstrated mutation of *disA* was able to restore swarming to a *speA* mutant (16). The authors also showed overexpression of *disA* completely abolished swarming and mutation lead to a modest but reproducible hyperswarming phenotype in a wild-type background (16). Our work has brought a new level of understanding to DisA both genetically and biochemically by defining a regulatory system, determining the start site of transcription, identifying the -10 promoter element, categorizing the effect of *disA* expression in other Gram-negative organisms and beginning the characterization of the physiological function of DisA.

As discussed in Chapter 4 we now know that *disA* is regulated by the Rcs phosphorelay, a non-canonical two component system well conserved among enterobacteria. In this work we showed mutation in *umoB*, the *P. mirabilis* homologue of the *E. coli igaA*, increased *disA* expression 2.5-3 fold (4, 6, 18) (Chapter 4, Figure 4). UmoB was previously shown to regulate the Rcs phosphorelay by inhibiting the autophosphorylation ability of the

sensor kinase, RcsC, when grown on a solid surface (11) (Figure 1B). Utilizing basic recombinant genetic techniques and epistatic studies we demonstrated the effect of *umoB* mutation on *disA* expression was mediated by the Rcs phosphorelay as evidenced by the restoration of *disA* to wild-type levels in *umoB rcsB/rcsC* double mutants. Further *in silico* analysis identified three near consensus RcsB binding sites upstream of the *disA* transcriptional start site (21). The identification of the transcriptional start site of *disA* 70 basepairs upstream from the ATG start codon as well as identification of the -10 promoter element will assist in future studies to demonstrate that RcsB~P directly regulates *disA* expression.

Identifying the Rcs phosphorelay as a regulator of *disA* was novel not only because regulators of *disA* were previously unidentified, but also because this implicates the system in swarming regulation at multiple levels. In *E. coli* direct regulation of *flhDC* by RcsB has been observed (7). It is proposed regulation of *flhDC* by RcsB is also direct in *P. mirabilis* due to the conserved nature of this system as well as the presence of a near consensus sequence (13 of the 14 basepairs are conserved) of an Rcs binding box upstream of *flhDC* (21). In *P. mirabilis*, the Rcs phosphorelay had not been shown to regulate swarming independently of *flhDC*. We now know that the Rcs phosphorelay regulates swarming at multiple levels by affecting the transcription of *flhDC* as well as *disA*.

We propose that co-regulation of the master swarming regulator, *flhDC*, and a potent inhibitor of swarming, *disA*, would provide the cell greater control over the swarm cycle.

Cells deficient in *umoB* result in an increase in RcsC autophosphorylation, causing the cell to mimic liquid growth even when on agar. The non-swarming phenotype observed in *umoB* mutants can be attributed to constitutive repression of *flhDC* by RcsB~P (phosphorylated RcsB) in these strains (Figure 1A versus 1B). However, the increase in *disA* observed in an *umoB* mutant may also contribute to the non-swarming phenotype. In theory, the simplest way to regulate two genes of divergent function is by having a single protein responsible for their inverse regulation.

There are two possible mechanisms of *disA* regulation by RcsB given the current data: unphosphorylated RcsB represses *disA* or RcsB~P activates *disA*. Despite having insufficient data to determine whether *disA* is under positive or negative regulation by RcsB, based on our current data it can be hypothesized that RcsB~P activates *disA* (Chapter 4, Figures 4 and 5; Figure 1A). Bacteria have been observed in several conditions to utilize the same DNA binding protein to regulate two genes of divergent functions in inverse manners. Thus, it would appear that the simplest way for the cell to regulate an activator of swarming (*flhDC*) and an inhibitor of swarming (*disA*) is to use RcsB~P to repress production of Flh_4C_2 and activate *disA* transcription. This mechanism would ensure *disA* expression in liquid culture where swarming is not desirable and may decrease *disA* expression sufficiently upon encountering a solid surface to allow for the onset of swarming. The direct regulation of *disA* by RcsB could be assessed in the future via DNA binding assays utilizing the phosphorylated and non-phorphorylated forms of RcsB to determine whether the hypothesis presented here or counterhypothesis is correct.

Furthermore, employing DNaseI protection assays in conjunction with Sanger sequencing would demonstrate if any of the predicted Rcs boxes were indeed RcsB binding sites.

It has become clear through this study that *disA* expression is not "on" or "off" but rather there are varying degrees of *disA* levels in the cell at different points in swarming. RcsB (phosphorylated or not) acting as the sole regulator of *disA* cannot account for the varying levels of *disA* transcription observed over the swarm cycle. Furthermore, one would expect the *umoB* mutation to elicit a greater change in *disA* expression than the observed 2.5-3 fold increase if RcsB is the sole regulator. Taken together, these data strongly suggest at least one additional, unidentified *disA* regulator exists. We have identified a spontaneous mutant that decreases *disA* expression thirteen-fold by β-galatosidase assay. Recent attempts to identify the location of this mutation via complementation with a *trans* over-expression library have been unsuccessful though the work is still in its infancy. Given the mutation is unmarked, there are two reasonable approaches to identifying this elusive regulator: complementation with an over-expression library or whole genome sequencing. While over-expression library complementation may be the most cost effective approach it comes with a myriad of challenges, not the least of which is ensuring the library contains a complete random sampling of the *P. mirabilis* genome. Even if the library is successfully generated, this approach may not successfully identify the location of the mutation. For example, if the spontaneous mutation is in an early gene in an operon the library may not contain the full operon making complementation impossible. While whole genome sequencing is a more assured method of identifying the mutation the genome of PM7002, our wild-type strain, itself is not yet sequenced making

this option both more difficult and resource intensive. Identifying the unmapped regulator is a project worth considering in the future.

As previously mentioned, *disA* expression is not all or none, but demonstrates variation over the course of the swarm cycle. The expression of *disA* peaks 3-4 hours in to the swarm cycle, this increase in *disA* levels coincides with the peak of *flhDC* expression as well as the onset of swarming. If *disA* is involved in regulating the periodicity of the swarm cycle, that is if the accumulation of the metabolite of DisA is involved in consolidation, concurrent expression with *flhDC* is logical. Allowing DisA to accumulate in the cell with maximal genetic expression at the peak of the swarm cycle should give the enzyme sufficient time to produce the swarming inhibitor molecule and repress swarming. However, this is extremely speculative given the product and function of DisA remain unknown. Furthermore, these data do not indicate whether the function of DisA is to produce a swarming inhibitor or sequester a signal required for swarming. The possibility of signal sequestration will be discussed later in the chapter.

Attempts at identifying the product of DisA have been informative but inconclusive. Several years ago it was put forth by Stevenson et.al. that DisA may be a phenylalanine decarboxylase (16). This hypothesis was based on the homology of DisA to an aromatic amino acid decarboxylase, specifically a tyrosine decarboxylase, and the observation that addition of phenethylamine (PEA) exogenously inhibited swarming as observed by *disA* over-expression. While these observations were sufficient to form the decarboxylase

hypothesis, they by no means serve as proof of decarboxylase activity in DisA. This work has assisted in the accumulation of suggestive evidence that DisA acts as a decarboxylase by demonstrating the existence of a negative feedback loop between phenethylamine (PEA) and *disA*. Dose dependent repression of *disA* by exogenously providing 4 mM-25 mM PEA was observed in a chromosomal *disA*::*lacZ* fusion (Chapter 4, Figure 3A). Given PEA was able to repress *disA* expression, we proposed if PEA is the metabolic product of DisA, overexpressing *disA* should lead to the same repression of the *disA*::*lacZ* fusion. To test this hypothesis, we overexpressed *disA* on a multicopy plasmid and measured β-galactosidase using the chromosomal *lacZ* fusion, the results are shown in Chapter 4, Figure 3B. Supplementing growth with 25 mM PEA decreased *disA* expression 3.1-fold (Chapter 4, Figure 3A) while overexpressing *disA* decreased *disA*::*lacZ* fusion activity 1.4-fold (Chapter 4, Figure 3B). The disparity between the decrease observed in *disA* expression when PEA is provided and when *disA* is over-expressed may be due to the inability of *disA* over-expression to reach equally high intracellular concentrations of PEA as exogenous provision. On the other hand, the disparity may be due to the fact DisA produces a molecule similar to, but not PEA. Nonetheless, this is indicative of a regulatory connection between PEA and *disA*.

In an attempt to more conclusively demonstrate the phenylalanine decarboxylase activity of DisA, an ornithine decarboxylase biochemical assay was modified to function under our required conditions (14). The technicalities of this assay are discussed extensively in Chapter 5 and conditions are summarized in Table 1. In summary, we utilized purified DisA as well as whole cell lysate (raw and partially purified) to attempt to show

decarboxylation activity with phenylalanine and tyrosine as substrates. We altered reactions by using a range of pH 5 – 9, varying concentrations of each substrate, changing the reaction temperature, modifying co-factor concentrations, and altering the amount of protein or lysate used. Under all conditions no decarboxylation activity was observed using DisA (Table 1); functional positive controls suggest the lack of decarboxylation is not a limitation of the assay but a legitimate lack of decarboxylase activity.

The initial argument for DisA acting as a decarboxylase arose from conserved domain results from NCBI BLAST showing homology to the *Enterococcus faecalis* tyrosine decarboxylase Tdc and the conservation of the pyridoxal 5'-phosphoate (PLP) binding pocket (3, 15). However, upon closer analysis of the conserved domain features it becomes apparent that of the 8 residues constituting the PLP binding site, DisA only contains 2. Furthermore a second theme is present in the conserved domains. There are multiple homologues to glutamate decarboxylase and DisA is classified as a potential member of the Aspartate aminotransferase superfamily (AAT) fold type I. The AAT-I superfamily has been demonstrated to have four potential functions: transamination, racemization, decarboxylation, and facilitating side-chain reactions, but homology alone is insufficient to determine which of these four functions DisA may provide.

Identifying these other domain homologies as well as the negative data obtained from the biochemical decarboxylation assay lead to performing bacterial metabolomics in an

attempt to identify the product of DisA. While untargeted metabolomics does not provide a clear answer to the function of DisA, when taken in conjunction with newly published literature, it does suggest a new route of investigation. Alteri *et. al.* demonstrated *P. mirabilis* requires aspects of both aerobic and anaerobic respiration for swarming motility and mutations in these pathways cause changes in swarming (1). A second publication by Armbruster *et. al.* identified several signals that induce swarming, including fumarate, and showed glutamine is essential for swarming initiation (2). These publications demonstrate a role for central metabolites in regulating swarming. Our metabolomics data indicate two intermediates in the TCA Cycle were significantly altered wheb *disA* was over-expressed: citric acid and succinic acid. The metabolome profile also indicates pantothenic acid is decreased in *disA* over-expressing strains and aspartic acid is significantly increased. The role of citric acid and succinate in central metabolism is obvious due to their place as intermediates in the TCA cycle. Pantothenic acid is also important in central metabolism as it is a component of coenzyme A, a critical coenzyme for a plethora of cellular processes, including the TCA cycle. Finally aspartic acid, aside from its role as an amino acid, also ties in to the TCA cycle as a component of the reversible conversion of α-ketoglutarate to oxaloacetate and glutamate by aspartate transaminase. Fumarate, another TCA intermediate, can also be converted into aspartate by aspartate ammonia lyase.

When taken together the observations that 1) DisA has not be shown to decarboxylate phenylalanine, 2) over-expression of *disA* alters levels of several metabolic intermediates, 3) *P. mirabilis* requires aerobic and anaerobic respiration for swarming motility and 4)

the natural habitats for *P. mirabilis* (soil, gastrointestinal and urinary tract) are all anaerobic environments raise the question of whether DisA functions to regulate swarming by controlling the levels of one or more metabolic intermediates in anaerobic conditions. These experiments remain in their infancy but initial data, reported in Chapter 5, demonstrate that supplying 10 mM succinate, fumarate and malate to a *disA* mutant strain increases swarming radius and restores the formation of swarm rings in an anaerobic condition. This change in motility is not observed in wild-type under anaerobic conditions and there is no change in motility with supplementation in aerobic conditions.

P. mirabilis does not naturally find itself in aerobic environments on rich media at neutral pH. More frequently, especially during human colonization, *P. mirabilis* encounters anaerobic, nutrient deplete, non-neutral (acidic in the gastrointestinal tract, alkaline in the urinary tract) environments. In these environments, the bacterium must be cautious with the expenditure of energy and use of metabolites. Swarming is a highly energy intensive process; the cells must elongate, replicate their genomes to maintain the DNA:cell ratio, and produce and assemble an enormous number of flagella. Recently it has also been shown that *P. mirabilis* utilizes both aerobic and anaerobic cellular metabolism to power swarming. Given the energy intensive nature of swarming, it seems logical that sensing levels of metabolic intermediates would act as a regulatory mechanism for determining whether committing to swarming was physiologically sound. If DisA is involved in regulating central metabolites to exert control over the swarm cycle, it must be

considered that, rather than producing a molecule to inhibit swarming, DisA may deplete the cells of a metabolite required for swarming.

The basis of the TCA cycle is it is self-regulating (a diagram of the TCA cycle is available in Chapter 5, Figure 4). Early steps, such as synthesis of citrate, isocitrate and α-ketoglutarate occur spontaneously providing the momentum for the cycle to continue through non-spontaneous reactions (those with a positive Gibbs Free Energy co-efficient) such as conversion of succinate to fumarate and fumarate to malate. Furthermore "late" intermediates, such as fumarate and $FADH_2$, can act as competitive inhibitors of "early" reactions when an intermediate is in surplus to stall the cycle. In this way, the cycle is auto-regulatory and creating an over-abundance or deficit in any intermediate will affect the preceding and following reactions. A depletion of fumarate due to conversion to aspartate by aspartate ammonia lyase could lead to the depletion of succinic acid due to a shift in the balance of the reagents and the natural drive of the reaction to achieve equilibrium. If aspartate is then underoing a transamination with α-ketoglutarate to produce oxaloacetate and glutamate, an increase in citric acid may be observed due to an overabundance of oxaloacetate driving the equilibrium of the reaction forward. In this way, single enzymes and small changes in their expression can cause quantitatively small alterations in central metabolism that manifest in overt phenotypes.

During aerobic respiration the TCA cycle functions as shown in Chapter 5, Figure 4. However, under anaerobic conditions the cycle functions as a branched biosynthetic pathway, where one branch operates as a reductive pathway and the other as an

oxidatively (Figure 2). *P. mirabilis* lacks the reductive pathways for anaerobic respiration, thus it is unable to convert malate to fumarate to succinate. A way to recycle these accumulated metabolites is to biochemically convert them to other molecules that can be utilized by the cell during anaerobic growth, such as the converting fumarate to aspartate by aspartate transaminase. Aspartate can then be utilized in glutatmate and oxaloacetate synthesis. *P. mirabilis* has demonstrated that it uses both aerobic and anaerobic respiration to power swarming (1), suggesting that it may have mechanisms to utilize intermediates from both forms of respiration in the production of energy and facilitating swarming.

Of the metabolites identified as significantly altered in our metabolomic study, aspartic acid may be the most reasonable candidate as a product of metabolic sequestration. Aspartic acid levels showed the second greatest fold change (~1.4-fold increase; Chapter 5, Figure 3) when *disA* is over-expressed, indicating it may be directly related to DisA function. The second observation implicating aspartic acid is that it is at most two chemical conversions away from two important swarming signals: fumarate and glutamine (Figure 2). If DisA assists in the production of aspartatic acid, as its homology to AAT-I might suggest, it may be accumulating aspartatic acid by depleting fumarate or glutamate. The hypothesis that DisA assists in aspartate production by depleting fumarate is supported by both the metabolomics data as well as the anaerobic motility assays using TCA intermediates (Chapter 5, Figure 3 and Table 2). Fumarate acts as a swarming signal, in a wild-type strain DisA will function as normal levels converting fumarate to aspartate and consequently decreasing succinate (Figure 2A). When *disA* is

non-functional fumarate will accumulate, increasing the intracellular levels of a swarming signal. Providing 10mM fumarate in the media may be sufficient to push fumarate levels over a signaling threshold, leading to enhanced swarming in the mutant strain. Similarly, over expressing *disA* would further deplete cellular fumarate levels, impedeing swarming. Creating an over-abundance of aspartic acid in the cell by depleting fumarate decreases the concentration a swarming signal while increasing the concentration of a swarming inert molecule that can easily be converted back into swarming signals at an appropriate time.

Searches for aspartate ammonia lyase and aspartate transaminase in the *P. mirabilis* genome demonstrate that *Proteus* encodes *aspA* as an aspartate ammonia-lyase but no distinct aspartate transaminase has been identified. However, twenty-four matches, including *disA*, return when we search the *P. mirabilis* NCBI gene databank for "aspartate transaminase". In support of the hypothesis that DisA increases aspartic acid levels by depleting fumarate or glutamine we have demonstrated that providing 10 mM fumarate restores terrace formation to a *disA* mutatnt when grown under anaerobic conditions (Chapter 5, Figure 5). Additionally, recent research demonstrated that an abundance of glutamate is required for swarming motility in *P. mirabilis*. We have not tested the ability of glutamine to restore terrace formation in a *disA* mutant or to restore motility to a *disA* overexpression strain; this should be examined in the future.

Glutamate and glutamine were not significantly altered in the metabolomics screen and many of the metabolites that were altered during *disA* overexpression were not

significantly changed in PM2199, but this does not necessarily invalidate the data. Due to the critical nature of many of these molecules, it is likely the cell contains multiple synthetic pathways to produce them. Thus we may not observe a change in metabolite levels in a *disA* mutant strain due to functionally redundant systems. We do observe a change during *disA* overexpression, however, because the redundant systems continue to function even when there is an overabundance of DisA. However, because so many cellular metabolites are synthesized and catabolized by multiple pathways, it is possible that identifying the metabolite of DisA by metabolomics may be impossible. If DisA is affecting the level of a critical central metabolite altering *disA* expression would change the equilibrium in the cell, causing other pathways to compensate. Depending on the efficiency of this compensation, we may not see concentration changes in the true product of DisA. However, identifying other metabolic intermediates that are affected by *disA* augmentation can at least direct research in the correct general direction.

Finally, it should be noted the effect of *disA* manipulation has always been addressed under laboratory conditions and has only very recently been considered in partially physiological conditions. It is possible the swarming and metabolic phenotypes observed when *disA* is altered under laboratory conditions do not represent the full function of *disA* in the natural environments. To address the possibility that laboratory growth masks an aspect of DisA function both overexpressing and mutant strains need to be more rigorously tested in better defined conditions. The nutrient deplete conditions encountered in the human host could be replicated by growth on a minimal, chemically defined media. In this method pH, iron levels, temperature, and oxygen abundance can

all be altered to physiological levels as well. However, the best way to assess the function of *disA* during gastrointestinal growth and urinary tract infection would be the use of the ascending mouse model of urinary tract infection. This would be of particular interest also due to the co-regulation of virulence factors and swarming motility, as discussed in Chapter 1. The *disA* over-expressing strain provides a unique opportunity to assess the role of swarming in virulence without modification of *flhDC* given that *disA* over-expression inhibits swarming without affect *flhDC* transcription and, in light of the recent unpublished data shown here, may exert its regulation without affecting $FlhD_4C_2$ at any level. Given the specificity of DisA to *Proteus*, it may represent a good antibiotic target for *P. mirabilis* infection though the regulatory mechanisms and chemical product of DisA would first need to be determined.

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Figure 1. Regulation by the Rcs phosphorelay. A schematic of Rcs regulation during growth in liquid culture (A) and on a solid surface (B). Regulation that is not active in a specific growth condition are shown in grey. Ultimately RcsC, the sensor kinase, autophosphorylates during liquid growth leading to the phosphorylation of RcsB. RcsB~P represses *flhDC*, preventing swarming. On a solid plate surface sensing, mediated by O-antigen, activates the integral membrane protein UmoB. Activated UmoB prevents RcsC from autophosphorylating leading to reduced levels of RcsB~P and consequently swarming by depression of *flhDC*. The hypothesized role for RcsB~P regulation of *disA* is also shown by dashed lines in both figures.

Figure 2. Chemical reactions utilizing TCA cycle intermediates. The TCA cycle is broken into two distinct pathways under anaerobic conditions, an oxidative branch and a reductive branch (A). While *P. mirabilis* has a functional oxidative branch, it does not utilize the reductive branch. The chemical reaction by which aspartate is converted to fumarate and ammonia and vise versa is shown in (B). Aspartase converts aspartate to fumarate and NH_3 by cleaving the C-N bond. Fumarate has been shown to act as a swarming signal by Armbruster et. al. (2) and our metabolomic screen identified aspartic acid as significantly increased when *disA* is overexpressed. In (C) one pathway for conversion of aspartase to glutamine using TCA intermediates and vise versa is shown. Glutamine has been demonstrated to be absolutely essential for swarming (2), disruption of TCA cycle intermediate levels also affects swarming (1). Enzymes are in italics.

Figure 1.

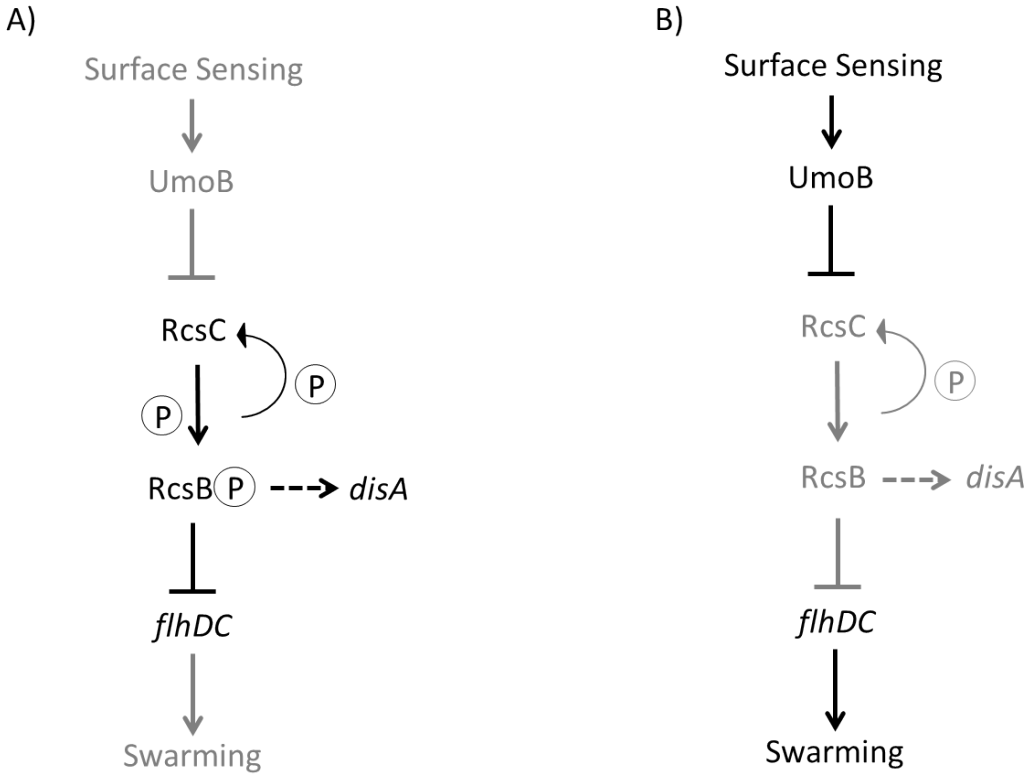
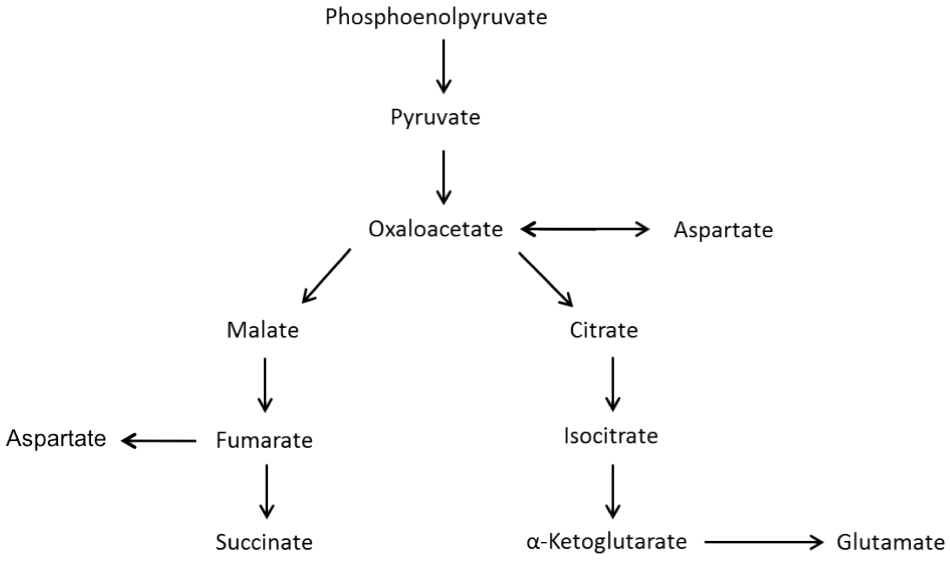
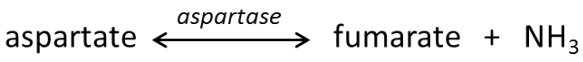

A)
Surface Sensing
UmoB
RcsC
P
P
RcsB P
disA
flhDC
Swarming
B)
Surface Sensing
UmoB
RcsC
P
RcsB
disA
flhDC
Swarming

Figure 2.

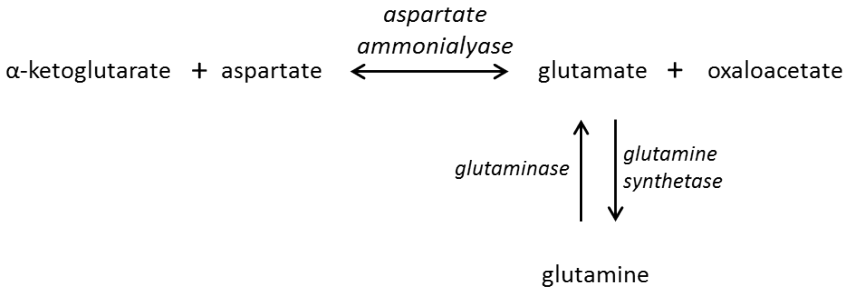
A)


Phosphoenolpyruvate
Pyruvate
Oxaloacetate
Aspartate
Malate
Citrate
Aspartate
Fumarate
Isocitrate
Succinate
α-Ketoglutarate
Glutamate

B)


aspartate
aspartase
fumarate + NH3

C)


aspartate
ammonialyase
α-ketoglutarate + aspartate
glutamate + oxaloacetate
glutaminase
glutamine
synthetase
glutamine